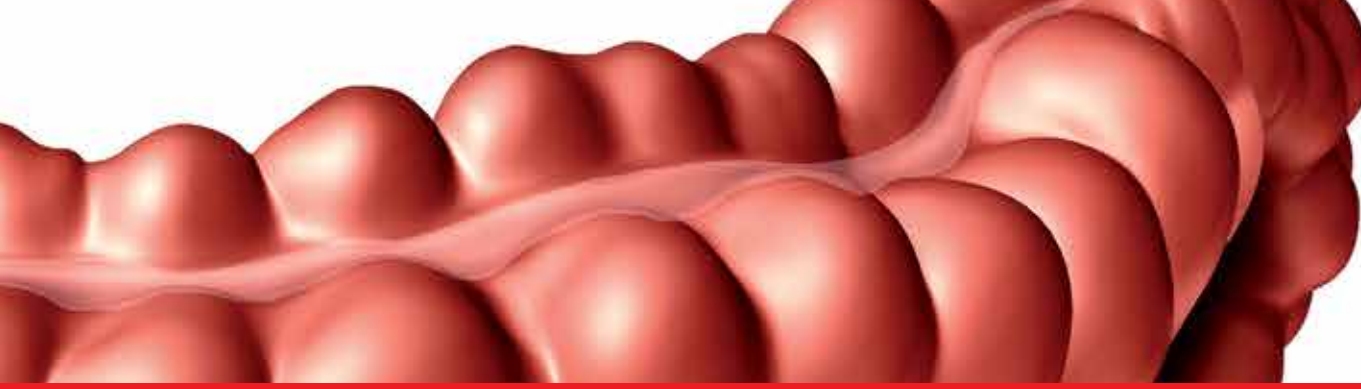

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Colorectal Cancer

From Prevention to Patient Care

Edited by Rajunor Ettarh

COLORECTAL CANCER – FROM PREVENTION TO PATIENT CARE

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http://dx.doi.org/10.5772/2443
Edited by Rajunor Ettarh

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Notice

First published in Croatia, 2012 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

Additional hard and PDF copies can be obtained from orders@intechopen.com

Colorectal Cancer - From Prevention to Patient Care
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p. cm.

ISBN 978-953-51-0028-7

eBook (PDF) ISBN 978-953-51-6808-9

Meet the editor

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Contents

Preface

When a patient are presented with symptoms that eventually lead to a diagnosis of colorectal cancer, it is the clinician who ultimately has to deliver the management and treatment of the condition based on what is known about the disease. The clinician synthesizes and brings together an understanding of the basic scientific facts available, and the information about effective clinical management and treatment – all for the patient's maximum benefit. Is the search of answers complete? No. There is still a very long way to go, but in terms of information, we are further ahead than we were a less than decade ago. Is there more to do? Yes. Our understanding is improving, but translation of basic scientific evidence to its application in terms of clinical treatment and management of patients remains a challenge. One thing is clear, a complete understanding of colorectal cancer and how it affects patients involves the continuing cooperation between research science and clinical practice. There are a number of positive examples resulting from searching and querying: monoclonal antibody therapy, pharmacologic agents and treatments, low dose aspirin, better risk management for colorectal cancer, and continually emerging targets.

This second volume of the book presents two sections that address aspects of epidemiology, psychology and nutrition as they relate to colorectal cancer from a patient illness and care perspective. Section 3 deals with the clinical management and treatment of the disease, while Section 4 explores different management approaches to colorectal cancer metastasis. Section 5 presents a collection of short reports that outline findings from studies on probability modeling, dietary risks and the prognostic value of metastatic lymph nodes.

Basic scientific researchers need to know where success has been registered, where failures lie and where the challenges in patient management and care remain. This volume represents an attempt to bring together much of what is known about colorectal cancer and provide a synoptic source of information that serves as a reference point for scientists, clinicians, researchers, students and patients.

The cure for colorectal cancer can only be discovered when research science and clinical evidence collectively arrive at the right cocktail of information.

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Acknowledgements

The publication of this book would not have been possible without the support of my family. I am also especially indebted to Publishing Process Manager Tajana Jevtic whose infinite patience, timely reminders, and never-ending assistance and support made the task of editing this book easier.

Part 1

Introduction

1

Tumor Engineering: Finding the Brakes

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1. Introduction

There is sufficiently detailed understanding about how an automobile works such that starting or running the engine can be selectively disabled. Applying this analogy to colorectal cancer, the question researchers and clinicians ask is: can colorectal cancer be prevented from starting? Once started, can the disease be prevented from running? These two aims fall broadly into the categories of prevention and treatment respectively. Many of the aspects of colorectal cancer that provide focal points for clinical management of patients of the disease are included in Figure 1 (below). This volume provides insights into aspects of disease incidence and presentation, some of the advances and developments in diagnosis and patient management, and examines prevention and therapeutic targets and regimes. This chapter provides a general overview of some of the aspects of colorectal cancer that affect clinical management of the disease and explores incidence of the disease, diagnosis and treatment as well as preventive screening programs.

2. Epidemiology

As a disease, the statistical data for colorectal cancer are disturbing. Every year, there are over 1 million new cases worldwide, half of them in men; over 200,000 new cases in Europe; and 1.5 million new cases in the United States (Jemal et al, 2010). Over 700,000 patients die each year.

Expanded surveys and studies show that the incidence of colorectal cancer is increasing worldwide, along with cancer detection rates. Other studies suggest that these rates may also be dependent on anatomic site along the intestine at which the cancer occurs. However, although absolute numbers of patients affected by the disease is increasing in the US, the trend for colorectal cancer is downward: age adjusted incidence has declined steadily since 1976 (Ji et al, 1998; Chen et al, 2011; Eser et al, 2010; Merrill & Anderson, 2011). Genomic instability is present in 15% of colorectal cancer, and forms the basis for those who advocate the need for screening programs for colorectal cancer patients (Geiersbach et al, 2011).

Incidence of colorectal cancer around the world per 100,000 of population varies between 3-43 and is influenced by age, gender, socioeconomic status, and ethnicity (Center et al, 2009; Hao et al, 2009). Younger patients have greater susceptibility if there is an associated family history and tend to present at a more advanced stage of the disease. Long and short-term incidence of colorectal cancer is also affected by aspirin intake and this effect may be

dependent on dosing regime and patient history (Dube et al, 2007; Flossmann et al, 2007; Rothwell et al, 2010).

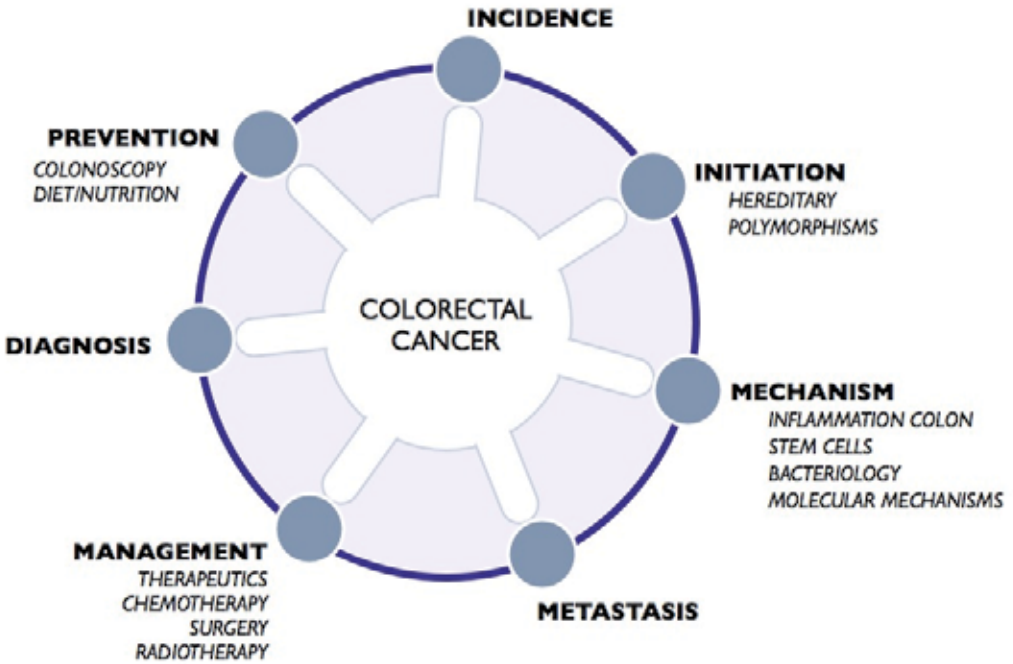

Fig. 1. Basic science studies and clinical management continue to improve our understanding of colorectal cancer. This volume considers incidence, diagnosis and clinical management of the disease as well as metastatic disease. Aspects of initiation and mechanisms are dealt with in the first volume of the book.

3. Diagnosis and treatment

There has been steady improvement in survival rates in colorectal cancers that are diagnosed early. Prognosis for patients who present with late stages of the disease remains poor. Treatment options include surgery for localized tumors, chemotherapy and immunotherapy. When resectable, surgical removal of the tumor remains the treatment of choice for localized colorectal disease. Surgery may be curative or palliative and is sometimes combined with chemotherapeutic regimes to achieve pre-operative tumor shrinkage (Zhao et al, 2010). Minimally invasive approaches such as laparoscopic surgery for colonic tumors are reported to offer improved short-term clinical outcomes (Hiranyakas & Ho, 2011). Chemotherapeutic regimes include infusional combination therapies such as FOLFIRI that combine irinotecan, 5-fluorouracil and leucovorin, and FOLFOX that combines oxaliplatin, 5-fluorouracil and leucovorin (Lee & Chu, 2007; Garcia-Foncillas & Diaz-Rubio, 2010). Studies suggest that overall survival time and progression-free survival are significantly improved with the addition of cetuximab to FOLFIRI.

Better understanding of some of the molecular mechanisms in colorectal cancer has led to the development of targeted therapy that modulate specific pathways and pathway

components. Biological treatment with bevacizumab, a recombinant antibody to vascular endothelial growth factor (VEGF) receptor, cetuximab and panitumumab has improved clinical outcomes for patients, prolonged survival times and is recommended in metastatic disease (Koukourakis et al, 2011). Despite these improvements in treatment, the number of patients who develop metastatic disease is significant and the prognosis for such patients is poor. Metastatic disease is thought to be related to epigenetic mechanisms and the development of cancer stem cell-mediated chemoresistance (Anderson et al, 2011). Treatment for metastatic disease is complex and requires careful patient evaluation and selection from single and combination treatment options that include surgery for resectable metastases, chemotherapy and biological therapy. Fluoropyrimidine 5-fluorouracil (5-FU) has been joined by cetuximab, an IgG antibody whose efficacy has been documented in several clinical trials (Lee & Chu, 2007). Improving regimes have led to better 2-year survival rates in patients.

New therapeutic approaches and targets are emerging from research studies. One promising approach currently being explored is the prospect of therapeutic vaccines to combat colorectal cancer (Kabaker et al, 2011; Kameshima et al, 2011).

4. Screening for prevention

A reduction in the morbidity and mortality from colorectal cancer can only be achieved through effective screening for the disease. Screening allows for early detection of cancer and early treatment of detected cancers. It is estimated that up to 60% of deaths from colorectal cancer could be prevented by routine screening after the age 50 years (Byers, 2011; He & Efron, 2011). Approaches to screening for colorectal cancer include stool-based tests (fecal immunochemical testing FIT, fecal occult blood testing FOBT), endoscopy (sigmoidoscopy and colonoscopy) and radiologic examinations (barium radiography, and colonography) (de Wijkerslooth et al, 2011). Studies suggest that stool-based testing is more cost effective than colonoscopy (Hassan et al, 2011; Wilschut et al, 2011).

Colonoscopy remains the gold standard for screening and while it offers advantages for treatment such as removal of premalignant lesions, this approach may not be as protective for right-sided disease as it is for left sided disease (Baxter et al, 2009; Brenner et al, 2010; Singh et al, 2010). Other advanced colonic imaging techniques include capsule colonoscopy, computed tomographic colonography, virtual colonoscopy and magnetic resonance colonography (Liu et al, 2011). All screening programs are complicated by social and community factors (such as culture, level of knowledge about the disease) that affect participation rates (O'Donnell et al, 2010; Ramos et al, 2011; Reeder, 2011).

5. Conclusion

Colorectal cancer remains a major health challenge. Trends for geographically distributed fluctuations in incidence point towards the need for developing strategies to tackle increasing colorectal disease in the population under age 50 years, the relationship of the disease with socioeconomic status, and the increasing incidence of the disease in Asia.

Treatment options are still dictated by the stage of the disease in the patient at presentation but evidence from basic science research studies are providing a better understanding of the disease process, drivers for improvements in therapeutic options for patients, and new therapeutic targets for impeding the progression of the disease.

Despite the remarkable improvement in our understanding of certain aspects of colorectal cancer, the best approach to combating the disease remains a preventive one. Prevention and screening programs need to be more efficient and more effective. Cost benefit analyses preclude early adoption of newer screening methods but advances in colonoscopic and colonographic approaches are helping to reduce morbidity and mortality for colorectal cancer.

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Part 2

Epidemiology and Psychology

2

Colorectal Carcinoma in the Young

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1. Introduction

Colorectal cancer (CRC) is the most common malignancy of the gastrointestinal tract. In the United States, it is the third most commonly diagnosed cancer, next only to breast and lung. It is the second most common cause of cancer-related death both in the USA and in the UK. (www. cancer. org, O'Connell et. al. 2004[a], Leff et. al. 2007). Its incidence has risen rapidly in Asia to pose a problem (Yuen et. al. 1997, Huang et. al. 1999, Mohandas et. al. 1999, Yiu et. al. 2004, Goh et. al. 2005,Gupta et. al. 2010). Sung et. al. (2005) in a review on CRC in Asia stated that many Asian countries, e. g., China, Japan, South Korea, Singapore have experienced an increase of two to four times in CRC incidence during the past few decades. In Hong-Kong CRC is the second most common cancer and the third most common cause of cancer death (Yuen et. al. 1997). Tamura et. al. (1996) in a Japanese study reported that age adjusted incidence for CRC per 100,000 population were 12. 6 and 8. 7 for males and females respectively in 1974, 20 and 13. 6 in 1980, 42. 5 and 25. 6 in 1991. Bae et. al. (2002) estimated on the basis of Korean data, that the expected number of cancer deaths in Korea showed an increasing trend for CRC, although the same did not hold for all cancers. In Iran, age adjusted CRC incidence per 100,000 population per year increased from 1. 61 in 1970-80 to 4. 2 in 1990-2000 in men and 2. 35 to 2. 72 for women (Hosseini et. al. 2004). The rising trend is more striking in affluent than in poorer societies and differs substantially amongst ethnic groups. Changes in dietary habits and lifestyle are recognized causes. Genetic characteristics of a population mediate the effect of life style change into disease propensity (Lin et. al. 2010).

Although the common perception is that it is a disease of an older person, there have been many reports from different parts of the world on CRC in the young adults (Bulow 1980, Denmark; Ohman 1982, Sweden;Jarvinen and Turunen 1984, Finland; Ibrahim and Karim 1986, Lebanon; Adloff et. al. 1986, France; Isbister and Fraser 1990, New Zealand; Yuen et. al. 1997, Hong-Kong; Fante et. al. 1997, Italy; Ashenafi 2000, Ethiopia; deSilva et. al. 2000, Srilanka; Paraf and Jothy 2000, Canada; Turkiewicz et. al. , 2001, Australia; Singh et. al. 2002[a], Nepal; Kam et. al. 2004, Malayasia; Frizis et. al. 2004, Greece; Guraya and Eltinay2006, Saudi Arabia; Fazeli et. al. 2007, Iran; Karsten et. al. 2008, USA; Gupta et. al. 2010, India). O'Connell et. al. (2004[a]) have reviewed the literature. The proportion of patients in the young group in a population of CRC patients was significantly larger in reports from Asia and Africa, as compared to the Western reports.

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The definition of 'Young adults' varies, to a small extent, in the literature. Majority of articles defined 'young' as <40 years, although upper limits of 50 years, 35 years and 30 years have also been used. O'Connell et. al. (2004[a]) estimated the average value of incidence of CRC in the young adults (<40 years) in the population of all CRC patients as 7% and adjusted it to 6%, when outliers were removed. It has been suggested (Hamilton 2005) that the adjustment was 'too small' and a more realistic estimate was an average of 2. 2%. Leff et. al. (2007) gave an estimate of 2-3%. About 0. 1% of all CRC patients were diagnosed <20 years of age, ~1% between 20-34 years, ~4% between 35-44 years and a further ~12% between 45-54 years. These average figures reflect the extent of the problem in the West. The figures from Asian and African countries are considerably higher, a quarter or a half of a study group of CRC patients may belong to the under-40 group (Ashenafi 2000, Ethiopia; deSilva et. al. 2000, SriLanka; Singh et. al. 2002[a], Nepal; Guraya and Eltinay 2006, Saudi Arabia; Gupta et. al. 2010, India). Numerical values given later will establish that the problem of CRC in the young adult in the developing world is alarming.

We now cite reports, from the West (USA, France, Scotland) and from Asia (Iran, Hong Kong), in which the incidence of the disease amongst the young adults has been studied in the same population over a period of time. O'Connell et. al. (2003) noted that in the USA, colon cancer incidence in older patients (60 + years) remained stable in the period 1973-1999 while rectal cancer incidence decreased by 11%. In the group of younger patients (20-40 years) colon cancer incidence increased by 17%, while rectal cancer incidence rose by 75% in the period 1973-1999. The improvement in the older age group is a reflection of more efficient cancer screening in the USA, a result of improved awareness of the disease. It is possible that relative ignorance about the problem of CRC in the young adult is responsible for the fact that the problem has worsened over the years. Other issues namely difference in molecular genetics, may also be present. In Iran, Hosseini et. al. (2004) defined the younger group as <60 years, compared figures in two 10 year periods 1970-1980 and 1990-2000 and found an increased proportion of < 60 years CRC patients (in a population of all CRC patients) in the latter decade, 37. 5% as against 70%. An increase in proportion of the young CRC patients was noted over a prolonged time span. Mitry et. al. (2001) from France reported that below-45 age standardized incidence rates doubled in the period 1976-1982 and then again in the period 1983-1989, in both genders and stabilized thereafter. In Hong-Kong, the overall incidence in > 50 years group increased at a rate of 4% a year during 1978-87, whereas in Scotland a higher overall incidence remained stable during this period (Yuen et. al. 1997).

O'Connell et. al. (2004[b]) in a study of American patients found that young (20-40 years) colon cancer patients tend to have later-stage and higher-grade tumours. However they have equivalent or better 5 year cancer-specific survival compared to 60+ older group, an apparently paradoxical result. Although most reports agree on a more severe advanced disease at presentation in the young (Adloff et. al. 1986, Cusack et. al. 1996, Nath et. al. , 2009) and many also agree with the opinion that prognosis is not poorer in the young (Jarvinen and Turunen 1984, Turkiewicz et. al. 2001, Karsten et. al. 2008) some reports (Moore et. al. 1984, Adkins et. al. 1987, Okuno et. al. 1987, Singh et. al. 2002[a]) do not share the view that prognosis is 'equivalent or better'. Inspite of this difference in assessment, a favourable prognosis in many studies should inspire more aggressive detection and treatment for the young.

The genetic basis of CRC has been investigated in recent years. A satisfactory understanding of the disease, tumour characteristics, relationship of disease susceptibility with age and issues related to survival rely on an understanding of the link between molecular genetics and disease. A complete resolution of this relation is a tall order, but a modest beginning is

being made. Intelligent choice of treatment protocol, surgical as well as chemotherapeutic is also influenced by research on molecular genetics of CRC (Liang and Church 2010). Hereditary CRC usually occurs at a relatively young age, between 25 and 55 years in individuals with family history of CRC. Individuals who inherit the predisposing cancer gene have a greater chance of developing the disease (Murday and Slock 1989, Lynch et. al. 1991, Lynch and de la Chapelle 2003, Ewart Toland, 2012). The importance of family history in determining susceptibility to CRC in the young has been stressed in the literature (St. John et. al. 1993, Fuchs et. al. 1994, Turkiewicz et. al. 2001). There exist literature reports that identify genetic factors in younger CRC patients which differ from those in older patients and may be responsible for greater cancer susceptibility of the younger patients (Farrington et. al. 1998, Chan et. al. 1999, Morris et. al. 2007, Berg et. al. 2010, Lin et. al. 2010).
In this essay, we focus on the issue of CRC in young age, with particular reference to developing countries. The relative incidence figures of CRC in the young patients as compared to older patients in different parts of the world are given. These figures, in greater detail are given in the Indian context (section 2). Disease stage at presentation and tumour characteristics of younger patients, often in comparison with the older ones in different countries are then summarized (section 3). A brief reference to de novo cancer in Asians (section 4) is followed by a discussion of some recent genetic studies in the young (section 5). Section 6 contains a discussion on prediosposing factors and section 7 has focus on prognosis in the young. The paper concludes (section 8) with a brief reference to the effect of recent molecular genetic research on treatment protocol.

2. Incidence amongst young adults

The relative incidence of CRC in the younger group varies significantly from one country to another. As cited above, it is typically 2-3% in the West. Other European figures are: Fante et. al. (Italy): 1%; Endreseth et. al. (Norway):6%**;** Ohman (Sweden): 4%; Adloff et. al. (France): 3%; Yilmazlar et. al. (Turkey): 20%. The corresponding figures are much higher from several Asian and African countries: Nath et. al. (India): 35. 6%, <40 yrs; Gupta et. al. (India): 39%,<40 years; Singh et. al. (South Asia): 23%,<40 years (with a maximum incidence in 40-60 years, a decade earlier than Western figures): study period 1975-1981; Soliman et. al. (Egypt): 35. 6%,<40 yrs; Ashenafi (Ethiopia): mean age 47 years (61. 4% <50years, 36% <40 yrs,16% <30 yrs) in two 5 year periods with a 10 year gap; Guraya and Eltinay (Saudi Arabia): study period 1999-2004,63% <40 yrs, mean age 44years, peak incidence 30-39 years; Hosseini et. al. (Iran): 70% (<60 years):study period 1990-2000; Chew et. al. (Singapore):25% <40 years; Singh et. al. (Nepal): 28. 6% <40 years; de Silva et. al. (Sri Lanka): 19. 7% <40 years. Some of these references are detailed in Table 1. In Egypt, more than half of all CRC patients are below-50, patients under-30 constitute 22% of the population of all CRC patients (Soliman et. al. 1997). Qing et.al. (2003) in a comparative study of American and Chinese patients (1990-2000) reported that the mean age at diagnosis of 690 American patients was 69 years (20-91 years) and that of 870 Chinese patients was 48. 3 years (13-84 years); peak incidence was 70-79 years in Whites and 50-59 years in Orientals. The conclusion is that the Orientals are affected by the disease at a younger age. The same theme emerges from recent data from several Indian hospitals which includes our own recent work (Gupta et. al. 2010). In a period spanning 8 years (2000-2008), we found the ratio of under-40 to above- 40 years age group to be 0. 64. The study group comprised of 305 patients in SSKM Hospital, Kolkata, India, a premier referral Hospital. The values reported by three premier Oncology centers located in two cities in India and in another report by Pal (2006), based on work done

Sr. No.	Reference, Period of study	Age profile	Disease stage	Tumour characteristics
1	Lee 1968-91	62 patients, <40yrs	Dukes' A:8%, B:20%, C:23%, D:48%.	Half of stage D patients and 20% of lower stage patients (p=0. 037) had high grade lesions.
2	Minardi, 1976-97	37 patients, <40yrs	Dukes' C:37%, D: 22%.	Mucinous tumour : 42%; moderate and poor differentiation : 84%
3	Cusack	186 patients, <40yrs	Dukes' C & D: 65. 6%.	Poorly differentiated tumour in 41%, signet-ring cell tumours in 11. 1%, infiltrating tumour leading edges in 69% of young patients. Aggressive tumour biology with higher frequency in <40yr patients (p<0. 001), potentially metastatic.
4	Bedikian, 1944-1977	2609 patients,<50yrs age; 183 aged<40 yrs. Comparison between<30yr and 30-39yr age group and with yet older age group	96% of < 40 years group had carcinoma extending beyond colonic wall.	Moderate and poorly differentiated neoplasms (80%) and mucinous variety (33%) in young.
5	Beckman 1943-1977	69 patients: 20-39yrs	67% Dukes' C and D	Mucinous variety (28%).
6	Varma	A review: all age groups	Advanced stage more frequent in the young.	Greater frequency of mucinous tumour in the young.
7	Cozart, Unusual Case Registry 1992-93	55 patients, <30yrs	62% Dukes' C and D	Poorly differentiated /mucinous variety: 33%
8	Howard	801 patients including <40yrs group	Advanced signs and stages more frequent in the young.	Greater frequency of mucinous variety in the young

9	Adkins, 1973-1984	705 patients; 45 patients, <35yrs	In the younger group: Dukes' A:2,B:8, C: 28, D: 7 patients.	19: poorly differentiated,19: well or moderately differentiated tumour
10	Moore, 1967-1981	3. 2% of 1909 patients <40yrs	Higher incidence of advanced disease, especially in second or third decades.	Greater incidence of mucinous variety (32. 3% in young vs. 8. 6% in the whole study group). Poorly differentiated tumour: 98%; distant metastases in one-third patients. Vascular (24%) and perineural (11%) invasion in the young.
11	Karsten. , 1998-2005	Younger group: 41 patients <40yrs Older group : >60 yrs	Advanced stage: T-3/4 lesion in 87. 8% of young/63% in older group (p=0. 002).	Poorly differentiated, (p=0. 003), mucin secreting/ signet ring (p=0. 005), more common in the young
12	Fairley, 1998-2001 Cancer Registry (NPCR,SEER)*	All age groups Young:20-49yrs	Less localized, more aggressive disease in terms of stage in the young (20-49yrs)	Incidence of poorly differentiated tumour in young (<50yrs) (i) twice as high as well differentiated ones in the young (ii)60% higher than that for well differentiated cancers in the old
13	Lichtman 1987-1991	57. 2% <70yrs	Dukes' C and D more frequent at a lower age (p=0. 03). Mean Age A/B-1 67. 7 yrs, B-2 70. 1yrs, C/D 63. 9yrs	Grade not related to age

14	Dozois, 1976-2002	1025 patients,<50yrs; Mean age 42. 4±6. 4 years 51% colon, 49% rectal (largest cohort of young-onset patients without genetic predisposition)	70% colon, 60% rectal: stage C&D	Colon Cancer: Mucinous(11%) & Signet cell (2%) Grade 2+3 for both rectum & colon cancer: ~87%
15	Behbehani 11 yrs period pre-1980	<40 years group: 56 patients	Advanced stage C&D: ~90% in young ~50% in general population	Poor differentiation: 21% in young, 8% in general population

*NPCR: National Program of Cancer Registries; SEER: Surveillance, Epidemiology and End Results

Table 1. Summary of references in the literature on stage and tumour characteristics in the USA

in the same referral hospital where Gupta et. al. (2010) worked are 0. 58, 0. 63, 0. 45, 0. 62. Average of these five ratios is 0. 52, which is equivalent to ~34% of < 40 years CRC patients amongst all CRC patients. This figure is of the same order as the values from several Asian and African countries cited above. They are also substantially larger than values recorded in National Cancer Registry (PBCR) in four Indian metropolises. The PBCR ratio is 0. 20 and has remained stable over 16 years (1988-2004).

The difference between PBCR values and those reported by five premier hospitals in India, irrespective of their location and specialty, cited by Gupta et. al. (2010) has a clear message. The concern and facilities for cancer detection in the premier hospitals is greater than those in district hospitals. The data of the district hospitals are reflected in the PBCR values. This is the reason for the larger proportion of under-40 patients reported by the premier hospitals. The reason for delay in diagnosis of a young patient in either the premier hospitals or the district hospitals, particularly in the developing world, is that unless there is a family history these patients are not screened. So cancers are usually symptomatic at presentation. Even when symptoms occur, they may initially be misdiagnosed. Rectal bleeding for example is often put down to an anorectal cause. O'Connell et. al. (2004[a]) report an average delay in diagnosis of 6. 2 months, the reasons for which include a delay in presentation on the part of the patients, limited access to care and misdiagnosis on the part of the physician. This delay is larger in the developing world. Minimizing delay in diagnosis means not taking such symptoms lightly. Rectal bleeding usually has an anorectal cause, but when no such cause is obvious and the bleeding persists, colonoscopy is mandatory, regardless of patient's age. The same concern must apply to other less obvious symptoms.

In a review on CRC in Asia, Sung et. al. (2005) placed India at the bottom of the list amongst Asian countries, in order of decreasing CRC incidence. The data we provide does not contradict this assessment, but if relative incidence in the young is an indication, India has joined the rest of Asia.

3. Disease stage and tumour characteristics in the young adults

The most powerful predictor of outcome for young adults, as it is for older patients is disease stage. Two staging systems are in use and are cited in Table 1-3. One is the tumour-node-metastases (TNM) staging system of the American Joint Committee on Cancer (AJCC). Microscopic extent of tumour invasion (T stage) and nodal involvement (N stage) from histological assessment are combined with assessment for metastatic disease (M stage) to specify a tumour stage. Brief description of TNM stages are: Tumour stages (T): Tumour in T1, invades submucosa, T2: invades muscularis propria, T3 and T4 are more extensive, T3 indicates invasion through muscularis propria into subserosa or into nonperitonealised pericolic or perirectal tissues while T-4 invades adjacent organs. Regional Lymph node stages: N1: 1-3 positive nodes, N2:4 or more positive nodes. Distant metastases stages (M): M1: Distant metastases present. The other classification system known as Dukes' system is: A: limited to bowel wall, B: penetration of bowel wall, C: lymph node involvement, D: distant metastatic disease present (Fry et. al. 2008).

Mucinous adenocarcinoma is one of the histological subtypes of colorectal cancers. It accounts for 5-15% of all primary CRC and is defined as a tumour with >50% of its body showing a mucinous pattern on histological examination and with a large amount of extra cellular mucin produced by secreting acini. This is distinct from signet ring adenocarcinoma, a rare variant in which mucin remains inside the cell, which is well known for its aggressiveness. It has been suggested that mucinous adenocarcinoma behaves differently from more common histological subtypes of CRC. However, its clinical implications remain unclear. According to published series, mucinous adenocarcinoma affects younger patients, is more frequent in proximal part of the colon and tends to present at a more advanced stage (Negri et. al. 2005).

In Table 1, 2 and 3 we tabulate data on disease stage and tumour characteristics, in particular its mucinous nature, of CRC patients in the USA (Table 1), in Europe, inclusive of Turkey and the UK and Australia (Table 2) and in Asia and Africa (Table 3).

Several reports cited in Table 1 (Sr No 1,2,3,5,7,9,14) were entirely on features of CRC in the younger patients. In several other reports (Sr No. 4,6,8,10-13,15), both the younger and the older patient groups were studied and comparative features were assessed. The size of the younger group was mostly ~50, was ~200 in two reports (Sr No 3 and 4) and was 1025 in the work of Dozois et. al. (2005) (Sr no 14), the largest cohort of young CRC patients. In reports that included older and younger patients, older patients were much larger in number (Sr No 4,8-10). In all studies that were on younger patients alone, a high incidence of advanced stage (C+D: >70%)was reported. In studies that included both groups, the frequency of advanced disease in the young was as high or higher (Sr No. 4,11,15). In all of them, advanced disease stages were found to be more frequent in the young than in the old. In studies on younger patients alone, a significant proportion of patients had aggressive lesions, namely mucinous, poorly differentiated tumours with infiltrating leading edge. The frequency of aggressive tumour biology varied from one study to another but remained significant in all of them. In the comparative studies (Sr No. 6,8,10-12,15), the younger patients showed a higher frequency of aggressive tumour biology. Only one report (Sr No. 13) concluded that grade was not related to age.

Country	Sr No.	Reference, Period of study	Age profile	Disease stage	Tumour characteristics
France	1	Adloff 1973-1980	1037 patients; 3% <40yrs	No significant difference in stage between <40 and >40 Yrs group.	Greater frequency of mucinous and poorly differentiated carcinoma in the young
Finland	2	Jarvinen 1970-1979	249 patients, <40yrs	53% Dukes' C and D.	Premalignant condition more common in young
Greece	3	Frizis 1994-2003	Two groups: 11 young <40yrs; 45 old > 80 yrs.	Dukes' C 54. 5% in the young and 44. 4% in the elderly group.	Undifferentiated tumour: 36. 3% of the young and 8. 8% of elderly.
Sweden	4	Ohman 1950-1979	48/1061 patients are <40 years (21-39 years)	Dukes' A same proportion in young and old, Dukes' B fewer, Dukes' C more in young	
Norway	5	Endreseth 1993-1999	2283 patients with rectal cancer <70 years, <45 yrs: 132, 45-49 yrs: 153 50-69 yrs:1998	Dukes' C&D : under 45: 73/132(~55%) 45-69yrs: 998/2152(~46%)	Higher frequency of poorly differentiated tumours (27 vs. 15%) & N-2 stage (37 vs. 15%) with distant metastases (38 vs. 20%); 56% of under-40 years: developed metastases (20-26% of older group) after tumour resection
	6	Berg 2010	181 patients, 45 of them < 50 yrs	Dukes' C &D 54% in < 50 yrs group 46% in 51-70 yrs 36% in > 70 yrs	

Country	Sr No.	Reference, Period of study	Age profile	Disease stage	Tumour characteristics
UK	7	Leff 1982-1992	49 patients all < 40 yrs: 67% in 31-40 yrs, 2 in their teens	Among all patients: 60% Dukes' C&D. Among patients at risk (family history /predisposing factor): 56% Dukes' C	Among all patients: 59% moderately & 22% poorly differentiated . Among patients at risk: 53% moderately & 20% poorly differentiated
Italy	8	Fante 1984-1992	Three groups <40, 41-50, 51-55 years: ~1%, 6%, 6% of 1298 patients	Stage did not differ	Histological features did not differ
Turkey	9	Yilmazlar 1986-1993	237 patients; 46 below 40yrs	76%of the young: Dukes C&D	48% tumours are poorly differentiated or mucinous in young.
Australia	10	Turkiewitz 1971-1999	61/2384 below 40 years	Distribution of stage not significantly different in younger and older group	35%tumours in the young are poorly differentiated

Table 2. Summary of references in the literature on stage and tumour characteristics from Europe (inclusive of UK & Turkey) and Australia

Two of the reports from Europe listed in Table 2 (Sr.No.2,7) are entirely on young patients. One of these (Sr No. 2) has the largest study group of young onset patients (~250), while the other reports have ~100-150 (Sr Nos. 5,6,8) or less ~50 (Sr No. 1,4,7,10)young patients. The report, Sr No. 3 is on a much smaller population of 11 patients. A significant frequency of more advanced (C+D) tumour in the young (50-60%) was reported in several studies (Sr No. 2,3,5-7). This frequency was larger (76%) in a study from Turkey (Sr No. 9). Comparative assessment showed a higher frequency of advanced stages in the young as compared to that in the older patients (Sr No. 3-6). Significant frequency (~ ≤50%) of high grade tumours were reported in the young in several publications (Sr Nos. 3,5,7,9,10). Higher frequency of high grade tumours in the young as compared to the older group were cited in several other papers (Sr No. 1,3,5,7). Three studies (Sr No. 1,8,10) however, reported no difference in disease stage and one report (Sr No. 8) found no difference in tumour grade, between the younger and older patient groups. A significant occurrence of premalignant conditions in the young was reported in only one paper (Sr No. 2).

Country	Sr No	Reference, Period of Study	Age profile	Disease Stage	Tumour Characteristics
ASIA Iran	1	Fazeli 1995-2001	403 patients in two age groups, <40yrs and >40yrs	Older group: 53. 2% in stage II; younger group: 45%in stage III.	Poorly differentiated tumours found in larger proportion in younger patients (22% vs. 5. 9%)
Singapore	2	Kam 1989-2001	39 patients <30yrs, mean age 25yrs	Advanced disease stage in 70% patients.	Mucinous histology: 18%; differentiation: moderate 61%, poor 36%
	3	Chew 1997-2005	523 Asian cohorts 19-50 years Of them <40 yrs:134; >40yrs:389	63% Advanced stage (III-IV) <40yr group: 89/134;66% >40yr group: 245/389;63%	Predominantly poorly differentiated: (30% in <40 years 12% in > 40 years) mucinous, signet ring cell histological subtypes (16% vs. 9%).
Malaysia	4	Shahruddin 1990-94	21 patients <30yrs	Extensive disease	Mucinous histology
Israel	5	Shemesh-Bar, 1997-2007	406 patients, 203 in < 50 years	More advanced stage III-IV at diagnosis (56 vs. 41%) higher rate of N-2 disease (29 vs. 16%)	No difference in other features
	6	Neufeld 1999-2005	<50 years 90; 190 > 50 years	40% Advanced stage (III-IV) <40yrs:47/90;52% >40yrs:61/190;32%	Mucinous tumour in 11% in early onset group, 7% in late onset group

Country	Sr No	Reference, Period of Study	Age profile	Disease Stage	Tumour Characteristics
Taiwan	7	Chiang 7 year period	5436 patients 7% <40 years	Dukes' stage improves with age (A & B 31% < 30 years, 49% > 80 years)	Poorly differentiated tumours tended to decrease with age, 16. 9% < 30 years. 6. 2% > 80 years. Similar trend in Mucin producing characteristics (36% vs7. 5%)
India	8	Nath 2003-2007	287 patients 35. 6% < 40 yrs	Advanced T stage (T 0-2: 18. 9% T -3: 62. 3% T-4: 19. 7% vs. 34. 5%, 56. 0%, 9. 5%) and N-stage (N 0: 31. 1%, N1: 41%, N2: 27. 8% vs. 53. 9%, 26. 7%, 17. 2%)	Poorly differentiated and / or mucinous or signet cell carcinoma (52% vs. 20. 5%)
	9	Gupta 2000-2008	305 patients 40% < 40 yrs	60% presented in Dukes' stage III & IV	Mucinous tumour 80% Poor differentiation 50%
Nepal	10	Singh 2002[a]	91 patients 28. 6% < 40 yrs	92. 3% present in Dukes' stage III-IV vs. 61. 5% in older patients	Significantly higher poorly differentiated and mucinous carcinoma in the young.
SriLanka	11	de Silva 15 yr period	305 patients 19. 7% < 40 yrs	No significant difference in Dukes' stage with older group	Significant presence of mucinous (13. 3%) or signet ring type (5%) tumours.

Country	Sr No	Reference, Period of Study	Age profile	Disease Stage	Tumour Characteristics
AFRICA Egypt	12	Soliman* 1982-1996	1608 patients; 35. 6% <40 yrs	Dukes' stage is worse in > 40 years group (72% vs. 57%)	Tumour grade comparable in two groups; mucin producing tumours: 31% in younger group, 14% in older group

*Soliman et. al. (1997)

Table 3. Summary of references in the literature on stage and tumour characteristics from Asia and Africa.

Reports from Asia and Africa are listed in Table 3. Features of only the younger patients were assessed in four reports (Sr. No. 2-4,9). The younger groups were larger in several studies (523:Sr No. 3; 203:Sr No 5; 370: Sr No. 7and 576 : Sr No. 12) from Asia and Africa as compared to ones from the USA (Table 1) and Europe (Table 2). Higher incidence of CRC in the young in Asia and Africa was found to be consistent with these figures. In two studies (Sr No. 11,12) the disease in the young was assessed as less advanced at presentation and less aggressive. In one report (Sr No. 5),a more advanced disease stage was noted but no difference in tumour grade was found. A more advanced disease and tumour grade was reported in the young as compared to the older patients (which is usually the case in Table 1 & 2) in 5 of 12 reports (Sr No. 1,6-8,10). In a report by Chew et. al (2009, Sr No. 3) the same conclusion was reached; 'older' patients were however in the age group 40-50years. The frequency of advanced disease and high tumour grade in the young in these reports were similar to that in reports restricted to only the young patients (Sr No. 2,4,9).

Irrespective of the country, the size of the study group, time span and the year of study, the dominant result is the same. Young CRC patients present at a more advanced clinical stage, the tumours are mucinous and poorly differentiated, more so in comparison with the older patient group. The features in India and neighbouring Nepal and Sri Lanka are the same as in the rest of the world. We have noticed some difference in disease pattern in Asia and Africa as compared to the West in our discussions of the data in Tables 1-3. The issue of ethnic differences in determining the difference in disease characteristics is important. This issue, without specific reference to the disease in the young, received attention in several papers, e. g., Isbister (1992; New Zealand and Saudi Arabia), Soliman et. al. (2001; Egypt and the West), Fireman et. al. (2001;Arab and Jewish neighbours in Israel),Qing et. al. (2003;USA and China), Sung et. al. (2005;Asia and the West),Goh et. al. (2005;Asian patients of different races in Malaysia) and Fairley et. al. (2006;Blacks,Asians/Pacific Islanders and Whites).

The advanced stage at presentation of many colorectal cancers in young patients is not just a result of a delay in diagnosis. It may also be that the cancer in younger patients is more virulent by nature. This feature is rooted in subtleties of genetic differences. More aggressive' tumour characteristics, as evidenced by its mucinous nature and poor

differentiation have also been linked to molecular genetic differences. Recent molecular biology studies have shown characteristic features of mucinous carcinoma, e. g., lower expression of p53, more frequent DNA replication errors expressed as microsatellite instability and specific codon 12 K-ras mutations and, when ploidy has been determined, a higher index if diploidy was found than for non-mucinous carcinoma (Negri 2005).

Tumour subsite: The issue of subsite location is important in screening strategies and in choice of treatment protocols. In the literature (e. g. , Breivik et. al. 1997) preference for subsite location has been associated with molecular genetic roots of CRC. Molecular genetic findings classify CRC into two groups. The first class of tumours show microsatellite instability (MSI), occur more frequently in the right colon, have diploid DNA, behave indolently, of which Hereditary Non polyposis Colorectal Cancer Syndrome (HNPCC) is an example. The larger incidence of proximal colon cancer in patients with HNPCC syndrome highlights the importance of genetics in preference for subsite location in colon cancer. In the other group belong tumours which tend to be left sided, show aneuploid DNA, behave aggressively, of which Familial Adenomatous Polyposis (FAP) is an example. Each group has its own characteristic gene mutations (Lynch and de la Chapelle, 1999).

Breivik et. al. (1997)in a study of 282 patients from 7 hospitals in Norway in the period 1987-9 concluded that proximal and distal CRC evolve by different genetic pathways and that these pathways are influenced by sex-related factors. Their results, analyzed by statistical models, pointed to hormonal mechanisms with important clinical implications. They found that presence of TP 53 mutations was dependent on tumour location only, with a positive association to cancers occurring distally (p=0. 002). Microsatellite instability was found almost exclusively in proximal colon cancers.

Stigliano et. al. (2008) compared a cohort of 40 HNPCC cases with 573 sporadic CRC cases in the period 1970-1993. Median age of diagnosis was 46. 8 years in HNPCC cases and 61 years in sporadic CRC cases. 85% had right sided lesion in HNPCC group as opposed to 57% in sporadic cancer group.

Slattery et. al. (1996) studied age, sex and tumour sub-site distribution in 1709 CRC patients from three geographic areas in the USA. Approximately 50% of CRC in men and greater than 50% of CRC in women were in the proximal segment of the colon. Men who were diagnosed prior to age 50 and both men and women diagnosed at age 70 or older had predominantly proximal cancers. People with proximal cancers and those diagnosed prior to age 50 were likely to have more advanced disease. In general, both men and women had more proximal cancers with advancing age, which were associated with more advanced disease.

Ionov et. al. (1993) showed that 12% of CRC patients carried ubiquitous somatic deletions in poly (dA. dT) sequences and other simple repeats. Tumours with these mutations showed distinctive genotypic and phenotypic features. Patients with these deletions showed a predominance of right sided tumours while those without deletions had a predominance of left sided lesions.

Thibodeau et. al. (1993)studied the association of microsatellite alterations with preference for tumour subsite. All four sites of alteration studied showed a dramatic change in preference from distal to proximal colon in the mutated form (typical values: proximal/distal; (26,49), (11,1 in the mutated form)).

Fancher et. al. (2011) studied 45 young patients, 20 males and 25 females,mean age 43. 6 years, in the USA and found preference for left sided lesions in females (16/8)and a preference for right sided lesions (12/10)in men (p=0. 35; small sample size);right sided cancers had a higher stage at presentation.

Kaw et. al. (2002) studied 1277 Filipino patients of whom 218 (17%)were <40 years, a mean age of 31. 3 years. Cancers of the right colon were noted to be more common in females (55%)and rectal tumours were seen more frequently in males (55%;p=0. 014),but when analysed in relation to age, right colon cancers were actually more common in men <40 years of age (p=0. 013);the incidence in women was higher only above the age of 50 years. The proportion of CRCs located on the right side was 28% for <40 years patients and 20% for the 40+ group. On the other hand, left colon cancers were seen in 30% of the older age group compared with 18% in the younger population (p=0. 001). For rectal cancer, there was no significant difference in proportion between the young and the old (p=0. 414).

Elsaleh et. al. (2000) in an older patient group (mean age 66. 7 ±12. 9 years) in Australia reported that MSI positive tumours were slightly more frequent in women than in men (10 vs 7%). Right sided tumours were more frequently MSI positive than left sided tumours (20 vs 1%). Men with right sided tumours benefited from chemotherapy (37 vs 12%) but men with left sided tumours did not.

Mahdavinia et. al. (2005), Fazeli et. al. (2007) and Malekzadeh et. al (2009) found that in Iranian patients with positive family history of CRC, the most frequently affected site of colon was the right side. Malekzadeh et. al. (2009) found that MSI was more frequent in early-onset patients and in proximal tumours. They reported that proximal and distal tumours harbor different p53 mutational spectra;distal CRCs showed a higher frequency of G to A transitions at CpG whereas G to A transitions at non-CpGs were more frequent in proximal tumours. Fazeli et. al. (2007) found that 62. 5% of patients with proximal colon tumours were males.

Nelson et. al. (1997) and Saltzstein et. al. (1997) showed that there was an increase in the relative proportion of proximal colon cancers with increasing age 'a shift to the right'. Thus with increasing age, full length colonoscopy will be a better screening tool. The exact age at which the shift occurs will vary with gender and ethnicity. There is a predominance of African-Americans amongst those at risk for proximal colon carcinoma and predominance of white males amongst those at risk for distal CRC.

Goh et. al. (2005) in a study of different races in Malaysia observed that demographic differences between Asia and the West may exist. No difference in anatomic distribution was found in Malay, Chinese and Indian races. They noted that in general CRC tends to be located distally in areas with a lower incidence of disease (parts of Asia) and migrated proximally with increasing incidence, as in Japan or Korea. They suggested that this may be related to a decrease in rectal cancer and an increasing proportion of elderly patients in the population. Young patients had a higher probability of having distal lesions as compared to the older patients.

Qing et. al. (2003) in a comparative study on Chinese and American patients, noted that the proportion of left sided lesions in Oriental patients (74%) was significantly higher than that in Whites (63. 7%) and that rectal cancers were significantly more common among Orientals (p<0. 001).

O'Connell et. al. (2004[a]) in their review quoted average values of subsite location in <40 years young patients as follows: ascending 22%, transverse 11%, descending colon 13%, rectum and sigmoid (including rectosigmoid junction) 54%,a dominance of left sided tumour in the young.

We summarize reports on preference for tumour sub-site from different countries in Table 4. Some of these are cited in Table 1-3 where patient groups are detailed. The others are detailed in Table 4.

	Sr. No.	Author, Country	Sub-site
Asia & Africa	1	Gupta, India	69. 7% distal tumours in < 40 yr group (rectal 57. 9%,left colon 11. 8%). Of patients with colon cancer proximal tumours constitute 72%.
	2	Singh, South Asia*	Rectum: commonest (83%) site of the lesion in young patients (21-30 yrs). No comparison with older patient group.
	3	Singh, Nepal	Rectum: most frequent site of tumor (76. 9% vs. 36. 9% in older age group)
	4	de Silva, Sri Lanka	No significant difference in tumour distribution between the young and the old.
	5	Shahruddin, Malaysia	Rectosigmoid region:most common (29%)' Left colon 19%,Splenic flexure 4%,Transverse colon 9%,Hepatic flexure 4%, Cecum 24% ;all patients<30yrs
	6	Goh, Asian patients of different races in Malaysia	No significant difference between <and > 65 years group; predominance (~90%)of left sided lesion in both age groups.
	7	Kam, Singapore	46% rectal and rectosigmoid; right-sided tumour:20%; patient group, all young <30yrs
	8	Ashenafi, Ethiopia	66. 7%rectal lesions; younger patients; mean age 47 years (61. 4% <50years, 36% <40 yrs,16% <30 yrs)
	9	Shemesh-Bar, Israel	Higher proportion of left side tumour in the young (82% vs. 71%)
	10	Chew, Singapore (Asian patients)	Predominantly left sided tumour (~80%)in <40 years and 41-50 years age group; no effect of age.
	11	Malekzadeh, Iran	Predominantly right sided tumour, general population
	12	Ibrahim, Lebanon	Rectosigmoid most common site in general population (553 patients),70. 7%;also in 32,<29 years younger group: 84. 4%
	13	Fazeli , Iran	Subsite distribution nearly independent of age group (< & > 40 years), distal ~ 80% in both groups.
	14	Chiang, Taiwan	No change in subsite preference from < 30 years to > 80 years
	15	Soliman**, Egypt	No change in subsite preference in < 40 years vs. > 40 years group, larger proportion of distal tumours (~65%) in both age groups

	Sr. No.	Author, Country	Sub-site
U. S. A.	16	Bedekian, USA	Increase in primary lesions in the right colon with increasing age at diagnosis; <40 yrs group compared with general population.
	17	Cozart, USA	Dominance of left sided lesions (12 right colon, 24 left colon,11 rectum) and left colon amongst colon cancer patients; study group comprises of only young patients<30yrs. No comparison with older group.
	18	Nelson, USA & Saltzstein, USA	Significant shift to right sided lesion with increasing age;<50 vs. >50yrs.
	19	Slattery, USA	Proximal cancers more frequent (>50%) in men<50 years and in both men and women >70 years(details in text).
	20	Fairley, USA	Rectal cancers more frequent in <50yrs group (37% vs. 26%); proximal colon cancer more frequent in >50 age group (42. 6% vs. 32. 1%),remaining <50%in both groups.
	21	Lichtman, USA	Older patients: more transverse/right sided lesions (p=0. 003). 138 patients;mean age of patients with different sites of tumour: Right colon 72 yrs,left colon 66. 1 yrs, rectum 61. 6 yrs
	22	Karsten, USA	Right sided lesion more frequent (44%)in young compared to 21% in older group, p=0. 004.
	23	Minardi, USA	Tumours evenly distributed in colon and rectum (under-40 group). Older group not compared.
	24	O'Connell[a] (International Review)	Rectum and sigmoid colon most frequent sites (54%) in the young <40 yrs patient group.
	25	Dozois, USA	Predominantly rectum (49. 1%) or left colon (29. 1%) than proximal colon (21. 9%). All young patients <50yrs. No comparison with older patient group.
	26	Behbehani, USA	Colon: Right 21%,Transverse 21%, Left 14% Sigmoid & Rectum 44% in the <40 yrs group; these figures are 34%,4%,8%,54% respectively in the older group.
Europe	27	Leff, UK	Only 12% right-sided colon cancer,<40 yrs patients, no comparison with older group.
	28	Fante, Italy	Majority of tumours in left colon and rectum in the whole patient group <40 – 55 years. Right colon: 37% in <40 years, 18% in 41-50 years, 14% in 51-55 years group.

*Singh et. al. 1984; **Soliman et. al. (1997)

Table 4. Tumour Sub-site in the young in different countries

In some of these papers, (Sr. No. 1, 2, 5, 7, 8, 10, 17, 23-25, 27), a preference for distal lesions in the young patients were cited, but were not compared with the older patient groups. In some others (Sr. No. 3, 9, 12, 16, 18-21) this comparison was made and a change in preference for tumour sub-site with increasing age was noticed. Shemesh-Bar et.al. (2010, Sr. No. 9) and Ibrahim et. al. (1986, Sr. No. 12) found that although left sided lesions formed the majority of tumours, their proportion decreased in the older group. Singh et. al (2002[a], Sr. No. 3) and Fairley et. al. (2006, Sr. No. 20)found that the proportion of rectal cancers decreased with increasing age. In several reports preference for right sided lesions showed an increase with increasing age (Sr. No. 16, 18, 20-21). Slattery et. al. (1996, Sr. No. 19)reported an increase in proportion of proximal tumours with increasing age for women, exceeding 50% (62. 3%), only in the age group 70-79 years. Amongst men, proportion of proximal tumours exceeded 50%in the <50 yr groups (62. 5%, 30-39 yrs; 51. 1%, 40-49 yrs), falls below 50%in the 50-59 and 60-69 yrs groups and then rises again to 54% in the 70-79 yrs group. A decrease in proximal tumours with increasing age was reported by Karsten et. al. (2008, Sr. No. 22) and Fante et. al. (1997, Sr. No. 28). Both studies reported a dominance of distal tumours in different age groups (two in Sr. No. 22, three in Sr. No. 28), but proximal tumours decreased with increasing age. In a few papers (Sr. No 4, 6, 10, 13-15, 26) sub-site preference was found not to depend on age. Fazeli et.al. (2007, Sr. No. 13) reported that~ 80% of the tumours were distal in the young (<40 years) and also in the older age group. In these reports which did not find any effect of age on subsite preference, distal tumours were >50% in the young and in the older group. A preference for proximal tumours in a population of colon cancer patients were reported in several papers (Sr. No. 11 and Mahdavinia et. al. (2005) in general population of colon cancer patients from Iran, where incidence is lower than in the West and in Sr. No. 1 in young colon cancer patients < 40 years in India). Cozart et. al. (1993, Sr. No. 17) found tumour sub-site preference for left colon (24/12) in a small population of colon cancer patients; Dozois et. al. (2005, Sr. No 25) found the same preference in a much larger (1025 patients) young (<50yrs)population. We cite several prospective reports on change in relative preference of tumour sub-site over a long time period. Fazeli et. al. (2007, Sr. No. 13) reported that the nearly equal preference for distal tumours (~80%) in the <40 years and in the >40 years group in Iran, remained unchanged for two decades (1970-80, 1990-2000). In contrast, it was reported in a study on patients from New Zealand, in the period 1974-83 (Jass 1991), that the incidence of right colon cancers remained stable in younger patients (<50 years), that in older patients showed an increase and a marked reduction in left colon and rectal colon cancer in <50 years group was observed. An increase in proximal CRC relative to distal tumours was reported in another retrospective study in the period 1940-79 in the US (Beart et. al. 1983).

4. de novo CRC in Asia

The problem arising from inability to detect cancer early because of hospital infrastructure and relative lack of awareness of the disease may not be the only problem peculiar to the developing world in Asia and Africa. Sung et. al. (2005) pointed out that non-polypoidal (flat or depressed) lesions and colorectal neoplasm arising without preceding adenoma (de novo cancer) seemed to be more common in Asian than in other populations. Although most cases of colorectal cancer are thought to arise from a sequence of adenoma to carcinoma, evidence from Asia, in particular Japan suggests another mechanism. Clinicopathological studies have shown that there are two groups of colorectal cancer,

polypoid and non-polypoid (superficial) tumours. The latter are flat lesions with a raised or depressed surface. Since these tumours are small (<1cm in diameter) and there are no adenomatous elements in their vicinity, they were proposed not to have originated from any precursor lesion and were termed de-novo carcinomas. These non-polypoid tumours are less likely to have K-ras mutations than are CRC arising from the adenoma-carcinoma sequence. Non-polypoid tumours of the colorectal regions tend to reach deeper layers of the intestinal wall in the early stage of the disease and with a higher degree of dysplasia. They are therefore more invasive than the polypoid adenomas (Sung et. al., 2005). Reports on de novo cancer have been published from Japan (Goto et. al 2004) and from Taiwan (Chen et. al 2003). About one-third of CRC patients in both countries have de-novo cancer. One study from UK also reported this feature (Rembacken et. al., 2000). Whether this feature is unique to Asia or whether it shows any preference for the younger or the older group of patients is not reported. Because of their flat appearance they are harder to identify by conventional colonoscopy**.** Chromoendoscopy and the use of magnifying colonoscopy may be necessary. The absence of polypoid growth preceding malignancy has posed difficulties in screening for early CRC by radiological imaging or even endoscopic techniques.

5. Early onset CRC and genetics

Colorectal tumours provide an excellent model system for understanding the molecular events that control the process of initiation and progression of human tumours. Rate of random mutational events alone cannot account for the number of genetic alterations found in most human cancers and it has been suggested that destabilization of the genome may be a prerequisite early in carcinogenesis. In CRC there are two separate destabilizing pathways. The more common involves chromosomal instability (CNI). The second mutational pathway in CRC displays increased rate of intragenic mutation characterized by generalized instability in microsatellites (MSI). Defects in mismatch repair genes (MMR) lead to high frequency MSI in CRC. National Cancer Institute definitions of MSI-L (L=low), MSI-H (H=high) and MSS (microsatellite stable) in CRC are given in Boland et. al. (1998). A recently recognized molecular alteration found frequently in MSI cancers is the CpG island methylator phenotype (CIMP). Colon cancer is usually observed in one of three specific patterns: sporadic, inherited or familial. Fewer than 10% of patients have an inherited predisposition to colon cancer. Sporadic cancer is common in persons older than 50 years of age, probably as a result of dietary and environmental factors as well as normal aging. Patients with inherited disease have CRC at a younger age, 10-20 years earlier than general population and are of interest in this essay. The area of hereditary CRC has been reviewed by Lynch and de la Chapelle (2003)and earlier by Lynch et. al (1991). Different aspects of molecular genetics of CRC have been discussed in this series (Ewart Toland, 2012) and elsewhere (Fearon and Volgenstein 1990; Loeb 1994; Jass 1995; Lynch 1996; Baba 1997; Gryfe et. al. 1997; Lengauer et. al. 1998; Lynch and Smyrk 1998; Lynch and de la Chapelle 1999; Yang 1999; Potter 1999; Jass et. al. 2002; Calvert and Frucht 2002; Zbuk 2009). In this section we discuss several recent papers which highlight the difference in genetic characteristics of younger CRC patients and those of the older group.

Morris et. al. (2007) showed that the incidence of tumours with microsattelite instability was significantly higher in patients aged ≤ 40 years, 18. 3% compared to 6. 6% in those aged 41 – 60 yrs ($p<0.0001$). TP53 mutations were also more frequent ($p=0.002$). However K-ras mutations were less common ($p=0.0001$) when comparing the same age groups. They

concluded that major age related differences in the clinical and molecular features of CRC exist.

Farrington et. al. (1998) pointed out that germ-line mutations in DNA mismatch-repair (MMR) genes impart a markedly elevated cancer risk, often presenting as autosomal dominant HNPCC. Not all gene carriers have a family history. Young probands with early onset CRC irrespective of family history were genetically tested and it was found that an appreciable proportion of young colon cancer probands carry a germline mutation in a DNA MMR gene.

Losi et. al. (2005) evaluated clinical features and molecular pathways, chromosomal instability (CNI) and MSI in early onset CRC. Of 71 patients (<45 years), 14 showed both MSI and altered expressions of MMR proteins. In the 57 MSI -negative (-) lesions, altered expression of APC, β-catenin and p53 genes were found more frequently than in MSI-positive (+) tumours. 7/14 MSI (+) tumours were associated with clinical features of HNPCC and in all but one, constitutional mutations in MLH-1 and MSH-2 genes could be detected. The same mutations were found in other family members. Involvement of chromosomal instability was demonstrated in a majority of early onset CRC.

Chan et. al. (1999)studied 59 Chinese patients <45 yrs and 58, >45 yrs in Hong Kong. The incidence of MSI-H varied statistically significantly with age, being observed in >60% of those <31 years at diagnosis and in <15% of those ≥46 years. More than 80% of Chinese CRC patients <31 years had germline mutations in MMR genes. In a novel case, mutation in hMSH-6 was present but MSI was absent.

Ho et. al. (2000) in a study on 124 young (<50yrs) Hong Kong Chinese CRC patients concluded that MSI occurs in a significant proportion of the subjects. Young age at CRC diagnosis, proximal tumour location, increasing number of first degree relatives with CRC and a personal history of metachronous cancer were independent predictors of MSI status in the patient group. In patients <30 years, MSI tumours were more likely to be located in distal large bowel. In a proportion of patients with MSI tumours, germline mutation in the two MMR genes hMSH2 and hMLH1 was identified. The authors opined that this observation suggests a differential activity of the MMR pathway in colorectal carcinogenesis in different age groups. They observed that the inconsistency between MSI-H and a family history in the early onset patients deserves further attention.

Liang et. al. (2003) studied 138 below-40 CRC patients and 339 patients who were 60+. They found a higher percentage of normal p53 expression (61. 1 vs. 46. 8%, p=0. 023) and high frequency microsatellite instability (MSI-H) (29. 4 vs. 6. 3% p<0. 001) in the young. The family history of the two groups was similar.

Durno et. al. (2005, 2006) found evidence of MSI in 73% cancers from individuals in 9-24 years of age, 50% of whom had features of HNPCC. Other reports found MSI in 46% of under- 21 patients with only 1/3 having a clear family history.

Sanchez et. al. (2009) performed a molecular classification of CRC based on microsattellite instability (MSI), CpG-island methylator phenotype (CIMP) and mutations in the K-ras and BRAF oncogenes. There were four classes, combinations of MSI-H and MSS with CIMP–H or CIMP (-). 69. 8% of tumours (391 subjects) were MSS-CIMP(-) and less likely to be poorly differentiated (p=0. 009). CIMP-H tumours were more common in older patients (p<0. 001). MSI-H/CIMP-H tumours had a high frequency of BRAF mutation and a low rate of K-ras mutation, the opposite was true for MSS-CIMP(-) tumours (p<0. 001). The four molecular phenotypes tended towards divergent survival. MSI-H cancers were associated with better disease free survival.

Alsop et. al. (2006) investigated association of young age in below-45 patients with somatic mutation of K-ras gene, a common event in CRC tumorigenesis. The role of these mutations was found to be comparatively minor in the younger group, in contrast to its significant role in CRC of older age of onset.

Soliman et. al. (2001) compared molecular pathology of CRC in Egyptian (44% <40 years) and Western patients. They found MSI-H carcinoma in 17% (2/12) of under-40 and 46% (12/26) of 40+ Egyptian patients; K-ras gene mutation in 0% (0/18) of under-40 group and in 17% (5/29) of 40+ group; p-53 overexpression in 57% (13/23) of under-40 group and 39% (13/33) of 40+ group. These data show that molecular pathology of CRC in young Egyptians differed from that in the old; in particular, K-ras mutation played a distinctly minor role in the younger group. Unique differences in molecular pathology of CRC between the Egyptian and Western patients were also discussed.

Breivik et. al. (1997) found that the presence of K-ras mutation was dependent on age and gender of the patient, with an especially low frequency amongst young males. Microsatellite instability was rare in tumours with K-ras and TP53 mutations.

Berg et. al. (2010) focused on the somatic tumour development in young patients with no known inherited syndromes. They studied mutations in oncogenes K-ras, BRAF, PIK3CA and the tumour suppressor gene PTEN and in TP53, in three age groups in 181 patients (45, < 50 yrs; 67, 51-70 yrs; 69, >70 yrs). Distinct genetic differences were found in tumours in the young and the elderly patients, who were comparable for known clinical and pathological variables. This result indicated that young patients had a different genetic risk profile for CRC development than older patients. Clinical implications of these differences were discussed by the authors. The total gene mutation index was lowest in tumours from the younger patients. In contrast the genome complexity assessed as copy number aberrations was highest in tumours from the youngest patients.

Casper et. al. (1994) showed in a study on 225 FAP patients that deletion of 5 base pairs at codon 1309 within exon 15 (the most common mutation) was identified in 20 families. Other mutations within exons 7-15 were found in 49 families. The 1309 mutation leads to development of colonic polyps at a younger age thus giving rise to an earlier malignant transformation. In patients with 5 base pair deletion at codon 1309, gastrointestinal symptoms and death from CRC occurred about 10 years earlier than in patients with other mutations.

Khan et. al. (2008) studied 35 patients with CRC diagnosed at <30 years age. They found no mutations in exons 4-10 of the p53 gene. The frequencies of polymorphism in p53 and in MDM2SNP309 did not differ from rates previously reported for normal control populations and no polymorphism in either gene could be associated with early onset CRC.

Ahmed et. al. (2005) reported a study on 363 CRC patients of whom 18 were of Bangladeshi origin. 22% of Bangladeshi patients presented with a locally advanced or a metastatic CRC, whereas the same figure for non-Bangladeshi patients was 11%. Sixty one percent of the Bangladeshi patients were below 40 years of age and did not report any family history. Microarray profiling between these two groups demonstrated 1203 differentially expressed genes ($p<0.05$). The patient groups studied by Nath et. al. (2005) and by our group, (Gupta et. al. 2010) (Table 3) and by Pal (2006) belong predominantly to West Bengal in India, which is adjacent to Bangladesh. These studies reported dominance of younger patients in their study groups, advanced disease stage and aggressive tumour characteristics.

Liu et. al. (1995) studied the prevalence of DNA replication errors (RER) associated with genetic instability in relation to age among patients without HNPCC. RER was found in

cancers of several different types, particularly in HNPCC. CRC in majority of <35 years group (58% of 31 patients) exhibited instability whereas CRC in > 35 years group uncommonly did (12% of 158). In 12 of <35 years group, instability was evaluated for alterations of MMR genes and in 5, it was found to harbor germline mutations. These data suggested that the mechanisms underlying tumour development in young CRC patients differ from those in most older patients.

Lin et. al. (2010) showed in a study cohort of 950 patients (2000-2005) that carcinogenic effects of Western lifestyle might be mediated via insulin-like growth factor-1 (IGF-1). IGF-1 is a peptide growth factor that promotes cell proliferation and inhibits apoptosis. Both in vitro and in vivo studies suggested that IGF-1 could promote CRC growth. Further, circulating levels of IGF-1 were associated with various cancers including CRC. It was shown that genetic variation controls variability of circulating IGF-1. The expression of IGF-1 was reported to vary in different ethnicities. In turn it was speculated that polymorphisms of the genes involved in the IGF axis might affect IGF-1 expression and possible cancer risk. The age at onset of CRC varied considerably. Extreme age at the CRC onset, very young or very old seemed to be associated with different carcinogenesis. It was shown that some genetic polymorphism affects age of onset of cancers. For example IGF-1 polymorphism plays a significant role in affecting disease onset in Lynch syndrome. These authors showed that older patients have a higher frequency of AA genotype of IGF-1 (-2995C/A), significantly higher (12. 7%) than that in younger patients (4. 2%). Mucinous differentiation, but not other clinicopathological factors was associated with the CA /AA genotype of IGF-1. The authors concluded that the genotype of the IGF-1 promoter was different in young CRC patients compared to older CRC patients and that IGF-1 SNP was associated with mucinous adenocarcinoma.

Yantis et. al. (2009) provided data to show that post translational regulation of mRNA and subsequent protein expression may be particularly important to the development of CRC in young patients. They compared 24 patients <40 yrs of age with 45 patients ≥ 40 yrs of age, who served as controls. Cases were evaluated for clinical risk factors of malignancy and pathologic feature predictive of outcome. More aggressive features in tumours of young patients, namely more frequent lymphovascular (81%) and venous (48%) invasion, an infiltrative growth pattern (81%) were reported. Significantly increased expression of miR-21, miR-20a, miR-145, miR-181b, and miR-203 was noted in the younger group.

6. Predisposing factors

Family history of CRC at a young age is a significant risk factor. Johns and Houlston (2001) performed a meta-analysis of 27 case-control and cohort studies of colorectal cancer risk and found that a family history of one affected first degree relative diagnosed before the age of 45 carried a 3. 87 fold (95% confidence interval 2. 40 – 6. 22) increased risk for the disease. Fuchs et. al. (1994) concluded that a family history of CRC is associated with an increased risk of disease, especially amongst the young. The relative risk factor of an under-45 yrs person with one or more affected first degree relative as compared with those without a family history was 3. St. John et. al. (1993) performed a case-control study of relatives of CRC patients and of matched control patients. They concluded that first degree relatives of patients with CRC have an increased risk of colorectal cancer. The risk was greater if diagnosed at an early age and when other first degree relatives were affected. Winawer et. al. (1996) observed that siblings and parents of patients with adenomatous polyps were at

an increased risk for CRC particularly when adenoma was diagnosed at < 60 yrs age. Despite limited accuracy and compliance, family history is still the most easily obtainable risk factor for colorectal cancer.

Deficiency in host response to carcinogenesis is less easily recognized and treated. A personal history of other cancers, especially chronic immunosensitive cancers such as melanoma, if occurring at a young age, may indicate an increased susceptibility to CRC. Chronic immune suppression or clinical suggestions of impaired immunity may also mean the same.

FAP and HNPCC patients have a lifetime risk of 100 and 80 percent respectively, of developing CRC. In FAP, the affected persons develop hundreds to thousands of colonic polyps. Although the rate of transition to cancer is slow, the vast number of polyps virtually assures colon cancer development at a young age. Average age of developing cancer is 39 years, with 7% diagnosed by the age of 20 and 15% by 25. In HNPCC, the affected persons have a very high risk for CRC but do not develop the hundreds of polyps seen in FAP. These polyps are very likely to make a transition to cancer. Although sporadic colon cancer usually arises in colon polyps after a 5-10 years period of growth and transformation, in HNPCC this progression can occur within 1 -2 years. HNPCC occurs at a relatively young age, median 42-45 years, with 35%-40% diagnosed before 40 years of age. The proportion of HNPCC or familial colorectal cancer among all CRC varies by country from 1-10% with a median of 2-5% (Mecklin and Ponz de Leon 1994). HNPCC has been reported from many different populations, Europeans, white and Indian Americans, Asians, Australasians, South Americans and Egyptians (Sarroca et. al. 1978; Bamezai et. al. 1984; Ushio 1985; Lynch et. al. 1985; Mecklin 1987; Vasen et. al. 1990; Mecklin and Jarvinen 1991; Jass and Stewart 1992; Soliman et. al. 1998).

Ulcerative colitis (UC) is another important predisposing factor. The most important risk factors for development of CRC in UC patients are prolonged duration of disease, pancolonic disease, continuously active disease and severity of inflammation. Eaden et. al. (2001) performed a meta-analysis of the risk of CRC in UC. 116 of 194 reported studies were included in this analysis. Overall prevalence of CRC in UC patients was 3. 7%. The risk of CRC in UC patients was determined by decade of disease and a non significant increase in risk over time was observed.

7. Prognosis and survival of young patients

Opinion on the issue of survival of younger patients is not unanimous. We have divided literature reports on this issue, pre-2000 and post-2000, in two separate sections. The reports in which prognosis for the young is shown to be poorer and the ones in which they are not so, are separately grouped.

Pre-2000, poor prognosis: Moore et. al. (1984) concluded that poorer survival in younger (<40 years) patients was a result of an inherently more virulent lesion, a conclusion supported by a greater incidence of mucinous tumours, an indicator of poor prognosis and a higher incidence of advanced disease, especially in the second and third decades. They did not find delay in diagnosis as an important factor in determination of survival. Adkins et. al. (1987) ascribed poorer prognosis in the young (<35 years) to unfavourable histological features of the tumours and advanced disease at the time of presentation in these patients. Of 45 under-35 patients, 19 patients with poorly differentiated tumours survived for an average of 1 year, whereas 19 with well or moderately differentiated tumours survived for

an average of 4. 5 years. Those few patients who presented early in the course of their disease responded well to radical resection. Okuno et. al. (1987) reported frequent occurrence of mucinous carcinoma, lymph node involvement and advanced stage according to Dukes' classification in the younger group (<39 years). The overall survival rate was poorer in the younger group (41% vs. 55. 9%), whereas the difference between the two groups in rates of curative resection was not statistically significant.

Pre-2000, favourable prognosis: Howard et. al. (1975) found that younger patients had a greater frequency of advanced signs, later stages of cancer and mucoid carcinoma, but when compared by clinical stage, they did as well or better than older patients. 5-year survival rates were 31% in <40 years group and 32% in>40 years group. Clinical staging was the most important prognostic factor irrespective of age. No inherent difference was found in the virulence of cancer in the young, survival rate being essentially the same. Adloff et. al. (1986) in a paper published much later reached identical conclusion. Walton et. al. (1976) in a study on 70 under-40 patients reported that survival time was shorter in patients with mucinous and anaplastic tumours and their incidences increased in this age group. Overall survival rates, however, did not significantly depend on age. Early diagnosis and prompt aggressive surgical treatment produced survival equivalent to that in patients of other age groups. Scarpa et. al. (1976) in a study on 47 adults in the age group 20-40 years found smaller tumours and depth of invasion as important prognostic factors but tumour grade had no correlation with survival. They concluded that there was no difference in survival rate between the young and the old. Bulow (1980) found, in an extensive study spread over 25 years (951 <40 patients, all <40 patients in Denmark in the period 1943-1967) that stage according to Dukes' classification and presence of intestinal obstruction and/or perforation and not age, determined prognosis. Ahlberg et. al. (1980) in a study group of 27 patients, aged <30 years, in 1969-70 in Scandinavia, concluded that prognosis was good, if predisposing factors were absent (9/15 survived 5 years), but not so otherwise. Ohman (1982) in a study group of 1061 patients, of whom 48 were below 40, in Sweden reported a five year survival rate in the overall population and in curable cases. Both rates were equal in the two age groups. Age factor had no impact. Five year survival was 100% in stage A, 50% in B, 33% in C. Proportion of Dukes' A lesion was equal in the two groups; there were fewer B and more C lesions amongst the young. Survival was not altered if ulcerative colitis was superimposed on carcinoma. Beckman et. al. (1984) studied 69 patients, 20-39 years and reported good prognosis. Neither age, sex, tumour size, location, mere presence of lymph node metastases, depth of tumour invasion nor predisposing disease of the colon was a strong prognostic factor. Metastases of six or more lymph nodes and distant spread of the tumour at the time of initial surgery were ominous findings; so was mucinous carcinoma, a relatively frequent occurrence. Jarvinen and Turunen (1984) in a study on 249 under-40 year patients between 1970 and 1979 found no difference in their 5 year survival rate from that of the general population. A premalignant condition was more common as age decreased. Family cancer syndrome, FAP and other predisposing diseases were observed in a significant proportion of study group. It was suggested that more emphasis should be placed on identification, family screening and treatment of conditions predisposing to colorectal cancer. LaQuaglia et. al. (1992) analysed their experience with 29 histologically verified cases of whom 20 were resected for cure. The predictors for survival were resectability, regional nodal involvement, depth of invasion, grade (Signet ring (45%) or anaplastic lesions (24%) were considered high grade) and interval from symptom onset to diagnosis. Median age at diagnosis was 19 years (10-21 years), median survival was 16

months, that for those undergoing complete resection was 33 months. In those undergoing resection for cure, tumour grade, regional nodal involvement and depth of invasion were the only factors that affected prognosis. Hidalgo (1995) in Spain studied 26, under-45 CRC patients (17. 2% of the whole group) whose potential risk factors were no different from those of the general population. Clinical presentation, tumour site and Dukes' stage were similar in the younger group and in the general population, but morbidity, mortality and post operative complications were lower. There were no differences in resection or survival rates. Chung et.al. (1998) in a study on 101 under 40 patients and 2064 older patients found no difference in tumour characteristics, Dukes' stage and overall 5 year survival, but reported a higher adjusted hazard ratio and adverse outcome in the <40 years group compared to 40-59 years group. They noted that a significant family history and predisposing conditions in the young warrants aggressive screening, surveillance and treatment. Heys et. al. (1994) in a review reported histological evaluations of the cancers in the younger age group patients and found that approximately four times as many tumours were of the mucinous type. This was associated with an increased risk of local recurrence. Dukes' staging and vascular invasion by tumour were prognostic indicators for overall patient survival. However survival rates for young patients with CRC were comparable to those of older patients, when equivalent Dukes' stage was considered.

Post-2000, favourable prognosis: O'Connell et. al. (2004[b]) used SEER (Surveillance, Epidemiology and End Results) database in the period 1991-1999 in the USA (1334 younger patients, 20-40 years; 46,457 older patients 60-80 years) to conclude that 5-year stage-specific survival was similar for stage I and III patients and better for younger patients in stage II and IV ($p<0.01$). The same patient group showed later stage (more of stage III and IV) and higher grade tumours for younger patients. The authors noted that their population-based finding contradicts earlier single institution reports. Stigliano et. al. (2008) compared a cohort of 40 HNPCC cases with 573 sporadic CRC cases in the period 1970-1993. Median age of diagnosis was 46. 8 years in HNPCC cases and 61 years in sporadic CRC cases. Early stage cancer (Dukes' A & B) was 70% in HNPCC group and 61. 6% in sporadic group. The crude 5-year cumulative survival for primary CRC was 94. 2% in HNPCC vs. 75. 3% in sporadic cancer patients ($p < 0.0001$). The influence of age on prognosis is apparent.

Berg et. al. (2010) studied 181 patients (45, < 50 yr; 67 (51-70 yr); 69, > 70 yr) and found no difference in survival while comparing age groups, even when adjustment for tumour stage at diagnosis had been made. Younger patients however presented at a more advanced disease stage (54, 46, and 36% in three groups). Tumour stage was the most powerful prognostic variable ($p < 0.001$). Turkiewicz et.al. (2001) in a study spanning 29 years in Australia concluded that young patients with CRC had the potential to do just as well. The overall 5-years survival among younger patients in Stage A and B (53%) was found to be better than their counterparts in the older group. With influence of a family history of CRC being very apparent in this group, the authors conclude that emphasis must be on screening. Makela et. al. (2002) in a study of 102 under-50 patients in Finland over a 20 years period (1980-1999) concluded that young age is not a poor prognostic marker in colorectal cancer. Radical operation, venous invasion and tumour grade were good predictors of survival in patients below 50 years. Kam et.al. (2004) in a study in Singapore on 39 under-30 years patients inferred that age did not affect survival and recommended early endoscopy for all with persistent symptoms. They concluded that early diagnosis, radical resection and adjuvant therapy still form the cornerstone in management of colorectal cancer in this age group. Karsten et.al.

(2008) in the USA performed a comparative study of two groups, < 40 years and > 60 years of age, ethnically diverse, between 1998 and 2005. Fifty one percent of 41 young patients were Hispanic. Young patients were more likely to have a family history. Aggressive nature of tumour in the young was noted, but operative intervention and survival was similar in the two groups. Tohme et. al. (2008) in a study of 325 patients, 13. 2% of whom were below 45 concluded that age by itself was not a significant prognostic factor. The independent prognostic factors were delay in consultation, which was more frequent in younger patients (29. 7 vs. 18. 6 weeks, p=0. 01), positive family history in the young (44. 1% vs. 18. 2%), right sided tumour and peritoneal carcinomatosis. Leff et. al. (2010) in a British study of 49, <40 patients reported a 5-year and overall survival of 58% and 46% respectively. They concluded that prognosis in the young was not worse than that for CRC in the population as a whole. Mitry et. al. (2001) reported, in both overall and stage for stage comparisons that patients below the age of 45 years had a better survival rate than older patients, mortality rate was lower in the younger group (2. 1% vs. 8. 4%) although advanced stage presentation was more frequent and predisposing conditions were significantly higher in the below 45 group (11. 7 vs. 0. 4%, p<0. 001). Lin et. al. (2005) studied 45 histologically confirmed under-40 patients, 90% of whom reported with advanced (C+D) stage, between 1992-2002 in Taiwan. They reported that disease stage was an important prognostic factor, 5 year survival in B, C and D stage patients being 25, 16, and 0% respectively. Karnofsky performance status (KPS ≥ 70%), lymph node involvement and preoperative LDH levels were major determinants of survival. Surgical resection and adjuvant chemotherapy improved survival of advanced stage patients, but the improvement achieved does reach the level of a patient who reports early. Liang et. al. (2003) reported that although the younger patients with colorectal cancer had more mucin producing (14. 7 vs. 4. 7%, p<0. 001) tumours and a more advanced tumour stage at presentation (p<0. 001) than older patients, the operative mortality rate was lower (0. 7 vs. 5%) and cancer specific survival was similar (p<0. 05) in stage I, II, III disease or better in stage IV disease (22-28 vs. 12-17 months, p< 0. 001).

Post-2000, poor prognosis: Endreseth et. al. (2006) in a study on 2283 rectal cancer patients found overall 5-year survival to be 54% for patients younger than 40 years compared to 71-88% for the older patients (p=0. 029). Among those treated for cure, 56% of <40 group developed distant metastasis compared to 20-26% in the older group. Age younger than 40 years was a significant prognostic factor in this group and increased the risk of metastasis and death. A study from Nepal by Singh et. al. (2002[a]) reported a more aggressive disease in the younger group (<40 years) and a significantly lower 2 years survival rate (4% vs. 55%). Singh et. al. (2002[b])in a study on 18 under 40 patients in India found that the tumor was unresectable in 5 patients (28%). Fourteen patients (78%) had advanced cancer indicated by TNM stage III or IV disease. Among the 13 patients subjected to surgical treatment followed by adjuvant chemotherapy, only 3 had long term disease free survival beyond 2 years.

Prognosis and Genetics: There have been reports in the literature that suggest that the survival of MSI-H CRC patients is longer than that of patients with MSS CRC. This latter group constitutes the majority. In some studies however no survival advantage was detected and a National Cancer Institute workshop held in 1998 (Boland et. al. 1998) concluded that MSI had not been shown to be an independent predictor of prognosis (Gryfe et. al., 2000). We cite a number of mostly post-2000 papers that link prognosis to MSI tumour pathway.

Gryfe et. al. (2000), in a study on 607 under-50 patients found MSI in 17% of patients and concluded that MSI was associated with a significant survival advantage independent of all standard prognostic factors including tumour stage. Regardless of depth of tumour invasion, MSI-H CRC had a decreased likelihood of metastasis to regional lymph nodes. Elsaleh et. al. (2000) (mean age 66. 7±12. 9 years) in Australia report striking survival benefits for patients with MSI tumours (90 vs 35%, p=0. 0007) and also for patients with right sided lesions, who received adjuvant chemotherapy as compared to those who did not (48 vs 27% alive at end of study, p<0. 0001) and for women (53 vs 33%, p<0. 0001). Suh et. al. (2002) in a comparative study of MSI(+) and MSI(-) sporadic young (<40years) CRC patients showed that the former had better prognosis (p=0. 051). Their results suggested that sporadic MSI(+) CRC in the young had different histomorphologic features as compared to MSI(-) CRC and HNPCC cancers. Samowitz et. al. (2009)in a study of 990 rectal cancer patients in the US showed that even though MSI-H has been associated in many studies with improved prognosis of colon cancer, the effect of MSI-H and K-ras mutations posed significantly higher risk of death for rectal cancers. Liang et. al. (2003) reported that there was a higher percentage of normal p53 expression (61 vs. 48%) and high frequency microsatellite instability (MSI-H) (29. 4 vs. 6. 3%, p, 0. 001) in the young. Lukish et. al. (1998) in a study group of 36 patients in the <40 year age group determined their DNA replication error (RER) status (expressed as MSI) and compared the clinical and pathologic characteristics of RER(+) and RER(-) cases. They concluded that RER(+) tumours were common (47%) in young patients and patients with RER(+) tumours had a significantly improved prognosis:5 year survival probability 68% in RER(+), 32% in RER (–) tumours (p<0. 05). Knowledge of RER status therefore could affect initial therapy, postoperative chemotherapy and follow up.

The paradoxical good survival after surgery for patients with young age at diagnosis of CRC supports the idea that many cancers in the young are microsatellite unstable. A number of studies linked high frequency MSI to poor tumour differentiation or mucinous histology, a signature of many tumours in the young (Sanchez et. al. 2009; Kim et. al. 1994, Lin et. al 2010, Suh et. al., 2002). Ionov et. al. (1993) in their study of mutations involving poly (dA. dT) sequences (Section 5 for details) found that the presence of mutations was accompanied by an increase in the proportion of poorly differentiated lesions (6/9 vs 17/90, poor/well, moderate)and also in an increase in proportion of Stage A +B disease (2/14 vs 53/68; C+D/A+B). Crude survival was expected to be better than usual in young patients because of their youth and the improved tolerance to surgery and complications that youth confers (Liang and Church 2010).

Berg et. al. (2010) in a study of patients in different age groups (Section 5 for details), found that patients with TP53 mutated tumours had poorer survival rates than patients with wild type TP53 (938 vs. 1016 days, p =0. 04); however the difference was not significant when corrected for tumour stage. TP53 mutation were of higher prognostic significance in right sided tumours (883, 1051 days; mutated, wild type; p = 0. 005). Among patients in the younger age group, those with K-ras mutation had significantly shorter survival than patients with K-ras wild type samples (841, 1033 days, p=0. 02).

Barnetson et. al. (2006) studied a group of 870 below-55 years CRC patients for germline mutations in DNA MMR genes, proposed a model for prediction of the presence of mutations in these genes and validated the model in an independent group of 155 patients. Survival in carriers and non carriers was similar.

8. Molecular genetics and treatment protocol

Chemotherapeutic treatment protocol will progressively become more specific as the genetic basis of CRC gets better understood and the heterogeneity of the disease is better characterized. There are several literature reports that show these connections.

Fallik et. al. (2003) studied response to irinotecan in 72 patients, of whom 1 responded completely and 11 partially. Among the 7 tumours that displayed MSI-H phenotype, 4 responded to irinotecan whereas only 7 out of 65 MSI-L tumours did (p=0. 009). A better response to irinotecan was observed in the patients whose tumours have lost BAX expression (p<0. 001). 7 of 72 tumours had inactivating mutations in the coding repeat of the target genes. Amongst these seven, five responded to irinotecan, whereas only 6 of the other 65 tumours did (p<0. 001) indicating that MSI-driven inactivation of target genes modifies tumour sensitivity. It has been shown that tumours with mucinous histology, a common feature of many tumours in the young(section 7) and whose molecular genetic signatures have been referred to earlier (section 3), show poor response to fluorouracil-based first line chemotherapy (Negri et. al. 2005) and first-line oxaliplatin /irinotecan based combination chemotherapy (Catalano et. al. 2009).

9. Acknowledgement

We thank Mr. Anil Kumar Verma and Dr. Nilanjana Bose.

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3

Early Detection of Colorectal Cancer and Population Screening Tests

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1. Introduction

Issues of early detection of colorectal cancer with reference to the value of screening programmes and the role of the primary care practitioner

Colorectal cancer (CRC) is the most common newly-diagnosed cancer, one of the leading causes of illness and death in the Western world, and the second most common cause of cancer morbidity in Europe. Yet, CRC is a preventable disease and, if detected early, highly treatable. Early detection and prevention are health care strategies of critical importance for the reduction of CRC morbidity and mortality. In a number of countries, screening programmes have been implemented on nationwide scale since the 1960s for other forms of cancer. The early detection of cancer increases the likelihood of successful outcomes, but in order to have early detection, education and training promoting early diagnosis and resulting in increased screening, participation is needed. Additionally, the effectiveness of screening can be measured by the reduction on mortality, but it greatly depends upon tangible and sometimes intangible factors, contingent on setting and target population; it is essential, for example, to identify and screen the appropriate target population and to overcome implementation and uptake barriers. All of these issues, with emphasis on obstacles encountered at the level of general and family practice are highlighted in a recent editorial in Family Practice (Lionis and Petelos, 2011).

Although the screening is performed in the context of public health, and for the benefit of the community, the rights and welfare of the individual should also be respected. The role of the General Practitioner/Family Practitioner (GP/FP) and generally of the Primary Care Provider (PCP) is challenging yet instrumental in achieving this balance, as it is at that level screening is initiated (Viguier et al, 2011). The involvement and the role of GPs and PCPs in convincing patients to participate and initiate CRC screening should be further explored and elucidated, as it is of key importance in cultural and organisational context and health policy issues (Sarfaty, 2006). CRC screening of asymptomatic population groups is currently recommended in the USA and many European countries, and a number of pilot and nationwide programmes have been developed for this purpose. More specifically, mass screening programmes are currently established in 13 of 39 European countries (Pox et al, 2007; Manfredi et al, 2011) with feasibility studies undertaken as pilot actions in many more.

Although many of these screening programmes, both opportunistic and population-based are already implemented, and screening and early detection of adenomatous polyps has been shown to be effective in the reduction of CRC morbidity and mortality, the rate of screening participation remains low in many population groups at risk for the disease. Good news have recently arrived from across the Atlantic, where decision analysis tools were employed to inform recommendation updates and "microsimulation modelling demonstrated that declines in CRC death rates are consistent with a relatively large contribution from screening" (Edwards et al, 2010), nevertheless, similar efforts are lacking in some European countries, an issue that is given its due importance in this chapter. The success in the US can be attributed to the efforts of international organisations and national task forces, as they have resulted in a level of high awareness of CRC screening among US primary care providers (PCPs) in the US (Klabunde et al, 2003; Levin et al, 2008), but also in certain European countries. However, there is a variation in the evidence that explains the low rate of CRC screening, especially in younger patients (Walsh et al, 2009), while, few physicians recommend screening for the majority of their patients (McGregor et al, 2004). Compounding this effect is evidence that close to a quarter of physicians report not following national screening guidelines, and only half reported the adoption of recommendations that was consistent with the guidelines (Meissner at al, 2006), another key issue that requires special attention.

Additionally, very few PCPs use chart reminders or outreach programmes to contact patient populations most likely to benefit from screening (Klabunde et al, 2009). There is limited research focusing on obstacles and barriers, and the role of the physician-patient relationship plays in determining participation in screening programmes, especially when it comes to ethnic and culturally diverse groups (Lionis and Petelos, 2011). The importance of culturally relevant strategies for designing and implementing screening programmes has been already highlighted (Tu et al, 2006). Additionally, the role of socioeconomic disparities in CRC screening has been highlighted and documented (Meissner et al, 2011) if not explored in detail (Aubin-Auger et al, 2011), thus indicating a need for a close collaboration between medical and social care scientists in order to improve the requisite understanding for increased compliance to CRC screening recommendations. To compound the increasing complexity of national guidelines and the sensitivity of implementing them to culturally and linguistically varied patients, support through interventions focusing on organizational changes and further education and training for PCPs on early diagnosis, prevention and health promotion is needed. These are all issues that this chapter attempts to address. All of these factors are relevant for and have an impact on the ongoing debate about the role of GPs/FPs and PCPs, as well as the contribution these have on the effective implementation of screening programmes, opportunistic and population-based.

From all of the above one can surmise that the early detection of CRC is an issue of complexity requiring clear messages to increase the awareness and performance of the health care actors. This is another objective of the present chapter. Thus, the particular aims of this chapter are: (a) to provide information about the recommendations issued by certain large national and international organizations, including those issued by the U.S. Preventive Services Task Force (US PSTF) on the use of the available screening tests for the early detection of CRC and adenomas for average-risk subjects, (b) to critically review the role of clinical physicians and mainly PCPs in the early detection of CRC, (c) to explore issues with an impact on CRC screening, and, finally, (d) to highlight some quality issues relevant to CRC screening and relevant guidelines for quality assurance mechanisms in the relevant

processes. The chapter starts with concepts and definitions, proceeds with the recommended screening tests and concludes by outlining main points of interest and corresponding recommended tasks and actions for PCPs, for the purpose of increasing uptake and facilitating implementation of CRC screening programmes.

2. Concepts and definitions

Population screening is the systematic application of a suitable test with the aim of identifying individuals at a risk of a specific condition or disorder, but who have not sought medical attention on account of symptoms for that particular condition or disorder, and who can benefit from further investigation or direct preventive action (Wald, 1994). The notion differs from opportunistic screening, and it is a systematic process that includes certain steps from call or recall to screening, feedback of the results and follow-up in well-defined intervals. For population screening, the organised framework in which it takes place provides opportunities for more effective management, quality assurance and evaluation.

In our empirical view, understanding of the notion of screening, population or opportunistic, greatly varies between health care practitioners with the result of adversely impacting the effective implementation of the early detection programmes for CRC. It is for this reason we have decided to provide an extensive review on the existing literature and consensus criteria to define screening, focusing on CRC screening.

As stated by Wilson and Jungner in their seminal paper (Wilson and Jungner, 1968) "*the central idea of early disease detection and treatment is essentially simple. However, the path to its successful achievement (on the one hand bringing to treatment those with previously undetected disease, and, on the other, avoiding harm to those persons not in need of treatment) is far from simple though sometimes it may appear deceptively easy*". On the basis of whether early detection is possible at an early stage of the disease and taking into consideration whether an appropriate treatment is available, they attempted to formulate criteria that could help guide the selection of conditions and population groups suitable for screening. They also noted case-finding differences, depending on whether it is performed by a public health agency or by a general practitioner, and, almost four decades ago, emphasised the aspect of cost by underlining the importance of assessing effectiveness not only from an individual, but also from a public health perspective.

The fast pace of genetic research and the advent of new therapies has resulted in the generation of many other lists of screening criteria; most of them based to a greater or lesser degree on the Wilson-Jungner criteria. Additionally, even when consensus at the national or regional level is reached on which set of criteria to apply, there are other social, ethical and even logistical considerations to be examined. More recent trends on patient-centric and evidence-based health care, as well as cost-effectiveness and quality assurance, have resulted through a series of consultations to the modified Wilson and Jungner criteria (Andermann et al, 2008). In these amended criteria, opportunistic screening, essentially case-finding performed outside a framework of an organised programme as the one required to ensure such criteria are met, is, therefore, not a valid alternative; additionally to being less efficient it is also more costly, and, most importantly, quality assurance mechanisms cannot be embedded in a standardised fashion in such a process.

The definition of an "organised" screening programme according to the International Agency for Research on Cancer (IARC) includes: 1) an explicit policy with specified age categories, method and interval for screening; 2) a defined target population; 3) a

management team responsible for implementation; 4) a health-care team for decisions and care; 5) a quality assurance structure; and 6) a method for identifying cancer occurrence and death in the population (IARC 2005). Such organised population-based screening programmes have a predefined specific population, according to epidemiological data and on the basis of target age and geographical area, and during all the stages, from the invitation of the eligible individuals to the assessment procedures following testing, a specific protocol is followed. As mentioned, quality aspects of the process can be better addressed, as for example during follow-up (Miles et al, 2004). Furthermore, such screening programmes usually do not incur any costs for the participants.

It is important to have also a concrete idea regarding more abstract terms determining the usefulness and, even, the effectiveness of screening tests, and to keep such definitions in mind. Although these terms have been widely used, there is also great variation of their understanding and usage in clinical decision-making. For example, the ability of a measure or test to predict a subsequent event is a form of validity. On the basis of such a criterion we determine the predictive value of a test. The positive predictive value (PPV) in terms of detection through FOBT screening is defined as "*the percentage of people with detection of at least one lesion/adenoma/advanced adenoma/cancer at follow-up colorectal screening among those with positive tests who have attended follow-up colorectal screening*" [Adapted from the European guidelines for quality assurance in colorectal cancer screening and diagnosis. 2011. European Commission, Directorate General for Health and Consumers, EAHC — Executive Agency for Health and Consumers, World Health Organisation], whereas a positive test is, effectively, an abnormal result leading to further investigation (i.e. colonoscopy) or the removal of a lesion, for example, according to the protocol of the organized screening programme. By alluding to a false-positive result, we effectively mean that although the test indicated disease is present this is not the case. In a true-positive test, the result is correct and the disease is really present. Similarly a false-negative test indicates a disease-free subject has been tested, but the disease is present and might remain undetected if there is no further testing, symptoms, etc., whereas a true-negative test indicates a disease-free subject has been tested. Prevalence of the disease affects not only the positive predictive value of a screening test but also its negative predictive value, i.e. the probability that the person subjected to the screening test is truly free from the disease when a negative (normal) test result is obtained.

Two concepts often discussed in relation to true- and false- positive and negative results are sensitivity and specificity and both terms have an important impact on the PCP decisions on which of the available tests for the early detection of CRC should be recommended. The sensitivity refers to the number of cases the test can identify or in more simple words the probability of one diagnostic test telling the truth when the disease exists. It gives us a certainty that true positives will not be missed. Specificity refers to the accuracy of the finding or in simple words the probability in telling the truth when the disease is absent. Ideally, a test should be both highly sensitive and specific. To that direction, the US Preventive Services Task Force and the Institute of Medicine (IoM) recommend the fecal occult blood test (FOBT) test, and more specifically the guaiac test (gFOBT) for screening programmes. Nevertheless, when used on its own, it has relatively low sensitivity, whereas the a combination with a more sensitive test, such as the fecal immunochemical test (FIT) could help to render screening programmes more effective (Allison, et al, 2007). Another interesting consideration, especially given the public health context of mass screening, is cost-effectiveness and how it correlates to specificity and sensitivity. Although uncertainty

remains, the assessment of a screening program based on FIT for a one-year period in France seemed to be the most cost-effective approach (Hassan, et al, 2011). More research is necessary, as for example indicating what the best cut-off levels for colonoscopy referral are, without compromising sensitivity, to determine optimal public health approaches.

3. Screening tests, guidelines for CRC and the importance of early detection

As previously mentioned, CRC can be curable when diagnosed at an early stage. Also, CRC mostly develops from colorectal non-malignant precursor lesions, thus, rendering it a preventable disease through the removal of premalignant lesions. Systematic early detection and removal at the "adenoma-phase" can prevent the occurrence of CRC and markedly decrease overall population incidence (Winawer et al, 1993), as human colon carcinogenesis progresses to the carcinoma pathway via the dysplasia-adenoma phase.

The readers of this chapter are aware from previous chapters of this book that there are various tumour staging systems, the ones mainly used in Europe being Duke's classification and TNM (Tumour, Node, Metastasis) classification of malignant tumours, introduced by the Union Internationale Contra le Cancer (UICC) and the American Joint Committee on Cancer (AJCC). Despite the fact TNM yields greater information, there are several major issues due to the reclassification of the system. Most importantly, there seems to be great disparity between the therapeutic decision-making and the TNM staging, with multiple TNM staging versions being used in different countries and great variance in reporting. It has been argued that changes should only occur after extensive discussion within the scientific community (Quirke et al, 2010), and it is essential to note that the reporting on a nationwide scale for any given CRC screening programme should be performed on the basis of the same staging system. Lesion reporting within the frame of the screening programme should be standardized to allow for better evaluation and reporting, and, consequently, improved outcomes. TNM stages and version, frequency of CRC and distribution of TNM stages should be reported along with the presence of non-neoplastic lesions. According to the report of the EU on CRC quality guidelines (European Commission, Directorate General for Health and Consumers, EAHC — Executive Agency for Health and Consumers, World Health Organisation, 2011), without explicit criteria for the diagnosis and staging of early adenocarcinoma unnecessary radical resection would result in severe overtreatment, raising the morbidity and mortality in the context of the programmes.

Various screening technologies are currently available, from the more established Guaiac faecal occult blood tests (gFOBT) and immunochemical FOBT to sigmoidoscopy and colonoscopy, as well as combinations (i.e. combined FOBT with sigmoidoscopy) to the new screening technologies, as CT colonography, stool DNA and capsule endoscopy. Early reports during the previous decade suggested that biennial screening by FOBT reduces CRC mortality, as for example in the French (Faivre et al, 2004) and Danish populations (Jørgensen et al, 2002). This fact lead WHO (World Health Organization) and OMED (World Organization for Digestive Endoscopy) to suggest a choice of FOBT that should take into account dietary compliance to recommendations, but also colonoscopy resources (Young et al, 2002). Prior to any recommendation for screening tests for CRC, the PCPs should be able to recognise whether the individual visiting the practice/office is at average, increased or at high risk for CRC. In other words, to be able to identify whether the particular individual truly belongs to the target population of the screening programme or –should there not be one available– whether there is reason for referral in the context of opportunistic screening.

In Australia, the Department of Health and Aging has issued clinical practice guidelines for the prevention, early detection and management, and national population-based screening programs are in place for various cancers, from breast cervix and to bowel (CRC) (Australian DoHA, 2011). Also, the National Bowel Cancer Screening Program Register plays an important role in the programme, as it assists participants through the screening pathway, allows for reminders and follow-ups without taxing local resources and GPs. There are online tools and decision-aids available to GPs and information in twenty languages targeted at patients. Pre-invitation, invitation, follow-up letters, FOBT kit instructions and an information booklet are all provided in all of these languages and are also available online. Additionally, a qualitative evaluation of opinions, attitudes and behaviours influencing CRC were examined in the pilot phase of the national screening programme and the report was published and integrated in future planning [A Qualitative Evaluation of Opinions, Attitudes and Behaviours Influencing the Bowel Cancer Screening Pilot Program: Final Report August 2005]. Most interestingly, the invitation is sent directly to the candidate participant and it is not necessary to nominate a physician in the forms submitted, although participants are encouraged to nominate a doctor in the context of follow-up if the FOBT is positive:

a. If no doctor is nominated, the FOBT results will only be sent to the participant.
b. If a doctor is nominated, the results of the FOBT will be sent to the participant and their doctor.
c. If the FOBT result is positive it is explained that it will be necessary to discuss the result with a doctor.

The American Cancer Society (ACS) and the National Colorectal Cancer (NCC) in cooperation with the Thomas Jefferson University have edited a Primary Care Clinician's Evidence-Based Toolbox and Guide (Sarfaty, 2008). According to this guide, an individual is at an average risk when s/he has no first-degree relatives with a history of either CRC or adenomatous polyps and no illness or past health problems have been reported (Sarfaty, 2008). For individuals at average risk, GPs are recommended to initially take a medical history, including age, symptoms, family medical history, and also individual history with a focus on bowel diseases and dietary habits, and to perform a clinical examination including a digital rectal examination. Also, various CRC screening guidelines and recommendations have been issued, both by national and international organisations and institutions. In 2008, a joint effort of the ACS and the American Gastroenterology Association was released regarding certain modalities including stool tests, flexible sigmoidoscopy (FS), colonoscopy (CS), double-contrast barium enema (DCBE), computer tomography, colonography (CTC) (McFarland, et al 2008). Those joint guidelines also stressed the importance of prevention of CRC important tasks for PCPs. The US Preventive Services Task Force (US PSTF) recommends routine asymptomatic screening for three cancer sites, including that of breast, CRC and cervix, mainly because they are asymptomatic to a high degree in early staging, have a high 5-year survival rate when the cancer is localised, and as there is a strong evidence on the screening effectiveness (Cardarelli, 2010).

In terms of a recommended start and stop age for screening, the ACS has issued guidelines for the early detection of CRC and polyps with recommended screening beginning at age 50 for both men and women (ACS, 2011). The US PSTF recommends a screening for average-risk men and women 50 years of age and older, with colonoscopy every 10 years, flexible sigmoidoscopy or DCBE every five years and faecal occult blood test every year (U.S. Preventive Services Task Force, 2011). In a supporting document, this Task Force

summarises its recommendations and recommends screening for CRC using an FOBT, sigmoidoscopy, or colonoscopy in adults beginning at the age of 50 years and continuing until the age of 75 (Grade: A Recommendation). However, US PSTF recommends against screening for CRC in adults over 85 (Grade: D Recommendation), while it concludes that the evidence is insufficient to assess the benefits of CT colonography and faecal DNA testing for CRC as screening modalities. Judging the benefits against harms, the American Task Force discusses among the benefits of the less invasive CRS screening the number of colonoscopies that may be reduced. However, it recommends that for any positive test there is a follow-up with colonoscopy. The Task Force Recommendation statement underlines that the benefits of CRC detection and early intervention decline at the age of 75 years, thus it leaves the decision for a routine screening at individual level. There is, as described, a lot of information, but slightly conflicting evidence and advice. Nevertheless, participation of the 50-75 years age group increased by 13.1% reaching 65.4%, whereas a significant CRC incidence decline was noted in 35 states and mortality declined in 49 states and DC (CDC, 2011). Further efforts are currently being made in the field of patient engagement and patient-reported outcomes, and in the context of comparative effectiveness research (PCORI, 2011).

In the United Kingdom, Cancer Research UK underlines the importance of screening for the reduction of CRC mortality and has elaborated upon the role of FOBT and flexible sigmoidoscopy (Cancer Research UK, 2011). This institute refers to evidence provided by four RCTs where the use of FOBT every two years reduced CRC mortality by 15% to 18% in people aged 45-74 years. Centralised systems, such as the Australian and, to a certain extent, the UK system, remove pressure from the individual GP and the organisational capacity at practice level, but could potentially result in a loss of involvement and a lowered feeling of responsibility.

An individual is at increased risk when s/he has a personal and family history of CRC or adenomatous polyps but without reporting any of the high-risk familiar syndromes. Those hereditary syndromes include: the hereditary non-polyposis CRC (HNPCC), the familiar adenomatous polyposis (FPP) and the attenuated PAP (APAP). In this group, the clinical physicians and the PCP should change their strategy from screening to regular surveillance, and the tests that primarily detect cancer should be replaced by more sensitive diagnostic approaches and particularly colonoscopy, which should start at age 40 or younger (Sarfaty, 2008). The National Institute for Health and Clinical Excellence (UK) has recently published a new guideline on colonoscopic surveillance for the prevention of people with ulcerative colitis, Crohn's disease or adenomas (NICE, 2011). At the third category where the probability of developing CRC is high, the PCPs should be more cautious when recommending screening and surveillance. A family history of an adenomatous polypus or CRC in a relative under the age of 50 is suggesting a high probability of the presence of any of the above high-risk hereditary syndromes and the clinical physician requires genetic testing; a close collaboration with hospital specialists at a centre with expertise should be established (Sarfaty, 2008).

In Europe, the high degree of heterogeneity in health care systems, policy, roles, screening programme resources and very different values in local and regional settings had previously created a rather fragmented picture. There are ongoing efforts toward harmonisation, for example, recently developed guidelines (2011) in an effort under the auspices of the European Commission, focus on quality assurance and provide clear and concise information to facilitate decision-making at the GP/FP and PCP levels. As illustrated by

Table 1, an evidenced-based brief overview of various conventional screening methods is given, although new technologies resulting in more modern forms of screening are not assessed for lack of evidence; some information regarding cost-effectiveness is provided along with the recommendations and examined in more details in the report (European Commission, Directorate General for Health and Consumers, EAHC — Executive Agency for Health and Consumers, World Health Organisation, 2011)

Finally, it is important to underline that early detection is directly dependent on acceptance of the screening test by both provider and patient, as well as the uptake of the screening programme. For example, early versions of stool DNA (sDNA) testing lacked the requisite sensitivity and markers, but improved sDNA tests are now available. It is important to understand patient preferences regarding screening options for selecting the right tool for a given population; for example, whether a non-invasive test is preferred to colonoscopy or whether accuracy is considered much more important than discomfort. In a study by Schroy et al, (2002), those preferring colonoscopy to sDNA or FOBT rated accuracy as the most important factor, whereas those rating concerns about discomfort or frequency of testing as the most important parameter preferred sDNA. Most subjects preferred a shared (54%) or patient-dominant (34%) decision-making process.

As previously highlighted, removal of all adenomas, without accurately distinguishing between those which will become malignant and those which will not, will effectively result in excessive overtreatment, and it is for this reason that newer screening tests, such as the sDNA, focusing on genomic changes affecting associated biological and metabolic processes should not be overlooked as options necessitating further research -particularly because of their potential to avoid iatrogenic care, but also because they might better reflect patient preferences for certain population groups (Sillars-Hardebol, et al, 2012).

[Adapted from the European guidelines for quality assurance in colorectal cancer screening and diagnosis. 2011. European Commission, Directorate General for Health and Consumers, EAHC — Executive Agency for Health and Consumers, World Health Organisation.]

Guaiac FOBT

There is good evidence that invitation to screening with FOBT using the guaiac test reduces mortality from colorectal cancer (CRC) by approximately 15% in average risk populations of appropriate age

RCTs have only investigated annual and biennial screening with guaiac FOBT (gFOBT) (II). To ensure effectiveness of gFOBT screening, the screening interval in a national screening programme should not exceed two years

Circumstantial evidence suggests that mortality reduction from gFOBT is similar in different age ranges between 45 and 80 years. The age range for a national screening programme should at least include 60 to 64 years in which CRC incidence and mortality are high and life expectancy is still considerable. From there the age range could be expanded to include younger and older individuals, taking into account the balance between risk and benefit and the available resources

Immunochemical FOBT

There is reasonable evidence from an RCT that iFOBT screening reduces rectal cancer mortality, and from case control studies that it reduces overall CRC mortality; Additional

evidence indicates that iFOBT is superior to gFOBT with respect to detection rate and positive predictive value for adenomas and cancer
Given the lack of additional evidence, the interval for iFOBT screening can best be set at that of gFOBT, and should not exceed three years
In the absence of additional evidence, the age range for a screening programme with iFOBT can be based on the limited evidence for the optimal age range in gFOBT trials
Sigmoidoscopy
There is reasonable evidence from one large RCT that flexible sigmoidoscopy (FS) screening reduces CRC incidence and mortality if performed in an organised screening programme with careful monitoring of the quality and systematic evaluation of the outcomes, adverse effects and costs
The available evidence suggests that the optimal interval for FS screening should not be less than 10 years and may even be extended to 20 years
There is limited evidence suggesting that the best age range for FS screening should be between 55 and 64 years. After age 74, average-risk FS screening should be discontinued, given the increasing co-morbidity in this age range
Colonoscopy
Limited evidence exists on the efficacy of colonoscopy screening in reducing CRC incidence and mortality. However, recent studies suggest that colonoscopy screening might not be as effective in the right colon as in other segments of the colorectum
Limited available evidence suggests that the optimal interval for colonoscopy screening should not be less than 10 years and may even extend up to 20 years
Indirect evidence suggests that the prevalence of neoplastic lesions in the population below 50 years of age is too low to justify colonoscopic screening, while in the elderly population (75 years and above) lack of benefit could be a major issue. The optimal age for a single colonoscopy appears to be around 55 years. Average risk colonoscopy screening should not be performed before age 50 and should be discontinued after age 74
Combination of FOBT and sigmoidoscopy
The impact on CRC incidence and mortality of combining sigmoidoscopy screening with annual or biennial FOBT has not yet been evaluated in trials. There is currently no evidence for extra benefit from adding a once-only FOBT to sigmoidoscopy screening
New screening technologies under evaluation
There currently is no evidence on the effect new screening tests under evaluation on CRC incidence and mortality. New screening technologies such as CT colonography, stool DNA testing and capsule endoscopy should therefore not be used for screening the average-risk population
Cost-effectiveness
Costs per life-year gained for both FOBT and endoscopy screening strategies are well below the commonly-used threshold of US$ 50 000 per life-year gained (LYG)
There is some evidence that iFOBT is a cost-effective alternative to gFOBT
Available studies differ with respect to what screening strategies are most cost-effective. No recommendation of one screening strategy over the others can be made based on the available evidence of cost-effectiveness

Table 1. Recommendations and conclusions

Finally, the concepts of colonoscopic surveillance and screening for recurrent CRC should receive attention by PCPs. The adenomatous precursors of CRC are present in over 30% of individuals over 55 (Eide, 1991), placing them at higher risk of developing CRC, but the removal of these lesions reduces risk to that of the general population (Citarda et al, 2001). Recurrent CRC, as for example following resection, also necessitates an intensive surveillance programme, as the detection at an asymptomatic stage can result in survival benefit (Renehan, et al, 2002). This means that surveillance and follow-up programmes should also be combined or evaluated along with a screening programme.

4. Primary care and CRC: Tasks and steps for screening implementation in primary care

One of the most important factors for the effective implementation of a CRC screening programme is the involvement of a PCP, particularly of the GP or the FP, in convincing targeted individuals to participate and to initiate the screening. The PCPs have multiple and varying tasks, more specifically to (Sarfaty, 2008):

1. Assess the risk of developing CRC and increase the risk awareness, as described above.
2. Discuss options with patients/individuals and effectively engage in shared decision-making (SDM) – this would ensure patient perspectives and preferences are consistent to decisions made.
3. Convince to participate – this task requires communication and consultation skills, as well as an established continuity of care.
4. Implement the initial tests: those primarily used to detect cancer, including the annual Guaiac-based occult blood test (gFOBT), the annual faecal immunochemical test (FIT), or stool DNA test (sDNA).
5. Consider and assess the available screening resources and capacity: it is an important task for PCPs, who should be aware of the available resources in their district or health region capacity, as well as patient limitations (e.g. socioeconomic, mobility, etc.), to determine the optimal referral pathway for the test(s) that detect adenomatous polyps and CRC (FS, CS, DCBE, CRC).
6. To make the necessary arrangement to complete the CRC screening.

One of the most challenging issues that the PCPs encounter is to convince the average risk individual to use a simple and inexpensive test to initially detect if any hidden blood is present in stools, constituting a strong indication of the presence of an adenomatous polyp or CRC. To achieve it, an effective doctor-patient communication should be established, and the purpose of the GPs/PCPs might also need to be re-assessed by further education or training on early diagnosis, prevention and promotion. The role of a multidisciplinary team is also essential. FOB Testing serves this role, although it has received criticism because of the lack of specificity, particularly when the test is dehydrated, and because of the subsequent increase of the associated costs of screening programmes (WHO, Rudy and Zdon, 2000). FIT, also, fulfils this purpose; it is a simple procedure: the stool sample is collected by the individual/patient at home, and the completed test is sent to a laboratory or to the PCPs office. Usually two samples from different bowel movements are required and the instructions on sampling procedures on how the water sample should be transferred by the brush onto the test card are clear and readily understood.

Another important task for PCPs and other practitioners is to educate their patients/clients to contact the PHC services when some warning signs are experienced and among them are (Rudy and Zdon, 2000):

- Hematochezia
- Melaena
- Anaemia resulting from occult blood loss
- Change in bowel habits

Prior to the decision of the PCP to refer the subjects to either CS/FT or CT should be explored the access to that screening method and consider the existing diagnostic capacity resources (Sarfaty and Wender, 2007).

Finally, another essential consideration in the PCP decision to implement screening tests for early detection of CRC is that of quality of life. Quality of life in evidence-based medicine should always reflect the preferences of patients, as patient-centeredness is its cornerstone. Despite the fact everyone values particular aspects of life differently, all aspects of life that may be affected adversely or in a beneficial manner by aspects of health and illness should be taken into consideration. For screening programmes, it is important to understand the cultural context in which it is performed or is to be performed and to ensure the values of the patients are taken into consideration when determining and/or assessing outcomes.

5. Obstacles to implementing CRC screening in primary care

Obstacles in primary care

As mentioned above, the CRC screening rate increase does not seem to apply in many countries and regions and the associated obstacles and barriers that have already been reported in the literature (Lionis and Petelos, 2011) could be classified as follows:

- Obstacles at doctor level: Obstacles reported by the GPs were relevant to the difficulties in being convinced especially when signs and symptoms were lacking. There was, in other words, confusion in addressing difficulties stemming from conflict between personal experiences and public health implications (Aubin-Auger et al, 2011). Also, there is research indicating that even in countries with established screening programmes only 50% of the GPs considered themselves to be sufficiently trained, as for example in France (Viguier et al, 2011).
- Obstacles at patient level: Researchers examined obstacles at patient level and how these were linked to the physician-patient interaction and communication. For example, cancer screening did not fall in with the perception of some patients regarding health care, and they failed to identify benefits outside the context of familiar high-risk groups. Potentially inadvertently reflecting specificity and sensitivity issues, participants were afraid of poor technical skills, and taking ownership of the risk for performing the test, resulting in false positive or false negative results (Aubin-Auger et al. 2011). Mirroring the high number of GPs who do not feel they are sufficiently trained, patients cited the absence of recommendation as one of the most important reasons for not undergoing screening (Viguier et al, 2011).
- Obstacles at doctor-patient level: GPs and patients agreed the lack of symptoms and lack of familial risk were two of the main reasons for doubting how useful such a test could be, the GPs thought that the patients misunderstood the process and were

afraid of reactions to false negative results, whereas the patients complained about time, as well as the constipation effect from repeating the test, and did not express fears about such results (Aubin-Auger et al, 2011). Further evidence (Schroy et al, 2011) indicates that screening intentions and test ordering are adversely affected when patient and provider preferences differ. Interestingly, compounding previously reported data (Serra et al, 2008), having a screening habit (e.g. mammography) proved to be a positive factor for women, whereas increased participation was reported for those with a higher educational level, particularly for male patients. Without diminishing the importance of facilitators, a patient having a relative having already performed gFOBT was more likely to accept the test, but friends and family were not identified as obstacles.

Further barriers:

Cultural and linguistic barriers were also touched upon by these researchers, but not explored in detail; it is highlighted that even the wording a doctor uses has an effect and that further research is necessary (Lionis and Petelos, 2011). There is evidence that by employing culturally and linguistically relevant approaches for FOBT promotion, screening participation increases in target populations of low-income and/or less acculturated minority patients (Tu et al, 2006). Indeed, a challenge of equal significance to guideline adherence and compliance in screening is ensuring equity of access to screening. Part of ensuring equity of access is to ensure awareness issues have been addressed for all ethnic and culturally diverse groups. A study of all the patients aged 50-60 registered in general practices for a UK region (West Midlands), with a total number of over eleven thousand respondents, examined factors that contributed positively or negatively on behaviour toward screening (Taskila et al, 2009). People without a screening habit (men), older people, and those with Indian ethnic backgrounds were more likely to have negative attitudes, whereas Black-Caribbean ethnic background people reporting abdominal pain, bleeding or tiredness were more likely to have a positive attitude. This great variation in attitudes indicates that there are different needs to be addressed for increasing awareness and highlight the importance of culturally relevant strategies for designing and implementing screening programmes (Taskila et al, 2009). Evidence amasses from various countries, with a study focusing specifically on FOBT use, along with the subsequent investigation of a positive result (Bampton et al, 2005). Researchers established that both indications for use and follow-up of a positive result varied according to the ethnicity of the GP and independently of the medical training received (Koo et al, 2011). Additionally, it was indicated that the ethnicity of the patient and, similarly to results of other research, associated linguistic and cultural barriers affect screening uptake and was noted that this may adversely affect the health of immigrant populations.

To address all the obstacles and barriers previously mentioned, it is necessary to embrace the perspective of the users of screening programmes, and also to examine screening under the prism of public health perspective. A recently conducted review highlights the need for policy supporting both screening delivery and organisational transformation in a manner that promotes improvement of operational features for preventive services (Senore et al, 2010). The researchers examined recently proposed conceptual frameworks that were aimed at identifying key elements and, thus, potential targets for interventions aiming to improve screening (Cole et al, 2009 and Federici et al, 2005). The models developed conceptualised these potential targets at various levels: the organizational

context in which health care delivery and provision are taking place, the practice itself, and the structural and operational characteristics of given settings, and also examined the provider and patient levels. The researchers concluded that although a given intervention may be implemented at one or multiple levels, the factors determining uptake and participation are, indeed, correlated with all of these levels in an interconnected and interdependent manner.

In concluding this section, the role of the PCPs is extremely complex, and although research on obstacles, barriers and limitations is starting to create a more robust evidence base, further qualitative and translational research is required to identify best practices and intervention transferability. Additionally, policy measures for the purpose of supporting screening delivery mechanisms are required, and, similarly, policy should aim to facilitate the organisational changes necessary for creating and supporting the operational features of preventive services.

6. Increasing the CRC screening rate

Although messages about the effectiveness of CRC screening have been widely available, there are still concerns in terms of both physician involvement and PC user participation in CRC screening. This is not a message that concerns CRC screening per se, but prevention and health promotion activities undertaken by GPs in Europe. There are significant gaps between GP knowledge and practice in Europe, already reported upon (Brotons et al, 2005). Evidence from the literature indicates that less than one third of the PC physicians use chart reminders and 15% use outreach mechanisms to contact patients needing screening (Klabunde et al, 2009). Investment has been made on efforts and research programs to assess the impact of quality improvement intervention programs. One of them combined diverse components, such as performance activities, delivery system design, electronic medical record tools and patient activation (Ornstein et al, 2010), and reported promising results in the Evidence-Based Toolbox and Guide we currently have (Sarfaty, 2008). Thus, the implementation of educational programmes for PCPs and patients in addition to the development of shared-decision making tools, given the differing perspectives between doctor and patient, seem imperative, as otherwise lack of consensus could adversely impact the CRC screening rate (Schroy et al, 2011).

A page invitation to the health practitioners to avoid certain errors has been made and among them the following:

- To screen for CRC with only a digital rectal exam or with a single sample from a stool blood test
- Recommend screening with colonoscopy at average risk more often than every 10 years or CT colonography, DBCE or flexible sigmoidoscopy more often than five years

A toolkit for a systematic approach in tracking and increasing screening for public health improvement of CRC intervention was prepared for the Agency for Health Care Research and Quality (AHRQ). It delivers tools, process guidelines, tips and evidence of the intervention effectiveness (Harris et al, 2010). It is strongly recommended for PCPs, health care planners and managers. However, the role of PCPs in increasing the CRC screening rate remains a key component. According to the ACS and NCS, the positive impact of its

advice is well documented, and the magnitude of the doctor's impact is considerable (Sarfaty, 2008).

7. Designing a national CRC screening programme/framework

We have seen the importance and potential of CRC screening in detail. The importance of screening taking place in an organised framework for optimal results, as for example in nationwide programmes, has also been examined and noted. As many European countries are still in the process of designing such a programme and many other countries globally are far from implementing such interventions, it is important to see how to best learn from other experiences and how to use lessons already learned to help us morph a flexible and robust model that can be adapted according to regional and local needs to ensure high acceptance, uptake and, indeed, equal access and reduced disparities. Thus, we decided to include in this section some key issues that health planners, health policy makers and public health decision makers should take into account when considering the design and implementation of a CRC screening programme.

Many of the countries in which a nationwide programme is implemented have extensively reported on the outcomes and evaluation of such programmes. Australia has introduced such a programme, but reporting indicated the needs and beliefs of minority groups, as for example indigenous Australians, were not always taken into consideration, with stronger drives, as for example economic benefit at country level, determining the approach undertaken and the strategy selected (Christou and Thompson, 2010).

To start, the need of conducting a feasibility study should be evaluated. According to Bowen et al, (2009) performing a feasibility study may be indicated when "*(a) community partnerships need to be established, increased, or sustained, (b) there are few previously published studies or existing data using a specific intervention technique, (c) prior studies of a specific intervention technique in a specific population were not guided by in-depth research or knowledge of the population's socio-cultural health beliefs, by members of diverse research teams, or by researchers familiar with the target population and in partnership with the targeted communities, (d) the population or intervention target has been shown empirically to need unique consideration of the topic, method, or outcome in other research or (e) previous interventions that employed a similar method have not been successful, but improved versions may be successful; or previous interventions had positive outcomes but in different settings than the one of interest*". By quickly reviewing these grounds it becomes apparent CRC programmes, independently of whether they are still being designed or already implemented, are prime candidates for qualitative research via feasibility studies. These pilot actions can help elicit patient preferences and elucidate obstacles adversely affecting participation. In terms of results, even small modifications to existing programmes or design can greatly affect outcomes. For example, a sound review being the starting point for deploying such a pilot action, recent systematic reviews of interventions in Australia, indicated that organisational level changes were the most effective in terms of screening behaviour enhancement. It is important to note necessary modifications for increased effectiveness were those that included non-physicians in the screening process (Christie et al, 2008, Wardle et al, 2003, Vernon 1997). Language and literacy barriers are, of course, the most difficult to overcome, as illustrated by the Alaskan and Australian Aboriginal examples, and can only be adequately researched and addressed

through engagement of local actors and community leaders, strong community orientation and high level of awareness of the PHPs, including most importantly nursing and all other available healthcare personnel –especially in rural or remote areas.
Additionally, elements of the care pathway ought to be assessed in context and real settings, and, especially where pragmatic trials are impossible, difficult or unethical. Participation in and completion of the screening test is not the only necessary part to ensure successful outcomes, as there has to be a follow up with the appropriate diagnostic testing. (Christou and Thompson, 2010).
Screening utilisation is also influenced by behavioural factors and health economics parameters, as well as by the organizational and cultural settings (Senore et al, 2010). A theoretical framework to explain the adoption of health-related behaviours is needed to underpin any given implementation effort. Models conceptualizing elements of the health care provision have been proposed, providing targets for intervention at patient and provider levels (Stone et al, 2002; Bastani et al, 2004, Senore et al, 2010). However, it is important to note that although any given intervention component may act upon more than one levels, screening uptake is interdependent on all these factors (Senore et al, 2010).
Consideration of multidisciplinary teams is also essential. These teams can help direct resources along a predefined, according to the evidence-base, care pathway ensuring effective, efficient and sustainable implementation and, thus, better results. As we have previously discussed, CRC screening is complex and comprises of different stages involving the participation of different health care actors. For example, all abnormal results should be followed-up and after-care service following treatment should be available. Nevertheless, only a small portion of health plans monitor follow-up care (Klabunde et al, 2003). Multidisciplinary participation can help implement interventions that have added value at patient, provider and even public health-health care system level. For example same day follow-up for abnormal FS, offering on-site colonoscopy seems to lead to better compliance (Stern et al, 2000; Senore et al, 2010). Another benefit in the involvement of nursing and clerical staff is the integration of quality indicators and quality assurance mechanisms in delivery processes and their monitoring as part of standardised care delivery without draining on valuable resources.

8. CRC screening: An issue of quality assurance in modern health care systems

Quality issues in colorectal cancer screening have been previously discussed in editorials in the journals of Quality in Primary Care and Family Practice (Lionis, 2007, Lionis and Petelos, 2011). These editorials address issues relevant to improvement of uptake of CRC screening with the use of cognitive methods and the translation of the Health Belief Model into education and training programs for health care providers. The authors call for a closer collaboration between medical and social care scientists, and reveals another important challenge that PCPs face: addressing health inequalities in a changing and financially restrained world, where for example, minority groups showcase low adoption rates of preventive measures and screening tests.
As previously mentioned, the Directorate General for Health and Consumers, with tasks funded by the EU Health programme (CRC screening grant No 2005317), led an effort

aiming to develop EU guidelines on best practice in CRC screening, which resulted in the publication of the first edition of the European guidelines for quality assurance in colorectal cancer screening and diagnosis in February of 2011 (European Commission, Directorate General for Health and Consumers, EAHC — Executive Agency for Health and Consumers, World Health Organisation). The guidelines systematically examine the evidence for efficacy and effectiveness of CRC screening and outline the guiding principles for organising CRC screening programmes. Most importantly, the authors underline the importance of the availability of comprehensive, evidence-based quality assurance guidelines that address all the steps of a screening programme, including invitation, information, surveillance and any other subsequent care, as a key factor to the success of any cancer screening programme. Finally, the authors advocate the widespread application of standardised indicators, as recommended and elaborated upon in the guidelines, to facilitate quality management and promote information exchange in the context of continuous quality improvement.

9. Epilogue

This chapter serves as an overview of the guidance available for CRC screening in the US, UK, Europe and Australia and briefly discussed the important role of GPs/FPs and PCPs, in general, in increasing the CRC screening rate. Although the literature is rich in information, guidelines and recommendation for CRC screening, there is room for improvement. It is to important invest in translating primary research into practice and combine qualitative and quantitative evidence for relevant, contextualised training and educational interventions, both at patient and provider levels.

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4

Turning Intention Into Behaviour: The Effect of Providing Cues to Action on Participation Rates for Colorectal Cancer Screening

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1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and second in females; throughout the world over 1.2 million new CRC cases and 608,7000 deaths are estimated to have occurred in 2008 (Jemal et al., 2011). The only developed country to have demonstrated a significantly decreasing incidence in both males and females is the United States, and this is largely due to the early detection and removal of pre-cancerous lesions through CRC screening (Jemal et al., 2011). Thus, an understanding of the variables that encourage people to participate in CRC screening is important because early detection and treatment of precancerous lesions and adenomas results in a significantly higher survival rate than if treatment is delayed until physical symptoms of the condition are apparent. Population screening using a Faecal Occult Blood Test (FOBT) can facilitate the detection of CRC at its early stages. FOBT is the collective term for a guiaic FOBT (gFOBT) or a faecal immunochemical test (FIT). Both are home-based tests which, although differing in the technology utilised, involve a stool sample being sent to a laboratory to be analysed for occult blood, ideally followed by colonoscopy for those with a positive result. The cost effectiveness of FOBTs for the screening of CRC, measured as Quality Adjusted Life Years gained, is comparable to other screening procedures (Frazier et al., 2000) and more cost-effective than treatment after physical symptoms are evident (Fisher et al., 2006). Randomised clinical trials have shown that both biennial and annual screening using FOBT screening reduces CRC incidence (Mandel et al., 2000) and mortality (Hardcastle et al., 1996; Kronborg et al., 2004; Mandel et al., 1993), and a systematic review concluded that FOBT screening is likely to avoid 1 in 6 colorectal cancer deaths (Hewitson et al., 2007). Effectiveness, however, depends upon yield and is critically dependent upon participation rates, which for population-based screening programs have been low, often despite high levels of intention to participate. For example, in Australia the National Bowel Cancer Screening Program, which provides people turning 50, 55 and 60 years with a free FOBT, had a participation rate in 2008 of 41% of the eligible population (AIHW, 2010). In England, the second round (2003–2005) of the pilot bowel cancer screening program had a

significantly lower uptake than in the first round (52% vs 58%) (Weller et al., 2006) and reported participation rates in 2008 in other countries with an established or pilot population FOBT screening program ranged mostly from a moderate 45–50% (Italy and Denmark, respectively) to a low 16–18% (Korean Republic and Japan, respectively) level (International Cancer Screening Network, 2008). Understanding motivators to intention to participate and motivators to test completion are critical issues that need to be addressed.

The central question in research within health psychology is identifying and understanding the range of influences that prompt an individual to take up healthy behaviours or reject patterns of behaviour which compromise their health. Many social cognitive health behaviour models include a measure of intention to behave in a specific way as a precursor to action (e.g., Theory of Planned Behaviour; (Ajzen, 1985). Stage models focus specifically on the importance of addressing intention as a core component of public health interventions. For example, the Transtheoretical, or Stages of Change, Model (Prochaska, 2008; Prochaska et al., 1988) suggests that people can be characterised in terms of their readiness to make a change. Stages include precontemplation (benefits of lifestyle change are not being considered), contemplation (starting to consider change but not yet begun to act on this intention), preparation (ready to change the behaviour and preparing to act), action (making the initial steps toward behaviour change), and maintenance of the behaviour over time; with both contemplation and preparation measuring aspects of intention.

One of the most difficult questions for researchers examining screening participation has been the question of how to move people along these stages to the performance of the actual behaviour and, ideally, maintenance of the behaviour. A range of social cognitive models of health behaviour have proven effective in describing individual motivation to perform a variety of health behaviours, including screening, by identifying a range of attitudinal predictors (Conner & Norman, 2005). Each of these deliberative models can successfully map variables that describe individual differences in the intention to perform a behaviour. However, the relationship between behavioural intention and actual behaviour is less than perfect; it has been shown that around 50% of people with positive intentions to engage in health behaviours successfully translate those intentions into action (Sheeran, 2002), and a medium-to-large change in intention leads to only a small-to-medium change in behaviour (Webb & Sheeran, 2006).

This 'gap', the difference between an individual's commitment to act and initiation of the necessary processes to actually carry out the behaviour, needs to be bridged—in other words, research that influences 'intention to try' (Bagozzi & Warshaw, 1990) needs to also identify cues that will enable people to link to the means for achieving the intended behaviour. Some health behaviour models incorporate a stimulus to action in their operationalisations in an attempt to capture this intervening, or additive, influence that prompts individuals to actually implement behaviour. For example, Becker and colleagues (1977) incorporated 'cues to action' as additional, independent predictors of health behaviour, over and above attitudinal variables. Although incorporated in the earliest descriptions of the Health Belief Model, a cue to action, or strategy to initiate "readiness", is a variable that has received limited attention in the empirical literature. Nevertheless, research does suggest that certain acts may serve to stimulate health behaviour including physician advice, advertising campaigns, and postcard reminders (Sheeran & Orbell, 2000).

Research originating outside the health area has examined the notion of volitional control and how it might be used to explain the problematic nature of the relationship between

behavioural intention and behaviour (Gollwitzer, 1993). This model suggests that individuals achieve volitional control of their intention to act by the development of implementation intentions; the plans made to achieve a specific behavioural target (e.g., a statement describing when, where and how a specific behaviour will be carried out). These plans serve to provide the cue to action identified by the Health Belief Model but go beyond this by providing the plan for goal achievement.

Recent empirical work suggests that the approach of providing cues to action in the form of a specific implementation intention improves prediction of behaviour over and above the intention to act alone. Thus, Milne, Orbell and Sheeran (2002) reported improved exercise participation; Sheeran and Orbell (2000) reported beneficial effects on the uptake of cervical cancer screening; Verplanken and Faes (1999) described improved dietary regimens; and Orbell et al. (1997) cited improved rates of breast self-examination.

A study examining uptake in the National Health Service Breast Screening Program (NHSBSP) in the UK (Rutter et al., 2006) has highlighted the importance of providing guidance on how to plan for a behaviour in order to ensure that people move from intention to actual behaviour (i.e., from the preparation to the action stage of the Transtheoretical Model, TTM). In this study, women invited to screen for breast cancer were asked to make specific plans for attending. The plans consisted of organising their travel, arranging to take time off work if necessary and changing the appointment if it was inconvenient. The results indicated that when women produced a *written* plan, actual rate of compliance with the screening appointment was 15% greater than in the control condition (no intervention) and 7% greater than women who failed to write down a plan although instructed to do so. Moreover, the influence from the production of cues to action in the form of a written plan was greatest for those who initially had a high intention to comply but a weak sense of control over making the necessary arrangements to put that intention into effect. This research suggests that uptake of FOBT might be significantly improved by providing a cue to action that seeks to stimulate people to do more than simply express their intention to screen. An effective informational intervention that results in the development of implementation intentions in the form of a plan describing the when, where, and how of faecal occult blood testing, and which enables the individual to deal with their own personal and environmental constraints, should provide those with the intention to act the further resources necessary for achieving their goal.

One possible mechanism for explaining the effectiveness in previous studies of asking participants to form implementation intentions is that doing so forces people to think through the steps necessary for actually completing the screening. This 'thinking through', in turn, may serve to raise people's confidence about their ability to successfully carry out the screening behaviour. Confidence in one's own capacity to act is known in the literature as 'self efficacy' and is widely reported as predicting health behaviour participation (Schwarzer & Fuchs, 1995). People's feelings of self efficacy are likely to be a particular consideration in using the FOBT because the test is performed by the individual and not administered, like mammography or Pap smear, by a health care professional. Previous studies looking at consumer-initiated screening behaviours have shown that feelings of confidence in one's ability to correctly perform the behaviour bear a strong relationship to people's performance of these behaviours. This includes performance of breast self-examination (Luszczynska, 2004), testicular self-examination (Lechner et al., 2002), and FOBT (DeVellis et al., 1990).

2. Aims

This study was designed to investigate the effect of the formulation of implementation intentions upon people's participation in screening using FOBT. We chose to examine uptake of FOBT rather than colonoscopy because, in comparison to the United States, usual CRC screening practice in Australia is by FOBT followed by colonoscopy for those with a positive result—in other words, colonoscopy is regarded as a diagnostic test rather than a screening test.

An additional aim was to monitor the impact upon participation of differing levels of directedness in formulating these intentions and to determine the impact of self efficacy and prior levels of generalised intention upon both implementation intention formation and participation.

Consistent with prior research, it was anticipated that the formulation of implementation intentions (regardless of level of directedness) would increase participation in FOBT over levels of participation in the control group. Furthermore, previous work in the area of preventive health behaviour suggests that people's feelings of self efficacy, or confidence to use the test (the terms 'self efficacy' [SE] and 'confidence' will hereinafter be used interchangeably) can be increased in response to appropriate cues to action, and it was anticipated that the provision of directions for the formulation of implementation intentions would increase people's feelings of self efficacy. It was further hypothesised that those who were already strongly intending to use an FOBT were expected to differ in implementation intention formation and participation from those whose intentions to test were initially weaker.

We conducted two randomised controlled trials to test these hypotheses. Study 1 was a trial conducted amongst a group of eligible, randomly selected males and females who were approached and agreed to participate in the trial. Study 2 was also a randomised controlled trial to examine the generalisability of results to population settings and which differed from Study 1 in that prior commitment to trial participation was not obtained and eligibility was unknown.

3. Study 1

3.1 Methods

3.1.1 Study design

The study was a parallel, randomised, controlled trial, stratified by sex, comparing return of FOBT between three intervention groups and one control group. People in the intervention groups received an FOBT of the immunochemical type (FIT) in the mail together with instructions on how to construct a (1) participant-determined and retained plan, (2) participant-determined and shared plan, or (3) researcher-directed and shared plan. The control group received the FOBT only.

3.1.2 Sample size and selection

Previous studies of implementation intentions have demonstrated that the effect of their formation upon behaviour is medium to large (Gollwitzer & Sheeran, 2006). To achieve statistical power of .80 to detect a medium-sized effect (allowing for the possibility of self efficacy and generalised intention as co-variants) and an alpha of 0.05, we aimed to recruit a minimum of 80 participants in each of the four groups described above. Accordingly,

allowing for non-contactability by telephone, a subsequent rejection rate of 30% and ineligibility, we needed to recruit at least 1600 participants to achieve a final sample size of 320 (160 men and 160 women).

A random sample of 6000 (3000 males, 3000 females) potential invitees aged between 50 and 76 years and residing in southern urban Adelaide, South Australia, was provided by the Australian Electoral Commission (AEC). The Australian Government was conducting a pilot National Bowel Cancer Screening Program (NBCSP) at the same time (2004) so individuals with postcodes within the Federal screening program were deleted from the sample provided.

Telephone contact numbers for the remaining sample were obtained by comparing the list against information contained in the electronic White Pages telephone directory. Those persons for whom telephone contact details were not indicated were excluded from the list, as were those whose address indicated that they resided in a hostel or nursing home; such individuals were unlikely to be in the position of deciding for themselves whether they should screen for CRC. The remaining sample was randomized separately by sex using a random number generator (Microsoft ® Office Excel 2003) and 400 (200 m; 200 f) names were assigned sequentially to one of 4 groups. In total 1642 names were allocated.

3.1.3 Study conduct

The trial proceeded through a number of phases, as described below and illustrated in Table 1.

Phase 1: All potential participants were mailed an advance notification letter and accompanying information, to the effect that an attempt would be made to contact them by telephone to invite them to participate in a study on how best to encourage people to participate in screening for colorectal cancer. Potential participants were advised that they were ineligible to participate if they had ever participated in CRC screening or been diagnosed with CRC or polyps. This exclusion criterion was because in Australia such diagnoses normally follow a positive FOBT and subsequent colonoscopy, and we wanted to target those who had not displayed overt symptoms but were of average risk (that is, based solely on the fact that they were aged 50 years or more) of developing CRC. An opportunity was provided at this point for individuals to decline participation or to indicate that they were ineligible.

Phase 2: One week after the advance notification letter, attempts were made (to a maximum of 3 occasions) to telephone individuals and recruit them to the study. A Computer Assisted Telephone Interview (CATI) format was used by trained interviewers to collect interview responses (Microsoft ® Office Access 2003). For those who were contactable and agreed to participate, informed consent was formally requested and recorded before commencement of the CATI. The recruiting interviewers were blinded to an individual's group allocation until they reached that part of the CATI (after having determined eligibility) that, as part of obtaining informed consent, provided details of the particular intervention to which the participant had been assigned. To those that agreed to participate, the interviewer briefly described what an FOBT was and asked whether they had heard of it: "*Before we contacted you, had you ever heard of a screening test for colorectal cancer, where you are given a set of cards to take home and asked to smear a part of your stool on the cards on two separate occasions, and then return the cards to be tested for blood? This is called a Faecal Occult Blood Test, or FOBT. This is the type of screening test we will be sending you*". Baseline measures were obtained: background demographics, level of commitment to using an FOBT, and confidence to use the kit.

Phase 1	**Recruitment Phase 2**	**Interventions Phase 3**	**Measures Phase 4**	**Measures Phase 5**
N=1642 Potentially eligible participants randomised to study arm then notification of intention to contact by telephone + information sheet mailed	N=994 contactable. N=364 agreed to participate in CATI interview: *Baseline measures:* Demographics Commitment to screen Self efficacy to use FOBT	N=364 *Control* FOBT screening package only (n=91) *Aide to retain* FOBT screening package + implementation plan to be formulated and retained by participant (n=81) *Aide to return* FOBT screening package + implementation plan to be formulated and returned to researcher (n=95) *Checklist to return* FOBT screening package + implementation plan devised by researcher completed and returned to researcher (n=97)	N=350 after exclusions (All groups) *Measures:* Return of kit within 6 weeks Return of kit after 6 weeks	N=328 after exclusions (All groups) *CATI interview measures:* Commitment to rescreen Self efficacy to use FOBT Reasons for not screening (if applicable) Reasons for screening (if applicable)

Table 1. Study 1 interventions by phase and arm, with attrition rates

Phase 3: The day following the recruitment interview, all participants were mailed a screening package which included an immunochemical FOBT. Accompanying the package, intervention groups also received an implementation plan to serve as a 'cue to action' to provide a strategy for goal achievement (completion and return of the FOBT). Two intervention groups received a participant-directed plan in the form of an 'Aide' that *invited* participants to think about, and write down, how they were going to deal with potential barriers to using the FOBT. Suggestions were made as to how these barriers could be addressed. Participants in one of these two groups were asked to retain their completed plan ('*Aide to retain'*); the other group were sent two copies of the plan and requested to

return one copy of the completed plan to CSIRO ('*Aide to return'*). The third intervention group received a plan in the form of a researcher-directed 'Checklist' ('*Checklist to return'*) which *directed* participants to think about how they were going to deal with potential barriers. This group was also provided with two copies of the checklist and asked to return one completed checklist to CSIRO. Thus, those in the intervention groups were invited to formulate implementation plans at differing levels of directedness, and the researchers, through their requirement that two of the intervention groups return a completed plan, were able to verify that in fact a plan had been completed. The control group received a screening package without any accompanying plan.

Phase 4: Receipt of completed FOBTs was recorded by the Bowel Health Service (Repatriation General Hospital, Bedford Park, South Australia) and participation data relayed to the researchers. People who did not return their test after six weeks were sent a reminder letter. Participation in screening was defined as receipt of kit within 6 weeks (before reminder) or after 6 weeks.

Phase 5: Approximately 7 weeks following FOBT despatch, participants were contacted by telephone. Confidence to use the FOBT was again measured, as was (for those who had returned their FOBT) commitment to screen every two years in the future, following recommended screening guidelines. Additionally, participants' reasons for screening or not screening were elicited, depending on whether a completed FOBT had been returned at the time of interview (data not included in these analyses).

3.2 Materials

3.2.1 Development of implementation plans

Two versions of implementation plan were designed; one as an 'aide' and the other as a more prescriptive 'checklist'. Each version was introduced to the participant with the words "*Many people find that they intend to complete the FOBT but then forget or 'never get around to it'. It has been found that if you form a definite plan of exactly when and where you will carry out an intended behaviour you are more likely to actually do so and less likely to forget or find that you don't get around to doing it. It would be useful for you to plan when, where and how you will complete the FOBT. To help you do this, we would like you to use the attached sheets we have provided"* (adapted from Milne et al., 2002). Both plans were designed to support confidence and addressed practical aspects of completing the test (reading the instructions; deciding the most convenient time to use the FOBT; deciding the most convenient location to use the FOBT; preparing for the test; using the FOBT; remembering to use the FOBT; sending the FOBT for analysis). Both versions commenced with the instruction: "*Using this plan, decide when you will use the screening kit, where you will use the kit, and the procedure you will use to carry out the screening test and obtain your result from the Bowel Health Service*". They thereafter differed in their level of directedness in covering the practical aspects. For example, for 'remembering to use the kit' the aides contained the following instruction: "*It is easy to forget to do things unless we have a way to remind us. Decide now how you can make it easier for you to remember – for example, by leaving the kit or this plan in a prominent location, or writing yourself a note. Write below how you will remind yourself to use the kit on two separate occasions"*. In contrast, for the same instruction the checklist stated "*Place a reminder in a prominent place so that you do not forget to use the kit*" with two check boxes (1st sample done; 2nd sample done) to indicate that this instruction had been carried out. The complete documents are available from the first author on request.

3.2.2 Development of self efficacy scale

Self efficacy was measured using 4 items derived from terms developed by Vernon et al. (1997) and our clinical experience of the challenges and impediments surrounding FOBT use. Participants were asked to rate their degree of confidence in surmounting the barriers described. The items were scored on a 5-point Likert scale ranging from *strongly disagree* (1) to *strongly agree* (5). The items were: "I feel confident that I would be able to carry out an FOBT"; "I feel confident that the test will not be overly distasteful or embarrassing"; I feel confident that I would be able to find time in the day to complete the test"; "I feel confident that I could complete the test correctly". The scale had good internal consistency, with a Cronbach alpha coefficient of .86.

3.2.3 Commitment to screen

Commitment to screen was measured in Phase 2 by asking "*Right now, how strongly committed are you to doing this test, where 1 is undecided and 5 is very committed?*". The follow-up interview measured commitment to screen again (for those who had returned their FOBT): "*Now that you have done this screening test once, do you think you'll go on doing it every two years?*" (yes/no answer) and "*Right now, how strongly committed are you to doing this test again, where 1 is undecided and 5 is very committed?*"

3.2.4 Screening offer

The screening package, or kit, included (a) a bowel cancer screening information pamphlet; (b) an immunochemical FOBT ((iFOBT also known as a faecal immunochemical test for haemoglobin [FIT], InSure™, Enterix Australia) that does not require dietary or drug restrictions; (c) a combined Participant Details and Consent Form confirming personal details, nominating a preferred doctor for follow-up, and consent to obtain clinical follow-up reports if required; and (d) a reply-paid return envelope.

3.3 Data analysis

Random missing values on pre- and post self efficacy (SE) variables (17/2800, 0.61%) were imputed using the expectation maximisation method, so that as many observations as possible were available for computing self efficacy total scores. The scores were split at the median baseline SE score of 17; scores ≤16 were designated 'low' and scores ≥17 'high' SE. Participation rates were viewed as 'early' or 'late' at a cut-off point of 6 weeks following despatch of FOBT, at which time a reminder was sent to non-responders. Chi-square analysis was conducted to assess FOBT awareness, FOBT participation and return of implementation plans between groups; Fishers exact test was utilised where cells contained <5. Paired samples t-tests and one-way ANOVAs compared score means for self efficacy and commitment to screen. A median split was not performed for commitment to screen as the majority of people had high intention to screen. Binary logistic regression was used to examine the ability of self efficacy and commitment to screen to predict return of FOBT, and Generalised linear models (GLM) were used to assess interactions between variables. All tests were conducted using a two-sided alpha level of 0.05.

3.4 Results

Recruitment and participation attrition rates are shown at Table 1. From a sampling frame of potential participants (3,000 men and 3,000 women), n=1642 were notified that they would

be contacted and invited to participate. Of n=994 able to be contacted and eligible, n=364 individuals (36.6%) agreed to participate in the study. Subsequently n=14 were excluded from analysis because they didn't receive an FOBT (n=3); had undergone screening since joining the study (n=4); reported symptoms that precluded them from using the FOBT (n=4), or were unable to participate due to barriers unrelated to the study (n=3). Baseline and screening participation data were therefore available for n=350/994 participants (35%).

	Control N=90 (%)	Aide to retain n=79 (%)	Aide to return n=91 (%)	Checklist to return n=90 (%)	Test of difference
Male	48 (53)	41 (52)	44 (48)	34 (38)	X^2 (3)=5.270,
Female	42	38	47	56	p=.153
Age, mean	61.1	60.5	61.2	61.7	NS
Age group**					X^2 (6)=2.236, p=.897
Age 50–59	43 (48)	38 (48)	45 (49)	37 (41)	
Age 60–69	31 (34)	29 (37)	32 (35)	39 (43)	
Age 70–76	15 (17)	11 (14)	14 (15)	13 (14)	
Highest level of education					X^2 (6)=5.894 p=.435
Some high school	46 (51)	35 (45)	39 (43)	52 (58)	
Completed high school/trade	32 (36)	27 (35)	36 (40)	26 (29)	
University qualification	12 (13)	16 (20)	16 (18)	12 (13)	
Country of birth: Australia	67 (74)	57 (72)	71 (78)	61 (68)	X^2 (3)=2.539, p=.468
Never heard of FOBT prior to participation	64 (71)	65 (82)	59 (65)≠	65 (72)	X^2 (3)=5.618 p=.132

*percentages have been rounded so may not be equivalent to 100%
** n=3 missing values for age group
≠n=2 missing values

Table 2. Study 1 Participant demographic characteristics*

At follow-up (post intervention and mailing of FOBT), n=13 participants declined or were unable to be interviewed and n=9 were unable to be contacted; follow-up data were therefore available for n=328/994 (33%) participants.

At recruitment, the groups (n=350 participants) were balanced for gender, mean age, age group, level of education and Australian birth, and awareness of FOBT. The majority of participants had never heard of an FOBT before they were approached, i.e. they were in pre-contemplation stage (Table 2).

3.4.1 FOBT participation

Completed FOBTs were returned by n=286/350 (81.7%) of eligible participants over a period of 15 weeks (mean = 3.12 weeks). Contrary to the hypothesis that formation of implementation plans would improve FOBT uptake, there was no significant difference

between the groups in FOBT participation or return within 6 weeks (i.e., before and after reminder) (Table 3).

	Control N=90 (%)	Aide to retain n=79 (%)	Aide to return n=91 (%)	Checklist to return n=90 (%)	Test of difference
FOBTs returned	76 (84)	66 (84)	70 (77)	74 (82)	X^2 (3)=1.980, p=.577
Return of kits within 6 weeks	67 (74)	61 (77)	62 (68)	66 (73)	X^2 (3)=.869, p=.833
Plans returned*			62	66	X^2 (1)=.367, p=.545

*These numbers do not correspond with participants who returned FOBTs within 6 weeks

Table 3. Study 1 return of kits and implementation plans by group

3.4.2 Return of implementation plans

Most participants who returned a completed FOBT and were also required to return a completed implementation plan did so. There was no significant difference in rate of return between aide and checklist (Table 3), suggesting that differing levels of directedness had no impact on whether the plans were completed. There were no cases of a plan being returned without an accompanying completed kit.

3.4.3 Self Efficacy (SE)

A mixed between-within subjects analysis of variance was conducted to assess the impact of the different interventions on follow-up SE scores. There was no significant interaction between intervention group and time [F(3, 324) = .874, p=.455]. There was a substantial main effect for time [F(1,324) = 46.424, p=<.005), η2 = .125] with groups showing an increase in self efficacy (Time 1, M = 17.45, SD = 1.95; Time 2, M = 18.3, SD = 1.91). The main effect comparing the groups was not significant [F(3,324) = .156, p=.93], suggesting that provision of assistance with planning did not influence SE (Table 4).

	Control mean (SD)	Aide to retain mean (SD)	Aide to return mean (SD)	Checklist to return mean (SD)
Time 1	17.21 (1.81)	17.67 (2.03)	17.50 (1.73)	17.45 (2.22)
Time 2	18.39 (2.04)	18.26 (1.98)	18.32 (1.93)	18.36 (1.73)

Table 4. Study 1 group mean self efficacy scores pre- and post intervention

Subsequent analyses compared self-efficacy between those who returned FOBTs and those who did not. Table 5 shows that when we compared SE over time for FOBT non-returners using a paired samples t-test there was a decrease in confidence that approached significance (p=.08). In other words, the confidence of non-participants to screen was impacted negatively by the provision of the FOBT. By contrast, confidence among those who returned an FOBT

increased significantly, regardless of group assignment. This result suggests that, in general, confidence to complete the test in the future is likely to decrease for those people who don't complete initial screening, regardless of initial level of confidence.

		M	SD	df	t
SE score non-returners (full sample)	Time 1	16.77	1.893	47	1.758
	Time 2	16.15	2.278		
SE score returners (full sample)	Time 1	17.57	1.944	279	8.674***
	Time 2	18.71	1.561		
Low baseline SE score non-returners	Time 1	15.46	1.208	25	0.220
	Time 2	15.35	2.279		
Low baseline SE score returners	Time 1	15.69	.978	116	15.388***
	Time 2	18.32	1.711		
High baseline SE score non-returners	Time 1	18.32	1.287	21	2.752**
	Time 2	17.09	1.925		
High baseline SE score returners	Time 1	18.92	1.202	162	0.489
	Time 2	18.99	1.383		

** p<.01
***p<.001

Table 5. Study 1 mean self efficacy scores pre- and post-intervention, overall and by return/non return of FOBTs

In order to determine whether confidence at baseline influenced reaction to the various interventions, participants were characterised as having a low or high SE score at baseline (determined by a median-split between 16 and 17), and change in confidence over time compared (See Table 5). Low SE non-returners did not significantly change their SE scores post intervention, whereas low SE returners' scores significantly increased post intervention. Similarly, for those with a high SE score at baseline, non-returners' scores significantly decreased post intervention but did not significantly change if they returned an FOBT. This latter result is likely to reflect ceiling effects given that the maximum score possible for SE was 20. These results suggest that self efficacy was increased when the test was completed but the initial level of confidence to complete the test was low, and conversely confidence was decreased when the initial level was high but the test was not completed.

3.4.4 Commitment to screen and maintain screening

At baseline, the majority of people (n=217/343, 63%) were committed or very committed to doing the test (M=4.39, SD=.924; median=5) and there were no group differences (Table 6). Those who returned an FOBT were asked their level of commitment to maintain screening, and just over half (n=137/239, 57.3%) were "very committed" to screening again (M=4.38, SD=.840, median=5, n=47 missing values), regardless of intervention assignment. For those that did return an FOBT, a paired-sample t-test indicated that for the sample as a whole there was a statistically significant decrease in commitment to screen from baseline, ie after exposure to the intervention and FOBT (Table 7). When we examined the relationship between commitment and self efficacy by comparing commitment level between those who had a low or high SE baseline score, it was apparent that the decrease in commitment came from those that had a high initial SE score (Table 7).

	Control mean (SD)	Aide to retain mean (SD)	Aide to return mean (SD)	Checklist to return mean (SD)	ANOVA	
					F, (df)	p
Time 1* (n=343)	4.48 (.844)	4.57 (.854)	4.33 (.974)	4.23 (.984)	2.21 (3, 339)	.087
Time 2** (n=239)	4.41 (.938)	4.33 (.816)	4.53 (.704)	4.24 (.878)	1.25 (3,235)	.294

*Includes non-returners and returners; 7/350 missing values
**Includes only those who returned an FOBT; 47/286 missing values

Table 6. Study 1 mean commitment to screen by group pre- and post intervention

		M	SD	df	t
Commitment to screen (full sample, n=233)	Time 1	4.52	0.804	232	2.15*
	Time 2	4.38	0.843		
Low baseline SE score commitment to screen (n=99)	Time 1	4.20	0.947	98	-1.522
	Time 2	4.36	0.814		
High baseline SE score commitment to screen (n=134)	Time 1	4.76	0.578	133	4.485***
	Time 2	4.39	0.866		

*<.05
***<.001

Table 7. Study 1 FOBT returners' commitment to screen pre- and post-intervention, overall and by SE level at baseline

3.4.5 Effect of self efficacy and commitment to screen on use of FOBT

Logistic regression was used to assess the independent and joint effects of baseline SE and baseline commitment to screen on return of FOBT. SE alone made a statistically significant contribution, X^2 (1, n=350)=11.535, p<.001, OR=1.27, CI 1.10-1.47), predicting 5.3% of the variance (Nagelkerke R squared) in screening uptake. Commitment to screen alone also made a statistically significant contribution X^2 (1, n=343)=13.837, p<001, OR=1.67, CI 1.28-2.18), and explained 6.4% of the variance. When these predictors were entered together into the logistic regression model, there was a statistically significant effect, X^2(2, n=343)=17.487, p<.001), but only commitment to screen displayed a unique and statistically significant contribution (p=.012, OR=1.46, CI 1.07-1.97); baseline self efficacy was marginally significant (p=.06, OR=1.17, CI.993-1.37). This suggests that those who are committed to using the FOBT will do so regardless of their level of confidence. The total variance explained by the combined model was R^2=8.0%, indicating that factors other than these also contribute to the likelihood of completing an FOBT.

4. Study 2

Study 2 was conducted to examine the generalisability of Study 1's results to the broader population. This approach more closely approximated that undertaken in current population screening programs utilising FOBTs.

4.1 Methods

4.1.1 Sample size and selection

Sample selection proceeded as described for Study 1. A separate sample of 6000 men and women aged between 50 and 76 years, randomly selected from four South Australian electoral divisions, was obtained from the AEC. People residing in postcodes included in the pilot NBCSP were omitted from the sample, as were those whose address indicated they resided in a hostel or nursing home. The remaining sample was randomised separately by sex and 400 men and women were assigned sequentially to one of 4 groups. In total 1600 names were allocated.

4.1.2 Study conduct

Phase 1: All potential participants were mailed an advance notification letter (which aligns with the protocol adopted by the NBCSP) and accompanying information as for Study 1, and were informed that they would shortly be receiving a screening package in the mail. Exclusion due to ineligibility was dependent upon self-identification and communication of this fact to the researchers before despatch of FOBT. Willingness to participate was not deliberately ascertained.

Phase 2: Three weeks after the advance notification letter, a screening kit including an immunochemical FOBT was sent to individuals. As for Study 1, intervention groups also received a discrete implementation plan. The nature of this approach precluded us from ascertaining willingness to participate and from obtaining pre- and post measures of self efficacy and commitment to screening.

Phase 3: Receipt of completed FOBTs was recorded by the Bowel Health Service and participation data relayed to the researchers.

4.1.3 Data analysis

Participation rates were viewed as 'early' or 'late' at a cut-off point of 6 weeks following despatch of FOBT, when a reminder was sent to non-responders. Chi-square analysis was conducted to assess FOBT participation between groups.

4.2 Results

N=1600 men and women were sent an advance warning letter. Those who did not identify themselves as ineligible or not wishing to participate were then mailed a screening kit and accompanying material according to intervention group. In total, n=225 were excluded from the study (n=118 identified themselves as ineligible; n=83 didn't wish to participate; n=24 packages were undeliverable). Analyses were therefore conducted for n=1375 men and women. Recruitment and participation attrition rates are shown at Table 8.

At baseline, the groups were balanced for gender (Table 9). It wasn't possible to ascertain age group breakdowns because the AEC supplied a random sample within an age range (50–74 years) which wasn't broken down into groups (for Study 1 we ascertained age from the participant). The study design also precluded us obtaining other demographic information (mean age, education, country of birth) as we did for Study 1. However, given that the underlying sampling mechanism was identical (i.e., supplied by the AEC), there is some confidence that the groups were balanced on these other factors.

Recruitment Phase 1	Interventions Phase 2	Measures Phase 3
N=1600 Potentially eligible participants randomised to study arm then mailed information sheet and notification that they would shortly receive an FOBT kit. Ineligibility was defined and dependent upon self-report	*Control* FOBT screening package only (n=400) *Aide to retain* FOBT screening package + implementation plan to be formulated and retained by participant (n=400) *Aide to return* FOBT screening package + implementation plan to be formulated and returned to researcher (n=400) *Checklist to return* FOBT screening package + implementation plan devised by researcher to be completed and returned to researcher (n=400)	(All groups, n=1375) Return of kit within and after 6 weeks

Table 8. Study 2 interventions by phase and arm, with attrition rates

	(Control) n=345 (%)	Aide to retain n=350 (%)	Aide to return n=334 (%)	Checklist to return n=346 (%)	Test of difference
Male Female	176 (51.0) 169	176 (50.3) 174	170 (50.9) 164	178 (51.4) 168	X^2 (3)=0.96, p=.992

Table 9. Study 2 participant demographic characteristics

4.2.1 FOBT participation

Completed FOBTs were returned by 548/1375 (39.9%) of participants over a period of 26 weeks (mean = 5.51 weeks). This rate is similar to that achieved in the NBCSP in 2008 (i.e., 41% (AIHW, 2010). As for Study 1, contrary to our hypothesis that the formation of implementation plans would improve FOBT uptake, there was no significant difference between the groups in FOBT participation or return within 6 weeks (before and after reminder) (Table 10).

	Control n=345 (%)	Aide to retain n=350 (%)	Aide to return n=334 (%)	Checklist to return n=346 (%)	Test of difference
FOBTs returned	144 (41.7)	131 (37.4)	131 (39.2)	142 (41.0)	X^2(3)=1.633, p=.652
Return of kits within 6 weeks	106 (30.7)	98 (28.0)	97 (29.0)	94 (27.2)	X^2 (3)=3.269, p=.352
Return of plans with FOBT			83/131 (58.4)	62/142 (43.6)	X^2 (1)=9.389, p=.001

Table 10. Study 2 overall return of kits and within 6 weeks (i.e. before reminder) by group

4.2.2 Return of implementation plans

A considerable proportion of those who returned an FOBT and were also required to return a completed implementation plan did not do so, and significantly fewer people returned the prescriptive plan (i.e., checklist) than the aide (Table 10), suggesting that level of directedness may have an effect on whether the plans were completed—those who were required to formulate their own plan based on suggestions for action were more likely to return a plan compared to those given a prescriptive checklist. Notwithstanding this result, given that the requirement for return was to act as an indicator of whether plans had actually been formulated, it appears that around half the participants used the FOBT without adhering to planning instructions, particularly those who received a prescriptive plan.

5. Discussion

We hypothesised that the formation of implementation plans would assist return of FOBT kits by providing a physical cue to action. In addition we hypothesised that the process of completing a plan would increase confidence in ability to complete the test and that those who were strongly committed to screening at baseline would differ in formation of implementation plans and participation to those with a less strong initial commitment.

Notwithstanding the difference in overall participation figures between Study 1 (81.7%) and Study 2 (39.9%), we found that for both studies provision of assistance with planning, regardless of directedness, had no influence on completion of an FOBT. The lack of influence of an implementation plan concurs with the conclusions of other researchers who have also found no effect of implementation planning on subsequent behaviour (Jackson et al., 2005; Michie et al., 2004; Rutter et al., 2006; Skar et al., 2011). Even so, this result goes against the large body of evidence suggesting that formulating action plans has a positive effect on the intention-behaviour gap. It has been suggested, however, that there exists sparse evidence for a positive effect of implementation intentions on behaviours outside student samples, who are more likely to comply with task demands (actually formulating the plan) (Jackson et al., 2005; Schweiger Gallo & Gollwitzer, 2007). It has also been argued that implementation plans are only effective where there is motivation to achieve a goal (Sniehotta, 2009) and that where goal intentions are positive, so will be the effects of implementation intentions (Gollwitzer, 1993; Oettingen et al., 2000). The majority of FOBT returners in Study 1 already had a high intention to screen, which may be attributable to the fact that that they had made a conscious decision to participate and were presumably more motivated to act, but in any case there was no evidence of a differential effect of combining high commitment with formation of implementation plans on FOBT return. Indeed, the high proportion of implementation plans returned by Study 1 participants (82%) may be indicative only of compliance with the study requirements (i.e., to return plans) rather than evidence of the use of these plans.

However, and in contrast to Study 1, it is evident that nearly half the FOBTs returned in Study 2 were completed without making a plan, a result which could reasonably be extrapolated to the group that was asked to retain their formulated plan. It has been suggested that non-completion may reflect ambiguity of study instructions (Michie et al., 2004) but, given that nearly all Study 1 participants returned identically-constructed implementation plans with a completed FOBT, this was not the case in our population. Rather, this outcome suggests that some felt they had no need to complete plans, perhaps because their intentions were sufficiently strong to make the use of plans unnecessary.

Indeed, we found from Study 1 that commitment had the most significant influence on FOBT use—because the majority of participants were strongly committed, we were unable to determine if having a weak level of commitment would influence formulation of an implementation plan or use of the FOBT. Of those Study 2 participants that did formulate and return plans, significantly fewer used the prescriptive 'checklist' format. Participants may have been "turned off" by the directedness of the checklist, particularly since they were a population sample and had not made a mindful decision to participate in a study. Study 1 demonstrated that provision of directions did not increase people's self efficacy. These results accord with a meta-analysis of 66 randomised controlled studies that concluded that forming implementation intentions had negligible effects on self efficacy and goal intentions (Webb & Sheeran, 2008).

For the group as a whole, baseline self efficacy did not have a strong influence on whether people used the test; rather, the act itself of completing the FOBT determined confidence—self efficacy was increased when the initial level of confidence to complete the FOBT was low, and conversely confidence was decreased when the initial level was high but the test was not completed. Rather than confidence to use the FOBT, from Study 1 it appears that being initially committed to screening had a more significant influence on whether people actually did use the FOBT, confirming the general consensus that intention to perform a behaviour is a necessary precursor of action. Even so, we found in Study 1 that commitment to screening, while a significant predictor of FOBT use, in conjunction with self efficacy explained only 8.0% of the variance, indicating that other factors exist which contribute to the likelihood of completing an FOBT. For example, Gregory et al. (2011) found that social-cognitive predictors of intention to screen for CRC and actual screening behaviour, although overlapping, were not the same, and Power and colleagues (2008) in their study of CRC screening found that life difficulty variables were better predictors of action than intention.

It is puzzling to note that there was a significant decrease in commitment to repeat screening by those that did use the FOBT and had a high initial level of confidence, in contrast to those with low confidence whose level of commitment to screening did not change. It may be that initial commitment was high for most because the participants were an 'interested' sample, and that those with high SE who screened reinforced their view that they were capable of completing an FOBT without necessarily moving from that conclusion and forming a commitment to rescreen. Conversely, those with low confidence but who did complete their test, thereby increasing their confidence, could have felt 'motivated' to repeat the experience again and so not changed their level of commitment. Interestingly, the same lessening of intention by those with high self efficacy was noted in a study examining the role of self efficacy in testicular self-examination (Umeh & Chadwick, 2010). The researchers found that those with high self efficacy appeared to have worsened attitudes toward self examination when both vulnerability and severity estimates were low. The same situation could well apply to CRC screening, particularly as perceived susceptibility is a Preventive Health Model (PHM) construct demonstrated to be associated with CRC screening ((Flight et al., 2010; Tiro et al., 2005). Commitment to future CRC screening in one or 2 years would perhaps, as Umeh and Chadwick (2010) have suggested, be temporarily rejected if the penalties of inaction are deemed insignificant, a viewpoint which may stem from a defensive reaction activated by anxiety. This view suggests that an emphasis on the development of messages designed to increase perceptions of personal risk of CRC without raising anxiety are warranted.

The low rate of participation in Study 2 may reflect a dissonance of messages appropriate to an individual's stage of readiness to screen (Prochaska, 2008). The differences in study design, particularly recruitment strategy, between studies 1 and 2 may have resulted in basic sample differences in stage of readiness to screen at baseline. Specifically, including only participants prepared to complete questionnaires in Study 1 resulted in a highly committed sample, likely to be in contemplation or preparation to act stage, characterised by a high participation rate. By contrast, Study 2 invitees were a population sample, most of who were probably in pre-contemplation on receipt of the FOBT, with participation rates comparable with those achieved by the national screening program (i.e. ~ 40%). Pre-contemplation is a stage where it could be argued that a person's knowledge, attitudes and intentions are in a more unstable state. People in this stage have been shown to have higher barriers, higher chance health locus of control, low powerful others health locus of control, lower perceived susceptibility and lower CRC knowledge (Gregory et al., 2011). It follows that these factors should be addressed to facilitate movement through contemplation to the action stage. However, our implementation plans as formulated were aimed at those with an intention to act and focused on the where, when and how of successful completion of the FOBT. It could be daunting for those who had never heard of FOBT screening to receive a test and accompanying material designed to assist with completing the test without first being given information aimed at overcoming barriers and lack of knowledge associated with the pre-contemplation stage.

6. Conclusion

The provision of assistance with the preparation of implementation plans, regardless of their level of directedness, had no influence on FOBT participation in the 2 studies conducted. One reason for their lack of effect may be that the majority of participants were likely to be in pre-contemplation stage in Study 2 and in the action stage in Study 1. Thus ceiling effects limited the potential for cues to impact behaviour among participants in Study 1, and Study 2 participants may have benefited from an intervention that tackled Contemplation as an intermediary to Action. This stage mismatch has implications for population-based screening programs and may contribute toward less than optimal screening uptake rates. Future research could usefully address the potential for the communication within a population setting of material targeted to specific decision stages, designed to progressively move an individual toward action and maintenance of action. Our research indicated that confidence to screen and commitment to screen separately and together exerted a greater influence on actual FOBT participation; however, these factors accounted for a small amount of variance and future research should address the contribution of other factors.

7. Acknowledgements

We would like to thank Ian Zajac, CSIRO, for timely statistical advice.

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5

Psychological Impact and Associated Factors After Disclosure of Genetic Test Results Concerning Hereditary Nonpolyposis Colorectal Cancer

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1. Introduction

Advances in genetics in recent years have made major contributions to the development of medical genetics. The existence of "familial tumors" has been recognized, and genetic testing, with a potentially incalculable benefit to humanity, is being attempted (Offit, 1998). Numerous gene analyses related to the genesis and development of colorectal cancer have been conducted, and the existence of hereditary colorectal tumors in the form of hereditary nonpolyposis colorectal cancer (HNPCC) and familial adenomatous polyposis (FAP) has been identified.

HNPCC is caused by inherited germline mutations in mismatch repair genes and accounts for 2 -5% of colorectal cancers. The condition is characterized by young-onset, synchronous and metachronous tumors, and a predisposition to gynecologic, urinary tract, and extracolonic gastrointestinal cancers. Genetic testing usually begins with a family member who has been diagnosed with an HNPCC syndrome-related cancer (proband). If a deleterious mutation is identified, testing can be offered to the proband's family members, since they are at risk of carrying the mutation. Knowing one's genetic risk for hereditary cancers may facilitate the early detection or prevention of cancer.

However, in contrast to the advances in scientific techniques, a great deal of apprehension exists with regard to the psychological or ethical, legal, and social issues (ELSI) associated with the application of these techniques. Since important personal genetic information that does not change throughout one's lifetime is handled during genetic diagnosis and an individual's genetic information is partly shared with blood relatives, with the impact of such genetic information not being limited to the individual, we find ourselves in a situation where new life health-care norms that also take psychosocial aspects into consideration are required. For this reason, a variety of studies have been conducted regarding the psychosocial aspects involved in the screening-test-taking behavior of high-risk people, the psychological aspects of high-risk people, interest in genetic counseling and genetic testing, and the psychosocial effects of genetic counseling. Studies on psychosocial aspects after being informed of the test results have also been reported recently, but many of these studies are concerning hereditary breast and ovarian cancer, and very few studies

examining the impact of genetic testing for hereditary colorectal tumors have been performed.

In this article, the psychological consequences related to HNPCC are reviewed with regard to the following four points: (1) attitude toward genetic testing, (2) risk perception, (3) psychosocial effects of genetic counseling, and (4) psychosocial aspects after undergoing genetic testing and being informed of the test results. I have reviewed and selected nearly all the articles regarding these themes using the PubMed database.

2. Attitude toward genetic testing

Many subjects who undergo genetic counseling for HNPCC also wish to undergo genetic testing. However, some subjects refuse to undergo genetic testing, despite its potential benefits. Some previous studies investigated the relationships between the intention to undergo genetic testing and psychosocial variables.

Hadley et al. (2003) investigated attitudes, intention, and the completion of genetic testing among 111 newly identified family members (first-degree relatives) of individuals with HNPCC. Most (97%) stated their intention to pursue testing. Fifty-one percent reported that learning about their children's risks was the most important reason to consider testing. The participants' intentions to pursue genetic testing were significantly affected by concerns regarding their ability to handle the emotional aspects of testing and the psychosocial effect on family members. On the other hand, 39% identified the potential effect on their health insurance as the most important reason not to undergo testing.

Wakefield et al. (2007a) qualitatively assessed 22 individuals' attitudes toward genetic testing for HNPCC. The most frequently reported pros were "to help manage my risk of developing cancer", "to help my family", and "to know my cancer risk." The participants expressed concern about the potential psychological impact of genetic testing. The authors also found that some affected individuals may not fully comprehend the meaning of their potential test results.

Wakefield et al. (2008) conducted a randomized trial to measure the effectiveness of a tailored decision aid designed specifically to assist individuals to make informed decisions regarding genetic testing for HNPCC. The decision aid explains the evidence available regarding HNPCC-related cancer risks, the differences between a mutation search and predictive testing, and the potential benefits, risks, and limitations of testing (Wakefield et al., 2007b). One hundred and fifty-three individuals were randomly assigned to a group who received the decision aid or a group who received a control pamphlet. Evaluations were conducted 1 week after consultation and 6 months after the completion of the intervention using a questionnaire, and 95 subjects completed the 6-month follow-up questionnaire. Although the decision aid had no significant effect on the actual genetic testing decision, the participants who received the decision aid had significantly lower levels of decisional conflict regarding genetic testing and were more likely to be classified as having made an informed choice concerning genetic testing than participants who received a control pamphlet. Furthermore, men who received the decision aid had significantly higher knowledge levels regarding genetic testing than men who received a control pamphlet.

These reports suggest that most individuals pursue genetic testing to help manage their own risk of developing cancer and to learn about their children's risks. On the other hand,

however, concerns about psychological and psychosocial issues may present barriers to undergoing genetic testing. The development of patient education tools, such as the decision aid, is needed.

3. Risk perception

HNPCC mutation carriers have a life-time risk of colorectal cancer of about 80%, while female carriers have a 40-60% risk of endometrial cancer and a 10-15% risk of ovarian cancer. Communicating cancer risk and assessing the perceived risk is very important for genetic counseling because of subsequent cancer prevention behavior or cancer-related distress. Four reports were extracted regarding risk perception among individuals at risk for HNPCC.

Codori et al. (2005) assessed the effect of genetic counseling on perceived lifetime risk and cancer-distress among 101 adult first-degree-relatives of colorectal cancer patients from families with known or suspected HNPCC. Most persons overestimated their cancer risk, and a higher perceived risk was associated with believing that colorectal cancer cannot be prevented. The individual perceived risk changed after counseling, although the mean perceived risk was unchanged.

Domanska et al. (2007) investigated the perceived cancer risk among 47 HNPCC mutation carriers and correlated the findings with individual characteristics. A perceived risk of colorectal cancer above 60% was reported by 49% individuals, and only one reported a perceived risk > 80%. Female mutation carriers, individuals under the age of 50 years, and individuals who received their counseling within 1 year prior to the study reported a higher perceived risk of colorectal cancer. Individuals who had lost a parent to HNPCC-related cancer at an early age also reported a higher perceived risk. Regarding gynecological cancer, 33% of the women reported a perceived risk of 40-60% for endometrial cancer, whereas the remaining 67% either underestimated or overestimated their risk.

van Oostrom et al. (2007) studied the difference in cancer risk perception among 271 individuals who opted for genetic cancer susceptibility testing for a known familial BRCA1/2 or HNPCC related germline mutation. The assessment was conducted before, 1 week after, and 6 months after disclosure of the test results. Individuals from BRCA1/2 and HNPCC mutation families did not differ with regard to their risk perceptions over time. Individuals from BRCA1/2 families perceived hereditary cancer as being more serious.

Grover et al. (2009) examined colorectal cancer risk perception among individuals tested for mismatch repair genes mutation and identified factors associated with an appropriate interpretation of their cancer risk. In this study, in particular, the authors paid attention to individuals with an indeterminate genetic test result. Pathogenic mutations in *MLH1* and *MSH2* have been identified in only 30% to 64% of families who meet the clinical criteria for HNPCC and have undergone testing. Genetic testing may not yield a definitive result because of the lack of an identifiable mutation in one of the known genes or a mutation of unclear pathogenic significance. In the absence of an identified family mutation, these results are considered indeterminate or uninformative. Patients remain at an increased risk for colorectal cancer, and intensive cancer screening recommendations are made based on their personal and family cancer histories. A total of 159 individuals who met the Revised Bethesda Guidelines and had previously undergone genetic testing participated in this study. Ninety individuals with a pathogenic mutation (true positive) correctly estimated their cancer risk. However, only 62% of individuals with an indeterminate genetic test result

correctly estimated their risk. Individuals with a history of HNPCC-associated cancer or indeterminate genetic test results were significantly less likely to estimate their cancer risk as being increased.

These reports suggest that despite educational efforts and an increasing amount of data on the risk of cancer associated with HNPCC, few individuals report a perceived risk that is actually correct. In particular, individuals at risk for HNPCC who receive an indeterminate genetic test result may be falsely reassured. It is important that health care providers continue to device a counseling approach for promoting a correct understanding of cancer risk and for discussing the implications of uninformative results on the lifetime cancer risk.

4. Psychosocial effects of genetic counseling

Cancer genetic counseling has become popular as a result of the recent development of genetic tests that pinpoint familial cancer risk. Such counseling is composed of presymptomatic risk assessment and management (cancer risk counseling) and reproductive risk counseling. The former has two components: risk assessment and counseling regarding behavioral, medical, and surgical options to decrease risk. A basic goal of cancer risk counseling is to derive and explain an individual's cancer risk in clear terms, and the counselor's role is to educate and enumerate options for patients and clinicians, answer questions regarding what is known, and suggest appropriate referrals to help individuals reach difficult decisions.

A cancer risk counseling session is comprised of the following components: 1) baseline risk perception; 2) medical history and exposure history; 3) pedigree construction and pedigree documentation; 4) empiric risk assessment and genetic risk assessment; 5) options for early detection and prevention; 6) options, risks, and benefits of genetic testing; and 7) response to questions, support, and plans for follow-up. Throughout these discussions, a sensitivity to the psychological and ethical aspects of counseling is essential. Therefore, continued follow-up by the counselor after the session is the best way to limit the potential for adverse effects as a result of the knowledge of an inherited cancer risk, and ready access to liaison mental health professionals with experience in cancer genetics is thought to be a valued asset of cancer risk counseling.

Psychological research on aspects of cancer genetic counseling has focused on three broad areas: factors predicting interest in cancer genetic testing (Lerman et al., 1996), the psychological impact and effect of genetic counseling and testing for inherited cancer risk (Lerman et al., 1997), and the relationship between psychological distress and preventive behaviors (Kash et al., 1992). In each of these areas, the results have implications for the management of at-risk individuals. However, such data is unlikely to be applicable to every case because of cultural differences among study populations and the complexity of the instruments used in research studies, in addition to the fact that most of these studies have been performed for hereditary breast cancer. In this section, four studies on the psychological impacts of genetic counseling regarding HNPCC are reviewed.

Keller et al. (2002) explored distress before and after comprehensive interdisciplinary counseling in families at risk for HNPCC. Sixty-five individuals (31 patients with colorectal cancer and 34 unaffected at-risk persons) participated in this study. Data were collected from semi-structured questionnaires before, as well as 4-6 weeks after counseling. Distress declined after counseling, as did worries related to HNPCC. A trend toward a greater anticipated ability to cope with a positive gene test was also observed after counseling. Changes after counseling were generally more pronounced for persons at risk, compared

with those for patients with cancer. A substantial minority, however, said that they experienced increased worry and physical symptoms after counseling.

Bleiker et al. (2007) examined: 1) levels of cancer-specific distress more than one year after genetic counseling for HNPCC; 2) associations between sociodemographic, clinical and psychosocial factors and levels of distress; 3) the impact of genetic counseling on family relationships; and 4) the social consequences of genetic counseling. One hundred and sixteen individuals who participated in this study completed a self-report questionnaire by mail an average of 4 years after the last counseling session. Among all the subjects, 6% had clinically significant levels of cancer-specific distress (Impact of Event Scale). Having had contact with a professional psychosocial worker for cancer risk in the past 10 years was significantly associated with higher levels of current cancer specific distress. Only a minority of the subjects reported any adverse effects of genetic counseling on communication regarding genetic counseling with their children, family relationships, obtaining life insurance, choice or change of jobs, and obtaining a mortgage.

Keller et al. (2008) conducted a prospective study that examined the impact of multidisciplinary risk counseling on the psychosocial outcome of 139 affected cancer patients and 233 family members without cancer but at risk for HNPCC. Participants completed questionnaires specific to HNPCC before and 8 weeks after attending the cancer clinic. The levels of distress among affected patients exceeded those of unaffected individuals, as did worry regarding their relatives' risk. A significant reduction in general anxiety (Hospital Anxiety and Depression Scale), distress specific to familial colorectal cancer (Impact of Events Scale), and general cancer worry (Distress due to Hereditary Disorder) was demonstrated after counseling among both the affected patients and unaffected individuals. The reduction in distress was more pronounced among affected patients given a high risk of HNPCC than among those with an intermediate risk.

Hasenbring et al. (2011) prospectively examined the impact of an initial interdisciplinary genetic counseling on feelings of anxiety with a special focus on subgroups related to personal cancer history, sex, age, and education. A significant interaction between time, sex, and age was identified for change in anxiety. While women in general and men older than 50 years revealed a significant reduction in anxiety, younger men did not show any change over time. A logistic regression analysis indicated that clinical Hospital Anxiety and Depression Scale-A cases could be predicted based on general distress (Brief Symptom Inventory) as well as by HNPCC-related cognitions of intrusion and avoidance (Impact of Event Scale) with a correct classification of 86%.

These studies indicate that anxiety and cancer-specific distress are reduced after genetic counseling, suggesting an overall beneficial impact of comprehensive counseling. On the other hand, a minority of individuals, such as cancer-affected younger men, exhibited adverse effects of genetic counseling on psychosocial variables. Thus, healthcare providers (genetic counselors, human geneticists, oncologists, and psycho-oncologists) should always be aware of psychosocial issues after genetic counseling. However, as little data is available on the psychosocial effects of genetic counseling regarding HNPCC, further data accumulation is needed.

5. Psychosocial aspects after being informed of genetic test results

Since 1991, when a gene for hereditary cancer was first identified, studies expressing concern about the psychosocial aspects of gene diagnosis began in Western countries, with

the results starting to be reported in 1993. Although studies investigating psychosocial aspects after the subjects had undergone actual genetic testing and had been informed of the test results have been reported, many of these studies have concerned hereditary breast and ovarian cancer, and only a few studies have been performed for HNPCC. Furthermore, little is known about the factors associated with psychosocial aspects. However, HNPCC testing might offer more benefit than hereditary breast and ovarian cancer testing because of the differences in the risk management options available to mutation carriers. In HNPCC, a colonoscopy every 1–2 years is more effective for detecting and preventing adverse health outcomes than measures available to carriers of hereditary breast and ovarian cancer mutations. Therefore, identifying the psychosocial situations in which individuals at risk for colorectal cancer have lived after the disclosure of genetic information or the way in which healthcare providers are able to support the mental states of these individuals are important.

Ten original articles (review articles were not included) assessing psychosocial aspects after individuals had been informed of genetic test results regarding HNPCC were extracted. In this chapter, cross-sectional studies that assessed psychosocial aspects at one time point after disclosure and prospective studies that followed-up psychosocial aspects for 1 year or longer after disclosure are described separately. A summary is shown in Table 1.

5.1 Cross-sectional studies assessing psychosocial aspects after the subjects had been informed of the test results

Four articles were extracted. Esplen et al. (2001) investigated psychosocial function in 50 individuals who were engaged in the genetic test process for HNPCC (the period between the psychosocial assessment and the disclosure of the test results was 1 – 48 months). Twenty-three individuals were identified as carriers (13 had a previous history of CRC), seven were non-carriers and 20 individuals were still awaiting their test results. The psychosocial scores demonstrated that a subgroup of individuals exhibited distress, with greater distress for those individuals awaiting results or testing positive. A high level of satisfaction was associated with the experience of testing.

Claes et al. (2004) assessed the short-term impact (1month after test result disclosure) of genetic testing using a semi-structured interview and self-reported questionnaires. The subjects were 40 cancer-unaffected relatives who had undergone predictive testing for HNPCC. Distress was within the normal ranges. Distress decreased significantly from pre- to post-test in non-carriers but not in carriers.

Murakami et al. (2004) identified the prevalence rates and predictors of psychological distress and evaluated the feelings of guilt at one month after the disclosure of test results in Japanese probands and unaffected relatives. The prevalence of major and minor depression, acute stress disorder (ASD), posttraumatic stress disorder (PTSD), and posttraumatic stress symptoms (PTSS) were assessed using the Structured Clinical Interview based on the Diagnostic and Statistical Manual of Mental Disorders, 3rd edition revised (DSM-III-R) or the DSM-IV; feelings of guilt were investigated using a numeric scale and a semi-structured interview. Forty-two participants completed the 1-month follow-up interview. Although none of the participants met the criteria for major depression, ASD, or PTSD at the time of the follow-up interview, 7% of the participants met the criteria for minor depression and 5% had PTSS. The only predictor of psychological distress was the presence of a history of major or minor depression. Twelve percent of the participants had feelings of guilt.

Author, year	Subjects	Study design	Assessment period after disclosure	Study method / outcome measures	Main study findings	Associated factors
Esplen et al, 2001	50 affected and unaffected individuals	Cross-sectional	1 – 48 months	Questionnaires / CES-D, IES, STAI	The psychosocial scores demonstrated that a subgroup of individuals exhibited distress, with greater distress for those individuals awaiting results or testing positive.	Disclosure their test results to family and non-family members
Aktan-Collan et al, 2001	271 unaffected individuals	Prospective	1 and 12 months	Questionnaires / STAI	The mutation-positive subjects were more anxious than their counterparts immediately after the test disclosure, but the differences had disappeared at the follow-ups. In other variables, neither differences between the groups defined by mutation status nor changes with time were detected.	Not shown
Claes et al, 2004	40 unaffected individuals	Cross-sectional	1 month	Questionnaires / SCL-90, STAI	Distress was within normal ranges. Distress decreased significantly from pre- to post-test in non-carriers and did not in carriers.	Not shown
Murakami et al, 2004	42 affected and unaffected individuals	Cross-sectional	1 month	Semi-structured interview / major depression, minor depression, ASD, PTSD, PTSS, guilt	Although none of the participants met the criteria for major depression, ASD, or PTSD at the follow-up interview, 7% of participants met the criteria for minor depression and 5% had PTSS. Twelve percent of participants had feelings of guilt.	Presence of history a major depression

Table 1. (continued)

Meiser et al, 2004	40 unaffected individuals	Prospective	2 weeks, 4 months and 12 months	Questionnaires / HADS, IES, STAI	Carriers showed a significant increase in mean scores for intrusive and avoidant thoughts 2 weeks and a significant decrease in mean depression scores 2 weeks and 4 months. For non-carriers, significant decreases in mean scores for intrusive and avoidant thoughts, depression scores and mean state anxiety scores were observed at all follow-up assessment time points.	Not shown
Gritz et al, 2005	155 affected and unaffected individuals	Prospective	2 weeks, 6 months and 12 months	Questionnaires / CES-D, IES-R, QLI, STAI	Mean scores on all outcome measures remained stable and within normal limits for cancer-affected participants. Among unaffected carriers, mean depression and state anxiety scores increased from baseline to 2 weeks and decreased from 2 weeks to 6 months. Among unaffected non-carriers, mean depression and anxiety scores did not differ..	Baseline mood disturbance, lower quality of life, and lower social support
Claes et al, 2005	72 unaffected individuals	Prospective	1 month and 12 months	Questionnaires / IES, SCL-90, STAI	Mean levels of distress (cancer-specific distress, state anxiety, psychoneuroticism) were within normal ranges and none of the participants had an overall pattern (on all scales) of clinically elevated levels of distress.	Not shown

Table 1. (continued)

Collins et al, 2007	73 unaffected individuals	Prospective	2 weeks, 4 months, 1 year, and 3 years	Questionnaires / HADS, STAI, IES-R	Mean cancer-specific distress in carriers increased at 2 weeks with a return to baseline levels by 12 months. This level was maintained until 3 years. Non-carriers showed sustained decreases after testing with a lower level at 3 years compared with baseline. Mean depression and anxiety scores did not differ between carriers and non-carriers and, at 3 years, were similar to baseline.	Not shown
Yamashita et al, 2008	46 affected and unaffected individuals	Cross-sectional	1 month	Questionnaires / IES-R	Comparison of the IES-R scores showed that they tended to be higher in the mutation-positive group, but the differences were not statistically significant.	Personality tendency "nervousness", Verbal memory
Shiloh et al, 2008	253 affected and unaffected individuals	Prospective	6 months and 12 months	Questionnaires / CES-D, IES-R	Mean reductions were indicated in distress and depression levels within the first 6 months after testing. The interaction between time and mutation was neither significant for distress nor for depression.	Coping style (high monitors)

CES-D: Center for Epidemiological Studies-Depression, IES: Impact of Event Scale (IES-R: Impact of Event Scale-Revised), HADS: Hospital Anxiety and Depression Scale,
QLI: Quality of Life Index, SCL-90: Symptom Checklist, STAI: State-Trait Anxiety Inventory
ASD: acute stress disorder, PTSD: post-traumatic stress disorder, PTSS: post-traumatic stress symptoms

Table 1. Characteristics of studies on psychosocial aspects and associated factors after being informed of genetic test results regarding HNPCC

Yamashita et al. (2008) elucidated the psychological impact at one month after the disclosure of genetic test results regarding HNPCC and assessed the associated factors, focusing on memory function in particular. The subjects were persons who were suspected of having HNPCC and had been given the choice of undergoing genetic testing. The post-genetic testing psychological impact was evaluated using the Impact of Event Scale-Revised (IES-R), and personality tendencies and memory function were evaluated. Final data were obtained from 46 Japanese probands and unaffected relatives (mutation-positive in 18 subjects, uninformative in 18 subjects, and mutation-negative in 10 subjects). A comparison of the IES-R scores showed that they tended to be higher in the mutation-positive group, but the differences were not statistically significant. The personality tendency "nervousness" and the verbal memory assessed prior to disclosure were significantly associated with the total IES-R score.

5.2 Prospective studies assessing psychosocial aspects after the subjects had been informed of the test results

Six articles were extracted. Aktan-Collan et al. (2001) assessed general anxiety, fear of cancer and death, satisfaction with life, and attitude regarding the future using a questionnaire survey in 271 individuals with no personal cancer history who were tested for HNPCC. Measurements were made before the first counseling (baseline), at the test disclosure session, and 1 and 12 months after disclosure. Although the mutation-positive individuals were more afraid of cancer than those who were mutation negative at every measurement point, the fear of cancer decreased significantly from the baseline until after disclosure in both groups. The mutation-positive subjects were more anxious than their counterparts immediately after the test disclosure, but the differences had disappeared at the follow-up examinations. Regarding the other variables, no differences among the groups defined according to mutation status or changes over time were detected.

Meiser et al. (2004) assessed the psychological impact of predictive genetic testing for HNPCC in 114 individuals with no personal cancer history (32 carriers and 82 non-carriers) using mailed self-administered questionnaires prior to and 2 weeks, 4 months and 12 months after the disclosure of the test results. Compared with the baseline results, carriers showed a significant increase in the mean scores for intrusive and avoidant thoughts regarding colorectal cancer at 2 weeks after test result disclosure and a significant decrease in the mean depression scores at 2 weeks and 4 months after test result disclosure. For non-carriers, significant decreases in the mean scores for intrusive and avoidant thoughts regarding colorectal cancer were observed at all follow-up assessment time points relative to the baseline. Non-carriers also showed significant decreases from the baseline in the mean depression scores at 2 weeks, 4 months and 12 months after test result disclosure. Significant decreases in the mean state anxiety scores from the baseline were also observed for non-carriers at 2 weeks after test result disclosure.

Gritz et al. (2005) examined the impact of HNPCC genetic test results on the psychological outcomes of cancer-affected and -unaffected participants up to 1 year after test result disclosure. A total of 155 persons completed the study measures before HNPCC genetic testing and at 2 weeks and 6 and 12 months after the disclosure of the test results. The mean scores for all the outcome measures remained stable and within the normal limits for cancer-affected participants, regardless of the mutation status. Among unaffected carriers of HNPCC-predisposing mutations, the mean depression, state anxiety, and cancer worry scores increased from baseline to 2 weeks after test result disclosure and decreased from 2 weeks to 6 months after test result disclosure. Among unaffected non-carriers, the mean depression and anxiety scores did not differ, but the cancer worry scores decreased during

the same time period. Affected and unaffected carriers had higher mean test-specific distress scores at 2 weeks after test result disclosure, compared with non-carriers, in their respective groups; the scores decreased for affected carriers and all unaffected participants from 2 weeks to 12 months after test result disclosure. Higher levels of baseline mood disturbance, a lower quality of life, and lower social support were associated with a risk for both short- and long-term increases in distress.

Claes et al. (2005) evaluated distress one year after the disclosure of a predictive genetic test result for HNPCC in 72 cancer-unaffected relatives (36 carriers and 36 non-carriers). The mean levels of distress (cancer-specific distress, state anxiety, and psychoneuroticism) were within the normal ranges and none of the participants had an overall pattern (on all scales) of clinically elevated levels of distress. Carriers had significantly higher cancer-related distress one year after test result disclosure than non-carriers. In both groups, colorectal cancer-related distress decreased. Non-carriers additionally showed decreased endometrial cancer-related distress and state anxiety.

Collins et al. (2007) conducted a 3-year study of individuals who received predictive genetic test results for previously identified familial mutations regarding HNPCC. Questionnaires were sent before attendance and 2 weeks, 4 months, 1 year, and 3 years after receiving the test results. Psychological measures were included each time. The study included 73 individuals with no personal cancer history (19 carriers and 54 non-carriers). The results showed an increase in mean cancer-specific distress in carriers at 2 weeks with a return to baseline levels by 12 months. This level was maintained until 3 years. Non-carriers showed sustained decreases after testing with a significantly lower level at 3 years compared with at baseline. These scores tended to be lower than those for carriers at 3 years. The mean depression and anxiety scores did not differ between carriers and non-carriers and, at 3 years, were similar to the baseline scores.

Shiloh et al. (2008) assessed the emotional effects of genetic testing for HNPCC at baseline before testing and again at 6 and 12 months after testing. The subjects were 253 cancer-affected and -unaffected individuals. Negative emotional reactions were evaluated using the Revised Impact of Event Scale and the Center for Epidemiological Studies-Depression Scale. Monitoring coping style was assessed at baseline using the Miller Behavioral Style Scale. Mean reductions were indicated in distress and depression levels within the first 6 months after testing. High monitors (individuals who vigilantly attended to threatening cues in their environment in an attempt to emotionally process the situation and who actively engaged in information seeking and cognitive problem solving with the intention of taking precautions) were generally more distressed than low monitors, specifically if they had indeterminate or positive results.

5.3 Summary

Many studies have shown that genetic testing does not result in short- or long-term significant adverse psychological outcomes, including depression, anxiety, and posttraumatic stress disorder (PTSD), in either carriers or non-carriers or in either cancer-affected or cancer–unaffected individuals. However, healthcare providers should assess psychological responses, such as minor depression, posttraumatic stress symptoms (PTSS), and feelings of guilt, particularly in individuals who have a history of major or minor depression, nervous personality tendencies, baseline mood disturbances, a lower quality of life, or lower social support.

Lastly, two cases that showed adverse psychological reactions after being informed of genetic test results will be presented. The first case is a man who was diagnosed as having

acute stress disorder at a 1-month follow-up examination after the disclosure of a genetic test result, despite the fact that the test result had been negative. The second case is a man who felt guilty after hearing of the positive test results of family members of individuals belonging to his support group.

5.4 Cases exhibiting adverse psychological reactions

[Case 1] Mr. A was a 39-year-old married man without children who came for genetic counseling and testing because of a family history of colon cancer. He had no history of cancer, but his father had a history of colon cancer and his sister had died of the disease at an early age. To confirm the diagnosis of HNPCC, a blood sample was obtained and mutations in the hMSH2 and hMLH1 genes were analyzed. He then consented in writing to participate in our study, and a baseline interview was conducted. He did not meet any of the criteria for any psychiatric disorders.

Approximately two months after the blood test, he underwent post-test counseling and was informed that no mutations had been detected in either the hMSH2 or hMLH1 gene. Four weeks after the disclosure of the test result, at a 1-month follow-up examination, he was diagnosed as having acute stress disorder according to a structured clinical interview based on the DSM-IV. The total score of the Impact of Event Scale-Revised was high. The score for Total Mood Disturbance in the Profile of Mood States was higher than that at the baseline interview. He reported that although he felt emotional relief to learn the negative result, his worries regarding colon cancer had increased instead of disappearing.

Mutation-negative individuals often choose not to participate in follow-up counseling after genetic testing. However, this case suggests that it is important to evaluate the psychological outcome after genetic testing regardless of the test result, and that psychiatrists or psychologists should support the genetic counseling system.

[Case 2] Mr. B, a 59-year-old man, underwent a total colectomy for the resection of colorectal cancer. He and his 25-year old son requested predictive genetic testing 3 years later to reduce uncertainty and to help plan his son's future, since Mr. B's mother had died of colon cancer secondary to HNPCC. Mr. B and his son were provided with both an educational session explaining the genetics of hereditary diseases and counseling regarding the possible impact of positive test results. The tests revealed the presence of a mutation in the father but not in the son. Mr. B was relieved that his "bad blood" had not been passed on to his son. Later, however, he began to experience anhedonia and became depressed for several days. His primary care physician could not determine the reason for his feelings.

Mr. B was the chairperson of a hereditary cancer patient support group run by patients, their families, and health care providers. The group had been established to help families with hereditary cancer exchange information and experiences. Mr. B began to feel guilty because his son had tested negative while the family members of others in his support group had tested positive for the disease.

6. Conclusion

Cancer genetic counseling and genetic testing for HNPCC are now conducted in ordinary clinical settings. However, as mentioned above, few studies have examined the psychosocial aspects of genetic testing for HNPCC, and psychosocial assessments and long-term follow-up care for individuals who have undergone genetic counseling or testing and at-risk relatives with no personal history of cancer remain insufficient. To develop this field, the following problems should be examined: 1) the development of a cancer genetic counseling

model, including psychosocial support; 2) the education of cancer genetic counselors; 3) the availability of appropriate information concerning cancer genetics; 4) the recruitment of subjects at risk for cancer susceptibility; and 5) the accumulation of further psycho-oncology research results. While it is by no means easy to deal with these problems, it is essential that medical oncologists, surgical oncologists, psycho-oncologists, medical geneticists, nurses, and all other health care providers involved in cancer care vigorously approach this new area in collaboration with one another.

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Part 3

Nutrition

6

Physical Activity, Dietary Fat and Colorectal Cancer

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1. Introduction

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers worldwide, with over 1.2 million new cases being recorded in 2008. Global cancer statistics show that there is great (10-fold) variation in the occurrence of CRC worldwide, with the highest incidence rates in economically developed countries and regions, such as Australia, New Zealand, Europe and North America. The latest report shows that CRC incidence rates are rapidly increasing in countries within Eastern Europe and Eastern Asia, which were formerly considered low-risk areas. In some countries, e.g., the Czech Repubic and Japan, the incidence of CRC has already exceeded the peak observed in the high-risk areas. Epidemiological studies have demonstrated that the increasing incidence of CRC in these developing countries is mostly due to a higher incidence of CRC in younger age groups, which readily adopt new lifestyle habits (Jemal et al., 2011). In addition, reports have shown that persons who were born in Asia and later migrated to the United States have a higher risk of CRC than their counterparts who have remained in Asia (Flood et al., 2000).

Changes in worldwide variations in the incidence rates, together with the results of migrant studies, provide convincing evidence that the incidence rates depend largely on environmental (i.e., non-genetic) risk factors, including lifestyle. It is estimated that most cases of CRC occur sporadically (70-80%). Approximately 15% of CRC cases develop as a result of inherited factors and 5-10% of them result from known genetic syndroms, e.g., familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal carcinoma (HNPCC) (Souglakos, 2007).

There are different approaches and strategies concerning how to reduce the incidence of and mortality due to CRC. Those directed toward the treatment of CRC, i.e., surgical and therapeutic measures, are mostly costly, painful and the prognosis is not promisig. Efforts have also recently been directed toward the identification and removal of precancerous lesions (visible polypoid adenomas) through screening programs, which is a promising approach and is an important step in reducing mortality due to CRC (Orlando et al., 2008). On the other hand, efforts invested into strategies directed toward public health promotion campaigns for the prevention or reduction of risk factors in populations at high risk of CRC have been few and obviously ineffective. Recent studies have shown that there is low level of awareness of the role that physical activity plays in preventing CRC among adults in the USA and Europe (Coups et al., 2008; Keighley et al., 2004).

Epidemiological studies have shown that around 30-40% of CRC may be preventable by maintaining a healthy lifestyle and suitable diet. The available evidence suggests that the population attributable risk of physical inactivity is 13-14%, which is the same as the risk due to a Western eating pattern (Coups et al., 2008). These data, and all the aforementioned facts, show that the majority of sporadic CRC cases are preventable by adjustment of appropriate environmental and lifestyle factors (dietary habits, low body index, physical activity) and that there is a need to improve strategies of public health promotion campaigns in countries with increased risk of CRC. Public health promotion campaigns, if adopted, could have a major impact in the fight against sporadic CRC and would address many health and financial challenges.

This chapter is therefore an attempt in this direction and provides a current review of the literature on the relation between physical activity or dietary fat and CRC and the mechanisms of their interaction.

2. Physical activity and CRC

The first evidence of the preventive role of physical activity against cancer appeared in 1922, when two groups of investigators, independently of each other, observed that cancer mortality rates declined with increasing physical activity required for an occupation (Lee, 2003). In spite of these results, further investigations in this area were not undertaken until the 1980s, when investigators observed that men with sedentary jobs had a higher colon cancer risk than men with jobs requiring strenuous activity (Larsson et al., 2006). Since then, the link between physical activity and cancer risk has been examined extensively, not only for cancer of the colon and rectum but also for breast and prostate cancer and, to a lesser extent also for cancers of the endometrium, lung, ovary, testis, pancreas and kidney. Nevertheless, cancer of the large bowel is the most frequently investigated cancer in relation to physical activity in humans. There have today been over 60 epidemiological studies investigating effects of physical activity on CRC in humans. Studies have been conducted in different parts of the world (North America, Europe, Australia, New Zealand and Asia-Japan) and among different populations and races. The results of these publications are convincing and clearly indicate that physical activity protects against colon cancer in all age groups, in various racial and ethnic groups and in diverse geographic areas around the world (Friedenreich & Orenstein, 2002; Kruk & Aboul-Enein, 2006; Lee, 2003; Thune & Furberg, 2001).

2.1 Physical activity and rectal cancer

Although colon and rectal cancer share some environmental risk factors, evidence of an association between rectal cancer and physical activity is inconsistent. A meta-analysis of 19 cohort studies even estimated that physical activity provides no protection against rectal cancer (Samad et al., 2005). It is therefore currently suggested that there is no association between physical activity and rectal cancer (Friedenreich & Orenstein, 2002; Kruk & Aboul-Enein, 2006; Lee, 2003; Thune & Furberg, 2001).

2.2 Physical activity and colon cancer in humans

Summarized observational epidemiological evidence on the association between physical activity and cancer suggests that the average risk reduction for colon cancer is 30-40% (Lee,

2003) or even 40-50% (Friedenreich & Orenstein, 2002). However, estimates from meta-analyses are little lower. A meta-analysis of 19 cohort studies estimated that physical activity may reduce the risk of colon cancer on average by 20-30% (Samad et al., 2005). The World Health Organization conducted a meta-analysis using data from studies prior to 2000 and estimated that around 16% of the global colon cancer disease burden can be ascribed to physical inactivity (Bull, 2004, as cited in Wolin et al., 2009). The most reliable results are probably those from the most recent meta-analysis, which included all available case-control (24) or cohort studies (28) that had been published to June 2008, but only those that investigated the association between physical activity and colon cancer or colon and rectal cancer separately. Studies on the association between physical activity and rectal cancer or colorectal cancer combined were not included. However, this meta-analysis showed a 24% average risk reduction for colon cancer when comparing the most to the least active individuals across all studies (Wolin et al., 2009).

The results of some studies suggest that the beneficial effects of exercise may be attenuated or less consistent in women (Kruk & Aboul-Enein, 2006; Thune & Furberg, 2001). However, most studies have found no difference in colon cancer risk according to gender (Friedenreich et al., 2006), which is in agreement with the latest meta-analysis. It was estimated that the protective effect of physical activity on colon cancer is similar for men (24%) and women (21%). The risk appeared smaller in cohort studies (men=19%; women=11%) than in case-control studies (men=28%; women=32%) (Wolin et al., 2009). It has been suggested that surveys used to measure physical activity were developed mainly for men and are thus less precise in estimating household work. Women may spend between 30 minutes to 6 hours a day on household chores and family care activities and from 4 to 16 hours a day on occupational activities, which makes it challenging to assess their physical activity accurately (Howard et al., 2008).

2.2.1 Type, duration and intensity

Although it is clear that physical activity is associated with a decreased risk of developing colon cancer, details of the relationship are less clear. It is known that the frequency of muscle contraction (e.g., number of activities performed per day, week, month), duration (number of minutes or hours per day), intensity (how much energy is expended) and activity levels throughout the participant's entire lifetime, are important components of activity that can significantly affect the protective effects of physical activity on colon cancer risk (Friedenreich & Orenstein, 2002). Few studies have examined the type, intensity and duration of physical activity required. Since they used different criteria of physical activity in their tests for trends, meta-analyses of trends across these studies have not yet been conducted (Wolin et al., 2009).

Physical activity is often thought of as recreational activity or exercise but it is much more than this. Physical activity is any bodily movement produced by the skeletal muscles that results in a quantifiable expenditure of energy and thereby comprises all leisure-time activities as well as occupational activities. Leisure-time physical activity refers to sports, conditioning exercises (structured and planned activity in order to improve or maintain physical fitness), household activities, self-care activities (e.g., dressing, eating, talking, standing, walking, climbing stairs) etc. (Kruk & Aboul-Enein, 2006).

Evidence is consistent that both occupational and leisure-time physical activity can affect colon cancer risk (Wolin et al., 2009). A dose-response relationship has been noted. Higher

activity has been related to a reduced risk of colon cancer for both leisure-time and occupational physical activities (Friedenreich & Orenstein, 2002; Kruk & Aboul-Enein, 2006; Thune & Furberg, 2001; Wolin et al., 2009). Since little information is available, conclusions cannot be made about the type, intensity, frequency and duration of physical activity that is most beneficial.

Based on the current level of knowledge, it is believed that 30-60 min/day of regular physical activity of moderate to vigorous intensity is sufficient to decrease the risk of colon cancer in the general population (Friedenreich & Orenstein, 2002; Lee, 2003; Thune & Furberg, 2001).

2.2.2 Other factors

More detailed investigations on the effects of physical activity in relation to colon cancer site have recently been conducted. A few studies have evaluated the association between physical activity and colon cancer risk by anatomic site (proximal versus distal) and produced contradictory results. One study found no significant difference in risk estimates among different parts of the colon (Mai et al., 2007), while other studies found a reduction of risk only in transverse or sigmoid segments of the colon (Nilsen et al., 2008) or predominantly in the proximal colon (Lee et al., 2007).

It is believed that physical activity is associated with a reduced risk of colon cancer independently of diet or other environmental risk factors. This is supported by studies that have found that adjustment for potentially confounding factors, such as age, diet and obesity or body mass index, does not diminish the observed protective association (Friedenreich & Orenstein, 2002; Thune & Furberg, 2001).

However, it has been suggested that determination of potentially confounding variables is not always easy. When there are multiple hypothesized mechanisms, some of which may be in the causal path, the determination of confounding variables may be especially difficult. "For instance, if physical activity is associated with colon cancer through its ability to help maintain body weight, adjustment for body mass index would be inappropriate. If physical activity acts through other mechanisms, body mass index may be an important confounding variable because it is associated both with physical activity and colon cancer." An understanding of the biological mechanisms involved in the association between physical activity and colon cancer is therefore fundamental to evaluating confounding factors. In order to identify and understand the modifying effects of physical activity on other risk factors, the use of effect modification is advised (Slattery & Potter, 2002).

Slattery et al. examined confounding effect modification and observed the relative importance of high-risk diet, body mass index, energy intake and glycemic index in colon cancer prevention, which were found to be dependent on the level of physical activity (Slattery & Potter, 2002). Some studies have suggested a greater protective effect of physical activity in lean than in obese persons (Friedenreich et al., 2006; Larsson et al., 2006; Thune & Furberg, 2001). The findings of one cohort study even indicated that sedentary behavior, in particular television or video watching, is associated with an increased risk of colon cancer, independent of the time spent participating in physical activity and body mass index (Howard et al., 2008). Little is actually known about how physical activity may modify or be modified by other dietary and lifestyle factors, so conclusions based on currently available evidence can be misleading. Additional research in this direction is needed to provide public health recommendations regarding physical activity as a means of primary prevention of CRC.

2.2.3 Physical activity may affect cancer treatment and outcome

As shown above, physical activity has an important role in the prevention of colon cancer. Does physical activity also have a beneficial effect in CRC patients and survivors, though? A literature search shows that most attention on the efficacy of physical activity in colon cancer has been paid to cancer prevention. There have been few studies investigating the efficacy of activity in CRC patients. Nevertheless, available evidence suggests that physical activity may affect cancer treatment and outcome. It has been proposed that exercise during the pretreatment period may increase (boost) physical and psychological functioning, resulting in better physical preparation for treatment (shown in detail in Friedenreich & Orenstein, 2002). A study evaluating the benefits of physical activity in cancer patients and survivors shows improved functional capacity and quality of life (Johnson et al., 2009).

2.3 Physical activity and CRC in animal models

The effect of physical activity on CRC has mainly been evaluated using two rodent models of CRC, i.e., DMH/AOM animal model and Apc^{Min} mice. The first model is a chemically induced animal model. Animals develop colon lesions after the application of a carcinogen (dimethylhydrazine (DMH) or azoxymethane (AOM)). Colorectal carcinogenesis in this model is a multistep process with molecular, morphological and histological features similar to those seen in human sporadic colon carcinogenesis (Perse & Cerar, 2011). The second model is a genetically predisposed model. Apc^{Min} mice carry a dominant heterozygous nonsense mutation at codon 850 of the mouse homologue of the human tumor suppressor gene, APC, which results in the development of multiple adenomas throughout the intestinal tract. This mutation is implicated in both sporadic and inherited human colorectal carcinogenesis.

The first studies on carcinogen treated rats were directed at evaluating the effects of different voluntary or forced exercise (swimming, treadmill running, voluntary wheel-running) on colorectal lesions in the later stages of development (tumors, adenomas, carcinomas). These studies found that exercise significantly reduced the incidence and multiplicity of tumors, as well as the incidence and multiplicity of adenocarcinomas but had little or no effect on the incidence and multiplicity of adenomas (Basterfield et al., 2005).

Various studies have recently evaluated the effects of exercise on aberrant crypt foci (ACF), which are the first microscopically visible precursor lesions of CRC. They found that moderate-intensity exercise reduced the number of ACF in colons of DMH–treated rats (Fuku et al., 2007), low intensity exercise had no significant effect on the incidence of ACF (Lunz et al., 2008), while excessive and exhausting exercise significantly increased the number of ACF and, consequently, also the risk of development of colon cancer (Demarzo & Garcia, 2004).

In contrast to the results obtained from the chemically induced model, the results of various studies on Apc^{Min} mice are inconsistent. Two studies reported that exercise (treadmill running) had no effect on the incidence of intestinal adenomas in females, while a tendency toward a reduced incidence in males was observed (Colbert et al., 2000; Colbert et al., 2003). It was then found that the beneficial effects of exercise may be related to the exercise mode (treadmill/wheel running), gender (Mehl et al., 2005) and energy balance (Colbert et al., 2006). A recent study found that voluntary wheel running exercise also inhibited tumor formation in female Apc^{Min} mice (Ju et al., 2008).

The reasons for the inconsistent results are not clear. In has been suggested that different types of exercise may elicit different physiological changes related to stress hormone release

and may alter the inflammatory effects (Mehl et al., 2005). Another possible reason is the large variation in tumor yield among individual Apc^{Min} mice, which may have resulted in false-negative or non-significant results when a small number of animals were used (Ju et al., 2008). Finally, it is likely that this model may be suitable for investigating and assessing the effect of physical activity on CRC development in organisms with an inherited mutation or genetic predisposition.

However, experimental studies investigating the modifying effect of other dietary and lifestyle factors in relation to the beneficial effect of exercise are scarce. One study investigated the effect of exercise in animal models maintained on different types of high-fat diet. It was found that a different type of high-fat diet (21% coconut + 2% corn oil versus 23 % corn oil) may be associated with a different outcome of colon carcinogenesis in carcinogen treated rats exposed to exercise (Thorling et al., 1994). A second study reported that 6 weeks and 9 weeks of voluntary exercise (wheel running) successfully decreased the number of intestinal polyps in Apc^{Min} mice on low and high fat diets, respectively (Ju et al., 2008). We recently found that exercise has a protective role in colon carcinogenesis in carcinogen treated rats, in the case of both low and high fat consumption diets. However, in terms of the combined effect of dietary fat and exercise, our results suggest that the protective role of exercise on colon carcinogenesis may be reduced in relation to the amount and type of fat in the diet (Perse, 2010). The lack of understanding of the biological mechanisms operating between physical activity and other risk factors warrants further research.

2.4 Mechanisms of physical activity modulation

A number of plausible biological mechanisms for the protective effect of physical activity against colon cancer have been suggested. They are mostly based on various experimental results. There are currently few empirical clinical data to support any of the hypothesized biological mechanisms for the protective effect of exercise on colon cancer.

2.4.1 Effects on gastrointestinal transit time and gut microbiota

The most frequently quoted explanation for reduced colon cancer among physically active people is that physical activity accelerates the movement of stool through the colon and shortens the gastrointestinal transit time, thereby reducing the contact of potential carcinogens and cancer promoters with colon mucosa (Kruk & Aboul-Enein, 2006). Although plausible, the epidemiological evidence of an association between gastrointestinal transit time and colon cancer risk has so far been inconsistent and this explanation has not yet been directly confirmed (Friedenreich et al., 2006).

The colon contains a vast population of many types of bacteria, which have potentially important functions and may contribute to cancer development (Tammariello & Milner, 2010). It was recently found that voluntary wheel running influnced the composition of cecal microbiota, which in turn produced higher concentrations of n-butyrate. Butyrate is a short-chain fatty acid end product of bacterial fermentation in the intestines, which has been related to intestinal motility and an inhibitory effect on tumor development (Matsumoto et al., 2008).

2.4.2 Effects on blood insulin, IGF-1 and body weight

Similarities in geographic patterns and dietary and lifestyle risk factors for CRC and type 2 diabetes have led to the suggestion that there is an association between the two diseases

(Giovannucci, 2001). Based on meta-analysis of case-control and cohort studies, individuals with diabetes have an approximately 30% increased relative risk of developing CRC compared to non-diabetic individuals, regardless of gender or the anatomical site of CRC (Larsson et al., 2005). Preliminary results have shown that CRC is more common in people with increased circulating levels of insulin and glucose. A long-term increase in circulating levels of insulin may influence every step of colon carcinogenesis by stimulating cell proliferation or inhibiting apoptosis (Pisani, 2008). In addition to type 2 diabetes, obesity may cause problems with insulin metabolism and an alteration in blood glucose (explained in Murthy et al., 2009).

Physical activity can contribute to increased insulin sensitivity in skeletal muscles, both directly and indirectly through its influence on body weight (Giovannucci, 2001). Regular physical activity significantly lowers insulin levels by stimulation of the signaling pathways that contribute to increased expression and translocation of GLUT 4, which is responsible for basal and insulin-stimulated glucose uptake into the cells (explained in detail in Kramer & Goodyear, 2007).

An increasing body of evidence suggests that variations not only in the levels of insulin but also in the levels of insulin-like growth factors (IGF) may account for colon cancer and for its high incidence in Western countries. The IGF family of proteins (peptide ligands, receptors, binding proteins and proteases) are involved in the regulation of somatic growth, cell proliferation, transformation and apoptosis. Among them, IGF-1 and IGFBP-3 have been most frequently investigated in relation to CRC. It has been hypothesized that IGF-1 is implicated in the etiology of CRC as a potent mediator of cell survival and growth (for more detail see Sandhu et al., 2002). In spite of this, current evidence does not support an association between the blood level of IGF-1 and CRC. Among exercise studies in humans, 50% have found no change in IGF-1, 37% an increase in IGF-1 and 13% a decrease in IGF-1 (Friedenreich & Orenstein, 2004). Likewise, no significant association between circulating levels of IGF-1 and exercise in animal models of CRC has been found (Colbert et al., 2000; Colbert et al., 2003; Colbert et al., 2006; Ju et al., 2008; Mehl et al., 2005).

2.4.3 Effects on inflammatory modulators

A number of studies have demonstrated that regular exercise has anti-inflammatory effects, which may play a significant role in the prevention of colon carcinogenesis, as well as many other diseases, such as atherosclerosis, type 2 diabetes and breast cancer. A marked increase in circulating levels of interleukin (IL)-6 after exercise, without any muscle damage, has been observed. It was found that the level of circulating IL-6 increases in an exponential fashion (up to 100-fold) in response to exercise and declines after exercise. It has been demonstrated that plasma IL-6 increase is related to exercise intensity, duration, the mass of muscle recruited and one's endurance capacity. Recent data demonstrate that IL-6 released from contracting human skeletal muscle has anti-inflammatory, immunosuppressive, metabolic and hypertrophic effects in humans (Petersen & Pedersen, 2005; Petersen & Pedersen, 2006).

Until recently, IL-6 was generally considered to be a pro-inflammatory cytokine released primarily from immune cells. However, dramatic increases in circulating IL-6 during exercise have led to the finding that skeletal muscles are a primary source of IL-6. Skeletal muscle has thus been found to be an immunogenic and an endocrine organ, which by contraction stimulates the production and release of cytokines, which can influence

metabolism and modify cytokine production in tissues and organs (Mathur & Pedersen, 2008).

It has been found that IL-6 induces the production of cytokine inhibitors, such as IL-1 receptor antagonist (IL-1ra) and IL-10, which are anti-inflammatory molecules. IL-1ra inhibits signaling transduction through the IL-1 receptor complex, while IL-10 inhibits the production of cytokines (IL-1α, IL-1β, TNF-α) and chemokines (IL-8, protein α), which play a critical role in the activation of granulocytes, monocytes, natural killer cells and T and B cells and in their recruitment to sites of inflammation (Petersen & Pedersen, 2005; Petersen & Pedersen, 2006).

IL-6 may increase basal and insulin-stimulated glucose uptake via increased GLUT 4 translocation. IL-6 has been shown to enhance AMP-activated protein kinase (AMPK) in both skeletal muscle and adipose tissue, which stimulates fatty acid oxidation and increases glucose uptake (Nielsen & Pedersen, 2008). TNF-α has been implicated in the pathogenesis of insulin resistance related to obesity (Steinberg, 2007). Evidence exists that TNF-α blocks AMPK signaling. However, exercise may also suppress TNF-α production via IL-6 independent pathways (Petersen & Pedersen, 2006).

2.4.4 Effects on immune function

It has been suggested that the immune system plays a role in reducing cancer risk by recognition and elimination of abnormal cells through immune components. Increased inflammation and/or depressed immune function are important risk factors that may lead to several cancers, including CRC. This is in accordance with the finding of an increased incidence of cancers among patients with inflammatory bowel disease (IBD) or AIDS. AIDS patients show an increased risk not only of AIDS-related malignancies (e.g., Kaposi's sarcoma) but also other cancers, such as lung and colon. An intact immune system is usually able to destroy cancer cells as soon as they appear.

It has been demonstrated that lifestyle factors can significantly affect immune function. Regular physical activity can enhance both the functionality and number of innate immune cell components, such as cytotoxic T lymphocytes, natural killer cells, lymphokine-activated killer cells and macrophages. Moderate physical activity results in enhanced immune function, whereas exhausting exercise, overtraining or high-intensity exercise may lead to suppression of the immune function (Pedersen & Hoffman-Goetz, 2000).

2.4.5 Effects on arachidonic acid metabolism

There have been studies suggesting that exercise affects enzymes that are implicated in arachidonic acid metabolism. Arachidonic acid is part of the phospholipids in the membranes of cells and in its free form serves as a precursor in the production of eicosanoids. After liberation (by the enzyme phospholipase A2 (PLA_2)), arachidonic acid is available as a substrate for cyclooxygenases (COX) and lipoxygenases (LOX) to form prostaglandins (PG) and leukotriens (LT). Increased levels of COX-2 and PGE_2 have been found to promote the development of CRC by increasing proliferation and decreasing colonic motility and apoptosis and have been associated with aggressive tumor progression. A relationship between PG and CRC is also supported by studies showing a reduced risk of CRC with aspirin and other non-steroidal anti-inflammatory drugs (NSAID), which inhibit COX-2, thereby inhibiting PG production (Jones et al., 2003).

It has been reported that physical exercise decreased COX-2 expression in the colon mucosa of healthy untreated rats (Buehlmeyer et al., 2007) and DMH-treated rats (Demarzo et al., 2008). Exercise was found to inhibit one of the products of COX activities, PGE_2, in intestinal tumors and serum of Apc^{Min} mice (Ju et al., 2008). In rat colon mucosa, exercise was found to reduce the expression of $iPLA_2$, which is one of the PLA_2 implicated in arachidonic acid release (Buehlmeyer et al., 2008). Exercise has also been found to reduce PGE_2 levels in rectal tissue among individuals with higher levels of self-reported exercise. On the other hand, in another study, exercise had no significant effect on PGE_2 levels in colon mucosa (Abrahamson et al., 2007).
The body of evidence is currently too limited to reach any final conclusions.

2.4.6 Effects on apoptosis, proliferation, gene expression

An alteration in the control of cellular proliferation and survival may be an important step in the development of colonic neoplasms. New cells are produced rapidly and continuously from stem cells at the base of the colonic crypt. Older cells undergo apoptosis (programmed cell death) and are sloughed into the colonic lumen. To maintain crypt organization and structure, cellular proliferation and apoptosis must be tightly controlled. Failure of these controls may lead to the formation of colonic neoplasms. It has been hypothesized that exercise-induced colon cancer risk reduction might be through alterations to colon crypt cell architecture and proliferation. It has been reported that a 12-month moderate-to-vigorous intensity exercise program (60 minutes per day, 6 days per week) increased colon crypt height and decreased proliferation in men (McTiernan et al., 2006) and changed the expression of Bcl-2 and Bax protein in colonic crypts (Campbell et al., 2007).

2.4.7 Effects on oxidative status

There is growing support for the concept that reactive oxygen species (ROS), which are already implicated in a range of diseases, may be important progenitors in the pathogenesis of colon cancer. Namely, an excess of intracellular ROS results in an environment that modulates gene expression, damages cellular molecules, including DNA, which ultimately leads to mutations. In order to counteract these deleterious actions of increased levels of ROS, cells possess an antioxidant defence system, which plays a central role in protecting cells from oxidative injury. It is belived that exercise may help to prevent colon cancer due to an improvement in the cell's antioxidant defence system. It has already been demonstrated that exercise improves the antioxidant defence system in various tissues. Exercise stimulates various signaling pathways in cells, such as MAPK and NFκB, which results in increased expression of important enzymes associated with cell defence (MnSOD and GPx) and adaptation to exercise (eNOS and iNOS). Many of the biological effects of antioxidants appear to be related to their ability not only to scavenge deleterious free radicals but also to modulate cell-signalling pathways. The modulation of signalling pathways by antioxidants could thus help to prevent cancer by preserving normal cell cycle regulation, inhibiting proliferation and inducing apoptosis, inhibiting tumor invasion and angiogenesis, suppressing inflammation and stimulating detoxification enzyme activity (Kramer & Goodyear, 2007; Scheele et al., 2009; Valko et al., 2007). Exercise has been found to decrease the expression of inducible nitric oxide synthase (iNOS), as well as TNF-α, in the colon of AOM-treated mice (Aoi et al., 2010).

3. Dietary fat and CRC

In contrast to physical activity, the association between fat intake and CRC is less conclusive. In the past, dietary fat has received considerable attention as a possible risk factor in the etiology of CRC but subsequent analysis of case control studies has indicated that the positive association was at least in part due to increased energy intake (Johnson & Lund, 2007).

While epidemiological studies have produced contradictory results (Johnson & Lund, 2007), experimental studies under isocaloric conditions have provided unequivocal evidence that a diet high in saturated fatty acids (SFA), such as lard or beef tallow, and n-6 polyunsaturated fatty acid (PUFA), such as corn or sunflower oil, increases the risk of developing CRC (Dai et al., 2002; Reddy, 2000). It was recently shown that long-term consumption of a high-fat, low-calcium and vitamin D diet induces colon neoplasia in mice, without any other treatment (Erdelyi et al., 2009). Interestingly, a recent expert review on nutrition and cancer published by the World Cancer Research Fund (American Institute for Cancer Research, 2007) found suggestive evidence that food rich in animal fat (rich in SFA) is associated with an increased risk of CRC. This means that epidemiological studies are mainly supportive but are limited in quantity, quality or consistency.

In contrast, diets high in olive oil or n-9 monounsaturated fatty acid (MUFA) have shown a protective or no effect on colon carcinogenesis in animal models, while diets with fish oil or n-3 PUFA have been shown to reduce colon tumorigenesis in both initiation and post-initiation phases (reviewed in Perše, 2010). Epidemiological reports investigating the effect of n-3 PUFA on CRC are scarce. However, some studies have shown that an n-3 PUFA-rich diet suppressed the risk of colon cancer in humans. The preventive or inhibitory effect of n-3 PUFA on experimental colon carcinogenesis has been widely evaluated (Biondo et al., 2008). All these results suggest that the composition of ingested dietary fatty acids is a more critical risk factor than the total amount of fat. This is further supported by their different mode of action, which is described in the following section.

However, at the same time, it is worth emphasizing that studies on animal models have shown that the promoting effects of SFA and n-6 PUFA on CRC can be modified by various dietary factors. A relatively small fraction of n-3 PUFA (25%) in total dietary fat or supplemental calcium or antioxidants, such as vitamin D (Pence & Buddingh, 1988), vitamin A (Delage et al., 2004), as well as green tea, vitamin B6 (Ju et al., 2003) and poliphenolic extract of red wine (Femia et al., 2005), have shown an appreciable beneficial effect in lowering the risk of CRC in animal models on a high fat diet. The influence of different amounts of calcium, antioxidants and other beneficial compounds in combination with dietary lipids may therefore be complex and difficult to elucidate, particularly in epidemiological investigations. Because many dietary, as well as environmental or lifestyle factors such as physical activity, can modify the promoting effects of dietary fat on CRC, results obtained from animal models under standardized conditions may represent an important contribution to understanding the mechanisms of dietary fat involvement in colorectal carcinogenesis (Hoffman-Goetz, 2003).

3.1 Mechanisms of dietary fat modulation

Dietary fats are an important energy reserve in an organism. However, this is not their only function. Linoleic acid (n-6 PUFA) and linolenic acid (n-3 PUFA) are considered essential, since they can not be synthesized by mammals and must therefore be obtained from diet.

Lipids and fatty acids obtained from dietary fats are metabolized and incorporated into the phospholipids of the cell membranes of many cell types and serve as precursors for many biologically active molecules, as well as being important for cell signaling (Jones et al., 2003). It is generally accepted that the balance of n-6 to n-3 PUFA in the diet is of importance to human health and disease, including CRC. An alteration in fatty acid composition in the cell as a result of altered dietary fat consumption may lead to changes in all these functions, which are briefly outlined below.

3.1.1 Effects on the concentration of secondary bile acids

Experimental and epidemiological studies have shown that diets high in beef tallow, lard or corn oil increase the concentration of colonic luminal (fecal) secondary bile acids, i.e. deoxycholic acid (DOC) and lithocholic acid, whereas high dietary fish oil has no such enhancing effect. It has been found and confirmed that these secondary bile acids induce cell proliferation and act as promoters in colon carcinogenesis. Recent experiments have provided new insight into their effects on colonic epithelial cells. The results indicate that secondary bile acid DOC may act as a carcinogen, not merely a promoter (explained in detail in Bernstein et al., 2011).

3.1.2 Effects on energy balance

Energy balance has become an important concept in exploring the etiology of a number of chronic diseases, including cancer, because of its close association with weight gain and overweight - conditions known to increase the risk of many chronic diseases.

The amount of energy that is required depends in part on the composition of the food. In this regard, it is worth noting that dietary fats are more readily converted to body fat and require less energy for transformation than carbohydrates. A high fat diet therefore contributes indirectly to CRC due to increased body mass index.

3.1.3 Effects on immune function

One of the most thoroughly evaluated associations between nutrition and the immune system is that related to dietary fat. Although total fat intake has been found to increase the risk of various types of cancer, it is the type of fat that has a more important effect on the immune response and, consequently, on cancer development. PUFA have been shown to modulate cytokine production, lymphocyte proliferation, expression of surface molecules, phagocytosis, apoptosis and natural killer cell activity (the last two are closely related to cancer development). An increase in n-3 PUFA helps control the production of pro-inflammatory eicosanoids, as well as cytokine production (Valdes-Ramos & Benitez-Arciniega, 2007).

3.1.4 Effects on arachidonic acid metabolism

It has been suggested that dietary n-3 PUFA has an anti-carcinogenic role in reduction of n-6 PUFA-derived eicosanoid biosynthesis and direct inhibition of COX-2.

Dietary n-6 PUFA incorporates into the membrane phospholipids as arachidonic acid (AA), while dietary n-3 PUFA does so as eicosapentaenoic acid (EPA). AA and EPA compete for prostaglandin and leukotriene synthesis. Pro-inflammatory eicosanoids of AA metabolism are released from membrane phospholipids in response to inflammatory activation. EPA is released to compete with AA for enzymatic metabolism, inducing the production of less

inflammatory and chemotactic derivatives. Eicosanoids produced from EPA are much less potent (up to 100-fold) than the analogues produced from AA and even have anti-thrombotic and anti-inflammatory properties. The relative amounts of n-6 and n-3 PUFA provided by the diet, and so present in blood and tissues, may thus be of importance in the development of inflammatory diseases and cancers. The production of inflammatory eicosanoids is increased in response to many inflammatory stimuli (Simopoulos, 2002a). When the production of these substances is excessive, it may lead to chronic inflammation and an increased risk of cancer, since inflammation has been linked to the promotion phase of carcinogenesis (Federico et al., 2007). The increased n-6/n-3 ratio in Western diets probably contributes to reduced levels of EPA in phospholipids and, consequently, to an increased incidence of cardiovascular disease and inflammatory disorders (Simopoulos, 2002b).
Another indication of the importance of diet and n-6 PUFA in the induction and progression of CRC may be the upregulation in fatty acid binding protein (FABP)-5 during tumorigenesis, with concomitant inhibition of Δ6 desaturase activity, which are important steps in the production of AA (explained in Jones et al., 2003). Most AA in the human body derives from dietary linoleic acid (essential n-6 PUFA), which comes from vegetable oils and animal fats.
Animal studies have demonstrated that a high fat diet significantly increases the expression of PLA_2, COX-2 and PGE_2 in colon mucosa and tumors of carcinogen treated rats (Rao et al., 1996; Rao et al., 2001).

3.1.5 Effects on cell proliferation and apoptosis

The expression of Polo-like kinase-3 (PLK-3) results in cell cycle arrest or induces apoptosis. It is significantly suppressed in tumor tissue of the colon but has been found to be unchanged in colon mucosa isolated from rats on different diets. Suppression of PLK-3 was lower in tumors from rats fed n-3 PUFA than those fed n-6 PUFA (Dai et al., 2002).
Dietary corn oil and beef tallow increased BrdU incorporation and decreased apoptosis of the colon mucosa. Long-term (44 wks) high intake of corn oil and beef tallow enhanced cell proliferation through Wnt signaling and modulated the distribution of proliferating cells (Fujise et al., 2007).
High corn oil consumption decreased apoptosis and increased cell proliferation in colon of AOM treated rats (Wu et al., 2004). On the other hand, studies have indicated that n-3 PUFA has an inhibitory effect at least in part due to increased apoptosis in colonic mucosa (Hong et al., 2000; Wu et al., 2004).

3.1.6 Effects on cell signaling pathways

Studies have suggested that different types of fat may be implicated in different cell signaling pathways, rather than at the level of mutations. n-3 PUFA may interfere with Ras activation by decreasing its membrane localization and may thereby potentiate the effects of anti-Ras therapies. EPA and/or docosahexaenoic acid (DHA; another n-3 PUFA) have also been reported to prevent Akt phosphorylation or activation. n-3 PUFA incorporation into rafts or caveolae may alter the distribution or function of raft-associated signaling proteins – reduced epithelial growth factor receptor (EGFR) levels in the rafts, decreased levels of H-ras and eNOS in colonic caveolae. Alterations in raft lipid composition by PUFA have also been shown to displace signaling proteins from rafts in immune cells. n-3 PUFA decreases NFκB activity or expression in cancer cells, as well as monocytes, macrophages and T cells (Biondo et al., 2008). Peroxisome proliferators and retinoic acid–activated receptors (PPAR

and RAR, RXR) are key transcription factors regulating gene expression in response to nutrient-activated signals. A high-fat diet containing various sources of fat, such as commonly consumed in Western countries (the majority SFA), induced PPARγ and RARβ expression, concomitant with an increase in levels of COX-2 and β-catenin in colon mucosa of DMH treated rats (Delage et al., 2004). Various fatty acids have different effects on the Wnt signaling pathway (Kim & Milner, 2007). Long-term (44 wks) high intake of corn oil and beef tallow enhanced Wnt signaling. Dietary corn oil and beef tallow increased the expression of cytosolic β-catenin and cyclin D1. Expressions of Wnt2 and Wnt3 in rats fed with beef tallow and Wnt5a in rats fed with corn oil increased, with or without AOM-treatment (Fujise et al., 2007).

3.1.7 Effects on oxidative status

It has been demonstrated that dietary fatty acids affect the lipid content of tissue and the lipid peroxidation process, due to the ratio of polyunsaturated versus saturated fatty acid. A substantial increase in the PUFA content may overcome the protective action of the antioxidant system and increase susceptibility to lipid peroxidation (Avula & Fernandes, 1999). We have recently demonstrated that long-term consumption of an high-fat mixed-lipid (HFML) diet together with physical inactivity significantly increased the production of lipid peroxides in the skeletal muscle (Perse et al., 2009), suggesting that an HFML diet is an important contributor to the development of chronic diseases, including CRC. There is growing support for the concept that an excess of intracellular ROS, results in an environment that modulates gene expression, damages cellular molecules, including DNA, which ultimately leads to mutations (Valko et al., 2006). On the other hand, fish oil has been found to reduce oxidative DNA damage (Bancroft et al., 2003; Wu et al., 2004).

The large intestine is constantly exposed to ROS originating from endogenous and exogenous sources, due to oxidized food debris, toxins and high levels of iron. It has been demonstrated that dietary fatty acids affect the lipid content of tissue and result in differential susceptibility to peroxidation (Kuratko & Pence, 1991; Kuratko & Becker, 1998; Kuratko & Constante, 1998; Wu et al., 2004). It was recently shown that a high-fat, low-calcium and vitamin D diet induces oxidative stress in the colon (Erdelyi et al., 2009).

3.1.8 Beneficial role of n-3 PUFA before or during chemotherapy

Based on considerable evidence showing different beneficial effects of n-3 PUFA, it has been suggested that n-3 PUFA may improve the outcome of patients undergoing abdominal cancer surgery (Valdes-Ramos & Benitez-Arciniega, 2007). Since n-3 PUFA enrichment can affect the physical properties of cell membranes, altering membrane fluidity and increasing the permeability of tumor cells, it has been proposed that n-3 PUFA consumption may modify the influx and efflux of drugs into or out of tumor cells. However, elucidation of mechanisms is essential for ensuring both the optimal efficacy of a drug and for identifying the target level at which to modify the diet or supplement with n-3 PUFA, in order to optimize the benefits to the patient (Biondo et.al, 2008).

4. Conclusion

Evidence that physical activity affects the risk of colon cancer has been provided by numerous epidemiological and experimental studies and reviewed extensively. Although strong evidence exists that regular physical activity is associated with decreased risk of

colon cancer, little is known about the type, intensity, frequency and duration of physical activity that is most benefical. Evidence is consistent that both occupational and leisure-time physical activity can affect the risk of colon cancer. Based on the current level of knowledge, it is believed that 30-60 min/day of regular moderate to vigorous intensity physical activity is sufficient to decrease the risk of colon cancer in the general population.

The relation between dietary fat and CRC is less conclusive. Experimental studies suggest that the composition of ingested dietary fatty acids is a more critical risk factor than the total amount of fat. This is further supported by their different modes of action. It has been proposed that the increased n-6/n-3 ratio in Western diets probably contributes to an increased incidence of cardiovascular disease and inflammatory disorders, as well as at least in part CRC. There is increasing body of evidence that the consumption of n-3 PUFA can impact on immune functions, as well as alter gene expression and transcription factor activity in normal and cancer cells. It has also been found to reduce CRC risk and is suggested to have a beneficial role before or during chemotherapy, and even improve drug uptake.

5. Acknowledgement

This work was in part supported by ARRS (Slovenian Research Agency, Program P3-054).

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7

Dietary Anthocyanins: Impact on Colorectal Cancer and Mechanisms of Action

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1. Introduction

Colorectal cancer is the third most common malignancy in males and the second most common in females, with significant variations in the worldwide distribution, and remains among four leading causes of cancer deaths overall, shows global cancer statistics. The highest incident rates are found in economically developed countries, whereas the lowest rates are noted in Africa and South-Central (Jemal et al., 2011). However, striking increase in colorectal cancer incident trends is observed in areas historically at low risk, such as Spain and some Eastern European (the Czech Republic and Slovakia) and Eastern Asian countries (Japan). On the other hand, generally high incident rates over the past several decades are going down in the Unites States (Center et al., 2009). These recent "perturbations" in colorectal cancer trends probably result from a combination of risk factors, including obesity, sedentary lifestyle, increased prevalence of smoking, excessive alcohol consumption and "westernization" in dietary habits - a diet rich in red and processed meat and low intake of fruits and vegetables (Center et al., 2009; Chao et al., 2005; Jemal et al., 2011). Decreasing incident and mortality rates are mainly associated with colorectal cancer screening and improved treatment.

Prognosis of these patients depends on the stage of the cancer at diagnosis. As the AJCC (American Joint Committee on Cancer) stage increases from stage I to stage IV, the 5-year overall survival rates decrease dramatically, reaching 90% if the disease is detected early when still localized, though just 39% of colorectal cancers are found at this stage. Almost 25% of patients have a metastatic disease at diagnosis, with a 5-year survival of less than 10% (Goldberg et al., 2007). The primary treatment for colorectal cancer is surgical resection. More than two-thirds of patients undergo radical surgery, but 30-50% of patients who present with stage II or III tumors ultimately experience disease recurrence and distant metastases (Rodriguez-Moranta et al., 2006). Although a broader base of treatment options for metastatic colorectal cancer (mCRC) has evolved in recent years, 50 - 70% of mCRC

patients still cannot be subjected to radical resection of metastases and are candidates for palliative therapy only (Fornaro et al., 2010).
The drugs commonly used to treat mCRC are fluoropyrimidines (fluorouracil and capecitabine), irinotecan – a semisynthetic derivative of the natural alkaloid camptothecin, and oxaliplatin – a diaminocyclohexane platinum compound. More recently, two monoclonal antibodies have been approved for the treatment of advanced stages of colorectal cancer. Bevacizumab, a humanized monoclonal antibody against vascular endothelial growth factor (VEGF), is broadly used in combination with fluoropyrimidine-based chemotherapy. Cetuximab, a chimeric monoclonal antibody against the epidermal growth factor receptor (EGFR), is used as monotherapy or together with irinotecan in irinotecan-resistant patients (Hess et al., 2010; Tol and Punt, 2010; Van Cutsem et al., 2009). These chemotherapy agents have significantly improved the prognoses and median overall survival. However, chemotherapy drug resistance occurs in nearly all patients and remains the most frequent cause of treatment failure (Candeil et al., 2004; Dallas et al., 2009), calling for finding novel agents capable of killing drug-resistant colorectal cancer cells.

2. Dietary compounds and cancer

2.1 Cancer prevention by diet

The possibility that fruit and vegetables might help to reduce the risk for various types of cancer raised great interest already in the 1970s. The first studies conducted to assess differences in cancer rates and diet between countries suggested that various dietary factors might have important effects on cancer risk (Armstrong and Doll, 1975; Bjelke, 1975).
In 1992, an epidemiological research with 156 studies on connection between the consumption of fruit and vegetables and cancer concluded that persons with a low fruit and vegetable intake face up to twice the risk of developing cancer compared to those with a high intake (Block et al., 1992). Several years later, a joint report by the World Cancer Research Fund together with the American Institute of Cancer Research found 'convincing' evidence that a high fruit and vegetable intake would reduce cancer of the colon and rectum (AIRC, 1997).
Unfortunately, 10 years later, an updated report released by the same organization and based on large prospective studies instead on case-control studies, downgraded these previous conclusions. The evidence that high intakes of fruit and/or vegetables decrease the risk for cancers of the mouth and pharynx, esophagus, stomach, colorectum and lung were judged 'probable' or 'limited- suggestive', so researchers did not confirm the earlier results (AIRC, 2007).
In a randomized dietary intervention trial, called The Polyp Prevention Trial, it was examined the effectiveness of a low-fat, high-fiber, high-fruit, and high-vegetable diet on adenoma recurrence. This study was the first to examine the association between flavonoid intake and colorectal adenoma recurrence. It was found that total flavonoid intake was not associated with colorectal adenoma recurrence, but they also detected during the trial a reduced risk of advanced adenoma recurrence with greater flavonol consumption (Bobe et al., 2008).
The European Prospective Investigation into Cancer and Nutrition in 2009 suggested that a high consumption of fruit and vegetables is associated with a reduced risk of CRC, especially of colon cancer but differs according to smoking status. An inverse association for

never and former smokers and a statistically non significant positive association for current smokers was observed (van Duijnhoven et al., 2009).
Key, on the other hand, by summarizing data recorded from large prospective studies or pooled analyses, recommended a diet which contains moderate amounts of fruit and vegetables in order to prevent deficiencies of any nutrients. Nevertheless, the available data suggest that, at least in relatively well-nourished populations, general increases in fruit and vegetable intake would not have much effect on cancer rates (Key, 2011).
Due, at least in part, to their anti-oxidant and anti-inflammatory activities, epidemiologic studies suggest that the consumption of anthocyanins lowers the risk of cardiovascular disease, diabetes, arthritis and cancer (Prior and Wu, 2006). Their activities are associated to their action at different molecular level: direct ability to scavenge reactive oxygen species (Wang and Jiao, 2000) or to induce phase II antioxidant and detoxifying enzymes (Shih et al., 2005; Shih et al., 2007).

2.2 Dietary compounds and tumor progression

Cancer cells differ from normal cells due to the following properties: unlimited replication potential, the absence of apoptosis, the absence of telomere shortening, angiogenesis and metastasis. Dietary compounds have been shown to affect molecular events involved in the initiation, promotion and progression of cancer, thereby inhibiting carcinogenesis. Furthermore, their inhibitory activity may ultimately suppress the final steps of carcinogenesis as well, namely angiogenesis and metastasis. The relationship between the frequency of consumption of vegetables and fruit and cancer risk is linked to a class of phytochemicals which flavonoids belong to.
The unlimited replication potential of cancer cells is a result of the inactivation of tumour suppressor genes. For instance, mutated p21 gene products are no longer able to bind to cyclin, thus cyclin-dependent kinase remains active and cell division becomes uncontrolled. Targeting these protein kinases using natural products has been seen as a promising approach in solving the cancer menace (Omura et al., 1995; Yasuzawa et al., 1986). Although research on protein kinases is still at an early stage, there is enough evidence that dietary compounds have useful potency and specificity against protein kinases of medicinal importance.
Resveratol has been shown by numerous reports to inhibit cell proliferation through the inhibition of cell-cycle progression at different stages (Aggarwal and Shishodia, 2006; Liang et al., 2003; Takagaki et al., 2005). Down-regulation of the cyclin D1/Cdk4 complex by resveratrol has been reported in colon cancer cell lines (Wolter et al., 2001) as well as resveratrol-induced G2 arrest through the inhibition of Cdk7 and Cdc2 kinases in colon carcinoma HT-29cells (Liang et al., 2003). Furthermore, an anthocyanin-rich extract caused cell cycle arrest and increased expression of the p27kip1 and p21WAF1/Cip1 genes and a 60% cancer cell growth inhibition (Malik et al., 2003).
Abnormalities in the ubiquitin-proteasome system have been implicated in many protein degradation disorders, including several types of cancer. This has made the proteasome an important target for anti-cancer drug discovery. Proteasome inhibitors can be categorized as synthetic and natural, where natural molecules are often more specific and potent than synthetic ones (D'Alessandro et al., 2009). Chen and colleagues showed that dietary flavonoids apigenin and quercetin inhibit proteasome, and this inhibition may contribute to their cancer-preventative effects (Chen et al., 2005). Furthermore, Kazi and colleagues also

showed that the tumor cell apoptosis-inducing ability of genistein (a soy flavonoid) is associated with its inhibition of proteosome activity (Kazi et al., 2003).

Apoptosis is triggered when normal cells are worn out. In cancer cells, the telomerase activity allows them to evade apoptosis by stabilizing and elongating telomeres through synthesis of de novo telomeric DNA (Naasani et al., 2003). Telomerase activity has been identified in most human tumors (Kim et al., 1994). A high telomerase activity has been linked to the degree of malignancy and likelihood of tumor progression (Fujiwara et al., 2000; Hiyama et al., 1995). Tea catechins, especially the degradation products of epigallocatechin gallate, epicatechin, quercetin, naringin and naringinin, have been found to inhibit telomerase activity (Naasani et al., 1998).

Angiogenesis, one of the hallmarks of cancer, vital to tumor growth and metastasis, is characterized by growth of new capillaries from preexisting vessels (Folkman, 1995). Cancer cells release vascular epithelial growth factor (VEGF), an angiogenic cytokine which stimulates blood vessel growth. Inhibition of VEGF has therefore become a primary target for anti-angiogenic strategies, and inhibitors directed against either VEGF or its receptor VEGFR-2, have been demonstrated to prevent vascularization and growth of a large number of experimental tumor types (Labrecque et al., 2005; Underiner et al., 2004). Ellagic acid (naturally occurring phenolic constituent in fruits and nuts) has been shown to inhibit VEGF-induced migration of endothelial cells (Labrecque et al., 2005). Green tea catechins inhibit vascular endothelial growth factor receptor phosphorylation (Lamy et al., 2002), and resveratol also inhibits vascular endothelial growth factor (VEGF)-induced angiogenic effects in the human umbilical vein endothelial cells through the abrogation of VEGF-mediated tyrosine phosphorylation of vascular endothelial (VE)-cadherin and its complex partner, b-catenin (Aggarwal and Shishodia, 2006; Lin et al., 2003). In addition, the flavonoid luteolin also inhibited both VEGF-induced survival and proliferation of the human umbilical vein endothelial cells (Bagchi et al., 2004). *In vitro* studies have shown that anthocyanin-rich berry extract formula exhibited a potent inhibitory effect on H_2O_2-induced VEGF expression. Anthocyanins suppress angiogenesis through the inhibition of H_2O_2- and tumor necrosis factor alpha (TNF-a)-induced VEGF expression, as well as through the inhibition of VEGF and VEGF receptor expression (Bagchi et al., 2004).

Metastasis occurs when cancer cells invade blood and lymphatic vessels and are transported to other cells and tissues in the body. Cancer cells produce proteinase enzymes that allow them to invade blood and lymphatic vessels. The matrix metalloproteinases (MMP) are a group of proteolytic enzymes that degrade the extracellular matrix (ECM) components (Nabeshima et al., 2002). MMP-2 and MMP-9 are two important MMPs in cell invasion as cancerous tissues and tumor cells have shown increased levels and activities of both MMP-2 and MMP-9 (Bernardo and Fridman, 2003).

Proanthocyanidins and flavonoids from cranberry and other *Vaccinium* berries have been shown to inhibit the expression of MMPs involved in remodeling the extracellular matrix (Pupa et al., 2002). Curcumin inhibits MMP-2, which is implicated in the formation of loose and primitive looking meshwork formed by aggressive cancers such as melanoma and prostate cancers (Aggarwal and Shishodia, 2006). Resveratrol has been found to cause a dose-dependent inhibition of PMA (Phorbol Myristate Aacetate)-induced increases in MMP-9 expression and activity and also the suppression of MMP-9 mRNA expression. Furthermore, Rose and colleagues found that phytochemicals from broccoli and rorripa have anti-invasive and anti-metalloproteinase activities (Rose et al., 2005). Purified ursolic

acid and hydroxycinnamate esters from cranberry fruit strongly inhibited expression of MMP-2 and MMP-9 activities at micromolar concentrations in fibrosarcoma cells (Cha et al., 1996). Anthocyanins from mulberry fruits and highbush blueberry (*V. angustifolium*) inhibited MMP-2 and MMP-9 activities (Huang et al., 2008; Matchett et al., 2005; Matchett et al., 2006). Delphinidin can inhibit invasion of human fibrosarcoma cells through down-regulation of MMP-2 and MMP-9, expression (Nagase et al., 1998). More recently, it has been demonstrated that black rice anthocyanins, cyanidin 3-glucoside and peonidin 3-glucoside, significantly reduce the expression of MMP-9 in diverse types of cancer cells (Chen et al., 2006). Furthermore, it was also demonstrated that the activities of MMP-2 and -9 were dose-dependently suppressed by anthocyanin treatment on HT-29 human colon cancer cells (Yun et al., 2010) and in HCT-116 human colon cancer cells through the activation of 38-MAPK and suppression of the PI3K/Akt pathway (Shin et al., 2011).

2.3 Flavonoids

Bioactive compounds that impart protective properties to plants against various pathological conditions are grouped under the name of phytochemicals. Active components of dietary phytochemicals which have been identified to protect against cancer include curcumin, resveratrol, diallyl sulfide, S-allyl cysteine, allicin, lycopene, capsaicin, diosgenin, 6-gingerol, ellagic acid, ursolic acid, silymarin, anethol, eugenol, isoeugenol, dithiolthiones, isothiocyanates, indole-3-carbinol, protease inhibitors, saponins, phytosterols, inositol hexaphosphate, Vitamin C, D-limonene, lutein, folic acid, beta carotene, selenium, Vitamin E, flavonoids, and dietary fiber (Aggarwal and Shishodia, 2006).

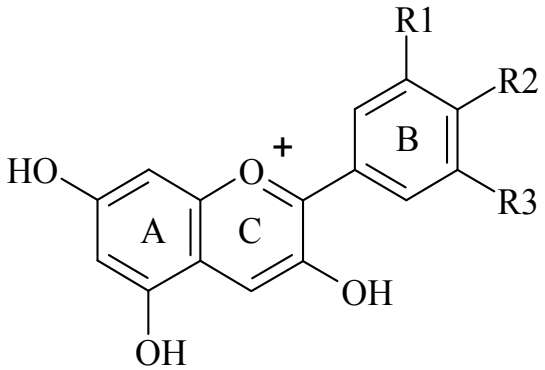
Flavonoids represent one of the largest groups of secondary metabolites whose name refers to a class of more than 6500 molecules based upon a 15-carbon skeleton (Harborne and Williams, 2000; Ververidis et al., 2007). They are divided into six major classes: flavanols, flavonones, flavones, isoflavones, flavonols and anthocyanins. Flavonoids are not synthesized in animal cells, thus their detection in animal tissues is indicative of plant ingestion (Mennen et al., 2008). Dietary flavonoids play an important role in cancer prevention and inhibition influencing various cellular processes, such as reactive oxygen species production and cell signal transduction pathways related to cellular proliferation, apoptosis, and angiogenesis (Yao et al., 2011).

Flavonoids compounds are the most studied anticarcinogens among phytochemicals. Anthocyanins, a particular class of this group of molecules, are the most abundant flavonoid constituents of fruits and vegetables (Wang and Stoner, 2008).

2.3.1 Anthocyanins: Chemistry

Anthocyanins (Greek anthos = flower and kyanos = blue) are water-soluble pigments in fruits and vegetables, responsible for red, blue and purple colors. In plant cells, they are present in vacuoles in the form of various sized granules. Their basic anthocyanidin aglycone structures consist of an aromatic ring A bonded to a heterocyclic ring C that contains oxygen, which is also bound by carbon-carbon bond to a third aromatic ring B (Figure 1). Anthocyanins normally occur in nature in glycoside forms. The sugar moiety is mainly attached to the C ring (in the 3-position) or to the A ring (in the 5, 7-position). Glucose, galactose, arabinose, rhamnose and xylose are the most common sugars bonded to the anthocyanidins. These glycosylated forms are known as anthocyanins. More than 500 different anthocyanins have been found, among which the most common is cyanidin

3-glucoside. The most common anthocyanidins (anthocyanins aglycones) found in nature are pelargonidin, peonidin, cyanidin, malvidin, petunidin and delphinidin (Figure 1) (Castañeda-Ovando et al., 2009; Manach et al., 2004; Szajdek and Borowska, 2008).



Anthocyanidin	R1	R2	R3
Pelargonidin	H	OH	H
Cyanidin	OH	OH	H
Delphinidin	OH	OH	OH
Peonidin	OCH_3	OH	H
Petunidin	OCH_3	OH	OH
Malvidin	OCH_3	OH	OCH_3

Fig. 1. Chemical structures of anthocyanidins (Prior and Wu, 2006).

2.3.2 Fate of anthocyanins in the gastro-intestinal tract

The lack of the knowledge of anthocyanin metabolism in the gastrointestinal tract has been studied by many authors (Aura, 2005; Hassimotto et al., 2008; He et al., 2009; McGhie and Walton, 2007; Vitaglione et al., 2007). The fate of anthocyanins in the gastrointestinal tract is summarized in Table 1.

Part of gastrointestinal tact	Anthocyanin fate
Mouth	Deglycosylation? (McGhie and Walton, 2007; Selma et al., 2009)
Stomach	Chemical stability (Hassimotto et al., 2008; McDougall et al., 2005) Absorption (Felgines et al., 2008; Passamonti et al., 2003b)
Small intestine	Deglycosylation, degradation, absorption
Large intestine	Deglycosylation, degradation (Talavera et al., 2004)

Table 1. Fate of anthocyanins through the gastrointestinal pathway

There are no data of the effect of saliva on anthocyanins but some publications suggest that flavonoid glycosides are hydrolyzed to corresponding aglycons (McGhie and Walton, 2007; Selma et al., 2009).

In the stomach, anthocyanins remain intact due to the low pH that shifts the molecules toward the most stabile flavylium cation. Anthocyanins absorption takes place in the stomach through active transport (including transport carriers such as bilitranslocase (Passamonti et al., 2003b) and sodium dependent glucose transporter (Felgines et al., 2008)) and continues in the small intestine (Talavera et al., 2004).

At the intestine neutral pH anthocyanins exist in equilibrium of four molecular forms (flavylium cation, quinoidal base, carbinol pseudobase and chalcone pseudobase) thus they can be easily exposed to degradation (McDougall et al., 2005). First studies showed degradation of anthocyanins from tart cherries to phenolic acids (Seeram et al., 2001). Later, their degradation was demonstrated by two steps. The first step is deglycosylation of anthocyanins to anthocyanidin aglycon while the second step is degradation of the formed aglycon to phenolic acid and aldehyde (Ávila et al., 2009; Fleschhut et al., 2006).

Deglycosylation is the cleavage of the glycosyl moiety from anthocyanins structure to form anthocyanidin aglycons.

These reactions could take place due to intestinal microflora (Aura et al., 2005b; Ávila et al., 2009; Fleschhut et al., 2006), under intestinal conditions at pH 7 (Fleschhut et al., 2006), or spontaneously in the presence of intestinal epithelial cells (Hassimotto et al., 2008; Kay et al., 2009).

Degradation of anthocyanidin aglycon, achieved spontaneously or by microflora (Ávila et al., 2009; Fleschhut et al., 2006; Forester and Waterhouse, 2008), represents the breakdown of its heterocycle and cleavage of the C-ring to form phenolic acid and aldehyde (Keppler and Humpf, 2005). Spontaneous degradation is a consequence of the neutral pH because anthocyanidin aglycones are observed in chalcone form which is rather unstable and can be easily degraded (Fleschhut et al., 2006; Keppler and Humpf, 2005). Data showed that major degradation products of anthocyanidin aglycons degraded to corresponding phenolic acids (Table 2), as well to some other less present products still unidentified (Ávila et al., 2009; Fleschhut et al., 2006; Forester and Waterhouse, 2008). Further phenolic acids can be transformed to the benzoic acids in the presence of intestinal bacteria by cleavage of the hydroxyl group in the 4-position (Aura et al., 2005a; Selma et al., 2009).

Anthocyanidin aglycon	Corresponding phenolic acid
Cyanidin	Protocatechuic acid
Delphinidin	Gallic acid
Pelargonidin	4-hydroxybenzoic acid
Malvidin	Syringic acid
Peonidin	Vanilic acid
Petunidin	3-*O*-methylgallic acid

Table 2. Degradation products of anthocyanidin aglycons

The fastest degraded were anthocyanidin aglycons, much faster than anthocyanin monoglycosides (Keppler and Humpf, 2005). As well anthocyanin degradation by intestinal microflora was much faster than spontaneous one (Forester and Waterhouse, 2008).

Anthocyanins that are not absorbed or degraded in the gastrointestinal tract can be excreted as intact forms. Unchanged anthocyanins were detected in human fecal samples 24 hours after blood orange juice consumption (Vitaglione et al., 2007), as well as in fecal samples collected from rats previously fattened by chokeberries, bilberries and grapes (He et al., 2005).

3. Citotoxicity/apoptosis of anthocyanins on colon cancer cells

As mentioned above, naturally occurring dietary substances, in particular, flavonoids, have gained increased attention as agents interfering with processes involved in cancer development and progression. Among them, anthocyanins might be of particular interest since their daily intake is remarkably high compared to other flavonoids - it is estimated to vary between 180 and 215 mg (Hou, 2003) whereas the intake of other flavonoids reaches only 20-25 mg/day. Numerous recent studies indicate that anthocyanins are able to inhibit the growth of embryonic fibroblasts and of different cancer cells derived from malignant human tissues, suggesting their possible role as chemopreventive agents. This brings in focus their possible importance for public health as dietary components with preventive impact on cancer as well as effective, cheap and safe anticancer supplements.

3.1 Cytotoxicity in colon and other cancer cell lines

There are few reports on the inhibitory effects of anthocyanins on colon cancer cell growth. Extracts of grapes, bilberries and chokeberries rich in anthocyanins have been shown to inhibit the growth of human malignant HT-29 colon cancer cells but did not affect the growth of non-malignant colon-derived cells (Zhao et al., 2004). Similar effect was observed in highly and low tumorigenic colon cancer cell lines, LoVo/Adr and LoVo. While delphinidin and cyanidin were cytotoxic and induced apoptosis in the former, they failed to demonstrate a similar effect in the latter (Cvorovic et al., 2010). Anthocyanins from tart cherries significantly reduced proliferation of human colon cancer cells HT29 and HCT-116 as well (Kang et al., 2003; Marko et al., 2004). An anthocyanins extract from Vaccinium uliginosum suppressed the growth of human colorectal cancer cells DLD-1 and COLO205 in a dose-dependent manner through the induction of apoptosis. It was hypothesized that the anticancer efficacy might be attributed to its high percentage of malvidin (Zu et al., 2010). On the other hand, the antiproliferative and the anti-cancer potential of several berry extracts containing different profiles of phenolic compounds (anthocyanins, flavonols, and ellagitannins) was studied in human colon cancer HT-29 cells. All the berry extracts studied decreased the proliferation and the number of HT-29 cells in the G0/G1 phase of the cell cycle. This correlated with their anthocyanin concentration supporting the fact that the inhibitory effect of berry extracts is based on the concentration rather than the composition of anthocyanins (Coates et al., 2007; Johnson et al., 2011; Wu et al., 2007).

Numerous studies reported antiproliferative activity of anthocyanins in human cancer cells derived from malignant tissues of various origins such as breast, lung, uterus, stomach, central nervous system, vulva, prostate (Lazze et al., 2004; Meiers et al., 2001; Olsson et al., 2004; Seeram et al., 2004; Zhang et al., 2005). Anthocyanins were potent and selective in inhibiting human promyelocytic leukemia cell proliferation as well (Feng et al., 2007; Hou et al., 2003; Katsube et al., 2003).

Animal studies have also reported anticarcinogenic properties of anthocyanins. In induced rat colon cancer cell models they significantly decreased total tumors as well as aberrant crypts (Hagiwara et al., 2001; Hagiwara et al., 2002; Harris et al., 2001; Lala et al., 2006; Magnusson et al., 2003). Cai and colleagues demonstrated that Red grape pomace extract (oenocyanin) interferes with adenoma development in the Apc^{Min} mouse by affecting tumor burden more prominently than tumor number. Oenocyanin efficacy was accompanied by the decreased adenoma cell proliferation and down-regulation of expression of the PI3 pathway component Akt, which supports cell proliferation (Cai et al., 2010). It was also

demonstrated in the same animal model that anthocyanin-rich tart cherry extract added to the drinking water was associated with fewer and smaller tumors in the cecum, but none of the tested treatments influenced the number of tumors in the small intestine or the number or burden of tumors in the colon. It was supposed, therefore, that lack of effect of anthocyanins on colonic tumor development may be a consequence of their metabolism by intestinal bacteria or their spontaneous degradation in the cecal and colonic environment (Bobe et al., 2006; Kang et al., 2003). Moreover, it was shown in Apc^{Min} mice that dietary consumption of anthocyanins in the form of either a mixture (Mirtoselect) or as a pure compound (cyanidin-3-glucoside) interferes with small intestinal adenoma development in a dose-dependent fashion. Authors remarked the presence of measurable levels of anthocyanins in the target organ and in the urine, and in concentrations near or below the detection limit in the systemic circulation. Unfortunately, the dietary dose, at which either agent was significantly efficacious when extrapolated by dose/ surface area comparison, suggested that equivalent for humans can be found in 740 g bilberries, that is a hefty dose. In terms of absolute dose of agent, cyanidin-3-glucoside was less efficacious than the Mirtoselect mixture. Furthermore, authors suggested that different results obtained, in comparison with other studies (Kang et al., 2003) were possibly due in part to the higher dose of anthocyanins employed but also due to the different way of administration since anthocyanins tend to be unstable in aqueous solution at neutral pH (Cooke et al., 2006). Recently, bilberry [(BB),Vaccinium myrtillus], lingonberry (LB, Vaccinium vitis-idaea), and cloudberry (CB, Rubus chamaemorus), rich in anthocyanins, proanthocyanidins and ellagic acid respectively, proved to be chemopreventive as demonstrated by a significant reduction in the number of intestinal tumors in Min/1 mice. Concerning their different chemical composition, authors suggested that the effects seen, may rather be a result of a mixture of compounds acting in synergy than an effect of a single active substance. Moreover, since the cellular levels of b-catenin are increased at all stages of colon carcinogenesis, it was demonstrated that two of these berries, LB and CB, markedly inhibited the growth of the adenomas and accumulation of nuclear b-catenin and cyclin D1. Unfortunately, also in this study, the amount of berries in the diets was high and could not be easily reached in a human diet (Misikangas et al., 2007).

Concerning other tumor models, the incidence, multiplicity and final mass of mammary tumors were significantly reduced in rats that would receive grape juice containing 15 different anthocyanins (Singletary et al., 2003). Cyanidin-3-glucoside reduced the size of lung cancer xenografts and significantly inhibited metastasis in nude mice (Ding et al., 2006). Lyophilized black raspberries prevented the development of NMBA (N-nitrosomethylbenzylamine)-induced esophageal tumors (Stoner et al., 2007), just like anthocyanin-containing pomegranate extract delayed the onset and reduced the incidence of DMBA (7,12-dimethylbenzanthracene)-induced skin tumors in CD-1 mice.

Epidemiological studies in humans are, however, still scarce and contradictory. Biopsies of tumor and normal-appearing tissues in colon cancer patients consuming black raspberry powder daily during several weeks, showed reduced proliferation and increased apoptosis in cancerous but not in normal tissue. Antiangiogenic effect was also observed in these patients (Wang et al., 2007). A phase I pilot study in colorectal cancer patients demonstrated that treatment with black raspberries caused positive modulation of biomarkers of tumor development, including cell proliferation, apoptosis, angiogenesis and Wnt pathway in both colorectal adenocarcinomas and adjacent normal tissues (Wang et al., 2007). In a clinical pilot study twenty-five colorectal cancer patients, scheduled to undergo resection of primary

tumor or liver metastases, received different amount of mirtocyan. This is a standardized anthocyanin mixture extracted from bilberries administered daily for 7 days before surgery. In the immunohistochemical observations of colorectal tumors from all patients who had received mirtocyan, in comparison with the preintervention biopsy, the proliferation index, reflected by Ki-67 staining, was significantly decreased by 7%. The apoptotic index in colorectal cancer samples from all patients increased from 3.6% to 5.3% of epithelial cells. However, in the absence of a zero dose control group, authors couldn't determine if this increase could, at least, to some extent, be the consequence of inherent procedural differences in measurements. Nevertheless, the pharmacodynamic changes observed seemed to be more prominent in patients at a dose of anthocyanins, which elicited target tissue levels below the detection limit, than at higher one, which furnished detectable anthocyanin levels in colorectal tissue (Thomasset et al., 2009).

However, an Italian study aimed at investigating the relationship between anthocyanidins intake and risk for oral or pharyngeal cancer did not show any significant association (Rossi et al., 2007). There was no protective effect demonstrated on the development of prostate cancer either (Bosetti et al., 2006). Optimal tumor inhibition occurs when the berry anthocyanins are added to the diet before, during and after treatment with carcinogens, suggesting that consumption of berries throughout life may maximize their chemopreventive effectiveness in humans. The fact that berry diets show a variable effect on tumorigenesis suggests that the inhibitory components of berry extract are not completely absorbed and/or that molecules housed in berry extracts do not affect certain critical signaling pathways of carcinogenesis (Stoner, 2009). Although further proves are needed, these studies open a possibility for anthocyanins to be considered for use in cancer treatment in combination with other therapeutic methods.

3.2 How anthocyanins work – The mechanisms

Antimutagenic and anticarcinogenic activity of anthocyanins is generally ascribed to their antioxidant properties conveyed by their phenolic structure. The double bonds in the ring and the hydroxyl side chains confers them potent free-radical scavenging activities (the positively charged oxygen atom in their molecule makes them more generous hydrogen-donating antioxidants compared to other flavonoids), but also enables their metal chelation and protein binding properties (Kong et al., 2003). Apart from acting as direct free-radical scavengers, anthocyanins have been demonstrated to affect the activity of phase II enzymes well-known for their detoxifying and antioxidant properties and therefore important in cancer prevention. *In vivo* studies showed that the diet supplemented with freeze-dried blueberries or black raspberries, both rich in anthocyanins, led to increased glutathione S-transferase (GST) activity in rats (Boateng et al., 2007; Reen et al., 2006). On the other hand, intake of an anthocyanin-rich mixed berry juice reduced oxidative DNA damage in peripheral-blood mononuclear cells and significantly increased total glutathione (GSH) level and GSH status in whole blood in male healthy non-smoking probands (Weisel et al., 2006). All this speaks in favor of a multi-level antioxidant activity of anthocyanins.

However, numerous recent studies, on the anthocyanins role in tumor growth inhibition, point at their prooxidant properties. It has been shown that the apoptotic effect of anthocyanins in malignant cells could be result of their ability to induce ROS accumulation in these cells. Moreover, the apoptotic activity was directly correlated to the number of hydroxyl groups at the B-ring (Hou et al., 2003). Interestingly, ROS generation was observed in leukemia cells treated with cyanidin-3-rutinoside, but not in normal human peripheral-

blood mononuclear cells. Parallel with the accumulation of ROS, Feng and colleagues demonstrated the increase of peroxides, but not superoxides in these cells, suggesting the reaction with the glutathione antioxidant system as one of the possible mechanisms for this prooxidant activity, together with ROS-dependent activation of p38 and JNK (Feng et al., 2007). Similarly, both delphinidin and cyanidin, showed prooxidant activity and induced apoptotic changes and cytotoxic effect in metastatic colorectal drug-resistant cells (LoVo/ADR), but not in cells originating from primary tumor site, Caco-2 (Cvorovic et al., 2010). This "inconsistent" behavior of the anthocyanidins might be influenced by cellular energy metabolism changes associated with neoplastic transformation (Warburg, 1956b). Indeed, the rate of lactate production is significantly higher in highly tumorigenic LoVo/ADR than in low tumorigenic LoVo cells (Fanciulli et al., 2000), and, presumably, in CaCo-2 as well. And even a slight decrease of pH might favor protonation of anthocyanidins, a mechanism causing loss of their free-radical scavenging activity (Borkowski et al., 2005). However, it is not clear if anthocyanidins directly promote oxidative stress in LoVo/ADR cells. One of the possible mechanisms proposed in this study is the interference with the glutathione antioxidant system. Delphinidin and cyanidin inhibited glutathione reductase (GR) activity in LoVo/ADR cells and significantly depleted their intracellular glutathione levels, while failing to induce any similar effect in CaCo-2 cells. These studies give evidence that anthocyanins preferentially kill cancer cells with high malignant characteristics and resistant to conventional treatment regimens, which could set the basis for the development of new sensitizing agents in the treatment of metastatic disease.

4. Biochemical features accompanying cytotoxicity/apoptosis

4.1 Membrane transport of anthocyanins

All the metabolic actions exerted by anthocyanins imply their cellular bioavailability. Previous *in vivo* studies have reported that anthocyanins are absorbed in the stomach and small intestine (Passamonti et al., 2003a; Talavera et al., 2003; Talavera et al., 2004). Felgines and colleagues administered an oral dose of a radiolabelled cyanidin 3-O-glucoside, demonstrating that the major site of absorption in mice is the intestine with a minimal accumulation of the radioactivity in tissues out of the gastrointestinal tract (Felgines et al., 2010).

Intestinal barrier is impermeable to most flavonoid glucosides because, based on their molecular structure, anthocyanins and their aglycones cannot cross the cell membrane passively (Dreiseitel et al., 2009). Among the influx carriers, the hexose transporters SGLT1 and GLUT 5 are expressed on apical membrane of the intestinal epithelium. Different groups suggested that anthocyanins, based on their glycosides moiety, could be transported by glucose carrier SGLT1. However, the involvement of this protein it is not completely understood (Milane et al., 2007; Talavera et al., 2004; Wolffram et al., 2002).

Recently, it was also demonstrated that GLUT2, another glucose transporter, is expressed not only at the basolateral but also at apical membranes of intestinal cells. Faria and colleagues showed that kinetic parameters of ^{3}H-2-deoxy-D-glucose-uptake of GLUT2 are changed after acute treatment with anthocyanins, supporting a favorable use of anthocyanins in diabetic population. Interestingly they also observed an increased GLUT2 expression (not for SGLT1 or GLUT5) after a chronic exposure to anthocyanins speculating that this behavior could increase their own bioavailability (Faria et al., 2009).

Bilitranslocase (BTL) is an organic anions transporter specific for bilirubin, initially found in the membranes of hepatic sinusoidal cells, but present also at the gastric mucosa and in renal tubules (Baldini et al., 1986; Elias et al., 1990; Sottocasa et al., 1989). Some of its substrates are nicotinic acid, bromosulfophthalein, cibacron blue and some flavonoids (Passamonti et al., 2009; Passamonti et al., 2002). Among flavonoids family, 17 anthocyanins showed competitive inhibitory behavior to specific transport assay with delphinidin as the most active molecule (Passamonti et al., 2002). It was also demonstrated that the BTL is directly involved in the vasoactivity of flavonoids: vasorelaxation induced by both, cyanidin 3-glucoside and bilberry anthocyanins, was significantly decreased in aorta rings pre-treated with anti-BTL antibodies (Ziberna et al., 2011). Recent studies have revealed that bilitranslocase is also expressed at the intestinal epithelial level, in particular, at the apical domain. Caco-2 cells express BTL as well and the uptake of BSP into these cells is strongly inhibited by anti-bilitranslocase antibodies (Passamonti et al., 2009).
The results reported should be further implemented to clarify the involvement of the influx membrane transporters.
More data are available on flavonoids efflux transporters. Major interest on these proteins is linked to their involvement in cancer resistance. These proteins belong to the class of the ABC transporters (ATP-binding cassette), and their role in cancer cells is to prevent the accumulation of anticancer drugs. Some ABC transporters are MRP1 (multidrug resistance-associated protein 1, ABCC1) (Cole et al., 1992), MRP2 (ABCC2) and MRP3 (ABCC3) (Borst et al., 1999) as well as BCRP/MXR1 (ABCG2) (Doyle et al., 1998; Miyake et al., 1999) and Breast Cancer Resistance Protein BCRP (ABCG2) however, P-glycoprotein (ABCB1) is overexpressed to the highest level and plays the major role. It was shown that flavonoids interact with these transporters but their effects are often contradictory depending on the type of cancer cells (Di Pietro et al., 2002). Moreover, a different behavior depending on the molecular structure was also demonstrated. Dreiseitel and colleagues showed that, depending on the sugar moiety, some flavonoids can act as BCRP stimulators while others act as inhibitors (Dreiseitel et al., 2009; Morris and Zhang, 2006).
However, the significance of these flavonoid–efflux transporter interactions has not been unequivocally demonstrated since it is impossible to exclude the involvement of other drugs transporters and of intracellular metabolizing enzymes that modify the substrate disposition (Morris and Zhang, 2006).

4.2 Oxidative stress and apoptosis

Reactive oxygen species (ROS) and reactive nitrogen species (RONS) are a collective term that broadly describes O_2-derived free radicals such as superoxide anions ($O2^{\bullet -}$), hydroxyl radicals (HO•), peroxyl (RO_2•) and alkoxyl radicals (RO•), nitrogen monoxide (NO•), peroxynitrite ($ONOO^-$), nitrogen dioxide (NO_2•) as well as O_2-derived non-radical species such as hydrogen peroxide (H_2O_2) (Halliwell and Cross, 1994). Both reactive species are important mediators in the normal regulation of different physiological processes such as cellular proliferation or activation. On the other hand, the imbalance of cellular redox homeostasis is described at the base of many chronic diseases and is also involved in cancer development (Acharya et al., 2010).
Specific ROS such as H_2O_2 or superoxide have been implicated as crucial mediators of apoptotic cell death (Casado et al., 2002; Circu and Aw, 2010; Madeo et al., 1999). ROS tend to enhance survival or promote cell death by activating different factors such as members of

the mitogen-activated protein kinases (MAPKs), phosphatidylinositol-3-kinase (PI3K)/Akt pathway, phospholipase C-g1 (PLCg1) signaling, protein kinase C, p53 signaling, ataxia-telangiectasia-mutated (ATM) kinase, nuclear factor-kappaB (NF-kB) signaling, and Jak/Stat pathway. ROS modulate the apoptotic signaling pathway through the cellular redox status by activating key protein kinases (Chan et al., 2010; Noguchi et al., 2005). Pro-oxidants such as H_2O_2 or other stressors, could induce apoptosis (or programmed cell death) by activating the intrinsic or "mitochondrial" apoptosis pathway that results in the damage of this sub-cellular compartment and the pro-apoptotic factors release (Circu and Aw, 2010; Mates et al., 2008). ROS involved in apoptosis derive both from environmental pro-oxidants or from intracellular respiratory dysfunction since mitochondria are the main site of intracellular source of ROS production. It was reported that oxidative stress plays an important role in the molecular mechanism of colorectal cancer (Keshavarzian et al., 1992) Flavones in HT-29 colon cancer cells increase the uptake of pyruvate or lactate into mitochondria, which is followed by an increase in O_2^- production that finally leads to apoptosis (Wenzel et al., 2005).

The prooxidant activity of anthocyanins through the increase of intracellular ROS production has been clearly explained in several studies (Feng et al., 2007; Hou et al., 2005).

4.3 GSH role in apoptosis

Intracellular glutathione (GSH) is a major buffer of cellular redox status due to its active SH-group that has reducing nucleophilic properties (Meister, 1983; Meister, 1991; Meister and Anderson, 1983). It acts as reducing agent, antioxidant and free-radical scavenger against ROS generated during oxidative metabolism and/or oxidative stress (Donati et al., 1990; Hall, 1999a; Hall, 1999b; Sies, 1999) and is also involved in the metabolism of xenobiotics and some cellular molecules (Wu et al., 2004). Free glutathione is present mainly in its reduced form maintained by the action of glutathione reductase (GR), but chemical oxidation of GSH to GSSG can occur as a result of numerous enzyme-catalysed reactions that use GSH to reduce hydrogen peroxide or other peroxides to water or the corresponding alcohol (Diaz Vivancos et al., 2010). GSH is preferentially (85-90%) located in the cytosolic-nuclear compartments and only a small amount is present in mitochondria and endoplasmic reticulum (Hwang et al., 1992; Meredith and Reed, 1982). The free-radical and antioxidant action of GSH depends on its involvement in different enzymatic reactions as those catalyzed by glutathione peroxidases (GPxs) (Lei, 2002), glutathione-S-transferases (GSTs), formaldehyde dehydrogenase, maleylacetoacetate isomerase, and glyoxalase I (Arrigo, 1999; Dickinson and Forman, 2002; Dickinson et al., 2002; Hayes and McLellan, 1999). Cancer cells present elevated GSH levels that generally increase antioxidant capacity and resistance to oxidative stress and regulate different mechanisms linked to carcinogenesis, sensitivity against cytotoxic drugs, ionizing radiation, and some cytokines, DNA synthesis, and cell proliferation (Estrela et al., 2006). There are yet a few reports on the possible role of flavonoids, as well as other phytochemicals, in modulating the glutathione antioxidant system activity, including regulation of GSH intracellular levels through targeting its synthesis (Ramos and Aller, 2008), induction of MRP-1 mediated GSH efflux (Kachadourian and Day, 2006), or inhibition of glutathione peroxidase enzyme activity (Trachootham et al., 2006). Upon grape seed extract treatment, HT29 colon cancer cells showed increased ROS production (that might result in oxidative stress in cells) and a decreased level of intracellular reduced glutathione (Kaur et al., 2011). In addition, after delphinidin and

cyanidin treatment in primary (Caco-2) and metastatic (LoVo and LoVo/ADR) colorectal cancer cell lines, no significant changes in the total GSH levels were observed in Caco-2 and LoVo cells, while both were shown to deplete intracellular glutathione levels in LoVo/ADR cells. GSSG content was not measurable in Caco-2 and LoVo cells, suggesting a normal cellular GSH/GSSG ratio (30:1–300:1) (Cvorovic et al., 2010). Cells undergoing apoptosis appear to rapidly and selectively release GSH into the extracellular space (Ghibelli et al., 1995; Ghibelli et al., 1998; Hammond et al., 2007). Hammond and colleagues demonstrated that apoptotic GSH export is directly linked to MRPs. Indeed basal and apoptotic GSH releases were decreased after RNAi reduction of MRP1 expression in Jurkat cells, indicating that MRP1 is a major player in both processes (Hammond et al., 2007). MRP1-channelled GSH export from cells can be also increased by different xenobiotics, including arsenite, verapamil (VRP), and some naturally-occurring flavonoids (Leslie et al., 2003; Loe et al., 2000).

GSTs are known as a family of Phase II detoxification enzymes that catalyze the conjugation of GSH (S-glutathionylation) with different compounds as xenobiotics and drugs or their metabolites, to form mercapturates (Hayes et al., 2005).

It has been recently shown that anthocyanin fractions from selected cultivars of Georgia-Grown Blueberries at 50-150 íg/mL do induce apoptosis in HT-29 colon cancer cells but these same concentrations decrease GST activities rather than induce it (Srivastava et al., 2007).

There are several studies, in normal cells and tissues, in which it was demonstrated that anthocyanins, probably involving some protein kinases, modulate the activity of some GSH-dependent enzymes, thus ameliorating the antioxidant response (Hou et al., 2010; Suda et al., 2008; Veigas et al., 2008).

GSSG formed intracellularly is continuously reduced to GSH by the activity of GR. If oxidative stress or other factors limit the GR activity (e.g., glucose-6-phosphate dehydrogenase deficiency may limit NADPH supply), GSSG will accumulate (Deneke and Fanburg, 1989). In this respect, Cvorovic and colleagues showed that delphinidin and cyanidin did inhibit GR activity in LoVo/ADR cells but not in Caco2 and Lovo cells (Cvorovic et al., 2010). This has two important consequences: (i) the thiol redox status of the cell will shift, activating oxidant response transcriptional elements; and (ii) GSSG may be preferentially secreted out of the cell. (i) The protein sequences of many transcription factors contain cys residues, mainly localized in the DNA-binding domain that, when oxidized, cause a different modulation of gene expression (Arrigo, 1999). (ii) GSSG may be reduced back to GSH, but when GSSG is present in excess, it is also eliminated from the cell by export into the extracellular space. Strong evidence that this export step is mediated by MRP2 was provided by studies of GSSG transport with canalicular membrane-enriched vesicles derived from normal and EHBR (Eisai hyperbilirubinuric rats) rats (Ballatori et al., 2009).

4.4 Intracellular pH and apoptosis

Despite the genetic variability, two phenotypes common to all tumor cells are cellular alkalinization and a shift to glycolytic metabolism. In the first decade of the 20th century, Otto Warburg found that cancer cells, even in the presence of oxygen disposition and a higher request of ATP for fast growing cells, prefer to metabolize glucose via glycolysis instead of oxidative phosphorylation (Warburg, 1956a). The oxygen levels within a tumor

vary both spatially and temporally. The elevated glycolytic pathway of cancer cells appears to be a response to hypoxia due to the growth of the tumor surpassing the available vascular supplied oxygen (Mathupala et al., 2001) and seems to be controlled directly by the antiapoptotic protein Akt that generates apoptotic resistance *in vitro* (Elstrom et al., 2004).
Then, the decreased dependence on aerobic respiration becomes a selective advantage for survival and proliferation escaping from the apoptotic event. Cell metabolism is shifted toward the increased expression of glycolytic enzymes, glucose transporters, and inhibitors of mitochondrial metabolism that result in a transitional intracellular acidification (Hsu and Sabatini, 2008) and increased glucose uptake is observed coincident with the transition from colon adenomas to invasive cancer (Yasuda et al., 2001). Nevertheless, evidence that intracellular acidification is associated with the progression of apoptosis, has been steadily accumulating (Barry et al., 1993; Gottlieb et al., 1996; Li and Eastman, 1995; Rebollo et al., 1995).
An important role in the intracellular acidification could be due to alterations of membrane pHi-regulating mechanisms, including the Na^+/H^+ exchanger (NHE) that might favor accumulation of the protons produced by energetic metabolism. NHE is ubiquitously expressed transporter in the plasma membrane with a main function to extrude H^+ from the cytoplasm.
Multidrug resistant tumor cells exhibit an altered pH gradient across different cell compartments, which favors a reduced intracellular accumulation of antineoplastic drugs and a decreased therapeutic effect. In fact, the activity and expression of NHE are increased in doxorubicin-resistant (HT29-dx) human colon carcinoma cells in comparison with doxorubicin-sensitive HT29 cells (Miraglia et al., 2005). On the other hand, it was demonstrated that activation of the NHE-1 and the resulting cellular alkalinization play a key role in oncogenic transformation (Reshkin et al., 2000). Cyanidin (10 microM), but not its glycosides, could inhibit the neurotensin- and EGF-induced increased rate of extracellular acidification in HT-29 human colon adenocarcinoma cell line probably by inhibiting cellular metabolism, rather than directly altering Na+/H+ exchange (Briviba et al., 2001).
The effect of anthocyanins on metabolism involved in pH modulation of apoptosis is anyway a poor-trodden path.

5. Roadmap for further investigations

5.1 Role of flavonoids on DNA methylation

In humans, multistage carcinogenesis was previously considered a consequence of genetic alterations that cause activation of oncogenes and inactivation of tumor suppressor genes. In addition to genetic events, epigenetic events are another leading player in carcinogenesis (Link et al., 2010). Indeed, it is believed that majority of cancers result from changes that accumulate throughout the life due to the exposure to various endogenous factors and arguably diet and environment-mediated epigenetic perturbations play a crucial role in cancer progression in humans (Herceg, 2007). It was first recognized more than 25 years ago that in colorectal cancer cells, global DNA methylation patterns differed considerably from those in their normal counterparts (Venkatachalam et al., 2010).
The developmental biologist Conrad H. Waddington coined the term 'epigenetics' in 1942, trying to describe reversible heritable changes in gene expression that occur without alteration in DNA sequence sufficiently powerful to regulate the dynamics of gene expression (Waddington, 1951 as cited in (Hitchler and Domann, 2009).

One of the "epigenome" processes is DNA methylation, a covalent chemical modification resulting in addition of a methyl (CH3) group at the carbon 5 position of the cytosine ring in CpG dinucleotides (Kanai and Hirohashi, 2007). This process plays important roles in chromatin structure modulation, transcriptional regulation and genomic stability, and is essential for the development of mammals (Ducasse and Brown, 2006; Li, 2002). CpG dinucleotides are not uniformly distributed throughout the human genome, but are often enriched in the promoter regions of genes. Short CpG-rich regions are also called as ''CpG islands'', and these are present in more than 50% of human gene promoters and can lead to gene silencing and proliferation or to affect the metabolic processes associated with energy metabolism. (Link et al., 2010). This mechanism is an enzymatic process mediated by DNA methyltransferases (DNMT): DNMT1, also called a "maintenance methyltransferase", preserves existing methylation patterns following DNA replication; DNMT3a and DNMT3b, on the other hand, serve as *de novo* methyltransferases, which act independently of replication on both strands, altering the epigenetic information content (Yu et al., 2011).
Recent studies havedemonstrated that all three DNMTs are overexpressed in several tumor types, including tumors of the colon and rectum, bladder, and kidney. When DNMT1 and DNMT3b are knocked out in colon cancer cell lines, methylation of tumor suppressor genes such as p16 is almost entirely eliminated and the gene is re-expressed (Rhee et al., 2002), as well as it has been established that the inhibition of DNA methyltransferase activity can strongly inhibit the formation of tumors (Stresemann et al., 2006).
It is known that some nutrients like folic acid, B vitamins and SAM (S-adenosylmethionine) and anthocyanins are key components of the methyl-metabolism pathway (Vanzo et al., 2011). Their methyl-donating mechanism can rapidly alter gene expression by modulating the availability of methyl donors as well as DNMT activity (Ross, 2003). There is a growing interest in the role of polyphenols in prevention of DNA methylation. It was demonstrated that epigallocatechin-3-gallate (EGCG), a tea polyphenol, through its methylation exerted by catechol-O-methyltransferase (COMT), indirectly inhibited DNMT. Indeed, S-adenosyl-L-homocysteine (SAH), produced by COMT reaction is a potent inhibitor of DNMT (Fang et al., 2003). On the other hand, EGCG can directly inhibit DNMT through the hydrogen bonds formation with different residues in the catalytic pocket of the enzyme (Lee et al., 2005). Moreover, Fang et al. showed that reactivation of some methylation-silenced genes by EGCG was also demonstrated in human colon cancers and prostate cancer cells (Fang et al., 2003).

5.2 Apoptosis and ATP/ADP ratio

Oxygen consumption in cells is regulated by a respiratory control system which depends on ADP and Pi. When the amount of ATP is high, the amount of ADP is limited and therefore, use of oxygen declines. In other words, oxygen consumption increases as the need for ATP arises (Valle et al., 2010). ATP generation through oxygen conversion is not a fully efficient process because a percentage of the energy of the electrochemical gradient is lost and not coupled to ATP production (Matsuyama and Reed, 2000). This situation arises due to a phenomenon called 'proton leak' which causes protons to return to the mitochondrial matrix via alternative pathways that by-pass ATP synthase (Brand, 1990; Brown and Brand, 1991; Valle et al., 2010). Lynen suggested that the increased dependence of cancer cells on glycolysis stemmed not from their inability to reduce oxygen, but rather from their inability to synthesize ATP in response to the mitochondrial proton gradient (Lynen, 1951 as cited in (Samudio et al., 2009).

Although, some explanations for the 'proton leak' come from the biophysical properties of the inner membrane, much of the explanation comes from the activities of a family of mitochondrial proteins termed uncoupling proteins (UCPs) (Klingenberg, 1999; Valle et al., 2010). UCPs exploit the gap in pH concentration to transfer the proton through the inner membrane into the matrix where they are released. Consequently, the mitochondrial membrane potential decreases, reduction of O_2 via the respiratory chain is no longer linked to ATP synthesis and ATP/ADP exchange is not longer maintained (Vander Heiden et al., 1999).
The influence of anthocyanins on ATP/ADP ratio and on UCPs role could be the aim of further studies.

5.3 Apoptosis and oxygen consumption

Cancer cells seem to show high glycolytic rates even when oxygen is sufficient for oxidative phosphorylation (OXPHOS). This condition leads to a survival benefit of the tumor providing protection from oxidative stress and resulting in apoptosis avoidance (Kondoh et al., 2007a; Kondoh et al., 2007b). The importance of glycolysis in the survival of cancer cells was demonstrated by Bonnet and colleagues. Their experimental approach aimed at inhibiting the anaerobic glycolisys by repressing the activity of pyruvate dehydrogenase kinase (PDK) with dichloroacetate (DCA). PDK acts as a negative modulator of pyruvate dehydrogenase, a gate-keeping mitochondrial enzyme which controls the glucose oxidative fate into the cell. DCA changes the metabolism of cancer cells from the cytoplasm-based glycolysis to the mitochondria- based glucose oxidation. This led to increased ROS production and decreased mitochondrial membrane potential, efflux of pro-apoptotic mediators from mitochondria, and induction of mitochondria-dependent apoptosis only in cancer cells (Bonnet et al., 2007). On the other hand, in the majority of mammalian cells, glycolysis is inhibited by the presence of oxygen, which allows the mitochondria to oxidize pyruvate to CO_2 and H_2O.
The transcription factor p53 regulates cellular energy metabolism and antioxidant defense mechanisms. Emerging evidence has shown that these two functions of p53 contribute greatly to p53's role in tumor suppression (Bensaad and Vousden, 2007; Matoba et al., 2006; Sablina et al., 2005). Loss of p53 results in decreased oxygen consumption and impaired mitochondrial respiration and promotes a switch to high glucose utilization in aerobic glycolysis in cells (Maddocks and Vousden).
It was shown that p53 regulates the OXPHOS dependence of cell by modulating the assembly of a key complex in the mitochondrial electron chain transport: cytochrome c oxidase (COX) (Ma et al., 2007; Matoba et al., 2006). It was demonstrated, in fact, that in HCT116 cells, p53 controls the expression of SCO 2 (Synthesis of Cytochrome c Oxidase 2). SCO2 is required for the assembly of mitochondrial DNA-encoded COX II subunit (MTCO2 gene) into the COX, so, p53 directly regulates mitochondrial oxygen consumption. p53 mutations in cancer cells induce a loss in SCO2, thereby resulting in a switch from an aerobic mitochondrial respiration to anaerobic glycolysis. p53 induces SCO2 expression to enhance mitochondrial respiration and induces TIGAR expression to slow glycolysis (Won et al., 2011).
The metabolic implications of anthocyanins through the oxidative use of glucose could be appreciated indirectly. In fact, it is known that anthocyanins induce p53 expression (Fimognari et al., 2005; Lo et al., 2007; Renis et al., 2008), but a direct involvement of this compounds on glucose metabolic use it is not yet demonstrated.

6. Conclusions

The different observations found in epidemiological studies in comparison to the *in vitro* ones are linked, partly to the relatively low flavonoid intake and complexity of metabolism in humans, and partly to the lack of adequate molecular biomarkers for monitoring the earliest stages of disease development in humans (Pierini et al., 2008). Moreover, the relevance of the *in vitro* studies to the *in vivo* situation needs to be confirmed in view of the high concentrations of polyphenols employed in the *in vitro* studies.

All the data recorded about the role of polyphenols and flavonoids has been obtained through the use of classical cell biology and biochemistry methods. Maybe nutrigenomics, that is the study of the effects of foods and food constituents on gene expression could deepen our understanding of these and other phytochemicals (Corthesy-Theulaz et al., 2005; Davis and Hord, 2005; Mariman, 2006).

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8

Polyunsaturated Fatty Acids, Ulcerative Colitis and Cancer Prevention

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1. Introduction

Fatty acids (FA) – lipid constituents – are carboxylic acids that can be represented by the form RCO2H. Most often, the group R is a long carbon chain, unbranched and with an even number of carbon atoms and may be saturated or contain one (monounsaturated) or more double bonds (polyunsaturated) (Calder et al. 2002). Fatty acids are often referred to by their common names, but they are correctly identified by a systematic nomenclature. This nomenclature indicates first the number of carbon atoms in the hydrocarbon chain, followed by the number of double bonds, and the position of the first double bond from the terminal methyl group, which is indicated by n-9, n-7, n-6 or n -3 (Figure 1). There are two main families of polyunsaturated FA (PUFA), n-6 (or w-6) and of n-3 (or w-3) (Curi et al. 2002).

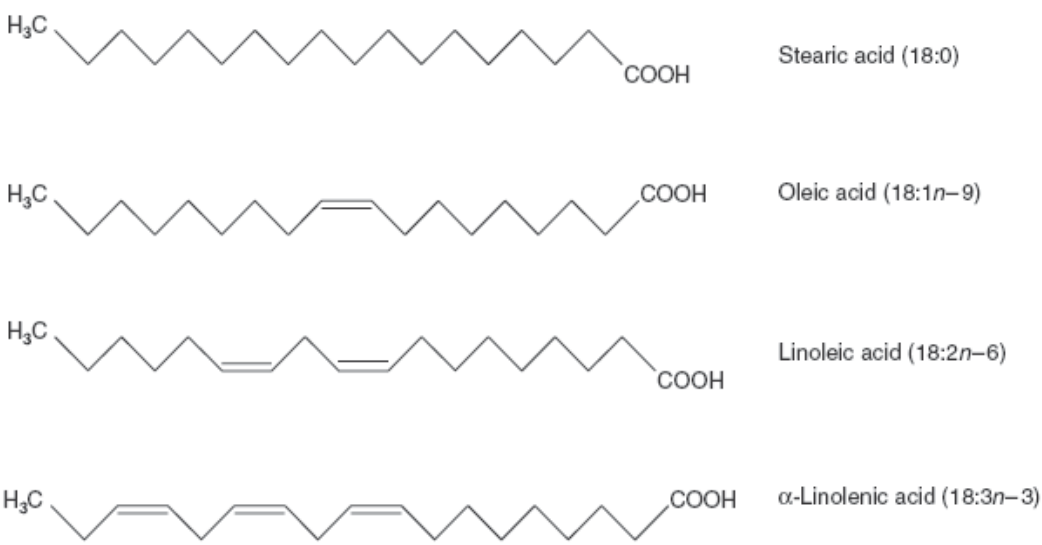

Fig. 1. Structure of some fatty acids (Sala-Vila et al. 2008).

Triacylglycerols (TAG), formed by three FA esterified to glycerol, are the main form of fat present in the human diet. TAG of animal origin are rich in saturated fatty acids and are characterised by being solid at ambient temperature (fats), while those of vegetable origin are rich in unsaturated fatty acids and liquid at room temperature (oils). TAG act as reserve lipids found in the form of oily microdroplets, emulsified in the cytosol (Lanning 1993). In addition to TAG, other lipids are present in small amounts in the diet, such as phospholipids, cholesterol, cholesterol esters and traces of free FA. Phospholipids are the major lipid components of the cell membrane, acting as structural elements, precursors of second messengers, and affecting the activity of some enzymes, such as phospholipase A2 and protein kinase C. Thus lipids, besides being a source of energy (immediate or reserve), act as key components of our body, both in terms of structure (cellular constituents) and function (Burr & Burr 1929, 1930).

Mammals synthesise saturated fatty acids from non-lipid precursors and unsaturated n-9 series and n-7; normally the diet provides adequate amounts of these fatty acids. However, the cell membrane also needs unsaturated FA of n-3 and n-6 families to maintain their structure, fluidity and function measures. As mammals lack the enzyme delta-12 desaturase and delta-15 (found in most plants), which insert double bonds at positions 3 and 6, they do not synthesise n-3 or n-6 PUFA. As such, these FA have to be consumed in the diet and are therefore called essential fatty acids (Semplecine & Valle 1994, Burr & Burr 1929).

The PUFA most commonly consumed are linoleic acid (LA, 18:2 n-6) and α-linolenic acid (ALA, 18:3 n-3). These two FA can be converted to other unsaturated derivatives. Linoleic acid can be converted to γ-Lilolênico (18:3 n-6), Dihomo-γ-linolenic (20:3 n-6) and arachidonic acid (AA, 20:4 n-6) sequentially. Similarly, the α-linolenic acid (18:3 n-3) is converted into eicosapentaenoic acid (EPA, 20:5 n-3) and Docosapentaenoico acid (DHA, 22:5 n-3) (Calder 2003) (Figure 2). The main dietary sources of acids LA and ALA are oils which are rich in polyunsaturated fats. The PUFA of n-6 series are derived from plants found, for example, in soybean, sunflower and evening primrose oils. The PUFA of n-3 series are predominantly found in fish oils and marine mammals, and deep cold water fish, such as mackerel, sardines, trout, salmon and tuna (Connor 1996). This occurs because many marine plants, especially phytoplankton algae, also synthesize EPA and DHA from-linolenic acid-α. This synthesis of long-chain PUFA n-3 by marine algae, and their transfer through the food chain to fish, explains their abundance in some fish oils and marine mammals (Semplecine & Valle 1994).

Up until 1929, the FAs were viewed exclusively as efficient energy storage. Between 1929 and 1930, George and Mildred Burr published articles reporting the essentiality of PUFA. The authors found that the administration of diets completely devoid of fat in rats caused severe changes in relation to growth and the physiological functions of various organs, which were attributed to the lack of long-chain PUFA. Similar changes were observed in newborns undergoing a diet based on skimmed milk and then reversed by the administration of whole milk. These findings led to a systematic study being carried out by Hensen et al. In 1958, it was found that the administration of skimmed milk to infants was associated with diarrhoea and skin abnormalities, among other things. The supplementation of milk with linoleic acid reversed all symptoms. These observations therefore characterise the effects of PUFA deficiency in humans (Hensen et al. 1958, Holman et al. 1998). With the development of parenteral nutrition, which initially did not contain essential fatty acids, it became evident that a deficiency of n-type PUFA-6 caused the death of patients. This led the

FDA (Food and Drug Administration), in 1982, approving the supplementation of parenteral nutrition with PUFA n-6 (Holman et al. 1998).

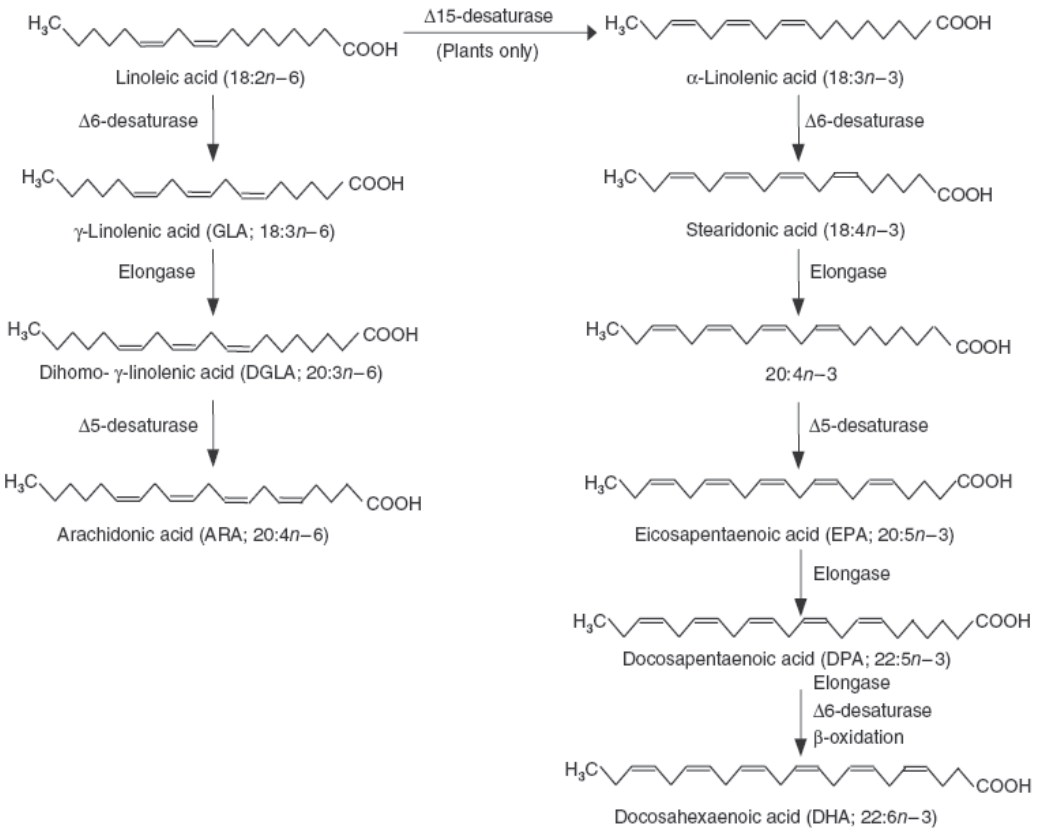

Fig. 2. Biosynthesis of some fatty acids (Sala-Vila et al. 2008)

2. Inflammation and PUFA

The relationship between inflammatory response and PUFA enriched diets has been investigated in recent years. Several studies show that PUFA can modify immunological and inflammatory reactions, and that it can be used as a complementary therapy in chronic diseases (Kinsella et al. 1990, Serhan et al. 2004).

Inflammation is a body's response to tissue injury, which can be triggered by mechanical stimuli, chemical or microbial invasion, as well as hypersensitivity reactions. This response includes complex processes that involve the immune system cells and biological mediators (Rankin et al. 2004). The acute phase response is characterised by increased blood flow and vascular permeability, increased accumulation of fluid, leukocytes and inflammatory mediators; meanwhile the chronic phase is characterised by the development of specific cellular and humoral immune responses against pathogens present at the site of injury (Saadi et al. 2002). Inflammation is characterised by redness, swelling, heat and pain. These signs occur primarily due to: vasodilatation, which allows increased blood flow to the

affected area; increased vascular permeability, which facilitates the diffusion of molecules such as antibodies; cytokines and other plasma proteins to the site of injury and cellular infiltration, which occur through chemotaxis and diapedesis and the direct movement of inflammatory cells through the vessel wall towards the site of inflammation. In addition, during the inflammatory response catabolic and metabolic changes may occur, as well as biosynthetic activation in various organs and enzyme systems and cells of the immune system.

The inflammatory response begins the process of immune elimination of invading pathogens and toxins for the repair of damaged tissue (Rang & Dale 1995). The nonspecific inflammatory response can be seen, for example, in the phagocytosis of bacteria or leftover tissue, the secretion of proteolytic enzymes, the production of reactive oxygen species and the secretion of molecular modulators. It can also be immune-mediated, where there is the participation of lymphocytes and antigen-presenting cells. This second type is closely associated with the onset and maintenance of chronic inflammation (Pompei et al. 1999).

The inflammatory process is controlled by cellular and molecular components. Among the cellular components are neutrophils, monocytes, lymphocytes, macrophages fixed, dendritic cells, mast cells and eosinophils. These cells accumulate in inflamed tissues and interact with the endothelial cells of the microcirculation. Different adhesion molecules participate in these interactions, including selectins, integrins and intercellular adhesion molecules (ICAM) (Rang & Dale 1995). Neutrophils constitute 60% of circulating leukocytes and act as the first line of cellular defence, and they may participate in both reactions as a nonspecific defence and as specific antigen reactions (Curi et al. 1997). Monocytes represent approximately 3-6% of circulating leukocytes in human blood, and they migrate to different tissues where they differentiate into macrophages in response to different stimuli. These cells participate in a variety of functions related to the host's defence, the most well known being the phagocytosis of microorganisms and cell debris, and cytotoxic activity against microorganisms, virus-infected cells and tumour cells (Curi et al. 2002).

The molecular components of inflammation include vasoactive substances (kinins, histamine), proinflammatory cytokines (such as Tumour Necrose Factor (TNF), Interleukin (IL)-1 and IL-6), anti-inflammatory cytokines (such as IL-4, IL-10 and IL-13), chemokines, acute phase proteins, bioactive lipids (such as eicosanoids derived from AA), Platelet Activating Factor, diacylglycerol, ceramides, cAMP, and inositol triphosphate, amongst others.

3. Inflammatory bowel disease and carcinogenesis

Inflammatory bowel diseases (IBDs) are chronic disorders of the gastrointestinal (GI), which generally refer to two conditions, namely ulcerative colitis and Crohn's disease (Galvez et al. 2006). IBDs are characterised by chronic diarrhoea, malabsorption, mucosal barrier dysfunction and inflammatory intestinal process, being incurable clinically (Benedetti & Plum 1996). Ulcerative colitis encompasses a spectrum of diffuse inflammation and the continuous surface of the colon, which begins in the rectum and may extend to the proximal level. Crohn's disease is characterised by transmural inflammation affecting any asymmetric portion of the GI tract, from the mouth to the anus (Benedetti & Plum 1996).

IBDs cause nutritional deficiencies, such as calorie and protein malnutrition, and deficiencies in vitamins, minerals and trace elements. This underscores the importance of

nutritional therapy in their treatment (Ferguson et al. 2007, Pizato et al. 2005, Razack et al. 2007). Malnutrition is common in these patients, and interventions through adequate nutritional therapy so as to restore the nutritional status have been associated with an improved recovery process involving the improvement of the immune system during periods of the exacerbation of the disease (Razack et al. 2007). Several characteristics contribute to the malnutrition observed in patients: 1) there is a decrease in the oral intake of nutrients associated with abdominal pain and anorexia; 2) the mucosal inflammation associated with diarrhoea leads to a loss of protein, minerals, blood, electrolytes and trace elements. In addition, multiple resections or bacterial overgrowth in the colon can cause adverse effects, such as the poor nutritional absorption of micronutrients: 3) drug therapies can lead to malnutrition. For example, sulfasalazine reduces the absorption of folic acid, and corticosteroids reduce calcium absorption and adversely affect the protein metabolism (Wild et al. 2007).

Although much progress has been made in understanding IBD, its aetiology is not fully elucidated. However, it is believed that there is involvement of immune factors, both genetic and environmental (Laroux et al. 2001, Cheon et al. 2006, Sainathan et al. 2008). Some studies have suggested that IBDs represent an inappropriate and exaggerated response of the intestinal mucosal immune system to normal intestinal microflora – in genetically susceptible individuals – which can be attributed in part to an imbalance between effector T cells (T eff) cells and T regulatory cells (T reg). (Sanchez-Muñoz et al. 2008, Ma et al. 2007). Effector T cells are helper T lymphocytes (lymph CD4 +) and cytolytic T lymphocytes (lymph CD8 +) that are activated during the adaptive or acquired immune response. The helper T cells secrete cytokines, whose function is to stimulate the proliferation and differentiation of T cells, as well as other cells including B lymphocytes, macrophages and other leukocytes (Sanchez-Muñoz et al. 2008, Sainathan et al. 2008). Cytolytic T lymphocytes destroy cells that produce antigens, such as cells infected by viruses or other intracellular microbes. Since regulatory T cells are cells capable of blocking the activation and effector function of T lymphocytes (Abbas & Lichtman 2005), some studies indicate that the suppressive action of these cells is linked to the secretion of immunosuppressive cytokines, such as IL-10 and Transforming Growth Factor Beta (TGF-β). TGF-β inhibits the proliferation of T and B cells, whereas IL-10 inhibits macrophage activation and is the main antagonist of Macrophage Activating Factor and Interferon Gamma (IFN-γ) (Sanchez-Muñoz et al. 2008).

The innate immune response in IBDs also plays an important role. This response is the first line of defence of the immune system, attended by phagocytic cells, natural killer cells, blood proteins, and including fractions of complements and other mediators of inflammation such as cytokines (Abbas & Lichtman 2005). Cytokines are polypeptides – produced mainly by immune cells – that facilitate communication between cells, stimulate the proliferation of antigen-specific effector cells, and mediate systemic inflammation and local roads in the endocrine, paracrine and autocrine (Muños-Sanchez et al. 2008). Dendritic cells and activated macrophages secrete various cytokines that regulate the inflammatory response. Once secreted, these cytokines promote the differentiation of T cells, activating the adaptive immune response (Abbas & Lichtman 2005). The T-helper cells or CD4 + T cells can differentiate into subpopulations of effector T cells that produce different sets of cytokines and, therefore, play different effector functions. The most well-defined sub-populations of effector T cells are T helper cells type 1 (Th1) and type 2 (Th2) (Abbas &

Lichtman 2005, Fuss et al. 2004). IFN-γ is associated with Th1 cells, while IL-4 and IL-5 are associated with Th2 cells. Today it is clear that individual cells can express various mixtures of cytokines, and that there may be many sub-populations with heterogeneous patterns of cytokine production. However, chronic immune reactions are often dominated by either Th1 or Th2 populations (Kampen et al. 2005). These sub-populations show differences in the expression of several cytokine receptors, and these differences may reflect the activation state of the cell, determine their effectors' functions, and participate in the development and expansion of their sub-populations (Abbas & Lichtman 2005). IBDs can cause an imbalance between regulatory T cells and T effector cells Th1/ Th2. The lack of appropriate regulation of T cells and the overproduction of effector T cells are related to the development and exacerbation of IBDs (Muños-Sanchez et al. 2008, Zhang et al. 2005).

Patients with IBDs, particularly ulcerative colitis, are at risk of developing cancer that is 10 times higher than that of the general population, indicating that chronic intestinal inflammation is an important risk factor for developing colon cancer (Gommeaux et al. 2007). Some studies have shown that the risk of developing cancer increases exponentially with the duration of the illness, and the extent and intensity of inflammation in the intestinal mucosa (Burstein & Fearon 2008).

The process of carcinogenesis seems to involve a sequence of events, where the chronically inflamed and hyperplastic epithelium progresses to initially flat foci of dysplasia, adenoma and finally to adenocarcinoma. Uncontrolled inflammation is associated with oxidative stress and oxidative cell damage. During cell proliferation, oxidative DNA lesions induce mutations that are commonly observed in oncogenesis and tumour suppressor genes, such as p53 (Gommeaux et al. 2007, Seril et al. 2003). It is likely that the cells of the colonic mucosa, persistently subjected to oxidizing agents, suffer progressive oxidative damage in their DNA, which can cause mutations in tumour suppressor genes (p53), oncogenes (k-ras) and genes that encode the repair of proteins (MSH2 and MLH1) (Gommeaux et al. 2007). The initiation of carcinogenesis is caused by an irreversible alteration of the DNA through the reaction of this molecule with carcinogenic substances. Thus, mechanisms of carcinogen detoxification, DNA repair, and the elimination of cells that have modified DNA (apoptosis, for example), are important for protection against cancer initiation (Brown et al. 1994). For initiation to occur requires not only the modification of DNA, but also its replication and cell proliferation, so that the original mutation can be fixed. Most human cancers originate from epithelial cells (carcinoma), as these are exposed to carcinogens (in the air or in food) and they are rapidly proliferating (Bartsch et al. 1996). In general, electrophilic substances are carcinogens or are metabolised to carcinogens substancesduring the process of detoxification. Such substances are attracted to molecules with high electron densities – such as DNA bases – which end up calling and leading to the formation of adducts (Bartsch et al., 2006).

The basis of the DNA which is more susceptible to this type of attack is guanine, but the adducts thereby formed have been reported in other bases. Being formed in DNA by specific chemical mechanisms, such adducts may lead to mutations in proto-oncogenesis or tumour suppressor genes, and they start the process of carcinogenesis (Lehman et al. 1994, Kinzler et al. 1996).

It is well established that inflammation facilitates the progression of normal cells to malignant cells, the production of pro-inflammatory cytokines such as TNF, IL-1, IL-6, IL-23 and reactive oxygen species (ROS) and nitrogen (Bartsch et al. 2006, Roessner et. al. 2008).

ROS – which are the cellular consequences of oxidative stress – may cause DNA oxidation, resulting in damage to all four bases and in the deoxy-ribose-molecule triggering the appearance of genetic mutations and initiating colorectal carcinogenesis (Chapkin et al. 2007).

With the large number of cytokines and growth factors released during inflammation, the immune cells and nonimmune cells may influence the process of carcinogenesis (Fantini et al. 2008). These mediators activate NF-kB, inducible nitric oxide synthase, and cyclooxygenase-2-related signalling pathways, which are associated with the delay or suppression of the apoptosis of intestinal epithelial cells and the modulation of angiogenesis (Chapkin et al. 2007, Fantini et al. 2008). Apoptosis – programmed cell death – is the mechanism by which the intestine eliminates cells with irreparable DNA damage, and the inhibition of this response is a characteristic of colon cancer (Bancroft et al. 2003).

The integrity of DNA is vital for cell division, and oxidative changes may interfere with transcription, translation and DNA replication, and may also increase mutations, senescence and cell death (Miranda et al. 2008).

4. Inflammatory bowel disease and dietary fatty acids

Epidemiological studies have been conducted in an attempt to correlate nutritional factors with chronic diseases and carcinogenesis on set. In this context, we can observe in recent years a drastic alteration in dietetic habits, mainly in lipids' composition and contents (Wild et al. 2007), leading to an association with the type and amount of fatty acid intake by diet, and the development of diseases (Figler et al. 2007). Asian countries that have changed from a traditional diet (i.e. high in fish and cruciferous vegetables) to a Western diet lifestyle (i.e. high in red meat and saturated fat), such as Singaporean Chinese (who have had a historically low risk for colorectal cancer), have doubled this risk in the past three decades, after dietetic modification (Stern et al. 2009).

Linoleic acid intake, in western countries, increased considerably in the 20th century, followed by vegetable oil and margarine introduction, which resulted in a significant rise in the n-6:n-3 PUFA ratio in the diet (Calder 2008). The incidence of IBDs is higher in western populations and has increased in developing countries which have adopted industrialised urban lifestyles associated with changes in dietetic habits, including an increased fast food intake with high lipids content (Wild et al. 2007).

PUFA n-6 and n-3 are incorporated in cell membrane phospholipids and can influence immunological and inflammatory responses by modifying fluidity, the antioxidant defence system and the inflammatory mediators (Calder 2008, Kinsella et al. 1990, Simopoulos 2003).

N-3 PUFA, EPA and DHA competitively inhibit AA oxygenation by cyclooxigenase, decreasing the synthesis of eicosanoids from series 2 and 4 from AA, with a concomitant increase in prostaglandin (PG), tromboxanes (TX) from 3 series and leukotrienes from 5 series (Yaqoob & Calder 1995). On the other hand, an excessive amount of n-6 PUFA, in diet poor in n-3 PUFA, can contribute to PGE_2, TXA2 and LTB_4 overproduction – potent inflammatory mediators. Eicosanoids produced from EPA (n-3 PUFA) are, in general, less active in inflammatory process than derived AA eicosanoids (Calder 1996, 1998, Kikuchi et al. 1998).

The inflammatory response is designed to remove the inciting stimulus and resolve tissue damage. However, excessive inflammatory response can cause local tissue damage and

remodelling, which may lead to a significant and chronic injury. Therefore, acute inflammation in healthy individuals is self-limited and has an active termination program (Seki et al. 2009). In the past, it was believed that this termination program was a passive mechanism but, nowadays, it is known that the process of the resolution of inflammation is an active and well controlled event. In part, this is due to the formation of newly endogenous mediators that act as local autacoids stimulating proresolving mechanisms (Serhan 2007, Gilroy et al. 2004). These proresolving mediators are derived from essential fatty acids, and include lipoxins (LX) from AA and resolvines (Rv) and protectins from EPA and DHA (Gilroy et al. 2004), that are biosynthesised in inflammatory exudates during spontaneous resolution (Figure 3).

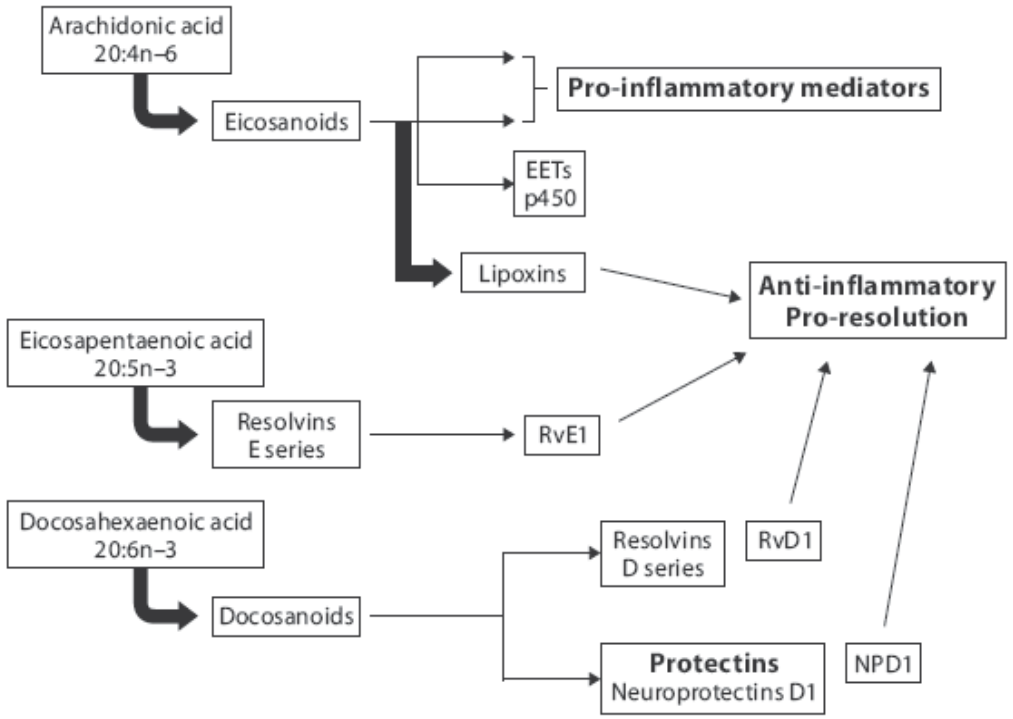

Fig. 3. News inflammatory mediators (Galli & Calder 2009)

The process of the resolution of inflammation has become a topic of interest because of expanding views of their action, particularly in chronic disorders where unresolved inflammation is a key factor leading to colon carcinogenesis. These newly identified LXs and Rvs have proven to be potent regulators of both leukocyte and cytokine production, thereby regulating the events of interest in inflammation and resolution. In light of the existing knowledge of the interconnected pathways of pro-inflammatory mediators (leukotrienes, chemokines (IL8, SDF-1α, MIP-1α, MCP-1,2 etc), and cytokines (IL3, IL6, IL12, IL-1β, GM-CSF, B94, TNF-α etc)), the anti-inflammatory properties of pro-resolving mediators in preventing the chronic inflammation which leads to carcinogenesis requires further study.
Clinical trials have demonstrated the beneficial effects of fish oil supplementation – rich in EPA and DHA – in chronic and acute inflammatory conditions (Innis et al. 2006, Simopoulos

et al. 2002, Harbige 1998, MacLean 2005). Fish oil supplementation seems to increase apoptosis on top of colonic crypts, where tumours and polyps are usually developed (Paulsen et al. 1997; Courtney et al. 2006, Hong et al. 2005). Bégin et al. (1991) showed that under some specific conditions, long chain PUFA – mainly GLA, AA, EPA, and DHA – are the most effective for inducing tumour cell death. However, this effect depends upon the type of cancer cells tested and the concentration of the fatty acid used.

The role of n-3 and n-6 PUFA on cancer development has been extensively investigated in epidemiological and experimental studies. The contrasting role of these fatty acids in carcinogenesis – n-3 as protectors and n-6 as promoters – remains as an intriguing question in the fields of nutritional and cancer research (Eder et al. 2008).

In rats with colitis induced by Dextran Sulphate Sodium (DSS), our group showed that a normal fat PUFA rich diet, with a balance on the n-6:n-3 ratio, can increase IL-10 cytokine – an immunoregulatory cytokine that influences the immunological system – both on the innate and cell-mediated response, reduce disease activity and the loss of weight, improve the histological score and protect against DNA damage (Barros et al., 2010). IL-10 is considered an immunoregulatory cytokine which exerts effects in both the innate immune response and in the adaptive immune response. IL-10 Knochout animals, for example, develop colitis spontaneously, and 30 to 60% of these animals show invasive carcinoma of the colon between 3 and 6 months of age (Hegazi et al. 2006, McCafferty et al. 2000). These animals have two important characteristics: 1) an increased intestinal permeability in an early age, and before the onset of the disease; 2) the development of colitis, dependent on the microbiological presence in the intestinal lumen. These characteristics suggest that the colitis observed in these animals can develop as a consequence of the high intestinal permeability that increases in the luminal agent's mucosal immune system (Arrieta et al. 2008). Some studies have demonstrated the role of IL-10 on gastrointestinal mucosal homeostasis maintenance. The mechanism by which this cytokine regulates mucosal inflammation is probably multifactorial; however, it is associated with reduced antigen presentation (Hegazi et al. 2006, McCafferty et al. 2000), an increased release of IFN-□ and IL-12 – a cytokine that inhibits the differentiation of T lymphocytes into Th1 lymphocytes (Rennick & Fort 2000). There is strong evidence that IL-10 promotes the differentiation and the increase of the activity of the regulatory T cells (Hegazi et al. 2006). *In vitro* studies have demonstrated that the administration of IL-10 reduces the release of pro-inflammatory cytokines in lamina propria mononuclear cells amongst patients with Crohn's Disease. In addition, high doses of IL-10 administered intraperitoneally into mice with colitis, induced by Trinitrobenzenesulphonic acid (TNBS), are able to restore the tolerance of the lamina propria mononuclear cells (Duchmann et al. 1996).

Considering the abundance of fatty acids in cells and its susceptibility to oxidation, PUFA are – for the oxidants – more likely targets than DNA (Shimizu et al., 2001, Wagner et al., 1994). It is estimated that approximately 60 molecules of linoleic acid and 200 of arachidonic acid are consumed by oxidants that react with the lipid bilayer. Autocatalytic oxidation triggers a cascade that generates numerous genotoxic substances, and such damage to lipids has important implications for the integrity of DNA (Wagner et al., 1994). The peroxidation of membrane lipids initiates autocatalytic breaks with the consequent formation of cytotoxic and genotoxic metabolites, such as malondialdehyde and hidroxinomenal. The degradation of these products can interfere with intracellular signalling cascades, involving replication and cell death (Eder et al. 2008).

The dietary lipids that are related to the pro-oxidative attack of the colonic epithelial cells may be an important contributor to carcinogenesis (Nowak et al. 2007, Udilova et al. 2003). So far, there is still no specific treatment for IBDs and the best strategy to regulate the exacerbated inflammatory response is to interfere with the multiple phases of the inflammatory cascade with anti-inflammatory and immunosuppressive drugs. These drugs, however, have serious side-effects that limit their use (Stein et al., 2000). Dietary treatment may be an alternative to drug therapy (Camuesco et al., 2005, Nowak et al., 2007).

Although the high intake of PUFA has been related to colorectal cancer, several studies show that, besides the genotoxic effects of lipid peroxidation, epigenetic factors may also be responsible for an increased cancer risk after excessive PUFA intake (Nystrom et al. 2009). Using a model of DSS colitis and a high fat diet (20%), in our laboratory, we did not observe an exacerbation of experimental ulcerative colitis in relation to the diet control group (5%) Besides, the great balance in the n-6:n-3 PUFA ratio (2:1) caused beneficial effects on both pro- and anti-inflammatory cytokine balance and protected the DNA against damage (Barros et al. 2010).

Sasasuki et al. (2010) in an epidemiological study where it was inquired as to whether the intake of n-3 and n-6 PUFA are related to a decreased risk of colorectal cancer development. They found that, in a population with high fish consumption and a wide range of n-3 PUFA intakes, the PUFAs originating with marine consumption may be inversely related to the risk of cancer in proximal sites of the large bowel. On the other hand, Dahm et al. (2010), in a case-control study nested within seven prospective UK cohort studies, comprising 579 cases of the incidence of colorectal cancer and 1996 matched controls, did not find any association between total dietary fat, saturated, monounsaturated and PUFA intakes, and colorectal cancer risk.

5. Polymorphisms

Conclusive evidence between colorectal cancer and PUFA in epidemiological studies may be related to genetic influence. The relationship between genes and the environment has been recognised as central to knowledge of disease and health. During the last two decades, advances in molecular biology have demonstrated that genetic factors determine disease susceptibility, while environmental factors determine whether or not genetically susceptible individuals will be affected (Simopoulos et al. 2008, Paolini-Giacobini et al. 2003). In this context, nutritional aspects are beginning to be considered as one of the most important environmental factors (Simopoulos et al. 2008). Several studies have shown the mechanisms by which genes may influence the metabolism of nutrients, as well as the mechanisms by which nutrients can influence gene expression (Simopoulos et al. 2008, Paolini-Giacobini et al. 2003, Calder 2007). With advances in science, and emphasis on the study of nutrigenomics and nutrigenetics, it has been shown that certain nutrients can influence the inflammatory response, accelerating or regressing the development of many diseases (Heller et al. 2002, Weiss et al. 2002, Mayer et al. 2003, Paolini-Giacobini et al. 2003, Simopoulos et al. 2008).

Stern et al. (2009), from the Singapore Chinese Health Study, through analyses taking into account variants in genes that are relevant for the proposed PUFAs mechanism of action – hypothesised that the genes which play key roles in the pathways that repair PUFA-induced damage might modify the effect of these FA on colorectal cancer. This study also showed that diets high in marine n–3 PUFA were positively associated with colorectal cancer risk

(Stern et. al. 2009). However, using a subset of this prospective cohort, Stern et al. (2009) reported that the marine n–3 PUFA association with rectal cancer is confined to those who carry the PARP codon 762 Ala allele. The PARP protein plays an important role in maintaining genomic stability, apoptosis, and in regulating transcription.

In this regard, some studies have shown that genetic variability in the FADS1-FADS-2 gene cluster, and the encoding delta-5 (D5D) and delta-6 (D6D) desaturases, have been associated with plasma long-chain PUFA and lipid levels in adults (Bokor et al. 2010). Desaturases and elongases catalyse the conversion of PUFAs in humans. The D5D and D6D desaturases are known to be the key enzyme of this pathway. Both desaturases are expressed in a majority of human tissue, with the highest levels in liver, but also with major amounts in the brain, the heart and the lungs. The hypothesis that they play a key role in inflammatory diseases is strengthened by functional studies in mice, where selective D5D and D6D inhibitors showed an anti-inflammatory response.

Several single nucleotide polymorphisms (SNP) in FADS genes were reported in humans, and some showed association between FADS SNPs and fatty acids in serum or plasma phospholipids, and erythrocyte membrane and adipose tissue (Schaeffer et al. 2006, Malerba et al. 2008, Rzehak et al. 2009), demonstrating that these concentrations are influenced not only by diet, but also to a large extent by genetic variants common in the world population (Koletzko et al. 2011).

6. References

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9

The Molecular Genetic Events in Colorectal Cancer and Diet

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1. Introduction

Compelling evidence suggests that dietary intakes directly influence colorectal cancer (CRC) risk. Initial observations that CRC incidence is not ubiquitous worldwide, with incidence rates varying up to twenty-five fold between populations (Parkin et al., 2005), indicate the large degree to which this cancer type is influenced by diet and environment. Additionally, observations that migration of individuals confers rapid (within one generation) adoption of the CRC incidence of the host population (Boyle & Langman, 2000; McMichael & Giles, 1988), suggest that dietary and environmental factors determine the risk of colorectal neoplasia to a degree similar to, or in excess of, genetic predisposition.

As diagnosis and treatment of CRC have improved, the study of the pathogenesis of colorectal neoplasia has increased. The most frequent precursor of CRC is the adenoma. As a proportion of adenomas, those of large size, with villous architecture and high grade dysplasia often progress to invasive adenocarcinoma, and this progression is associated with accumulation of mutations and other genetic and epigenetic changes. In the effort to understand the mechanisms and causes of colorectal cancer development, molecular genetic analyses have identified a variety of molecular changes and protein targets involved in colorectal tumourigenesis. The greater understanding of genetic, epigenetic and expression changes that occur during the development and progression of CRC has shown that these neoplasms do not comprise a single disease. Instead, colorectal cancers comprise a collection of distinct and independent neoplastic pathways, such as those pathways displaying chromosomal instability (CIN), microsatellite instability (MSI) or gene promoter activity changes due to the epigenetic phenomenon of methylation at CG dinucleotides (referred to as CIMP: CpG island methylation phenotype, whereby CpG describes dinoculeotides of cytosine and guanosine, separated by the characteristic phosphate group in the DNA structure). Each pathway subtype is characterised by individual genetic and molecular characteristics (Poulogiannis,

Ichimura, Hamoudi, Luo, Leung, Yuen, Harrison, Wyllie & Arends, 2010; Poulogiannis, McIntyre, Dimitriadi, Apps, Wilson, Ichimura, Luo, Cantley, Wyllie, Adams & Arends, 2010). Dietary constituents have been studied in relation to the major genetic and molecular changes occurring in CRC development, including alterations in the proto-oncogenes, *K-RAS* and *BRAF* and the tumour suppressor genes *p53* and *APC*. Many studies have analysed a wide variety of dietary components in an effort to elucidate which, if any, dietary constituents may contribute to their mutation in CRC progression. Furthermore, in addition to these genetic lesions, the epigenetic phenomenon of CIMP and MSI have similarly been analysed in relation to dietary constituents.

This review is intended to summarise the currently available literature describing the associations between the molecular genetic changes seen most prevalently in colorectal cancer and dietary intakes. This report does not attempt to assess dietary associations with total CRC incidence. The objective is to highlight consensus observations, where several sources of data exist, suggestive of causative or protective effects of dietary constituents regarding specific molecular genetic changes frequently observed in colorectal neoplasia. Throughout, an emphasis is placed on the number of cases analysed in individual studies, but notably absent are descriptions of odds ratios, hazard ratios or p-values. Throughout, all associations discussed are statistically significant (all $p \leqslant 0.05$). However, due to the varying methodology of data collection and statistical analysis across studies, the inclusion of differing variables in adjusted models and the lack of consensus regarding the degree to which analyses should be adjusted following multiple statistical tests, detailed statistical aspects are not discussed. In order for an assessment to be made of the potential statistical power of each analysis, the number of cases involved in each study is instead highlighted when a statistically significant association is discussed. Full details of all statistical analyses can be found in the original reports, referenced in the text and listed at the end of the chapter.

2. Dietary influences on the major genetic and epigenetic perturbations leading to colorectal cancer development and progression

2.1 *K-RAS* and *BRAF* in colorectal cancer: the MAPK signalling pathway

Mitogen activated protein kinase (MAPK) signal transduction pathways are present in all eukaryotes, six versions of which have been distinguished in mammals (Robinson & Cobb, 1997). MAPK signal propagation is responsible for regulating a variety of cellular processes, which include potentially pro-tumourignenic properties such as proliferation, apoptosis and transformation (Arends et al., 1993; 1994; Peyssonnaux & Eychene, 2001; Robinson & Cobb, 1997). The best characterised of these pathways is the LIGAND RECEPTOR-RAS-RAF-MEK-ERK pathway (Figure 1), which consists of core modules including the RAS and RAF proteins. Although three *RAS* genes have been identified (*H-RAS*, *N-RAS* and *K-RAS*), the *K-RAS* gene is the only one mutated at significant frequency in CRC (Bos, 1989). Similarly, of the three *RAF* genes identified (*ARAF*, *BRAF* and *CRAF/RAF-1*), only the *BRAF* gene is mutated at significant frequencies in human cancers (Fransen et al., 2004).

Experimental mouse models have provided direct evidence that mutated *K-RAS* genes expressed in the intestinal epithelium do not significantly initiate intestinal adenoma growth, but they can cooperate either with other mutant genes or carcinogens to accelerate intestinal tumour formation (Luo et al., 2007; 2009; Luo, Poulogiannis, Ye, Hamoudi & Arends, 2011;

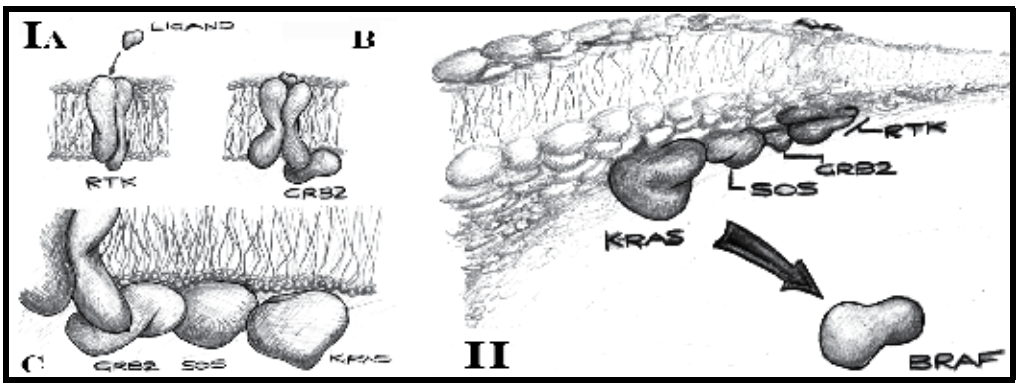

Fig. 1. **RAS, RAF and the MAPK signalling pathway: frequently perturbed in colorectal neoplasms.** Initially, RAS is inactive in a RAS-GTP bound state. **I:** Initiation of signalling through the MAPK pathway occurs at the plasma membrane. Upon extracellular ligand binding to membrane receptor tyrosine kinases (RTK), such as epidermal growth factor binding to the epidermal growth factor receptor (IA), receptor conformational change gives rise to receptor autophosphorylation. Subsequently, src-homology 2 (SH2) domains present in the GRB2 adaptor protein bind the phosphate moieties on the activated receptor (IB). Src-homology 3 (SH3) domains in GRB2 bind proline-rich motifs present in son of sevenless (SOS), localising SOS to the inner surface of the plasma membrane. SOS, a guanine nucleotide exchange factor (GEF) interacts with RAS proteins, catalysing the exchange of GDP for GTP, thus activating RAS to a RAS-GTP state (IC). **II:** Upon activation, RAS phosphorylates cytosolic RAF. The resulting activation of RAF in turn phosphorylates cytosolic MEK, which then phosphorylates ERK, leading to induction and repression of distinct transcription programmes, promoting cell proliferation and modulating cell death by apoptosis, among other processes. The vast majority of mutations in the *K-RAS* or *BRAF* genes are in distinct hotspot regions: *K-RAS* at codons 12 and 13, and also, but much more infrequently at codons 61 and 146 (Forbes et al., 2008). Additionally, mutations observed at lower prevalences at other sites in the gene have been described and their functional significance determined (Naguib, Wilson, Adams & Arends, 2011). Mutations in *BRAF* occur most frequently at codons 463-468 and codon 600 (Forbes et al., 2008). Activating mutations in the *K-RAS* and *BRAF* genes render their protein products constitutively active, leading to increased transduction through this signalling axis. Additionally, mutationally active *K-RAS* can also propogate signalling through other pathways, including the PI3K/AKT axis.

Luo, Poulogiannis, Ye, Hamoudi, Zhang, Dong & Arends, 2011). *K-RAS* mutations are observed 20-50% of sporadic human CRC and *BRAF* mutations are observed in 5-15% of CRC (Forbes et al., 2008). The high frequencies at which *K-RAS* and *BRAF* mutations are observed in CRC has prompted several analyses of dietary intakes in relation to these genetic lesions.

2.1.1 *K-RAS* mutation and meat consumption

Specific types of meat consumption have been identified as associated with general CRC incidence (Norat et al., 2005; Santarelli et al., 2008) with plausible mechanisms postulated as to the manner in which these consumptions may influence colorectal carcinogenesis (Kuhnle & Bingham, 2007; Kuhnle et al., 2007). Consequently, several studies have attempted to identify

the nature of these associations in relation to *K-RAS* mutations. Some reports have identified associations with meat consumption and *K-RAS* mutation, although not all.

A single study analysing 390 *K-RAS* wildtype and 218 *K-RAS* mutated CRC identified an increased consumption of beef with higher incidence of *K-RAS* wildtype colonic cancers (Brink, Weijenberg, de Goeij, Roemen, Lentjes, de Bruïne, Goldbohm & van den Brandt, 2005). In this same report, a reduction in pork consumption was found to be linked to reduced frequency of both colonic and rectal cancers harbouring mutated *K-RAS*. Another report, assessing *K-RAS* mutations and diet in 155 *K-RAS* wildtype and 41 *K-RAS* mutated CRC, identified an increased white meat consumption associated with higher incidence of *K-RAS* mutated CRC (Naguib et al., 2010). Although positive associations were identified in these two analyses, there appears to be little consistency between these independent findings. The report by Naguib and colleagues also analysed red and processed meat consumption in relation to mutation status and found no statistically significant association between the two, although, beef consumption was not tested independently of other meat types, as in the report by Brink and co-workers. The study by Naguib and colleagues did not test pork consumption in isolation: this meat type was included in the 'red' or 'processed' meat categories. Similarly, Brink and coworkers did not identify an association between white meat and increased incidence of *K-RAS* mutations. This analysis tested the consumption of chicken in isolation, not in a combined 'white meat' category containing other meat types, such as turkey etc.

Notwithstanding the identified statistically significant associations between meat consumption and *K-RAS* mutation status described above, the majority of studies which have attempted to address this question have failed to identify any link between meat intake and the mutation status of this gene. An analysis testing 67 *K-RAS* wildtype and 39 *K-RAS* mutated CRC assessed animal protein intake and found no link between this and *K-RAS* mutation status (Bautista et al., 1997) although clearly, 'animal protein' as a variable makes no distinction between meat types and is an assessment of protein, not animal product, consumption. A large analysis testing 971 *K-RAS* wildtype and 457 *K-RAS* mutated CRC (Slattery et al., 2000) identified no assocaition between total *K-RAS* mutations and meat intake. A small study (28 wildtype, 15 mutated rectal cancers) failed to identify an association between red meat intake and *K-RAS* mutation (O'Brien et al., 2000). An assessment of a larger cohort of rectal cancers (535 *K-RAS* wildtype and 215 *K-RAS* mutated) corroborated this observation of lack of association with red meat intake and rectal cancer (Slattery, Curtin, Wolff, Herrick, Caan & Samowitz, 2010).

In addition to colorectal cancers, pre-cancerous adenomas have also been tested in order to identify dietary assocaitions with *K-RAS* mutation status in the early stages of colorectal neoplasia. An assessment of 558 *K-RAS* wildtype and 120 *K-RAS* mutated adenomas failed to identify an association between red meat intake and mutation status (Martínez et al., 1999). Another study, testing 453 *K-RAS* wildtype and 81 *K-RAS* mutated adenomas also failed to identify a statistically significant association between red meat, processed meat or poultry and *K-RAS* mutation status (Wark et al., 2006).

Published reports assessing *K-RAS* mutation status in CRC in relation to meat intakes provide limited evidence to suggest that total *K-RAS* mutations are either positively or negatively associated with meat consumption. The majority of studies have categorised meat types according to shared properties (such as haem content or preservation methods) and have generally failed to identify links between these groups and *K-RAS* mutation status. It is

Study	*K-RAS* WT CRC/RC/adenomas	*K-RAS* mutated CRC/RC/adenomas	dietary association
Bautista *et al* 1997	CRC: 67	CRC: 39	↑ MUFA with *K-RAS* mutation, ↓ calcium with *K-RAS* mutation
Bongaerts *et al* 2006	CRC: 385	CRC: 193	no association between alcohol and *K-RAS* mutated or wildtype cancers
Brink *et al* 2004	CRC: 390	CRC: 218	↑ PUFA (specifically linoleic acid) with *K-RAS* mutated colonic, but not rectal, cancers
Brink *et al* 2005	CRC: 390	CRC: 218	↑ folate reduced risk of *K-RAS* mutated rectal, not colonic, cancer in men only
Brink *et al* 2005	CRC: 390	CRC: 218	↑ beef, ↓ pork with *K-RAS* wildtype colonic tumours, ↓ pork with *K-RAS* wildtype rectal tumours
Laso *et al* 2004	CRC: 68	CRC: 49	*K-RAS* codon 12 mutation was associated with ↓ vitamin A, B1, D and iron
Martinez *et al* 1999	Adenomas: 558	Adenomas: 120	↑ folate reduced risk of developing *K-RAS* mutated adenomas
Naguib *et al* 2010	CRC: 155	CRC: 41	↑ white meat consumption with *K-RAS* mutation
O'Brien *et al* 2000	RC: 28	RC: 15	no association between red meat consumption and *K-RAS* mutation
Schernhammer *et al* 2008	CRC: 427	CRC: 242	no association between folate intake and prevalence of *K-RAS* mutated or wildtype cancers
Slattery *et al* 2000	CRC: 971	CRC: 457	↓ cruciferous vegetables with reduced risk of *K-RAS* mutation
Slattery *et al* 2010	RC: 535	RC: 215	no association between calcium and vitamin D and *K-RAS* mutation
Slattery *et al* 2010	RC: 535	RC: 215	↑ vegetables and dietary fibre with a reduced risk of *K-RAS* mutations
Wark *et al* 2006	Adenomas: 453	Adenomas: 81	↓ MUFA and ↑ vitamin B2 associated with *K-RAS* mutation

Table 1. Summarised description of literature analysing *K-RAS* mutations in colorectal neoplasms (case numbers provided) in relation to dietary intakes and the statistically significant findings described. *WT*: wildtype, *CRC*: colorectal cancer, *RC*: rectal cancer, *MUFA*: monunsaturated fatty acid, *PUFA*: polyunsaturated fatty acid, ↑ and ↓ denote an increase or decrease in consumption respectively.

plausible that if specific meat types, as suggested in at least one study (Brink, Weijenberg, de Goeij, Roemen, Lentjes, de Bruïne, Goldbohm & van den Brandt, 2005), are linked to the incidence of *K-RAS* mutated CRC, that grouping of meat types together may have failed to identify associations where they existed. However, in practical terms, it should be noted that similarities in the composition of meat types, such as in terms of haem content, a postulated carcinogen intermediate (Kuhnle & Bingham, 2007), justify a grouping of types in order to minimise multiple statistical testing and to test consumption levels large enough to be likely to affect bowel carcinogenesis.

Several reports have analysed the relationship between base changes at specific positions in the *K-RAS* gene, types of mutations (i.e. transition *versus* transversion) or specific types of base changes (i.e. G→A) in relation to meat intakes. It is entirely plausible that the nature of the mutation, not the gene in which it arises, is linked to dietary constituents. However, due to the very limited number of studies instigated with objectives of such an analysis, and the often low numbers of different mutation subgroups existent in the studies which do attempt such an assessment rendering lower statistical power, such analyses are not discussed in this review.

2.1.2 *K-RAS* mutation and folate consumption

Several studies have described an association between folate intake and the prevalence of *K-RAS* mutations in CRC. A report analysing 390 *K-RAS* wildtype and 218 *K-RAS* mutated CRC identified an increased consumption of folate associated with a reduced prevalence of *K-RAS* mutated rectal, but not colonic, cancers in males only (Brink, Weijenberg, de Goeij, Roemen, Lentjes, de Bruïne, van Engeland, Goldbohm & van den Brandt, 2005). Testing in this study demonstrated that in the male participants of this cohort, increased intake of folate was linked to reduced prevalence of rectal cancer incidence, however, this link, when considering mutation status, seemed only to reduce the risk of *K-RAS* mutated rectal cancers. A large analysis of colorectal adenomas (558 wildtype, 120 *K-RAS* mutated) also identified increased folate intake associated with a reduced incidence of *K-RAS* mutation (Martínez et al., 1999).

However, in addition to these positive associations in relatively large cohorts, several other studies have failed to identify a link between folate intake and *K-RAS* mutation status in

colorectal neoplasms. Reports describing the testing of 67 *K-RAS* wildtype and 39 *K-RAS* mutated CRC (Bautista et al., 1997), 68 *K-RAS* wildtype, 49 *K-RAS* mutated CRC (Laso et al., 2004), 155 *K-RAS* wildtype, 41 *K-RAS* mutated CRC (Naguib et al., 2010), 427 *K-RAS* wildtype, 242 *K-RAS* mutated CRC (Schernhammer, Giovannuccci, Fuchs & Ogino, 2008), 971 *K-RAS* wildtype 457 *K-RAS* mutated CRC (Slattery et al., 2000) and 453 *K-RAS* wildtype, 81 *K-RAS* mutated adenomas (Wark et al., 2006) failed to identify folate intake as associated with *K-RAS* mutation status.

Increased consumption of folate offering some degree of protection against *K-RAS* mutation was observed in two independent studies. The failure to confirm this link in many other reports may potentially be explained several ways. Firstly, many of the studies described which identified no link between *K-RAS* mutation and folate intake contained relatively few mutated samples ($<$100). It is plausible that in these instances too few cases were analysed to detect any association, although this does not explain the studies which failed to identify a link using relatively large sample sets (Schernhammer, Giovannuccci, Fuchs & Ogino, 2008; Slattery et al., 2000). Secondly, some dietary constituents have been described to affect folate utilisation, such as alcohol (Eichholzer et al., 2001; Freudenheim et al., 1991). It may be possible that the protective effect of folate against *K-RAS* mutation is only prevalent in the context of certain dietary patterns, possibly explaining why associations are not observed in all epidemiological studies. Finally, Martinez and colleagues identified an increased protective effect against *K-RAS* mutation as provided by supplement derived intake relative to natural dietary intake of this macronutrient (Martínez et al., 1999). The nature of folate consumption, i.e. bioavailablilty, may also determine the degree to which it offers a protective effect in colorectal carcinogenesis.

Although not observed in every analysis, increased intake of folate is associated with a reduced prevalence of total CRC incidence, which is observed in approximately half of the analyses testing this link (Eichholzer et al., 2001). It is probable, that at least to a limited degree and in certain circumstances, that this may be due to the ability of folate to protect against *K-RAS* mutation during development of colorectal neoplasia.

2.1.3 *K-RAS* mutation and fat consumption

Consumption of several forms of fat intake have been described to affect the prevalence of *K-RAS* mutations in CRC. However, there is no consensus in the literature to date, regarding both the manner of the association and type of fat involved. Independent studies have identified monounsaturated fatty acid (MUFA) consumption as associated with the prevalence of *K-RAS* mutations in CRC. One report, analysing 67 *K-RAS* wildtype and 39 *K-RAS* mutated CRC, identified an increased MUFA consumption as linked to an increased prevalence of *K-RAS* mutated CRC (Bautista et al., 1997). MUFA, mostly derived from olive oil in this population, reduced the risk of CRC harbouring wildtype *K-RAS*, but offered no protection against *K-RAS* mutated cancers. However, contradictory findings of an increased MUFA consumption being associated with a higher prevalence of *K-RAS* wildtype neoplasia in a study assessing adenomas (453 wildtype, 81 mutated) (Wark et al., 2006) challenges the observation made by Bautista and co-workers. Other published reports have failed to identify any link between *K-RAS* mutation status and MUFA intake (Brink et al., 2004; Laso et al., 2004; Naguib et al., 2010; Slattery et al., 2000; Slattery, Curtin, Wolff, Herrick, Caan & Samowitz, 2010).

In addition to these observations, one report describes an increase in polyunsaturated fatty acids (PUFA), specifically linoleic acid, as associated with increased prevalence of *K-RAS* mutated colonic, but not rectal, cancers (Brink et al., 2004). However, this association with PUFA has not been identifed in any other report (Bautista et al., 1997; Laso et al., 2004; Naguib et al., 2010; Slattery et al., 2000; Slattery, Curtin, Wolff, Herrick, Caan & Samowitz, 2010; Wark et al., 2006).

Taken together, the published data describing the association of dietary fats with *K-RAS* mutations have failed to identify a convincing association, and have generated conflicting results. Presently, the evidence suggesting that the *K-RAS* mutation status of colorectal neoplasia may be affected by fat intakes is weak: the limited data available suggest that the mutation status of this gene is largely independent of this dietary consumption. It should be noted however, that although fat intake itself is probably not associated with this mutation type, increased body mass index (BMI), which may be associated with fat intake, is associated with overall CRC risk.

2.1.4 *K-RAS* mutation and other dietary constituents

The mutation status of *K-RAS* in CRC has also been linked to several other dietary variables in addition to meat, folate and fat. Testing of 971 *K-RAS* wildtype and 457 *K-RAS* mutated CRC identified an increased risk of *K-RAS* mutations with reduced consumption of cruciferous vegetables (Slattery et al., 2000). Another analysis of rectal cancers (535 wildtype, 215 mutated) identified a reduced incidence of *K-RAS* mutated rectal cancers with increased vegetable and fibre intake (Slattery, Curtin, Wolff, Herrick, Caan & Samowitz, 2010). Although corroborative, these two analyses were performed on the same test cohort and are yet to be identified in other independent populations. In this cohort at least, the data of Slattery and colleagues suggest that increased vegetable intake reduced the prevalence of *K-RAS* mutations in CRC, with an overt association identified in rectally located neoplasia.

Increased vitamin B2 intake has been identified to reduce the prevalence of adenomas harbouring *K-RAS* mutations. In an analysis of 453 *K-RAS* mutated and 81 *K-RAS* wildtype pre-cancerous adenomas an inverse association suggested a protective effect against *K-RAS* mutated adenomas. This protection was not found in relation to the prevalence of *K-RAS* wildtype adenomas (Wark et al., 2006). This association has not been identified in cohorts testing colorectal cancers.

Some dietary intakes have been repeatedly tested with no link to the prevalence of *K-RAS* mutation in CRC having been identified, notably alcohol. Many studies have included assessment of this dietary factor, with some studies analysing alcohol consumption independent of any other dietary factors (Bongaerts et al., 2006).

In summary, current literature describing the assessment of *K-RAS* mutation status in colorectal neoplasia has identified many associations with dietary intakes (summarised in Table 1). Very few of these associations have been repeatedly identified in independent cohorts, making assessment of their general validity challenging. Presently, few dietary components seem to be strongly linked to *K-RAS* mutation status in CRC across many populations, environments and genetic backgrounds. Furthermore, it is problematic to directly compare different studies. Other than folate, which has been described by the World Cancer Research Fund as having a 'limited' protective effect against CRC, which may impart this limited protection through reduced prevalence of *K-RAS* mutation, there is a lack of strong

evidence to firmly suggest any other dietary intakes affect the prevalence of *K-RAS* mutations in CRC.

2.1.5 *BRAF* mutations and dietary associations

Relative to *K-RAS*, far fewer data exist describing the association between *BRAF* mutations in CRC and dietary influences. A prospective study involving 186 colorectal cancers, of which 29 harboured *BRAF* mutations, analysing meat, fruit and vegetable, fat, vitamin and macronutrient intakes identified no potential dietary associations with *BRAF* mutation in CRC (Naguib et al., 2010).

Other analyses have centred on analysing dietary constituents which may act as methyl group donors, such as folate, or vitamins, such as B6 and B2, which function as co-factors in the pathway responsible for DNA methylation (de Vogel et al., 2008; Kim, 2005). Based on observations that *BRAF* mutation has been linked previously to the CIMP phenotype (Lee et al., 2008; Samowitz et al., 2005; Velho et al., 2008) and has been linked to 60-80% of CRC demonstrating the highest levels of CIMP with concurrent MSI (Kambara et al., 2004; Samowitz et al., 2005), this mutation type may be influenced by dietary factors thought to influence DNA methylation. One such analysis used data and tissue samples from 648 individuals, of which 101 harboured CRC with mutations in the *BRAF* gene. This report identified a positive association between *BRAF* mutation in males and the highest tertile of folate consumption (de Vogel et al., 2008). This same report also identified an inverse correlation between methionine intake, as well as no association between vitamin B2 and alcohol consumption and *BRAF* mutations in the male portion of the cohort. In the female cohort members, no dietary consumptions were identified which were associated with *BRAF* mutations. An additional assessment of 86 *BRAF* mutated and 300 *BRAF* wildtype colonic cancers failed to identify an association between alcohol, folate, vitamins B6 and B12 or methionine consumption and *BRAF* mutation status (Schernhammer et al., 2011).

Another study population, of which 1108 cases of CRC were assessed for the presence of *BRAF* mutations, identified no associations between the 114 cancers harbouring this genetic lesion and intake of either vitamins B6, B12, folate, methionine or fibre consumptions, when compared with non-cancerous controls (Slattery et al., 2007). Similarly, the determination of *BRAF* mutation status in 189 CRC cases in another study cohort identified no associations between mutations in this gene and plasma levels of folate, vitamin B12 and homocysteine (Van Guelpen et al., 2010).

At present, few analyses of dietary intake in relation to the incidence of *BRAF* mutations in CRC have been attempted, and the majority of the limited data which do exist generally fail to identify strong associations between CRC harbouring *BRAF* mutations and any dietary constituent. In only one study to date, limited, sex specific dietary associations with *BRAF* mutation have been identified (de Vogel et al., 2008), but these observations are yet to be validated and corroborated in other studies.

The lack of identification of any of dietary component associated with *BRAF* mutation in CRC may have several causes. Primarily, only one study, analysing a very limited number of *BRAF* mutated tumours (n=29) has attempted a broad analysis of many dietary factors (Naguib et al., 2010). The remaining limited data has involved anlaysis of only a selected spectrum of dietary components hypothesised to be involved in the DNA methylation process. The limited scope of these studies in terms of dietary factors tested does not exclude the possibility that other

dietary factors may be associated with *BRAF* mutated CRC. Secondly, *BRAF* is identified at higher frequency in CRC demonstrating CIMP and MSI. Definitive evidence is yet to be provided describing the order in which tumours displaying CIMP and MSI acquire these instabilities and when *BRAF* mutations are acquired during progression. It is plausible that mutation in the *BRAF* gene is secondary to the acquisition of these global genomic alterations. As such, the question of diet and any relationships with this mutation may be redundant, if following the acquisition of CIMP and MSI status, *BRAF* mutation may arise independent of dietary influences. Thirdly, the limited number of studies available addressing the question of dietary associations and *BRAF* mutation may be too few in number to identify any dietary associations with this lesion, or, the majority of the studies performed are correct and that in this instance, dietary components do not affect the prevalence of *BRAF* mutations in CRC.

2.2 *p53* mutations in colorectal cancer

The *p53* tumour suppressor gene is the most commonly mutated gene in all human cancers, mutated in approximately 50% of human malignancies, including 50-60% of CRC (Forbes et al., 2008). Subsequent to its activation following DNA damage, oxidative stress or other cellular insults, wildtype p53 protein accumulates in the cell nucleus and acts as a transcription factor, capable of activating and suppressing transcription programmes leading to cell cycle arrest, DNA damage repair and apoptosis (Aylon & Oren, 2011; Bourdon et al., 2003). As such, perturbation of the normal role of p53 is highly selected for in cancer cells. The high prevalence of *p53* mutation in CRC, notably in later stage cancers, has led to various studies of mutations of this gene in the context of dietary consumptions.

2.2.1 *p53* mutations and dietary associations

Mutations in the *p53* gene have been linked to a variety of dietary intakes. Low folate and vitamin B6 intakes have been linked to p53 over-expressing cancers (Schernhammer, Ogino & Fuchs, 2008). This report, analysing 143 p53 over-expressing and 256 colonic cancers demonstrating low or absent p53 expression used an immunohistochemical (IHC) analysis to assess p53 accumulation following mutation. p53 over-expression or accumulation is the result of reduced protein degradation, mostly due to point mutations in the *p53* gene, greatly increasing the half-life of the gene's protein product (Melhem et al., 1995). This fast method of assessment of a range of activating *p53* mutations should be interpreted with some caution however, as less commonly observed mutations giving rise to truncated protein, such as those introducing premature *stop* codons, are not identified using this method. The observation linking low folate intake to an increased prevalence of cancers of the colon exhibiting over-expression of p53 is yet to be corroborated. Two reports using DNA sequencing, testing 62 *p53* mutated and 123 *p53* wildtype CRC (Park et al., 2010) and 686 *p53* mutated and 772 *p53* wildtype colonic cancers (Slattery et al., 2002), identified no link between p53 status and folate intakes. Little or no apparent other data exist describing vitamin B6 intakes and possible relationships with *p53* mutation status.

Specific meat intakes have been linked to *p53* mutation status in several independent studies. One report by Park and colleagues identified an increased consumption of red and total meat (all types, including poultry) as associated with increased prevalence of *p53* mutations in CRC, however, this was only present in advanced stage CRC (Dukes' C or D), not in those of less advanced stages (Dukes' A or B) (Park et al., 2010). In addition to this, an assessment by

Slattery and co-workers identified high glycaemic load, increased red meat, increased fast food and increased trans fatty acid intakes as associated with increased prevalence of *p53* mutations in colonic cancers (Slattery et al., 2002). These two independent studies suggest that red meat in particular may promote mutations in *p53* in neoplasia of the large intestine. However, these data do not completely overlap: the study by Park and colleagues only found this association in advanced stage cancers and the report by Slattery and co-workers assessed only colonic, not rectal cancers. Opposed to the above observations of meat intakes promoting *p53* mutations in CRC, an IHC based analysis (73 p53 over-expressing, 90 p53 absent CRC) identified increased beef consumption as associated with reduced prevalence of p53 over-expressing cancers (Freedman et al., 1996). Further data are needed to evaluate the potential association of meat, and meat types, with *p53* mutations in CRC, with particular emphasis on cancer location and stage.

In a study of colonic cancers assessing both p53 expression and *p53* gene mutations, total and saturated fats were identified as linked to tumours not over-expressing p53 or harbouring gene mutations (Voskuil et al., 1999). Of the 185 colonic cancers tested in this study, 81 displayed p53 overexpression by IHC, of which 59 were found to harbour mutations in the sequenced region (exons 5-8) of these cancers. Mutations in *p53* were not found to be linked to total fat intake in other reports assessing either CRC as a general subgroup (Park et al., 2010) or rectal cancers in particular (Slattery, Curtin, Wolff, Herrick, Caan & Samowitz, 2010).

An analysis of 340 *p53* mutated and 410 *p53* wildtype rectal cancers reported an increased consumption of vegetables, whole grains and fibre associated with reduced prevalence of *p53* mutation (Slattery, Curtin, Wolff, Herrick, Caan & Samowitz, 2010). Conversely, a high intake of refined grains was found to increase the prevalence of rectal cancer harbouring *p53* mutations. Increased intakes of cruciferous vegetables have also been described to be associated with reduced prevalence of p53 over-expressing CRC (73 p53 over-expressing CRC, 90 p53 absent CRC) (Freedman et al., 1996). The observation of increased vegetable intakes associated with reduced frequency of *p53* mutations in CRC was not observed in another study analysing general CRC (Park et al., 2010). Fibre was not observed to be associated with a protective effect in analyses combining colonic and rectal cancers (Park et al., 2010) or assessing colonic cancers in isolation (Slattery et al., 2002; Voskuil et al., 1999).

Alcohol intakes and *p53* mutation status in CRC have been assessed in several reports. A study analysing 340 *p53* mutated and 410 *p53* wildtype rectal cancers identified increased beer consumption as being associated with higher prevalence of *p53* mutations when compared with non-beer drinkers (Slattery, Wolff, Herrick, Curtin, Caan & Samowitz, 2010). No associations between alcohol intakes and *p53* mutation status have been identified in several analyses of colonic cancers (Schernhammer, Ogino & Fuchs, 2008; Voskuil et al., 1999), however, neither of these studies assessed specific alcoholic beverages, just total alcohol intake. Total alcohol intake was found to be linked to increased prevalence of *p53* mutations in CRC of advanced Dukes' stage (C and D), but not in CRC of less advanced stage (Dukes' A or B) (Park et al., 2010). Another report analysing Dukes' stage C cancers by IHC (42 p53 over-expressing CRC, 65 p53 absent CRC) did not identify total alcohol intake as linked to p53 expression status (Zhang et al., 1995).

Presently, the limited data on *p53* mutation status in CRC and dietary intakes are inconsistent. As a result, several consumptions have been linked to *p53* mutation status but none have been corroborated by other studies performing a similar assessment in an independent cohort.

Further evidence is needed to substantiate these isolated observations. Future studies should focus on the analysis of the potential association of vegetable and meat intakes in relation to p53 status as several data exist suggesting a possible link between these intakes and p53 aberrations, although contrary observations have been published.

2.3 *APC* mutations in colorectal cancer

The *adenomatous polyposis coli* (*APC*) gene is one of the most frequently mutated genes in colorectal cancer (Sjöblom et al., 2006; Wood et al., 2007), with some studies reporting 50-80% of CRC harbouring mutations in this gene (Forbes et al., 2008). The majority of mutations identified in CRC in the *APC* gene are located in exon 15 in the central third of the coding sequence, the *mutation cluster region*, which corresponds to the β-catenin-binding region of the protein (Goss & Groden, 2000). Mutations in *APC* most frequently result in truncation of the protein, corresponding with a reduction in the ability of APC to bind β-catenin (Figure 2). In addition to its role as a modulator of WNT pathway signalling, APC also has a role in mitosis and cytokinesis: cells harbouring truncated APC undergo abnormal chromosomal segregation and may develop aneuploidy (Ceol et al., 2007). Wildtype APC functions as a regulator of apoptosis, differentiation and migration and functions during cell division (Ceol et al., 2007; Fodde et al., 2001; Goss & Groden, 2000).

Although mutations in other genes, such as *p53*, may be almost as frequent as those in *APC* in CRC, *APC* mutations seem to be particularly prevalent from the earliest stages of CRC initiation and progression. Dysplastic aberrant crypt foci (ACF), monocryptal or oligocryptal adenomas, which are the lesions considered to be the earliest forms of colorectal neoplasia, frequently display *APC* mutations (Jen et al., 1994) and can develop into CRC through the adenoma-carcinoma sequence (Suehiro & Hinoda, 2008; Takayama et al., 1998). Intriguingly, the more frequently occurring heteroplastic ACF, which possess limited, if any, potential to develop to malignancy, very rarely harbour *APC* mutations but frequently exhibit *K-RAS* mutations (Jen et al., 1994). These data suggest that initiating genetic lesions in CRC determine malignant potential, and that if the initial mutations occur in the *APC* gene, there is a high probability of subsequent adenoma formation. In concordance with observations in dysplastic ACF, *APC* mutations are very frequently observed in colorectal adenomas (Kinzler & Vogelstein, 1996) and when inherited as germline *APC* mutations allow formation of hundreds of colorectal adenomas in the Familial Adenomatous Polyposis Coli syndrome. Hence, there have been several analyses of APC mutations in CRC relation to dietary intakes, with the purpose of identifying links between this early genetic lesion and dietary carcinogens.

2.3.1 *APC* mutations and dietary associations

APC mutations have been linked to several dietary constituents. One report, analysing 121 *APC* wildtype and 63 *APC* mutated colonic cancers, identified alcohol as inversely associated with *APC* mutated and positively associated with *APC* wildtype cancers (Diergaarde, van Geloof, van Muijen, Kok & Kampman, 2003). Additionally, red meat, fish and fat, notably unsaturated fat, were shown to be associated with development of *APC* mutated colonic cancers. Conversely, another report assessing 347 *APC* wildtype CRC and 184 *APC* mutated CRC identified increased consumption of saturated fat, but not unsaturated fats, as associated with *APC* mutated rectal cancers (Weijenberg et al., 2007). Furthermore, the analysis by

Fig. 2. **APC and the WNT signalling pathway**. **A:** In the absence of WNT signal, free β-catenin is bound by APC, in a complex with axin/conductin and glycogen synthase kinase 3β (GSK3β) and this complex acts as a scaffold, bringing β-catenin into close proximity with GSK3β. This results in GSK3β mediated phosphorylation of β catenin. **B:** Phosphorylated β-catenin is recognised by the SCF complex and is polyubiquitinated. **C:** Polyubiquitinated β-catenin is recognised by the proteasome and degraded. In the absence of WNT signalling, β-catenin is largely degraded, thus preventing β-catenin nuclear accumulation and subsequent co-activation of transcription programs. Upon binding of WNT ligand to membrane-located receptors, a subsequent signalling cascade prevents formation of the APC-axin-conductin-GSK3β complex. As a result, β-catenin avoids degradation and can translocate to the nucleus where it co-activates transcription of target genes, such as *c-myc*.

Weijenberg and co-workers identified specific types of *APC* wildtype CRC (i.e. those harbouring *K-RAS* mutations and showing no loss of MLH1 expression [see 2.4.2]) as being linked to increased intake of linoleic acid, a polyunsaturated fatty acid.

A further study has identified increased consumption of folate associated with reduced prevalence of *APC* wildtype colonic cancer, but increased prevalence of *APC* mutated colonic cancers in males (de Vogel et al., 2006). These associations were not observed in rectal cancers of men or in either colonic or rectal female cancer cases. This analysis, studying 347 *APC* wildtype CRC and 182 *APC* mutated CRC, also identified increased vitamin B2 and iron intakes in men associated with colonic cancers harbouring *APC* mutations compared with those men with colonic cancer not harbouring *APC* mutations.

These analyses are difficult to compare, notably as Diergaarde and colleagues did not stratify cases by sex or cancer location, which may possibly explain the lack of association between folate intake and *APC* mutation status in their report. The study by Diergaarde and co-workers did not analyse iron or vitamin B2, and de Vogel and colleagues did not assess meat and fish intakes. Alcohol association with *APC* mutation status was not observed in the testing by de Vogel and co-workers. Assessed in conjunction, these studies do not corroborate each other as direct comparisons are difficult to make.

Further analysis of *APC* mutation status has been performed in the context of specific meat intakes. In a study of 347 *APC* wildtype CRC and 184 *APC* mutated CRC, increased processed meat consumption was linked to an increased prevalence of *APC* mutated colonic cancers (Lüchtenborg et al., 2005). Additionally, increased beef consumption was linked to increased frequency of *APC* wildtype colonic cancers. Rectal cancers without *APC* mutations were found to be more prevalent amongst those with increased consumption of other meat types, which included horsemeats, lamb and mutton among other products. This detailed analysis of *APC* mutation status in the context of very specific meat types, with both positive and negative associations having been identified, is yet to be corroborated by similarly detailed meat-type subgroups testing in additional studies. This report does suggest however, that meat classification is important when testing for associations with *APC* mutations. In this

context, these observations partially confirm the increased consumption of general red meat that was observed to be associated with an increased risk of *APC* mutated CRC in the report by Diergaarde and co-workers.

In addition to reports assessing *APC* mutation status relative to dietary intakes in CRC, a single study has assessed these relationships in colorectal adenomas (Diergaarde et al., 2005). This analysis of 117 *APC* wildtype adenomas and 161 *APC* mutated colorectal adenomas identified a high intake of red meat and fat as associated with increased prevalence of *APC* wildtype adenomas. These observations are intriguing as identification of increased consumptions of certain red meat types being specifically associated with certain *APC* wildtype CRC has been described previously (Lüchtenborg et al., 2005).

Taken together, the available data describing *APC* mutations in CRC in relation to dietary intakes are too few and inconsistent to draw any strong conclusions. However, several analyses have identified certain meat consumptions as linked to either colonic or rectal cancers with a particular *APC* mutation status. These observations, although not in full agreement, indicate that certain red meat types, determined by both animal origin and preparation method, may affect the prevalence of mutation in *APC* in CRC. Further assessment of these particular dietary associations are warranted to determine the relationship between *APC* mutation status and specific red meat consumptions. Based on these somewhat conflicting data, some associations do seem plausible.

2.4 Microsatellite instability (MSI) and CpG island methylator phenotype (CIMP) in colorectal cancer

2.4.1 MSI and CIMP as genomic instabilities in colorectal cancer

Acquired variation in length of repetitive DNA sequences (microsatellites) can be detected as microsatellite instability (MSI) and is prevalent in approximately 15% of sporadic CRC and in almost all CRC in Lynch/Hereditary Non-Polyposis Colorectal Cancer syndrome (Soreide et al., 2006). MSI arises as a result of DNA replication errors that produce a change in length of repetitive sequences, which if not repaired (by the DNA mismatch repair (MMR) system), accumulate with increasing frequency (Martin et al., 2010; Soreide et al., 2006). In sporadic CRC, the most frequent inactivating cause of MMR is the methylation of the *MLH1* promoter on one or both alleles (Herman et al., 1998; Wheeler et al., 2000).

The MMR process is responsible for the correction of DNA replication errors which result in small insertions or deletions in the genome; these are especially prevalent at microsatellites due to increased frequency of DNA polymerase slippage at repetitive sequences. In humans, two major components comprise the MMR pathway: MutS (which is present in two heterodimers of MSH2/MSH6 and MSH2/MSH3) and MutL (which is also present in several heterodimer forms:- MLH1/PMS2, MLH1/PMS1 and MLH1/MLH3) (Martin et al., 2010). Disruption of the formation of the MutS and MutL dimers (by abrogation of the component proteins due to acquired promoter methylation or mutation) leads to a limited or defective MMR pathway, giving rise to genomic instability whereby DNA regions, most frequently repetitive sequences, increase or decrease in length (MSI). Such instability can lead to gene mutations, frequently of frameshift type, which can contribute to cancer progression.

CIMP is observed in 30-40% of proximal colonic and 3-12% of distal/rectal cancers (Curtin et al., 2011; Ibrahim et al., 2011). The exact causes of excessive methylation in DNA regions harbouring high levels of adjacent cytosine and guanine bases (CpG islands) are

unknown, although some evidence exists which suggest that such an increase in methyl group incorporation at these sites occurs during ageing in normal epithelial cells in the gut, and this is elevated in cancer (Toyota et al., 1999). Hypermethylation of gene promotors, in addition to or independent of methylation of other local DNA sequences, leads to transcriptional silencing of those genes. Such transcriptional silencing can be considered as one mechanism by which genes can be 'knocked out', in addition to mutation and deletion, in Knudson's model of tumour suppressor gene inactivation (Kondo & Issa, 2004). In this way, the aberrant methylation of genes can contribute to their inactivation in cancer epigenetically, such that in the absence of inactivating genetic changes tumour suppressor gene activity can be lost, leading to cancer progression.

2.4.2 MSI and dietary associations

MSI in CRC has been assessed in relation to dietary intakes in several reports, many of which did not identify a link between this type of genomic instability in CRC and specific dietary intakes (Chang et al., 2007; de Vogel et al., 2008; Jensen et al., 2008; Schernhammer, Giovannuccci, Fuchs & Ogino, 2008) (Table 2). However, a limited number of studies have described links between dietary intakes and MSI in colorectal neoplasms. An analysis of 144 microsatellite stable (MSS) and 40 MSI colonic cancers described an increased intake of red meat as associated with increased prevalence of MSS cancers (Diergaarde, Braam, van Muijen, Ligtenberg, Kok & Kampman, 2003). However, an assessment of 437 MSS and 49 MSI colonic cancers, failed to identify a similar association with red meat and MSS status (Satia et al., 2005). Additionally, a further report, testing 238 MSS and 35 MSI colonic cancers also failed to identify red meat intake as associated with MSI or MSS status (Wu et al., 2001). However, in the study performed by Wu and colleagues, heterocyclic amines were found to be associated with increased prevalence of MSI CRC. Heterocyclic amines can be produced during certain high-temperature methods of cooking of meats (Santarelli et al., 2008). Consequently, it is plausible that cooking method, independent of, or in conjunction with, certain meat types, may be associated with MSI status in CRC, potentially explaining the inconsistent observations between MSI and meat intakes.

Alcohol intake has been described as associated with MSI status in CRC. One report, analysing 1337 MSS and 227 MSI CRC identified increased alcohol intake as associated with a higher prevalence of MSS cancers (Poynter et al., 2009). Discordantly, a second analysis of 1244 MSS and 266 MSI colonic cancers identified increased alcohol consumption as linked to increased prevalence of MSI cancers (Slattery et al., 2001).

Folate intake has also been assessed relative to MSI status in CRC. Increased levels of plasma folate were associated with MSI cancer prevalence in a report assessing 166 MSS and 24 MSI CRC (Van Guelpen et al., 2010). However, assessment of dietary intake of folate in studies testing 179 MSS and 16 MSI CRC (Chang et al., 2007), 572 MSS and 76 MSI CRC (de Vogel et al., 2008), 111 MSS and 19 MSI CRC (Jensen et al., 2008) and 542 MSS and 127 MSI colonic cancers (Schernhammer, Giovannuccci, Fuchs & Ogino, 2008) all identified no association between folate intake and MSI status in CRC.

Presently, the data describing dietary associations and MSI status in CRC are contradictory and difficult to interpret. No strong associations have been identified and corroborated in independent cohorts. The difficulty in identification of plausible dietary constituents which may affect MSI prevalence in CRC may be due to the lack of such a relationship existing. It

Study	MSS/MSI-low CRC/CC	MSI/MSI-high CRC/CC	dietary association
Chang *et al* 2007	CRC: 179	CRC: 16	no statistically significant association between folate or vitamin B12 and MSI status
de Vogel *et al* 2008	CRC: 572	CRC: 76	no statistically significant association between folate, vitamin B2, methionine or alcohol and MSI status
Diergaarde *et al* 2003	CC: 144	CC: 40	↑ red meat associated with MSS cancers
Jensen *et al* 2008	CRC: 111	CRC: 19	no association between MSI and folate or vitamin B12
Poynter *et al* 2009	CRC: 1337	CRC: 227	↑ alcohol associated with MSS cancers
Satia *et al* 2005	CC: 437	CC: 49	no association between diet and MSI status [some associations comparing MSI/MSS cases vs controls]
Schernhammer *et al* 2008	CC: 542	CC: 127	no statistically significant association between folate, vitamin B6, B12, methionine or alcohol and MSI status
Slattery *et al* 2001	CC: 1244	CC: 266	↑ alcohol associated with MSI cancers
Van Guelpen *et al* 2010	CRC: 166	CRC: 24	Increased levels of plasma folate associated with MSI cancers
Wu *et al* 2001	CC: 238	CC: 35	↑ heterocyclic aromatic amines associated with MSI cancers

Table 2. Summarised description of literature analysing microsatellite instability (MSI) in colorectal neoplasia in relation to dietary intakes with the statistically significant associations described. *MSS*: microsatellite stability, *WT*: wildtype, *CRC*: colorectal cancer, *CC*: colonic cancer, ↑ and ↓ denote an increase or decrease in consumption respectively.

may also be plausible that such relationships exist and are particularly subtle. Methylation of the *MLH1* promoter, leading to gene silencing and subsequent DNA MMR deficiency, occurs in the vast majority, but not all, of MSI CRC (Kuismanen et al., 2000); suggesting that other components of the MMR system can be disrupted, such as mutations to the *MSH2* or *MSH6* genes, and that MSI may develop from a group of distinct initial aberrations in a small proportion of CRC. Furthermore, subsequent instability at microsatellites as a result may depend on other promoting factors. As such, it appears that a series of molecular events takes place leading to the MSI phenotype, which may arise from different epigenetic silencing or mutational events in different cancers. The multiple causes of MSI, and the different associated factors, may explain, at least in part, the lack of consistently identified dietary constituents which have been associated with this type of genomic instability. Alternatively, age-related susceptibility to promoter methylation, including the MLH1 promoter, may be the predominant risk factor for MSI in CRC rather than dietary factors.

2.4.3 CIMP and dietary associations

Studies assessing dietary associations with CIMP in CRC have centred largely on testing intakes of those compounds which may act as methyl group donors, or which function in the biochemical pathways responsible for methylation processes. Vitamin B6 has been described as associated with an increased prevalence of CIMP in CRC in one study assessing 496 CIMP-low/absent and 152 CIMP-high cancers (de Vogel et al., 2008). However, several other reports, assessing 288 CIMP-low/absent and 87 CIMP-high (Schernhammer et al., 2011) and 824 CIMP-low/absent and 330 CIMP-high (Slattery et al., 2007) colonic cancers failed to identify a similar association.

A similar lack of consensus has been observed when assessing vitamin B12. A single study assessing 107 CIMP-low/absent and 44 CIMP-high colonic cancers described an increased serum vitamin B12 concentration as associated with CIMP in this cohort (Mokarram et al., 2008). Schernhammer and colleagues (Schernhammer et al., 2011) and Slattery and co-workers (Slattery et al., 2007) did not identify a similar association in their studies. A report assessing 163 CIMP-low/absent and 27 CIMP-high CRC also identified no association between vitamin B12 intakes and CIMP status (Van Guelpen et al., 2010). Assessment of folate intake in relation to CIMP status has consistently failed to identify associations between the two in both colorectal and colonic cancer studies (Schernhammer et al., 2011; Slattery et al., 2007; Van Guelpen et al., 2010).

A single report, assessing 167 CIMP-low/negative and 17 CIMP-high colonic cancers identified reduced fruit intake as associated with an increased prevalence of CIMP-high colonic cancer (Diergaarde, Braam, van Muijen, Ligtenberg, Kok & Kampman, 2003). In an independent study, reduced consumption of vitamin A was identified as associated with increased prevalence of CIMP-high CRC (98 CIMP-low/absent CRC and 22 CIMP-high CRC) (Mas et al., 2007). These observations are yet to be corroborated in other studies. An additional report, assessing 776 CIMP-low/absent and 74 CIMP-high rectal cancers (Slattery, Curtin, Wolff, Herrick, Caan & Samowitz, 2010) failed to identify fruit intakes as associated with CIMP-high rectal cancer prevalence. Little additional data exists describing vitamin A intakes relative to CIMP status in CRC.

A limited number of additional associations have been observed relating CIMP status to certain dietary patterns. One report, assessing broad dietary patterns in addition to specific nutrient and foodstuff intakes identified increased fat-rich dairy products and omega-3 fatty acid consumption as associated with increased frequency of CIMP-high rectal cancers (776 CIMP-low/absent cancers and 74 CIMP-high cancers) (Slattery, Curtin, Wolff, Herrick, Caan & Samowitz, 2010). In an additional analysis, using this same patient cohort, long-term liquor/spirit intake was also found to be associated with an increased prevalence of CIMP-high status (Slattery, Wolff, Herrick, Curtin, Caan & Samowitz, 2010). Very few studies have assessed alcohol intake in terms of beverage consumed, as such, this observation awaits confirmation in an independent study. Additional data do not exist at present which validate the observed associations between increased consumption of fat-rich dairy products and omega-3 fatty acid with CIMP-high status.

There is no dietary intake which has been identified in several cohorts as associated with CIMP-high colorectal neoplasia. This may be due to the variety of methodologies used to assess CIMP status and the different criteria used to define CIMP-high status in these cancers, with no consensus method and definition having been used across studies (see Table 3). Furthermore, CIMP itself is the resulting phenotype of precursor genetic and epigenetic aberrations. As such, it may be plausible that this CRC subtype may not be linked to dietary risk factors, but instead diet may be linked to the causative precursor events, such as *MLH1* promoter methylation and MSI. Assessment of large study cohorts, in which CIMP-high cancers are categorised by causative lesions or processes, would in part help to understand dietary intakes and causation in the context of this phenotype.

3. Review limitations

This review has attempted to assess the available data describing the relationship between dietary factors and the molecular genetic events occurring during the development and progression of CRC. Published analyses have been summarised and where consensus between studies exists, this has been highlighted. Although providing a synopsis of the available information, several limitations are inherent in such a general discussion.

No detailed analysis or discussion of the methods of statistical analysis in each report has been provided. The wide range of methodology employed for this purpose across studies makes such a discussion in the present chapter impractical. Opinions on statistical methods vary across reports in terms of adjustment for multiple testing, inclusion of confounding variables in statistical models and the requirement for power calculations. In this context, no discussion or comparison of statistical methods has been attempted; notably, hazard and odds

Study	CIMP-low/absent CRC/CC/RC	CIMP-high CRC/CC/RC	dietary association
de Vogel *et al* 2008	CRC: 496*	CRC: 152*	↑ vitamin B6 associated with *MLH1* promoter methylation in males only
Diergaarde *et al* 2003	CC: 167**	CC: 17**	↓ fruit associated with *MLH1* promoter methylation and concurrent absence of MLH1 protein
Mas *et al* 2007	CRC: 98*	CRC: 22*	↓ vitamin A associated with *MLH1* promoter methylation
Mokarram *et al* 2008	CC: 107∓	CC: 44∓	increased levels of serum B12 associated with CIMP
Schernhammer *et al* 2011	CC: 288†	CC: 87†	no association between folate, vitamin B6, B12, methionine or alcohol consumption and CIMP
Slattery *et al* 2007	CC: 824††	CC: 330††	no association between folate, vitamin B6, B12, methionine or alcohol consumption and CIMP
Slattery *et al* 2010	RC: 776††	RC: 74††	no association between calcium and vitamin D consumption and CIMP
Slattery *et al* 2010	RC: 776††	RC: 74††	↑ fat-rich dairy products and ↑ omega-3 fatty acids associated with CIMP
Slattery *et al* 2010	RC: 776††	RC: 74††	long-term ↑ spirits/liquor with CIMP
Van Guelpen *et al* 2010	CRC: 163‡	CRC: 27‡	no association between plasma folate or plasma vitamin B12 and CIMP

Table 3. Summarised description of literature analysing CpG island methylator phenotype (CIMP) in colorectal neoplasia in relation to dietary intakes with the statistically significant associations described. *WT*: wildtype, *CRC*: colorectal cancer, *CC*: colonic cancer, *RC*: rectal cancer, ↑ and ↓ denote an increase or decrease in consumption respectively. * CIMP positive status defined by *MLH1* promoter methylation. ** CIMP positive status defined by *MLH1* promoter methylation and concurrent loss of MLH1 expression as determined by immunohistochemistry. ∓ CIMP positive status defined by methylation of one or more of the *p16*, *MLH1* or *MSH2* promoters. † CIMP positive status defined by methylation at 11 of 16 tested markers. †† CIMP positive status defined by methylation at 2 of 5 tested markers. ‡ CIMP positive status defined by methylation at 6 of 8 tested markers.

ratios should be further analysed in order to interpret the relative 'strength' of the associations highlighted here.

In addition to statistical methods, the methodology of dietary assessment in each individual report has not been discussed. Dietary intakes can be measured in a variety of ways, including person-to-person interview, food frequency questionnaires, food diaries and biomarker assessment. Such an assessment is beyond the scope of this chapter. Outside of this review, several specific reports have been published describing the merits, limits and practicality of some of the available options for dietary assessment (Bingham et al., 1995; Day et al., 2001). To fully interpret dietary associations identified in different studies, although not discussed herein, an appreciation of dietary assessment methodology, and the relative accuracy of such techniques, should be taken into account.

Further to the limits inherent in the compilation of this review, consideration of the nature of assessment of dietary intakes relative to characteristics of colorectal cancers is required. For example, considerably more data exist describing the relationship between mutations in *K-RAS* and diet than for *APC*. An 'assessment bias' exists, presumably due to the significantly simpler task of examining hotspot mutation regions of the *K-RAS* proto-oncogene compared with the longer lengths of sequencing required for mutational assessment of tumour suppressor genes. As a result, the molecular genetic changes which occur during CRC development have not been assessed at equal frequencies. Such 'assessment bias' should be noted when considering such a broad view, as presented in this chapter. This should be particularly considered when trying to interpret the genetic or molecular changes which have been tested in relation to diet in only a small number of studies.

It should also be understood that in many reports assessing dietary associations in CRC broad definitions are employed, in order to maintain the practicality and feasibility of studies. For example, often reports describing mutations in *BRAF* are actually describing mutations only in exons 11 and 15; reports describing *p53* mutations are frequently only describing mutational analyses of exons 5-8. Such limited analyses of coding regions is justified, with the significant majority of mutations in these examples being present in the regions described.

Furthermore, such limitations increase the practicality of these studies, in terms of both financial support and time investments required. Additionally, these limited regions of analyses are frequently selected based on biological evidence. Although justified, the limited extent to which genes are searched for the presence of mutations should be appreciated, and such variability between studies may in part explain inconsistent observations. In conjunction with this, different methods of mutational assessment provide different levels of sensitivity. For example, hotspot mutational assessment has been demonstrated to be more sensitively performed using pyrosequencing compared with dideoxysequencing (Naguib et al., 2010; Ogino et al., 2005). Such discrepancies between different reports were not discussed in this chapter, but should be considered when making side-by-side comparisons of studies.

In addition to the genetic and epigenetic changes giving rise to CRC development and progression described in this chapter, additional events occur during progression of these neoplasms. Furthermore, these events may be associated with dietary intakes, and data exist describing their associations with dietary consumptions; for example, loss of PTEN expression has also been tested for association with dietary intakes in CRC (Naguib, Cooke, Happerfield, Kerr, Gay, Luben, Ball, Mitrou, McTaggart & Arends, 2011). Studies of genetic and epigentic events beyond those discussed here were omitted due to the current low number of studies assessing their relationship with diet.

4. Future directions of the field

Next generation sequencing technology now affords the practical and accurate sequencing of entire genomes, with such strategies being employed to assess the genetic changes in several cancer types (Stratton et al., 2009). Furthermore, genomewide single nucleotide polymorphism analyses are being employed in a variety of settings. With these tools it is now possible to ask different questions relating diet to cancer. Are certain chemicals in the diet associated with an increased prevalence of any type of base change across the genome? Are transitions or transversions associated with intakes of specific compounds? The prospect of such investigations greatly expand the potential to understand the biochemical implications of certain dietary intakes, and provide an attractive avenue by which the identification of initiating factors in colorectal carcinogenesis might be pursued.

At present, a moderate number of studies have attempted to assess what impact, if any, dietary factors may have on CRC and the molecular subtypes of tumours which comprise this disease. With new technologies becoming available which have the power to expand this field of study, the underlying question of the purpose of such analyses should be clarified. Simply identifying dietary links to disease is only of limited use: how can this understanding be employed to reduce cancer-related mortality? It may be unrealistic to expect that if dietary constituents can be shown to be associated with increased prevalence of any particular molecular subtypes of colorectal cancer that these may be eliminated from the diet. The overwhelming evidence describing the strong association between tobacco use and cancer mortality fails to deter a significant number of smokers; although, the identification of such a link has undoubtedly provided individuals with knowledge upon which informed decisions have been made to refrain from tobacco use. Instead, a more 'protective' approach might be endorsed, such that dietary constituents which are found to confer protection against certain types of CRC might be promoted. This may be particularly useful in the attempt to lower the number of diagnoses of the molecular subtypes of CRC which confer a poor

prognosis. Some have suggested that excessive administration of dietary advice may prove to be counterproductive: advice should be administered sparsely and where the greatest potential to reduce cancer-related deaths exists. It is in this context that the understanding of diet and the molecular subtypes of CRC has the greatest potential and will provide the greatest impact in the effort to reduce the number of CRC-related deaths.

5. Conclusions and summary

At present, although data exist describing the association of particular dietary factors with the specific molecular genetic changes in CRC, very few consistently reproducible associations have been described. Several factors may contribute to this, including variations in study methodologies (dietary intake assessment, sequencing strategies), statistical assessment (variation in the statistical power/number of samples, inclusion of different confounding variables in models) and features of study design.

Assessment of the presently available data do describe some associations which warrant further study: *K-RAS* mutation appears to be less frequent in CRC in individuals consuming a high folate diet. Furthermore, *APC* mutation appears to be associated with meat intakes to some degree, although this exact relationship is unclear.

At present, the study of diet in relation to the specific subtypes of CRC is at an exciting stage. Sequencing technology advancements now provide an avenue by which the total genetic composition of CRC, and the specific molecular subtypes, can be assessed. Using such tools, detailed understanding of genomewide events can be correlated with dietary intakes. Such modern approaches, coupled with renewed efforts to improve, validate and employ the most reliable and accurate methods of dietary intake assessment, provide the keys to the success of this field, which will help to provide the sought after end goal of a reduction in the number of CRC-related deaths.

6. Acknowledgments

This work was supported by EPIC Norfolk and the Medical Research Council Centre for Nutritional Epidemiology in Cancer Prevention and Survival. Furthermore, the dedication and support of Professor Kay-Tee Khaw and the late Professor Sheila Bingham were essential in the completion of this chapter.

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10

Colorectal Cancer and Alcohol

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1. Introduction

Chronic alcohol consumption may lead to a variety of diseases and may deteriorate a great number of existing health problems. Among all these diseases the development of certain types of cancer is of major concern. Since decades it has been known that chronic alcohol consumption is a risk factor for cancer of the upper aerodigestive tract (oral cavity, pharynx, larynx and oesophagus), the liver and the female breast. Data with respect to alcohol and cancer concerning other organs do not show such clear correlations. In February 2007 an international group of epidemiologists and alcohol researchers met at the International Agency for Research on Cancer (IARC) in Lyon, France, to evaluate the role of alcohol and its first metabolite acetaldehyde as potential carcinogens in experimental animals and humans. The working group has concluded from the epidemiological data available that the occurrence of malignant tumours mentioned above is related to the consumption of alcoholic beverages. In addition, at this time epidemiologic and experimental data showed that alcohol is also a risk factor for colorectal cancer (Baan et al., 2007).
Worldwide a total of approximately 389,000 cases of cancer presenting 3.6 % of oral cancers (5.2 % in men and 1.7 % in women) derive from chronic alcohol consumption (Rehm et al., 2004). Besides the fact that alcohol is a co-carcinogen and may act as a promoter alcohol can also accelerate tumour spread as exemplified for liver metastases of colorectal cancer possibly due to immune suppression and induction of angiogenesis by the expression of vascular endothelial growth factor (VEGF) (Seitz & Stickel, 2010). In addition, it is important to know that ethanol also interacts with the metabolism of chemo-therapeutic drugs which can result in a decreased respond to medication and increased side-effects (De Bruijn & Slee, 1992).
This review focuses solely on the effect of chronic alcohol consumption on colorectal cancer, a cancer which is wide spread in Western societies and is No. 3 cancer in men and women in Germany. The present review will, therefore, discuss epidemiology of alcohol and colorectal cancer, will briefly address possible mechanism by which alcohol stimulates colorectal carcinogenesis and may finally give some suggestions and recommendations with respective to earlier detection and identification of high risk groups.

2. Epidemiology

An increased risk for the development of colorectal cancer associated with chronic alcohol ingestion has been considered for decades. In 1974 Breslow and Enstrom emphasized a

correlation between beer consumption and rectal cancer occurrence (Breslow & Enstrom 1974). In 1992 Kune and Vitetta summarized the results of more than 50 epidemiologic studies between 1957 and 1991 including 7 correlational studies, more than 40 case control studies and 17 prospective cohort studies on the role of alcohol on the development of colorectal tumours (Kune & Vitetta 1992). Most of these studies reported a positive association of large bowel cancer with alcohol consumption. In addition a positive trend with respect to dose-response was found in 5 out of 10 case control studies and in all prospective cohort studies in which a dose-response analysis has been performed.

In the nineties of the last century another 12 epidemiological studies have been published with inconsistent results (Seitz et al., 2006). Most importantly a prospective cohort study in Japan reported a positive dose-response relationship between alcohol intake and colon cancer risk in men and women (N. Shimizu et al., 2003), while a Danish population based cohort study showed no association (Pedersen et al., 2003).

A panel of experts at a WHO consensus conference on nutrition and colorectal cancer reviewed in 1999 the epidemiology on alcohol and colorectal cancer and it was concluded that alcohol ingestion even in a low dose-intake between 10 and 40 grams especially consumed as beer resulted in a 1.5-fold increased risk for colorectal cancer and to a lesser extend for colonic cancer in both sexes but predominantly in men (Scheppach et al., 1999).

Cho et al showed in a meta-analysis of 8 cohort studies from the US and Europe a trend between the increased amount of alcohol intake and the risk for colorectal cancer. In this meta-analysis a consumption of more than 45 grams per day was associated with an increased risk of 45 % (Cho et al., 2004).

A huge prospective follow-up study of more than 10,000 US citizens concluded that alcohol consumption of one or more alcoholic beverages per day is associated with a 70 % greater risk of colonic cancer with a strong positive dose-response relationship (Su & Arab, 2004).

Most recently it was proposed that the alcohol colorectal cancer association is more apparent in Japanese than in Western populations. A pooled analysis of results from 5 cohort studies from Japan showed a strong and highly significant association between alcohol intake and colorectal cancer not only in men but also in women (Mizoue et al., 2008). Twenty five per cent of colorectal cancer cases in men were attributable to an alcohol intake of more than 23 grams per day. A recent meta analysis from the IARC of 34 case control and 7 cohort studies provides strong evidence for an association between alcohol consumption of more than 1 drink per day and the risk for colorectal cancer (Fedirko et al., 2011). Similar results were reported from the Netherlands (Bongaerts et al., 2008, 2010) and the US (Thygesen et al., 2008) but not from Great Britain (Park et al., 2009, 2010).

The accumulation of all these convincing epidemiologic data on alcohol and colorectal cancer resulted by the IARC that chronic alcohol consumption is a risk factor for colorectal cancer (Baan et al., 2007).

3. Animal experiments

Various animal experiments have been performed to study the effect of alcohol on chemically induced colorectal cancer. The results of these experiments depend on the experimental design, the type of carcinogen used, its time duration of exposure and dosage as well as the route of alcohol administration. While alcohol alone does not induce colorectal tumours, the administration of alcohol together with a colorectal carcinogen does under certain experimental conditions result in a stimulation of carcinogenesis (Seitz et al., 2006).

This is especially relevant when a carcinogen such as acetoxymethylmethylnitrosamine (AMMN) is locally applied to the rectal mucosa (Seitz et al., 1990). Some evidence exist that acetaldehyde (AA) is an important factor since inhibition of its degradation stimulates colorectal cancer (Seitz et al., 1990).
For a detailed summary of the animal experiments performed so far we refer to the following review article (Seitz et al., 2006).

4. Risk factors in alcohol mediated colorectal carcinogenesis

Various risk factors for ethanol-mediated colorectal carcinogenesis exist. Five out of 6 studies of the effect of alcohol on the occurrence of adenoma polyps in large intestine showed a positive association with alcohol (Kune et al., 1992; Seitz et al., 1998). In addition, also the occurrence of hyperplastic polyps is enhanced when more than 30 grams of alcohol per day were consumed. The relative risk for men was 1.8 and for women 2.5 (Kearney et al., 1995).
Alcohol affects the adenoma/carcinoma sequence at the different early steps (Boutron et al., 1995). High alcohol intake favours high risk polyps or colorectal cancer occurrence among patients with adenoma (Bardou et al., 2002). It has also been reported that a reduction in alcohol consumption for individuals with genetic predisposition for colorectal cancer had large beneficial effects on tumour incidence (Le Marchand et al., 1999). Thus, patients with tendency towards colorectal polyps have an increased risk to develop carcinoma when they consume additional alcohol.
Another additional risk is possibly the presence of ulcerative colitis, although the data are not completely clear. Alcohol by itself may additionally enhance inflammation and may thus favour carcinogenesis.
Another important factor is that alcohol reduces the availability of folate which results in a decrease of methylation and thus, a decrease of thymidine generation, DNA synthesis and cellular regeneration, in a situation of enhanced need. Therefore, folate, methionine and vitamin B6 deficiency are risk factors for ethanol mediated colorectal carcinogenesis (Giovannucci et al., 1995; Larsson et al., 2005; Schernhammer et al., 2008; Weinstein et al., 2008; Yamaji et al.; 2009; Figueiredo et al., 2009; Lee et al., 2010).
It is well known that tobacco smoking is associated with a higher risk for colonic adenoma and hyperplastic polyp formation as well as increased incidence of colorectal carcinoma (Seitz & Cho, 2009).
Age, another risk factor for colorectal cancer may also affect ethanol mediated cancer development in the large intestine. It has been shown in animal experiments that mucosal damage induced by chronic alcohol ingestion is more pronounced with advanced age compared to youth (Simanowski et al., 1994).
Finally, genetic risk factor with respect to alcohol metabolism and colorectal cancer has to be taken into consideration. Alcohol is metabolised by alcohol dehydrogenase (ADH) to AA. Seven different ADHs exist and two of them (ADH1B and ADH1C) reveal polymorphism. Among the two ADH1C is of considerable interest (see below) (Edenberg, 1997). Individuals with increase metabolism of ethanol via ADH1C due to homozygosity of the ADH1C*1 allele seem to have a significantly increased risk for colorectal cancer when they consume more than 30 grams alcohol per day (Homann et al., 2006).

5. Possible mechanisms of alcohol mediated colorectal carcinogenesis

5.1 Acetaldehyde (AA)

Most recently the IARC has identified AA as an important carcinogen for humans (Secretan et al., 2009). AA is produced from ethanol via ADH. In the gastrointestinal mucosa various ADHs are present and capable to produce AA from alcohol. In addition, gastrointestinal bacteria of the upper gastrointestinal tract and of the large intestine can metabolize ethanol to AA (Salaspuro, 2003) (Figure 1).

AA is highly toxic and carcinogenic and causes point mutations in the hypoxanthine-guanine phosphoribosyltransferase localized in human lymphocytes, induces sister chromatid exchanges and cross-chromosomal aberration (Seitz & Stickel, 2010). AA forms stable adducts with DNA. One of these adducts is especially generated in hyperregenerative tissues (in the presence of spermine and spermidine) such as the upper gastrointestinal tract and the colon where chronic alcohol consumption results in tissue hyperregenerativity. This propane DNA adduct is highly carcinogenic (Brooks & Thiruvathu, 2005). There is significant evidence that AA is responsible for the carcinogenic effect of alcohol in the upper gastrointestinal tract, oesophagus, larynx, pharynx and oral cavity (Baan et al., 2007; Seitz & Stickel, 2010). For more details about the role of AA on upper gastrointestinal cancer we refer to the following review article (Baan et al., 2007; Stickel et al., 2006).

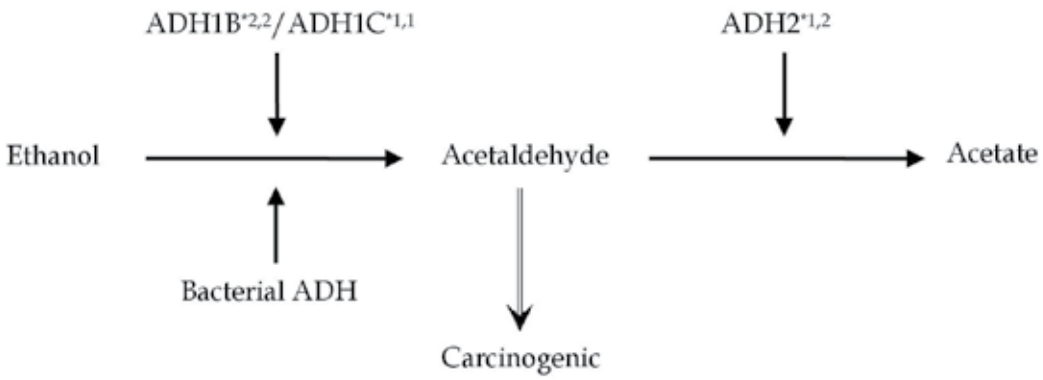

Fig. 1. Ethanol metabolism by mucosal enzymes and gastrointestinal bacteria. Ethanol is first metabolized by alcohol dehydrogenase (ADH) to acetaldehyde (AA) which is toxic and carcinogenic. AA is then further converted by acetaldehyde dehydrogenase (ALDH) to acetate which is non-toxic and is channelled into the intermediary metabolism of the cell. Accumulation of AA may either occur with rapid generation through ADH or slow degradation through ALDH. ADH1B and ADH1C are polymorphic. While the ADH1B*2 allele encodes for ADH enzymes approximately 40-fold more active as compared to the enzymes encoded by the ADH1B*2 allele, the ADH1C*1 allele encodes for an enzyme approximately 2.5-fold more active than the enzyme encoded by the ADH1C*2 allele. Thus, ADH1B*2,2 and ADH1C*1,1 homozygotes may accumulate AA. In addition, 40 % of Asians are heterozygote for the ALDH2 gene (ALDH2*1,2) encoding for an enzyme with very low ALDH activity resulting in AA accumulation.

In the colonic mucosa ADH1 and ADH3 are present and are involved in AA generation. On the other hand acetaldehyde dehydrogenase (ALDH), the enzyme responsible for AA

degradation is detectable at relative low activities only (Seitz & Oneta, 1998). The net amount of AA present in the tissue may determine its toxic and carcinogenic action. Thus, individuals with an increased production and decreased degradation of AA are especially at risk for colorectal cancer development. It has been proposed that the ALDH activity of colonic mucosa may be sufficient for the removal of AA produced by colonic mucosal ADH during ethanol oxidation but it is insufficient for the removal of AA produced by intracolonic bacteria.

The most striking evidence of the causal role of AA in ethanol-associated carcinogenesis derives from genetic linkage studies in alcoholics and / or heavy drinkers. Individuals who accumulate AA due to polymorphism and / or mutation in the gene coding for enzymes responsible for AA generation and detoxification have been shown to have an increased cancer risk. In Japan as well as in other Asian countries a high percentage of individuals carry a mutation of the ALDH2 gene which codes for an enzyme with low activity leading to elevated AA levels after alcohol consumption. While homozygotes are completely protected against alcoholism and alcohol associated diseases due to the fact that they cannot tolerate alcohol even at very small doses, heterozygotes (ALDH2*1,2) have an increased risk for alimentary tract cancer including the colon and the rectum (Yokoyama et al., 1998) (Figure.1).

In addition, polymorphism of the ADH1C gene may also modulate AA levels. ADH1C*1 transcription leads to an ADH isoenzyme 2.5 times more active than that from ADH1C*2 (Figure1). We evaluated whether the associated between alcohol consumption and colorectal cancer development is modified by ADH1C polymorphism. We recruited 173 individuals with colorectal tumours diagnosed by coloscopy and 788 control individuals without colorectal tumours and determined their genotypes. Genotype ADH1C*1/1 was more frequent in patient with alcohol associated colorectal neoplasia compared to patients without cancer (Homann et al., 2006). In addition, only individuals drinking more than 30 grams ethanol per day with a genotype ADH1C*1/1 had an increased risk for colorectal tumours. These data identify ADH1C homozygosity as a genetic risk marker for colorectal tumours in individuals consuming more than 30 grams alcohol per day and emphasize further the role of AA as a carcinogenic agent in alcohol mediated colorectal carcinogenesis.

It has been shown that after alcohol administration, the amount of AA per gram of tissue is highest for the colonic mucosa compared to all other tissues in the body (Seitz et al., 1987). This is primarily due to the production of AA by the faecal bacteria. AA has toxic effects on the colon mucosa resulting in secondary compensatory hyperregeneration with increased crypt cell production rates and an extension of the proliferative compartment towards the lumen of the crypt (Simanowski et al., 1994, 2001). This change in crypt cell dynamics represents a condition associating with increased risk for colorectal cancer.

The alcohol associated hyperregeneration of the colonic mucosa is especially pronounced with increasing age (Simanowski et al., 1994). As already pointed out, this may have practical implications since age by itself is a risk factor for CRC.

High AA levels have been found after alcohol administration in the colon of rats and these concentrations were significantly lower in germ free animals as compared to the conventional rats suggesting that faecal bacteria are capable of producing AA (Seitz et al., 1990). Indeed, the reversed microbial ADH reaction produces under aerobic or microaerobic conditions striking amounts of AA when human colonic contents or some microbes representing normal colonic flora are incubated in vitro at 37°C with increasing ethanol

concentrations (Jokelainen et al., 1996, 1994; Salaspuro et al., 1999). This reaction is already active at comparatively low ethanol concentrations (10-100 mg %) which exist in the colon following social drinking (Jokelainen et al., 1996). AA formation catalysed by microbial ADH takes place at a pH normally found in the colon and is rapidly reduced with decreasing pH (Jokelainen et al., 1994).
The administration of antibiotics to animals has significantly decreased colonic bacteria and colonic AA production (Jokelainen et al., 1997).

a. high AA levels occur in the colon due to bacterial and mucosal ethanol oxidation
b. animal experiments show an increased occurrence of colorectal tumours induced by the specific locally acting carcinogen AMMN when cyanamide, an ALDH inhibitor, is applied and AA levels are elevated,
c. crypt cell production rate correlates significantly with AA levels in the colonic mucosa, (d) colonic AA levels show a significant inverse relationship with mucosal folate concentration which supports in vitro data showing a destruction of folate by AA,
d. individuals with the inactive form of ALDH2 resulting in elevated AA concentrations exhibit an increased risk for CRC when they consume alcohol,
e. individuals homozygous for the ADH1C*1 allele coding for an enzyme with a 2.5 times higher AA production have also an increased risk for colorectal cancer, the action of AA seems the major mechanism of ethanol-mediated colorectal cancer development.

5.2 Oxidative stress

Chronic ethanol consumption results in the induction of cytochrome P4502E1 (CYP2E1) predominantly in the liver (Seitz & Stickel, 2010) but also in other tissues including the colorectal mucosa (Shimizu et al., 1990). This CYP2E1 induction is associated with an increased metabolism of ethanol through CYP2E1 and the generation of AA but also of reactive oxygen species (ROS). For more details we refer to the following review article (Seitz & Stickel, 2007). ROS may attack lipids and result in lipidperoxidation with the generation of lipidperoxidation products such as 4-hydroxy-nonenal or malondialdehyde. These lipidperoxidation products may bind to proteins but also to DNA and form exocyclic etheno DNA adducts with a high carcinogenic potency (Wang et al., 2009).
The effect of chronic ethanol consumption on the induction of CYP2E1 and the activation of procarcinogens has been convincingly demonstrated by the use of azoxymethane (AOM), a procarcinogen for the colon. The metabolism of AOM to its ultimate carcinogen has been inhibited in the presence of ethanol in the body since ethanol competes for the binding site at CYP2E1 but significantly enhanced when ethanol is withdrawn in a condition where CYP2E1 is induced and completely available for the metabolism of AOM (Sohn et al., 1987).
The induction of CYP2E1 in the colonic mucosa may lead to an enhanced activation of dietary (nitrosamines) and cigarette smoke derived (polycyclic hydrocarbons) procarcinogens and may be one mechanism by which ethanol enhances colorectal carcinogenesis (Seitz & Osswald, 1994).
In this context it is interesting that alpha tocopherol, a radical scavenger, prevents mucosal cell hyperproliferation induced by ethanol suggesting that ROS may be involved in this process (Vincon et al., 2003).

5.3 Epigenetics

There is increasing evidence that alcohol related epigenetic changes of DNA methylation and histone acetylation do occur which may potentially modulate tumour development (Stickel et al., 2006). Epidemiologic data have clearly shown that folate deficiency alone or together with methionine deficiency increases the risk for ethanol mediated colorectal cancer (Giovannucci et al., 1995; Larsson et al., 2005; Schernhammer et al., 2008; Weinstein et al., 2008; Yamaji et al., 2009; Figueiredo et al., 2009; Lee et al., 2010). Similarly, vitamin B6 deficiency also enhances tumour risk. All these factors are involved in methyl transfer. Their deficiency results in DNA hypomethylation, a condition relevant in carcinogenesis. In addition, histone acetylation is also favoured by chronic ethanol consumption (Kim & Shukla, 2006; Choudhury & Shukla, 2008) since ethanol metabolism leads to the accumulation of acetate on one hand and to a change in the intracellular redox potential with increasing concentrations of NADH and decreasing concentrations of NAD on the other hand. This change in redox potential also favours histone acetylation. In addition, histone deacetylation is blocked by ethanol through its inhibitory effect on histone deacetylase (HDA).

Indeed, animal experiments have shown DNA hypomethylation in the colon following chronic ethanol ingestion (Choi et al., 1999). However, site specific hypomethylation have not been demonstrated so far.

5.4 Other mechanisms

Other mechanisms of ethanol mediated carcinogenesis in the colon may including deficiency of retinoic acid (Wang X.D. & Seitz, 2004), effect of ethanol on intracellular signalling (Dunty, 2010) including various pathways such as Mitogenic signals (MAPK, RAS), Insensitivity to anti-growth signals (Rb and Cell cycle control, TGFβ), Apoptosis (p53, PTEN), Angiogenesis, Metastasis (ECM, Osteopontin, Wnt) and an ethanol effect on inflammation (Wang, J., 2010).

6. Summary, conclusion and recommendations

Chronic ethanol consumption at a dose of more than 30 grams ethanol per day is a risk factor for colorectal cancer. Chronic ethanol consumption also increases the risk for colorectal cancer in individuals with polyps and colorectal inflammation as well as in those with an ALDH mutation (ALDH2* 1,2, only Asians) and ADH1C*1 homozygosity since they accumulate AA following ethanol ingestion.

The mechanisms of ethanol mediated colorectal carcinogenesis may involve AA produced by mucosal ADH and intestinal bacteria. In addition, oxidative stress induced by ethanol may also play a role.

As a clinical consequence, chronic alcohol consumers should be screened earlier as the general population for colorectal cancer either by faecal blood test or colonoscopy depending on the methods available.

7. Acknowledgements

The authors wish to thank Mrs. Heike Grönebaum for typing the manuscript. Original research was supported by grants of the Manfred Lautenschläger- and Dietmar Hopp Foundation.

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11

Effects of Dietary Counseling on Patients with Colorectal Cancer

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1. Introduction

Cancers of the colon and rectum together are second most common tumor type worldwide. The prognosis for the survival after disease progression is usually poor (1). Cancer anorexia-cachexia syndrome is highly prevalent among patients with colorectal cancer, and has a large impact on morbidity and mortality, and on patient quality of life. Early intervention with nutritional supplementation has been shown to halt malnutrition, and may improve outcome in some patients (2).

The etiology of cancer-associated malnutrition appears to be related to the pathological loss of inhibitory control of catabolic pathways, whose increased activities are not counterbalanced by the increased central and peripheral anabolic drive (3).

The goals of nutritional support in patients with colorectal cancer are to improve nutritional status to allow initiation and completion of active anticancer therapies (chemotherapy and or radiotherapy) and improve quality of life (3, 4).

Cancer growth and dissemination but also cancer treatments, including surgery, chemotherapy, and radiation therapy, interfere with taste, ingestion, swallowing, and digest food which leads to hypophagia. Also, chemotherapy agents may cause nausea and diarrhea (3, 4). Although many new agents are on the market to combat these symptoms, prevalence of colorectal cancer is still high (1).

We studied the influence of nutritional support (counseling, nutritional supplements, megestrol acetate) on physical status and symptoms in patients with colorectal cancer during chemotherapy. The study was designed to investigate whether dietary counseling or oral nutrition commercial supplements during chemotherapy and/or BSC affected nutritional status and influence survival status prevalence in patients with colorectal cancer.

Results: Three hundred and eighty-eight colorectal cancer patients were included in the study. Nottingham Screening Tool Questionnaire, Appetite Loss Scale and Karnofsky Performance Status were taken to evaluate the nutritive status of patients. Group I consisted of 215 patients who were monitored prospectively and were given nutritional support and in this group weight gain of 1,5 kg (0,6-2,8 kg) and appetite improvement was observed in patients with colorectal cancer. In both groups Karnofsky Performance Status didn't change significantly reflecting the impact of the disease itself.
Nutritional counseling, supplemental feeding and pharmacological support do temporarily stop weight loss and improve appetite, QoL and social life, but this improvement has no implications on patients KPS and course of their disease.
Conclusion: These results encourage further studies with more specific nutritional supplementation in patients with colorectal cancer and probably in gastrointestinal oncology.

2. Colorectal cancer

The incidence and mortality rates for cancers of the colon and rectum are among the highest of all malignancies worldwide (1, 2). Colorectal cancer is second in global cancer incidence and it is the most common cause of cancer death among non-smokers. US and EU incidence figures exceed global averages, which is consistent with an increased risk in industrialized nations (2). Factors associated with increased risk of colorectal cancer are host susceptibility and a sequence of different carcinogenic exposures. Specific etiology for sporadic colorectal cancer is still elusive but predisposing hereditary and environmental factors have been clearly identified (5).

2.1 Etiology of colorectal cancer

Important causes of colorectal cancers are uncommon genetic syndromes. A small percentage of "sporadic" colon cancers cluster in families. Relatives of people with colorectal cancer have increased risk for colorectal cancer, and risk varies depending on the number of relatives affected and the age at which cancer occurred (5).
Colorectal cancer is a heterogeneous disease that can develop through only partly known complex series of molecular changes. It is a long-term, gradual process which, besides external factors (carcinogens), also involves ever more recognized hereditary factors that cause genetic changes capable of triggering the uncontrolled mucosal (epithelial) growth (1, 5). The sequence of events that leads to the development of disease are passage from normal mucosa to adenoma – malignantly transformed adenoma and invasive carcinoma is associated with a series of genetic events occurring over long periods (5-7 years), the knowledge of which keeps expanding for the past ten years (1). In other words, the malignant transformation of cells requires various types of genetic damage in the form of gene mutation, deletion, amplification or expression disorder (1). In the 1990-ies, Fearon and Vogelstein first developed an algorithm for genetic events in colorectal cancer. According to their model, sporadic colon cancer arises as a result of a series of genetic changes that affect the process progression from enhanced epithelial proliferation to metastatic disease. The ultimate outcome of the process depends more on the number of accumulated changes than on their chronology.
The syndromes of colorectal cancer are inherited in an autosomal dominant fashion and are categorized by phenotypic, histological and genetic findings. Familial adenomatous

polyposis, hereditary nonpolyposis colorectal cancer, Peutz-Jeghers disease, juvenile polyposis, Cowden disease are rare conditions (6, 7).

Ulcerative colitis, among other diseases in the medical history, is the top risk factor. The longer the disease and the segment of the colon affected, the higher the risk. The risk is also increased in individuals with Crohn's disease. The patient undergoing surgery for colorectal cancer has three times the risk of cancer recurrence (9).

The inheritance determines individual susceptibility to sporadic cancer but the lifestyle and environmental exposures are necessary for cancer expression. Colorectal cancer incidence varies between different geographic regions and incidence and mortality rates have been highest in developed western nations (10, 11). The basic argument that environment plays a huge role in colorectal cancer expression we get from observational studies in migrant populations. Migrants from low-incident regions to the high-incident regions of North America within one generation accept the incidence of the host country. Yet, studies with migrants also suggest that geographic variation in colorectal cancer incidences is due to environmental exposures and not due to the inherent predisposition (racial and ethnic group) (1, 10, 11).

Population based investigations have found many dietary and other environmental factors associated with colorectal cancer incidence (2). Most of these studies have methodologic limitations and therefore the interpretation of such studies has to be made with caution. Many studies have been conducted to investigate external factors that may increase or diminish the incidence of colorectal cancer. Many authors recognize four risk factor categories: epidemiological, intestinal, dietetic and mixed. The most frequently reported factors, among these shown to increase the risk of developing the disease, are diets rich in meat and animal fats (bile salts), physical inactivity, smoking and alcohol consumption (1, 2, 12). Between other diets ingested, consumption of red meat has the strongest correlation with colorectal cancer; over 30 case control studies report increased risk of colorectal cancer with higher red meat intake. Especially fried, barbecued and well-done meat is associated with colorectal cancer risk. Obesity and high caloric intake are the independent risk factors for colorectal cancer, excessive body mass gives a two fold increase in colorectal cancer, and this association in more expressed in men than women (12). Although studies carried out in humans and animal models have shown a positive correlation between the saturated fats/red meat consumption and the development of colorectal cancer, only a few of them are confirmed to be statistically significant. The total amount of fat in terms of daily caloric intake (>40%) and their form appears to have special significance. The conversion of dietary phospholipids to diacylglycerol by intestinal bacteria is assumed to be a potential mechanism of carcinogenesis. Diacylglycerol can enter the epithelial cell directly and by stimulating protein kinase C, it evokes intracellular signal transduction or mucosal proliferation. Another important mechanism is the formation of free radicals during fat metabolism and the mucosal damage induced by the secondary bile acids (lithocholic acid). Nitroso compounds, heat-generated heterocyclic amines and high protein intake (accelerated epithelial proliferation) have potential carcinogenic consequencies (12, 13).

Among the factors mentioned to reduce the risk are diet high in plant fibers and calcium, antioxidants (vitamin E, selenium etc.), menopausal hormone replacement therapy and administration of nonsteroidal anti-inflammatory drugs (12). In 1990 more than 13 studies showed significant reductions in colorectal cancer risk comparing the group with higher vs group with lower fibre intake. Some of the potential benefit mechanisms are: increased stool

weight, dilution of potential carcinogens and increased colon transit rate. But other studies did not confirm such results, and today in this field we have inconclusive and controversial results (12, 13).
A complex interaction between inherited predispositions and external factors is responsible for the development of colorectal cancer (Table 1).

RISK FACTORS FOR COLORECTAL CANCER	
Genetic factors	- inherited polyposis syndrome - syndromes: Gardner, Turcot - Peutz-Jeghers, juvenile polyposis
Family factors	- inherited colorectal cancer syndrome - hereditary adenocarcinomatosis syndrome - family history of colorectal cancer
Pre-existing diseases	- ulcerative colitis, Crohn's disease - colorectal cancer - radiation therapy to the small pelvis - colorectal polyps
External factors	- diet rich in meat and animal fat - physical activity - smoking
Other factors	- age over 40 years

Table 1. Risk factors for colorectal cancer development

2.2 Pathology

Molecular basis of disease are genetic mutations of somatic cells and the inner innervation of the colon is important in carcinoma pathogenesis and spread. According to their macroscopic appearance, colorectal cancers are divided into exophytic, ulcerative and stenosing tumors. Exophytic tumors are most often located in the right half of the colon, while stenosing tumors are mostly found in its left half. The majority (up to 75%) of colorectal cancer occur within the descending colon, sigmoid colon and rectum, 15% of cases are located in the cecum and ascending colon, and only 10% in the transverse colon (13, 14, 15). Adenocarcinoma accounts for more than 95% of colorectal cancer cases. The prognosis of the disease is associated with the depth of tumor invasion through the colonic wall, peripheral lymph node involvement and absence or presence of distant metastases. The Dukes staging system (Table 2) as used in clinical practice divides this cancer into three stages, depending on the depth of cancer invasion into the colorectal wall (16, 17).

DUKES A	- tumor confined within the colorectal wall
DUKES B	- tumor invaded through the colorectal wall B1-tumor limited to muscular mucosa B2-tumor protruding in/trough serosa
DUKES C	- metastases to lymph nodes

Table 2. The Dukes staging system for colorectal cancer

2.3 Clinical signs and diagnostic procedures

The symptoms of colorectal carcinoma depend on the anatomical location and size of the tumor. The tumor located in the cecoascendent portion will not necessarily produce obstruction since in this portion the stool has a liquid consistency, and the colonic lumen is wider than in the other parts. Patients complain of weakness, subfebrile temperature and blunt pain in the right lower hemiabdomen, and their laboratory tests show a high sedimentation rate and sideropenic anemia (1, 13).

Tumors of the transverse colon and on the left side of the half usually invade the colonic wall in a ring-shaped pattern mainly producing symptoms of the obstructive nature (cramping pain after meal, meteorism, change in stool form, occasional sudden ileus development and even bowel perforation). Symptoms of tumors confined to the rectosigmoid portion are most often false and/or painful urge to defecate (tenesmus), narrow stool and hematochezia (13).

The patient with suspicion of colorectal cancer should undergo a complete physical examination which must include digital rectal examination. In a large number of patients, the digital rectal examination already shows a hard lump inside the rectum, bleeding on touch. Colonoscopy is a procedure for visualizing colonic mucosa and obtaining samples for pathohistological analysis. Colonoscopy is the gold standard for detecting colorectal cancer. If for technical difficulties colonoscopy cannot be done, double-contrast irrigography may be considered although only 70-80% of lesions are detected by this method. Virtual colonoscopy and MR colonoscopy are also more and more often used. These radiology techniques use high-speed spiral CT and magnetic resonance imaging, and sophisticated software to process endoluminal images of the air-filled colon. Diagnostic techniques show a sensitivity of about 90% for tumors larger than 10 mm. Disadvantage is an inability to take biopsy samples and perform interventions available during colonoscopy. 'Colon capsule' for minutely detailed inspection of the colonic mucosa is also being gradually introduced, although this technique has the same disadvantage as the above mentioned techniques, and that is its inability to obtain biopsy samples (1, 13). Endoscopic ultrasound of the lower digestive tract is capable of providing assessment of tumor invasion into muscles and adjacent structures, as well as assessment of regional lymph node enlargement. The technique is employed to determine the extent of the spread of rectal tumors. Diagnosis of the spread of the disease involves imaging techniques (US, CT/MSCT/MRI of the abdomen and small pelvis, and CT/MSCT of the thorax). Serum CEA has limitations in sensitivity and specificity but was recommended for detection of recurrence. Molecular detection of tumor cells in circulation may prove to be more sensitive and specific than CEA (13, 18).

2.4 Treatment

Treatment for colorectal cancer depends on the extent of cancer spread. Surgery is the method of choice for treatment of localized tumors. Colon resection surgery for colorectal cancer must be as radical as possible. Chemotherapy, immunotherapy and radiotherapy used may be adjuvant, neo-adjuvant, curative or palliative in nature. Adjuvant chemotherapy aims to destroy micrometastases following surgery, and neo-adjuvant chemotherapy is aimed at reducing the tumor mass to allow surgery for either the primary tumor or distant metastases (usually to the liver or lungs) (1, 13). Systemic therapy for disseminated disease has been gaining popularity over the past few years. Treatment options for colorectal cancer include a variety of chemotherapy and immunotherapy

regimens, with 5-fluorouracil/leucovorin, which may be added irinotecan and/or oxaliplatin, and bevacizumab, cetuximab and panitumumab as biological therapy, still remaining the mainstay for the management of patients with disseminated disease (3). The addition of this molecularly targeted therapy to standard chemotherapy improves treatment response, prolongs both the time to disease progression and eventually, median survival for disseminated or metastatic colorectal cancer, which currently is over 30 months (3, 19). In the future, prognostic and predictive factors will allow individual identification of patients who may benefit most from adjuvant chemotherapy, and which therapy should be used for the treatment of disseminated disease (personalized medicine). Therapy of rectal cancer includes adjuvant chemotherapy combined with radiation therapy (19).

Despite huge advances in diagnostic and surgery and despite global and national programs of prevention, about 50% of colorectal carcinomas are diagnosed in advanced stage (11). Advanced disease is largely refractory to conventional therapy and 5 years survival is still poor. Patients with advanced disease suffer from many stress symptoms (pain, vomiting, diarrhea, anorexia-cachexia syndrome, and etc.) and the therapeutic goal for them is maintenance of quality of life (QoL) (13). Many of those symptoms have implications for diagnostic and therapeutic procedures and can heavily disturb the process of chemo-immunotherapy and radiotherapy (3).

3. Anorexia-cachexia syndrome

3.1 Pathophysiology of anorexia-cachexia syndrome

Anorexia is defined as an unintentional reduction in food intake and anticipated cachexia. Cachexia develops as a result of progressive wasting of skeletal muscle mass and to a lesser extent adipose tissue (20). In cachexia, progressive wasting of skeletal muscle mass is replaced with adipose tissue and this occurs even before weight loss. Anorexia-cachexia syndrome is highly prevalent among patients with malignant diseases. Depending on primary tumor site anorexia-cachexia syndrome is present in 8-88% of cancer patients. Tumors of head and neck, stomach and pancreas have highest percentage of cachexia (21). At the time of diagnosis weight loss is present in about 50% of patients. Weight loss is independent predictive factor of survival (21). Cachexia-anorexia syndrome includes clinical features which are associated with growth of cancer. In addition, it has a large impact on morbidity, mortality and on patients' quality of life. Cancer cachexia develops in a majority of patients with advanced disease (22) (70 %) and directly causes death in 20% of cancer patients. Clinical signs of cancer cachexia are anorexia and weight loss. Abnormalities in carbohydrates, fat, protein and energy metabolism are clinically manifested as weakness, fatigue, malaise, loss of skeletal muscle and adipose tissue. In serum chemistry and haematology tests we can find anaemia, hypertrigliceridaemia, hypoproteinaemia with low albumines, hyperlacticacidaemia and glucose intolerance (insulin resistence) (23). Metabolic aberration in cancer cells and cells and microenvironment (inadequate energy intake, increased energy expenditure, mucositis, nausea, vomiting, change in taste or psychological problems as reaction to cancer disease) cause primary cancer cachexia. There are several conditions that can contribute decreased food intake (gastrointestinal obstruction, post-chemotherapy nausea and vomiting, pain and etc) and cause secondary cancer cachexia (24, 25). Anorexia-cachexia syndrome often occurs or worsens after the administration of chemotherapy. Chemotherapeutic agents are toxic to malignant tissue, and also to the quickly proliferating cells. This group of cells also includes cells of the gastrointestinal

mucosa. Consequently, the absorption of nutrients is reduced. Some chemotherapeutics may affect the digestive system causing severe nausea, vomiting, abdominal pain, stomatitis and aversion to food. It should be noted that, in addition to the above mechanism for development of this syndrome, some chemotherapeutic agents also affect the taste buds of the tongue resulting in a changed and reduced sense of taste. It may also lead to reduced saliva production (26).

Sometimes we cannot find a reason for anorexia and weight loss may be unrelated to nutritional intake. In this cases weight loss is reflection of elevated resting energy expenditures and over expression of pro-inflammatory cytokines (27). The most common factors to stimulate the production of proinflammatory cytokines include: TNF-alpha, interleukins, interferon gamma and leukemia inhibitory factor. It should be noted that, due to such complex mechanism, energy supplementation in cachectic patients does not result in an increased body mass index (28).

The pathophysiologic mechanism is correlated with the production of catabolic factors either by the tumor or via factors produced by the host. Cancer cachexia differ from starvation. It is an unquestionable fact today that cancer cachexia is pro-inflammatory condition. The pathophysiology of cachexia involves very complex pathways; cachexia is caused with numerous metabolic changes mediated with pro-inflammatory cytokines. The most known mediators are tumor necrosis factor α, interleukin-1 (IL-1), interleukin 6 (IL-6), interferon-Y (from patients mononuclears) and molecules from tumor cells as lipid mobilisation factor (LMF) and proteolysis inducing factor (PIF). PIF is stimulating adenosine-triphosphate ubiquitin proteolitic pathway that is important in degradation of muscle mass and its stimulating synthesis of C-reactive protein (28, 29).

The result of these changes is impairment of immune functions, quality of life, and performance status. The worst consequence is inability of patient to endure chemo, immunotherapy and radiotherapy. Cachexia decreases response to therapy due to frequent toxicity and severe complications, what leads to shortened survival time (3, 20).

3.2 Nutritional support

Although increasing nutritional intake is insufficient to prevent the development of cachexia, nutritional support (taking into account the specific needs of the patient group), is required to reduce the consequences of nutritional decline and to improve quality of life and possibility to support the anticancer therapy (30). However, data from published studies are divided; some studies suggest that aggressive nutritional support can improve response to the antitumor treatment and decrease complications, but some deny any impact of nutritional support on tumor response, chemotherapy toxicity and survival (3, 30). Aggressive nutritional therapy does not significantly influence the outcome of patients with advanced cancer; „super"nutrition alone cannot reverse cachexia. But its use is still warranted because the patients QoL is significantly improved (3). The pharmacological treatments of cachexia antagonize the main symptoms (anorexia and chronic nausea) and improve the muscle metabolism. A significant number of studies (many uncontrolled) have suggested that anorexia and asthenia can be alleviated in cancer patient under corticosteroid treatment; also, the feeling of well-being is observed (31).

We must not underestimate advantage of nutritional treatment in improvement of asthenia and patients body image. Oral nutrition (after nutritional counseling) is ideal for cancer patients with a functional bowel. Enteral nutrition is useful in patients with advanced head and neck cancers or esophageal and gastric cancer and the use of parenteral nutrition (due

to high costs and morbidity of 15%) with exception of high selected cases has no major role in care of cancer patients, especially in terminal disease (30, 31). In patients with colorectal cancer, enteral nutrition is usually provided by administration of food and/or commercial nutrient solutions and formulas (32). They either supplement daily diet or provide basic nutritional needs to patients who are unable to ingest sufficient amounts of food. The baseline requirements to administer such feeding include preserved swallowing function and the ability of the esophagus and stomach. There is a wide range of enteral nutrition formulas available for everyday use (33). Enteral nutrition formulas are classified into the following categories: monomeric (elementary) formulas, oligomeric formulas, polymeric formulas (32, 34, 35). The essential difference between them is in their size and/or the amount and type of molecules present. Accordingly, formulas containing a larger number of molecules that are also shorter at the same time, have a higher osmolality and can therefore cause side effects, such as diarrhea. The osmolality of an enteral formula depends on the type and amount of carbohydrates. Polysaccharides account for the vast majority of carbohydrate types present in the enteral feeding formulas. According to their solubility, fibers in the digestive system are divided into two categories: soluble and insoluble. Soluble fiber absorbs water in the intestinal lumen and increase the volume of the stool. They thus help regulate bowel motility. Soluble fibers are fermented by bowel bacteria using the aerobic pathway. Pectin slows down the emptying of the stomach and prolongs the passage of contents through the colon resulting in formation of the stool of satisfactory consistency even in tube fed patients (37). Normal metabolism requires daily protein intake of 0.8-1.0 g/kg body weight, and in the hypercatabolic state daily protein needs range from 1.2 to 1.6 g/kg body weight. According to the presence of nitrogen-containing compounds the diet may be divided into three groups: polymeric diet (includes natural proteins), oligomeric diet (includes small peptides), elementary diet (containing amino acids). Patients with the preserved gastrointestinal function require a diet in which complete proteins prevail (38). In case of compromised digestion, peptides should be the most represented. Among the amino acids, glutamine should be singled out. Glutamine helps maintain normal intestinal integrity by stimulating RNA, DNA and protein synthesis, resulting in an increase in the number and size of intestinal villi. Glutamine also prevents damage to intestinal permeability, preserves mucosal structure and prevents translocation of bacteria and toxins in the intestine. Glutamine is an important nutritional substrate for the intestinal cell line. In catabolic conditions including colorectal cancer, the intestinal system's requirements for glutamine are increased. The deficiency can be compensated for by addition of glutamine to the enteral nutrition formula (39). Arginine is another amino acid that plays a significant role in the immune events. It stimulates nitrogen oxide (NO) synthesis and the CD4/CD8 ratio, as well as the release of insulin, glucagon, prolactin and somatostatin (40, 41). The use of arginine requires caution as increased NO synthesis may accelerate the synthesis of proinflammatory cytokines and thereby cause a number of side effects. The main role of lipids in enteral formulas is to ensure large amounts of energy stored in relatively small volumes and sufficient amount of essential fatty acids which are a vital component of cell membranes and organelles. Corn oil and soybean oil used in enteral formulas provide long-chain triglycerides (LCT), while coconut and palm oil provide medium-chain triglycerides (MCT). These products have a favorable effect on: 1) growth, differentiation and function of lymphocytes, macrophages and granulocytes; 2) release of trophic hormones or growth factors; 3) function of NK cells; 4) IL-2 synthesis; 5) improvement of mesenteric blood flow;

6) reduction of skeletal and visceral muscle proteolysis; 7) prevention of bacterial translocation; 8) reduction in the frequency and severity of infectious complications and 9) shortening hospital stay (42). Fatty acids are thus formed providing a basic substrate for the colonic mucosa. Formulas containing omega-3 fatty acids from fish oil have been recently introduced. Omega-3 fatty acids reduce the synthesis of immunosuppressive and proinflammatory mediators. Meta-analyses of several studies have shown that immunomodulatory formulas do not significantly reduce mortality compared with standard enteral formulas. Their administration, however, achieves a lower rate of infection and septic complications, reduces dependency on assisted ventilation and shortens length of hospital treatment (43).

Omega-3 polyunsaturated fatty acid, eicosapentaenoic acid (EPA) can down-regulate the production of pro-inflammatory cytokines such as IL-6, IL-1 and TNF in patients with cancer and in healthy individuals. EPA can also inhibit the effects of proteolysis inducing factor (PIF). EPA normalizes metabolic pathways changed due to malignant disease and stabilize weight gain through the competitive metabolism with arachidonic acid. EPA metabolites have lower inflammatory and immunosuppressive effect versus arachidonic acid metabolites. Especially interestingly is inhibitory effect of EPA on pancreatic and colorectal cancer cell line growth observed „in vitro" (44, 45). Wigmor and Bruera, like many other investigators, showed that EPA can stabilize body weight in cancer patients (46). We investigated if dietary counseling and oral nutrition supplement during chemotherapy affected nutritional status and symptom prevalence in our first study on 388 patients with colorectal cancer receiving chemotherapy for advanced disease (FOLFIRI/XELIRI/ FOLFOX) (47, 48).

Megestrol acetate is a type of medicine that comes in suspension form recommended in treatment guidelines for appetite and body weight loss in patients with malignant diseases. The drug belongs to a group of steroid hormones - progesterone. Its empirical formula is $C_{24}H_{32}O_4$. Progestational derivate megestrol acetate has been evaluated in many studies; conclusion is that megestrol acetate significantly increases appetite, caloric intake and nutritional status with mild side effects as edema and hypercalcaemia. It is not completely clear through which mechanisms megestrol is acting. It is assumed that megestrol acetate changes the cytokines which are inhibiting TNF effects. Stimulation of appetite is due to stimulation neuropeptide Y in lateral hypothalamus. Megestrol acetate enhances appetite and increases food intake, enables the administration of specific treatments, and improves both patient treatment tolerance and their quality of life. Implementation of megestrol acetate in nutritional support plan is necessary; according to the highlights of the 2004 Cachexia Cancer Conference anorexia preceding to weight loss and orexigenics are necessary even when weight loss is absent. Furthermore, patients with cancer cachexia do not react on isolated over caloric food intake. Mild side effects (edema) are not enough pronounced over the social benefits caused by appetite stimulation; patients do not withdraw megestrol acetate therapy (49). Therefore, the International Association for Hospice and Palliative Care, NCCN Guidellines and European Palliative Care Research Collaborative Group recommend megestrol acetate as a mandatory drug for treatment cancer cachexia (50). The recommended starting dose is 400 mg (10 ml) once a day. The dose may be increased up to 800 mg (20 ml) / day. The most common side effects of megestrol acetate include edemas, insomnia, impaired libido, and very rarely thromboembolic complications (48, 50).

The choice of enteral route depends on the underlying pathology, anticipated duration of enteral feeding and patient's preferences (30). In addition to the oral route of nutrition administration the transnasal route can also be considered. Indications for transnasal tube feeding include conditions or illnesses where normal feeding cannot be provided, and where the gastrointestinal tract maintains its function. For this purpose, nasogastric, nasoduodenal and nasojejunal types of tubes may be used. The tubes are usually placed 'blindly', however they may be placed by radiological and endoscopic means. The tubes are used when it is anticipated that tube feeding will be needed for up to 4 weeks. If enteral feeding is likely to be needed for more than 4 weeks, percutaneous endoscopic gastrostomy tubes or percutaneous endoscopic jejunostomies may be placed via an endoscopic access. The surgical placement of the gastrostomy or jejunostomy tube may also be taken into consideration. Two types of feeding can be used for patients requiring tube feeding: bolus (6 to 10 doses a day, each ranging from 50 to 200 ml, given over 5 to 30 minutes) or continuous feeding(20 to 150 ml per hour during 16-18 hours). The method of 'bolus feeding' is more frequently reported to cause side effects than continuous feedings (30, 35).
In some clinical situations, enteral feeding may be unsafe or contraindicated. Reasons for postponing enteral nutrition administration are as follows: persistent nausea/vomiting, intensive postprandial pain, diarrhea, mechanical obstruction, diminished bowel motility, malabsorption, gastrointestinal bleeding. In mentioned situations, parenteral feeding is used. Parenteral feeding may be administered by peripheral or central vein access. Risk-benefit assessment of parenteral nutrition is necessary for each patient (30, 31, 35).
We can evaluate nutritional status of the cancer patient with quick screening methods (NRS-2002, NSTQ, ect) or more detailed examination (laboratory findings, anthropometric measurement, body composition measurement, BMI). Nottingham Screening Tool Questionnaire is simple, quick, and proper for re-evaluating. Another simple model (Fearon) is suggested for quick evaluation: if patient unintentionally decrease in weight gain more than 5% in 3 to 6 months, if caloric intake is less than 1500 kcal/day and C-reactive protein value is 10 and higher. Based on these data we can assume that cancer cachexia is developing (34, 35, 36, 43, 46, 50)

3.3 Study results

Our study was conducted at the Gastrointestinal Oncology Department, Clinic for Internal Medicine, University Hospital Center Rijeka, from January 2001 to December 2007. The aim of the study was to evaluate the effect of nutritional support in patients with colorectal cancer. The follow-up included 338 patients divided into two groups. Group I: patients receiving nutritional support (215 patients), and group II patients who did not receive nutritional support (173 patients); retrospectively collected data. Visit 0 took place one week before initiation of chemotherapy. The nutritional status was evaluated according to body weight change. The body mass index (BMI) was calculated for all patients and all patients were also evaluated through three questionnaires: Nottingham Screening Tool (Table 3), Appetite Loss Scale and Karnofsky Performance Status. The reassessments were done at control visits, each visit taking place before the next chemotherapy course. There were, in total, 12 visits performed. The aim of the study was to assess the effects of nutritional support in colorectal cancer patients on chemotherapy. For all patients, the following parameters were monitored and statistically evaluated: selection of nutritional support regimen in group I, BMI in groups I and II, at

visit 0 and visit 12, Nottingham Screening Tool Questionnaire in groups I and II, at visit 0 and visit 12, Appetite Loss Scale in groups I and II, at visit 0 and visit 1, Weight loss in groups I and II , at visit 0 and visit 12, Karnofsky Performance Status in groups I and II, at visit 0 and visit 12, side effects of megestrol acetate. Evaluating the initial risk measurement according to BMI, decrease in weight gain and NST, we did not find any significant difference between the two groups. We performed 12 visits in follow-up according to chemotherapy schedule. Before initiation of chemotherapy, we re-evaluated nutritional status of our patients using evaluation tools. After chemotherapy was completed, in group I (consisted of 215 patients who were monitored prospectively and were given nutritional support) we observed weight gain of 1.5 kg (0.6-2.8 kg) and appetite improvement, the most commonly seen result after 4 weeks of therapy with megestrol acetate. The appetite also improved on Appetite Loss Scale from 3.1 (pre-chemotherapy) to 4.7 (post-chemotherapy). But KPS did not change significantly (74.2% before chemo versus 80.4% after chemo respectively) reflecting the impact of the disease itself. The most common side effects in patients receiving enteral nutrition were diarrhea (12% of patients) , abdominal pain (9%) and altered taste sensation(5%). The most frequently reported side effect in patients receiving megestrol acetate was the occurrence of edema (20% of patients).
This clinical study is ongoing and preliminary results from more than 600 patients are similar to this one.

BMI	Score
>20	0
18-20	1
<18	2

Has the patient unintentionally lost weight during last 3 months?	Score
No	0
A little, up to 3 kg	1
A lot, more than 3 kg	2

Table 3. Nottingham Screening Tool Questionnaire

Score: 0-2 Patient is not in nutritive risk and does not need nutritional support
3-4 Patient need re-evaluation weekly
≥ 5 Patient is in nutritive risk and needs nutritive support

4. Discussion

Anorexia-cachexia syndrome often occurs in patients with gastrointestinal cancers. Malnutrition has huge impact on outcome in patients who underwent major surgical resections, and also in patients who have chemo/radiotherapy treatment (3, 22).
Although manifestations of chemotherapy injury on nutritional status is well-known, the potential role of nutritional supplementing is still not explored in detail. When treating cancer patients with chemotherapy we observed two problems and one of them is general failure in recognition of the weight loss early enough to perform nutritional support (30).

But if we know that patients will undergo to stress-full procedure which can have impact on his nutritional status (diagnostic procedures, colonoscopy for example, major gastrointestinal surgery) we have to give adequate nutritional support according to different clinical algorithms (3).
Adequate substitution with metabolites, increased caloric intake, inhibition of catabolic and inflammatory mediators leads to decrease of surgery, chemo and radiotherapy complications, but still has no significant impact on survival. Nutritional counseling, supplemental feeding and pharmacological support do temporarily stop weight loss and improve appetite, QoL and social life but this improvement has no implications on patient´s KPS and course of their disease. An improved knowledge of the pathophysiology of cancer induced cachexia will lead to development of more effective treatments (26).
In clinical practice, the role of nutrition therapy is often assumed to be less important than role of chemo, immunotherapy and radiotherapy as outcomes are less clear in literature (22, 26, 30). Our study showed that early nutritional intervention can decrease course of weight deterioration in the early course or locally advanced or metastatic colorectal cancer. Karnofsky Performance Status did not change significantly, what we expected.
Taking food is not only a physiologic necessity, but also cultural and a social event reflecting life and religious philosophy. Nutritive support can facilitate life of oncology patienta, their family support and caregivers understand (1). Therefore we have to recognize nutrition-related issues and to implement strategies that will lead to a better outcome for patient and his caregivers. In the end of the life the wish of dying patient is most important factor regarding enteral/parenteral nutrition. The interaction between major syndromes in terminal disease (pain, cachexia, cognitive failure) should be better established because it seems that severity of them has impact on the others. If we improve pain and depression we can expect impact on cachexia syndrome (3, 21).

5. Conclusion

The role of nutritional therapy in oncology patients has been neglected. This mainly results from failure to recognize malnutrition and untimely introduction of nutritional support.
Our study shows that early introduction of nutritional support can decrease weight loss and in some cases even enable weight gain in patients with locally advanced and metastatic colorectal cancer.
To achieve better treatment results for patients with colorectal cancer, nutritional therapy should be considered as a highly important part of their treatment and more attention should be paid to timely recognition of malnutrition and introduction of nutritional support. Patients with anorexia-cachexia syndrome should undergo to individualized nutritional intervention where nutrition counseling is base for improvement of nutritional status, quality of life and social life. Anorectic patients have changes in taste and smell and do not support high-fat food and therefore frequent but small meals are highly recommended (20).

Future perspectives:

Cancer patients have increased level of growth hormone (GH), low serum concentrations of insulin growth factor-1 (IGF-1) and insulin resistence. Loss of lean mass and inflammatory processes are closely connected to the action of three signaling molecules: insulin, growth hormone and insulin growth factor-1 is essential (51). Basic stimuli of insulin, IGF-1 and GH does not provide response in muscle cells in cachexia, its reasonable to target post-receptor

pathways or using alternative pathways in muscle cells. A number of molecules exhibiting anti cytokines activity have been tested without significant clinical data (20). Ghrelin is a hormone that stimulates the release of GH and increases appetite. In a phase II clinical study, ghrelin agonist anamorelin produced an improvement in total body mass (52).
Despite cachexia is very common condition in cancers, there are still very few trials of drug therapies to reduce weight loss in cancer cachexia. Cachexia remains poorly studied and often undertreated condition that causes severe impairment of quality of life and increases mortality.

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Part 4

Management and Treatment

12

Therapeutic Targets in Colorectal Cancer

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1. Introduction

Colon cancer is common worldwide: nearly a million people develop the disease every year and in the United States, colorectal cancer ranks third for frequency of occurrence and mortality in both men and women, with projected estimates for 2011 for occurrence and mortality put respectively at approximately 140,000 and 49,000 (American Cancer Society, 2011; Jemal et al, 2005). The projection for total deaths from all cancers in 2010 was 569,490 (Aliperti et al, 2011).

Significant progress in understanding colon cancer has produced a wealth of information that has aided improvements in aspects of diagnosis and disease management, contributing in the process to reduced mortality rates. The mechanisms that facilitate colorectal carcinogenesis and sustain progression and metastatic spread have been extensively investigated. The cause of colorectal cancer is multi-factorial. Notwithstanding the various contributing elements to the disease, the primary manifestation of colorectal carcinoma is the relentless and uncontrolled proliferation of cells and tissues in the intestinal mucosal epithelium. This pattern of abnormal proliferation is a disruption of the normal balance between new cell production by the epithelial cells in the mucosal crypts, and the release and loss of epithelial cells into the intestinal lumen i.e. cell-producing proliferation is normally finely and properly counter-balanced by regulated apoptotic and physical cell loss (Raz, 2002).

Given the multistep, multifactor origins of colorectal cancer, the rationale for targeted therapies and the identification of therapeutic targets is that the disease can be (a) prevented prior to initiation (b) obstructed in its progression by blocking or inhibiting mechanisms that sustain progression and facilitate metastasis (c) reversed. The list of potential targets include microbes and bacteria that facilitate tumor initiation, molecular targets such as adenomatous polyposis coli (APC), and cancer stem cells (CSCs) where targeted destruction is thought to be central to preventing metastatic tumor spread.

As with all cancers, finding and delivering therapeutic targets in colorectal cancer is based on the premise that there is one originating cell type (van der Flier & Clevers, 2009). If this population of mutant originating cells is eliminated, the ability for new initiation, progression and distant seeding of tumor cells should be impaired and eventually abolished. Several therapeutic approaches have shown promising results in experimental

studies. However, this chapter will focus largely on molecular targets in Wnt signaling, the nuclear receptor peroxisome proliferator-activated receptor (PPAR), and cancer stem cells (also known as cancer initiating cells).

2. Colonic epithelial cell renewal

The colon is the distal part of the intestinal tract and is lined internally by a simple layer of columnar epithelial cells (colonocytes) that send tube-like extensions called crypts into the mucosal layer of the intestinal wall. The crypts provide a conducive environment for the regulation and renewal of the epithelial covering of the colonic mucosa.

The epithelial cells in the crypt divide continuously and rapidly, achieving a turnover rate of epithelial renewal of between 5-6 days in mammals, with much shorter cell kinetic data reported for rodents (Di Garbo et al, 2010; Hall et al, 1994; Heath, 1996; Giles et al, 2003; Li et al, 1994; Loeffler et al, 1986; Potten & Loeffler, 1990; Okamoto & Watanabe, 2004; Wright & Alison, 1984). In the small intestine, between 8-9 cells are produced by each crypt epithelium every hour in mice; 2-3 dividing cells per crypt support cell production in the proximal intestine while up to 5 dividing cells are required to maintain cell production in the distal intestine (McGarvey et al, 2007a, 2007b). The renewal mechanism is sustained by a hierarchical arrangement of epithelial cells within the crypts, exemplified by the model described by Tomlinson and Bodmer (1995), with stem cells thought to reside in the lower part of the crypts, while differentiated cells populate the upper part of the crypt. By dividing and supplying transit (semi-differentiated) cells that migrate up the crypts, the stem cells are capable of and responsible for producing the various cell types that are found in the colonic epithelium. Differentiated cells at the top of the crypt and colonic mucosal surface eventually undergo spontaneous apoptosis and are released into the intestinal lumen (Fig 1).

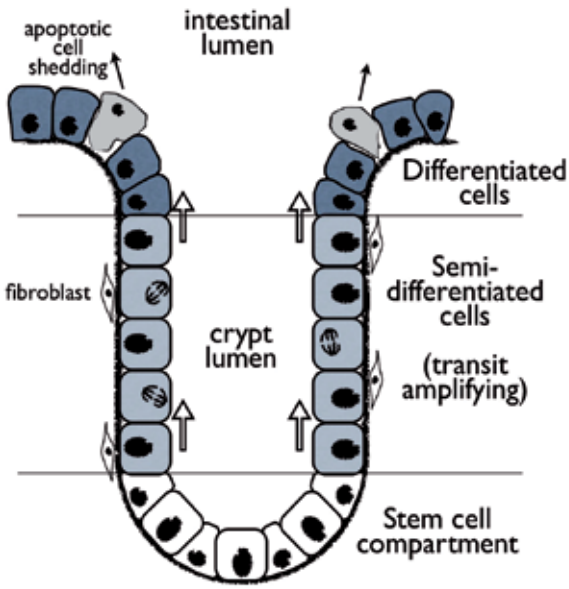

Fig. 1. Schematic diagram of colonic crypt, illustrating the three zones and cell categories that constitute the kinetic framework for cell production and regeneration.

The maintenance of functional and structural integrity and viability of the enteric mucosal epithelium depends on the preservation of the crypt cell renewing and emigration mechanisms for repopulating the continuously shedding epithelial cell cover (McGarvey et al, 2007a, 2007b). Several models for investigating the dynamics of colon cell regulation have been described (Boman et al, 2001; Hardy & Stark, 2002; Lander, 2009; Michor et al, 2004; Paulus et al, 1992; van Leeuwen et al, 2006; Wodarz, 2007). Many of these models have been employed in studies of the mechanisms that underlie normal colonic epithelial cell regulation and regeneration, as well as the dysregulated proliferation in colorectal cancer.

3. Apoptosis

All of the new cells that are produced by proliferation of the cells in the stem cell compartment of the crypt, and numerically amplified in the semi-differentiated compartment, are distributed to the colonic mucosal epithelium to provide functionally important roles in absorption and secretion as well as providing a selectively permeable surface cover (Hall et al, 1994). The supply of new cells towards the upper crypt and surface epithelium is designed to satisfy the losses caused by cell injury, loss and programmed death (apoptosis). Surface cover cells are therefore removed or shed by processes that are as controlled and as balanced as the crypt-mediated cell renewal mechanism, and involves a cessation of proliferative processes in conjunction with the initiation of disposal and cell loss pathways (Leblond, 1964; Wright & Alison, 1984; Hall et al, 1994). Because the enteric epithelium is associated with underlying connective tissue fibroblasts, the accompanying fluxes in these cells are also correspondingly regulated in a controlled manner for proliferation and for cell loss (Marsh & Trier, 1974a, 1974b; Parker et al, 1974; Pascal et al, 1968a, 1968b). Together, the careful balance of cell production and cell loss maintains homeostasis in the colonic epithelium. Apoptosis does not occur randomly, rather it is seen towards the distal end of the cell migration route up the crypt (Hall et al, 1994). In colon cancer, proliferation is elevated and apoptosis is dysregulated, making the restoration of apoptosis an attractive proposition for therapeutic control of colon cancer growth (Evan & Vousden, 2001, Johnstone et al, 2002).

A number of cyclooxygenase (COX) inhibitors induce apoptosis by activating mechanisms that are either upstream (via the lipid metabolite 13-S-hydroxyoctadecadienoic acid) or downstream (via 14-3-3ε proteins) of the nuclear hormone receptor PPAR∂ (Liou et al, 2007; Shureiqi et al, 2003), indicating that the pro-apoptotic effect of COX inhibitors on cancer cells is dependent on down-regulation of PPAR∂. In APC min mice, short-term treatment with nitric-oxide-donating aspirin (NO-ASA) induces apoptosis in differentiated intestinal epithelial cells while prolonged treatment with sulindac reverses the anti-apoptotic effect of APC (Mahmoud et al, 1998; Ouyang et al, 2006). In contrast, celecoxib administration produces no effect on apoptosis (Williams et al, 2000).

Other agents that have been shown to reduce colorectal cancer growth in vitro include CDDO-Me, an oleanane synthetic triterpenoid that achieves its apoptotic effect partly through the generation of reactive oxygen (ROS) and the activation of procaspases (Gao et al, 2011), green tea polyphenols that achieve their apoptotic effect through the induction of caspases (Oz & Ebersole, 2010), and tocotrienol, a member of the vitamin E family of compounds that induces morphological changes similar to apoptosis (paraptosis) and an accompanying reduction in Wnt signaling and its down-stream genes (Zhang et al, 2011).

4. Wnt and colorectal cancer

One of the primary regulators of epithelial cell proliferation is Wnt signaling (Di Garbo et al, 2010). This signaling pathway involves the intermediate elements beta catenin, glycogen synthase kinase 3 beta (GSK3β), casein kinase I (CKI), axin, adenomatous polyposis coli (APC) and T-cell factor/lymphoid enhancer factor (TCF/LEF). Inappropriate activation or disruption of Wnt signaling upsets the careful regulatory balance in epithelial kinetics, leads to disorderly proliferation, and is an important contributor to the process of colorectal carcinogenesis. Wnt signaling helps to control the levels of cytoplasmic beta catenin, between pools bound to APC and to the cell adhesion molecule E-cadherin. The APC-bound pool of beta catenin is held in a stable complex of axin, GSK3β, CKI and APC that serves to regulate its cytoplasmic levels via targeted ubiquitin-mediated proteasomal degradation (Kikuchi et al, 2003; Pinto & Clevers, 2005). Wnt ligand signaling via membrane receptor proteins triggers a cascade that alters the relationship between the scaffold protein axin and GSK3β, interrupts regulated destruction of beta catenin, and leads to accumulation of non-phosphorylated beta catenin in the cytoplasm that then reaches the nucleus. Translocation of beta catenin into the nucleus after binding with TCF/LEF leads to the activation of target genes that regulate proliferation, differentiation and apoptosis (Araki et al, 2003; Coghlan et al, 2000; DiGarbo et al, 2010; Fagotto et al, 1998; He et al, 1998; Kishida et al, 1999; Shtutman et al, 1999; Tetsu & McCormick, 1999; van der Flier & Clevers, 2009; Yamamoto et al, 1999; Yanagawa et al, 1995; Yost et al, 1996). Direct binding of TCF to regulatory elements in downstream genes have aided identification of target genes and suggest that Wnt-activated gene expression shows a gradient-wise concentration of activity in intestinal crypts with the highest expression in the bottom of the crypt (Gregorieff et al, 2005). Most of these target genes are expressed in normal crypts and in adenomas (van der Flier et al, 2007; van der Wetering et al, 2002).

5. Wnt and COX inhibition

Colon cancer is associated with dysregulation and overexpression of COX, a key enzyme in the biosynthetic conversion of arachidonic acid to eicosanoids (Botting, 2006). Increased levels of expression of COX-2 are seen in up to 85% of colorectal adenomas and carcinomas (Eberhart et al, 1994; Fujita et al, 1998; Rigas et al, 1993; Sheng et al, 1997).

COX inhibitors demonstrate an ability to disrupt proliferation in several CRC cell lines. In HT29 colorectal adenoma cell lines, suppression of proliferation is evident as early as 48 hours after treatment with naproxen and piroxicam and at later timepoints with aspirin, indomethacin, aspirin and NS398 (Shiff et al, 1996; Shureiqi et al, 2000). But in some studies, naproxen and salicylic acid showed no effect on proliferation in the same cell lines pointing to differing potencies for inhibition of COX as well as effects on growth and apoptosis (Piazza et al, 1997). Although anti-proliferative effects have been reported in studies using HCA7, HT115 and SW620 cell lines which all express COX, the non-COX expressing cell line HT116 also shows reduced growth when treated with celecoxib for 72 hours (Shureiqi et al, 2003). Most of the evidence allows the conclusion that the anti-proliferative effects of COX inhibitors on colon cancer cell lines are not related to COX expression or activity.

When COX inhibitors are administered to APC min mice, initiation and progression of intestinal and colonic polyps is inhibited and polyp load is reduced (Jacoby et al, 1996, 2000; Kohno et al, 2005; Mahmoud et al, 1998, Moorghen et al, 1988, 1998; Narisawa et al, 1983;

Rao et al, 1995, 2009; Reddy et al, 1993). Prevention of tumorigenesis or tumor load reduction reflects either decreased cell proliferation or increased cell death but findings from animal studies are inconsistent (Table 1). For example, celecoxib treatment reduces tumor numbers and inhibits cell proliferation but data from studies using various sulindac preparations point to a variability that may be rodent species dependent (Jacoby et al, 2000; Mahmoud et al, 1998; Moorghen et al, 1988, 1998; Rao et al 1995, 2009).

Model	Inhibitor	Dose & duration (wks)		Inhibition effect	Reference
APC					
mouse	sulindac	160ppm	10	none	*Shiff et al 1996*
mouse	sulindac S_2	20mg/kg	11	none	*Swamy et al 2006*
mouse	celecoxib	1500ppm	6	tumor number	*Han et al 2008*
DMH					
mouse	sulindac	5mg/kg	24	tumorigenesis	*Shureiqi et al 2000*
mouse	sulindac	5mg/kg	18	n/a	*Kim et al 2009*
AOM					
mouse	nimesulide	0.04%w/w	14	n/a	*Shureiqi et al 2003*
rat	celecoxib	300ppm	46	n/a	*Guo et al 2009*
rat	aspirin	200-400ppm	52	tumorigenesis	*Piazza et al 1997*
NMNU					
rat	indomethacin	10ppm	1-30	tumorigenesis	*Hanif et al 1996*

Table 1. Effect of COX inhibitors on initiation and progression of experimental colon cancer in vivo. S_2 = sulfide, NMNU = n-methyl-N-nitrosourea, AOM = azoxymethane, DMH = 1,2-dimethylhydrazine, APC = adenomatous polyposis coli, n/a = not measured

Some of the inconsistency in findings from animal studies is reflected in the results from clinical investigations in patients. Treatment with aspirin and celecoxib shows beneficial prevention of colorectal cancer in patients, and treatment with 150mg sulindac twice daily for nine months reduces number and size of colorectal adenomas. However, treatment with standard sulindac doses (25-150 mg twice daily) for 48 months did not prevent adenomas in patients (Giardiello et al, 1993, 2002; Giovannucci et al, 1994; Lanas & Fernandez, 2009; Thun et al, 1991).

6. PPAR and COX inhibition

Peroxisome proliferator-activated receptors (PPAR) are part of the nuclear hormone receptor superfamily. While PPARα and PPARγ have been shown to be involved in various aspects of dietary lipid and glucose metabolism, PPAR∂ is implicated in the control of cell proliferation, differentiation and colorectal carcinogenesis (Desvergne & Wahil, 1999; Michalik et al, 2003; Wang & Dubois, 2010). Ligand activation of PPAR∂ is associated with suppressed induction of colon cancer (genetic and chemical treatment models) in mice via mechanisms that are linked to colonocyte differentiation and apoptosis (Harman et al, 2004;

Marin et al, 2006). Conversely, inactivation of PPAR∂ in APC-min mice enhances predisposition to multiple intestinal and colorectal polyps (Harman et al, 2004; Reed et al, 2004). Such evidence suggests that PPAR∂ attenuates colon cancer. However, Park and colleagues found a reduction in the ability of PPAR∂-/-(null) cells to form tumors in nude mice and they concluded that PPAR∂ might function to assist the tumor-suppressing function of adenomatous polyposis coli (APC) protein (Park et al, 2001).

Despite significant insights into the role of PPAR∂ in colorectal cancer, the physiological role of PPAR∂ in epithelia is still not completely understood. The unresolved nature of the available data has not prevented studies that have explored the possibility of targeting PPAR∂ therapeutically in colorectal cancer. Prostacyclin I_2 can act as a natural ligand for PPAR∂ (Gupta et al, 2000), and because COX-2 inhibitors can suppress carcinogenesis and reduce intestinal polyposis (Hollingshead et al, 2008; Jacoby et al, 1996; Mahmoud et al, 1998), a number of studies examined the use of COX inhibtion to influence PPAR∂ activity. Sulindac and indomethacin inhibit colorectal carcinogenesis in vitro by rapidly downregulating transcriptional activity of PPAR∂ via disruption of DNA binding to PPAR∂-response elements (He et al, 1999). A similar effect on PPAR∂ is also observed following administration of sulindac and celecoxib but this is preceded by induction of the enzyme 15-lipoxygenase-1 (Shureiqi et al, 2003). Administration of nitric-oxide-donating aspirin reduces PPAR∂ expression and intestinal polyp numbers in mice but neither nimesulide nor GW0742 (a PPAR∂ ligand) has an effect on PPAR∂ mRNA levels, despite the fact that both agents reduce intestinal polyp numbers (Gupta et al, 2004; Hollingshead et al, 2008; Kohno et al, 2005).

COX-2 inhibitors and PPAR∂ ligands can separately attenuate cancer growth, however combinatorial protocols have so far failed to produce potentiated inhibition of colon cancer indicating that COX-inhibitory and PPAR∂ pathways are mechanistically separate (Hollingshead et al, 2008). In addition, concurrent expression of PPAR∂ and COX-2 in colorectal tumors has poor prognostic implications for patients (Yoshinaga et al, 2011).

Ligand activation of PPARγ is also anti-neoplastic in several tissues, but the data regarding its role in colorectal cancer is just as conflicting as the data for PPAR∂. PPARγ activation inhibits colon cancer cell growth in vitro whereas a mutation-dependent pro-tumorigenic effect has been reported in vivo (Girnun et al, 2002; Yoshizumi et al, 2004). The mechanistically interrelated and inter-dependent nature of colorectal cancer is illustrated by the finding that PPARγ agonists induce apoptosis by suppressing activation of NFκB and GSK3β (Ban et al, 2010). Other investigators have shown that PPARγ induces apoptosis via inactivation of survivin and activation of caspase-3 in colorectal cancer cell lines and were able to inhibit PPARγ-ligand induced apoptosis by activating PPAR∂ (Wang et al, 2011).

7. Clones and stem cells

The crypt structure of the colonic epithelium is maintained by the putative presence of pluripotent intestinal crypt stem cells (Schmidt et al, 1988). Initially crypts are polyclonal and subsequently become monoclonal. Two kinetic models of the stem-cell-sustained intestinal crypt have been described. In the classic model, intestinal stem cells are thought to reside in the 4th cell position from the bottom of the crypt (the +4 cell). These stem cells supply daughter cells to the proliferative, transit-amplifying zone of the crypt; stem cells can be replaced by these daughter cells if necessary (Marshman et al, 2002; Pottten, 1977; Potten et al, 1974, 2002). The zone model localizes stem cells to the bottom of the crypt; these cells are proposed to be the undifferentiated crypt base columnar (CBC) cells (Bjerknes & Cheng

1981a, 1981b, 1999, 2006). On the basis of modelling studies, it is proposed that stem cells and crypts can suffer losses and be replaced (Cairnie & Millen, 1975; Nicolas et al, 2007; Yatabe et al, 2001).

Unequivocal stem cell identification has long remained elusive but, using genetic lineage tracing experiments, Barker et al (2007) showed that Lgr5, a G-protein-coupled receptor, is expressed in CBC cells. The study followed Lgr5-positive daughter cells up intestinal crypts and on to the intestinal villous epithelium, where all differentiated epithelial cell types could be demonstrated. The ability of Lgr5-positive stem cells in the crypt to give rise to crypt-villus units appear to be dependent on proximity to CD24+ cells at the bottom of the crypt (Sato et al, 2011). Stem cells have also been identified in mammalian epidermal hair follicles where they express Lgr6 (Snippert et al, 2010). Deletion of the APC gene in crypt stem cells in Lgr5 knock-in mice facilitates intestinal microadenoma growth; deletion of APC in transit-amplifying, semi-differentiated crypt cells in Lgr5 knock-in mice significantly reduces the growth of intestinal adenomas. Together this suggests that APC loss needs to be stem cell specific to propagate unrestrained tumor growth (Barker et al, 2009). The finding that single isolated Lgr5-positive stem cells can give rise to self-organizing crypt-villus units (Sato et al, 2009) raises the possibility that these cells may be useful in treatment strategies that aim to repopulate enteric epithelia.

There is experimental evidence for several proposed colon cancer stem cell markers including CD133, CD44, CD166, the extracellular matrix protein olfactomedin-4 (OLFM4), aldehyde dehydrogenase (ALDH1A1), Lgr5, and pleckstrin homology-like domain family A member 1 (PHLDA1). Some of these markers are associated with IL6-STAT3-JAK2 signaling (Becker et al, 2008; Dalerba et al, 2007; O'Brien et al, 2007; Ricci-Vitani et al, 2007; Sakthianandeswaren et al, 2011; Sanders & Majumdar, 2011; Shmelkov et al, 2008; Tsai et al, 2011; Uchida et al, 2010; van der Flier et al, 2009).

In contrast to the idea that carcinogenic mutations can occur in any cell, the cancer stem cell model (first described in 1997 for hematologic malignancies) proposes that tumor transformation, progression and metastatic initiation is driven by the acquisition of oncogenic self-renewal properties by tissue stem cells, contributing to differentiation and the cellular heterogeneity of tumors (Chen et al, 2011; Sanders & Majumdar, 2011). This has led to the idea that conventional cancer therapies that target only proliferating cells in tumors may not necessarily be effective against cancer stem cells that mediate metastasis (Abdul Khalek et al, 2010, Sanders & Majumdar, 2011; Soltanian & Matin, 2011), and that these therapies may therefore be ineffective in producing long-term remissions. CSCs have greater DNA repair capacity and expression of ABC transporter genes, both of which contribute to relatively higher resistance to chemotherapy and radiation (Bao et al, 2006; Cho & Clarke, 2008; Hirschmann-Jax et al, 2004; Zhou et al, 2009). GO-Y030, a curcumin analogue has been shown to inhibit STAT3 phosphorylation signaling in colon cancer stem cells, offering the possibility of targeting STAT3 signaling in colon CSCs (Lin et al, 2011). The clonogenic and proliferative properties of CSCs are significantly interrupted by histone deacetylase (HDAC) inhibitors and this effect is associated with apoptotic cell death and modified Wnt signalling (Sikandar et al 2010).

8. Conclusion

1. When applied to colorectal cancer, the concept of hierarchical compartmentalization (as described in crypt kinetic models) offers target environments for stemness, proliferation

and differentiation. Potential targets in each compartment include dividing cells, apoptotic mechanisms and cancer stem cells.

2. Wnt signalling has been targeted for inhibition because of its relationship with proliferation. Activity in this pathway is highest in the stem zone which provides the source of new cells.
3. COX inhibitors have variable effects on proliferation that may be related to differing potencies, and the evidence suggests that these effects may not be due to any inhibitory action by the compounds on COX. Inconsistencies remain in trying to reproduce in patients the experimental outcomes on tumor loads seen following treatment with COX inhibitors.
4. A range of compounds, including nutritional and synthetic substances, induce apoptosis in colorectal cancer cell lines. Not all COX inhibitors induce apoptosis.
5. Some COX inhibitors down-regulate PPAR∂, other inhibitors do not. However, combination treatments do not produce the expected potentiation effect. The conflicting evidence of the roles of PPAR∂ and PPARγ in colorectal cancer remains unresolved.
6. Stem cells markers are increasingly being identified and involvement in signalling pathways such as IL6-STAT3 point to new targets that may be modulated using therapeutic agents or genetic manipulations.

9. References

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13

Anti-EGFR Treatment in Patients with Colorectal Cancer

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1. Introduction

The survival of patients with colorectal cancer (CRC) has increased constantly for many years due to superior surgical techniques, improved postoperative care, regular follow-up and an increased use of effective systemic therapy in the adjuvant and the palliative setting [1,2]. All of these advancements are important, but the establishment of multidisciplinary teams which facilitate optimal selection of therapy for individual patients may have been the most important concept on its own.

In recent years a number of biologically active substances attacking specific signalling pathways in cancer cells (targeted therapy) have been developed and included in the treatment of patients with CRC. Three monoclonal antibodies (cetuximab, panitumumab, bevacizumab) have by now been approved for therapy in metastatic CRC (mCRC) [2,3].

Angiogenesis is necessary in tumour development and controlled in part by the vascular endothelial growth system which is inhibited by bevacizumab (Avastin ®) and many other anti-angiogenic drugs.

Cetuximab (Erbitux®) and panitumumab (Vectibix®) block the extracellular portion of the epidermal growth factor receptor (EGFR) and these two drugs will be discussed in detail in this chapter.

2. Targeted therapy - Inhibition of EGFR

EGFR is a trans-membrane glycoprotein that is involved in signaling pathways affecting cellular growth, differentiation, and proliferation and EGFR is expressed in many types of normal tissues. The EGFR is up regulated in a large number of cancers, in CRC in 60-80% of cases, and might be associated to a poor prognosis. Once a ligand binds to the extracellular domain of EGFR, receptor-dimerization occurs and down-stream signaling cascades are activated. Amongst the downstream effectors are the RAF/MEK/MAPK pathway and the PI3K/PTEN/AKT pathway.

Two anti-EGFR monoclonal antibodies are approved by US Food and Drug Administration and European Medicines Agency for the treatment of CRC – cetuximab and panitumumab. Both are directed against the ligand-binding site of EGFR and competitively inhibiting ligand-induced activation, and thereby inhibiting EGFR induced cell growth, survival, and

proliferation. In addition, cetuximab may act by inducing an antibody-dependent cell-mediated cytotoxicity reaction as cetuximab is an IgG1 antibody.
Cetuximab is a human-murine chimeric monoclonal antibody with terminal half-life around 4-5 days (range 3-7 days) whereas panitumumab is a fully humanized IgG2 monoclonal antibody with terminal half-life around 7 days (range 4-11 days).

3. Predicting efficacy of anti-EGFR therapy

Unfortunately only a fraction of patients will benefit from the EGFR inhibition and therefore much research is ongoing to identify predictive markers in order to tailor therapy for the individual patient.
Several studies have shown a clear correlation between the severity of skin rash and outcome of therapy of the anti-EGFR antibodies [4-7].
In addition, development of hypomagnesaemia may be a surrogate marker for outcome of therapy [8,9].
Until recently, the development of skin rash during therapy was the most promising predictive factor, but focus has now changed towards assessment of tumour tissue.
Predicting efficacy of anti-EGFR treatment is naturally focused on the EGFR and effectors in the down-stream-signaling pathways in the tumour. Even though EGFR is the target of the anti-EGFR antibodies there is no difference in efficacy in patients with EGFR positive and EGFR negative tumours as assessed by immunohistochemistry [10] and therapy is therefore not restricted to tumours overexpressing EGFR.
KRAS is a member of one of the intra-cellular signal-transduction cascades. If KRAS harbors a mutation (KRASmut), then the growth signal is constitutively activated independently of ligand binding to the extracellular part of the receptor.
KRASmut is found in approximately 40% of mCRC patients [11].The KRAS mutation is an early event during the colorectal adenoma-carcinoma carcinogenic process [12] and thus there is a high concordance between KRAS mutations in the primary tumour and the metastasis [13]. Analyses of clinical trials with anti-EGFR antibodies in mCRC have demonstrated that the KRAS mutational status is central for the effect of anti-EGFR treatment. Patients with KRASmut do not or hardly ever respond to anti-EGFR therapy and progression-free survival (PFS) and survival is definitely shorter than in patients with KRAS wildtype tumours (KRASwt) [5,11,14-19]. However, recently it has been suggested that the different KRAS mutations might have different biological potentials, and that patients with codon 13 mutated tumours may be sensitive to therapy with cetuximab [20].
Normal expression and mutation status of other members of the down-stream signaling pathways (e.g. BRAF PTEN and PI3K), are also needed for normal function of the EGFR pathway. In a recent study including more than 600 patients with mCRC treated with cetuximab and irinotecan in the third line setting response rates were as high as 41 % in the population of patients with wildtype KRAS, BRAF, NRAS, and PIK3CA exon 20 compared to 24% in the unselected population [11].
Furthermore many other attempts have been made in order to identify other predictive marker including of expression of ligands to the EGFR, mutations in the EGFR resulting in structural changes in the receptor, expression of other members of the EGFR-family; however currently the K-RAS gene mutational status is the only established marker of sensitivity to panitumumab and cetuximab, and the use of anti-EGFR antibodies should be restricted to patients with KRASwt tumours.

4. Adjuvant therapy

4.1 Adjuvant chemotherapy after radical resection for colon cancer

Adjuvant fluoropyrimidine-based chemotherapy for 6 months is standard of care in patients with radical resected stage III colon cancer, whereas it is more controversial in patients with stage II colon cancer. Modest but definite benefit of 4-5 % in 5-years survival has been demonstrated in pooled analyses and in the Quasar study [21-24].

Three large phase III trials have documented that addition of oxaliplatin to fluoropyrimidine (FOLFOX, FLOX, XELOX) are superior compared to single agent fluoropyrimidine in terms of disease-free survival and overall survival in stage III patients and probably also in high-risk stage II [25-27].

4.2 Adjuvant targeted therapy after radical resection for colon cancer

Cetuximab has been tested in the adjuvant setting. The US Intergroup N0147 study assessed the potential benefit of cetuximab added to adjuvant FOLFOX after resection in patients with colon cancer stage III. The primary end point was 3 year disease-free survival. The initial concept was to treat patients regardless of KRAS status, but when the impact of KRAS status in the metastatic setting was established NO147 was amended to include only patients with KRAS wild type tumours. In 717 patients with KRAS mutations included before amendment both 3-year disease-free survival (FOLFOX: 75.8% versus FOLFOX + cetuximab: 72.3%) and 3-year survival (88.0% versus 80.4%) favoured FOLFOX alone [28]. Surprisingly, the addition of cetuximab to FOLFOX in the KRAS wild-type population did not add any benefit as well , with a 3-year disease-free survival of 75.8% in the FOLFOX arm versus 72.3% in the FOLFOX-cetuximab arm [29].

The FOxTROT trial is presently evaluating a neo-adjuvant strategy with oxaliplatin-based chemotherapy with or without panitumumab in patients with high-risk but resectable colon cancer.

Data from ongoing or completed adjuvant trials are awaited, but currently cetuximab, panitumumab and other targeted therapies should not be used outside clinical trials.

5. Systemic treatment of metastatic colorectal cancer

Since the introduction of 5-fluorouracil in 1957, numerous well-conducted phase III studies have proven its efficacy and even nowadays fluoropyrimidine is the backbone of systemic therapy [30,31]. The era of modern combination therapy started when it was shown that irinotecan prolonged survival in patients with fluoropyrimidine-resistant disease. Since then irinotecan, oxaliplatin, and two oral formulations of 5-fluorouracil (capecitabine and uftoral) have been approved [32] and are used in the routine clinical practise.

Combination chemotherapy with fluoropyrimidine and irinotecan (e.g. FOLFIRI or XELIRI) or oxaliplatin (e.g. FOLFOX or XELOX) produces tumour regression in approximately half of patients with mCRC. PFS is prolonged from 6 to 9 months and the use of several sequential lines of chemotherapy has improved median survival from 6 months to more than 18 months.

When planning the treatment strategy for an individual patient in the daily clinic it is important to realize the goal of treatment – is there a possibility for cure or is the treatment of palliative character – which depends on the resectability of the metastases and on patient-related factors as performance status and co-morbidity.

Patients with mCRC may be grouped according to the resectability of their metastases: resectable at diagnosis and initially unresectable. Patients with initially unresectable mCRC can be further subdivided into two groups: potential resectable mCRC which may become resectable after tumour shrinkage and non-resectable which is defined as unresectable despite major tumour regression [33]. This classification has to be done in a close collaboration between surgeons, oncologists, radiologist and pathologist – in a multidisciplinary team. For patients with non-resectable mCRC therapy is primarily of palliative character.

In patients with potential resectable or symptomatic mCRC, tumour shrinkage is absolutely mandatory and therefore the most effective combination should be used as initial therapy. However, in patients with unresectable mCRC AND no tumour-related symptoms a sequential approach (single agent immediately followed by combination therapy upon progression) seems to be a safe strategy.

Targeted therapy enhance efficacy of chemotherapy but should be restricted to selected patients.

6. EGFR inhibition in patients with chemoresistent mCRC

There are no established cytotoxic drugs or combination in the third-line settings after progression to irinotecan, oxaliplatin and fluoropyrimidine, but this changed dramatically when efficacy of EGFR inhibition was proven in patients with chemo-resistant mCRC [2,3]. Data are summarized in Table 1.

The promising activity observed in phase I and II studies was first confirmed in the pivotal BOND study [34] where 329 patients with irinotecan-resistant mCRC were randomised to receive either weekly single agent cetuximab alone or cetuximab in combination with irinotecan. This combination significantly increased response rate from 11% to 23% and prolonged PFS from 1.5 months to 4.1 months. Survival was not significantly prolonged, perhaps due to cross-over and use of combination therapy as salvage therapy. As a result of the BOND study, cetuximab was approved for patients with irinotecan-resistant disease in US and Europe in 2004.

One of the criticisms of the BOND study was the lack of a control group and therefore NCIC-CO.17 was planned and completed [35]. Patients pre-treated with irinotecan and oxaliplatin were randomised to receive best supportive care (BSC – no crossover upon progression) or cetuximab monotherapy. Compared to BSC, cetuximab prolonged OS from 4.6 months to 6.1 months (Table 1).

In a parallel study, a similar benefit in terms of response and PFS was established for panitumumab [6]. In contrast to NCIC-CO.17, OS was not significantly prolonged perhaps due to the possibility of cross-over to panitumumab after progression in patients randomized to BSC. Based on these data, panitumumab was approved for monotherapy of refractory mCRC by the US Food and Drug Administration in September 2006 and conditionally approved in patients with tumours harbouring wild-type KRAS by the European Medicines Agency in December 2007. Presently there are more data on the combination of irinotecan and cetuximab as salvage therapy but it may be expected that efficacy of irinotecan and panitumumab will be comparable. Indirectly these data suggested that irinotecan with cetuximab (and perhaps irinotecan with panitumumab) increase response rate to more than 20%, prolong PFS from less than 2 months to more than 4

months and that OS is prolonged from around 5 months to 9 months, in patients treated unaided by KRAS status.

Author. year	Regimen	KRAS	No of patients	RR (%)	Median PFS (months)	Median OS (months)
Third line therapy						
Jonker et al NEJM 2007	BSC	?	285	0	1.8	4.6
	Cet	?	287	7*	1.9*	6.1*
Karapetis et al NEJM 2008	BSC	WT	113	0	1.9	4.8
	Cet	WT	117	13*	3.8*	9.5*
Van Cutsem JCO 2007	BSC	?	232	0	1.7	6.5
	Pan + BSC	?	231	10*	1.8*	6.5
Amado et al JCO 2008	BSC	WT	119	0	1.7	7.6
	Pan + BSC	WT	124	17*	2.8*	8.1
Cunningham et al NEJM 2004	Cet	?	111	11	1.5	6.9
	Cet + Iri	?	218	23*	4.1*	8.5
Di Fiore et al ASCO 2008	Weekly Cet + Iri	MUT	281	0	2.7	8.0
	Weekly Cet + Iri	WT		43*	5.5*	13.2*
Jensen et al ASCO 2010	Biweekly Cet + Iri	MUT	165	3	3.9	7.9
	Biweekly Cet + Iri	WT		23*	5.5*	12.1*
Second line therapy						
EPIC Sobrero et al JCO 2008	Iri	?	650	4	2.6	10.0
	Cet + Iri	?	648	16*	4.0*	10.7
181 Peeters et al JCO 2010	FOLFIRI	WT	294	10	3.9	12.5
	FOLFIRI + Pan	WT	303	35*	5.9*	14.5
	FOLFIRI	MUT	248	14	4.9	11.1
	FOLFIRI + Pan	MUT	238	13	5.9	11.8

Table 1. Selected studies evaluating efficacy of EGFR-inhibition (cetuximab or panitumumab) in patients with chemo-resistent mCRC.

In the second line setting, the EPIC and "181" studies (Tables 1) showed that irinotecan + cetuximab or FOLFIRI + panitumumab, respectively, significantly increased response rate. PFS was prolonged significantly in both studies but the higher response rate and longer PFS did not translate into an improvement in OS [36,37].

7. EGFR inhibition in patients with chemo-naïve mCRC

Excellent efficacy in patients with chemo-resistent mCRC started logically a number of phase II studies for chemotherapy-cetuximab or panitumumab combinations with response rates as high as 80%, high liver resection rates and long survival [38]. As a consequence of these promising data, phase III studies were planned and conducted (Table 2). All published randomized trials were initiated and started before the importance of KRAS was known and therefore these studies have included patients with both KRASwt and KRASmut. As described efficacy of EGFR monoclonal antibodies is restricted to patients with KRASwt however for comparison, data on patients with KRASmut are included in Table 2 but only data on KRASwt will be discussed.

Most trials combining anti-EGFR treatment with chemotherapy confirmed a much higher response rate (absolutely 10-20% difference) in the combination arm and most trials also showed that PFS was prolonged absolutely 1-2 months but this difference was not as long as anticipated or hoped. However, at this time only one phase III study could confirm that the benefit in response and PFS was translated to a significant and clinical meaningful improvement in survival [19].

In the CRYSTAL study more than 1200 patients with EGFR-expressing mCRC were randomised to FOLFIRI or FOLFIRI + cetuximab [19]. The investigators managed to collect tumour tissue and analyze KRAS status in an astonishing 89% of all patients. Response rate and resection rate was significantly higher and both median PFS (8.4 vs. 9.9 months) and median survival were significantly prolonged (20.0 vs. 23.5 months). A higher response rate and longer PFS were also observed in the OPUS [39] and PRIME [40] studies. In PRIME, median survival was non-significantly prolonged (19.7 vs. 23.9 months) at the same level as in the CRYSTAL trial. In the large COIN study only response rate was increased [41] and in the smaller NORDIC VII trial cetuximab did not improve efficacy of the Nordic bolus regimen [42].

Since addition of cetuximab or panitumumab improve response rate to combination chemotherapy, there has been a particular interest in the use of these agents in patients with potential resectable liver-only metastasis if it was anticipated that a major response could lead to potentially curative surgery. In the CELIM study, a randomized phase II trial with 111 patients with unresectable liver- metastasis, patients were randomized to FOLFOX + cetuximab or FOLFIRI + cetuximab [43]. In these selected patients the R0 resection rate was impressing 38% and 30%, respectively, which show the importance of selecting and evaluation patients at a multidisciplinary conference but also that the patients should receive the most effective systemic therapy to enhance the chance for curative surgery. In a retrospective analysis of response by KRAS status, a partial or complete response was noted in 70% of patients with KRASwt.

When cetuximab or panitumumab is chosen for patients with KRASwt, it must be concluded, that the chemotherapy combination should be carefully selected. A combination of fluoropyrimidine with oxaliplatin and cetuximab seem to have no or less additional

Author. year	Regimen	KRAS	No of patients	RR (%)	Median PFS (months)	Median OS (months)
First line therapy						
CRYSTAL van Cutsem et al NEJM 2009 & JCO 2011	FOLFIRI	WT	350	40	8.4	20.0
	FOLFIRI+Cet	WT	316	57*	9.9*	23.5*
	FOLFIRI	MUT	183	36	7.7	16.7
	FOLFIRI+Cet	MUT	214	31	7.4	16.2
PRIME Douillard et al JCO 2010	FOLFOX	WT	331	48	8.0	19.7
	FOLFOX+Pan	WT	325	55	9.6*	23.9
	FOLFOX	MUT	219	40	8.8*	19.3*
	FOLFOX+Pan	MUT	221	40	7.3	15.5
NORDIC 7 Tveit et al ASCO GI 2011	FLOX	WT	97	47	8.7	20.1
	FLOX + Cet	WT	97	46	7.9	22.0
	FLOX	MUT	58	40	7.8	20.4
	FLOX + Cet	MUT	72	49	9.2	21.1
COIN Maughan et al Lancet Oncol 2011	"Ox"	WT	367	50	8.6	17.9
	"Ox"+Cet	WT	362	59*	8.6	17.0
	"Ox"	MUT	268	41	6.9	14.8
	"Ox"+ Cet	MUT	297	40	6.5	13.6
OPUS Bokemeyer et al Ann Oncol 2011	FOLFOX	WT	97	34	7.2	18.5
	FOLFOX + Cet	WT	82	57*	8.3*	22.8
	FOLFOX	MUT	59	53*	8.6*	17.5
	FOLFOX + Cet	MUT	77	34	5.5	13.4
Second line therapy						
181 Peeters et al ECCO 2009	FOLFIRI	WT	294	10	3.9	12.5
	FOLFIRI+Pan	WT	303	35*	5.9*	14.5
	FOLFIRI	MUT	248	14	4.9	11.1
	FOLFIRI+Pan	MUT	238	13	5.9	11.8

Table 2. Recent studies evaluating EGFR-inhibition (cetuximab or panitumumab) as first line therapy according to KRAS-status.

benefit over chemotherapy alone [41,42] and presently capecitabine or bolus 5-fluorouracil in combination with oxaliplatin can not be recommended outside clinical trials (Table 2). Until otherwise proven, cetuximab or panitumumab should be combined with FOLFIRI or FOLFOX.

7.1 Combinations of targeted therapies

In vitro studies have shown that simultaneous inhibition of angiogenesis and EGFR systems have additive and perhaps even synergistic effect, but surprisingly this benefit could not be confirmed in first line randomised studies (Table 3).

In a small randomised phase II study, a triple-combination of cetuximab, irinotecan and bevacizumab was more effective than cetuximab + bevacizumab alone [44]. Even more interesting, PFS and survival for the triple-combination were considerably longer than the historical double-combination in the BOND1 trial [34]. It was therefore expected that a similar combination would increase efficacy also as first line therapy.

Author. year	Regimen	KRAS	No of patients	RR (%)	Median PFS (months)	Median OS (months)
CAIRO2 Tol et al NEJM 2009	CapOx+Bev	WT	156	50	10.6	22.4
	CapOx+Bev+Cet	WT	158	61	10.5	21.8
	CapOx+Bev	MUT	108	59*	12.5*	24.9*
	CapOx+Bev+Cet	MUT	98	46	8.3	17.2
PACCE Hecht et JCO 2009	"Ox"+Bev	WT	203	56	11.5*	24.5*
	"Ox"+Bev+Pan	WT	201	50	9.8	20.7
	"Ox"+Bev	MUT	125	44	11.0	19.3
	"Ox"+Bev+Pan	MUT	135	47	10.5	19.3
	"Ir"+Bev	WT	58	48	12.5	19.8
	"Ir"+Bev+Pan	WT	57	54	10.0	NR
	"Ir"+Bev	MUT	39	38	11.9	20.5
	"Ir"+Bev+Pan	MUT	47	30	8.3	17.8

Abbreviations in the tables:
RR = response rate, PFS = progression free survival, OS = overall survival, BSC = best supportive care, cet = cetuximab, pan = panitumumab, WT = wildtype, mut = mutant, iri = irinotecan, ox = oxaliplatin, bev = bevazicumab

Table 3. Recent studies evaluating double targeted therapy (inhibition of angiogenesis and EGFR) according to KRAS-status.

In the PACCE study more than 1000 patients were randomised to a combination of chemotherapy with bevacizumab (optional oxaliplatin-based regimen (n = 823) or irinotecan-based regimen (n = 230) with or without panitumumab [45]. The four-drug combination of oxaliplatin-based therapy with bevacizumab and panitumumab resulted in several serious adverse events and also a shorter PFS and survival, while there was no significant difference in efficacy data in the smaller group where therapy was based on irinotecan. Even in patients with KRAS wild-type there was evidence of a harmful effect of double targeted therapy.
In the CAIRO-2 study, 734 patients were randomised to XELOX + bevacizumab with or without cetuximab. Similar to PACCE study, PFS was significantly shorter in patients receiving double targeted therapy and subgroup analysis of patients with KRAS mutations showed that efficacy (response, PFS and survival) was significant worse [46].
Double targeted therapy against angiogenesis and EGFR should not be used as first line treatment outside of controlled studies.

8. Weekly or biweekly cetuximab

Cetuximab is approved as weekly administration with an initial loading dose of 400 mg/m^2 followed by weekly administration of 250 mg/m^2. However, as most cytotoxic regimens are administered in two-weeks (or longer) schedules it would be more convenient if cetuximab could be administered as a two-week schedule. Based on a study showing that there is no major differences in the pharmacokinetics and pharmacodynamics between the standard weekly cetuximab schedule and cetuximab 500 mg/m^2 given every second week [47,48] a simplified biweekly administration schedule of cetuximab has been developed [47-49]. The biweekly regimen has efficacy and safety profile similar to the weekly schedule [49-52] and ongoing studies will prospectively evaluate the biweekly regimen (www.clinicaltrial.org NCT00660582). In many institutions the biweekly schedule is used in the daily clinical setting based on the above-mentioned experiences.
Panitumumab is administered as an intravenous infusion at 6 mg/kg every 14 days or 9 mg/kg every 3 weeks. There is no loading dose.

9. Toxicity of anti-EGFR therapy

Toxicity of the anti-EGFR therapy is related to the blockade of the EGFR in the normal tissue. The most often reported side-effect is a papulo-pustular rash primarily in the seborrheic areas seen in up to 90% of patients [6]. The onset is usually within the first three weeks after start of therapy and with spontaneous improvement within the next 4–5 weeks [53]. Most cases are mild to moderate but severe in 5% to 20% of patients [6,7,34,35,54]. Prophylactic treatment with systemic tetracyclines reduces the severity of skin reactions but not the incidence of rash [55-57].Other dermatological reactions are xerosis, fissures of palm and foot, paronychia and extensive growth of both eyelashes and eyebrows [53,54,58,59], but these side-effects are primarily seen after many months of exposure to anti-EGFR therapy.
Furthermore, cetuximab and panitumumab may induce severe hypomagnesaemia in as many as 25% of patients, but fortunately it is seldom symptomatic. Hypomagnesaemia results from inhibition of the EGFR in the kidneys - particularly in the ascending limb of the

loop of Henle. The hypomagnesaemia may be corrected by oral or IV supplements [60,61]. Anti-EGFR antibody therapy may as well cause nausea and diarrhea due to affection of the EFGR in the gastro-intestinal tract [34].

In addition, administration of chimeric antibodies also may give rise to severe allergic reactions in 1.4-4.5% [3]. The incidence of infusion reactions is reduced by the prophylactic use of antihistamines and corticosteroids as premedication [62]. No study has compared side effects of cetuximab and panitumumab, but cross-trial comparison shows that the spectrum of side effects is similar. However, as panitumumab is a human antibody anaphylactic reactions are rarely seen with panitumumab, and treatment with panitumumab does not require premedication [63]. A switch to panitumumab may be used after severe hypersensitivity reaction to cetuximab [64-66].

10. Conclusion

Optimal therapy of patients with CRC has increased in complexity with the introduction of targeted therapies, but unfortunately our expectations for these new drugs have not quite been settled. The largest benefits have been achieved with modern chemotherapy, which remains the backbone of treatment of patients with mCRC. However, targeted therapy has clinically significant effect, but we must learn to identify the correct regimes for the right patients. KRAS status is currently the most important predictive marker for efficacy of anti-EGFR therapy. To ensure the optimal treatment strategy, every patient with mCRC must be assessed by a multidisciplinary team.

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Pharmacogenetics and Pharmacogenomics of Colorectal Cancer: Moving Towards Personalized Medicine

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1. Introduction

Colorectal cancer (CRC) remains one of the most deadly diseases in the western world, and starts to become a concern in developed countries (Labianca et al., 2010). However, significant steps have been made recently in CRC therapy. Until the 80's, 5-fluorouracil (5-FU) was the only available drug to treat patients, with limited efficacy. Today, 4 cytotoxic agents (5-FU associated with folinic acid, capecitabine, oxaliplatin, irinotecan) and three monoclonal antibodies (cetuximab, panitumumab, bevacizumab) are available, mostly as part of combinations (Koutras et al., 2011). In particular, the rise of targeted therapies in digestive oncology has fueled a new hope by significantly stretching the therapeutic options available so far. Despite these improvements, treatment of metastatic CRC (mCRC) remains a challenging task, and it is acknowledged now that although improving response rates, the introduction of the latest targeted therapies only marginally impacts on either progression free survival (PFS) or overall survival (OS) of mCRC patients. Because of the cost of these new therapies, identifying biomarkers likely to sort patients on their ability to benefit or not, from these new drugs is paradigmatic of the current trend to move towards a more personalized medicine in oncology. Because genetic variability is one of the main factor regulating efficacy and toxicity of most anticancer agents, addressing the issues of pharmacogenomics and pharmacogenetics (PGx) in CRC patient becomes critical, far beyond the only use of costly targeted therapies. Although often used interchangeably, the term "pharmacogenetics" refers historically to inherited changes in genes coding for drug metabolizing enzymes or membrane transporters, thus impacting on the pharmacokinetic (PK) profile and exposure levels eventually, whereas "pharmacogenomics" is a broader definition encompassing genetic changes at the tumor level potentially affecting drug response (Amstutz et al., 2011). Whether they are somatic or found in the germline, all these mutations can potentially have deleterious impacts on the clinical outcome of patients with CRC cancer. At the tumor level, genetic changes affecting the expression of pharmacological

targets or downstream signaling pathways can lead to treatment failure, as highlighted by the now canonical KRAS mutational status in patients undergoing anti-EGFR therapies. Constitutive mutations are mostly associated with increased toxic risk, as largely publicized by the dihydropyrimidine dehydrogenase (DPD) deficiency syndrome, a condition that puts 5-FU patients at risk of life-threatening toxicities. Of note, when not directly life-threatening, inherited genetic mutations affecting drug disposition in the body and pharmacokinetics can ultimately lead to treatment failure too, because the induced-toxicities often require discontinuation of the treatments until the patient recovers. For all these reasons, developing pharmacogenetic and pharmacogenomic testing in routine clinical practice is now seen as a major issue in oncology.

2. Pharmacogenomics: A matter of life & death at the tumor level

2.1 Cytotoxics: Why should we not forget that they are targeted therapies too

Standard care of colorectal cancer includes the use of a variety of cytotoxic agents, used either alone or more frequently as part of combinations (e.g., the canonical Folfiri and Folfox4 regimen). Each of these drugs have their own specific target (e.g., thymidylate synthase for 5-FU, DNA for oxaliplatin, topoisomerase I for irinotecan) and in this respect, numerous studies have focused on the deregulations affecting these targets, either at the genetic or the molecular level, as an attempt to predict treatment efficacy. Indeed, variations in the expression level of the targeted protein, polymorphisms inducing conformation changes, or increase in the repair systems/salvage pathways have been identified as major causes for treatment failure in mCRC patients.

2.1.1 5-FU & Oral 5-FU: The older, the better

5-FU remains the pivotal drug for treating CRC. Initially used alone, it soon turned to be systematically associated with folinic acid so as to enhance its effect as an antimetabolite, before being introduced as the backbone of several polychemotherapies including irinotecan (a.k.a. Folfiri regimen) or oxaliplatin (a.k.a. Folfox regimen). 5-FU's main target is thymidylate synthase (TS), an enzyme essential to the DNA synthesis and cell replication. Several genetic polymorphisms can affect both *TYMS*, the gene coding for TS, and the folate cycle necessary for the synthesis of methylene tetrahydrofolate, the cofactor required for a complete inhibition of the target through the formation of a stable ternary complex between the enzyme, the cofactor, and fluorodeoxyuridine monophosphate (FdUMP). TS overexpression in tumors is generally associated with resistance to 5-FU treatment, both in vitro and at the bedside (Popat et al., 2004, Lenz et al., 2004). Conversely, another pivotal study has demonstrated that higher TS expression was predictive of higher response with adjuvant fluoropyrimidine (Edler et al., 2002). However, other clinical reports failed in demonstrating such relationship (Locker et al., 2006, Lurje et al., 2009), thus preventing substantial step to be undertaken for implementing screening for TS expression in tumors in routine clinical practice. Variations in TS expression are, at least in part, related to mutations affecting the *TYMS* gene promoter. For instance, the TSER*3 genotype has been associated with increased mRNA production, thus potentially leading to lower response rates in mCRC patients treated with 5-FU (Uetake et al., 1999). Beside the issue of over-expressing TS tumors likely to resist to 5-FU, constitutive polymorphisms in the 5′ and 3′UTRs of the *TYMS* gene responsible for downregulation of TS, have been associated with increased toxicities in patients treated with 5-FU or oral capecitabine (Larguiller et al., 2006). However,

as for TS expression level in tumors, the actual clinical relevance of these polymorphisms is far from being consensual. Lower response rates have been reported in colorectal cancer patients with the TS 5'-UTR 3R genotypes (ie, TSER*3), as compared to individuals harboring the homozygous TS 5'-UTR 2R/2R genotype (Salgado et al., 2007). Of note, other groups (Stoehlmacher et al., 2004, Kostopoulos et al., 2009) failed in observing any significant difference in the clinical outcome according to the TS 5'-UTR genotypes, whereas conversely, other authors (Jakobsen et al., 2005; Dotor et al., 2006) found longer survival in carriers of TS 5'-UTR 3R genotypes as compared with those carrying the TS 5'-UTR 2R/2R genotypes. Such conflicting results for predicting outcome from *TYMS* genomic status is not surprising. Several factors such as genetic and epigenetic regulations may interfere with the genotype-to-phenotype relationships (Pullmann et al., 2006). For instance, the loss of heterozygosity in tumours at the TS locus may cause the heterozygous TS 5'-UTR 2R/3R risk genotype to acquire either the 2R/loss or the 3R/loss genotype. Consequently, individuals theoretically at risk of treatment failure on the basis of their TS 5'-UTR 2R/3R genomic status may harbor actually the favorable 2R/loss genotype in cancer cells and exhibit higher response eventually when treated with 5-FU (Ruzzo et al., 2007). In addition to target TS, other non-synonymous SNPs (677C>T: MTHFR*4 and 1298A>C:MTHFR*6 allelic variants) affecting methylene tetrahydrofolate reductase (MTHFR), one of the key-enzyme involved in the synthesis of reduced folate cofactor, could lead to lack of efficacy when down-regulated (Etienne-Grimaldi et al., 2007, Zintsaras et al., 2009, Braun et al., 2009). However, as for *TYMS*, the actual impact of *MTHFR* genetic polymorphisms on the clinical outcome with 5-FU or 5-FU-derivatives remains to be fully elucidated because inconsistent data have been generated so far (Sharma et al., 2008, Ruzzo et al., 2007). All these contradictory findings with *TYMS* and the associated *MTHFR* genomic status are better understood when one keeps in mind that TS is not the main locus of action of 5-FU. Incorporation into RNA and DNA can be alternative mechanisms of actions for the cytotoxic effects of 5-FU, depending on the way the drug will be metabolized within tumor cells (Ciccolini et al., 2000a). In this respect, the expression levels of activating/deactivating enzymes at the tumor level (eg, orotate phosphoribosyl transferase, thymidine kinase, thymidine phosphorylase, dihydropyrimidine dehydrogenase) have been associated with clinical outcome in patients treated with 5-FU-containing regimen, although once again the data collected so far proved to be rather conflicting (Ciccolini et al., 2004; Soong et al., 2008). For instance, thymidine kinase is implicated both in the activation of 5-FU to active metabolite FdUMP with subsequent theoretical better TS inhibition if highly expressed, and in the *de novo* salvage pathway likely to help cancer cells to survive to 5-FU-induced thymineless stress (Fanciullino et al., 2007). Similarly, thymidine phosphorylase (TP) is involved in the tumoral activation of both 5-FU and capecitabine, but could promote neoangiogenesis too, thus rendering the clinical impact of TP levels in tumors hardly predictable (Ciccolini et al., 2004). Furthermore, deregulation of downstream proteins involved in the transmission of apoptosis in cells exposed to thymineless stress can affect 5-FU or capecitabine antiproliferative efficacy, despite proper inhibition of target TS. Because 5-FU exerts its cytotoxic effects partly through a p53/Fas-dependent apoptotic pathway involving Bax translocation and mitochondrial permeabilization, deregulations affecting each of these steps can interfere with the actual upstream *TYMS* status or the extent of TS inhibition (Borralho et al., 2007). For instance, down-expression of Apo-1 Fas CD95 receptor has been associated with resistance to 5-FU or capecitabine in non-clinical colorectal models, including after that a near-total inhibition of TS activity was achieved (Ciccolini et al., 2000b; 2001).

However, subsequent clinical studies failed in demonstrating the role Fas expression could play as a predictive marker in patients with colorectal cancer (Backus et al., 2001; Bezulier et al., 2003).

2.1.2 Oxaliplatin: A metal precious to the patients

In clinical practice, oxaliplatin is given in mCRC patients in association with 5-FU/folinic acid, a combination known as the Folfox regimen. It can be further combined now with the latest monoclonal antibodies targeting VEGF or EGFR-1. Oxaliplatin is a third-generation platinum derivative that targets complementary DNA strands, thus inducing cell death eventually. However, the nucleotide excision repair (NER) system is designed to remove the oxaliplatin-induced DNA-adducts, and several factors (XPD (a.k.a. ERCC2), XPC and XPA) are implicated in the repair process of DNA helixes once adducts have been formed. In addition, XPG and ERCC1 are implicated in the cleavage of the damaged DNA strand and participate to the repairing pattern of cells exposed to oxaliplatin. Any changes in those repair mechanisms can lead to increase of sensitivity or loss of efficacy in patients. Several genotypes at the tumor level have been associated with clinical outcome in oxaliplatin-regimen. In particular, it has been demonstrated that polymorphisms affecting *ERCC1* and *XPD* genes are related to patient survival. For instance, ERCC1-118 T/T, or XPD-751 A/C and C/C genotypes have been associated with reduced disease-free survival in patients treated with oxaliplatin (Ruzzo et al., 2007). In another study, the Lys751Gln polymorphism of the *XPD* gene has been identified as a predictive marker in mCRC patients undergoing FolFox treatment (Le Morvan et al., 2007). Beside the NER, basepair excision repair is also involved in the chemosentivity to oxaliplatin. *XRCC1* gene is affected by several polymorphisms, and expression of the wild-type allele has been associated with better clinical outcome in patients with mCRC (Suh et al., 2006, Stoehlmacher et al., 2001), although subsequent studies failed in confirming the relevance of establishing *XRCC1* genotype as a predictive biomarker with oxaliplatin (Ruzzo et al., 2007). Along with the issue of efficacy, mutations affecting Glutathione-S Transferase (GST), the enzyme responsible for the cell detoxification of oxaliplatin, could have an impact on the clinical outcome with oxaliplatin. Overexpression of tumoral GSTP1 has been found in CRC patients, thus leading to lack of efficacy (Glasgow et al., 2005). However, the exact role the genetic status *GSTP1* plays in patients treated with oxaliplatin remains controversial. For instance, the GSTP1 ile105val genotype has been associated with improved survival in patients treated with Folfox regimen (Stoehlmacher et al., 2002), although the same genotype was predictive of reduced survival in another study (Sun et al., 2005) . In addition, the GSTP1-105 G allele, could explain higher incidence of severe neurotoxicities, the most common side-effect of oxaliplatin, observed in some patients (Ruzzo et al., 2007). Another polymorphism affecting the *AGXT* gene coding for the enzyme responsible for the metabolism of oxalate, which peaks during oxaliplatin infusion, could explain higher risk of neurotoxicity in patients (Gamelin et al., 2007).

2.1.3 Irinotecan: Twist again 'till double-strand DNA breakage

Irinotecan (CPT-11) is a topoisomerase-I (Topo-1) inhibitor usually combined with 5-FU/folinic acid regimen, an association known as the FolFiri regimen. Topo-1 relieves torsional strain in DNA, thus allowing DNA replication, recombination, and repair. Irinotecan prevents religation of the DNA strand by binding to topoisomerase I-DNA

complex, thus causing double-strand DNA breakage and cell death eventually. Expression levels of target topo-I has been associated with clinical outcome in multivariate analysis performed from large studies including several hundreds of patients undergoing irinotecan-based therapy (Braun et al., 2008; Kostopoulos et al., 2009). However, the lack of randomized, prospective trial prevents, for the time being, the evaluation of Topo-1 level in tumours to be proposed in routine clinical setting as a predictive biomarker for irinotecan efficacy, and little is known about the genetic or epigenetic events affecting the Topo-1 gene likely to modify expression levels of the target protein. However, in the Focus trial, Topo-1 expression level was found to be related to efficacy, although it remains unclear whether the expression level is to be considered as a predictive or a prognostic marker (Braun et al., 2008). In addition, as for oxaliplatin, deregulations affecting DNA-repairing enzymes like *XRCC1*, ERCC1 and *GSTP1* have been found to be predictive of the clinical outcome in irinotecan-treated patients. Polymorphism affecting the *XRCC1* gene (eg, the GGCC-G haplotype) was associated with improved response rates in patients given irinotecan, much probably in relation with loss of ability to repair DNA damage (Hoskins et al., 2008). Conversely, better response and, in some cases, improved PFS was observed in patients undergoing FolFiri regimen with tumors overexpressing GSTP1 and ERCC1 (Vallbohmer et al., 2006). This finding may be confusing because higher expression in DNA-repair enzymes is normally associated with resistance to DNA-targeting agents. Here, high ERCC1 levels could be indicative of a higher DNA damage, thus making the tumor cells more sensitive to Topo-I inhibition by irinotecan. In the same study, EGFR expression was found to be associated too with better response, although to date, no molecular mechanisms underlying this observation have been found.

2.2 Biotherapies: Where are my keys?

Treatment of colorectal cancer has taken benefit from the rise of the biotherapies in clinical oncology, because both anti-VEGF and anti-EGFR monoclonal antibodies can be used now in association with cytotoxics agents. However, the efficacy of most targeted therapies is generally contingent upon a number of biomarkers at the tumor level to be checked.

2.2.1 Anti-EGFR monoclonal antibodies: Why hitting the target is not enough

Cetuximab and panitumumab are two anti-Her1 monoclonal antibodies indicated for treating metastatic colorectal cancer. Initially proposed alone, both drugs showed better efficacy and improved survival when combined with standard Folfox4 or Folfiri regimen. Although cetuximab is a chimeric IgG1 and panitumumab a 100% human IgG2, these both antibodies target the extracellular domain of EGFR-1, thus blocking the downstream signaling pathway normally leading to cell proliferation and differenciation, neoangiogenesis and invasion patterns associated with colorectal cancer. Cetuximab and panitumumab prescription is contingent upon the completion of pharmacogenomics testing. Expression level of target EGFR is the first condition, although in clinical practice, the relevance of this test is more and more debated and controversial at the bedside. However, several studies have demonstrated how patients with elevated *EGFR* gene copy number are more likely to respond to cetuximab or panitumumab therapy (Moroni et al., 2005; Sartore-Bianchi et al., 2007, Heinemann et al., 2009). More interestingly and consensual, determination of the mutational status of KRAS soon turned to be the paradigm of implementing pharmacogenomic testing prior to initiating treatment with a targeted

therapy. The EGFR/KRAS/Raf pathway is implicated in signal transduction from receptors to the nucleus, thus promoting cell proliferation and differentiation. KRAS transmits signal after binding to guanosine triphosphate (GTP), and becomes inactive when GTP is converted to GDP. Mutations affecting *KRAS* will maintain the protein continuously activated in a switch-on position, even if the upstream receptor is inhibited by a monoclonal antibody. It was demonstrated in the mid-2000's that specific *KRAS* mutations (eg, codons 12/13) was associated with lack of response in cetuximab-treated patients (Lievre et al., 2006). Subsequent studies all confirmed the predictive value of wild-type (WT) *KRAS* for the response with anti-EGFR biotherapies, either cetuximab or panitumumab, regardless of their use as monotherapy or combined with cytotoxics (Heinemann et al., 2009, Asghar et al., 2010). However, WT *KRAS* is a mandatory but no sufficient condition to guarantee an optimal efficacy with anti-EGFR therapies. Mutations affecting *BRaf*, an effector of KRAS, has been associated with treatment failure, although it remains unclear whether *BRaf* mutational status should be used as a prognostic or a predictive marker (Di Nicolantonio et al., 2008). Similarly, correlation was found in cetuximab-treated patients between *EGFR* gene amplification, WT *KRAS* status, PTEN expression, and response. Of note, loss of PTEN expression was systematically associated with treatment failure, thus suggesting that PTEN could be a novel predictive biomarker for anti-EGFR therapies (Frattini et al., 2007). Along with PTEN, several other parameters like epiregulin and amphiregulin expression have been recently identified as putative biomarkers (Jacobs et al., 2009; Laurent-Puig et al., 2009; Di Fiore et al., 2010), although larger prospective studies will be necessary to validate their clinical relevance to predict clinical outcome with EGFR-inhibitors.

2.2.2 Anti-VEGF therapy: Desesperatly seeking biomarkers

Bevacizumab is the only *stricto-sensu* antiangiogenic therapy approved for treating mCRC patients in association with cytotoxics. This humanized monoclonal antibody targets circulating VEGF-A. To date, no predictive biomarkers have been identified with bevacizumab. Overexpression of VEGF is usually associated with poor survival in mCRC patients, but VEGF level is generally considered as a prognostic, rather than a predictive, biomarker. Even in a prognostic setting, the actual role *VEGF* polymorphism plays remains unclear. For instance, in some studies, the -460CC genotype was found to have a favorable impact on OS in gastric cancer patients (Kim et al., 2007), but deleterious in breast cancer patients (Lu et al., 2005). Beside, some studies in breast cancer patients have found a relationship between *VEGF* polymorphisms (eg, -2578A/A and -1154A/A genotypes) and better survival in patients treated with the paclitaxel + bevacizumab regimen (Schneider et al., 2008). Similar relationship between *VEGF-A* polymorphism and both toxicity and DFS has been evidenced more recently (Etienne-Grimaldi et al., 2010). A similar trend has been found with digestive cancers (Formica et al., 2010). Additionally, circulating PDGF could be implicated in resistance to anti-angiogenic drugs (Crawford et al., 2009), as well as SDF1 and FGF2 factors (Batchelor et al., 2007). Finally, plasma cytokines and vascular factors could be associated with clinical outcome in patients undergoing bevacizumab-based therapy (Kopetz et al., 2010). However in a recent study, Loupakis et al. have investigated the molecular and genetic markers likely to predict efficacy in mCRC patients treated with the Folfoxiri plus bevacizumab quadruple combination. Among the various bevacizumab-related biomarkers they monitored in plasma (ie VEGF, PIGF, sVEGFR2, TSP-1 plasma level) and the screening of several polymorphims affecting *VEGF* (eg., -2578C/A, -1498C/T, -1154G/A, 936C/T) and *VEGFR-2* (-604A/G, 1192C/T, 1719T/A), little relevant association

with PFS was found (Loupakis et al., 2011). This latter study illustrates the difficulty in identifying relevant biomarkers for response in heavily treated mCRC patients receiving several drugs in a row, the observed efficacy being the resulting combination of the numerous parameters affecting each drug.

3. Pharmacogenetics: When genetics help finding the right exposure

3.1 Cytotoxics: Improving the efficacy/toxicity balance

Beside those affecting tumors, several constitutive genetic mutations can impact on the disposition of anticancer drugs, especially when they concern genes coding for detoxifying enzymes in the liver. Although for years, such polymorphisms were mostly associated with increased risk of developing severe and sometimes deadly toxicities upon drug intake, they may impact as well on treatment efficacy eventually. Indeed, when they are not directly life-threatening, drug-induced toxicities and their management often require treatment discontinuation, delays in subsequent radiotherapy courses if scheduled, with a subsequent loss of chance and poor clinical outcome eventually.

3.1.1 5-FU & Oral 5-FU

Fluoropyrimidines pharmacokinetics is primarily dependent upon an intense liver first pass effect mediated by dihydropyrimidine dehydrogenase (DPD), the enzyme that converts uracil into dihydrouracil. It is generally estimated that about 90-95% of an administered 5-FU dose will be metabolized in the liver before being distributed throughout the body. DPD exhibits a similar pivotal role in the disposition of oral fluoropyrimidines like capecitabine or UFT, all generating 5-FU eventually. *DPYD* gene is highly polymorphic because several dozen of mutations have been described thus far (Van Kuilenburg, 2004). Mutational inactivation of the *DPYD* gene has been characterized as an autosomal recessive disease in Caucasians' population, with probably a higher impact in black American (Mercier C et al., 2006). Genetic and epigenetic regulations, such as promoter hypermethylation or variations in transcriptional factor expression, could play as well a critical role in *DPYD* dysregulations (Etienne MC et al., 1994, Zhang et al., 2006), although this issue remains debated today. Admittedly, three relevant mutations (canonical IV14+1G>A (*DPYD**2A), plus 2846A>T, and 1679T>G) should be screened at bedside to anticipate 5-FU-related side effects (Morel et al., 2006). Numerous clinical reports have demonstrated the deleterious effect of DPD genetic polymorphism in patients undergoing 5-FU based regimen. Regardless of the upstream genetic events leading to the loss of enzymatic activity, impaired DPD has been systematically associated with increased risk of developing severe/lethal toxicities upon 5-FU exposure. In a proof-of-concept study, DPD deficiency was retrospectively identified as the culprit for 70% of the severe toxicities and 80% of the toxic-death cases monitored over a two-year observation period, and when performed, drug monitoring confirmed strong overexposure to 5-FU in DPD-deficient individuals (Ciccolini et al., 2006). However, some reports failed in providing data for this pivotal role *DPYD* genetic polymorphism could play in the incidence of severe toxicities with 5-FU. In a gene-candidate study, Schwab et al. have investigated the role several polymorphisms, including the *DPYD**2A allelic variant, could play in the tolerance to 5-FU. Surprisingly, this genotype was found to be only marginally associated with toxicities, but it has to be underlined that in this study, no complementary functional investigations were undertaken to evaluate globally the DPD status in those

patients (Schwab et al., 2008). In addition to 5-FU, several reports have suggested that *DPYD* genetic polymorphism could be an issue with capecitabine too. The very first toxic-death case has been first observed in the late-2000' in a patient treated with capecitabine who was found to be profoundly DPD deficient after post-mortem investigations (Mercier et al., 2007a). Several other clinical reports have demonstrated how *DPYD* genetic polymorphism could put deficient patients at risk of experiencing severe toxicities if given capecitabine (Mercier et al., 2007b). Lastly, another genetic polymorphism could be a rising concern with capecitabine. Deregulations affecting cytidine deaminase (CDA), one of the three enzymes responsible for the conversion of prodrug capecitabine to 5-FU, could lead to severe toxicities. As for DPD, the gene coding for CDA is highly polymorphic with either loss (poor metabolizer) or gain (ultra-metabolizer, UM) of enzymatic activity. The first life-threatening toxicity in a patient displaying the UM phenotype was reported in the late 2000's (Mercier C et al., 2009). The role CDA could play in severe toxicities with capecitabine has been next confirmed in another larger study showing that deletion in the promoter region of the CDA gene with increased transcription was a predictive marker for hand-foot syndrome (Caronia et al., 2011). Lastly, the first toxic-death case in a capecitabine-treated patient harboring several polymorphisms on the CDA gene, including the Caronia deletion, has been published recently (Dahan et al., 2011), thus highlighting the fact that beside *DPYD*, other genetic polymorphisms should be screened to ensure a better safety when handling oral fluoropyrimidines.

3.1.2 Irinotecan

Irinotecan is a prodrug that can be either metabolized by the Cyp3A sub-family to form the inactive APC derivative, or be converted by carboxylesterase into SN38, a highly cytotoxic metabolite responsible for both the efficacy and the toxicity of irinotecan. SN38 is next mainly detoxified after conjugation by the UGT1A1 to yield inactive SN-38G that will be excreted by the kidneys and the bile eventually. Numerous polymorphisms have been described for the gene coding for UGT1A1, and variations in the promoter region consisting in 7 instead of 6 TA-repeats (UGT1A1*28) is admittedly associated with increased risk of severe toxicities in mCRC patients administered with high dose (e.g., above 250 mg/m^2) irinotecan (Kweekel et al., 2010). A strong influence of ethnicity has been observed with this allelic variant because its population frequency is as high as 43% heterozygotes in the Caucasians but much lower in the Asians (Innocenti et al., 2005; deJong et al., 2006). Several independent studies have demonstrated how individuals with the UGT1A1*28 genotype were up to 7-time more at risk to experience haematological or gastrointestinal severe toxicities when treated with irinotecan (Ando et al., 2000; Marcuello et al., 2004). Of note, some authors have reported an association between the UGT1A1*28 genotype and irinotecan efficacy (Toffoli et al., 2006), although other studies have failed in providing evidence for such a relationship (Kweekel et al., 2008). Along with the UGT1A1*28 genotype, other variations such has the UGT1A1*6 most frequently found in Asian populations has been associated with increased severe neutropenia after irinotecan intake (Han et al., 2006), although other studies failed in confirming such relationship (Ando et al., 2000). Additionally, polymorphisms affecting transmembrane pumps involved in the excretion of toxic metabolites could be related to drug resistance. Pharmacogenetics of the ATP-binding cassette proteins has been associated with changes in the pharmacokinetics of irinotecan, because they impact of the renal clearance of the drug and ultimatelly on

exposure levels. For instance, patients harboring the 34A>G SNP on the *ABCG2* gene could be more at risk of treatment failure, as compared with WT patients (Mc Leod et al., 2008). Conversely, other SNPs like the 421C>A polymorphism seems to have limited impact on irinotecan pharmacokinetics and clinical outcome (de jong et al., 2004) whereas some mutations were associated with higher incidence of drug-induced toxicities (Cha et al., 2009).

3.2 Pharmacokinetics of targeted therapies: The hidden biomarker?

For years, the importance of pharmacokinetic issues such as residual plasma levels or drug concentrations at the tumor site has been largely underestimated with targeted therapies. For instance, it took 5 years since its first approval in Chronic Myeloid Leukemia to acknowledge the fact that the residual concentrations of imatinib were predictive for the major molecular response in patients, thus highlighting the utility to perform drug monitoring and subsequently developing dose-tailoring strategies to ensure a better efficacy (Egorin et al., 2009). Although similar strategies are now developed with other small molecules such as pazopanib (Suttle et al., 2010), no such trend is currently proposed with monoclonal antibodies, despite the fact that dose/exposure/efficacy and dose/exposure/toxicities relationships have been described (Lu et al., 2009, Keiser et al., 2010). Both non-clinical and clinical studies suggest that 90% of target inhibition should be continuously achieved to ensure a maximum efficacy, thus stressing the usefulness to monitor residual concentrations of monoclonal antibodies such as panitumumab, as for other target therapies (Yang et al., 2010). For instance, plasma residual concentrations of 10-30 ug/ml are considered necessary with bevacizumab for an optimal efficacy (Data on File Genentech Inc). However, little is known about the pharmacokinetics of monoclonal antibodies and there is a clear lack for markers of inter-patient variability. Proteolytic degradation along with target-mediated drug disposition are the main patterns implicated in the clearance of monoclonal antibodies, and several factors such as antibodies anti-therapeutic antibodies, target expression, number of metastatic sites or inflammatory syndromes are likely to modify drug levels in plasma. Of note, genetic polymorphism affecting immunoglobulin G fragment receptor Fc-γ-R has been identified as a putative marker for rituximab clearance, but the clinical importance of *Fc-γ-R* genotype could be more related to the Antibody-Dependent Cell Cytotoxicity (ADCC) of rituximab that involves Fc-γ-R, rather than a pharmacokinetics issue (Cartron et al., 2002). In digestive oncology, Fc-γ-R genotype has been identified in mCRC patients treated with cetuximab as predictive for PFS, but as for rituximab, this could be related to changes in the ADCC described sometimes with cetuximab rather than changes in pharmacokinetics (Zhang et al., 2007), and other studies failed in confirming the impact this polymorphism could have with the anti-EGFR therapy (Graziano et al., 2008).

4. Conclusions: One patient, one disease, one drug, one dosage…. Can we finally do it?

Developing strategies to implement personalized medicine in digestive oncology is now an irreversible trend (Ciccolini et al., 2011). However, identifying predictive biomarkers associated with either treatment efficacy or tolerance remains an uneasy task, because CRC patients are usually treated with up to 6 different drugs in combination over several lines.

Consequently, and despite the abundant literature published, the heterogeneity in the clinical settings can hinder the relevance of some markers, thus preventing standardized guidelines to be issued. However, oncogenetic, pharmacogenetic and pharmacogenomic tools are now developed as a new mean to help oncologists to choose the optimal strategy for each patient, regarding the staging of the disease, the status of the various response markers, and eventually information about specificities in pharmacokinetics and detoxification patterns, once the right drugs have been chosen. However, this later and critical step remains today the forgotten item in routine clinical setting. Although implementing KRAS pharmacogenomic testing is now a systematic practice prior to administrate cetuximab or panitumumab to mCRC patients, little is done to further develop pharmacogenetics-based dose tailoring strategies to reach next the right exposure likely to ensure an optimal efficacy/toxicity balance. Screening for *DPYD* or *UGT1A1* genetic polymorphisms, despite countless clinical reports demonstrating their role in life-threatening toxicities, and therefore the usefulness of preliminary testing in patients undergoing 5-FU, capecitabine or irinotecan-based regimen to anticipate treatment-related toxicities, is far from being a common practice. However, when performed, pharmacoeconomic studies suggest that implementation of such screening is cost-effective, thus suggesting that routine pharmacogenetics should benefit both to the patients and to the institute ultimately, by dramatically cutting the costs dedicated to managing the treatment-related toxicities (Mercier et al., 2009). Of note, no regulatory official step has been undertaken to date to prompt oncologists to require such tests when prescribing cytotoxics to mCRC patients. Changes in the drug label informing physicians about the toxic risks with irinotecan related to the *UGT1A1* genetic polymorphism has been done by the F.D.A in the mid-2000's and official warning issued as a as a level-2 priority, but with little impact in clinical practice, partly because the UGT1A1 test is not reimbursed in the U.S. by most insurance companies (Ikediobi et al., 2009, Meckley et al. 2010), and partly because of the lack of tools to customize the irinotecan dosage once the *UGT1A1* status has been obtained. As of today, it is acknowledged that the UGT1A1*28 genotype is a concern in patients scheduled for irinotecan dosage above 200 mg/m² only, with little further guidelines made available about adaptive dosing strategies to treat patients harboring this polymorphism (Hoskins et al., 2007) because. Usually, an empirical 25-50% reduction in irinotecan starting dose is recommended in patients with the homozygous variant. Similarly, screening for *DPYD* genetic polymorphism is an exceptional, rather than a routine test in most institutes. DPD testing can be required at best once the severe toxicities have already shown in a patient treated with a 5-FU-containing regimen to keep or discard the fluoropyrimidine in the forthcoming course. As for UGT1A1, little tools are available to tailor dosage based upon the DPD status of the patient. However, a case-control study has demonstrated the immediate advantages patients could benefit from prospective DPD testing associated with adaptive-dosing, with a sharp reduction in the incidence of 5-FU-related toxicities in patients screened for DPD deficiency with tailored dosage as compared with patients treated with standard regimen (Yang et al., 2009). Of note, efficacy remained the same in this study despite markedly lower doses in patients with DPD deficiency, thus illustrating how pharmacogenetics-based adaptive dosing could improve indeed the efficacy/toxicity balance of canonical 5-FU. In this respect, prospective clinical trials investigating pharmacogenetics of drugs given in mCRC patients with strong PK, PK/PD and

PK/PD/PGx modeling support should help to develop easy-to-implement tools designed to individualize dosing based upon the patients genotypes or phenotypes.

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15

Animal Models of Colorectal Cancer in Chemoprevention and Therapeutics Development

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1. Introduction

Considering the complexity of genetic and epigenetic events that occurs during colorectal carcinogenesis and the uncertainty in relying solely on extrapolation from cell culture models, it is essential to use animal models relevant to the molecular characteristics of colorectal cancer (CRC). Requirements for a mouse model of CRC are that the model has relevance to the molecular pathways involved in human CRC, that there are correlates with factors that affect the frequency of the disease in human populations, and that the chemopreventive/therapeutic agent-induced signal is sufficient to carry out the project with an affordable and statistically significant number of mice. In this chapter we will discuss how animal models have not only advanced our understanding of CRC initiation and progression but have also greatly facilitated the development of newer chemopreventive and therapeutic strategies to reduce mortality and incidence.

2. Animal models of colorectal cancer

Use of animal models could significantly expedite not only the delineation of molecular pathogenesis of colorectal carcinogenesis but could also aid in the development of newer preventive and therapeutic strategies. Animal tumor models can be classified as spontaneous and artificially transplanted systems. Spontaneous tumor models are now being widely considered for studying the biology of carcinogenesis and development of chemopreventive or chemosuppressive drugs.

Initial animal models of CRC involved use of chemical carcinogens in mouse, rat, as well as in guinea pig. In the last two decades genetically modified mice such as $APC^{Min/+}$ (Min: multiple intestinal neoplasia) with germline APC mutations at different sites have been extensively used for the investigation of therapeutic, chemopreventive and dietary factors for management of colorectal cancer (Hu et al., 2006; Gerner, 2007). Furthermore, studies to identify genetic modifiers of CRC are undertaken by generating mouse models representing molecular events involved in colorectal carcinogenesis like mismatch repair deficiency (MSH2-/-) and crossing

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them with $APC^{Min/+}$(Kwong & Dove, 2009). Other models for CRC targeting relevant pathway are also being developed to elucidate chemopreventive and chemotherapeutic response to newer molecules (Kwong & Dove, 2009). Because there is no ideal animal tumor model, which can mimic all the complexities associated with human CRC, the selection of an appropriate experimental model is crucial to study specific biologic end points towards the understanding of mechanistic, preventive and therapeutic aspects of the cancer.

2.1 Rat models

In the last few decades many murine models have been established that are useful for the investigation of initiation, expansion and progression of gastrointestinal (GI) cancers. In most of these models, Wnt signaling, mismatch repair, and TGFβ pathways are targeted not only to understand initiation and progression of CRC but also to evaluate various pharmaceutical and biological agents for prevention and treatment of CRC.

Normally CRC is not observed in rats (<0.1%) (Goodman et al., 1980) except the Wistar-Furth/Osaka strain that spontaneously develops adenocarcinomas in 30-40% of the animal (Miyamoto & Takizawa, 1975). The rat models of colon cancer are developed using common carcinogens like AOM (azoxymethane), DMH (dimethylhydrazine), or PhIP (2-amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine)(Corpet & Pierre, 2003, 2005). Most of the carcinogen-treated rat models develop tumors in the colon and often progress to adenocarcinomas. However, long latency period of tumor development is a distinct disadvantage. Efforts have been made to generate target-selected mutations, including nonsense alleles by several laboratories resulting into development of a rat strain carrying a nonsense allele in codon 1137 of APC (Corpet & Pierre, 2005). Interestingly, multiple intestinal neoplasms mostly in the colon were observed in F344 rats, commonly known as PIRC (polyposis in the rat colon) model, heterozygous for this allele and these animals survive for about one year (Zan et al., 2003; Smits et al., 2006; Amos-Landgraf et al., 2007). The rat models due to their size allows investigators to perform procedures like endoscopy, microCT (Computerized Tomography), and microPET (Positron Emission Tomography) imaging to evaluate chemoprevention or therapeutic interventions without sacrificing the animal.

2.1.1 Rat models in chemoprevention and therapeutics development

Although, dimethylhydrazine and its metabolites azoxymethane (AOM) and methylazoxymethanol are commonly used in the induction of colonic tumors in rat models, other carcinogens, like nitrosomethyl urea (MNU), specific nitrosamines and heterocyclic amines are also in frequent use. Many potential chemopreventive agents of colorectal cancer have been assessed in rat models. The effects of chemopreventive and therapeutic agents on initiation and progression of carcinogen-induced colonic tumors can be studied by varying the time of intervention. In rat models over 160 compounds have been screened for chemopreventive properties (Corpet et al., 2008) and the compounds found to be of chemopreventive and therapeutic importance are summarized in Table 1. Complete inhibition of cancer induction has been detected in ursodeoxycholic, polyethylene glycol (PEG), methylmethanethiosulfonate (MMTS) treated rats and in rats given exercise. Also, celecoxib, acetoxychavicol, selenium, p53 vaccination, piroxicam with difluoromethylornithine (DFMO), cellulose, aspirin, S-allylcysteine, obacunone, sulindac sulfone and hesperidin (flavanone glycoside) reduced the incidence of adenocarcinoma more than 78% (Corpet et al., 2008). Moreover, a DMBDD (7-hydroxy-7'-methoxy-4,4'-

Rat model	Name of the compound	Reference
Azoxymethane (AOM) /Dimethylhydrazine model	Etoricoxib (selective COX-2 inhibitor)	(Kaur Saini &Nath Sanyal, 2010)
	Diclofenac (preferential COX-2 inhibitor)	(Kaur Saini &Nath Sanyal, 2010)
	Adlay bran ethanol extract (ABE-Ea)	(Chung et al., 2010)
	Soy isoflavones	(Min et al., 2010)
	Arabinoxylan-oligosaccharide	(Femia et al., 2010)
	Probiotic soy products	(Silva et al., 2009)
	Physical exercise	(Silva et al., 2009)
	Astaxanthine	(Prabhu et al., 2009)
	Soy isoflavones	(Raju et al., 2009)
	R-Flurbiprofen	(Martin et al., 2010)
	Copper-indomethacin	(Bonin et al., 2010)
	Naproxen	(Steele et al., 2009)
	Nitric Oxide-Naproxen	(Steele et al., 2009)
	CP-31398 (a p53 modulator)	(Rao et al., 2009)
	Celecoxib	(Rao et al., 2009)
	Symbiotic association of Bifidobacterium lactis and carbohydrate 'resistant starch	(Le Leu et al., 2010)
	Melatonin	(Tanaka et al., 2003)
	Prebiotic germinated barley foodstuff (GBF)	(Kanauchi et al., 2008)
	High amylose maize starch and butyrylated high amylose maize starch	(Clarke et al., 2008)
MNU model	TAC-101 (a retinobenzoic acid derivative)	(Nakayama et al., 2009)
DMBDD model	PJJ-34 (13 alpha, 14alpha-epoxy-3beta-methoxyserratan-21 beta-ol), a triterpenoid	(Doi et al., 2010)
Orthotropic model	Combined use of bevacizumab and irinotecan (CPT-11) as postoperative adjuvant chemotherapy	(Mizobe et al., 2008)
AOM model or Dimethylhydrazine model	Combinatorial therapy using HMG-CoA reductase inhibitor (HRI) lovastatin (LOV) and the selective apoptotic antineoplastic drug (SAAND) exisulind (EXS)	(Kim et al., 2004)
	Ursodeoxycholic acid (UDCA)	(Hess et al., 2004)
	Protective effect of Fullerenol on heart and liver toxicity induced by doxorubicin	(Injac et al., 2009)

Table 1. Rat models used in chemoprevention & chemotherapy.

bis(1,3-benzodioxole)-5,5'-dicarboxylic acid dimethyl ester) rat multiorgan carcinogenicity model has also been developed for carcinogen testing (Takahashi et al., 1992; Imaida et al., 2003). The DMBDD model can be used for prediction of intestinal carcinogenesis risk assessment as well as for chemoprevention studies.

2.2 Mouse models

2.2.1 APC related models

The first heritable mouse model of colon cancer, $APC^{Min/+}$, was reported in 1990 as a result of ethylnitrosourea (ENU)-induced germline truncating mutation at the codon 850 of APC (Moser et al., 1990, 1992; Su et al., 1992). In the C57BL/6J mouse background $APC^{Min/+}$ mice develop about 30 small intestinal polyps with occasional adenocarcinoma and essentially no tumor in the colon (McCart et al., 2008). Although this contrasts human FAP (familial adenomatous polyposis) where most of the adenomas are in the colon and these adenomas certainly progresses to invasive adenocarcinoma, the $APC^{Min/+}$ models due to their phenotypic and histopathological similarities to human intestinal neoplasm are used not only to test therapeutic and chemopreventive interventions but also to understand the role of APC gene in CRC (Fodde & Smits, 2001). The $APC^{Min/+}$ models also proved to be important for the study of genetic modifiers-of-Min (*Mom*) locus. When $APC^{Min/+}$ C57BL/6J mice were crossed to AKR and MA mice, only 6 to 7 intestinal adenomas were observed and backcrossing F1 hybrids to the C57BL/6J helped identify a number of loci modifying number and distribution of adenomas (Moser et al., 1992; Gould et al., 1996; Fodde & Smits, 2001). The adenomas in the small intestine of the $APC^{Min/+}$ mouse have dysplastic and hyperplastic crypts and villi but the colonic tumors are spherical and peduncular with dysplastic cells. Importantly, these adenomas display higher mitotic index than surrounding normal crypts (Kwong & Dove, 2009). The Min mice due to their many advantages have been extensively used by scientists to study molecular pathogenesis of CRC – i] Min mice contains a single genetic change that produces a organ-specific, consistent, and discrete tumor phenotype, ii] Adenomas in Min mice develop rapidly, with lesions visible as early as 60 days, iii] high tumor multiplicities (>100 /intestinal tract) providing strong statistical power, and iv] multiple pathways impacting tumorigenesis enable many entry points for basic or applied study (Kwong & Dove, 2009). Importantly, other mouse models with targeted genetic manipulations at different locations on APC have also been generated (summarized in Table 2). When heterozygous, the Δ474, Δ14, Δ716, lacZ, and Δ1309 mouse models show phenotypes similar to that of Min (Sasai et al., 2000; Oshima et al., 2001; Niho et al., 2003; Colnot et al., 2004). In contrast, heterozygosity for the 1638N allele results in 0-2 tumors (none in the colon) while the 1638T model is tumor-free and unlike any other truncating allele, 1638T homozygous is viable. The 1638N has only approximately 1-2% of the truncated protein and is referred to as leaky allele (Fodde & Smits, 2001). In contrast, 1638T has the full expression level of the truncated protein and is known as truncated allele. Furthermore, Li Q et al., (2005) inserted a neomycin cassette in either orientation (reverse (neoR) or forward (neoF)) into the 13^{th} intron of APC to generate full-length hypomorphic (expression reduced to 10-20%) alleles and showed that these heterozygous mice developed fewer than two adenomas per mouse (Li et al., 2005). The Cre/*loxP* conditional gene targeting system is developed to generate additional APC models to induce tumors specifically in the colon (Shibata et al., 1997; Colnot et al., 2004; Gounari et al., 2005; Hinoi et al., 2007).

Allele/truncation location	Average life-span	Genetic back-ground	Homozygous pheno-type	Heterozygous phenotype	Average no. of spontaneous tumors	References
Min/850	4-6 months	C57BL/6J	Embryonic lethality	Multiple adenoma in GI-tract (mainly small intestine)	50-70	(Moser et al., 1990; Su et al., 1992)
Min/850	3-5	BTBR/Pas	Embryonic lethality	Multiple adenoma in GI-tract (mainly small intestine)	Up to 600	(Kwong et al., 2007)
Δ716/716	5-7 months	C57BL/6J	Embryonic lethality	Multiple adenoma in GI-tract	Up to 300	(Oshima et al., 1995)
580S/580* (based on Cre-LoxP recombination system)	N/A	Mixed	N/A	N/A	No tumor up to 1year of age	(Shibata et al., 1997)
580D/580*	N/A	Mixed	Embryonic lethality	Multiple adenomas at 4 week after conditional deletion	7-10	(Shibata et al., 1997)
1638N/1638	>1Year	C57BL/6J	Embryonic lethality	Adenoma and adenocarcinomas in GI-tract, desmoids tumors	3-4	(Fodde et al., 1994)
1638T/1638	Up to 2 Years	C57BL/6J	Viable	Normal	0	(Smits et al., 1999)
Δ474/474	<6 months	C57BL/6J	Embryonic lethality	Develops tumor mainly in small intestine, but also in colon and stomach	120	(Sasai et al., 2000)
Δ1309/1309	N/R	C57BL/6J	N/R		25-40	(Niho et al., 2003)
Δ14/580*	N/A	C57BL/6J	N/A	Multiple adenomas	40-50	(Colnot et al., 2004)
Δ468/468	N/R	N/R	Embryonic lethality	Polyps starting after 2 months of age	N/R	(Gounari et al., 2005)
Ex13NeoR/ Full-length	>15 months	C57BL/6J	N/R	Small microadenoma	1.1	(Li et al., 2005)
Ex13NeoF/ Full-length	>15 months	C57BL/6J	N/R	Small microadenoma	0.3	(Li et al., 2005)

*Conditional expression; N/A=Not applicable; N/R=Not reported

Table 2. Adematous polyposis coli (APC) based intestinal tumor models

Because $APC^{Min/+}$ mice rarely develop invasive cancer, efforts were made to develop compound mouse models by introducing alterations of genes known to be involved in CRC signaling pathways and produce invasive tumors mimicking human disease. The oncogene Kras, which is mutated in 40-50% of human CRC, is not altered in $APC^{Min/+}$ polyps and the $APC^{Min/+}$ mice with the introduction of Kras develop aggressive adenocarcinoma (Fodde & Smits, 2001). Loss of EphB receptor is an important event in the progression of CRC and APCMin/+ carrying a dominant negative EphB2 transgene showed 10 fold more tumor formation with greater number of invasive adenocarcinomas (Fodde & Smits, 2001; Batlle et al., 2005). The $APC^{Min/+}$ mouse model has been quite useful as an experimental system for studying colorectal tumorigenesis and CRC chemoprevention strategies (Moser et al., 1990) because Min mice have two distinct advantages i] numerous adenomas with the same inherited APC mutation are available for analysis and ii] these adenomas develop in animals of uniform genetic background (Luongo et al., 1994).

2.2.2 Mismatch repair (MMR) deficient models

The HNPCC (hereditary nonpolyposis colorectal cancer) is an inherited condition with inactivated DNA mismatch repair (MMR) genes, like *MLH1, MSH2, MSH6*, and *PMS2* (Fishel et al., 1993; Lynch & de la Chapelle, 2003) and leads to the development of a variety of cancers including that of the colon (Lynch & Smyrk, 1996). Mouse models with loss of function of MMR genes have been generated and mice lacking *Mlh1*, *Msh2* and *Msh6* develop tumors in stomach, small intestine, and colon. However, these mice also develop cancers of the lymphatic system, skin and lung (Reitmair et al., 1996; Edelmann et al., 1997, 1999, 2000). Enhanced development of adenomas was observed in the $APC^{Min/+}$ mice lacking *Msh2* with increase in colonic adenoma numbers. Interestingly, these mice show normal growth and can reproduce but have reduced life span (Reitmair et al., 1996). Although loss of *Msh3* is not associated with increased tumors, loss of both the *Msh3* and *Msh6* leads to an increase in GI tumors at a younger age, similar to *Mlh1* or *Msh2-deficient mice* (Edelmann et al., 2000). Mice bearing mutations in the *Msh6* gene have a life span of 18 months and develop GI tumors within one year. The MMR mouse models carrying one functional copy of APC showed increasing mutation of APC and an enhanced frequency of intestinal neoplasia (Reitmair et al., 1996; Kuraguchi et al., 2001). Furthermore, mice lacking *Mlh1* in APC1638N model have a 40-fold increase in adenomas compared to APC1638N mice alone (Edelmann et al., 1999). Interestingly, the $PMS2^{-/-}$ mice are vulnerable to lymphomas but they do not develop GI tumors. However, the $PMS2^{-/-}$ mice in APCMin background showed an increased number of adenomas in the GI-tract compared to Min alone (Prolla et al., 1998; Prolla, 1998; Baker et al., 1995). In contrast, the Mlh1/APC1638N mice showed a greater percentage of tumors progressed to invasive carcinomas (Edelmann et al., 1999). The MMR models are useful for screening of agents known to interfere with DNA mismatch repair processes for their therapeutic or carcinogenic effects.

2.2.3 TGF-β models

The TGF-β (transforming growth factor-β) signaling pathway regulates a number of cellular processes including cellular differentiation, growth suppression, deposition of extracellular matrix and apoptosis (Figure 1). The TGF ligands through a heteromeric receptor mediate their effects on cells and dysregulation of the TGF-β receptor 2 (TGF-βR2) is the most common occurrence in the CRC (Bellam & Pasche, 2010; Grady et al., 1999). Although TGFβR2 has been suggested to have a tumor suppressor function in CRC, recent reports indicate that it could act as a tumor suppressor as well as a tumor promoter (Tang et al., 1998;

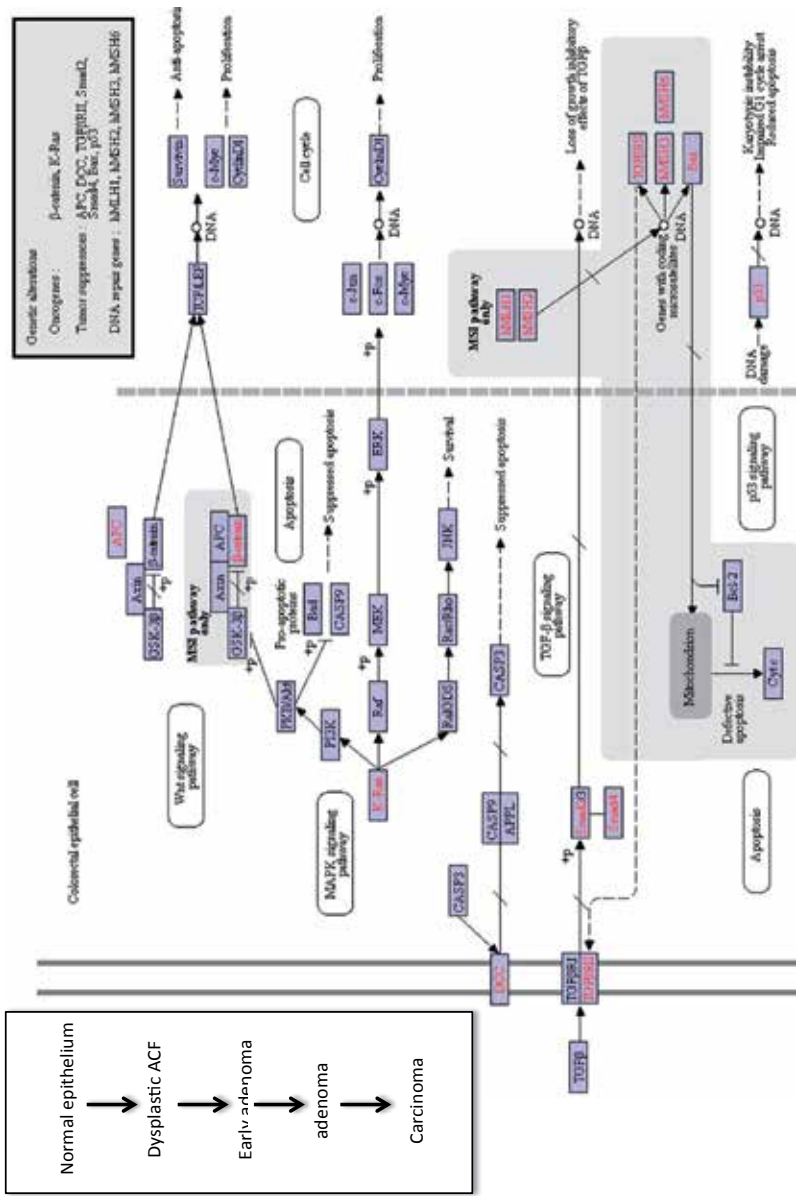

Fig. 1. Integrated molecular pathway implicated in development of colorectal cancer (Courtesy: KEGG pathway, www.genome.jp/kegg). Alterations in Wnt signaling including Wnt, APC, Axin and TCFs are associated increased β-catenin level and increased cell proliferation. The MAPK signaling is associated with the oncogenic activation of RAS and ERK signaling leading to increase cell proliferation). The TGF-β pathway is mainly a growth inhibitory pathway and any perturbations leads to suppressed apoptosis and increased cell proliferation. The mismatch repair (MMR) pathway maintains DNA homeostasis by facilitating post-replication repair and dysfunction results in accumulation of potential mutations and genetic instability implicated in the development of CRC. Important candidate proteins altered in CRC are highlighted in red color.

Yang et al., 2002a, b; Blobe et al., 2000). Mouse models related to the TGF-β pathway have been used to delineate the multifaceted role of this pathway during colorectal carcinogenesis. It has been reported that the *TGF-β1* deficient mice due to widespread inflammation die around three weeks of age (Shull et al., 1992; Kulkarni & Karlsson, 1993). Importantly, in the absence of *Rag2* the *TGF-β1*-deficient mice survive until adulthood (Diebold et al., 1995). Due to the significant incidence of carcinoma of the cecum and colon in these mice, the *Rag2/TGF-β1*-deficient mice remains a useful model to study the role of inflammation in CRC in relation to the TGFβ1 signaling (Engle et al., 1999; Maggio-Price et al., 2006). Mouse lacking TGF-β2 in the colonic epithelium showed increased adenoma and adenocarcinoma formation after carcinogen treatment. Apart from TGF-β, mouse models representing alterations in the other factors in this important pathway like SMAD2, SMAD4 and SMAD3 have been reported. (Eppert et al., 1996). Although mice lacking SMAD2 and SMAD4 are embryonically lethal, the SMAD3 deficient mice are viable and are a useful model of CRC (Zhu et al., 1998). Importance of the TGF-β related models lies in the fact that the APC remains intact in the adenomas of these mice and these models could serve as a valuable tool to investigate non-WNT/APC/β-catenin-mediated colorectal carcinogenesis (Kaiser et al., 2007). Interestingly, the TGF-β related models in the $APC^{Min/+}$ background showed increased incidence of invasive carcinoma specifically in the distal colon (Takaku et al., 1998; Sodir et al., 2006).

2.2.4 Inflammation mediated models

Inflammatory bowel diseases (IBD) like ulcerative colitis (UC) and Crohn's Disease are predisposing conditions of CRC (Itzkowitz & Harpaz, 2004; Itzkowitz & Yio, 2004). Prolonged administration of dextransulfate sodium (DSS) in mice resulting in chronic colitis and formation of high-grade dysplasia confirmed the involvement of chronic inflammation in colorectal carcinogenesis (Okayasu et al., 1990). Interestingly intestinal tumorigenesis was augmented by combined administration of AOM and DSS. (Tanaka et al., 2003). To demonstrate that deficiency of *Sigirr* (single immunoglobulin and toll-interleukin 1 receptor domain) along with bacteria-induced inflammation increases susceptibility to CRC investigators effectively utilized the combined AOM/DSS model (Wald et al., 2003; Xiao et al., 2007; Uronis & Threadgill, 2009). Furthermore, the combined AOM/DSS model was also an important instrument in defining the role of the JAK/STAT (Janus kinase/signal transducers and activators of transcription) and NFkB (nuclear factor of kappa light chain gene enhancer in B-cells) pathways in inflammation-induced CRC (Wirtz & Neurath, 2007). The role of the JAK/STAT pathway in colorectal carcinogenesis was further confirmed by the fact that dysfunctional *Socs1* and *Socs3* (suppressors of cytokine signaling) leads to enhanced activation of STAT1, STAT3 and NFkB and subsequent growth of colorectal tumors (Hanada et al., 2006). Although lack of only *Socs3* in the intestinal epithelial cells is not associated with chronic inflammation or tumor formation, these mice when treated with AOM/DSS showed distinct inflammatory response followed by colonic tumors (Hanada et al., 2006; Rigby et al., 2007). In contrast to increased nuclear β-catenin in the *Socs3* deficient mice, the colorectal tumors in *Socs1* deficient mice display enhanced expression of *Myc* (Sutherland et al., 2006). In addition, the $Muc2^{-/-}$ is an important animal model to study the role of inflammation in the colorectal carcinogenesis. This model specifically targets mucin-forming Muc2 and unlike other models tumor formation is also observed in the rectum (Mack & Hollingsworth, 1994; Yang et al., 2005; Femia et al., 2009).

2.2.5 Immune system related models

Models have been developed to investigate the role of immune system in colorectal carcinogenesis. Immune cells are involved in inflammatory response and inflammation is intimately related to CRC. Therefore, the IL-2 (interleukin-2), IL-10, and TCRα knockout models were developed and used in studying role of diet and inflammation in colorectal cancer initiation and progression. (Rudolph et al., 1995; Kullberg et al., 1998; Mizoguchi et al., 2000; Seril et al., 2005).

2.2.6 Carcinogen-induced models

Although important information on familial and sporadic colorectal carcinogenesis was obtained from genetic animal models, it is the carcinogen-induced animal models that were instrumental in delineating molecular events specifically in the sporadic CRC. It is worth mentioning that the colon-specific carcinogen dimethylhyrdrazine (DMH) along with AOM has been useful in developing our current understanding of the molecular mechanisms underlying sporadic colorectal carcinogenesis (Druckrey et al., 1966). To its advantage the carcinogen-induced mouse model develops tumor which show much similarities to the pathophysiology of the human CRC (Kaiser et al., 2007; Uronis et al., 2007). The carcinogen-mediated tumors show alterations in the WNT/β-catenin pathway (Takahashi et al., 2000). Interestingly, the AOM-induced colorectal carcinogenesis, unlike in the $APC^{Min/+}$, is mainly due to the mutations in the *Ctnnb1 gene*, which encodes β-catenin protein. Mutations in the *Ctnnb1* results in ubiquitination-resistant stabilization of the β-catenin leading to growth of colorectal adenomas associated with upregulation of proliferation markers like cyclin D1 and c*Myc* (Wang et al., 1998; Kaiser et al., 2007). The carcinogen-induced model was also a key player in the identification of the modifier loci like the *Ptprj* (a receptor-type protein tyrosine phosphatase), which has been shown to modify susceptibility to DMH and has shown frequent loss of heterozygosity in human colon cancer (Ruivenkamp et al., 2002). Additionally, the carcinogen-induced models led to the recognition of *Pref1* as a modifier of CRC and the promoter of the *Pref1* is suggested to contain a β-catenin/TCF response element (Dong et al., 2004; Ruivenkamp et al., 2002; Uronis et al., 2007).

2.2.7 Mouse models in chemoprevention and therapeutics development

The genetic mouse models as well as the carcinogen-induced models of CRC (APC, TGF-β and mismatch repair based models) are used to evaluate the effect of diets and chemopreventive agents. The chemopreventive and dietary interventions are usually started between 3 and 6 weeks of age and invariably the principal biological endpoint is the number and grade of the tumors. It is important to note that in contrast to human CRC where no small intestinal tumors are observed, the tumors in the mutant models are mostly in the small intestine. However, the concept that the non-steroidal anti-inflammatory drugs (NSAID) have a chemopreventive role in the CRC was first established in these models showing reduction in tumor number in the small intestine. The chemopreventive properties of NSAIDs like celecoxib and piroxicam were also validated in the carcinogen-induced models showing decrease in polyp number and size. Due to commercial availability, the $APC^{Min/+}$ is the model of choice in many studies and a list of agents screened in $APC^{Min/+}$ mouse model is shown in Table 3. Most of the other CRC mouse models are used for specific biological end points. But a few of them such as APC1638N model because of its longer life span and good signal to noise ratio is used for carcinogen testing and is suggested to be more useful than $APC^{Min/+}$ mice (Trani et al., 2010).

Name of compound	References
Aspirin	(Barnes&Lee, 1998)
Piroxicam	(Ritland&Gendler, 1999)
Phenanthridinone derivative (PJ 34)	(Mabley et al., 2004)
Non-steroidal anti-inflammatory drugs (NSAIDs)	(Gescher, 2004)
DMU-135 (3-4 methylenedioxy-3′4′5′-trimethoxy chalcone), a potent tyrosine kinase inhibitor prodrug	(Sale et al., 2006)
Atorvastatin	(Swamy et al., 2006)
Celecoxib	(Swamy et al., 2006)
Sulforaphane (Isothiocynate compound)	(Khor et al., 2006)
Anthocyanin	(Bobe et al., 2006)
Difluoro methylornithine (DFMO)	(Telang&Katdare, 2007)
Epigallocatechin 3- gallate (EGCG)	(Telang&Katdare, 2007)
Dibenzoylmethane	(Tammariello&Milner, 2010)
PPAR ligand MCC-555	(Yamaguchi et al., 2008)
Metformin	(Tomimoto et al., 2008)
Alpha-phenyl-tert-butyl-nitrone (PBN) and 4 hydroxy-PBN	(Floyd et al., 2010)
Chafuroside	(Tammariello&Milner, 2010)
Sodium Taurocholate	(Smith et al., 2010)
COX-2 inhibitors	(Nakanishi et al., 2011)
Scopolamine butylbromide (muscarinic receptor antagonist)	(Raufman et al., 2011)
Curcumin	(Murphy et al., 2011)
Silibinin	(Rajamanickam et al., 2010)
Ellagic acid	(Mutanen et al., 2008)
Epigallocatechin gallate (EGCG)	(Telang&Katdare, 2007)
Dietary sphingolipids	(Symolon et al., 2004)
Dietary Folate	(Song et al., 2000)
Dietary isoflavones	(Sorensen et al., 1998)
Apple polyphenol extract (APE)	(Fini et al., 2011)
Grape Seed extract (GSE)	(Velmurugan et al., 2010)
Berries (bilberry, lingonberry, cloudberry)	(Mutanen et al., 2008)
Green tea	(Issa et al., 2007)
Orange peel extract (OPE)	(Fan et al., 2007)
Anthocyanin rich tart cherry extract	(Bobe et al., 2006)
Fermented brown rice and rice bran	(Phutthaphadoong et al., 2010)
Fish oil	(Bose et al., 2007)
Dietary /caloric restriction	(Tammariello&Milner, 2010)
Exercise/physical activity	(Baltgalvis et al., 2008)

Table 3. Use of $APC^{Min/+}$ mouse model in chemoprevention development.

2.3 Xenograft models of colon cancer

Colon cancer xenograft models are created by implantation of cells subcutaneously, intrasplenically, or into the renal capsule. It is important to implant the xenograft into the immunocompromised mice and commonly the T-cell deficient ''nude'' mice or NOD-SCID (non-obese diabetic/severe combined immunodeficiency) mice are used (Rygaard & Povlsen, 1969). The xenograft models of CRC are commonly used to assess newer therapeutics and understand the pathogenesis of human disease. Indeed, subcutaneous xenografts have found an important place in CRC research due to the fact that anesthetics are not required and the tumors are accessible for external measurement. Some of the disadvantages of the subcutaneous model are i] lack of tumor microenvironment representative of the CRC, and ii] in contrast to the >50% hepatic metastatic incidence of CRC, no metastasis is observed in the subcutaneous xenograft models. However, xenograft models involving intrasplenic or intra-renal-capsule, although have shown metastasis similar to human CRC, does not represent tumor microenvironment of CRC and signaling pathway could be different than the human disease (Furukawa et al., 1993; Fidler, 1991a, b, c). Consequently, implantation of CRC xenografts into mouse colon, the orthotopic model, is much preferred by the investigators due to their similar characteristics of the human ailment.

2.4 Orthotopic mouse model

An orthotopic mouse model involves placing of colorectal cancer cell or tumor tissue into the intestinal sub-mucosa (Tseng et al., 2007). The orthotopic model, unlike the subcutaneous model, is associated with all of the components of the tumor microenvironment as well as all of the angiogenic and growth factors, and cytokines. In addition to mimicking the human CRC in terms of metastasis and microenvironments, the orthotopic model also allows assessment of the alterations in the microenvironment on tumor initiation and progression. From a technical point of view, generation of orthotopic models demands specific expertise and more time than subcutaneous models. Because of technical difficulties in the physical measurement of the tumors, the orthotopic model also requires that an appropriate reporter like luciferase be in place for measuring tumor growth to determine the efficacy of a drug treatment. As with any animal model of human diseases there are inherent shortcoming and the orthotopic animal model is no exception. Because the tumors are in the colon, the orthotopic model requires sacrifice of the animals at a predetermined time for quantitative and qualitative analysis of the tumor. However, the orthotopic model has the advantage of mimicking human CRC including tumor microenvironment.

3. Zebrafish – A non-murine model of colorectal cancer

Signaling pathways involved in colorectal carcinogenesis are conserved across species and zebrafish, a well-characterized simple model system for human disease, are widely used to understand the molecular basis of cancer including CRC. Water borne carcinogens induce a wide variety of benign and malignant tumors in many organs of zebrafish. Zebrafish due to its easy maintainence and breeding along with conservation of human cancer-relevant oncogenes, and tumor suppressor and cell cycle genes makes it a useful model to study carcinogenesis. Interestingly, the zebrafish mutants display phenotypes similar to many human disorders, including cancer, cardiovascular disease, and neurodegeneration. Zebrafish carrying a mutation in the region representing most of the observed human APC

mutations were identified recently and like murine models the heterozygous fish develop intestinal adenomas (12%), which resembles its murine counterpart (Goessling et al., 2007). The APC-heterozygous fish when exposed to dimethylbenzanthracene, showed significant increase in the tumor number with 44% developing liver tumors and 35% developing intestinal tumors and could serve as an important model system to screen carcinogens (Goessling et al., 2007).

3.1 Zebrafish model in chemoprevention and therapy

Zebrafish model is in use for the target selection, bioactive compound screening as well as in the drug toxicity and efficacy studies (Lieschke & Currie, 2007). Angiogenesis supports cancer progression including CRC and anti-angiogenic therapy inhibits cancer growth. On the contrary anti-angiogenic therapy has been implicated in inflammation a known risk factor of colorectal carcinogenesis. Our understanding of the correlation between tumor angiogenesis, inflammation, and metastasis was much enhanced by studies in the zebrafish model (Moshal et al., 2010). Furthermore, the zebrafish model has been used to study potential therapeutic agents like SKLB610 (inhibitor of angiogenesis related tyrosine kinase), which was reported to inhibit angiogenesis in zebrafish sub-intestinal veins (Cao et al., 2011). The zebrafish model was useful in determining the role of DNA demthylase in maintaining intestinal epithelial cells lacking APC in an undifferentiated state (Rai et al., 2010). Additionally, it has been reported that the zebrafish expressing a truncated form of APC with either retinoic acid or a selective COX-2 inhibitor decreased β-catenin in the cell. Curcumin-loaded biodegradable polymeric micelles (Cur/MPEG-PCL) has been shown to efficiently block angiogenesis in transgenic zebrafish model (Gou et al., 2011). Similarly, the incorporation of doxorubicin in MPEG-PCL micelles enhanced the anticancer activity and decreased the systemic toxicity of doxorubicin in Zebrafish and has implications for CRC treatment (Lee et al., 2006).

4. Animal models in chemoprevention and chemotherapeutics development

Rodent models have been used for CRC research providing insight into the complex oncogenic events contributing to the loss of cell growth and differentiation control. These models also offer prospects to identify and study both therapeutic and chemopreventive agents. In general, almost all popular human colorectal cancer prevention strategies, from dietary manipulations (such as folate or calcium supplementation), to drug testing (such as (NSAIDs) have been evaluated in both carcinogen-induced and genetically modified animal models. In most cases, suppression of polyps has demonstrated the preventive effects of these strategies, and in some cases, investigators have been able to dissect pathways where these agents block the development of aberrant crypt foci (ACF). Moreover, approaches that are effective in preventing the early stages of colorectal tumorigenesis have been shown to actually promote tumor growth in later stages of the adenoma-carcinoma sequence. This type of observation in mice is important in polyp prevention studies in humans where folate supplementation may actually be harmful in subjects already predisposed to colorectal neoplasia. Although it is important to note that caution has to be exercised in extrapolating animal model data to human, at least studies with NSAID have shown similar protective effects both in human and animal. However, some differential response of chemopreventive drugs has been observed between animal studies and human response. This could be because of higher genetic homogeneity of mice compared to humans, physiologic differences

in gut motility, hormones and immune surveillance, and differences in genetic events in somatic cells during adenoma to carcinoma transition in mouse compared to human colorectal neoplasia.

5. Comparison of human data with animal model data

With few exceptions, a significant correlation was observed between animal and human studies. Both the AOM rat model and the mutant mouse models supported the chemopreventive effects of the NSAID. This is supportive of the epidemiological studies proposing that, NSAIDs might reduce the colorectal cancer incidence by at least 45% in humans. Also supportive is the effects of celecoxib and sulindac shown to decrease the number of polyps in FAP patient trials. Similar to human data, rats and mice fed a high-fat diet showed increased adenomas than those fed a low-fat diet thus establishing the relationship between the colon cancer incidence and the intake of fat. Fatty diets with high linoleic acid content and n-6-polyunsaturated fatty acids seem to increase the number of tumors in rodents. Moreover, caloric reduction is a strategy that seems very efficient in animals. A reasonable agreement is observed between the results of these animal studies and the more limited clinical studies with few differences (Corpet & Pierre, 2003).

6. Limitations of animal models

Currently a number of animal models are available to dissect various facets of CRC and to undertake risk estimation studies. Mutant mouse models provide a unique opportunity in studying numerous adenomas under defined experimental conditions and uniform genetic background. However, use of animal models in studying human disease has its own limitations. For example, in carcinogen-induced models of CRC, the tumor incidence and latency period could be modulated by amount of carcinogen used – higher amount of carcinogen leading to higher incidence of tumors. However, high ethanol consumption reduced carcinogen (DMH)-induced tumorigenesis suggesting that DMH model is not useful in determining the role of alcohol in CRC. This discrepancy was resolved using the $APC^{Min/+}$ mice model where ethanol consumption was observed as a risk factor for CRC (Roy et al., 2002). Careful consideration is essential for the selection of animal model to study a particular agent and requires validation is two or more models for the unequivocal demonstration.

7. Conclusions

Future advances in animal model development will require combinations of dietary and genetic manipulation of rodents or other inexpensive animals to more accurately mimic the various factors that contribute to colorectal neoplasia in humans. As epidemiologic and molecular studies demonstrate the heterogeneity of colorectal tumor development in diverse populations (e.g. the microsatellite instability or CIMP pathways), it is expected that any one model will not answer all the questions about the CRC chemoprevention or therapeutic intervention strategies under investigation.

8. Acknowledgment

This work was supported by funding from NASA (Grant#NNX09AU95G).

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The Stem Cell Environment: Kinetics, Signaling and Markers

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1. Introduction

Colorectal cancer (CRC) is one of the commonest cancers and the third leading cause of cancer death. In the developed world more than 1 million individuals will develop colorectal cancer every year (Parkin et al. 2005). According to the Surveillance, Epidemiology and End Results (SEER) Program database, the prognosis of CRC has an improving trend. 5-year survival rates have risen from 56.5% for patients diagnosed in the early 1980s to as much as 63.2% for those diagnosed in the early 1990s and most recently to 64.9%. Currently, rectal cancer is usually discussed together with colon cancer and historically accounted for more than 50% of CRCs. However, this has now decreased to less than colon cancers; in a recent review the incidence in the European Union was approximately 35% of the total CRC incidence (Glimelius and Oliveira 2009). The prognosis of rectal cancers is worse than that of colon cancers (Enblad et al. 1988), and the clinical treatment of rectal cancers is different from that of colon cancers (Vo et al. 2010). Rectal cancer is a very different tumor from colon cancer because of the anatomical narrow confines of the pelvis, the proximity of the genitourinary organs and nerves, and the anal sphincter mechanism. Oncological cure remains the primary aim of treatment for rectal cancer, but sparing of the anal sphincters with adequate bowel, genitourinary, and sexual function is also taken into consideration.

Pre-operative staging is crucial to stratify patients into one of three treatment strategies: patients whose tumors are superficial require surgery alone, patients with operable tumors but at an increased risk of local recurrence require short course radiotherapy and then optimal surgery, and thirdly those with more locally advanced rectal cancers with close or involved circumferential resection margins require neoadjuvant chemoradiation (CRT) followed by surgery. Early detection and treatment is vital to better survival. The five year survival rate of patients diagnosed with early stage CRC is approximately 90% as opposed to close to 10% for those diagnosed with locally advanced or metastatic disease. Indeed, the median survival of patients with metastatic CRC is only two years despite multiple available treatment modalities, including surgical resection, chemoradiation, monoclonal

antibodies to tumor growth factors, and liver-directed therapies for metastatic disease. Few patients are sensitive to these therapies and even fewer are cured.

The early pathway to CRC tumorigenesis has been well elucidated by the seminal work of Vogelstein and colleagues in which a single colorectal epithelial cell acquires a mutation in the tumor suppressor APC gene (Jones et al. 2008). The cells subsequently acquire a complex array of molecular mutations and quickly acquire the potential to metastasize (Jones et al. 2008). The concept of clonal evolution which postulated that tumor progression results from acquired genetic variability within the original mutated clone allowing sequential selection of more aggressive sub-lines provided a ready explanation for the relentless advance toward ever more malignant behavior within established tumors (Nowell 1976) including colorectal cancer (Fearon and Vogelstein 1990). However, prior to the theory of clonal evolution, the cancer stem cell concept had been formulated to account for the heterogeneity, resistance to treatment, and dormancy exhibited by many solid tumors (Pierce and Speers 1988). The CSC concept postulated that, similar to the growth of normal proliferative tissues such as bone marrow, skin or intestinal epithelium, the growth of tumors is driven by limited numbers of dedicated stem cells that are capable of self-renewal. More recently, the CSC concept has gained more momentum due to studies in leukemia. These studies showed that engraftment of tumors in an immunodeficient mouse could only be initiated from a specific subpopulation of $CD34^{+}CD38^{-}$ cells and led to the identification of a CSC in acute myeloid leukemia (AML) (Bonnet and Dick 1997). In 2003, Clarke and colleagues (Al-Hajj et al. 2003) applied the same concepts and experimental approaches to a solid breast cancer tumor and showed that as few as 100 $CD44^{+}CD24^{-/low}$ cells were tumorigenic, whereas tens of thousands of cells with alternate phenotypes were not. CSC theory and clonal evolution are not mutually exclusive, and it is likely that a single tumor may contain multiple cancer stem cell clones that are genetically distinct. However, they will always have a common ancestor in the stem cell that sustained the first oncogenic mutation and became the origin of the tumor.

2. Cancer stem cell theory

Stem cell concepts and their application to cancer is not a new subject (Clevers 2011; Wicha et al. 2006). As far back as the nineteenth century, it was recognized that tumors exhibit profound histological heterogeneity. In the 1930's, it was discovered that a single cell from a mouse tumor could initiate a new tumor in a recipient mouse. Subsequently, several studies showed that the number of cells with tumor-initiating properties in solid tumors and leukemias was found to be variable but low (10^3 to 10^7 cells). The resulting tumors typically showed the morphologic heterogeneity of the original tumor.

CSCs possess several key properties of normal tissue stem cells including self-renewal, unlimited proliferative potential, infrequent or slow replication, resistance to toxic xenobiotics, enhanced DNA repair capacity, and the ability to give rise to daughter cells that differentiate. However, unlike highly regulated tissue stem cells, CSCs demonstrate deregulated self-renewal/differentiation processes and generate daughter cells that arrest at various stages of differentiation. The progeny of the stem cells make up the bulk of the tumor and are characterized by rapid replication, limited proliferative potential, and the inability to form a new tumor. Only the CSC is able to initiate tumor formation as it is solely capable of self-renewal (Figure 1). CSCs are thought to maintain their numbers by slow self-replication and produce other tumor cells by asymmetric cell division. In this process, cell division of a CSC generates a CSC and a transformed "progenitor-like" cell, which has limited self-renewal ability but is highly proliferative, similar to a transit-amplifying

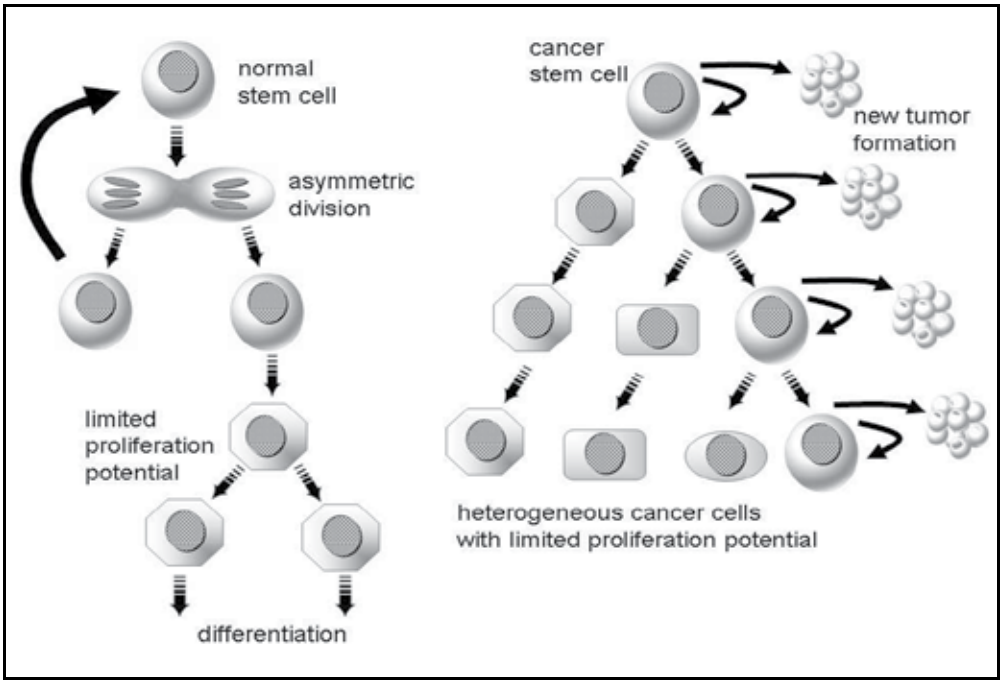

Fig. 1. The cancer stem cell hypothesis. In normal tissues, cell numbers are regulated by asymmetric division of stem cells to replace the loss of functional differentiated cells. In cancer, the mutated stem cell drives tumor heterogeneity through asymmetric division and aberrant proliferation/differentiation. Only the CSC has the capacity to form new tumors and accumulate further mutations leading to tumor progression.

population in normal tissues. These progenitors give rise to more or less partially differentiated bulk tumor cells through a combination of proliferation and abortive differentiation.

The existence of CSCs has been supported by seminal research performed on acute myeloid leukemia (AML), where it has been demonstrated that only a specific cellular subset that express antigenic markers similar to hematopoietic stem cells has clonogenic activity in immunocompromised mice (Lapidot et al. 1994; Bonnet and Dick 1997). The cell responsible for tumor initiation was identified as having a $CD34^+$ $CD38^-$ phenotype; interestingly the majority of AML cells tend to be $CD34^-$. It was observed that as few as 5×10^3 $CD34^+$ $CD38^-$ cells could successfully engraft an immunocompromised mouse, while 100 times more $CD34^-$ or $CD34^+$ $CD38^+$ cells were not tumorigenic (Bonnet and Dick 1997). Significantly, the resulting tumors were heterogeneous and composed of a mixture of tumorigenic and non-tumorigenic cells similar to the donor leukemia. Subsequently, studies on tumors of epithelial origin, such as breast cancer, also provided evidence for the presence of stem-like cells within the cancer (Dontu et al. 2005). Ponti and colleagues demonstrated that only $CD44^+/CD24^-$ cells, isolated from breast cancer, were able to produce tumors in immunocompromised mice (Ponti et al. 2005). The initial publications in leukemia and breast cancer were followed by reports showing the prospective isolation of CSCs in numerous malignancies including: brain, colon, head and neck, pancreatic, melanoma, mesenchymal, hepatic, lung, prostate, and ovarian tumors (O'Brien et al. 2010). The

existence of CSCs in rectal cancer has yet to be verified by stem cell isolation and xenotransplantation into immunodeficient mice.

3. Intestinal structure and the stem cell niche

The large bowel consists of a rapidly proliferating and perpetually differentiating epithelium. Unlike the small intestine, the mucosal surface of the colon has no villi. The ileocecal junction marks an abrupt transition from the villi of the small intestine to the smooth glandular pattern of colon. The crypts of Leiberkuhn continue, and these straight tubular glands are lined with simple columnar epithelium for the reabsorbtion of water and electrolytes, numerous goblet cells for mucus secretion, stem cells for replication, and occasional enteroendocrine cells. Stem cells located in the crypts of Lieberkuhn give rise to proliferating progenitor or transit amplifying cells that differentiate into the four major epithelial cell types (Figure 2). These include columnar absorptive cells or enterocytes, mucous-secreting goblet cells, enteroendocrine cells, and Paneth cells. Enterocytic, goblet, and enteroendocrine cell differentiation takes place during migration upward from the crypt to the surface epithelium, whereas Paneth cells complete their differentiation at the crypt base. The crypt is surrounded by the supporting lamina propria which contains cells of mesenchymal origin, the pericryptal myofibroblasts, which are derived from a mesenchymal lineage.

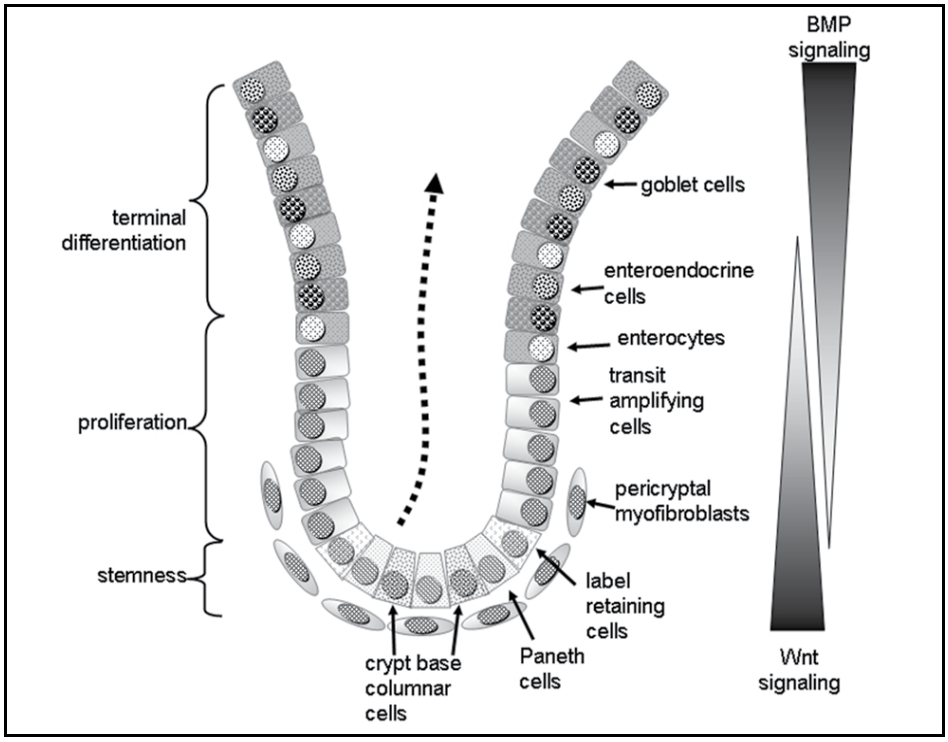

Fig. 2. The intestinal stem cell structure. Two putative populations of stem cells exist: (1) a quiescent/reserved population that consists of label retaining cells located above the basally situated Paneth cell and (2) an actively cycling/primed population that consists of crypt base columnar cells.

Much of the detailed research on intestinal stem cells has been carried out in the setting of the small intestine where the localization of putative intestinal stem cells (ISCs) was initially studied using indirect means. Based on the premise that stem cells may be slowly cycling, Potten and colleagues used long-term label retaining techniques (tritiated thymidine or bromodeoxyuridine) to mark putative ISCs in the small intestine (Potten et al. 1997). They detected long-term label-retaining cells (LRCs) in an annulus four cells up (+4 LRCs) from the crypt base. More recently, using in vivo lineage tracing, it was shown that cells expressing BMI1 predominantly mark +4 LRC position and are able to give rise to all four epithelial lineages (Sangiorgi and Capecchi 2009). BMI1 encodes a chromatin remodeling protein of the polycomb group that has essential roles in self-renewal of hematopoietic and neural stem cells. However, an alternative hypothesis was put forward by Barker and colleagues, who identified a WNT target gene, LGR5/GPR49, which is expressed exclusively in crypt base columnar cells (CBCs) at crypt positions 1–4 (Barker et al. 2007). They elegantly showed that LGR5- expressing CBCs fulfill all criteria of putative ISCs in that they can persist for a long time, self-renew, give rise to all mature intestinal epithelial cells, and are also apoptosis-resistant. However, in contrast to the putative +4 LRC/BMI1 cells, they are highly proliferative. This raises the possibility that there are two types of intestinal stem cells: quiescent stem cells +4 LRC/BMI1 reflecting their inhibitory microenvironment, and the active CBC/LGR5-positive stem cells, representing a population of stem cells able to respond to stimulating signals generated from adjacent mesenchymal cells (Scoville et al. 2008). Very recently, work from Clevers laboratory has implicated the Paneth cell as an important component of the CBC/LGR5 niche (Sato et al. 2011).

There are clear differences between the small and large intestine, apart from the lack of villi. Paneth cells are not generated in the large intestine, and there are differences in the enteroendocrine cell types. However, colonic stem cells have also been shown to reside in the base of the crypts within the stem-cell niche, which is formed by the stem cells themselves and mesenchymal cells that surround the crypt base (Potten 1998). Using bromodeoxyuridine injections into patients with various colorectal cancers, we were able to show marked differences in the proliferation characteristics of "normal" ileum, colon and rectal mucosa (Potten et al. 1992). The mean crypt height in sections of the human colon was 81.9 and 79.5 cells for the rectum. The mean crypt circumference was 41.6 cells in the colon, and 46.0 cells in the rectum. This gave a total of 2044 cells per crypt in the colon and 2194 cells per crypt in the rectum. In the colonic crypts 10% of cells were in S phase and 0 4% in mitosis. Ninety per cent of labeled cells were found between cell positions 4 and 43; we showed that the maximum labeling index was about 30% and occurred at cell position 15. The labeling index at the crypt base, the putative stem cell zone, was about 14%. The rectum showed significant differences. The rectal mucosal crypts contain approximately 30% fewer S phase and mitotic cells (Figure 3). This may indicate either that the cell cycle time of rectal mucosa cells is longer than in the colon, or that there are fewer proliferating cells in the rectum. Extrapolating from biologic studies in rodents suggests that ISCs in the human colonic and rectal crypts represent only a small proportion of crypt cells (approximately 20 cells per crypt, or approximately 1%) (Potten and Loeffler 1990). This finding is consistent with recent immunostaining studies in human colonic crypts for Musashi-1 protein, a putative ISC marker, indicating that there are, on average, 19 ISCs per crypt (Potten et al. 2003).

A key component of tissue architecture that is involved in the regulation of stem cells has been termed the "stem cell niche " (Spradling et al. 2001). The stem cell niche has been well characterized in hematopoietic and neural systems and is an intricate and dynamic milieu

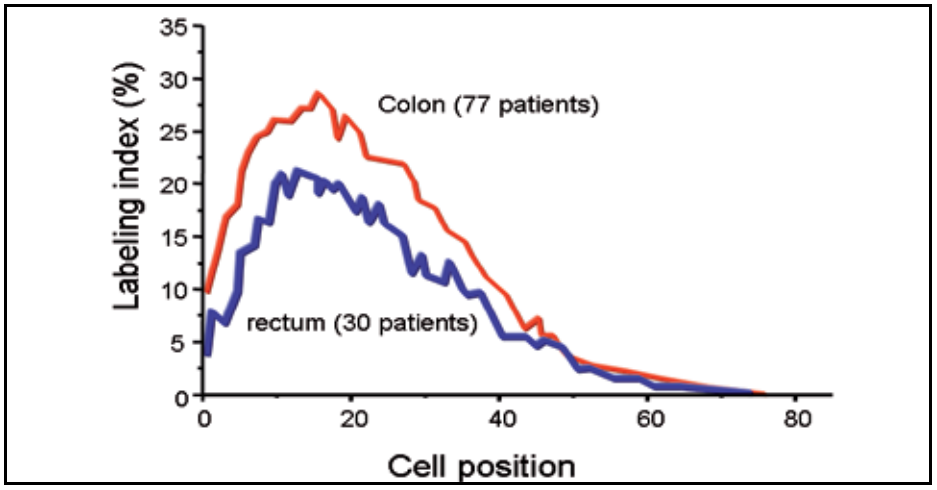

Fig. 3. A comparison of the bromodeoxyuridine labeling index frequency plots for the human colon and the rectum.

that adapts in response to environmental signals. The niche consists of a stromal microenvironment surrounding the stem cell population that can contain neural cells, lymphocytes, macrophages, endothelial cells, fibroblasts, smooth muscle cells and myofibroblasts in an extracellular matrix (Li and Xie 2005). As mentioned previously, the crypt is surrounded by the supporting lamina propria which contains pericryptal myofibroblasts. These myofibroblasts are thought to be strategic cells in the regulation of stem cell behavior through growth factor and cytokine signaling (Mills and Gordon 2001). Several key signaling pathways are common to stem cells and their niche including Wnt, BMP and Notch.

The discovery that mutations in the APC gene, the most important tumor suppressor in intestinal tumorigenesis, affects the control of Wnt signaling, indicated the importance of this pathway in intestinal stem cell regulation (Korinek et al. 1997). It is thought that the pericryptal myofibroblasts produce the Wnt signaling ligands that access Frizzled receptors on the basal epithelial stem cells (Fevr et al. 2007), and this prevents degradation of the main effector, β-catenin by a destruction complex containing APC and AXIN1/2. β-Catenin translocates to the nucleus, where it acts as a transcriptional activator after binding to TCF/LEF family members. Korinek *et al* showed that lack of the intestine specific β-catenin partner, TCF4, resulted in the depletion of the epithelial stem cell compartment in the small intestine (Korinek et al. 1998). Wnt signaling varies across the crypt (Kosinski et al. 2007) in that the crypt top is characterized by APC, WNT5B, and TCF4 whilst the crypt bottom, the putative stem cell niche, expresses AXIN2, TCF3, and several secreted Wnt inhibitors including DKK3, SFRP1, SFRP2, FZD2, FZD3, FZD7, and FZDB. The identification of many different Wnt/ β-catenin target genes indicates that Wnt signaling has different effects in different cell types depending on their localization along the crypt axis. The microenvironment surrounding the LGR5/GPR49 stem cell is characterized by prominent Wnt activity and inhibition of BMP signaling with the presence of BMP inhibitors noggin and gremlin whereas the microenvironment surrounding the +4 LRC is characterized by expression of the Wnt inhibitor sFRP5 and BMP4 (Pages et al. 2009).

This also highlights the importance of members of the BMP pathway as important contributors to the colorectal epithelial stem cell niche by modulation of the Wnt pathway. Bone morphogenetic proteins (BMPs) bind to BMP receptor types I or II (BMPR1 or BMPR2) leading to phosphorylation of SMAD1, 5 or 8, which then form a heterodimer with SMAD4, translocate to the nucleus, and act as transcriptional activators (von Bubnoff and Cho 2001). Active BMP signaling, indicated by phosphorylated SMADs, is found predominantly in differentiated intestinal epithelial cells. Several lines of evidence support the postulated inhibitory role of BMP signaling on stem cell self-renewal. These include the observations that conditional mutation of BMPR1A resulted in *de novo* crypt formation and a juvenile polyposis phenotype (He et al. 2004) and leads to reduced differentiation into the three secretory cells types, Paneth cells, goblet cells and enteroendocrine cells in mice (Auclair et al. 2007). Also, human juvenile polyposis has been shown to be associated with mutations in the SMAD4/DPC4 or BMPR1A genes (Sayed et al. 2002).

Several other pathways have been shown to be important in intestinal stem cell regulation including Notch signaling, hedgehog signaling, and the PTEN–PI3K–Akt pathway. Notch has been implicated in the control of cell fate in many tissues. The binding of the ligands Jagged or Delta to the Notch receptor induces proteolytic cleavage by γ–secretase which releases a fragment, NCID. This fragment translocates to the nucleus and acts as a transcription factor after dimerization with RBP-jκ/CSL. One of the key genes stimulated by this activation is a bHLH transcription factor termed hairy/enhancer of split (Hes), which has been shown to activate factors involved in the control of proliferation and differentiation (Bray 2006). Knocking out RBP-jκ or Hes1 leads to increased numbers of secretory epithelial cells (Jensen et al. 2000) whereas mutation in ATOK1, a transcription factor repressed by Notch signaling, leads to depletion of all three secretory lineages (Yang et al. 2001). Therefore, Notch functions by stimulating proliferation of crypt progenitor cells in the transit-amplifying units, and suppression of Notch signaling induces specific differentiation into the intestinal epithelial lineages. A role of the PTEN–PI3K–Akt in enhancing stem cell self-renewal in the intestine has been suggested as a result of the connection between this pathway and the Wnt pathway. p-Akt can phosphorylate β-catenin and thus enhance the transcriptional activity of β-catenin whilst PTEN exhibits strongest expression in lumenal epithelial cells and might be involved in the restriction of strong Wnt signaling to the crypt base (Persad et al. 2001). In contrast to the other signal pathways which seem to be regulated in response to ligands originating from the niche cells, the morphogens of the hedgehog (HH) pathway, sonic hedgehog (Shh), and Indian hedgehog (Ihh) are secreted by epithelial cells. Their receptor, Patched (PTCH), is expressed on the pericryptal myofibroblasts. Accordingly, HH signaling is not directly concerned with the fate of the epithelial cells but is important in shaping and regulating the proper overall structure of the intestinal mucosa into crypts and villi (Madison et al. 2005).

4. Stem cells and the development of colorectal cancer

The mechanisms underlying colorectal cancer initiation have yet to be fully elucidated (see section 5). It is clear that the APC gene is involved as APC mutations are found in 75% to 80% of sporadic CRCs (Powell et al. 1992). However, mutations in mitochondrial DNA and mutations in the genes encoding cytochrome c oxidase (COX), a component of complex IV of the respiratory chain, are also relatively common (Taylor et al. 2003).

When an initial mutation occurs in a basally situated crypt stem cell, it gives rise to a clone that migrates up the crypt, expanding as it progresses. At this stage the proliferation pattern of crypts are shifted toward the crypt top with a maximum labeling index (LI) at approximately crypt level 20. The mutant clone then begins to colonize the base of the crypt, in effect taking over and replacing the non-mutant cells in the stem-cell niche in a process that has been termed niche succession. Eventually the entire niche will be colonized with mutant stem cells and the crypt filled with their progeny, a result termed monoclonal conversion. Interestingly, crypts containing this proliferative abnormality do not show any discernible histological changes. Crypts only begin to show obvious abnormalities when they become dysplastic during later formation of premalignant adenomas. Boman and colleagues have postulated that only an increase in crypt SC number and not changes in cell cycle proliferation, differentiation, or apoptosis of non-SC populations, could explain the LI shift in these crypts (Boman et al. 2001). This led to the hypothesis that the link between APC mutation and the LI shift is crypt SC overpopulation caused by a decrease in the rate of degradation of cytoplasmic β-catenin and alteration of TCF-4 transcriptional activity and survivin expression leading to inhibition of apoptosis and promotion of mitosis (Boman and Huang 2008).

Inactivation of the second APC allele occurs during the development of intestinal adenomas, and it has been proposed that the next critical event may be the movement of the progeny of the mutated stem cell moving from the niche into the proliferative zone of the crypt where they are freed from the constraints of the niche cells and are able to undergo further symmetric divisions and clonal expansion forming monocryptal adenomas (Humphries and Wright 2008). These are the earliest histologically detectable precursor lesions of tumor development and are thought to precede adenoma development. Further clonal expansion seems to be through crypt fission. Crypt fission is a normal process that leads to crypt replication. This process is responsible for the increased number of new crypts that arise during a short postnatal period, after which the total number of crypts increases only gradually with age. During crypt fission, development of a fissure bisects the crypt base and ascends longitudinally. This bifurcation results in the symmetric creation of two identical daughter crypts and must therefore be a process that results from symmetric ISC division. Experimental evidence suggests that mutant APC and an increasing rate of crypt fission, leads to abnormal, asymmetric crypt fissioning during adenoma development resulting in characteristic crypt branching and budding (Wasan et al. 1998). Crypt fission is also the mechanism that leads to the spread of mutant crypt populations in normal colonic epithelium. Further development of the lesion may be through random collision between neighboring neoplastic clones or through clonal interaction in which active cooperation between multiple initiated clones promotes continued survival and growth of the adenoma leading to genetic heterogeneity (Axelrod et al. 2006). Indeed, it has been shown that genetic alterations occur in the stroma from an early stage of carcinogenesis and that these may induce microregional differences in tumor susceptibility promoting loss of heterozygosity in the associated epithelium (Thliveris et al. 2005).

5. Genetic differences between colon and rectal tumors

The most well known model for colorectal carcinogenesis (the Vogelstein model) describes the progression of normal epithelium into adenomatous polyps and neoplasia and finally into metastatic carcinoma (Fearon and Vogelstein 1990). A series of specific genetic

alterations are responsible for the transition to more tumorigenic phenotypes. While alterations in the APC (adenomatous polyposis coli gene)/β-catenin pathways as well as inactivation of mismatch repair proteins generally occur early, modifications to p53 and DCC/SMAD4/SMAD2 occur as one of the final steps in the progression to carcinoma. The step-wise progression of colorectal carcinogenesis through transitional dysplastic and adenoma stages is demonstrated by the high rate of success seen in preventing the development of colorectal cancer by removing polyps. Polyps represent the dysplastic and adenoma stages of colorectal carcinogenesis, and their removal prevents the development of carcinoma.

There are three main pathways that lead to the genetic alterations responsible for colorectal tumorigenesis, the chromosomal instability (CIN) pathway, the mismatch repair (MMR) pathway and the hypermethylation phenotype. The CIN pathway is characterized by alteration of APC tumor suppressor gene signaling. A germline mutation of the APC gene results in the development of familial adenomatous polyposis (FAP) which is typified by hundreds to thousands of colorectal polyps by age 20-30. Tumors that developed via the CIN pathway have a high level of chromosomal instability that results in large numbers of deletions, insertions, and loss of heterozygosity. The MMR pathway to colorectal carcinogenesis results from a failure of DNA repair genes, in particular MLH1 and MSH2. This malfunction in DNA repair results in an accumulation of errors throughout the genome, particularly in areas called microsatellites. Microsatellites are short nucleotide regions that are repeated hundreds of times within the genome; thus, the MMR pathway is characterized by microsatellite instability. Germline mutations in one of the MMR genes results in hereditary nonpolyposis colorectal cancer (HNPCC). Finally, the hypermethylation pathway is characterized by a high incidence of methylation of CpG islands which may result in gene silencing of the MMR genes.

In addition to these major pathways in colorectal carcinogenesis, there are specific genetic pathways that have a role in colorectal carcinogenesis. The gene K-ras is mutated in ~50% of sporadic colorectal cancer and in 50% of adenomas larger than 1 cm while rarely in smaller adenomas (Vogelstein et al. 1988). The lack of mutations in the smaller adenomas implies that the K-ras mutation is relevant to a later stage of progression. However, the presence of K-ras mutations in both nondysplastic aberrant crypt foci and hyperplastic polyps makes the role of these mutations unclear.

Another commonly mutated gene in colorectal cancer is p53. In response to DNA damage and other stress, p53 induces responses ranging from cell cycle arrest and senescence to differentiation and is inactivated in 50-70% of colorectal cancers. The p53 gene is located on chromosome 17p which is lost in up to 75% of colorectal cancers, but it is lost rarely in adenomas. This suggests that the loss occurs late in the progression (Baker et al. 1990).

One more common feature of colorectal cancers is the loss of chromosome 18q. The deletion is seen in 73% of sporadic colorectal cancers and 47% of large adenomas but less in smaller adenomas (Vogelstein et al. 1988). This divergence implies the chromosomal loss occurs later in tumorigenesis. Chromosome 18q contains three significant genes: DCC ("deleted in colon cancer"), SMAD4, and SMAD2. DCC functions as a tumor suppressor and has a role in cell-cell interactions. Loss of DCC expression is associated with a worse overall survival in colorectal cancer patients (Popat et al. 2007). As mentioned previously, SMAD4 and SMAD2 are both involved in BMP signaling as well as in the TGFβ signaling cascade which modulates cell proliferation, apoptosis, and differentiation.

However, while cancers of the colon and rectum have generally been grouped into the single category of "colorectal" cancer, it has long been speculated that cancers that develop in different anatomical areas of the colon and rectum should be considered as separate diseases. Differences in the biology and function and risk factors between proximal colon, distal colon, and rectum may lend to these divergent disease entities. Genetic evidence also supports etiological evidence that colon and rectal tumors are different entities. These differences between colon and rectal tumors include incidence of certain gene mutations, change in gene expression, even differences in the mechanisms of carcinogenesis. The frequency of mutations to K-ras and APC differs between sites. Mutations to K-ras were more widespread in tumors of the colon. Rectal cancer more often has mutations restricted to APC while colon cancers often contain mutations in several genes (Frattini et al. 2004). In fact, the number of genetic mutations regardless of gene were higher in colon than the rectum (Li and Lai 2009).

In addition to mutations, there are several genes that show different levels of expression between rectal and colon cancers including β-catenin, MMR proteins, p53, and COX2 (cyclooxygenase 2). β-catenin binds the APC protein and is involved in regulating cell growth and adhesion between cells. The cellular localization of β-catenin is altered between colon and rectal cancers. Nuclear β-catenin expression was found more often in cancers of the rectum than colon (Kapiteijn et al. 2001), and reduced membranous and cytoplasmic staining was associated with increased metastatic disease in rectal cancer (Fernebro et al. 2004). Unlike colon cancer, rectal tumors rarely show a loss of expression in MMR proteins including MLH1 and MSH2 (Fernebro et al. 2004). Over-expression of p53 was more common in rectal cancers than colon cancer; however, this may indicate a higher level of p53 mutation in rectal cancer (Kapiteijn et al. 2001). In addition, 90% of rectal tumors demonstrate up-regulation of COX2 while only 20% of colon cancers had increased levels (Li and Lai 2009).

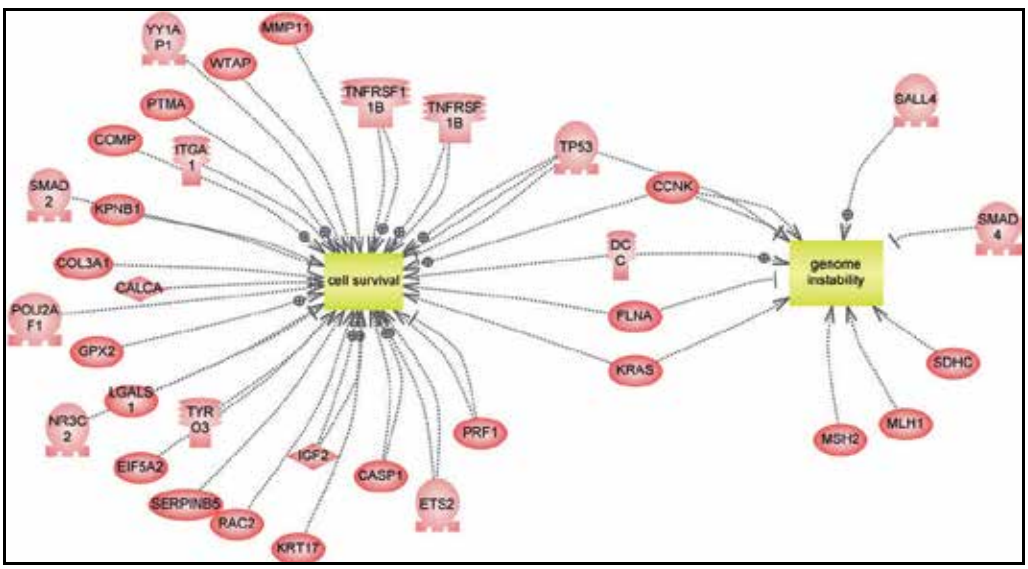

Fig. 4. Gene expression linked cell survival and genome instability in rectal cancer. Genes identified in 4 gene expression studies of rectal cancer were highly represented by genes involved in cell survival and genome instability. Pathway generated by Ariadne Pathway Studio.

These differences in mutational and gene expression patterns demonstrate a fundamental difference in the carcinogenesis of rectal and colon cancer. With the low rate of MMR disruption seen in rectal cancer, the incidence of MSI is quite low. However, the incidence of CIN is high in rectal cancer as exemplified by the mutations seen in APC. The importance of genomic instability in rectal cancer is further illustrated by examining gene expression studies which were done to differentiate rectal cancer patients that respond to radiation therapy (Watanabe et al. 2006; Ojima et al. 2007; Rimkus et al. 2008) or patients that have local recurrence (Kalady et al. 2010). These four studies identify a total of 120 genes that are differentially expressed between rectal cancer patient populations. Of these genes, 30 are associated with cell survival, and 10 have been linked to genome instability (Figure 4).

6. Current candidates as stem markers in rectal tumors

Various cell surface markers (Table 1) have been used for the identification of cancer stem cells. These markers are used to isolate sub-populations of cells that are characterized by the ability to reconstitute the original tumor by xenotransplantation using a limited number of cells. Breast cancer stem cells that are $CD44^+$ $CD24^{-/low}$ Lin^- are able to form tumors in mice using as few as 100 cells while injection of tens of thousands of cells with different phenotypes fail to form tumors (Al-Hajj et al. 2003). Similarly, CD133+ cells from brain tumors are able to form tumors with an original xenograft of only 100 cells (Singh et al. 2004). Even more astonishing, human ovarian cancer cells that have high ALDH activity and express the cell surface marker CD133 are able to form tumors in mice using as few as 11 cells (Silva et al. 2011).

Site	Markers
Brain / CNS	$CD133^+$ (Singh et al. 2004), ALDH1 activity (Wang et al. 2011)
Breast	$CD44^+$ $CD24^{-/low}$ Lin^- (Al-Hajj et al. 2003), ALD1 activity (Marcato et al. 2011)
Esophagus	Hoescht exclusion (Kalabis et al., 2008)
HNSCC	$CD44^+$ (Harper et al. 2007), $CD133^+$ (Yang et al. 2011), ALDH activity (Clay et al. 2010), Hoescht exclusion (Sun et al. 2010)
Lung	$CD133^+$ (Bertolini et al. 2009), ALDH activity (Liang and Shi 2011)
Melanoma	$CD20^+$ (Zabierowski and Herlyn 2008), $CD133^+$ (Gazzaniga et al. 2010)
Ovary	ALDH activity (Silva et al. 2011), Hoescht exclusion (Hosonuma et al. 2011)
Pancreas	$CD44^+$ $CD24^+$ ESA^+ (Lee et al. 2008), ALDH activity (Kim et al. 2011)
Prostate	$CD44^+$ $\alpha2\beta1^{high}$ $CD133^+$ (Miki et al. 2007)
Colon	$CD44^+$ ESA^{high} (Dalerba et al. 2007), CD133+ (Ricci-Vitiani et al. 2010), $CD166^+$ (Dalerba et al. 2007), ALDH activity (Huang et al. 2009), $Lgr5^{+ve}$ (Takahashi et al. 2011)
Rectum	$CD44^+$ (Nagata et al. 2011), $CD133^+$ (Nagata et al. 2011), $CD133^+ESA^+$ (Yang et al. 2010)

Table 1. Stem cell markers. CNS: central nervous system; HNSCC: Head and neck squamous cell carcinoma; ALDH: aldehyde dehydrogenase-1; ESA: epithelial-specific antigen

As mentioned previously, there are several critical pathways that are involved in the maintenance of cancer stem cells including the Wnt, Sonic Hedgehog (SHH), Notch, phosphoinositol-3-kinase (PI3K), and bone morphogenic protein (BMP) pathways (Brabletz et al. 2009). Interestingly, most of the known cancer stem cell markers are not directly related to these pathways; however, these signaling pathways are up-regulated in these cancer stem cell-enriched sub-populations. Pancreatic cancer stem cells that are CD44+ CD24+ ESA+ show an up-regulation of SHH and BMI-1 signaling. These pancreatic cancer stem cells represent less than 1% of all pancreatic cancer cells (Lee et al. 2008), and 100 of these pancreatic stem cells are able to form tumors that are indistinguishable from the original tumor (Chen et al. 2011).

In colorectal cancer, $ALDH^+$ cells are rare in the normal colorectal epithelium and located exclusively in the normal crypt base which is the proposed location for colorectal stem cells. As colorectal carcinogenesis progresses from normal through adenoma and carcinoma, the number of ALDH+ cells increases as well as being distributed more extensively (Huang et al. 2009). Colorectal tumor cells that express both CD44 and ESA are able produce tumors and reproduce the full heterogeneity of the original tissue. CD133+ colorectal cancer stem cells constitute 2.5% of all cells in the tumor (Ricci-Vitiani et al. 2010). These $CD133^+$ cells are able to reproduce the original tumor while the $CD133^-$ cells cannot form tumors. However, it is worth noting that a study using fractionating dilution revealed that 1 in 262 CD133+ cells are able to form tumors (O'Brien et al. 2007). While significantly enriched compared to the unfractionated cell population which form tumors at a rate of 1 in 57,000, it still illustrates that not all $CD133^+$ cells are able to reconstitute the original tumors.

Although CSCs have been studied in colon cancer, the existence and implication of stem cells has not been extensively studied in rectal cancer. In a case study, Yang et. al. found elevated levels of CD133+ ESA+ cancer stem cells circulating in the blood of a 75-year old rectal cancer patient who later developed liver metastasis (Yang et al. 2010). Immunohistological analysis of rectal cancer tissue demonstrated that local recurrence was greater for patients that were positive for either CD133 or CD44 (Nagata et al. 2011). Yasuda and colleagues (Yasuda et al. 2009) showed that elevated CD133, but not VEGF or EGFR, was a predictive marker of distant recurrence after preoperative chemoradiotherapy in rectal cancer while Wang et. al. (Wang et al. 2009) showed that the proportion of CD133+ cells was a significant prognostic factor for adverse disease-free survival and overall survival independent of TNM stage, tumour differentiation or lymphovascular invasion. More recently, Kojima and colleagues (Kojima et al. 2010) studied 92 cases of rectal cancer of which 43 patients received preoperative chemoradiation therapy and 49 patients underwent surgery alone. Forty pretreatment biopsy specimens from 43 patients in the preoperative chemoradiation therapy group were also analyzed. CD133-positive cases were more common in the preoperative chemoradiation therapy group than in the surgery-alone group. Furthermore, CD133-positive cases were more common in the preoperative chemoradiation therapy group than in pretreatment biopsy specimens. In the preoperative chemoradiation therapy group, the CD133-positive cases showed poorer prognosis than the CD133-negative cases. These studies suggest that the CD133+ population is important for outcome and that chemoradiation enriches this population.

The biological function of CD133 remains unknown. It is a transmembrane pentaspan protein that was initially described as a surface antigen which was specific to human hematopoietic stem cells (Yin et al. 1997). Utilizing the literature mining software found in Ariadne Pathway Studio, Figure 5 illustrates the known relationships found between CD133

and other genes and cellular processes. Cellular processes that are associated with CD133 include those that are expected to be involved with cancer stem cells: cell death, morphogenesis, apoptosis, cell differentiation, cell proliferation, and drug response (response to drug). Others may provide hints at important processes that have yet to be investigated: lipid metabolism, glucose metabolism, and vascularization. In addition, CD133 is linked with well-known cancer-related genes such as p53, Myc, Src, and transforming growth factor β1 and the TGFβ-associated SMAD6 and SMAD7. Expression of the gene HIF-1α in conjunction with CD133 is associated with tumor recurrence following chemoradiation (Saigusa et al. 2011). Also associated with recurrence of rectal cancer after chemoradiation is putative stem cell marker POU class 5 homeobox 1 (POU5F1). Expression of CD133, POU5F1, and the SOX2 gene following treatment was associated with poor disease-free survival.

The addition of other known cancer stem cell markers such as CD44 and ESA (EPCAM) to the diagram exemplifies the level of similarity in signaling. Figure 5 shows CD133 associated with 61 other genes and cellular processes. While ESA is associated with 10 of these entities, CD44 has been linked with almost half. CD44 has been associated with the inhibition of apoptosis, cell differentiation, and p53 signaling/expression. Both CD133 and CD44 are linked to CXCL12 (chemokine (C-X-C motif) ligand 12). CXCL12 and its receptor CXCR4 have previously been associated with the mobilization and homing of hematopoetic stem cells (Juarez and Bendall 2004). Interestingly, CXCR4 overlaps much of the CD133 signaling and has been implicated in cancer stem cell signaling at several other sites. Sustained CXCR4/CXCL12 signaling occurs in prostate cancer stem cells (Mimeault and Batra 2011) and is involved in the up-regulation of stem cell-related gene expression in breast cancer cells (Zhang et al. 2011). Additionally, CXCR4 was identified as a therapeutic target of glioblastoma stem cell-like cell lines (Schulte et al. 2011). This implies that, while many markers may be used to identify cancer stem cells, much of the signaling behind these superficial membrane markers may be quite similar.

7. Stem cells and treatment response

The mainline cancer therapies of conventional chemo- and radiotherapy target rapidly cycling cancer cells and can cause impressive, but usually temporary, clinical remissions. This initial remission followed by local recurrence would support the argument for the existence of a small subpopulation of resistant CSCs, while at the same time the majority of the non-CSCs being responsive to the treatment. Treatment failure could be explained by several CSC characteristics that would make them difficult to eradicate by conventional agents. First, they may be slow-cycling or quiescent rendering them less sensitive to agents that target actively cycling cells. Second, a characteristic of many normal stem cells is the increased activity of ABC transporter proteins as a protective mechanism against environmental toxins; these are also up-regulated in CSCs (Dean et al. 2005). Third, there are data suggesting that CSCs may be more resistant to radiation (Bao et al. 2006) although this has not been universally found (McCord et al. 2009).

CD133 has been extensively studied in the context of radiation sensitivity in the setting of glioma. An increase of the CD133+ fraction following irradiation of human glioma cells has been shown in vitro as well as in tumors in nude mice (Bao et al. 2006). The CD133+ cell fraction was found to have a reduced sensitivity to radiation-induced apoptosis. Interestingly, when CD133+ cells were irradiated with 3Gy, they were able to initiate tumors

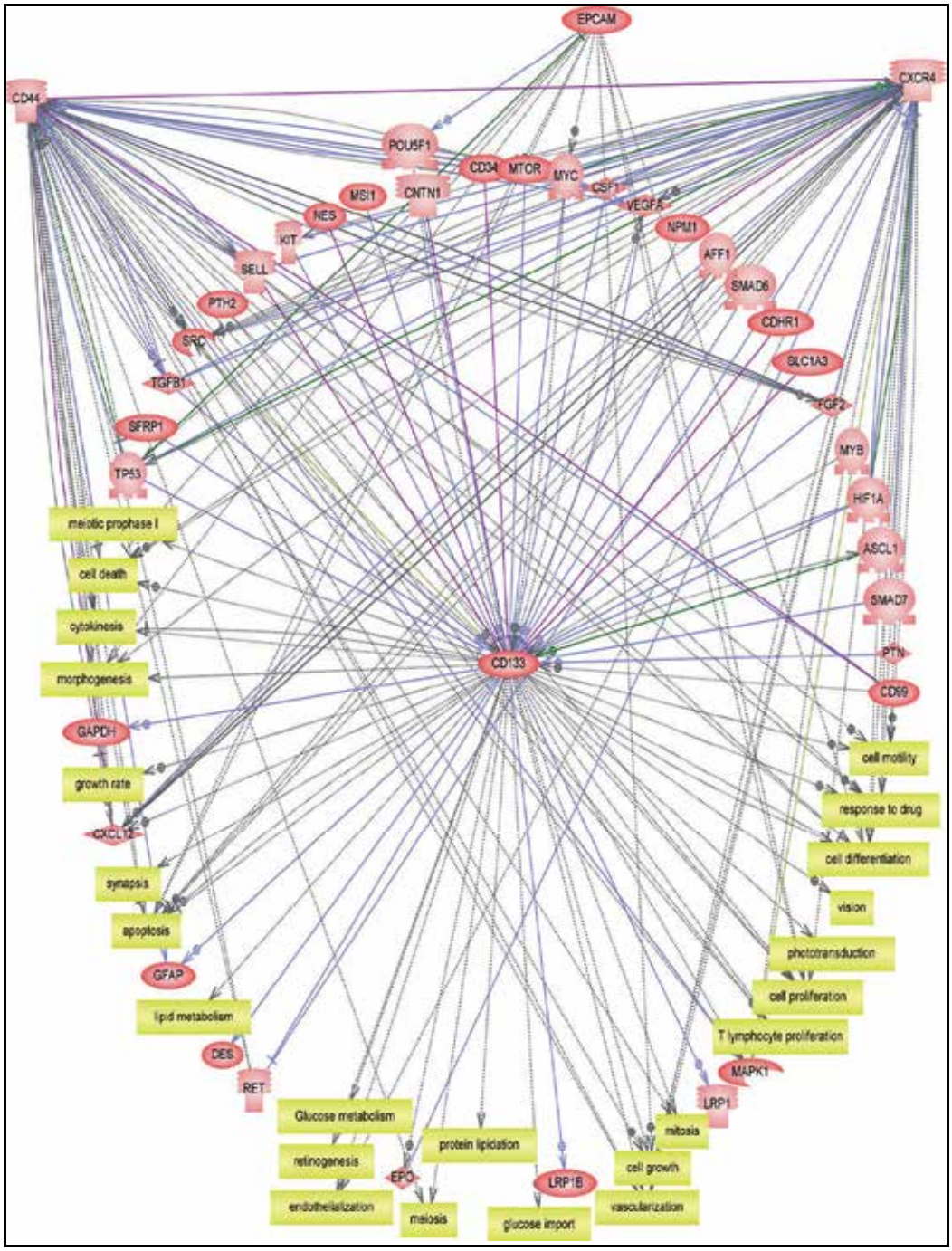

Fig. 5. Genes and cellular processes associated with CD133 expression. The literature mining software in Ariadne Pathway Studio software identifies genes and cellular processes that have been shown to be associated with CD133. This figure was supplemented by adding the stem cell markers CD44, ESA (EPCAM), and CXCR4.

with almost the same efficacy than the non-irradiated CD133+ cells. A clinical study in atypical teratoid/rhabdoid tumors demonstrated a correlation of the amount of immunohistochemically detected CD133+ cells with resistance to combined chemoradiation and decreased survival (Chiou et al. 2008). These data are supported by evaluations of glioma (Murat et al. 2008) and rectal cancer patients (Wang et al. 2009). All these studies show correlations between CD133 expression and efficacy of radiotherapy or combined treatment. Similarly, studies have also shown that CD133 positivity is associated with chemoresistance in glioma (Nakai et al. 2009), oral squamous cancer (Zhang et al.), and mesothelioma (Cortes-Dericks et al. 2010) amongst others. A large clinical study of 501 cases of human colorectal cancers showed that CD133-overexpressing tumors were more resistant to 5-FU–based chemotherapy and that CD133 expression was associated with poor prognosis (Ong et al. 2010). A recent study showed that treatment of human HT-29 colorectal cancer cells with high doses of 5-FU or oxaliplatin resulted in enrichment of CD133+ and CD44+ CSCs, which also exhibited decreased in vitro proliferation rate (Dallas et al. 2009). Interestingly in another colorectal cell line study, a recent publication has suggested that although CD133+ cells had higher *in vivo* tumor-forming ability than CD133− cells, it was the CD133− cells that were more resistant to 5-fluorouracil (FU) treatment (Hongo et al. 2011).

Although newer targeted agents such as cetuximab and bevacizumab are being tested in both frontline (Minsky et al. 2010) therapy and the metastatic setting (Cunningham et al. 2004; Hurwitz et al. 2004), they have modest effects on disease-free survival and overall survival. It would seem that the current combined modality therapies for rectal cancer will not be effective against CSCs no matter what combination is used. However, the most effective method to target CSCs has yet to be elucidated, but a number of possibilities exist including the administration of differentiating agents such as salinomycin (Gupta et al. 2009), targeting the specific signaling pathways of the CSCs (hedgehog, wnt, Notch) with drugs like cyclopamine (Merchant and Matsui 2010; Pannuti et al. 2010; Takahashi-Yanaga and Kahn 2010), targeting the microenvironmental niche of CSCs (LaBarge 2010), targeting the DNA checkpoint response (Frosina 2009), or using normal stem cells to home to the region of tumor (Hu et al. 2010). It is likely that future therapies will include inhibitors of survival pathways, along with immune cells, differentiation agents, and cytotoxic drugs, as a combination.

Immunological approaches have been demonstrated to be effective against CSCs in colorectal cancer. It has been shown that inhibiting the IL-4 signaling transduction pathway with an anti–IL-4 neutralizing antibody or an IL-4 receptor α antagonist sensitized CSCs to chemotherapeutics through down-regulation of anti-apoptotic proteins, such as cFLIP, Bcl-xL, and PED (Todaro et al. 2007). The same group has also shown that incubation of colon CSCs with the bisphosphonate zoledronate induced an efficient γδ T-cell response. These immune cells have been shown to be effective at killing different tumor cells in vitro, but this was the first report of using γδ T-cell to target CSCs (Todaro et al. 2009).

Another important area of future investigation will be to determine the optimal timing of CSC-targeted therapies with other modalities, i.e. should it be co-administration of agents for newly diagnosed tumor or sequential scheduling after a remission to standard treatment has been obtained or at the time of progression after a standard treatment (Al-Hajj et al. 2004). A crucial element in optimizing timing will be the development of novel imaging probes to develop strategies for robust and efficient tracking and validation of CSCs and their niche under in vivo conditions. This will pave the way to better elucidate the

underlying regulatory mechanisms of CSC and develop platforms for targeted theragnostics.

8. Conclusions

The CSC model, if generally correct, has important implications for the current paradigm of treatment for rectal cancer. The practice of small, successive improvements in survival by the refinement of current schedules and addition of newer agents is unlikely to result in significant advances in treatment outcomes. Gene expression analyses of CSCs populations have the possibility to identify novel diagnostic markers and novel therapeutic targets. A recent study identified EGR1 to the be the most highly expressed gene in CD133 positive colorectal cancer cells (Ernst et al. 2011). EGR1 is known to regulate Wnt through up-regulation of TCF4, which induces stem cell marker LGR5. Previous studies identified 100 candidate-genes, which were differentially expressed in the CD133 positive fraction (Regenbrecht et al. 2008) of which 10 genes were shown to be differentially regulated between the different studies. However, 9 of these 10 genes were shown to form an interactive network with each other and that these genes were positioned at the interface between proliferative pathways (JAK/STAT) and differentiating pathways (HOX, PBX, MEIS, GATA2). Of importance in this cascade was the gene KIT which encodes the receptor for stem cell factor (SCF). It is clear from Figure 5 that CD133 is involved in many signaling pathways and identifying the candidate pathways for future drug development will be challenging. Equally challenging will be targeting pathways of stem cell self renewal without affecting this crucial process in normal stem cells. Thus, the elucidation of the mechanisms regulating the survival, self-renewal, and differentiation of normal and CSCs could potentially lead to significant advances in the treatment of neoplastic diseases including rectal cancer.

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17

Endoscopic Diagnosis and Treatment for Colorectal Cancer

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1. Introduction

Colonoscopy plays an important role in the medical care of patients with colorectal cancer. It is generally used for both the diagnosis of different stages of colorectal cancer and the treatment of early colorectal cancer and its precursors. The recent progress in colonoscopy has been remarkable. Endoscopes with variable rigidity and small diameters provide efficient insertion to the cecum and result in lower distress for patients. Trained colonoscopists can insert endoscopes into the cecum within a few minutes, and it is not necessary to anesthetize patients without severe peritoneal adhesion.

We can obtain good-quality pictures and special images to assist in diagnosis by using high-vision endoscopes, magnifying endoscopes, dye spray, and narrow-band imaging (NBI). Determining whether a colorectal carcinoma can be curatively resected by endoscopic treatment or whether the carcinoma has a risk of lymph node metastasis is a very delicate and important task. In particular, the depth of cancer invasion is related to lymph node metastasis; therefore, endoscopic ultrasonography and the classification of pit patterns, capillary patterns via NBI, and the lesion-lifted condition are used to diagnose the depth of cancer invasion (Kato, 2001, Sano, 2008).

Treatment for colorectal neoplastic lesions begins with hot biopsy and snare polypectomy, and recently, endoscopic submucosal resection (EMR), piecemeal EMR (EPMR), and endoscopic submucosal dissection (ESD) have become available for large and flat lesions of the colon and rectum. Early colorectal carcinoma is defined as a carcinoma within the submucosal layer that is not invading the muscularis propria. Carcinoma in situ (mucosal carcinoma) and carcinoma that slightly invades the submucosa and without risk factors for metastasis do not metastasize into lymph nodes or distant organs. Nonmetastatic carcinoma is cured by local resection with colonoscope. It is important to make an accurate diagnosis by endoscopy and to perform confident resection for pathological evaluation.

In this chapter, we describe endoscopic diagnosis for colorectal carcinoma and differential diagnosis, and treatment options for early colorectal cancer without metastasis and for adenoma which is regarded as a precancerous condition. In addition, we briefly discuss risk factors for lymph node metastasis in early colorectal carcinoma.

2. Endoscopic diagnosis of colorectal carcinoma

Colorectal carcinomas are the most common malignancies in industrialized countries, and are classified as early or advanced according to the depth of invasion. In advanced cancers, the invasion reaches the muscularis propria (MP) or the deeper layers. In endoscopic diagnosis, macroscopic classification is the most basic information. In this section, endoscopic diagnosis of colon carcinoma is discussed.

2.1 Macroscopic classification of colorectal carcinoma

Colonoscopy is a valuable tool in the diagnosis and management of colorectal neoplasms. Advanced colorectal carcinoma can be divided into 4 groups based on endoscopic appearances (Fig. 1).

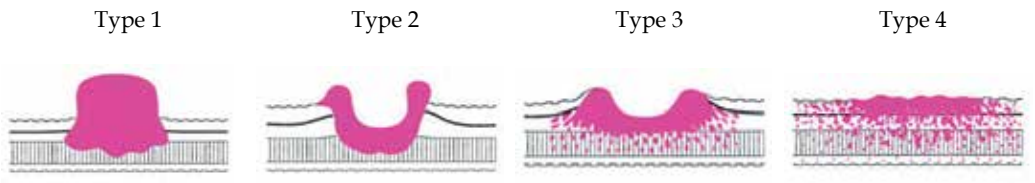
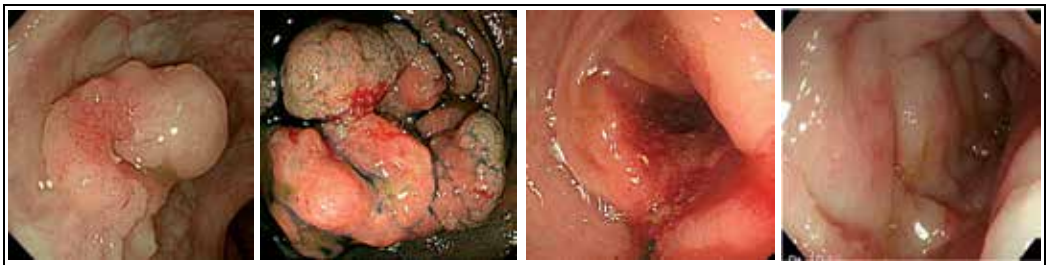


Fig. 1. Macroscopic types of advanced colorectal carcinoma



Type 1 lesion, protuberant tumor with fold convergence
Type 2 lesion, showing an irregular ulceration and clear marginal swelling
Type 3 lesion, showing an irregular ulceration and unclear marginal swelling
Type 4 lesion, showing an irregularly edematous mucosa with luminal stenosis due to diffuse infiltration, Ulceration is not pointed out on the lesion.

Fig. 2. Endoscopic view of each type of advanced colorectal carcinoma

Localized carcinoma is classified as a polypoid- (protuberant type) or ulcerative–type lesion. More than 90% of colorectal carcinomas are ulcerative-type lesions. To further distinguish early colorectal carcinoma and unclassified advanced colorectal carcinoma, the macroscopic type is subclassified from Type 0 to Type 5 (Sugihara, 2009). Early cancers are defined by the depth of cancer invasion into mucosal or submucosal layers. In this manual, early carcinoma is classified as Type 0, and advanced carcinoma is classified as Type 1 to 5.
The subclassification of Type 0 carcinoma is described in further detail in the next section. Type 1 lesions are protuberant. Type 2 lesions include ulcerative-type lesions with clear margins, and Type 3 lesions include ulcerative-type lesions with infiltration. Diffusely infiltrating lesions are classified as Type 4 lesions (Fig. 2). Type 5 lesions are an unclassified type. Type 2 is the most common type of advanced colorectal carcinoma. Circular carcinoma

of Type 2 is occasionally observed as a stenosis due to the tumor of the large intestine, and ulceration is not always detected by endoscopy. Pathological examination of biopsy specimen from the stenosis or edge of ulceration reveals adenocarcinoma. Type 4 lesion is observed like hard mucosal stenosis and neither obvious tumor nor ulceration is always recognized by endoscopy. And pathological diagnosis from biopsy is very difficult because the carcinoma is covered with normal mucosa.

2.2 Early colorectal carcinoma

Early colorectal carcinoma is defined as a carcinoma that is confined to the mucosa (M) and submucosa (SM). Early colon carcinoma may occur in an adenomatous polyp or may be difficult to distinguish from a nonmalignant adenomatous polyp by colonoscopy. For example, a 2-cm-wide villous adenoma has an approximately 40% chance of harboring cancer (Kim, 1997). Polyp risk factors for malignancy include villous rather than tubular histology, large size, sessile morphology, and high numbers of colonic polyps (Morson, 1972). Another route of carcinogenesis is "de novo" carcinogenesis, which produces small, aggressive carcinomas that do not appear to develop from adenomas (Kudo, 1997; Mueller, 2002). Macroscopic depressed type is the most common type of this carcinoma. This type is difficult to diagnose early using colonoscopy let alone barium enema; therefore, it is important to observe these lesions by endoscope extremely carefully. Early colorectal carcinoma is asymptomatic. It is usually revealed by screening colonoscopy or a positive stool occult blood test followed by colonoscopy. Colorectal screenings are important for detecting early colorectal carcinoma, which may be curatively treated by endoscopy.

2.2.1 Macroscopic type of early colorectal carcinoma

Regarding the classification of macroscopic-type lesions, the Japanese colorectal cancer handling protocol and Paris classification are representative classification systems. Both are used to judge endoscopic findings. Early colorectal carcinoma is classified as any Type 0 lesion judged to be a superficial carcinoma.

2.2.2 Japanese classification of colorectal carcinoma

Type 0 is subclassified into Type 0-I (tubercle type) and 0-II (surface type). Type 0-I is further subclassified as Ip (pedunculated), Isp (subpedunculated), and Is (sessile), whereas Type 0-II is further subclassified as IIa (surface tubercle), IIb (surface flatness), and IIc (surface depressed) (Fig. 3).

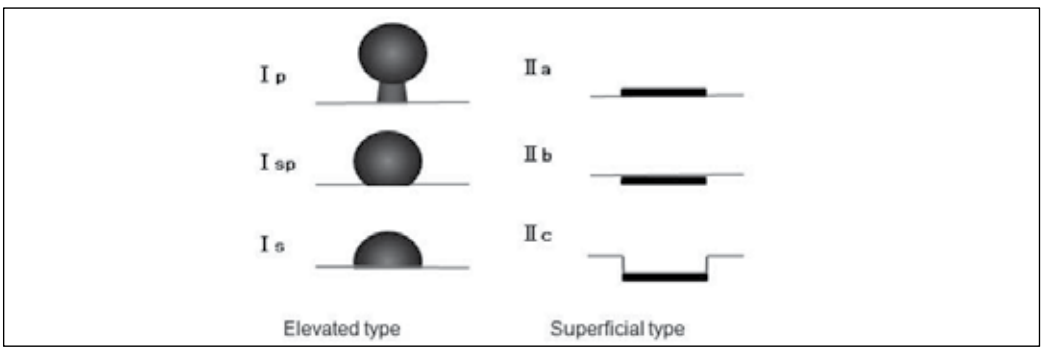


Fig. 3. Classification of superficial colorectal carcinoma (Japanese protocol)

Carcinomas can also be mixed-type lesions, which are lesions possessing elements of both Type 0-I and 0-II. Mixed-type lesions include types 0-IIc+IIa, 0-IIa+IIc, 0-IIc+Is, and 0-Is+IIc.

2.2.3 Paris classification

Terms used in the Paris classification of macroscopic-type lesions are unified by the terms used in a paper by Schlemper in 2002 (Fig. 4).

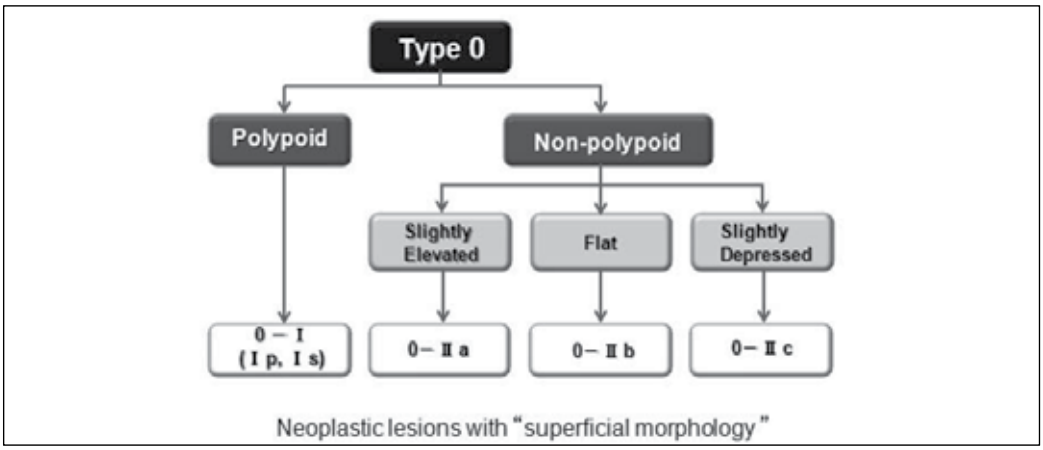


Fig. 4. Paris classification of superficial colorectal carcinoma

Firstly, lesions are divided into polypoid (Type 0-I) and non-polypoid (Type 0-IIa, IIb, IIc) lesions, and Type 0-I is subclassified as Type 0-Ip (pedunculated) and Type 0-Is (sessile). Type 0-III carcinomas comprise excavated-type lesions in the original classification, but these lesions are rare in the colon and rectum. Isp lesions are classified in the Japanese colorectal carcinoma handling protocol as Type 0-Is. Type 0-IIa lesions include those in which their height does not exceed that of closed biopsy forceps (about 2.5 mm), and lesions with heights exceeding this threshold are classified as type0-Is. Mixed-type lesions include Type 0-IIa+IIc, 0-IIc+IIa, 0-IIc+Is, and 0-Is+IIc (Fig. 5,6).

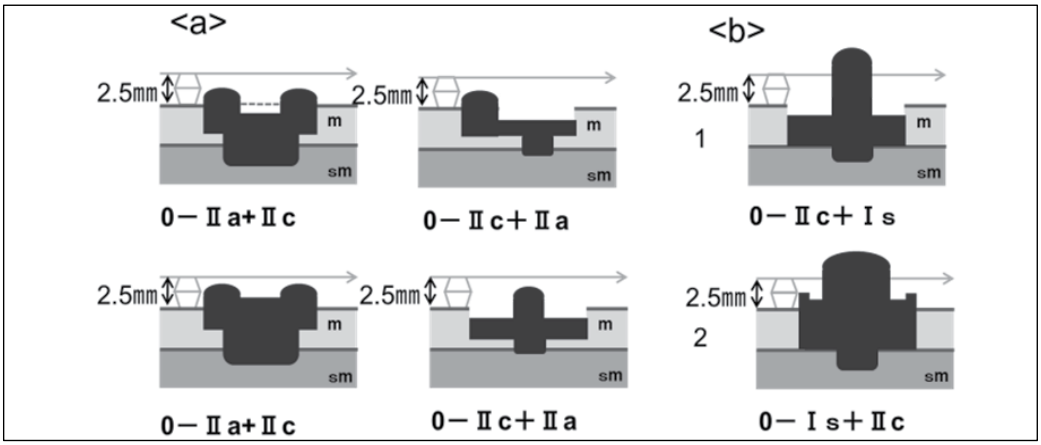


Fig. 5. Classification of mixed-type lesions (Paris classification)

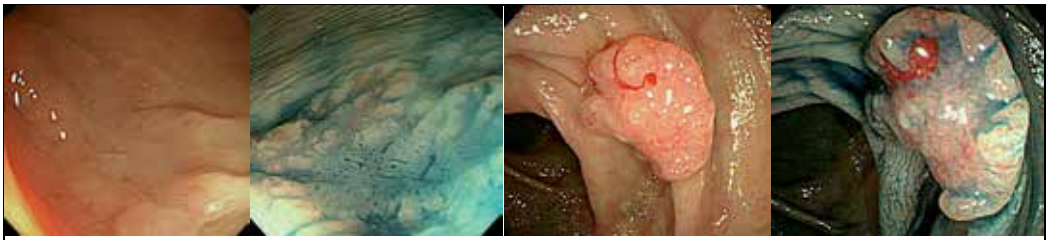


Fig. 6. (A) Type 0-IIc, Ordinary colonoscopic picture showing a depressed area with erosion (B) Type 0-IIc, Indigocarmine dye spraying view (C) Type 0-IIa+IIc, Ordinary colonoscopic picture showing a flat elevated lesion with irregular depressed area (D) Type 0-IIa+IIc, Indigocarmine dye spraying view

The importance of an endoscopic classification system for superficial lesions is that it permits endoscopic staging. In other words, we can predict the depth of invasion of a superficial carcinoma and predict the risk of lymph node metastasis, both of which assist in treatment selection (endoscopic treatment or surgical resection). Regarding Type 0-I lesions, if a lesion becomes large size, the risk of submucosal invasion is increased gradually. Conversely, Type 0-IIc lesions have deep invasion tendencies despite their small size. In addition, Type 0-IIa+IIc lesions frequently infiltrate the deep stratum submucosum, and their potential for progression is higher than that of other types.

2.3 Endoscopic ultrasonography (EUS) and diagnosis of depth invasion for colorectal carcinoma

EUS is an imaging technique for ultrasound scanning of the gastrointestinal tract lumen. It can depict lesions as vertical tomographic images. EUS can be used to evaluate the depth of invasion of epithelial tumors and carcinomas, as well as for qualitative diagnosis, such as the differential diagnosis of extramural lesions in patients with submucosal tumors. EUS is an important diagnostic procedure for deciding the treatment policy and assessing the status of diseases involving the lower gastrointestinal tract. This section focuses on the diagnosis of colorectal cancer.

The lower gastrointestinal tract has the highest incidence of colorectal cancer; EUS is indicated for the diagnosis of the depth of wall invasion and lymph node metastases. EUS is also indicated for the evaluation of submucosal tumors. Malignant lymphomas, gastrointestinal stromal tumors (GIST), lymphangiomas, and lipomas arise at a relatively high frequency in the lower gastrointestinal tract.

2.3.1 Instruments and ultrasonic probe (USP) of EUS

Ultrasonographic instruments specifically for the colorectum and ultrasonic probes are available for EUS of the colorectal region. Endoscopic three-dimensional ultrasonic probes are also commercially available.

An ultrasonic probe is attached to the tip of a direct-viewing electronic endoscope to perform mechanical radial ultrasonic scanning. One advantage of using a specialized device is the excellent ultrasonic resolution, allowing distinct tomographic images to be obtained throughout the entire intestine. Scanning can be performed at either of two frequencies (7.5 MHz, 20 MHz), and the frequency best suited for a given lesion can be selected. The higher

frequency is better suited for low and superficial lesions, whereas the lower frequency is recommended for the assessment of high and deep lesions and the examination of lymph node metastases and other lesions around the intestine. A disadvantage of specialized devices is the large outer diameter of the scope and the long hard tip, often precluding insertion into the proximal side of the sigmoid colon.
A USP can usually be inserted in the forceps channel of the endoscope. Either a 12-, 20-, or 30-MHz probe is selected, depending on the lesion. A USP is inferior to a specialized device in terms of lateral resolution and durability, but excels with respect to targeting because ultrasonic procedures can be done while directly viewing a lesion. Lesions associated with stenosis are also good indications for a USP.

2.3.2 Diagnostic technique of EUS for colorectal carcinoma

The colorectal wall is fundamentally depicted as a 5-layer structure on EUS. Starting from the lumen, the first, hyperechoic layer and the second, hypoechoic layer correspond to the mucosa, the third, hyperechoic layer to the submucosa, the fourth, hypoechoic layer to the muscularis propria, and the fifth, hyperechoic layer to the sabserosa and serosa (the tunica adventitia at sites with no serosa) (Fig. 7.).

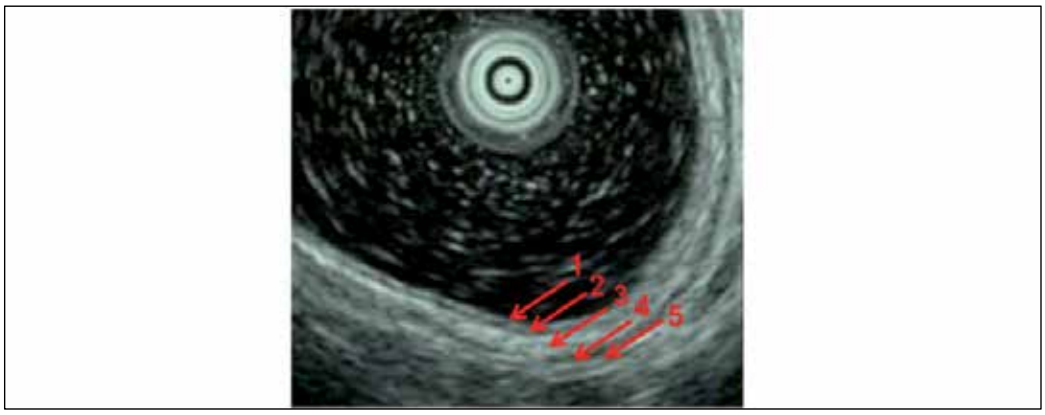


Fig. 7. Layer structure of the normal intestinal wall

With the use of high-frequency devices (20-30 MHz), the muscularis mucosae is depicted as a thin hypoechoic layer with a hyperechoic border at the upper margin of the third layer. In the fourth layer, the connective tissue of the muscularis is depicted as a thin hyperechoic layer, and the colorectal wall is sometimes depicted as a 9-layer structure. An understanding of these characteristics is essential for the diagnosis of lesions by comparison with the layer structure of the intestinal wall.

2.3.3 Diagnosis of colorectal carcinoma on EUS

On EUS, the depth of wall invasion is evaluated on the basis of what layers are preserved or destroyed by a hypoechoic mass. In M cancer (intramucosal cancer), the mass is confined to the first to second layers. In SM cancer (cancer invading the submucosa), the third layer is narrowed or ruptured by the mass, but the fourth layer remains intact. In MP cancer (cancer invading the muscularis propria), the fourth layer is narrowed or ruptured by the mass, but the fifth layer remains intact (Fig. 8.).

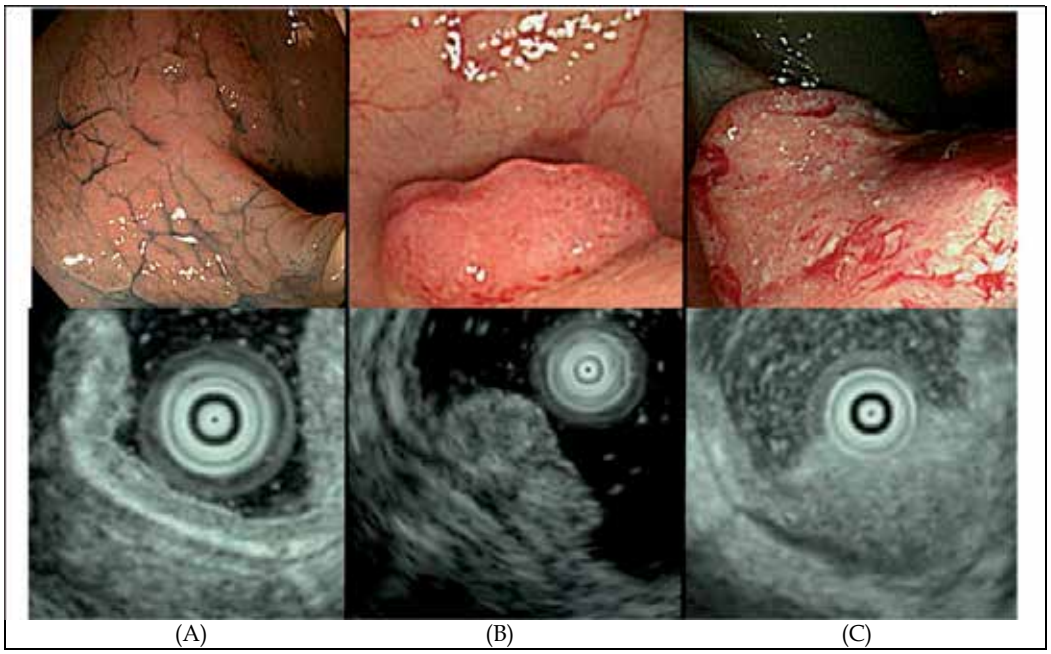


Fig. 8. Endoscopic view and EUS image, (A) Mucosal cancer, (B) SM cancer, (C)MP cancer

In SS to SE cancer (cancer invading the sabserosa or serosa) or A cancer (cancer invading the tunica adventitia), the fifth layer is narrowed or ruptured by the mass, but the border with the adjacent organ remains intact. In SI or AI cancer (cancer invading the adjacent organ), up to the fifth layer is destroyed by the mass, and the border with the adjacent organ is unclearly demarcated.

2.3.4 Diagnosis of invasion depth of SM cancer

Intramucosal cancer and SM cancer with mild invasion to a vertical depth of less than 1000 μm from the lower border of the muscularis mucosae have virtually no risk of metastasis, and cure can be expected after endoscopic treatment. Endoscopic therapy is thus indicated for such lesions (Sugihara, 2009). On EUS, SM cancers are classified into lesions with shallow invasion and those with deep invasion at the time of diagnosis based on Kudo's classification of SM cancer (Kudo, 2000). The third layer, corresponding to the normal submucosa adjacent to cancer, is subdivided into 3 equal layers, and the location of deepest region of the hypoechoic mass is determined. Masses that are confined to the shallowest third of the submucosa are classified as sm1, those that invade the second third are classified as sm2, and those that invade deeper than the second third, but do not extend beyond the region near the medial border of the fourth layer are classified as sm3. SM cancers with shallow invasion correspond to sm1, and those with deep invasion correspond to sm2 and sm3. EUS diagnosis thus plays an important role in the selection of treatment for early colorectal cancer.

2.3.5 Diagnosis of lymph node metastasis

Normal lymph nodes are not depicted on EUS. Lymph nodes visualized on EUS that have a shortest diameter of ≥5 mm, a hyperechoic and homogeneous internal echo, and are either

circular or irregularly shaped are considered positive for metastasis. However, differential diagnosis from enlarged lymph nodes associated with inflammation is challenging. The rate of correctly diagnosing lymph node metastases has been reported to be 70% to 80% (Tio, 1991, Cho E, et al. 1993). The diagnostic ability of EUS is thus not considered good.
EUS is useful for diagnosis of the invasion depth of colorectal cancer because it can depict lesions as vertical tomographic images. It is thus an important diagnostic procedure for deciding treatment policy and evaluating disease status.

2.4 Diagnosis of colorectal neoplastic lesions by chromoendoscopy and image-enhanced endoscopy

If a colorectal lesion is detected by conventional endoscopy, the location, size, macroscopic type, color, surface pattern, presence of fold conversion, and air-induced deformation can be observed. The indigo carmine dye-spraying method more clearly reveals the extent and surface pattern. In addition, magnifying endoscopy after staining with indigo carmine or crystal violet is useful for pit pattern classification (Kudo et al., 1994), as it enables the differentiation of neoplasms as well as histological grading and depth evaluation of early cancers. This leads to the selection of endoscopic therapy or surgery.
The combination of image-enhanced endoscopic techniques such as NBI with magnification is used to observe the capillary pattern of the tumor surface, and these techniques can also improve diagnosis (Sano et al., 2006).

2.4.1 Classification of pit pattern

Pit patterns in the large intestine were classified into 7 types by Kudo (Fig. 9). Type I includes round pits that are observed in normal mucosa. Type II includes stellar or papillary pits, and these pits always indicate hyperplasia. Type IIIs includes small tubular or round pit that are smaller than normal pits, and they indicate neoplastic lesions, occasionally including carcinoma that can be resected by endoscopy. Type IIIL includes

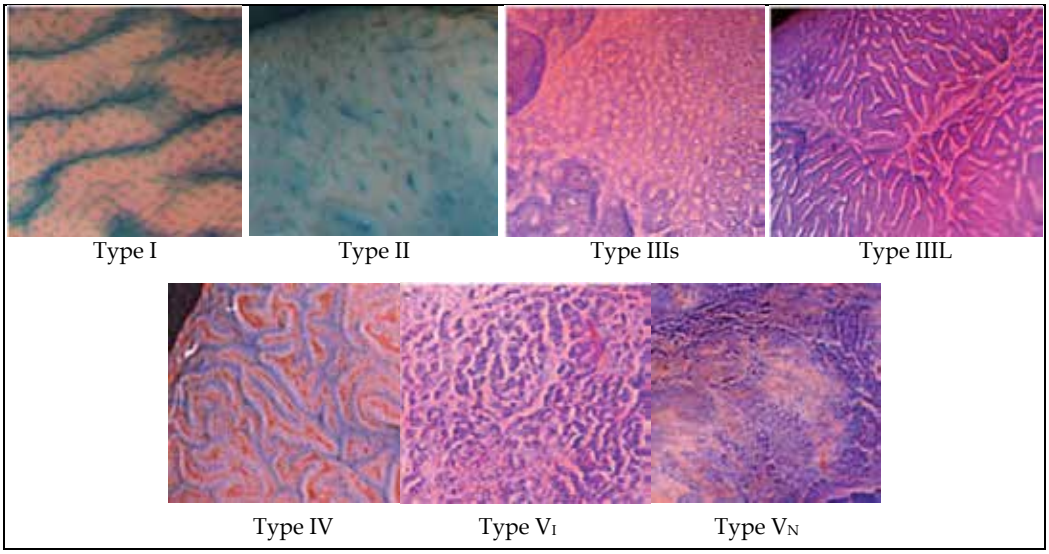


Fig. 9. Classification of pit pattern

tubular or roundish pits that are larger than normal pits. Almost all of Type IIIL lesions are tubular adenomas in pathology, which can be treated by polypectomy. Type IV includes branch-like or gyrus-like pits, most of which are tubulovillous adenoma. Mucosal carcinoma is present in 35% of these pits and can be treated by endoscopy. Type V_I includes irregularly arranged pits that may be submucosal invasive carcinoma, for which the proper treatment straddles the borderline between endoscopic and surgical therapy. Lastly, type V_N includes nonstructured pits, which indicate massive submucosal invasive carcinoma and require surgical resection with lymph node dissection.
Kudo reported that small round pit patterns (type IIIs) and non-pit patterns (type V) were common in depressed lesions and that these depressed lesions had invaded the deeper layers more rapidly than had protruding lesions.

2.4.2 Classification of capillary pattern by magnified NBI

The NBI system involves modifying spectral features by narrowing the bandwidth of spectral transmittance using various optical filters (Sano, 2001). This modification provides a unique image that emphasizes the capillary pattern, as well as the surface structure, by simple operation of a button on the control panel of the endoscope. Because of its similarity to chromoendoscopy, NBI can be referred to as optical or digital chromoendoscopy. Sano et al. (2006) classified 3 types and names (CP types I, II, and III) of microvascular architectures based on the magnified NBI pattern. CP type I has no meshed capillary vessels. CP type II has meshed capillary vessels surrounding the mucosal glands. CP type III lesions were further classified into 2 groups: types IIIA and IIIB. CP type IIIA has irregular meshed capillary vessels, whereas irregular meshed capillary vessels disappear or loosen in CP type IIIB (Fig.10). CP types I, II, IIIA, and IIIB are observed in nonneoplastic lesions, adenomas, mucosal or slightly invasive submucosal carcinoma, and massive invasive submucosal carcinoma, respectively. Capillary patterns, as assessed by magnifying NBI, are useful for differentiating small colorectal nonneoplastic polyps from neoplastic ones (accuracy, 95.3%; sensitivity, 96.4%; and specificity, 92.3%) (Sano, 2008), and they are highly accurate for distinguishing low-grade dysplasia from high-grade dysplasia/invasive cancer (accuracy, 95.5%; sensitivity, 90.3%; and specificity, 97.1%) (Katagiri, 2008). Therefore, capillary patterns can be used to predict the histopathology of colorectal neoplasia.

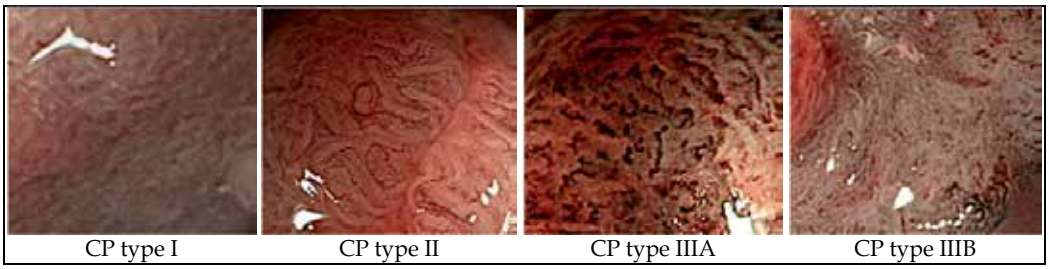


Fig. 10. Capillary pattern by magnified NBI

2.5 Risk factor for lymph node metastasis of submucosal invasive carcinoma

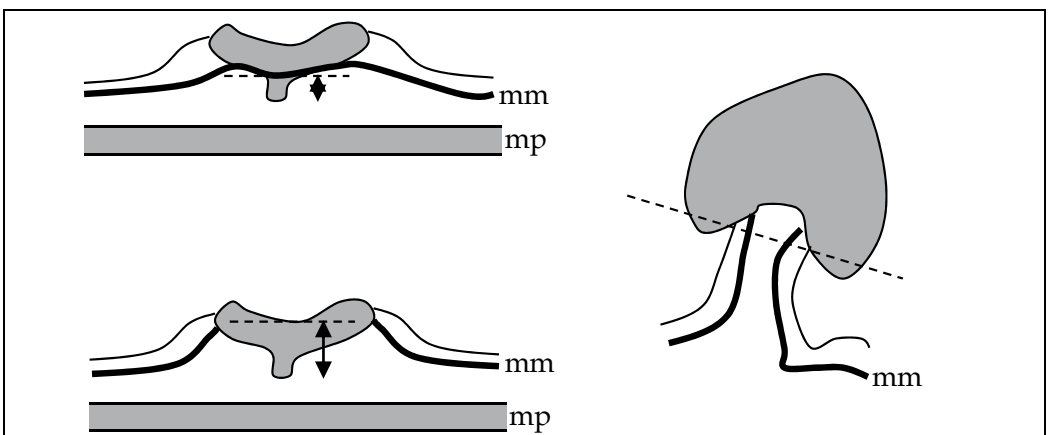
Lymph node metastasis is reported to occur in approximately 10% of SM cancers. SM cancer is a boundary lesion, and its treatment plan involves endoscopic treatment or surgical

resection with lymph node dissection. Therefore, investigation of the risk factors for lymph node metastasis is important. The risk factors for lymph node metastasis are described on the "Colorectal Cancer Treatment Guideline 2009," written by the Japanese Society for Cancer of the Colon and Rectum; these guidelines are listed below. When a risk factor for metastasis is revealed upon examination of an endoscopically resected SM cancer specimen, additional surgical resection with lymph node dissection is recommended.

1. Carcinoma that is histologically classified as poorly differentiated adenocarcinoma, signet ring cell adenocarcinoma, or mucinous carcinoma
2. Depth of submucosal invasion >1000 μm
3. Vascular invasion is positive
4. Grade 2/3 budding

In classifying the histological type of a carcinoma, the predominant pattern is adopted as its representative histological type in the Japanese classification of colorectal carcinoma (Sugihara, 2009). For example, for a tumor consisting mainly of well-differentiated carcinoma with a small portion of moderately differentiated carcinoma, a diagnosis of "well-differentiated carcinoma" should be made. High-grade carcinomas that are poorly differentiated adenocarcinomas, signet ring cell adenocarcinomas, or mucinous carcinomas have strong proliferative and metastatic capabilities.

When it is possible to identify the muscularis mucosae, the depth of submucosal invasion is the distance between the deepest edge of the muscularis mucosae and the deepest invasion. If the muscularis mucosae cannot be identified, the depth of submucosal invasion is the distance between the surface of the tumor and the deepest invasion. In Ip lesions with disrupted muscularis mucosae, the depth of submucosal invasion is the distance between the deepest invasion and the reference line, and it is defined as the boundary between the tumor head and the pedicle. When cancer does not invade beyond the reference line, it is defined as head invasion (Fig. 11). According to Kitamura, for pedunculated SM cancer, the rate of lymph node metastasis was 0% in cases of head and stalk invasion with depths <3000 μm if lymphatic invasion was not observed. For nonpedunculated SM cancer, the rate of lymph node metastasis was also 0% if SM depth was <1000μm (Kitajima, 2004).



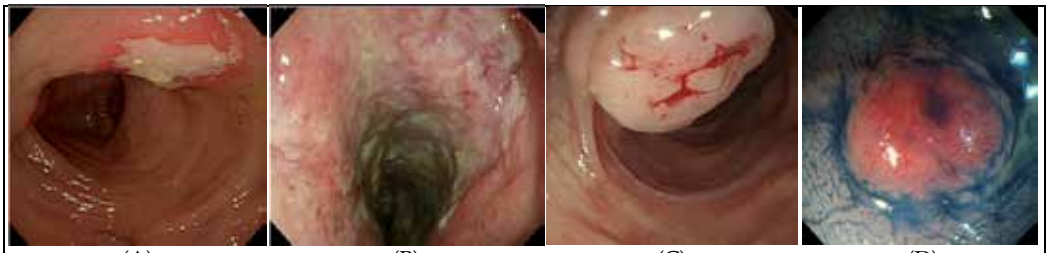
mm, muscularis mucosae; mp, muscularis propria

Fig. 11. Depth of submucosal invasion in SM cancers

Vessel invasions contain 2 types of lymphatic invasion and venous invasion. The distinction is not so easy. When cancer nests are located in lymphatic ducts lined by flat endothelial cells, it is considered positive lymphatic invasion. Cancer nests existing near an artery are very likely to represent venous invasion, which can be confirmed by determining the presence of an internal elastic membrane and plain muscle around the cancer nests. Occasionally, it is difficult to detect venous invasion by hematoxylin and eosin staining. Elastica van Gieson staining, however, is useful for detecting venous invasion.
Budding is defined as cancer nests comprising less than 5 cancer cells and invading the interstitial tissue of the cancer growth front. The area where budding appears most frequently is selected and the number of instances is counted in a ×200 field. Budding is classified into 3 groups (grade 1, 0–4 pieces; grade 2, 5–9 pieces; and grade 3, ≥10 pieces), and grades 2 and 3 are risk factors for lymph node metastasis.

2.6 Differential diagnosis

There are many diseases that must be differentiated from colorectal carcinoma. The characteristic appearance of colorectal carcinoma does not cause interpretive difficulties generally.
Metastatic lesions in the large intestine: Cancers that frequently metastasize to the large intestine include those of the stomach, pancreas, ovaries, lung, and breasts in descending order of frequency. The endoscopic appearance of metastatic lesions generally includes (1) extraluminal masses with or without hyperemia, (2) wall thickening, and (3) hyperemia. Extraluminal masses are often smooth, but the base is not distinct. Mucosal hyperemias are often multiple, and the border is obscure. Ulcerated tumors are also the findings of metastatic lesions, but the marginal elevation typical of primary cancers is rarely observed (Fig. 12A).
Peritonitis carcinomatosa: Peritonitis carcinomatosa does not affect the mucosal surface.
Malignant lymphoma and sarcoma: Polypoid-type lymphomas are often smooth-surfaced. Colon cancer ulcers are usually accompanied by irregular margins, but that of ulcerative-type malignant lymphoma often contains a smooth margin (Fig. 12B, C). Sarcomas are likely to be malignant lymphomas.



A, metastatic colon cancer from gastric cancer (poorly differentiated adenocarcinoma);
B, ulcerative type of malignant lymphoma; C, elevated type of malignant lymphoma;
D, carcinoid

Fig. 12. Colorectal neoplasmas

Carcinoids: Carcinoids are covered with the normal mucosa initially. In addition, carcinoids are usually elastic, hard, and yellowish. Endoscopic ultrasound staging (EUS) is useful for

diagnosing the internal state and present layer. If they invade mucosa, vessels and ulcer formation occur (Fig.12D)

Inflammatory disease: Inflammatory colonic diseases, such as Crohn disease (CD), ulcerative colitis (UC), and colonic tuberculosis (TBc), are sometimes likely to resemble colonic carcinoma regarding its endoscopic features. CD often includes longitudinal ulceration, but the ulcer is sharp and smooth. UC is rarely displayed as a self-limited ulcer, but ulcers of UC are soft and thin. Colonic TBc is rarely similar to Type 2 cancer, but the ulcers of TBc are not hard or irregular.

3. Endoscopic treatment for colorectal carcinoma and its precursors

There are many methods of treating colorectal tumors, such as hot biopsy and snare polypectomy, EMR, EPMR, and ESD. Advances in endoscopic instruments and techniques have led to a large increase in the number of endoscopically resected lesions. Safe and reliable endoscopic treatment for colorectal carcinoma requires diagnostic ability and skill in colonoscopy.

3.1 Hot biopsy and polypectomy

Colonic polyps less than 0.8 cm in diameter are usually removed by hot biopsy, particularly when they are sessile, whereas polyps more than 0.8 cm in diameter are usually removed by snare polypectomy, particularly when they are pedunculated (Mann, 1999). Hot biopsy involves grasping the top part of the polyp upward and moderately cauterizing the polyp. Hot biopsy is performed cautiously in the cecum using a low amplitude and brief duration of current because the colonic wall is the thinnest and most vulnerable to transmural necrosis in this region (Weston, 1995). One limitation of hot biopsy is that only a portion of the polyp can be examined pathologically because the polyp cannot be completed removed. Therefore, we also perform snare polypectomy for even polyps smaller than 0.8 cm in diameter.

Snare polypectomy is chiefly applied to pedunculated and sessile lesions of 0.5 to 2.0 cm in diameter. Sessile polyps between 2 and 3 cm in diameter may be removed by snare polypectomy after creating a pseudopedicle by injecting normal saline or other solution into the polyp base, as described in the following section (Waye, 1997; Kato, 2001, 2008). Sessile polyps more than 3 cm in diameter may be unamenable to conventional snare polypectomy but can be removed by sequential piecemeal polypectomy over several colonoscopies (Dell'Abate, 2001).

The complication rate of therapeutic colonoscopy is 1.4–2.0% (Jentschura, 1994; Nelson, 2002; Kato, 2008). The most common postpolypectomy complications are gastrointestinal bleeding, colonic perforation, and local peritonitis. In local peritonitis, a patient develops abdominal pain, leukocytosis, and localized peritoneal irritation from an almost transmural burn occurring during polypectomy. This occurs in up to 1% of polypectomies (Waye, 1996). This syndrome is usually managed medically by the cessation of oral intake, intravenous hydration, and antibiotic administration (Waye, 1993).

3.2 EMR and EPMR

EMR combines the classic principles of conventional snare polypectomy with submucosal injection to remove more deeply affected mucosa or submucosa by resecting the lesion

through the middle or deep submucosa. It is a less invasive treatment option for colorectal lesions even if the lesion is flat and difficult to remove by snare polypectomy. When we want to resect early colorectal carcinoma surrounded by the normal mucosa, EMR is a suitable procedure. After observation of the lesion, hypertonic saline solution with epinephrine is injected into the submucosal layer. At this time, the lesion-lifted condition is observed as follows (see section 3.3). When the lifted condition is complete, the bulging lesion is captured in a surgical snare and removed by cauterization with a high-frequency current.
A lesion less than 2.0 cm in diameter can generally be resected en bloc. The relationship between tumor size and the en bloc resection rate in our medical center between 2000 and 2010 is presented in Fig. 13. Lesions smaller than 20 mm in diameter were resected en bloc in more than 90% of colorectal carcinoma cases. Most lesions larger than 26 mm in diameter are treated by the piecemeal method.

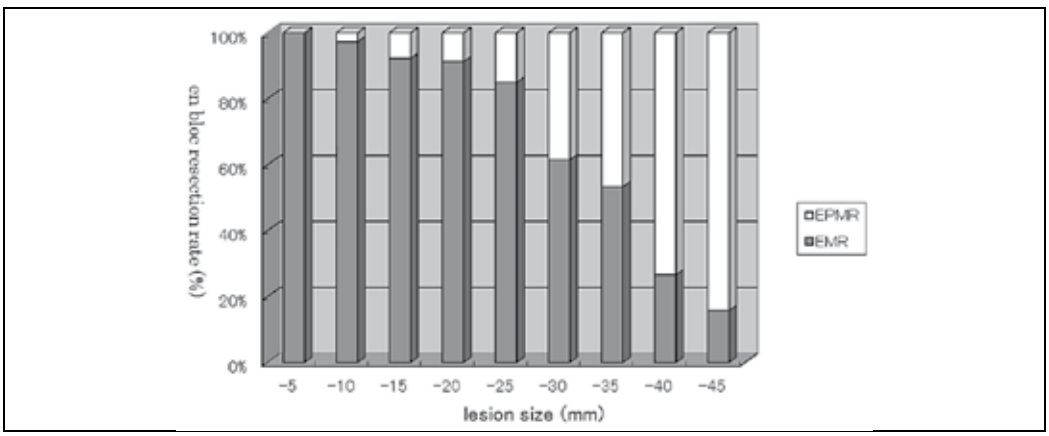


Fig. 13. Relationship between lesion size and en bloc resection (EMR) vs. EPMP

Among 281 cases of early colorectal carcinomas treated by EMR, only 2 cases recurred during the observation period of 5 years (recurrence rate, 0.8%). The recurrent cases could be retreated by endoscopic methods. Conversely, 8 cases among 148 cases treated by EPMR recurred (recurrence rate, 5.4%), and 2 of these cases were treated surgically. Therefore, the recurrence rate of EMR is significantly lower than that of EPMR ($p<0.01$). However, these methods are used to treat lesions of different sizes, and thus, simple comparisons of recurrence rates can be misleading. However, we believe that en bloc resection is better than piecemeal resection from the point of view of accurate pathological diagnosis. Large superficial carcinomas without lymph node metastasis requiring en bloc resection are currently resected using ESD at our medical center.
Bergmann (2003) reported that local recurrence after EMR was observed in 2 of 59 completely resected adenomas and in 0 of 6 early-stage carcinomas during a mean follow-up of 18 months. He concluded that advanced non-polypoid colorectal adenomas and early-stage carcinomas can be safely and effectively resected by EMR.
Jin et al. (2009) reported that recurrence was found to be related to piecemeal resection and diameters larger than 20 mm and that >20-mm-diameter is an independent risk factor for laterally spreading tumors (LSTs) treated by EMR. They stated that for the LSTs larger than 20 mm in diameter, another method, such as ESD or even a major operation, should be considered.

3.3 Classification of lesion-lifted condition

Special findings such as depression, ulceration, fold convergence, bleeding tendency, irregular shape, and a non-lifting sign indicate a deep invasion (Uno, 1994; Kobayashi, 2007). The "non-lifting sign" is a simple yes/no classification, and compared to EUS, it is a much easier method to determine whether EMR is indicated. However, because it is by no means rare for submucosal invasion to be found among lesions that exhibit a negative lifting sign, we have created a more detailed classification of the lesion-lifted condition. In a previous study, we focused on the tumor's lifted condition after submucosal injection and classified lesions into 4 types (Kato, 2001).

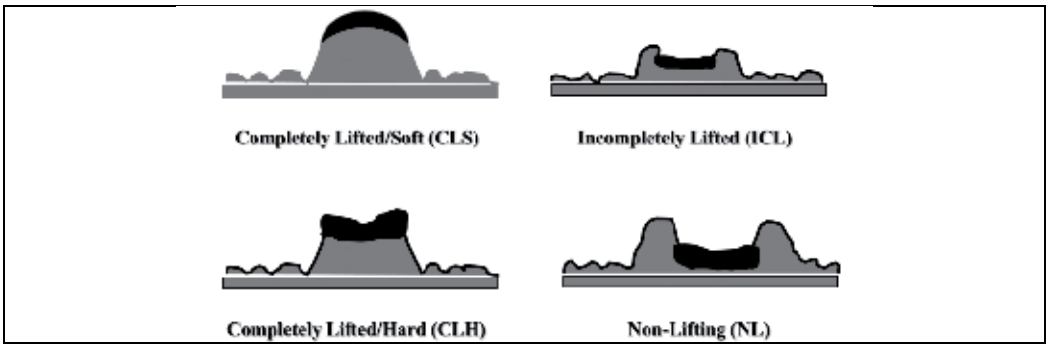


Fig. 14. Classification of the lesion-lifted condition (Kato, 2001)

This classification is closely related to the depth of invasion, and has proved to be particularly useful in the identification of early colorectal cancers that are good candidates for endoscopic treatment without requiring any special apparatus. We classified the lesion-lifted condition at the time of submucosal fluid injection into 4 categories (Fig. 14): (1) completely lifted/soft (CLS), (2) completely lifted/hard (CLH), (3) incompletely lifted (ICL), and (4) non-lifting (NL). A CLS lesion is completely lifted by submucosal injection, and it stretches softly like a dome. A CLH lesion is completely lifted, but it is rigid and maintains its original form. An ICL lesion is slightly lifted, but the surrounding mucosa lifts higher than the lesion. An NL lesion is not lifted, and only the surrounding mucosa is elevated (Fig. 15).

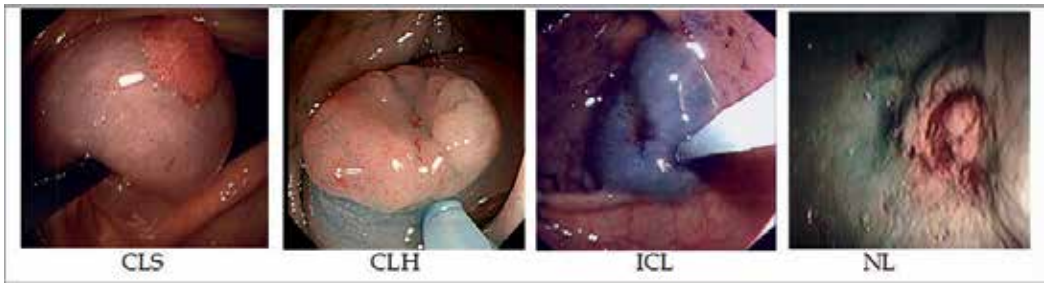


Fig. 15. Classification of the lesion-lifted condition (Kato, 2001)

Lesion-lifted conditions are related to tumor pathology and the extent of tumor invasion, and they often correspond to particular macroscopic types of tumors. The relationship between the lifted condition and macroscopic type is shown in Table 1. Type IIa predominates among CLS lesions, whereas elevated lesions such as types Ip, Isp, and Is tend

to fall into the CLH category. On the other hand, type IIa+IIc is relatively common type of morphology among ICL and NL lesions. The relationship between lifted conditions from CLS to ICL lesions and their corresponding macroscopic types are statistically significant ($p<0.001$).

	Ip	Isp	Is	IIa	IIc	IIa+IIc	LST
CLS	4	30	66	324	24	9	105
CLH	25	73	90	77	13	8	18
ICL	0	10	22	21	7	12	13
NL	0	0	8	2	1	5	2

Table 1. Relationship between lifted condition and macroscopic type ($p<0.001$)

Classification of submucosal invasion is based on the division of the submucosa into 3 layers from sm1 to sm3. sm1, sm2, and sm3 are lesions that are limited to the upper, middle, and lower thirds of the submucosal layer, respectively. sm1 lesions are further subdivided into 3 categories (a, b, and c) with regard to the degree of horizontal involvement of the upper submucosal layer (ratio of involved part and non-involved part). Whereas sm1a+sm1b lesions have a very low risk for metastasis, the malignant potential increases with increasing depth of submucosal invasion (Kudo, 1997; Kashida, 2006). The relationship between the lesion-lifted condition and the depth of invasion is shown in Table 2. We used Kudo's classification to subclassify the depth of SM cancer. All CLS lesions are found to be sm1 or shallower, whereas the CLH category included 20 sm2 and 14 sm3 lesions. The rate of SM massive cancer (sm2 or sm3) among CLH lesions was 11.0%. ICL lesions range from sm1 to sm3, and most of the NL lesions exhibit invasion to sm3 or deeper. Four noncancerous cases and 1 mucosal cancer case of NL lesions were recurred adenomas and cancers that had previously been treated by endoscopic therapy. The rates of SM massive cancer among ICL and NL lesions are 38.5% and 74.1%, respectively. The lesion-lifted condition well correlates with the depth of invasion.

	nc	cia	m	sm1	sm2	sm3-
CLS	451	65	34	3	0	0
CLH	123	75	58	18	20	14
ICL	20	14	7	7	10	20
NL	4	0	1	0	1	13

nc, noncancerous lesion; cia, cancer in adenoma; m, mucosal cancer; sm1, shallow SM cancer; sm2, moderate SM cancer; sm3, deep SM cancer
Chi-square test, $p < 0.0001$

Table 2. Relationship between the lesion-lifted condition and the depth of invasion (Number of the lesions)

When the lifted condition of a lesion is CLS or CLH, it is a good indication that endoscopic resection will be successful. When the lifted condition is ICL, however, endoscopic resection has a smaller chance of success. And almost all of NL lesion without previous endoscopic therapy had better receive surgical treatment.

3.4 ESD

ESD is a resection technique for superficial neoplastic lesions of the gastrointestinal tract without the use of snaring. It was developed for en bloc resection of large superficial mucosal tumors, and it was initially used in the stomach and later in the esophagus, colon, and rectum. ESD is superior to EMR for a more reliable en bloc resection of a targeted area of the mucosa. It also provides a higher complete resection rate with a lower recurrence rate compared with EPMR (Saito, 2010). The drawbacks of ESD include that it is a time-consuming procedure, has greater technical demands, and has a higher rate of perforation. For these reasons, ESD for colorectal tumor is performed as an advanced medical treatment because it is currently not recognized as treatment covered by the national health insurance system of Japan in 2011.

3.4.1 Method of ESD

Method of ESD is incising mucosa around a lesion lifted by injected fluid and dissecting the submucosal space under the lesion (Fig.16). ESD for colorectal tumors is considered more technically demanding than ESD in the stomach for a variety of reasons including the following: (1) the colonic wall is thinner and softer than the gastric wall; (2) endoscopic control is difficult in some parts of the colon because of its meandering form; and (3) there are limitations in the retroflex approach due to the narrow lumen of the colon, and tumors can be located on or behind a prominent fold of the colon.

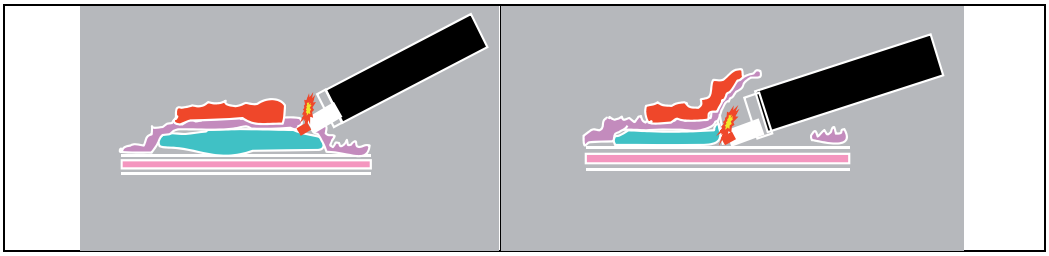


Fig. 16. Method of ESD

First, the borders of the tumors are determined by chromoendoscopy with indigo carmine spraying for enhanced or magnified observation using NBI. Marking around the tumor is not necessary in most cases because colorectal neoplasms typically have clear margins.

The use of 0.4% sodium hyaluronate solution for submucosal injection keeps the tumor lifted for long periods (Yamamoto, 1999). For successful ESD, the position of the patient should be selected such that the lesion is located at the top of the colonic lumen with regard to gravity. Because the lesion is naturally pulled down and blood flows down from the bleeding point by gravity, good visualization of submucosal space can be maintained.

Next, the mucosal incision in front of the tumor is made with a short needle knife such as FlushKnife BT™ (1.5 mm; Fujifilm Corp., Tokyo, Japan) (Fig.17). Only the needle part should be used for the incision, keeping the tip of the sheath touching the surface of the mucosa without pushing the sheath into the submucosal layer. We use the endcut mode of electric surgical unit for the mucosal incision. After repeated submucosal injection, submucosal dissection is performed parallel to the muscular layer by sliding the knife from the center to the side while hooking submucosal fibers with the knife. We use the swift coagulation mode of electric surgical unit at this time. When thick vessels can be observed in

the submucosal layer, grasping and soft coagulation are performed using coagulation forceps. Furthermore, a surrounding incision is made, and submucosal dissection is performed while lifting up the dissected part of the tumor with the edge of the transparent cap at the tip of the scope. Finally, hemostasis and the lack of a weak point of the muscularis propria are confirmed after resection.

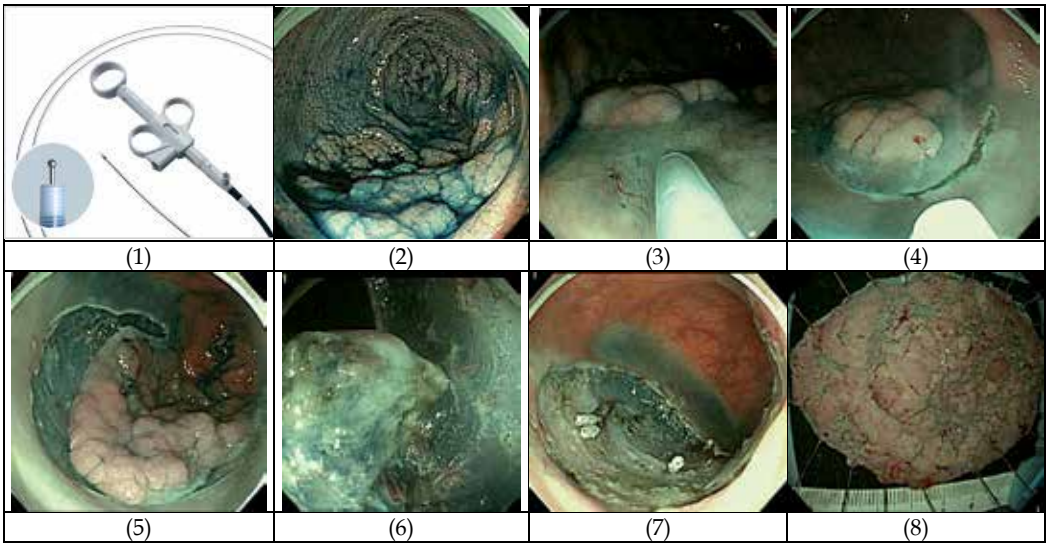


Fig. 17. ESD using FlushKnife BT™ for rectal mucosal carcinoma of 80mm in diameter

The usefulness of new grasping-type scissor forceps (GSF) such as ClutchCutter™ (Fujifilm Corp., Tokyo, Japan) was reported by Akahoshi et al. (2010). ESD using GSF is a safe (no intraoperative complication) and technically efficient (curative en bloc resection rate, 92%) method for the dissection of early gastrointestinal tumors. The use of GSF is a promising option for performing ESD in early-stage GI tract tumors both safely and effectively. We typically use GSF on the lesions that are difficult to approach or control by endoscopy. The ability to confirm that GSF is not grasping the muscle layer before coagulation or cutting is a point of safety (Fig.18).

Fig. 18. ESD using Clutchcutter™ (Fujifilm Corp., Tokyo, Japan)

3.4.2 Cases of ESD

We investigated 116 patients with colorectal lesions for whom ESD was performed between Jan 2005 and Mar 2011. The tumors were entirely located in the large intestine (27 in the

transverse colon and 25 in the rectum) (Table 3). Type IIa was the most common macroscopic type (Table 4). The average diameter was approximately 30 mm (range, 4–82 mm). The average operation time was 75 min. Regarding complications, an incision in the muscularis propria was found in 6% cases. Perforation was experienced in 7%. But all of the perforation hole could be closed by endoscopic clip without surgical procedure.
Approximately half the lesions were adenomas, and the rest were carcinomas. One patient with carcinoma in situ exhibited recurrence in the mucosa and received endoscopic treatment. Additional colectomy was performed in 8 patients with submucosal invasion. There are pathological residual cancer nests in 2 cases. One patient had persistent carcinoma in the colonic wall, and another had lymph node metastasis.

	C	A	T	D	S	R
No of Lesions (%)	12 (10)	19 (16)	27 (23)	8 (7)	18 (16)	25 (22)

C, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon; R, rectum

Table 3. location of the ESD lesions

	Is	IIa	IIc	SMT
No of Lesions (%)	24 (21)	82 (71)	4 (3)	6 (5)

SMT, submucosal tumor

Table 4. Macroscopic types of ESD lesions

3.4.3 Comparisons among EMR, EPMR and ESD

Clinicopathological data were compared among EMR, EPMR, and ESD between 2000 and 2011. The size in diameter of the lesions that were treated by each technique was compared. Very large lesions can be treated by EPMR and ESD. The mean sizes of lesions treated by EMR, EPMR, and EMR were 13.1 (range, 2–45), 24.6 (4–69), and 29.7 mm (4–82), respectively (Fig. 19).

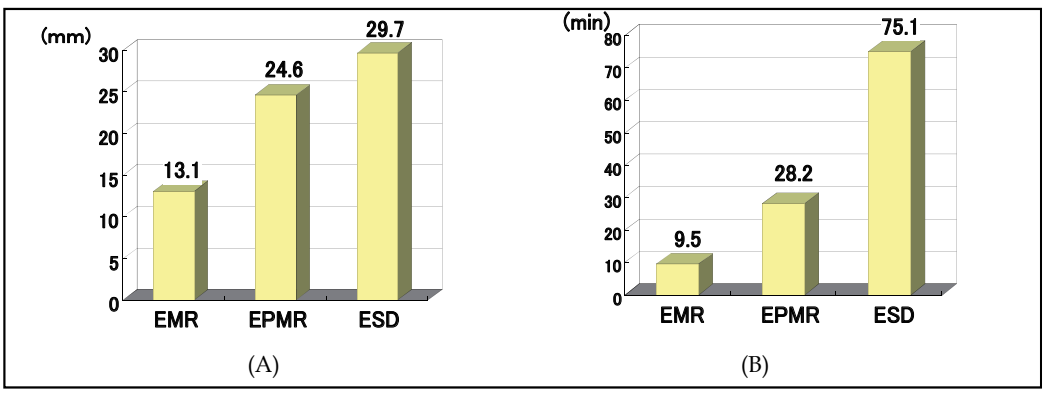


Fig. 19. (A)Mean diameter of the lesions treated by each method , (B) Mean operation time

The mean operation times of the 3 methods were also compared. ESD required about 75 minutes to perform and longer than the other techniques. Regarding ESD complications, postoperative hemorrhage is not frequent, but perforation and muscularis propria incision are more common with ESD than with EMR or EPMR. However, all perforations and muscle incisions could be closed by endoscopic clipping, and there was no negative effect in the clinical course (Table 5).

	EMR (n=1039)	EPMR (n=147)	ESD (n=116)
Postoperative hemorrhage: endoscopic hemostasis	16(1.5%)	6(4.1%)	3(2.6%)
Perforation: endoscopic closure	1(0.1)	0	8(6.9)
Perforation: surgical closure	1(0.1)	0	0
Incision of muscularis propria: endoscopic clipping	0	0	7(6.0)
Local peritonitis: conservative therapy	2(0.2)	2(1.4)	2(1.7)

Table 5. Complications of endoscopic treatments for colorectal tumor

Colorectal ESD can be performed in all sites of the large intestine, and even a large lesion could be resected en broc using ESD. However, the procedure was lengthy and involved more complications than did other treatments. Further technical proficiency and instrumental improvements are expected in the future.

4. Conclusions

New methods of endoscopic diagnosis and treatment have been recently developed. Patients with early-stage colorectal carcinoma can be diagnosed by colonoscopy. Endoscopic treatment facilitates healing, and the method is less invasive, more cost-effective, and less time-consuming for patients. Endoscopic apparatuses, devices, and techniques must be further improved in the near future. Endoscopy for colorectal carcinoma will remain important in medical education and practice.

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18

Peri-Operative Care in Colorectal Surgery in the Twenty-First Century

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1. Introduction

Under conventional circumstances, colorectal cancer resection has been associated with an often protracted recovery. Large published studies, randomized trials and meta-analyses suggest an average length of hospital stay of about ten days (Bokey et al., 1995; Abraham et al., 2004 & 2007). In an attempt to mimic the success of laparoscopic gall bladder surgery, laparoscopic colorectal resection was introduced in 1991 as a proposed less invasive alternative to the open technique (Jacobs, 1991; Redwine, 1991). Under conventional circumstances, in the first published series of 20 laparoscopic sigmoid colectomies, the authors reported that a five-day hospital stay was achieved in 70% of patients.

However, subsequent larger studies including randomized trials reported an average length of stay of about eight days which is still an improvement of about 20% compared with conventional open resections (Abraham et al., 2004 & 2007; Schwenk et al., 2005). The last published large randomized controlled trial of the topic (The ALCCaS) showed no statistically significant difference in postoperative complications, reoperation rate, or perioperative mortality between laparoscopic and open resections (Allardyce et al., 2010). However, a recent meta-analysis showed that laparoscopic colorectal resections were associated with higher intra-operative complication rates than open resections (Sammour et al., 2011). The ALCCaS group also reported that reviews show that the short-term advantages for laparoscopic resection for colorectal cancer are arguably relatively minor and often subjective (Allardyce et al., 2010).

In 1999, in a series of 16 open colectomies, the authors reported using a Fast Track (Enhanced Recovery after Surgery (ERAS)) Program with a median postoperative length of hospital stay of two days (Kehlet & Mogensen, 1999). However, subsequent larger studies reported a median length of stay of about five days (Abraham & Albayati, 2011). ERAS programs challenge the conventional approaches to peri-operative care in colorectal surgery in an evidence-based manner. These include conventional bowel preparation, peri-operative starvation, routine nasogastric decompression, routine prophylactic drainage, defunctioning ileostomy, vigorous intravenous hydration, narcotic analgesia, etc ... These traditional protocols and practices are replaced with evidence based protocols that enhance postoperative recovery.

2. General outline of an ERAS protocol

In an ERAS program, all the small steps that form the care package in colorectal surgery are "optimized" to achieve the best possible outcome. These include targeted preoperative interview to educate the patient on what to expect, preoperative nutritional assessment if required, minimal peri-operative starvation, preoperative carbohydrate and protein loading, no routine bowel preparation, transverse or oblique incision if seen fit by the operating surgeon, high oxygen concentrations and normothermia.

They also include avoiding excessive intravenous hydration and the routine use of nasogastric tubes and drains. Other important postoperative management issues include multimodal analgesia (epidural analgesia if seen fit by the anaesthetist, subcostal nerve block when possible, continuous wound infiltration with a local anaesthetic agent (wound soaker) and regular oral non-narcotic analgesia with minimal or no morphia and only using patient controlled applications.

Other elements of an ERAS protocol include the routline use of regular prokinetic agents, the routine use of regular anti-emetic drugs, a structured early postoperative mobilization program and early oral feeding (clear fluid intake on the evening of surgery, free fluid intake on day one and a soft diet on day two). This is all achieved through the co-operation of a team of clinicians, nursing staff, physiotherapists, stoma therapists, dieticians, etc … The general aim is to have the patient ready for discharge by postoperative day four or five.

3. Supportive evidence for the main ERAS practices

3.1 No routine bowel preparation

Mechanical bowel preparation was used for almost a century to cleanse the colon prior to surgery. The aim was to evacuate the colon, reduce the fecal load in the hope that this would – in a plausible way - reduce the bacterial load thus reducing the risk of postoperative infection and anastomotic leak rates. It was also believed that bowel preparation allowed better visualization of the lumen as well as making the anastomosis technically easier. Mechanical bowel preparation became "traditional". However, microbiological testing showed that bowel preparation did not reduce the microbial count in colonic mucosa (Jung et al., 2010). For a few decades, right hemicolectomies have been performed without bowel preparation.

Avoiding routine mechanical bowel preparation is an important component of any ERAS program. In the early seventies, Hughes showed that receiving preoperative bowel preparation made no difference to outcomes including anastomotic leak rates (Hughes, 1972). Multiple studies addressing the same questions have since been conducted (Scabini et al., 2010). A meta-analysis of outcomes following close to five thousand colorectal resections showed no evidence to suggest that bowel preparation reduced the incidence of anastomotic leakage (Guenaga et al., 2009). In fact there was a suggestion that routine bowel preparation could actually increase the risk of infections and overall complication rates associated with colorectal resections.

In an ERAS protocol, patients admitted for right sided resections receive no bowel preparation. For left sided resection, we use enema preparation the night before and the day of surgery to evacuate the rectum and the left colon to facilitate the surgery from the technical point of view. Others use normal saline enemas.

3.2 Minimal preoperative starvation

Preoperative starvation for eight hours or more before receiving an anesthetic has been implemented as an unchallenged rule for a very long time. This was meant to reduce the risk of aspiration pneumonitis if gastric contents were regurgitated during the course of induction of the general anesthetic. There is currently Level I evidence that shows that drinking clear fluids up to two hours prior to surgery does not increase the risk of aspiration or regurgitation as it does not increase gastric acidity or the amount of gastric secretions that could be regurgitated (Brady et al., 2003; Ljungkvist et al., 2003).

Patients were generally instructed to completely fast from midnight the night before their procedures are due to take place. This used to further complicate the semi-starvation state associated with bowel preparation. This could result in an increased catabolic state, dehydration and electrolyte imbalance especially if the procedure took place later in the morning or in the afternoon. This catabolic state is aggravated and complicated further by the surgery itself with the added negative nitrogen balance, insulin resistance and the release of stress hormones such as catecholamines, glucagon and cortisol (Nygren et al., 2001). Starvation also compromises the physiological response to hemorrhage and infection (Brady et al., 2003; Nygren et al., 2001). Patients receiving oral preoperative carbohydrate loading are more likely to have physiological postoperative insulin levels compared with those receiving glucose via the intravenous route and those fasting overnight and not receiving any carbohydrate loading (Kaska et al., 2010).

In an ERAS protocol, patients are typically allowed clear fluids up to two hours before the anesthetic and routinely "loaded" with oral carbohydrate and protein drinks and symbiotics preoperatively.

3.3 No postoperative starvation

Again, patients have traditionally been "fasted" postoperatively until they passed flatus. Even then, they were only allowed clear fluids until they had passed a bowel motion. It was believed that such practice would minimize the risk of an anastomotic leak or make such a leak more easily manageable than if the patient were allowed to eat. This further complicated any pre-operative malnutrition (Garth et al., 2010). Bowel preparation, the strict diet that goes with it and perioperative starvation further increase the catabolic state. Furthermore, the increased immediate postoperative need for nutrients is not met resulting in proteolysis, negative nitrogen balance and increased insulin resistance.

There is now Level I evidence that shows that there is no benefit in postoperative starvation in terms of reducing anastomotic leak rates (Lewis et al., 2009). It is likely that enteral nutrition reduces the overall risks of wound infection and intra-abdominal sepsis, probably through improving the capillary-intestinal barrier (Lewis et al., 2009).

In a standard ERAS protocol, patients are allowed clear fluids the evening after the procedure, free fluids on postoperative day one and a soft diet on postoperative day two regardless of the type of resection performed. We find this protocol to be well tolerated. We warn patients beforehand of the small risk of vomiting but reassure them that this would not be of serious consequence if it took place.

3.4 No nasogastric decompression

The traditional aim of routine nasogastric intubation is to achieve gastric decompression in order to reduce the risk of postoperative ileus, vomiting and abdominal distension. This

seemed to be a plausible means of improving postoperative peristalsis in an attempt to achieve an early return to bowel function. However, Cheatham et al showed in 1995 that routine nasogastric intubation did not reduce the risk of complications or length of postoperative stay in hospital following abdominal surgery (Cheatham et al., 1995). They also showed that for every patient requiring nasogastric intubation, 20 patients will not need it.
The results of this meta-analysis were reinforced by a recent Cochrane review of studies of close to six thousand patients (Nelson et al., 2007). Routine nasogastric decompression slowed the return of bowel function and did not reduce the risk of an anastomotic leak compared with no decompression. It was also associated with a more prolonged length of stay in hospital.

3.5 Consideration for a transverse or oblique incision

It has been suggested that a transverse or an oblique incision is an important part of the practices contributing to a quick recovery (Kehlet et al., 1999). A Cochrane review suggested an overall advantage in adopting a transverse over a midline incision (Brown & Goodfellow, 2005). This finding was supported by the results of a randomized controlled trial of transverse versus longitudinal incisions for cholecystectomy (Halm et al., 2009). Right hemicolectomies have probably been more commonly performed through a transverse rather than a vertical incision for a few decades.
A recent randomized trial suggested that there was no advantage in using a transverse incision over a longitudinal incision in terms of required analgesia, pain, pulmonary complications, median length of stay, median time to tolerating a diet or one year incisional hernia rates (Seiler et al., 2009). The sample size was small and a type II error could not be excluded. However, more wound infections occurred in the transverse incision group (15% vs. 5%, $P = 0.02$). It is the author's experience that left sided resections are overall easier to perform through a midline incision compared with an oblique incision. The choice between midline and transverse incisions may continue to be debated for some time yet.

3.6 No routine prophylactic drainage

It has been thought that prophylactic drainage of colorectal anastomoses would reduce the risk of anastomotic leakage. This was thought to be by a process of reducing the likelihood of a postoperative collection forming near the anastomosis with the plausible risk of infection and a subsequent anastomotic leak. The presence of a drain could also make it easy to detect an anastomotic leak guided by the amount and quality of drain output.
However, multiple randomized trials and a subsequent meta-analysis failed to demonstrate a benefit for routine drainage in colorectal surgery. The systematic review referred to above include the results of 1140 colorectal resections (Jesus et al., 2004). It showed no statistically significant difference between outcomes in patients receiving routine prophylactic drainage or no drainage for colorectal resections in terms of anastomotic leakage, wound infection and all complication rates (Qadan et al., 2009). There is probably no advantage for routine prophylactic drainage of low rectal or colo-anal anastomoses either (Merad et al., 1999; Yeh et al., 2005).

3.7 The limited role of a defunctioning ileostomy

A relevant randomised trial was published in 2008 (Chude et al., 2008). The authors compared routine defunctioning loop ileostomy versus no ileostomy for low rectal

resections within 5 cm of the anal verge. They reported clinically significant anastomotic leaks in 12 out of 120 (10%) in the "no ileostomy" group with two patients requiring Hartman's procedures. In the "ileostomy" group, clinically significant anastomotic leaks occurred in three out of 136 (2.2%) with no patients requiring a re-operation. The authors recommended the routine use of loop ileostomy for all anastomoses within five cm of the anal verge. Experience shows that this is particularly relevant if the patient has received neoadjuvant radiotherapy.

These results were confirmed in a Cochrane systematic review of six randomized trials of routine ileostomies for rectal resections with anastomoses within five cm of the anal verge (Montedori et al., 2010). A defunctioning ileostomy was associated with a reduced risk of reoperation for an anastomotic leak. In another systematic review of 27 retrospective studies and four randomized trials, the authors reported that the use of a defunctioning ileostomy after low rectal resections did not reduce the incidence of an anastomotic leak but was associated with improved outcomes in terms of a reduction in clinically significant leak rates (OR=0.32(0.17-0.59); (P<0.001)) and a reduction in associated reoperation rates (OR=0.27 (95% CI 0.14-0.51); (p<0.001)) (Huser et al., 2008).

3.8 Intravenous fluid restriction

The electrolyte imbalance, dehydration and hypotension resulting from preoperative starvation and the use of bowel preparation are often over-compensated for with the liberal use of perioperative intravenous isotonic fluids. However, this liberal use of perioperative intravenous rehydration has been shown to be associated with an increased risk of cardiopulmonary complications, a delay in the return of gastrointestinal function and an increased length of postoperative stay in hospital (Lobo et al., 2002). On the other hand, restricting perioperative intravenous fluid therapy has been shown to hasten gastrointestinal recovery, reduce postoperative complication rates and shorten the length of hospital stay (Nisanevich et al., 2005; Holte & Kehlet, 2006).

3.9 Multimodal postoperative analgesia

Routine spinal anesthesia was used in the original ERAS protocol described by Kehlet and his group in 1999 (Kehlet et al., 1999). However, this has evolved into the concept of multimodal analgesia as an integral part of the ERAS approach. The use of epidural analgesia with general anesthesia for major abdominal surgery has been shown to be associated with a reduced incidence of postoperative nausea and vomiting as well as lower rates of respiratory complications compared with intravenous narcotic analgesia, whether as a continuous infusion and/or patient-controlled boluses (White et al., 2007).

The use of a local anesthetic agent administered via an epidural catheter (usually as a continuous infusion and patient controlled boluses) following major abdominal surgery has also been shown to be associated with faster return of gastrointestinal function compared to intravenous and epidural narcotic analgesia to achieve the same analgesic effect (Jorgensen et al., 2000). Autonomic reflexes activated through a painful laparotomy incision cause inhibition of gastrointestinal functions. This is further aggravated by the use of narcotic analgesia and the nausea and vomiting associated with it.

3.10 Normothermia

Hypothermia is quite common with general anesthesia and abdominal surgical procedures. This is due to the combination of impaired thermoregulation, exposure and the use of air

conditioning and negative pressure ventilation in the operating rooms (Qadan et al., 2009). Hypothermia in a surgical setting is associated with an increased risk of bleeding due to coagulopathy as well as arrhythmias, myocardial ischemia and overall risks of complications (Diaz & Becker, 2010).
Under an ERAS protocol, hypothermia is actively prevented using warm and space blankets, warm intravenous infusions, avoiding unnecessary exposure, etc … The patient does not leave the recovery ward until normothermic.

4. Supportive evidence for ERAS protocols

As pointed out above, in the first published series of 16 open sigmoid colectomies under an ERAS (Fast Track) protocol, the authors reported a median postoperative length of hospital stay of two days (Kehlet & Mogensen, 1999). However, subsequent larger studies reported a median length of stay of about five days, three days longer than what was reported in the first series (Abraham & Albayati, 2011; Nygren et al., 2009).
Multiple published trials and systematic reviews have reported that ERAS protocols were associated with a faster recovery, reduced primary and overall lengths of hospital stay and complication rates after colorectal resections compared with the traditional approach. Wind et al reported that the use of an ERAS protocol in the care of patients having elective colorectal resections was associated with a reduced length of hospital stay by about one-and-half days as well as significantly reduced postoperative morbidity rates with no significant increase in readmission rates compared with conventional care (Wind et al., 2006).
These results have been further confirmed in a number of other meta-analyses. These reported a reduced overall length of postoperative hospital stay after elective colorectal resections by about 2.5 days with a reduced overall risk of postoperative complications with adopting an ERAS protocol compared with the traditional approach (Gouvas et al., 2009 & Eskicioglu et al., 2009).

5. Laparoscopic surgery under ERAS protocols

As pointed out above, meta-analyses of laparoscopic versus open colorectal resections showed that the postoperative length of hospital stay was reduced by about 20% by adopting the laparoscopic approach (Abraham et al., 2004 & 2007; Schwenk et al., 2005). The ALCCaS trial showed no statistically significant difference in postoperative complication, reoperation or peri-operative mortality rates between laparoscopic and open resections (Allardyce et al., 2010). The ALCCaS group also reported that reviews show that the short-term advantages of laparoscopic resection for colorectal cancer are arguably relatively minor and often subjective (Allardyce et al., 2010). They also reported that the benefit in adopting a laparoscopic approach in colorectal resections may be limited mainly to patients 70 years of age or older in whom the procedure was completed laparoscopically. An average length of stay of about eight days is common between those trials and meta-analyses. This is three days longer than what was initially reported in the first published series of laparoscopic colorectal resections (Jacobs 1991). A recent meta-analysis of the topic showed that laparoscopic colorectal resections were associated with higher intra-operative complication rates than open resections (Sammour et al., 2011).
To date, the role of laparoscopic resection within an ERAS protocol has not been established. Multiple studies have been conducted to assess whether adopting the laparoscopic

approach would complement ERAS rehabilitation programmes. In a small, 2:1 design, randomised trial of 62 patients (43 laparoscopic and 19 open resections), the authors reported an added benefit for adopting the laparoscopic technique in an ERAS protocol in terms of a reduced postoperative length of stay (King et al., 2006).
However, the results of a systematic review of two randomised controlled trials and three controlled clinical trials of laparoscopic versus open colorectal surgery under ERAS rehabilitation programs were inconclusive as no clear advantage for laparoscopic over open resection was demonstrated under ERAS protocols (Vlug et al., 2009). Further research was recommended.
Another recently published large review of 11 studies (four randomised trials and 11 controlled clinical trials) including 1021 patients reported a clear advantage for patients enrolled in an ERAS rehabilitation program in terms of length of hospital stay compared with those who were not (Gouvas et al., 2009). Although the authors reported that an added benefit to recovery rates in adopting the laparoscopic over the open approach was assumed, such a benefit could not be established. The authors concluded that ERAS programs should become a mainstay of elective colorectal surgery.
In a systematic review of three randomised trials and seven non-randomised studies of laparoscopic versus open colorectal resections under an ERAS protocol, Khan and colleagues reported that the currently available limited evidence suggests that the inclusion of laparoscopic surgery in ERAS protocols for colorectal resections does not confer an added benefit in terms of postoperative recovery rates and postoperative length of stay (Khan et al., 2009).

6. Standardisation of a colorectal ERAS protocol

A consensus statement on ERAS was published in 2005 (Fearon et al., 2005). The statement was written by colorectal surgeons and other specialists and professionals from five universities or tertiary hospitals in five European countries (Denmark, Scotland, Sweden, Norway and The Netherlands). The authors presented their methodology in the published article with a specific focus on colorectal resections. They also recommended their protocol as one that may provide a standard of care against which current and future novel elements of an ERAS approach can be tested or added to. Members of the same group published the outcomes of 169 colorectal resections under an ERAS protocol with very good results (Nygren et al., 2009).
Figures 1-4 show a summary of an ERAS colorectal program adopted at the Coffs Harbour Health Campus, a regional hospital in New South Wales, Australia in July 2006. The summary results of 111 ERAS consecutive open colorectal resections performed at that hospital by one surgeon have been recently published with outcomes similar to those in the North European experience (Abraham & Albayati, 2011).
The Australian Safety and Efficacy Register of New Interventional Procedures - Surgical (ASERNIP-S) under the auspices of the Royal Australasian College of Surgeons and the Department of Health and Aging – Victoria, assessed the experience of Australian and New Zealand surgeons with colorectal resection under ERAS protocols (Strum & Cameron, 2009). They concluded that ERAS programs can result in beneficial outcomes for patients by reducing the length of hospital stay with no significant increase in readmission rates. They also indicated that further work is required to assist in standardisation and implementation of ERAS protocols.

Protocol for Enhanced Recovery After Surgery (ERAS)
Coffs Harbour Health Campus
NSW Australia

Preoperative Care:

Surgeon's Rooms:

- Clinical pathway commenced
- Education brochure given to patient
- Nutritional screen
- Bowel preparation specified
  - Nil *or*
  - Enema preparation *or*
  - Colonoscopy preparation
- Referral to Cancer Co-ordinator, Dietician, Stoma Therapist as required

Preadmission Clinic:

- Preoperative investigations: FBC, UEC, LFTs COAG, CEA and others as required
- Preadmission process completed by RMO/RN.
- Once only medications prescribed:
  - Fleet enema the night before and the morning of procedure.
  - Carbohydrate loading: 6 tetra packs (4 between 9 and 10 pm the night before and 2 between 5 and 6 am the day of surgery)
- Anesthetic consultation: Anesthetic assessment and explanation of postoperative pain management.
- Perioperative nurse consultation: Patient education regarding symbiotics (e.g. Inner Health Plus), bowel preparation / enemas, low residue diet, carbohydrate loading drinks, postoperative pain management, etc …
- Other referrals: (cancer co0ordinator, stoma therapist, dietitian, etc)

Day Surgery Unit:

- Base line observations charted
- Skin preparation
- Enema if ordered
- Normothermia maintained

Fig. 1. Preoperative care in a typical ERAS program

Intraoperative Care:

Thoracic Epidural:

If planned

General Anesthetic: (guide only)

Induction Agent: Propofol

Narcotic: Fentanyl

Maintenance: 80% oxygen with air Sevo

Fentanyl as indicated

Muscle Relaxant: Rocuronium or Atracurium

Antibiotics: Ceftriaxone 1g, Metronidazole BP 500mg in 100mls

Antiemetics: Dolasetron (Anzemet) 12.5mg IVI stat plus

Dexamethasone 8mgs IVI

NSAID: Parecoxib sodium 40mgs IVI, single dose

Urinary catheter, TEDS & SCD

Fluid Replacement:

Hartmann's Solution 1-2mls/kg/hour (don't over hydrate)

Wound Soaker Placement:

Prior to wound closure, the fascia is grasped with two Moynihan tissue forceps and elevated. On each side of the incision, the introducer is placed at the superior end of the incision and tracks into the preperitoneal space. The introducer should be inspected through the parietal peritoneum to ensure the catheter is not placed deep into the muscle. Care must be taken to place the introducer greater than 1cm from the fascial edge to avoid incorporation with the fascial sutures. The introducer should be placed to its fullest extent. The needle is withdrawn and a soaker catheter is placed through the sheath.

Normothermia: not less than 36ºC

No routine drains or NG tube used

Fig. 2. Intra-operative care in a typical ERAS program

Postoperative Care:

Recovery Ward (PACU):

- Observations documented as per relevant chart or Recovery Ward protocol
- Maintain Normothermia
- Continue 80% Oxygen
- Maintain SCD
- Clexane as per anesthetic orders
- VAS score
- Commence i.v. PCA if no epidural
- Check Wound Soaker if no epidural

Thoracic Epidural:

- Clinician Initiated Dose/ Loading Dose: 5mls repeat after 20mins.
- Maintenance postoperatively: 0.2% Naropin with 2 mcgs Fentanyl /ml
- Dose Range: 2.5 to 5ml /hr continuous
- Patient Controlled (PCEA) Dose: 5mls with 20min lockout interval

Wound Soaker:

1. Bilateral Pain Buster for open abdominal wound: Naropin 0.375%: 270mls/5mls per hour each unit
2. Single Pain buster for laparoscopic wounds: Naropin 0.375%: 270mls/5mls per hour

Postoperative Medications:

- Movicol half sachet BD
- Ibuprofen 400mgs TDS for first 2 days then PRN oral
- Paracetamol 1gm QID oral
- Dolasetron PRN 12.5mgs BD IV
- Droperidol PRN 0.5 to 1.25mgs TDS IV
- Maxolon 10mgs TDS IV commence on arrival to ward
- Clexane 40mgs (at least 2 hours post epidural insertion) daily SC

Fig. 3. Early postoperative care in a typical ERAS program

7. Implementation of a colorectal ERAS protocol

A transverse incision has been used for right sided colonic resections for a few decades. There is an observation that many of the other components of ERAS protocols such as multimodal analgesia have been incorporated in traditional colorectal surgical care without necessarily implementing a complete protocol. The implementation of a structured complete ERAS program is less common (Lassen et al., 2005). The implementation of such a protocol requires coordinated training and a team approach by anesthetic, surgical, nursing and other staff (Fearon et al., 2005). This could explain the somewhat delayed uptake of the approach despite the available supportive evidence. It has been suggested that an ERAS protocol should be routinely implemented in colorectal surgical care (Gouvas et al., 2009).

As is the case with most innovations, it will probably take some time for the ERAS approach to be used widely.

Ward Care:

High Dependency Unit:

Day of surgery (0-24h):

- PCEA management as per epidural orders (if present)
- PCA/Wound soaker management (if present)
- Out of bed 6 hours postoperatively for 2 hours with physiotherapist
- Oral fluids and 2 protein drinks to 1000mls
- Bowel chart

Postoperative day 1 (24-48h):

- Daily weigh (day 1-4)
- Mobilize 8 hours, 100 meters of walking with physiotherapist
- Fluids: 2000mls including 4 protein drinks
- Normal diet and sit out of bed for all meals
- Bowel chart

Surgical Ward:

Postoperative day 2 (48-72h):

- Remove epidural 0600
- Remove wound soaker catheter when device is empty
- Remove urinary catheter 0800 (2 hours after epidural removal)
- Regular paracetamol & NSAID
- Maintain pain score <5
- Fluids, 2000mls including 4 protein drinks
- Mobilize 100 meters and out of bed 8hrs
- Bowel chart

Postoperative day 3 (72-86h):

- Maintain pain management, mobilization, fluids and diet.
- Remove IVC
- Bowel chart.
- Early Discharge Planner review and appointments confirmed

Postoperative Day 4: Discharge

Postoperative Day 10: Skin clips removed, histology Surgeon's Rooms

Postoperative week 4: Patient interview by phone

Fig. 4. Ward care in a typical ERAS program

8. Conclusion

In this chapter, the evidence (mainly Level I and II) against traditional peri-operative colorectal surgical practices was presented. These practices included mechanical bowel preparation, peri-operative starvation, the routine use of nasogastric decompression and prophylactic drainage, defunctioning ileostomy, aggressive IV hydration and the routine use of postoperative narcotic analgesia.

At the same time, supportive evidence for the individual aspects of an ERAS protocol and for such a protocol as a whole was also presented. The main emphasis was on avoiding mechanical bowel preparation and peri-operative starvation, ensuring nutritional support including preoperative carbohydrate and protein loading, transverse or oblique incisions if deemed appropriate by the surgeon, high oxygen concentrations, normothermia, minimal intravenous hydration, multimodal analgesia including non-narcotic epidural catheter analgesia if deemed appropriate by the anesthetist, prokinetic agents, anti-emetic drugs and early mobilization, feeding and discharge.

ERAS programs for colorectal resections have been shown to be associated with a faster recovery and a shorter length of hospital stay compared with traditional practices. Furthermore, a number of studies showed that ERAS programmes are also associated with reduced complication rates. Although further research may be required, the current evidence suggests that under an ERAS programme, there is no added benefit in adopting a laparoscopic approach over the open approach.

As with most other innovations, the use of ERAS programs might take some time to become widely spread. However, an ERAS protocol is recommended as a mainstay in colorectal surgical practice.

9. Acknowledgment

The author would like to acknowledge the assistance of Moya Anderson (Clinical Nurse Consultant), Tracey Moore (Data Manager), Dr WB Ross, Dr A Sutherland, Dr M Smith and others for their assistance in establishing the ERAS protocol at the Coffs Harbour Health Campus, NSW, Australia. The author would also like to acknowledge the assistance of Dr John Blaxland in reviewing the text.

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19

Follow Up and Recurrence of Colorectal Cancer

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1. Introduction

Despite optimal primary treatment, with adequate surgery with or without adjuvant chemotherapy, ~30%–50% of patients with colon cancer will relapse and die of their disease. The principal aim of follow-up programmes after curative resection of colorectal cancer is to improve survival (Gan et al., 2007). To achieve this goal, patients are screened for early recurrent disease with the intent of a second curative surgery. Patients with a history of colorectal cancer are also at risk to develop new primary colorectal cancers (CRC). The risk of development of new primary lesions has been estimated to 0,35% per year (Cali et al., 1993). Bouvier et al reported the incidence of metachronous cancer as being 1.8% at five years, 3.4% at 10 years, and 7.2% at 20 years with the greatest excess risk between one and five years post-surgery(Bouvier et al. 2008).
The main sites of colorectal cancer relapse are listed in table 1 (Figueredo et al., 1993).

Colorectal cancer relapse	Colon cancer	Rectum
	%	%
Liver	35	30
Lung	20	30
Peritoneum	20	20
Retroperitoneum	15	5
Peripheral lymphonodes	2	7
Local relapse	15	35
Other (brain, bones)	<5	<5

Table 1. Sites of colorectal cancer relapse

To evaluate the stage of the disease, treatment strategy and prognosis a combination of investigations is necessary. In the past there was no strong evidence that regular follow-up could improve the outcome for patients radically resected for colon cancer. As follow-up can be expensive and resource consuming in terms of both money and procedures, an intensive

surveillance needs to be justified with a good level of evidence. The more effective the treatment of patients with colorectal cancer, the less cost-effective is the follow-up with regard to diagnosis of a relapse.

Although most patients with relapsed colorectal cancer are inoperable at the time of diagnosis, one third of patients with isolated locoregional or distant metastases survive 5 years (Browne et al., 2005). Number of resected patients for relapse is increasing - about 20% of patients with liver metastases are indicated for surgery (Guyot et al., 2005). Other patients are operated after downstaging after chemotherapy or chemoradiotherapy. Long-term survival is also achieved after resection of pulmonary metastases, even when combined of liver and lung metastases resection (Ike et al., 2002). It is evident that high-risk patients (TNM II, III) with relapse diagnosed using imaging and endoscopic techniques have better survival than patients who had clinically evident relapse (Chau et al., 2004). Even patients, who are at the time of relapse diagnosis inoperable, had improved survival due to new palliative chemotherapy regimes (Ahmed et al., 2004).

Due to the different localization of possible recurrence, we cannot use one diagnostic tool, we need a combination of various imaging and laboratory methods. In the first two to three years after resection of primary tumor incidence of relapse increases exponentially, then passes into the plateau (Griffin et al., 1987; Scholefield et al., 2002). Therefore, diagnostic schedule must be adapted. Finally, the postoperative monitoring of patients after curative resection has psychological effects. It can be both positive and negative. Positive involves calming the patient and aid in further treatment. Negative effects includes a sense of false security when relapse is undetected.

Any follow-up system combines a number of tests, whether clinical, laboratory and imaging, as well as their frequency.

Meta-analyzes of studies concludes that intensive follow-up shows a statistically significant difference in overall 5-year survival rate of patients after curative surgery for colorectal cancer, and can diagnose relapse in curable stage, especially if located in the liver and lungs (Tjandra & Chan, 2007; Rosen et al., 1998). In the case of relapse in rectal cancer, studies indicate the minor importance of intensive follow-up (Secco et al., 2000). Analysis showed no significant difference in the incidence of relapse among patients in groups with minimal versus intensive monitoring system, however, closely monitored group had significantly higher number of surgical interventions for recurrence, which is given by an earlier diagnosis and thus a higher resectability of recurrence.

Studies previously conducted and their meta-analyses may be problematic because of non-standard combinations of investigations and also non-standard frequency. Intensive follow-up study in one study is very similar to that in other studies considered to be less intense (Jeffery et al., 2007).

2. Recomendations for follow-up

Diagnostic tools used for follow-up can be dividend in to:

- Imaging procedures
- Endoscopic techniques
- Laboratory tests
- History and physical examination

2.1 Recommendations arising from the meta-analyzes

- for patients at high risk of relapse (stages IIb and III)
  - clinical examination, carcinoembrionic antigen (CEA), chest radiograph, ultrasonography of liver or computer tomography scan (CT) every 6 months for first postoperative 3 years, 3 next years with a frequency of one year
- if the recurrence is detected the patient should be discussed in the multidisciplinary oncology team (surgeon, radiologist, oncologist) to consider the best course of treatment
- for patients at high risk of relapse with comorbidities or other barriers
- clinical examination every year
- for all patients with resected colorectal cancer
- colonoscopy with the polypectomy 1 x per year, in the absence of polyps every 3-5 years(Tjandra & Chan, 2007; Rosen et al., 1998).

2.2 American Society of Clinical Oncology (ASCO) recommendations

Imaging Procedures:

Computed tomography (CT). Patients who are at higher risk of recurrence, and who could be candidates for curative-intent surgery, should undergo annual computed tomography of the chest and abdomen for 3 years after primary therapy for colon and rectal cancer. A pelvic CT scan should be considered for surveillance after rectal cancer therapy, especially for patients who have not been treated with radiotherapy.
Chest x-ray. Annual chest x-rays are *not* recommended.

Endoscopic Techniques:

Colonoscopy. All patients with colon and rectal cancer should have a colonoscopy for the pre- or perioperative documentation of a cancer- and polyp-free colon. After the surgical treatment of colorectal cancer, ASCO recommends the surveillance guideline presented by the American Gastroenterological Association (AGA): a colonoscopy at 3 years and, if normal, every 5 years thereafter (Winter et al, 2003).
Flexible proctosigmoidoscopy (rectal cancer). For patients who have not received pelvic radiation, flexible sigmoidoscopy of the rectum every 6 months for 5 years is recommended.

Laboratory Tests

Tumor markers (Rocker et al., 2006): For colorectal cancer, it is recommended that carcinoembryonic antigen (CEA) be ordered preoperatively, if it would assist in staging and surgical planning. Postoperative CEA levels should be performed every 3 months for stage II and III disease for at least 3 years if the patient is a potential candidate for surgery or chemotherapy of metastatic disease. CEA is the marker of choice for monitoring the response of metastatic disease to systemic therapy. Data are insufficient to recommend the routine use of p53, ras, thymidine synthase, dihydropyrimidine dehydrogenase, thymidine phosphorylase, microsatellite instability, 18q loss of heterozygosity, or deleted in colon cancer (DCC) protein in the management of patients with colorectal cancer. For pancreatic cancer, carbohydrate antigen 19-9 (CA 19-9) can be measured every 1 to 3 months for patients with locally advanced or metastatic disease receiving active therapy. Elevations in serial CA 19-9 determinations suggest progressive disease but confirmation with other

studies should be sought. *Blood tests.* Routine blood tests (i.e., CBCs or liver fiction tests) are not recommended.
Fecal occult blood test. Periodic fecal occult blood testing is not recommended.
Laboratory-derived prognostic and predictive factors. Until prospective data are available, use of molecular or cellular markers should not influence the surveillance strategy (Desch et al., 2005).

2.3 European Group On Tumour Markers (EGTM) recommendations

For identifying recurrences in patients with previously diagnosed colorectal cancer, CEA has a sensitivity of about 80% (range 17-89%) and a specificity of approximately 70% (range 34-91%). Early studies showed that serial CEA levels could detect recurrent disease many months (usually 4-10 months) in advance of clinical evidence of disease (Fletscher, 1986). CEA testing was found to be most sensitive for diagnosing hepatic or retroperitoneal disease and relatively insensitive for either local, peritoneal or pulmonary involvement (Moertel et al., 1993). Some investigators have reported that a slowly rising CEA usually indicates a locoregional recurrence while rapidly increasing levels usually suggest hepatic metastasis (Begent, 1984).
In the follow-up of patients with colorectal cancer, the optimum interval between CEA measurements has not been established. In practice, most clinicians use intervals of 3 months, at least for the first 2 years after the initial diagnosis.
Clearly, further work is necessary to address the impact of CEA monitoring on patient survival, quality of life and cost of care. Ideally, this study should be carried out as part of a prospective randomised trial.
Although surgery remains the most effective therapy for colorectal cancer, chemotherapy is finding increasing use especially in patients with advanced disease. Administration of this therapy may however, cause transient elevations in CEA levels.
While CEA is the preferential biochemical test for colorectal cancer, a number of other markers such as CA19-9, CA242 and cytokeratins (e.g., TPA and TPS) have also been evaluated for this malignancy. While some of these markers have been found to complement CEA, further work will be required to see which marker is most complementary to CEA.

2.4 European Society for Medical Oncology (ESMO) recommendations

- Intensive follow-up must be performed in colon cancer patients [I, A].
- History and physical examination and CEA determination are advised every 3–6 months for 3 years and every 6–12 months at years 4 and 5 after surgery [II, B].
- Colonoscopy must be performed at year 1 and thereafter every 3–5 years looking for metachronous adenomas and cancers [III, B].
- CT scan of chest and abdomen every 6–12 months for the first 3 years can be considered in patients who are at higher risk for recurrence [II, B].
- Contrast enhanced ultrasound imaging (CEUS) could substitute for abdominal CT scan [III, C].
- Other laboratory and radiological examinations are of unproven benefit and must be restricted to patients with suspicious symptoms.(Labianca et al., 2010)

2.5 National Comprehensive Cancer Network (NCCN) guidelines

- History and physical examination every 3-6 months for 2 years, then every 6 months for a total of 5 years
- CEA every 3-6 months for 2 years , then every 6 months for a total of 5 years
- CT scan of abdomen and pelvis annually for 3 years
- Colonoscopy at 1 year , then as clinically indicated(NCCN, 2011)

2.6 Guidelines British Columbia Medical Associattion (BCMA)

Recommendation 1: Clearing colonoscopy

Ideally, colonoscopy should be performed pre-operatively. If this is not feasible, then it may be done three to six months post-operatively if no metastases were found. Air-contrast barium enema combined with sigmoidoscopy is an acceptable alternative where colonoscopy is not readily available.

Recommendation 2: Post-operative follow-up

After recovery from surgery, visits should only be scheduled as needed. The routine use of liver enzyme tests and abdominal ultrasound is not recommended in the absence of symptoms.

Recommendation 3: Tumour markers

The value of carcinoembryonic antigen (CEA) testing in the post-operative period is controversial and its usefulness is therefore limited. However, in individuals who would be candidates for resection of isolated hepatic or pulmonary metastases, serial measurement of CEA levels post-operatively (every three months for two years) may be of value in detecting recurrence that is treatable in up to 25 per cent of patients.

Recommendation 4: Prevention of new cancers

Repeat colonoscopy once every three years until no new adenomas are discovered. Thereafter, repeat colonoscopy every five years until the detection of new tumours is unlikely to influence the patient's lifespan. Air-contrast barium enema combined with sigmoidoscopy is an acceptable alternative where colonoscopy is not readily available.

Recommendation 5: Low rectal cancer

For patients who have undergone low anterior resection of rectal cancers, digital rectal examinations and proctoscopy or sigmoidoscopy should be undertaken at three months, six months, one year and two years to look for anastomotic recurrence. Thereafter, recommendation 4 should be followed.(BCMA, 2011)

2.7 Australian Clinical Practice Guidelines (CCA)

The Australian *Clinical Practice Guidelines for the prevention, early detection and management of CRC, 2nd edition, 2005* proposed that follow-up should be offered to all patients who have undergone curative surgery and are fit for further intervention if disease is detected. This includes patients who have had malignant polypectomy or curative endoscopic resection of Stage I CRC but excludes patients with Stage IV CRC if their treatment does not offer the possibility of cure.

Patients with proved Lynch syndrome (HNPCC or hereditary non-polyposis colorectal cancer), should continue to have annual surveillance colonoscopy performed post-

operatively because of the apparent rapid progression of neoplasia from adenoma to carcinoma.

Patients including those whose initial diagnosis was made younger than 40 years of age, with probable or possible HNPCC (i.e. patients whose tumours are MSI-high and less than 50 years old at time of initial cancer diagnosis but not proved by genetic testing to have HNPCC), with hyperplastic polyposis and BRAF mutation and with multiple synchronous cancers or advanced adenomas at initial diagnosis should be considered following surgery to continuing with more frequent surveillance than would otherwise be recommended (e.g. initial post-operative colonoscopy at one year and then annually, second-yearly or third-yearly. (CCA, 2011)

Summary in reccomendations for follow-up see in table 2.

	Metaanalyses	ASCO	EGTM	ESMO	NCCN	BCMA
CEA	every 6 months	every 3 months	every 3 months	every 3-6 months	every 3-6 months for 2 years , then every 6 months	every 3 months for two years
Colonoscopy	every 6 months	3 years after surgery		In 1 year, and thereafter every 3–5 years	at 1 year , then as clinically indicated	3-6 months after surgery
History, physical examination	every 6 months			every 3-6 months	every 3-6 months for 2 years, then every 6 months	as needed
Chest X ray		not reccomended				
CT	every 6 months	3 years after surgery		every 6–12 months for the first 3 years	annually for 3 years	
Ultrasound	every 6 months			CEUS could substitute for abdominal CT scan		

Table 2. Summary of follow up recommendations

3. Discussion

The optimal combination and frequency of investigations in follow-up of patients after CRC resection has not been determined. Importantly, the performance of annual colonoscopy has not been shown to improve five-year survival.

Studies comparing intensive and less intensive follow up programes were conducted in many countries around the World. In Finland(Makela et al., 1995) randomized more than 100 patients. Less intensive follow up system included either rigid sigmoideoscopy (for rectal or sigmoid cancer) or barium enema (for colon cancer) once a year. Patients in intensive arm underwent colonoscopy within 3 months of surgery and then yearly colonoscopy thereafter, liver ultrasound every 6 months and CT scans every year. Intensive follow up programe significantly earlier identified recurrence, there were no signifiant diference in resecability rates and five-year survival. Ohlsson et al. showed the same results on 107 patients (Ohlson et al., 1995). In Italy, (Pietra et al., 1998) randomized more than 200 consecutive patiens into low intensity follow up arm (physical examination, liver ultrasound and CEA at 6 month and then yearly, colonoscopy and chest X ray annually) and intensive arm (clinical controls, liver ultrasound, CEA each 3 months during the first 2 years, at 6 month interval for the next 3 years and then yearly, colonoscopy, chest X ray and CT scan yearly). Intensive group demonstrate statistically signifiant increase in five-year survival (73,1% vers. 58,3%). Kjeldsen and coleagues randomized big group up to 600 patients to frequent and minimal follow up arms and demonstrated significantly earlier detection of recurrence, but not improvement of overall or cancer-related five-year survival (Kjeldsen et al., 1997).

Then, metaanalyses were conducted. Renehan and colleagues involved 1342 patients and demonstrated a signifiant improvement in overall five-year survival in intensivelly followed patiens. The intensive follow-up groups were also associated with significantly earlier detection of all recurrences and isolated local recurrences (Renehan et al., 2002). A meta-analysis by Tjandra et al concluded that intensive follow-up increased the re-resection rate for recurrent disease and improved overall survival but the survival advantage was not due to earlier detection of recurrence and cancer-related mortality was no better (Tjandra & Chan, 2007). Forty-one centers have participated in the GILDAtrial. There are 39 centers in Italy, one in Spain, and one in the United States. Both the less intensive follow-up group and the more intensive follow-up group are well matched for distribution of sex, age, cancer stage (Dukes B or C) and primary site of cancer (colon or rectum). This trial will allow us to quantify the lead-time provided by the specifically defined intensive follow-up regimen, and to compare the likelihood of uncovering recurrent disease amenable to salvage therapy (Grossmann et al., 2004).

3.1 Imaging procedures – New possibilities

3.1.1 Computed Tomographic Colonography (CTC)

CTC has been introduced in the last decade for the identification of colorectal lesions, polyps and cancer (Reuterskiold et al., 2006). CTC is being increasingly used for the radiological evaluation of colorectal symptoms. There have been a few publications on the use of CTC in follow-up of these patients after surgery (Amitai et al., 2009). CTC has the advantage in demonstrating the inner surface of the colon tube simulating the endoscopic colonoscopic

view and demonstrating the pericolonic structures at the same time. It has a high accuracy in detecting colonic neoplasia (Halligan et al., 2005).

3.1.2 PET/CT scan

In past 10 years PET/CT scan was introduced as a standard method for colorectal cancer imaging, especially for distant metastases diagnosis. Studies comparing PET/CT scan with standard methods showed superiory of this paging method. In these studies (Deleau et al., 2011; Han et al., 2011) data of patients with suspected CRC recurrence and in whom both FDG-PET/CT and CT were performed were analyzed. All detected lesions were characterized according to their number, size, and localization. In Deleau´s study 171 true-positive lesions were identified in 71 patients. CT scan was positive in 58 (82%) patients and FDG-PET/CT in 70 (98%) patients. In per lesion analysis, the global accuracy of FDG-PET/CT in detection of lesions was of 88% (sensitivity = 95%, specificity = 54%), which was higher than that of CT (53%, sensitivity = 55%, specificity = 43%), particularly in case of lymph nodes metastases (100 vs. 35%) and locoregional lesions (100 vs. 39%). FDG-PET/CT modified the clinical management in 31 patients.

At present, whole-body (18)F-FDG PET/CT is an advanced diagnostic imaging technique in detecting loco-regional recurrence and metastasis in postoperative patients with colorectal carcinoma for its higher sensitivity and specificity and also appears to be useful modality in evaluating chemotherapy response and can differentiate responders from nonresponders in recurrent CRC patients (Shamim et al., 2011).

3.1.3 Contrast enhancement ultrasound scan

There were few studies done to compare the sensitivity and specificity of contrast-enhanced ultrasonography (CEUS) and computed tomography (CT) in the detection of liver metastases in patients with colorectal cancer (Larsen et al., 2009). In this Denmark study 365 patients were included. All patients had undergone preoperative US, CEUS and Multidetector CT and 65.5% had received Intraoperative US. Multidetector CT found significantly more metastases than CEUS. In a patient-by-patient analysis MDCT had a non-significantly higher sensitivity in the detection of liver metastases compared to CEUS . The specificity of was slightly better than that of MDCT. Multidetector CT found significant more metastases than CEUS. In previous study, held by same authors (Larsen et al., 2007), sensitivity and specificity of contrast enhanced ultrasonography (CEUS) with conventional ultrasonography (US) in detection of liver metastases in patients with colorectal adenocarcinoma were compared. In 461 patients contrast enhanced ultrasonography improved the sensitivity significantly in detection of liver metastases from 0.69 by US to 0.80 ($p=0.031$). In 24 patients, CEUS found a higher number of metastases than US ($p<0.001$). The specificity (0.98) and the positive predictive value (0.86) was the same.

In Italian study (Piscaglia et al., 2007) a total of 109 patients with colorectal (n = 92) or gastric cancer prospectively underwent computed tomography (CT) scan and conventional US evaluation followed by real time CEUS. A diagnosis of metastases was made by CT or, for lesions not visible at CT, the diagnosis was achieved by histopathology or by a malignant behavior during follow-up.

Of 109 patients, 65 were found to have metastases at presentation. CEUS improved sensitivity in metastatic livers from 76.9% of patients (US) to 95.4% , while CT scan reached

90.8%. CEUS and CT were more sensitive than US also for detection of single lesions In 15 patients (13.8%), CEUS revealed more metastases than CT, while CT revealed more metastases than CEUS in 9 patients (8.2%) Piscaglia concluded that CEUS is more sensitive than conventional US in the detection of liver metastases and could be usefully employed in the staging of patients with gastrointestinal cancer. Findings at CEUS and CT appear to be complementary in achieving maximum sensitivity.

3.2 Endoscopic techniques

Colonoscopy: Surveillance colonoscopy after CRC resection has the theoretical potential to improve patient outcome by finding metachronous cancers at an early stage, detecting luminal/ anastomotic cancer recurrences and removing metachronous adenomas. Nevertheless, studies have differed in their conclusions about the overall effectiveness of colonoscopic surveillance. Recommendations about the timing of colonoscopy after CRC resection should be based upon the "natural history" of metachronous colonic neoplasia, in order to meet the objectives of surveillance, namely early detection of metachronous cancer and timely polypectomy for metachronous adenomas.

Patients undergoing either local excision (including transanal endoscopic microsurgery) of rectal cancer or advanced adenomas or ultra-low anterior resection for rectal cancer should be considered for periodic examination of the rectum at six monthly intervals for two or three years using either digital rectal examination, rigid proctoscopy, flexible proctoscopy, and/or rectal endoscopic ultrasound. These examinations are considered to be independent of the colonoscopic examination schedule (CCA, 2011).

Two recent case-control studies of colonoscopy suggest that colonoscopy is not as effective in decreasing mortality (Baxter et al., 2009) or incidence of right-sided cancer (Lakoff et al., 2008). A recent investigation of surveillance colonoscopy found that subjects were more likely to develop recurrent adenomas in the same colon segment, suggesting that particular attention be paid to where a previous adenoma has been removed (Pinsky et al., 2009).

Leung and colleagues studied colorectal cancer risk despite surveilance colonoscopy and concluded that despite frequent colonoscopy there was a persistent ongoing risk of cancer in the years after the trial. Subjects with a history of advanced adenoma are at increased risk of subsequent cancer and should be followed closely with continued surveillance (Leung et al., 2010).

Importantly, the performance of annual colonoscopy has not been shown to improve five-year survival.

3.3 Laboratory tests

Based on literature and also our results (Lipská et al., 2007), it can be concluded that monitoring of the tumor markers is valuable, mainly in those cases where preoperative CEA and/or CA19-9 were elevated. The level of CEA and CA19-9 increases according to the pTNM stage of the disease. CEA or CA19-9 below the cut-off level does not exclude even a very advanced colorectal cancer. To evaluate the stage of the disease, treatment strategy and prognosis a combination of investigations is necessary. Surveillance based only on CEA and/or CA19-9 is cost-effective, but does not disclose more than 1/3 of patients with relapse. In general practice CEA is often used as the only parameter in the follow-up

regimens. Based on this system, CEA seems highly effective, but when other investigations are included, 1/3 of relapsed patients are diagnosed by a method other than CEA.
Non-invasive screening of molecular biomarkers(such as cell-free tumor DNA) may enable effective surgical intervention through an early diagnosis of the disease. To determine the most appropriate diagnostic and therapeutic strategy we need to know not only clinical and histological factors but also molecular factors of the tumor. New impromvent in molecular biology should open the way to new perspectives in research of carcinogenesis, medical (targeted therapy) and surgical treatment (in the appropriate moment and appropriate extent). There has been increased interest in identifying biologic indicators that may help better define patients at risk for recurrence.

3.4 Author´s experience

At Surgical Department of Thomayer University Hospital, Charles University Prague, there is for more than 15 years intensive system of follow-up applied. See table 3.

	Months after operation														
	3	6	12	18	24	30	36	42	48	54	60	72	84	96, 120	108, 144, 166
History	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Physical examination	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CEA, CA19-9	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Ultrasonography	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
CT			X		X		X		X		X	X	X	X	X
Coloscopy			X		X		X		X		X	X	X	X	X
Chest X ray					X				X			X		X	
PET, PET/CT	In the case of unsolved elevation in markers or before reoperation														

Table 3. Follow-up system in Thomayer University Hospital

Four follow-up studies (based on different tumor markers, CT scan, PET/CT scan, ultrasonography and other investigations) performed on author´s department are reported.

3.4.1 Study 1

Aim: To evaluate CEA and CA19-9 in a long-term follow-up after radical surgery for colorectal cancer.
Patients and Methods: A total of 1090 patients were operated on for colorectal cancer, 716 patients underwent R0 resection, 631 patients were under further surveillance, relapse was diagnosed in 122 patients (20%), 74 patients were indicated for reoperation The resectability of the relapse was 35%. An AxSYM instrument (Abbott) was used for analysis.

Results: At the time of relapse both markers were normal in 31% of the patients. When relapse was diagnosed, in patients with normal preoperative levels, CEA and CA19-9 were below cut-off in 48% and 79% of cases respectively, and in those with primary elevation, they were again elevated in 78% and 64% of cases respectively.
Conclusion: The surveillance based only on CEA and /or CA19-9 was cost-effective, but failed to disclose 1/3 of patients suffering from relapse; markers must be combined with liver and chest imaging methods and colonoscopy.

3.4.2 Study 2

The aim of the study was to investigate the clinical significance of serum tumor markers and biological activity markers - oncofetal tumormarker CEA, mucin tumormarkers CA19-9, CA242, proliferative tumor markers Thymidine kinase, soluble cytoceratines fragments TPS, TPA, adhesive molecules ICAM - 1, VCAM -1, IGF-1, and adipocytokinins Adiponectin, Leptin in patients with colorectal cancer before primary operation. The study included 142 patients between the ages of 35 - 89 years. We confirmed that CA19-9 is besides CEA an important marker in colorectal cancer. Comparing CA19-9 and CA242 in preoperative staging, CA242 is more specific. Statistical significant difference between early and metastatic stage of colorectal cancer was not confirmed in markers: ICAM-1, VCAM, adiponectin, leptin. Statistical significant difference between early and metastatic stage of colorectal cancer was confirmed in markers: CEA, CA19-9, CA242, TPS, TPA, TK, IGF-1. None of the used markers was able to distinguish stage II and III, in other words to identify patients with infiltration of lymph nodes. This fact is very important in our aspirations to find which marker from periferal blood could help to identify patiens at risk of lymphatic infiltration and select these patients for adjuvant therapy. Combination of CEA and either CA19-9 or CA242 can be recommended for preoperative investigation. CA 242 in this study seems to have slightly better results in preoperative staging (Levý et al., 2008).

3.4.3 Study 3

Aims: To investigate presence of cell-free tumor DNA and its correlation to clinical status of the patient, especially metastatic liver disease. There has been increased interest in identifying biologic indicators that may help better define patients at risk for recurrence after hepatic resection for colorectal metastases.
Methods: In a prospective study cohort of 108 patients we have initially acquired a tissue samples from primary tumor (n=108). Where available, additional tissue was collected from nodes and liver metastases. For each patient, multiple plasma samples were acquired over a period covering initial examination, immediately preceding the surgery, at the surgery, post-surgery and during a subsequent follow-up. We have used the most frequent colorectal somatic mutations (APC, TP53, BRAF, PIK3CA and KRAS) detected in primary tumors to trace cell-free tumor DNA in plasma samples.
Results: A total of 66 patients (66/108, 415%) had somatic mutation in primary tumor. From these 66 patients in 57 patients the plasma samples were examined. Where available, mutation from primary tumor was also confirmed in the metastatic liver tissue (4/4, 100%). Cell-free tumor DNA was detected in plasma according to TNM stages in 0%, 10%, 28% and 100% respectively. In 2 patients positivity was detected in subsequent plasma samples, following the course of the disease development.

Conclusion: Our results indicate a potential for the detection of cell-free tumor DNA as a non-invasive test of metastatic liver disease. Somatic mutations in additional genes (BRAF, PIK3CA) are now being explored as markers to increase the number of patients that can be evaluated from cell-free tumor DNA.

3.4.4 Study 4

Aim of the study:The conventional diagnostic techniques used to assess recurrence of colorectal cancer (CRC) often yield unspecific findings. Integrated FDG-PET/CT seems to offer promise for the differential diagnosis of benign and malignant lesions. The aim of this study was to compare the value of FDG-PET and PET/CT in the detection of CRCR subsequent to colonic resection or rectal amputation.
Methods:The population for this retrospective study comprised 84 patients with suspected CRC. The sensitivity, specificity and accuracy of PET and PET/CT were calculated for (a) intra-abdominal extrahepatic recurrences, (b) extra-abdominal and/or hepatic recurrences and (c) all recurrences, and tumour marker levels were analysed.
Results: The sensitivity, specificity and overall accuracy of PET in detecting intra-abdominal extrahepatic CRC were 82%, 88% and 86%, respectively, compared with 88%, 94% and 92%, respectively, for PET/CT. The corresponding figures for detection of extra-abdominal and/or hepatic CRC were 74%, 88% and 85% for PET and 95%, 100% and 99% for PET/CT. Considering the entire population, the sensitivity, specificity and overall accuracy of PET were 80%, 69% and 75%, respectively, compared with 89%, 92% and 90%, respectively, for PET/CT. FDG-PET/CT examination correctly detected 40 out of a total of 45 patients with CRC. Two of five patients with falsely negative FDG-PET/CT findings had local microscopic recurrences and one had miliary liver metastases. Of 39 patients without CRC, three showed false positive FDG-PET/CT results. Two of these cases were due to increased accumulation in inflammatory foci in the bowel wall, while one was due to haemorrhaging into the adrenal gland.
Conclusion:FDG-PET/CT appears to be a very promising method for distinguishing a viable tumour from fibrous changes, thereby avoiding unnecessary laparotomy.(Votrubova et al.,2006)

4. Conclusion

The goal of any surveillance program should be detection of recurrent disease at a early time to allow subsequent curative therapy. Periodic clinical examinations, laboratory tests, radiographic imaging, and markers testing is necessary. The optimal combination and frequency of investigations in follow-up of patients after CRC resection has not been determined. It seems that intensive follow-up increased the re-resection rate for recurrent disease and improved overall survival but the survival advantage was not due to earlier detection of recurrence and cancer-related mortality was no better.
All patients having recurrences should be assessed by a multidisciplinary team in a cancer centre.

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Part 5

Metastasis

20

Panitumumab for the Treatment of Metastatic Colorectal Cancer

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1. Introduction

According to data by GLOBOCAN, the worldwide incidence of colorectal cancer in 2008 was 1,234,000 (with 663,000 male and 571,000 female cases). The number of deaths due to this disease was 608,000 (320,000 men and 288,000 women). Given these figures, colorectal cancer is the third and second leading cause of mortality among men and women. In the recent year in Hungary with a population around 10 million the annual incidence among males was 4,415, whereas the number of afflicted women was 3,690. Mortality data is similar with deaths among men and women being 2,563 and 2,190 respectively. Therefore, the disease is the second leading cause of death for both genders worldwide and in Hungary as well (Gaudi & Kásler, 2002; Ottó & Kásler, 2005; World Health Organization [WHO] – International Agency for Research on Cancer [IARC], 2008). In international comparison Hungarian colorectal cancer mortality rates for 2008 were the highest in Europe for both men (31.4 per 100,000) and women (16.2 per 100,000). This is in striking contrast to comparable figures of Albanian men (6.2 per 100,000) and women (5.8 per 100,000), with the lowest registered numbers (WHO – IARC, 2008). Both frequencies of the disease and continuously improving treatment results highlight the accentuated place colorectal cancer takes in routine oncology practice and at the same time oblige health care services to provide the best possible treatment for patients.

As a result of organizational efforts in the last decades to improve professional cooperation, leading to the development of new drugs and to a more conscious treatment planning with a closer to optimal use of combinations, metastatic colorectal cancer (mCRC) has become a chronic disease (Haller, 2007; Khan et al., 2008; Khan et al., 2010; Phillips & Currow, 2010; van der Velden et al., 2009; van Kleffen et al., 2004).

Today median survival of CRC-patients from the diagnosis of distant metastases can reach 36 months on overall. Even in disseminated illness the chances of surviving more than five years are above 12% now (Blaser, 2010; Chau & Cunningham, 2009; Goldberg, 2007; Grothey, 2007; Michael & Zalcberg, 2000; National Cancer Institute [NCI], n. d.; Sudoyo, n. d.)
In 2004 Grothey and colleagues presented a diagram in the Journal of cilinical oncology which has been cited countless times ever since. The survival of mCRC patients was plotted on this diagram as a function of the proportion of patients treated with drug combinations considered "basic" (fluoropyrimidine, irinotecan, and oxaliplatin), and multiple linear regression was performed (Grothey et al., 2004) Based on the results it is clear that those the patients that had the greatest chance of survival who had received all three drugs during their treatment. Of course, it is not just "traditional" cytostatic remedies – antimetabolite fluoropyrimidines, the topoisomerase inhibitor irinotecan, and alkalizing agent oxaliplatin – that influence survival (Takimoto and Calvo, 2005). Based on new results, drugs aimed at biological targets do so, on their own and in different combinations with chemotherapy as well, which we will discuss later in detail.

2. Biological targeted drugs

2.1 Brief description of drugs affecting biological targets

Drugs currently in use in this category can be classified into two major groups.
A well known and characteristic representative of one of these groups is bevacizumab (Avastin®) (European Medicines Agency [EMA], 2011a) inhibiting neoangiogenesis, i.e. this drug slows down the pathological vascularization of tumours and thus inhibits their provision of oxygen and nutrition.
The other group consists of cetuximab (Erbitux®) (EMA, 2010) and panitumumab (Vectibix®) (EMA, 2011b), both influencing the effect of "epidermal growth factor receptors" (EGFR) located on the surface of tumours and in this way both interfere with the regulation of cell division and proliferation (Helbling & Borner, 2007; Mayer, 2009; Siena et al., 2009; Willet et al., 2007).
These are all monoclonal antibodies. As a result of advances in manufacturing technology "chimeras" containing more non-human amino acid sequences (cetuximab – "cmab") were followed by "humanized" antibodies like bevacizumab ("beva") with increased proportion of human sequences within the molecule. The ultimate result of this process is the development of monoclonal antibodies containing exclusively human amino acid sequences (panitumumab – "pmab"). The ratio of human and non-human amino acid sequences within a given therapeutic antibody medication is crucial—the presence of the latter usually necessitates the use of saturating doses, while fully human substances can be administered using the same dose from the start of therapy. Human versus non human composition of complex protein molecules administered via infusion is also a key determinant of the frequency of infusion related and other side effects caused by "foreign proteins" (Eng, 2010; de Bono & Rowinsky, 2002; EMA, 2009; EMA, 2011a, b; Hochster, 2006; LoBuglio, 1989; Yang et al., 2001).

2.1.1 Bevacizumab

Generally used in combination with traditional cytostatic drugs, bevacizumab has been approved in Europe for many types of tumors: mCRC, breast cancer, clear cell renal cell

carcinoma, and lung cancer (excluding planocellular or small cell carcinoma-types) (EMA, 2011a). In addition, the U. S. Food and Drug Administration (FDA) has also approved its use in brain tumour recurrences following "traditional" treatment and in advanced brain tumour cases as well (glioblastoma multiforme) (U. S. Food and Drug Administration, 2009).

Beva binds to "vascular endothelial growth factor" (VEGF), one of the most important angiogenesis regulators. By doing this, beva inhibits the binding of VEGF to its receptors Flt-1 (VEGFR-1) and KDR (VEGFR-2) on the surface of endothelial cells. The neutralization of VEGF's biological activity lowers tumour vascularisation, normalizes the tumour's surviving vasculature and inhibits the development of a new vascular system for the tumour. By blocking tumour growth beva thus lowers intra-tumour pressure helping anticancer drug delivery to tumour tissue (Bergers & Benjamin, 2003; Borgstrom et al., 1999; EMA, 2011a; Folkman, 1971, Kim al., 1993).

One of its main side effects is high blood pressure (usually successfully treated with ACE inhibitors, calcium channel blockers, or diuretics), and this usually does not necessitate ending or suspending the use of the drug. Therapy-resistant chronic hypertension however may mean a treatment contraindication. The frequency of proteinuria can vary considerably. Its severity can range from laboratory value deviations to development of nephritic syndrome. The severity of the detrimental side effect congestive heart failure can also cover quite a wide spectrum. Reduced left ventricle ejection fraction may ensue without any clinical symptoms but can be represented in a life-threatening form too. A wide variety of arterial and venous thromboembolic complications, as well as bleeding of any grade can occur. Bleedings may represent in the gastrointestinal system, primarily as perforations in patients with metastatic colorectal cancer treated with bevacizumab. Inflammatory intestinal diseases render patients especially susceptible to such perforations. Fistulae can also develop in different areas; perforations of the nasal septum are detected rarely. Reversible posterior leukoencephalopathy syndrome is a rare, neurological disorder which can also develop during beva treatment. Differential diagnosis can be challenging in such cases to rule out headaches, mental disorders, and possible cortical blindness frequently caused by cerebral metastases. (Allen et al., 2006; BC Cancer Agency Cancer Management Guidelines, 2006; Benson et al., 2003; EMA, 2011a; Fakih & Lombardo, 2006; Giantonio et al., 2004; Hamilton, 2008; Kilickap et al., 2003; Martel et al., 2006; Pereg & Lishner, 2008; Scappaticci et al., 2007; Traina et al., 2006; van Heeckeren et al., 2007; Widakowich et al., 2007).

2.1.2 Correlation between the therapeutic effect of EGFR inhibitor monoclonal antibodies (cmab and pmab) and K-ras mutation status

Before presenting the mechanism of action of cmab and later that of pmab in details, it is necessary to understand the importance of EGFR status and K-ras mutation. Awareness of EGFR and K-ras mutation status has proven to be essential not only for an apt evaluation and interpretation of clinical trial results, but for adequate patient selection and diagnostics planning as well. A precise determination of both is a prerequisite for an effective treatment in everyday clinical routine. EGFR, a superficial structure of epithelial tumours and also CRC cells is a glycoprotein composed of three subunits. The exodomain receiving the ligand is outside the cell membrane, while the hydrophobic transmembrane domain provides proper cell membrane integration. The cytoplasmic "endodomain" is a catalytic subunit with tyrosine kinase activity. It transmits signals to other proteins by phosphorylating

messenger routes. In a complex mechanism, EGF activation initiates cell division following the reception of an adequate external signal. It also assures survival and inhibits apoptosis. The resulting effect is cell proliferation. While this mechanism is strictly controlled in healthy cells, EGF activation is uncontrolled in a considerable proportion of epithelial tumours. The signal is transmitted to other proteins via the biochemical route of tyrosine kinase by phosphorylation. EGF activation can initiate cell division, proliferation, development of metastases and inhibition of apoptosis. Apparently, this leads to tumour progression (Cohenuram & Saif, 2008; Coutinho & Rocha Lima, 2003; EMA, 2009; EMA 2011b; Harari, 2004; Hamilton, 2008; Herbst & Shin, 2002; (Ritter & Arteaga, 2003; van Cutsem et al., 2009).

EGFR inhibitors (cmab and pmab) are licensed for the treatment of mCRC patients. They bind to the extracellular ligand-binding domain and thus inhibit transmembrane signal transmission and prevent EGF dependent signal transduction within the cell as well. Although the mechanism of action has already been established in theory, EGFR inhibitors yield clinical improvement to not more than approximately 50% of mCRC patients. This observation led to the assumption that a biological factor could have prevented these monoclonal antibodies from being effective in tumours expressing EGFR. The K-ras ("Kirsten rat sarcoma 2 viral oncogene homolog") gene belongs to the family of RAS proto-oncogenes. The K-ras protein coded by this gene plays a central role in growth-inducing signal transmission routes. By doing so it affects cell reproduction, differentiation and survival. If a mitogenic signal reaches the EGF receptor, the signal is forwarded to the nucleus by the K-ras. It is essential that this close correlation applies only to the "normal" (i.e. non-mutated or "wild type") K-ras. Mutant types of K-ras escape receptorial regulation and thus they autonomously stimulate cell proliferation.

For this reason K-ras mutation is not a genetic failure with "function loss", on the contrary, in this case RAS remains in "on" status (i.e. phosphorylation is continuous) and acts independently from EGFR (and other physiological signaling pathways). As a consequence, despite the signals reaching the cell surface being "blocked" by monoclonal antibodies at the receptor level, signaling tracks regulated by EGFR under normal conditions remain (chronically) activated (Amado et al., 2008; Benvenuti et al., 2007; Dahabreh et al., 2011; De Roock et al., 2010; (Engstrom et al., 2011a, b; Esteller et al., 2001; EMA, 2009; EMA 2011b; Hamilton, 2008; Heinemann et al., 2009; Malumbres & Barbacid, 2003; Normanno et al., 2009).

As the estimated incidence of K-ras mutation in CRC is 30-50%, it is expected that in about half to two thirds of patients the regulation of signal effect and signal transmission are preserved and drugs acting via the K-ras route can be used with success. (Amado et al., 2008; Benvenuti et al., 2007; Bardelli & Sien, 2010; Esteller et al., 2001; Garcia-Sáenz et al., 2009; Malumbres & Barbacid, 2003; Nagasaka et al., 2004). In an interesting re-evaluation of their primary study population Hurwitz et al. found that though bev combined with IFL as a first line treatment of mCRC was effective in both K-ras wild type and mutant subgroups, efficacy was by large affected by K-ras status, underlining a mixed predictive and prognostic function of this mutation (Hurwitz et al., 2009).

3. Characteristics, application and side effects of panitumumab

3.1 Characteristics of panitumumab (Vectibix®)

Pmab is a recombinant fully human monoclonal IgG2 antibody produced in a mammalian cell line (Chinese Hamster Ovary, CHO) by recombinant DNA technology. Vectibix has high

affinity and specificity to human EGFR. It inhibits receptor autophosphorylation caused by all known EGFR ligands by attaching to the ligand-binding domain. Binding of pmab to EGFR results in the internalization of the receptor, inhibition of cell growth, induction of apoptosis, and decreased interleukin-8 and vascular endothelial growth factor production (Berardi et al., 2010; EMA, 2011b; Harari, 2004; Helbling & Borner, 2007; Keating, 2010; Martinelli et al., 2007; Peeters et al., 2008; Pikó, 2009; Rakkar, 2007).

3.2 Using Vectibix

The recommended dose of Vectibix is 6 mg/kg of bodyweight once every two weeks both in monotherapy and when combined with cytostatics. Prior to infusion Vectibix should be diluted in 100 mL of 0.9% sodium chloride solution to a final concentration not exceeding 10 mg/mL. Vectibix must be administered as an intravenous infusion via an infusion pump using a low protein binding 0.2 or 0.22 micrometer in-line filter through a peripheral line or indwelling catheter. The recommended infusion time is approximately 60 minutes (Alberta Health Services, 2010; EMA, 2011b). The first dose injected over 60 minutes was well tolerated in clinical trials where Vectibix was combined with cytostatic agents; subsequent treatments were allowed to be given over 30 minutes (Douillard et al., 2010; Peeters et al., 2010). Doses higher than 1,000 mg should be administered as a 150 mL solution over approx. 90 minutes. No incompatibilities have been observed with 0.9% sodium chloride injection in polyvinyl chloride bags or polyolefin bags (EMA, 2011b; Knudson, 2007).

3.3. Side effects of panitumumab

3.3.1 Skin toxicity

The common pharmacological effect of EGFR inhibitors can lead to the following: EGFR inhibition in the skin, hair follicles, and periungual tissues can cause abnormal proliferation, migration and differentiation of target cells (i.e. basal keratinocytes), while changes in the skin structure attract inflammatory cells. Clinical symptoms emerge within 10 days following the introduction of pmab therapy and resolve in 28 days after the last injection on average. Skin symptoms are characteristic: papular skin rash, monomorphic pustular lesions, etc. presenting on skin areas exposed to the sun. Although signs may resemble those of acne for the first sight (labeled as "acneiform"), differentiation is easy and essential. Acne may manifest as non-inflammatory lesions on the basis of comedos or as inflammatory papules, pustules, or nodules. On the contrary, rash due to EGFR inhibitors is dominated by pustules. Non-inflammatory comedos are never seen in these cases. Skin rash is more widespread than classical acne as symptoms can often be observed on the upper and lower extremities and trunks of patients simultaneously. In order to prevent nail diseases it is important to avoid mechanical injuries (e.g. caused by tight shoes). Development of paronychia can be stopped by bathing the foot in diluted antiseptic agents and by using topical antiseptic ointments. Feet should not be soaked for a long time to prevent tissues from loosening. In some cases surgery cannot be avoided (Busam et al., 2001; Eaby, n. d.; EMA, 2011b; Moy & Goss, 2007; Pérez-Soler et al., 2005; Segaert & van Cutsem, 2005; Winkeljohn, 2008).

Conventional modalities to treat acne should not be used. On the contrary, advices and interventions are usually completely different from those applied during acne therapy. Sun bathing is prohibited, patients should protect themselves from any direct sunlight (hat, long-sleeved clothes, and sun screens are recommended). Dryness of the skin should be treated with neutral emollients. Caution is warranted if topical steroid drugs are used. Such

products are recommended solely to alleviate symptoms. Systemic antihistamines are more useful to cure itching. If rash is accompanied by superinfection, external use of either clindamycin or mupirocin, or internal use of tetracycline are to be considered (Eaby, n. d.; EMA, 2011b; Hoda et al., 2008; Lacouture, 2009; Lacouture et al., 2010; Melosky et al., 2009; Moy & Goss, 2007; Peeters et al., 2008; Pérez-Soler et al., 2005; Pikó, 2009; Potthoff et al., 2011; Saif & Cohenuram, 2006; Winkeljohn, 2008). Efforts to deal with skin toxicities via pre-emptive approach (i.e. applying emollients, hydrating and photoprotective creams, topical steroids and oral doxycyclin) in the STEPP ("Skin Toxicity Evaluation Protocol With Panitumumab") comparative clinical trial resulted in decreasing the frequency of Grade II or more severe forms already present from 62% to 28%. Quality of life improved significantly whereas the clinical efficacy of panitumumab treatment was unaffected. (Lacouture et al., 2010)

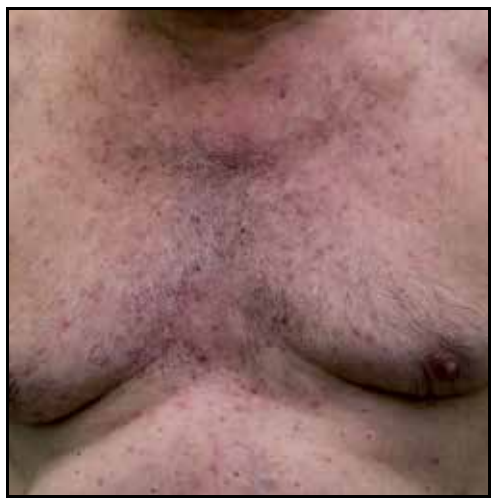

Fig. 1. 66-year-old male patient's acneiform rash after 2nd cycle (4th week) of pmab therapy for CRC with hepatic and pulmonary metastases.

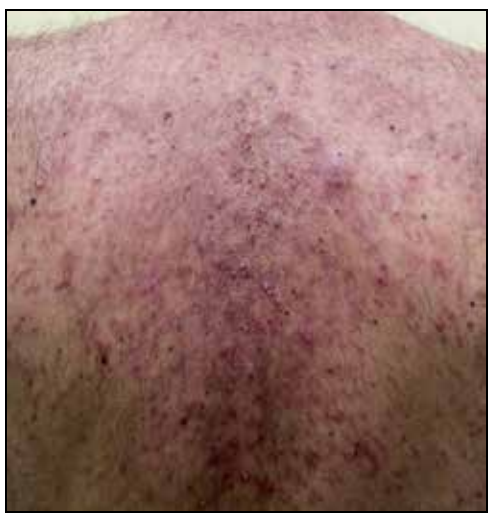

Fig. 2. Similar but more pronounced symptoms are visible on the back of the above patient.

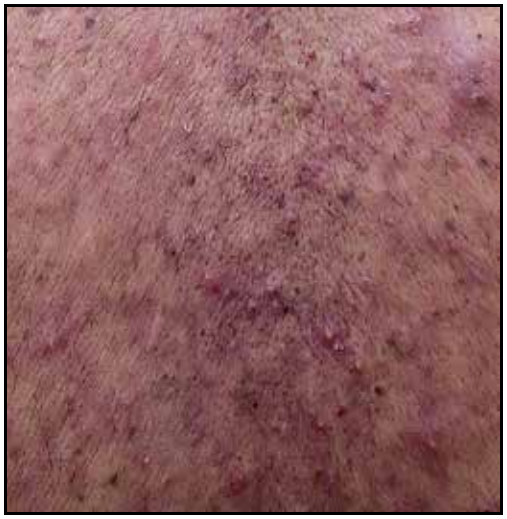

Fig. 3. Clearly visible inflammatory signs (pustules) differentiate EGFR-inhibitor therapy related rash from classical acne.

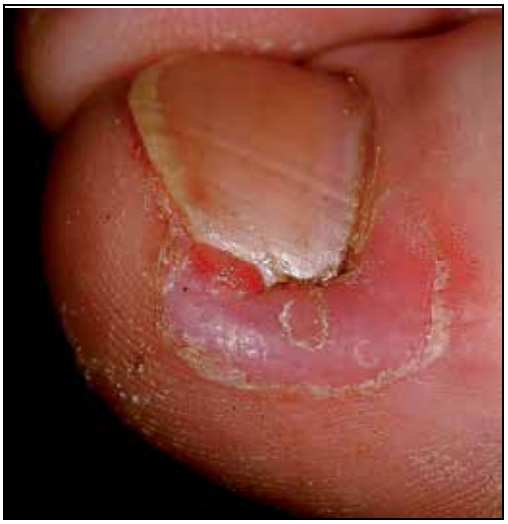

Fig. 4. Nail lesions (paronychia and overgrowth) developed on 6th week of pmab therapy. The disease did not resolve on conservative therapy, surgical treatment (exploration and drainage) was necessary.

It is important to modify or discontinue pmab administration according to the stage of rash. If the adverse events to (U. S. Department of Health And Human Services, U. S. National Institutes of Health, National Cancer Institute – Common Terminology Criteria for Adverse Events [NCI-CTCAE]) Grade 3 skin lesions emerge Vectibix should be suspended until the lesions resolve to Grade 2 or lower. In this case the product can be used by a 50% dose reduction; the dose can then be increased to the original in 25% increments every two weeks. If the rash persists or the symptoms recur in spite of dose reduction, pmab should be definitively discontinued (Alberta Health Services, 2010; EMA, 2011b; Pikó, 2009; Potthoff et

al., 2011; Widakowich et al., 2007). Nevertheless, skin and nail lesions are usually considered as positive predictive markers of efficacy and clinical response (Amado et al., 2008; Berardi et al., 2010; Busam et al., 2001; Eaby, n. d.; EMA 2011b; Grothey, 2006, 2007; Keating, 2010; Malik et al., 2005; Martinelli et al., 2007; Saif & Cohenuram, 2006; Siena et al., 2009; Widakowich, 2007).

3.3.2 Ophthalmologic complications

Since marketing authorization rare cases of keratitis and ulcerative keratitis has been reported, both representing a consequence of general mechanism of action of EGFR inhibitors (EMA, 2009; Burtness et al., 2009; Specenier et al., 2007; Thomas & Grandis, 2004; Xu et al., 2009). Retrospective analyses have shown that these complications were not severe in clinical trials, i.e. they did not reach Grade 2-4 (U. S. Department of Health And Human Services et al., 2009), and their incidence was between 0.2% and 0.7%. In clinical use as monotherapy, another case of severe keratitis and three cases of severe ulcerative keratitis have been reported (EMA 2011b). Care must be taken when the patients has a record of keratitis or ulcerative keratitis in his/her medical history. Consultation with an ophthalmologist is necessary in any instances the following symptoms are presented: inflammation of the eye, increased lacrimation, sensitivity to light, blurred vision, pain or redness of the eyes. The diagnosis of keratitis allows the oncologist to weigh the risk/benefit ratio of continuing or stopping Vectibix therapy, in cases of ulcerative keratitis however pmab treatment should be discontinued or suspended (EMA, 2011b; ManageCRC.com. 2011).

3.3.3 Pulmonary complications

Lung toxicity is a widely known complication of EGFR inhibitor therapies (interstitial lung disease [ILD], interstitial pneumonitis, fibrosis) (Eaby, n. d.; Cohenuram & Saif, 2007; Gandara et al., 2006; Grothey, 2006; Inoue et al., 2003 ; Nagaria et al., 2005 ; Pikó, 2009 ; Saif & Cohenuram, 2006 ; Yoneda et al., 2007).

As patients suffering from the above lung diseases were excluded from pmab clinical trials before randomization, there are no available data on lung complications in these patients during pmab therapy (EMA, 2011b). If patients experience chest symptoms (dyspnea, dry cough, clinical or ECG signs of hypoxia, abnormalities of pulmonary function tests), at least simple (posterior-anterior) chest radiography or a more appropriate chest CT should be performed. If these examinations are indicative of an interstitial pulmonary disease, Vectibix should be discontinued. Depending on the severity of symptoms, symptomatic treatment with corticosteroids or diuretics (NCI-CTCAE Grade 2), oxygen supplementation (Grade 3), or intubation, tracheostomy or assisted respiration (Grade 4) may be necessary (Alberta Health Services, 2010; Peeters et al., 2008; U. S. Department of Health And Human Services et al., 2009).

It is important to differentiate pulmonary changes due to pmab therapy from signs of an underlying malignancy (e.g. well-defined metastases, carcinomatous lymphangiosis). Besides scrutinizing radio-morphologic features, other helpful measures, e.g. obtaining earlier radiographs, considering the dynamics of the process and sharing exact data with the radiologist (about the disease, signs, physical examination results, applied therapy) and further personal consultations may be appropriate as well and would underline the necessity of multidisciplinary oncological team-work.

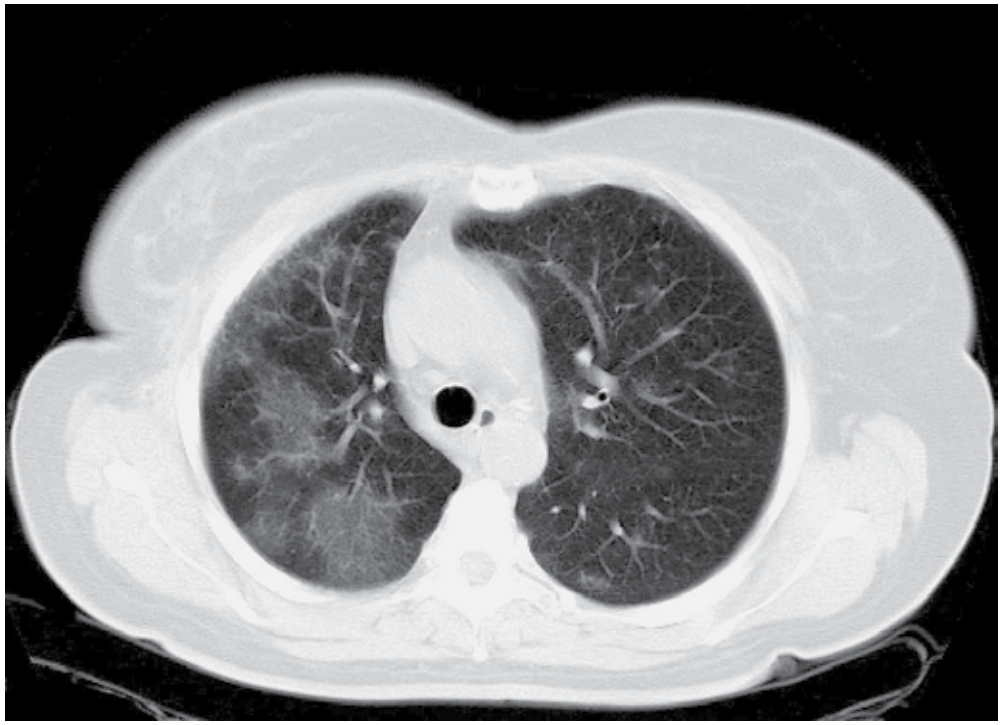

Fig. 5. Chest CT taken before starting planned pmab therapy of a mCRC patient who received therapy earlier in another institution. As the scan revealed pulmonary infiltration we did not administer Vectibix.

3.3.4 Hypomagnesaemia and hypocalcaemia

Symptoms are caused by the renal effects of EGFR inhibitors. Pronounced EGFR expression can be detected in the renal parenchyma (primarily in the ascending limb of loop of Henle, where magnesium and calcium are absorbed). Inhibition of EGFR in the renal tissue causes a decrease in the serum magnesium and calcium concentration. Following the recognition of these phenomenon patients involved in pmab studies have had their serum magnesium levels assessed. In 39% of cases the result proved to be abnormal, most often indicating mild hypomagnesaemia. The "Summary of Product Characteristics" requires regular assessments of serum magnesium and calcium levels before the treatment starts and for at least 8 weeks thereafter. Appropriate substitution is necessary for patients with mild-moderate disturbances, but the treatment may be discontinued in those who do not respond to substitution or present with severe clinical signs. Other electrolyte changes, such as hypokalaemia, have been detected as well. In such cases appropriate electrolyte substitution must be the primary step (Eaby, n. d.; EMA, 2011b; Pérez-Soler et al., 2005; Peeters et al., 2008; Pikó, 2009;, U. S. Department of Health And Human Services et al., 2009).

3.3.5 Diarrhoea

This is also a common side effect of EGFR inhibitors and indicates an injury of the intestinal mucosa similar to what is seen in dermatologic toxicities. Its frequency is not high; about 2% in patients with wild-type K-ras would develop diarrhoea. Its significance and its effect on

the continuability of pmab therapy depend on the severity of symptoms. Apart from lifestyle advices and loperamide administration, one should bear in mind that parenteral fluid replacement and normalization of electrolyte levels is essential in NCI-CTCAE Grade 3 diarrhoea (defecation more than 7 times per day or fecal incontinence, or necessity of hospitalization due to symptoms). If one fails to do so, calcium and magnesium electrolyte disturbances may increase in severity and acute renal failure may also develop (Berlin et al., 2007; Eaby, n. d.; EMA 2011b; Moy & Goss, 2007; Peeters et al., 2008; Pikó, 2009; Tuma, 2006; Widakowich et al., 2007).

3.3.6 General symptoms and infusion complications

Generally speaking, this term actually stands for adverse events (fever, chills and suffocation) which develop when a "foreign" protein is administered. Infusion complications emerge within 24 hours after administration. In most cases, premedication is needed to prevent general symptoms and infusion complications if human-animal chimeric or humanized monoclonal antibodies are used. As pmab is fully human, this is unnecessary when applying Vectibix. Nevertheless, infusion reactions might emerge during administration of fully human amino acid sequences despite using adequate protein filters to avoid complications. Several authors have reported however, that treatment with pmab may still be a viable and beneficial option for patients who suffered infusion reaction while being treated with the "chimeric" monoclonal antibody cetuximab (Cartwright & Genther, 2008; Chung, 2008; EMA, 2011b; Grothey, 2006; Helbling & Borner, 2007; Heun & Holen, 2007; Langerak et al., 2009; Lenz, 2007; Nielsen et al., 2009; O'Neil et al., 2007; Power et al., 2010; Saif et al., 2008).

Across all clinical studies, infusion-related reactions were reported in 3% of Vectibix-treated patients; of which < 1% were severe (NCI-CTC grade 3 or 4), i.e. required acute hospitalization or prolongation of hospitalization or was life-threatening. In the post-marketing setting serious infusion reactions have been reported, including rare reports of fatal outcome. If a severe or life-threatening reaction occurs during an infusion or at any time post-infusion, Vectibix should be permanently discontinued (U. S. Department of Health And Human Services et al., 2009).

4. Results of clinical studies with panitumumab

4.1 Phase 1 studies

At the Annual Meeting of the American Society of Clinical Oncology (ASCO) in 2002 Figlin and co-workers demonstrated the effect of a newly developed monoclonal antibody (called "ABX-EGF" in the presentation) on different tumors they evaluated in a phase 1 study (Figlin et al., 2002). The applied doses ranged from 0.01 mg/kg to 2.5 mg/kg. They found that the above therapy resulted in significantly long survival in certain cases. One patient with oesophageal cancer had stable disease for 7 months and minor response was reached in a patient with prostate cancer. No antibodies produced against ABX-EGF were detected, and its main side effect was rash.

In 2004, Rowinsky and co-workers published their results from a Phase 1 study with ABX-EGF (the agent later named pmab) used in renal cell carcinoma (Rowinsky et al., 2004). The highest dose used in this study was 2.5 mg per week. Although this dose could produce the highest rate of objective tumour response, the relationship between time to progression (median values were between 53 and 165 days) and the applied dose was unclear. It was

found that the most common side effects were dermatological symptoms (rash), already known in case of EGFR inhibitors. Presentation and severity of these symptoms were dose dependent and closely correlated with treatment results, while low haemoglobin and high alkaline phosphatase levels had a negative predictive value. No antibodies against ABX-EGF have been detected in this study.

4.2 Phase 2 studies

Based on the analysis of early Phase 1 study results subsequent studies with pmab were conducted in mCRC patients.

In 2004 and 2005 results of a phase 2 study with pmab monotherapy, involving CRC patients relapsing following a subsequent irinotecan and oxaliplatin therapy, were published (Hecht et al., 2004; Malik et al., 2005). Data of 148 patients were evaluable in the analysis. Median progression-free survival (PFS) was 3.4 (2.0-4.0) months and overall survival (OS) was 9.4 months (6.0-10.6). Results did not differ in EGFR positive or negative patients.

Berlin and co-workers (Berlin et al., 2004) and Hecht and co-workers (Hecht et al., 2006b) used pmab with combinations containing irinotecan (IFL or FOLFIRI) in Phase 2 studies. The main adverse effects were dermatological symptoms and diarrhoea. In the IFL arm partial remission fulfilling the "Response Evaluation Criteria in Solid Tumors" (RECIST) was seen in 48% of patients and stable disease could be reached in 26% that equated to a tumour control in 74% of cases (Jaffe, 2006; Padhan & Ollivier, 2001; Therasse et al., 2000). Median PFS and OS were 5.6 and 17 months, respectively. When pmab was used in combination with FOLFIRI rates of remission, stable disease and total tumour control were 33%, 46%, and 79%, respectively. Progression-free survival was 10.9 months, but overall survival results could not have been calculated (overlapping results of the study had been published by other authors in various forums).

Patients were later divided into groups with negative or "low" (1 to 10%) (Hecht et al., 2006a), and "high" (above 10%) EGFR-expression (Berlin et al., 2006). No significant differences were found: at low EGFR levels 48% response rate and the rate 7.9 weeks median PFS were detected, while in patients with high EGFR levels 42% tumour response rate and 12-14 weeks PFS was seen. The adverse effect profile was similar. Grade 3/4 adverse events were presented in 19-24% (dermatological symptoms prevailed), and the rate of hypomagnesaemia was similar (8 and 12%).

4.3 Phase 3 study and analysis of further results

Van Cutsem et co-workers were the first to publish a comparison of Vectibix and "best supportive care" (BSC): they treated a total of 463 patients with EGFR expressing mCRC, after failure of irinotecan- and oxaliplatin-containing therapies (van Cutsem et al., 2007). Patients were given either pmab (6 mg/kg every two weeks, without premedication) in combination (with symptomatic treatment) or BSC alone, in 1:1 ratio. Patients in the BSC group could have been switched to the active arm in case of progression. Thirty-five percent of patients had been on adjuvant chemotherapy earlier, and all of them had had at least two treatment options due to metastatic disease. Thirty-seven percent of the patients had a disease progression after the third line of drug therapy. Treatment efficacy was assessed after week 8, 12, 16, 24, 32, and 40, and every 3 months thereafter according to the RECIST (Jaffe, 2006; Padhan & Ollivier, 2001; Therasse et al., 2000).

The following chart represents the results of this study and shows the benefits of Vectibix compared to supportive care:

Studied parameters	pmab + BSC (232 patients)	BSC alone (231 patients)
PFS rate at week 24	18%	5%
PFS rate at week 32	10%	34%
Response rate (RR)	8%	0%
Stable disease (SD)	28%	10%
Overall response rate (ORR)	36%	10%
Median duration of response	17 weeks	NA

Table 1. Results of progression-free survival (PFS), response rate, stable disease, overall response rate treated with pmab + BSC vs. BSC alone (adapted from: van Cutsem et al., 2007)

In terms of all parameters (age, sex, site of primary tumour, ECOG performance status, former lines of chemotherapy applied, number of organs with metastases and degree of EGFR positivity), subgroup analyses unanimously showed that the active treatment arm (pmab) was superior to BSC. Degree of risk reduction was 46%, which was statistically significant ($p<0.000000001$). It is remarkable that among the 174 patients who were crossed over from BSC arm to the active (pmab) arm due to progression partial response (PR) could be reached in 9% and SD in 32% of cases, in spite of a more progressed disease (Cohenuram & Saif, 2007).

This study once again proved the correlation between side effects and efficacy, i.e. assessment of the results showed that skin symptoms are of good predictive value. These findings underline the fact that rash is one of the most important predictive factors of efficacy.

In the study designed to compare pmab and BSC, Siena and co-workers re-assessed response and survival data, and divided the group of responders into subgroups of patients with remission and those with stable disease. Differences between each group were statistically confirmed (Siena et al., 2007). Curves demonstrating treatment efficacy were also different, survival curve of patients with disease progression and that of those with no progression after 8 weeks of pmab therapy (equivalent with 4 treatment cycles) were compared. Based on these data the authors presumed with good reason that there must be another factor apart from the detectable EGFR expression (an inclusion criterion for all patients) that has an impact on treatment results.

The presumed factor was later proved to be the K-ras mutation status. Differences in treatment results could be explained by the presence of "normal" (wild type) or "abnormal" (mutated) K-ras genes. Amado and co-workers determined the frequency of mutations in the already known patient population (Amado et al., 2008). Although not all, 427 samples of the 463 patients were suitable for subsequent central laboratory evaluations and were eventually analyzed. Analyses showed mutations in 184 patients and "wild type" K-ras in 243 patients. Data analyses showed that no correlation can be detected between K-ras mutation and EGFR status (the latter determined by immunohistochemistry), neither by expression nor by the intensity of membrane staining.

Analyses of clinical results showed that (in accordance with the biological role of K-ras described earlier) tumour progression in mutation carriers is independent from the regulation of stimuli reaching the EGFR. Consequently, in these patients the EGFR inhibitor pmab is less effective and does not provide better results than BSC.

	pmab + BSC		BSC	
	wild type K-ras	mutant K-ras	wild type K-ras	mutant K-ras
Number of patients	124	84	119	100
Median PFS (weeks)	16	8	8	8
Median OS (months)	8,1	7,6	4,9	4,4

Table 2. Progression-free survival (PFS) and overall survival (OS) of pmab + BSC vs. BSC treated patients by K-ras status. (Source: Amado et al., 2008)

Side effects were more frequent and severe in the K-ras mutant subgroup that, apart from inefficacy, may lead to a worse tolerability and possibly higher treatment risks. Considering both efficacy results and side effects, it was proven that pmab should only be used in patients with the wild type K-ras.

4.4 Vectibix summaries of product characteristics: A reinforcement of treatment criteria and results of the clinical trials

Based on the consideration that the approval had been based on a clinical trial including pre-treated EGFR positive patients whose treatment was shown to be effective only in those with the K-ras wild type, the European Medicines Agency (EMA) summarizes treatment criteria in all of the Summaries of Product Characteristics congruently. „Vectibix is indicated as monotherapy for the treatment of patients with EGFR expressing metastatic colorectal carcinoma with non-mutated (wild-type) *KRAS* after failure of fluoropyrimidine-, oxaliplatin-, and irinotecan-containing chemotherapy regimens" (EMA, 2011b). The U. S. Food and Drug Administration **(**FDA) defines the same criteria in more detail: „Vectibix is an epidermal growth factor receptor antagonist indicated as a single agent for the treatment of metastatic colorectal carcinoma with disease progression on or following fluoropyrimidine, oxaliplatin, and irinotecan chemotherapy regimens. Approval is based on progression-free survival; no data demonstrate an improvement in disease-related symptoms or increased survival with Vectibix. Retrospective subset analyses of metastatic colorectal cancer trials have not shown a treatment benefit for Vectibix in patients whose tumors had *KRAS* mutations in codon 12 or 13. Use of Vectibix is not recommended for the treatment of colorectal cancer with these mutations" (U. S. Food and Drug Administration, 2009).

5. Combining panitumumab with cytostatic agents

The Summary of Product Characteristics of other anti-mCRC targeted biologic therapies states that these agents can be used either only in combination with "traditional" anti-tumour chemotherapies (e.g. beva), or both in combination and as a stand-alone therapy (e.g. cmab). In contrast, pmab could only have been used as a monotherapy and in patients who have already had a definite cytostatic pre-treatment. Supposing that such timing of treatments does not provide optimal circumstances for the efficacy of monoclonal antibodies, possible combinations of Vectibix and cytostatic agents have been evaluated in clinical studies.

5.1 Combination of pmab and chemotherapy as a first-line treatment

Following completion of a study involving 1183 patients titled "Panitumumab Randomized Trial in Combination with Chemotherapy for Metastatic Colorectal Cancer to determine

Efficacy" (PRIME), Douillard and co-workers presented results of the application of pmab with FOLFOX4 (5-fluorouracil, leucovorin and oxaliplatin) versus FOLFOX4 alone as the first-line treatment in mCRC patients in open label, randomized, multicenter, Phase 3 trial (Douillard et al., 2010). Eligible patients were individuals older than 18 years who did not receive chemotherapy for their metastatic disease. 5-fluorouracil was allowed in adjuvant chemotherapy in case the disease recurred within 6 months after discontinuing the adjuvant therapy, but oxaliplatin was not allowed under any circumstances.

Pmab was administered every two weeks in a dose of 6 mg/kg by intravenous infusion over one hour on the day before FOLFOX4 chemotherapy was scheduled. If patients tolerated the first pmab infusion, the consecutive doses could have been administered over 30 minutes. FOLFOX4 was administered every two weeks: on day 1 oxaliplatin was administered at 85 mg/m^2 and leucovorin at 200 mg/m^2 (or equivalent dose) via infusion. On days 1 and 2 this was followed by fluorouracil at 400 mg/m^2 by intravenous bolus and fluorouracil at 600 mg/m2 by a continuous 22-hour infusion. This treatment was continued until disease progression (adjudicated by an independent committee) or the occurrence of unacceptable side effects.

In terms of evaluation the study had four arms, as groups of K-ras mutant and wild-type patients were distinguished following previous laboratory assessment both in the FOLFOX4 alone and the pmab + FOLFOX4 arm.

The administration of the monoclonal antibody Vectibix to patients with wild-type K-ras increased PFS significantly from 8.0 to 9.6 months, while increase in overall survival (23.9 months as compared to 19.7 months) was clinically considerable and relevant nevertheless statistically non-significant, compared to FOLFOX4 alone arm. In K-ras mutated cases however, Vectibix with FOLFOX4 versus FOLFOX4 alone decreased the median PFS (7.3 vs. 8.8 months) and OS (15.5 vs. 19.3 months).

By a glance on the table summarizing side effects one can realize that apart from typical side effects of EGFR inhibitors in the Vectibix group no significant differences were revealed.

Antibodies against pmab were found in blood samples of 3.0% of patients (samples were drawn during treatment). After discontinuation, neutralizing antibodies were found in another 0.4% of patients.

A forest plot subgroup analysis with overlapping confidence intervals showed that pmab addition was generally beneficial in terms of improving progression-free survival. Treatment without pmab showed a tendency to be more beneficial in those with bad performance status (ECOG 2). Pmab seemed to be more beneficial in those with hepatic metastases, however in patients with dissemination in multiple organs and in cases presenting exclusively hepatic metastases no significant differences between the arms were shown. Subgroup analyses of overall survival revealed similar results, notably, poorer general condition (ECOG 2) seemed to be again more disadvantageous for Vectibix treated patients, age and gender showed marked but somewhat weaker interference than is PFS.

The authors claimed that adding pmab to FOLFOX4 increased PFS significantly in previously untreated mCRC patients with wild-type K-ras. Another clinically important feature of pmab is that severe infusion reactions are rare, and the standard 2-week protocol of Vectibix enables treating physicians to synchronize administration with chemotherapy schedules and decreases the number of visits to the minimum. As no premedication is required and no observation is necessary following treatment, the short outpatient therapy is advantageous for patients and caregivers as well.

An important aspect, also relevant for routine clinical practice, was investigated by Siena and co-workers in their subgroup analysis of the above study detailed in ASCO Annual Meeting 2011 (Siena et al., 2011). Patients with good performance status (ECOG 0-1) obviously profited from the addition of pmab to FOLFOX4 as PFS increased in these cases from 8.0 (7.5-9.3) to 10.4 months (9.3-11.3), OS from 20.7 (18.2-23.2) to 25.8 months (21.7-not estimable); whereas at ECOG2 (ambulatory and capable of all self-care but unable to carry out any work activities up and about more than 50% of waking hours) patients the addition a pmab decreased PFS from 7.6 (5.3-11.1) to 4.8 months (2.7-5.3), OS from 11.7 (8.0-15.7) to 7.0 (4.6-11.7) months. An adequate determination of performance status may serve as a simple and statistically convincing tool to predict the value of the addition of pmab to FOLFOX4 in the first line treatment of mCRC.

Notably, besides performance status, quality of life may be a further parameter worth evaluating when analysing treatment results. Primary results from a phase II study involving 142 patients evaluating the combination a pmab and FOLFIRI as a first line chemotherapy in mCRC were published by Kohne and co-workers in 2010 (Kohne et al., 2010). Results showed 48% response rate (RR) for wild type and 29% RR for mutant K-ras patients, with no differences in side effects. Results of a secondary analysis of initial quality of life measures were published during ASCO Annual Meeting 2011 (Karthaus et al., 2011). The results demonstrated that those patients with better quality of life had better tumour responses as well by week 8 and 24 of the combination therapy. It does not seem to be an overstatement that the combination of pmab with cytostatics in the first line treatment of CRC is a promising option for patients in better clinical (performance and quality of life) status.

5.2 Combination of pmab and chemotherapy as a second-line treatment

Peeters and co-workers compared the efficacy of pmab and FOLFIRI to FOLFIRI alone as the second-line treatment of mCRC patients in a phase 3, equally randomized trial (Peeters et al., 2010). The study was originally designed to compare the therapeutic effect in the entire population, but due to convincing external data it was modified before the efficacy assessments so that prospective assessments would be carried out as per the K-ras status of the tumour.

A total number of 1186 patients were treated after randomization. Five hundred-ninety-two (50%) patients were given pmab and FOLFIRI, and 595 (50%) were given FOLFIRI alone. The K-ras status of 1083 patients (91%) was known (based on central laboratory tests): 597 patients (55%) had wild-type K-ras tumour and 486 (45%) had K-ras mutant metastatic colon cancer.

The eligible patients were older than 18 years and their ECOG performance status was 0, 1 or 2. Only one earlier chemotherapeutic scheme, i.e. first-line fluoropyrimidine-based chemotherapy was allowed for the treatment of mCRC. A radiologically verified progression by RECIST was required during the course of treatment or within 6 months. Known EGFR expression or K-ras status were not required for enrolment. Patients previously treated with irinotecan or anti-EGFR therapy were excluded from the study (Jaffe, 2006), (Padhan & Ollivier, 2001), (Therasse et al., 2000).

Pmab (at 6 mg/kg) was administered over 60 minutes by infusion before chemotherapy; if patients tolerated the first dose, the following infusions were administered over 30 minutes. Every patient was given FOLFIRI: 180 mg/m^2 irinotecan and 400 mg/m^2 raceme leucovorin

(or 200 mg/m² l-leucovorin) by intravenous infusion on day 1 and 400 mg/m² fluorouracil by intravenous bolus on day 1, followed by 2400 mg/m² by continuous infusion on days 1 and 2. Patients were given chemotherapy with pmab or without pmab until the onset of progression or intolerance as per RECIST (confirmed by independent investigators) (Jaffe, 2006), (Padhan & Ollivier, 2001), (Therasse et al., 2000).

In terms of evaluation the study had four arms, groups of K-ras mutant and wild-type patients (as previously assessed) were distinguished both in the FOLFIRI (alone) and the pmab + FOLFIRI arm.

PFS improved significantly in the subgroup of wild-type K-ras patients if pmab was added to chemotherapy; the median PFS was 5.9 and 3.9 months in the pmab + FOLFIRI and the FOLFIRI alone group, respectively. A non-significant increase in OS was also observed, median OS was 14.5 and 12.5 months, the response rate improved from 10% to 35% with added pmab. Theoretical assumptions and earlier clinical experiences were confirmed by the fact that no difference was seen in terms of efficacy in patients with K-ras mutant tumors compared to chemotherapy alone.

Antibodies produced against pmab following therapy were found (by central laboratory) in less than 1% (4 out of 501) of patients. None of these antibodies had a neutralizing effect.

Subgroup analysis suggests that pmab was advantageous in every subgroup in terms of improved PFS with a similar age and gender tendency as seen in the "PRIME" study. In terms of OS, combination arm seemed equivocal with chemotherapy alone in patients previously treated with oxaliplatin, beside those overlapping confidence intervals and summary measures favouring panitumumab reinforced a positive tendency of improving OS.

The authors claimed that the study confirmed the efficacy of pmab with FOLFIRI in K-ras wild-type mCRC patients who were treated previously. PFS improved in a statistically significant manner in this group, which underlines the fact that K-ras status of the tumour can be considered as a predictive biomarker. With a Q2W administration, pmab was comfortably combined with FOLFIRI given at a similar dosing frequency. The toxicity profile was not different from that of EGFR inhibitors and chemotherapy combinations, toxicities could have been managed well.

Considering that, in Hungary bevacizumab is reimbursed only as a first line treatment by the state health fund - even though its use is not confined to a given line in mCRC by the effective Summaries of Product Characteristics (EMA, 2011a). Peeters and co-workers published data of critical relevance in ASCO Annual Meeting 2010 in this aspect (Peeters et al., 2010). The authors evaluated K-ras wild type patients from the above study previously treated with bevacizumab. According to the results, PFS was not different in bev pre-treated patients compared to the overall K-ras wild type study population (5.8 and 3.7 months vs. 5.9 and 3.9 months for pmab + FOLFIRI and FOLFIRI arms). In striking contrast OS improved when bev treatment preceded the pmab + FOLFIRI combination in second line from 14.5 months to 15.7 months.

6. Panitumumab in current therapeutic guidelines

6.1 Pmab in U. S. guidelines

From among clinical recommendations issued in the United States the first to review is the National Comprehensive Cancer Network's (NCCN) guidelines referring to the diagnosis and treatment of colon (Version 3.2011) and rectal carcinoma (Version 4.2011) (Engstrom et

al., 2011a, b). As the results of clinical studies with pmab concern distant metastatic diseases only, there is no significant difference between the two compilations. Like other agents affecting biological targets pmab is not allowed in any adjuvant indication except for clinical trials. Pmab is recommended in monotherapy or in combination with FOLFIRI in diseases with distant metastases whether or not resection of the primary malignancy was performed. It is considered reasonable to remove the primary malignancy (which has not been removed earlier) and the distant metastases in one or more surgeries following a 2- to 3-month treatment. (It is strongly highlighted in the recommendation, that K-ras evaluation must be performed and that the product should be administered only in patients with the wild type K-ras.) In non-resectable synchronous or metachronous distant metastases FOLFIRI ± pmab is an alternative of FOLFIRI ± bevacizumab or cetuximab as a first-line therapy at least 12 months after the administration of adjuvant FOLFOX.
In patients eligible for intensive treatment, pmab ± FOLFOX is considered as the first-line therapy of metastatic diseases (among other combinations), while pmab ± FOLFIRI acts as a second-line therapy. Monoclonal antibody panitumumab is indicated as monotherapy in case the patient has decreased chemotherapy tolerance. Biological targeted agents such as pmab (depending on the previously administered agents) can be administered following a new progression (i.e. as a third treatment possibility), mostly in patients who do not tolerate irinotecan. Vectibix is recommended as a monotherapy by NCCN in patients who are ineligible for intensive therapy.

6.2 Pmab in european guidelines

The European Society for Medical Oncology (ESMO) released guidelines in 2010. Pmab is not mentioned in the publications referring to the diagnosis, adjuvant therapy and follow-up of CRC (Labianca et al., 2010). This is compliant with the European Summary of Product Characteristics, which limits treatment possibilities much rigorously than the guidelines in the United States do. In guidelines detailing the treatment of advanced disease authors state (van Cutsem et al., 2010) that anti-EGFR antibodies pmab and cmab are effective as monotherapy for patients with chemorefractory mCRC, and wild-type state of K-ras is necessary to reach therapeutic effect. In comparison with BSC, pmab is considered beneficial in terms of PFS; this effect is not reflected in terms of overall survival (OS) due to the "cross-over" design of trials. Pmab and polychemotherapy (FOLFOX4 as a first-line, and FOLFIRI as a second-line therapy) but the absence of significant improvement in OS is emphasized. Evidence level of all recommendations for pmab therapy is IB.

7. Summary

Being a fully human monoclonal antibody not requiring a special pre-treatment or saturation dosage, pmab belongs to a new group of biological targeted agents used in the treatment of metastatic colon or rectal cancer. Pmab binds to EGF receptors, and the post-study pathologic evaluation of monotherapy registration trial samples provided convincing evidence of the crucial role K-ras status played in clinical efficacy: median progression-free survival was 16 weeks in the wild-type (vs. 8 weeks with best supportive care) patients group. Although pmab was practically ineffective in patients with mutant K-ras, side effects were more frequent and severe. According to effective Summaries of Product Characteristics the product can be applied in Europe as monotherapy in EGFR positive and K-ras wild-type

mCRC patients after fluoropyrimidine, oxaliplatin and irinotecan-based chemotherapeutic protocols had failed.

Based on clinical study results published in 2011, the addition of panitumumab to FOLFOX4 polychemotherapy as a first-line treatment in wild-type K-ras resulted in a significant increase in progression-free survival (PFS) (8.0 to 9.6 months), while increase in overall survival (OS) (19.7 to 23.9 vs. FOLFOX4 alone) was clinically considerable but non-significant. In K-ras mutant cases however, Vectibix with FOLFOX4 versus FOLFOX4 alone decreased the median PFS (8.8 to 7.3 months) and OS (19.3 to 15.5 months). PFS improved significantly in the group of wild-type K-ras patients if pmab was added to the FOLFIRI protocol as a second-line treatment; median PFS was 5.9 and 3.9 months in the pmab + FOLFIRI and the FOLFIRI alone groups, respectively. A non-significant increase in OS was also observed; median OS was 14.5 and 12.5 months, and response rate significantly improved from 10% to 35% with added pmab. In mutant K-ras, PFS was 5.0 months with added monoclonal antibodies and 4.9 months with FOLFIRI alone, while OS was 11.8 and 11.1 months, respectively, i.e. no difference could have been statistically confirmed. Following a positive EMA's Committee for Medicinal Products for Human Use (CHMP) opinion in the middle of 2011, both the FOLFOX4 (1st line) and the FOLFIRI (2nd line) combinations will be likely authorized in the EU for the treatment of mCRC.

The side effect profile matches other EGFR inhibitors (the spectrum as a whole being utterly different from that of conventional cytostatics), with dermatologic symptoms (rash), nail diseases, lung infiltration, diarrhoea and electrolyte disturbances of renal origin may develop. Infusion complications are not common. Panitumumab therapy is safe in cases where followed-up carefully, this may mean temporary suspension of treatment, dose reduction or therapy discontinuation if justified by above detailed side effect related signs and symptoms.

8. Acknowledgments

I would like to express my gratitude to all those whom I got the chance to learn from, my co-authors who are my co-workers as well, my wife, my daughter, and all my colleagues who did their best to provide a quiet environment for this work. I am grateful to my patients for the experiences I gained during their treatment encouraged me to write this chapter. Last but not least, I thank Amgen Inc. for the invaluable technical support.

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21

Resection for Colorectal Liver Metastases

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1. Introduction

Colorectal cancer is the third most frequent cancer in the Western world. About half of the patients develop synchronous or metachronous metastases. The liver is the most common site of such metastases and thus hepatic metastatic disease is a significant socio-medical problem. If it is not treated, the median patient survival is only some months. Surgical resection is the treatment of choice for patients with isolated colorectal liver metastases when feasible. For patients with four or fewer isolated hepatic lesions, five year relapse-free survival rates range from 24 to 58 percent and ten year survival rates vary between 17 and 33 percent. There is a convincing socio-epidemiological evidence of the dramatic unfavourable influence on population wealth of untimely diagnosis and inadequate treatment of the patients with advanced and metastatic colorectal cancer worldwide (Hata et al., 2010; Kostov & Kobakov, 2006a; Stillwell et al., 2011; Tsoulfas et al., 2011).

2. Purposes of the study

The purposes of the present paper are to define the variety of liver resections as an important component of the modern treatment of colorectal liver metastases, to describe their operative techniques and postoperative results, to illustrate some peculiar resection patterns from our own patients' contingent and, based on our own experience with the complex preoperative diagnostic algorithm and the individualized indications and contraindications for surgery and multimodal therapy, to outline the advantages of different types of hepatic resections in properly selected cases with colorectal liver metastases as manifested by improved patient's quality of life and survival.

3. Material

Patients' contingent included a total of 158 patients who have undergone liver resections for colorectal liver metastases in the Department of Surgery, Naval Hospital of Varna and in the Division of Surgery, Marko Markov Interregional Dispensary and Hospital of Oncological Diseases of Varna, Bulgaria, during a 10-year period (January 1, 2000 - December 31, 2010). Results concerning 108 patients dynamically followed-up for at least one year after the operation were illustrated in this comprehensive retrospective study. Demographic characteristics, preoperative clinical, laboratory and functional diagnosis of the patients,

types of surgical interventions and conservative therapy as well as metastatic tumour localization, volume, number and staging were systematized. Only some of our data could be presented in the present paper.
Number and mean age of male and female patients can be seen on Table 1.

Gender	Patients		Mean age	
	n	%	years	range
Males	68	63	58	36-81
Females	40	37	54	32-79
Total	108	100	59	32-81

Table 1. Patients' distribution according to gender and mean age

4. Methods

The algorithm for contemporary diagnostic evaluation comprised total colonoscopy, conventional chest radiography, conventional blood tests, serum levels of some tumour markers such as carcinoembryonic antigen (CEA), carbohydrate antigen CA 19.9 and carbohydrate antigen CA 242, abdominal preoperative and intraoperative ultrasonography (for estimation of the type and volume of liver resection), intraoperative cholangiography (for pre- and postoperative bile drainage evaluation in remnant hepatic parenchyma), methyene-blue injection through the portal vein (for assessment of afferent and efferent blood flow in remnant hepatic parenchyma), contrast-enhanced and spiral computed abdominal tomography, MRI in case of contradictory computer tomographic data and histopathology of enlarged hilar lymph nodes. The volume of the liver resection was determined not only by the number, size and localization of the metastases but also by the degree of compensatory hypertrophy of the intact hepatic volume.
The presentation of all the types of surgical interventions included the following:

i. types of surgical access, liver mobilization, hilar dissection, and hepatic-vein control,
ii. operative approaches, and
iii. operative volumes.

Patients' distribution according to the volume of liver resection is demonstrated on Table 2.
During the last 8 years, a total of 14 patients underwent repeated liver resections. A third resection was done in three of these patients, and a fourth resection was done in two of these patients.
Table 3 indicates the consecutive number and volume of primary and repeated liver resections.
Some essential parameters of colorectal liver metastases in these repeated resections of different consecutive number are summarized on Table 4.
Additionally, multimodal treatment of all the patients with colorectal liver metastases included a variety of chemotherapeutic protocols as aneoadjuvant and/or adjuvant chemotherapy along with radiofrequent ablation, portal vein embolization, and two-stage resection of bilobar colorectal liver metastases (Kostov & Kobakov, 2006). The effect of neoadjuvant chemotherapy was assessed according to the Response Evaluation Criteria in Solid Tumours (Eisenhauer et al., 2009).

Volume of resection	Type of resection	n=108
Monosegmentectomy n=22 (20%)	Sg1	3
	Sg2	2
	Sg3	3
	Sg4	5
	Sg5	2
	Sg6	3
	Sg7	2
	Sg8	2
Bisegmentectomy n=24 (22%)	Sg6,7	4
	Sg5,8	3
	Sg2,3	4
	Sg5,6	3
	Sg4b,5	2
	Sg1,4	2
	Sg7,8	2
	Sg4a,8	2
	Sg3,4b	1
	Sg4 + parts of Sg 1,2,3,5	1
Multisegmentectomy n=62 (57%)	Sg4,5,8	3
	Sg1,4,5,8	2
	Sg1,4b,5,6	1
	Sg5,6,7	1
	Sg3,5,6,7	1
	Sg3,4b,5	2
	Sg4 + part of Sg2,3 + metastasectomy of Sg8	1
	Sg4b,6,7	1
	Sg6,7,8	1
	Sg3+ parts of Sg4,6,8	1
	Left hemihepatectomy (Sg2,3,4±1)	13
	Right hemihepatectomy (Sg5,6,7,8±1)	32
	Right trisectionectomy (Sg1,4,5,6,7,8)	2
	Left trisectionectomy (Sg1,2,3,4,5,8)	1

Table 2. Type and volume of liver resections

Number of resection	Number of patients	Volume of resection					
		One Sg	Two Sg	Three Sg	Trisection-ectomy	Hemihepat-ectomy	Wedge resection
First	14	2	6	2	2	2	-
Second	14	-	-	-	-	-	14
Third	3	-	-	-	-	-	3
Fourth	2	-	-	-	-	-	2

Table 3. Consecutive number and volume of resections

Parameter	First resection	Second resection	Third resection	Fourth resection
CEA > 200 ng/mL	9	9	3	2
CEA ≤ 200 ng/mL	5	5	-	-
synchronous	5	-	-	-
metachronous	9	14	3	2
after < 12 months	6	-	-	-
after ≥ 12 months	8	-	-	-
total number				
One	2	8	2	-
two-three	7	6	1	-
≥ three	5	-	-	2
unilobar	8	12	3	2
bilobar	6	2	-	-
diameter < 20 mm	2	7	2	-
diameter of 20-50 mm	8	5	1	1
diameter ≥ 50 mm	4	1	-	1
nodes in *lig. hepato-duodenale*	-	-	-	2
positive margins	-	-	-	2
negative margins	14	14	3	-
MSKCC-CRS				
0-2 factors	8	8	1	-
3-5 factors	6	6	2	2

Table 4. Characteristics of colorectal liver metastases in repeated liver resections

One- and three-year survival data were retrospectively recorded up to December, 2010. Memorial Sloan-Kettering Cancer Center Clinical Risk Score (MSKCC-CRS) was used to evaluate the postoperative prognosis of the patients (Arru et al., 2008).
Kaplan-Meier estimates outlined differences with Kaplan-Meier curves. Comparisons of sex and age between segmentectomy and major hepatectomy patients applied chi-square and *t*-test. The *t*-test compared mean blood loss, diameter of colorectal liver metastases, duration of surgery, length of hospital stay, and resection margins. The postoperative complications were compared by means of Fisher's exact test. Patients' homogeneity was comparatively assessed by means of the log rank and Wilcoxon tests.

5. Operative techniques of liver resections

5.1 Types of surgical access

The following types of surgical access for liver mobilization, hilar dissection, and hepatic-vein control can be used (Kostov & Kobakov, 2010):
Upper medial laparotomy with transversal enlargement to the right until 9th intercostal space along with Makuushi incision is most commonly performed to access right or left hemiliver while Mercedes-Benz incision is suitable to access both left and right hemilivers.
Complete liver mobilization passes through five stages: i) interruption of *lig. teres hepatis* between two ligatures, ii) cutting of *lig. falciforme hepatis* up to the subdiaphragmatic part of *vena cava inferior*, iii) search for an accessory left hepatic artery as a branch of *a. gastrica sinistra* when cutting *lig. hepatogastricum*, iv) cutting to the left of both *lig. triangulare sinistrum* and *lig. coronarium hepatis* to left hepatic vein trunk and v) cutting to the right of both *lig. triangulare dextrum* and *lig. coronarium hepatis* to right hepatic vein trunk.
Hilar dissection aims at dividing the vessels designed for the left and right hemiliver that enables the application of hemi-Pringle maneuver. Right hepatic vein extrahepatic part can be reached by interruption of Makuushi ligament. Right hepatic vein is lifted on rubber holder (Fig. 1).

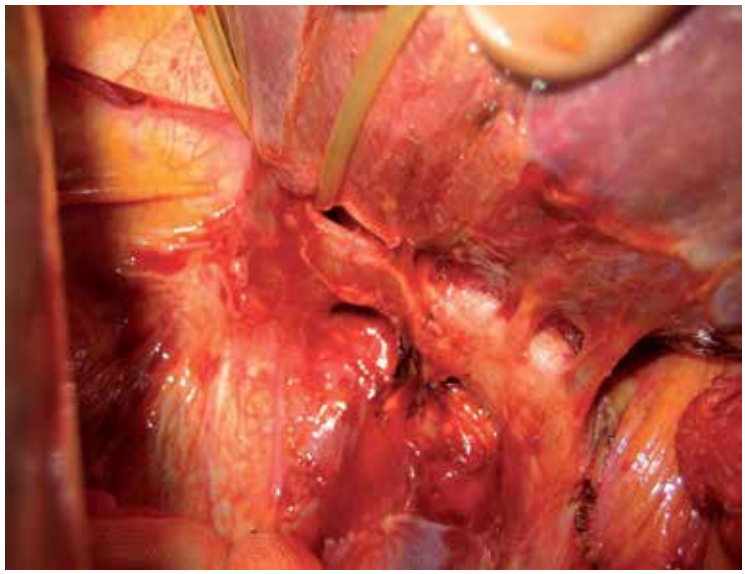

Fig. 1. Right hepatic vein mobilization and short retrohepatic veins

Usually, both left and middle hepatic veins present with a common trunk as their bifurcation is intraparenchymally located (Fig. 2).

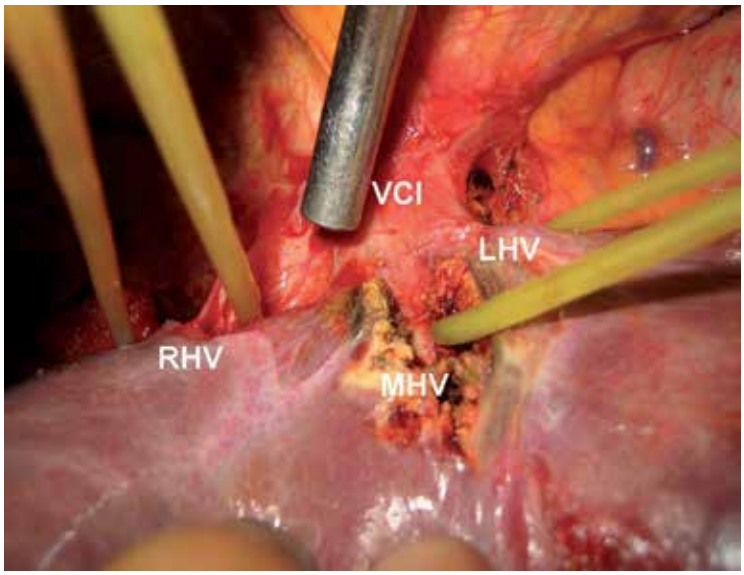

Fig. 2. Extrahepatic mobilization of three hepatic veins enables a complete vascular exclusion of the liver and preserves blood flow through *vena cava inferior*

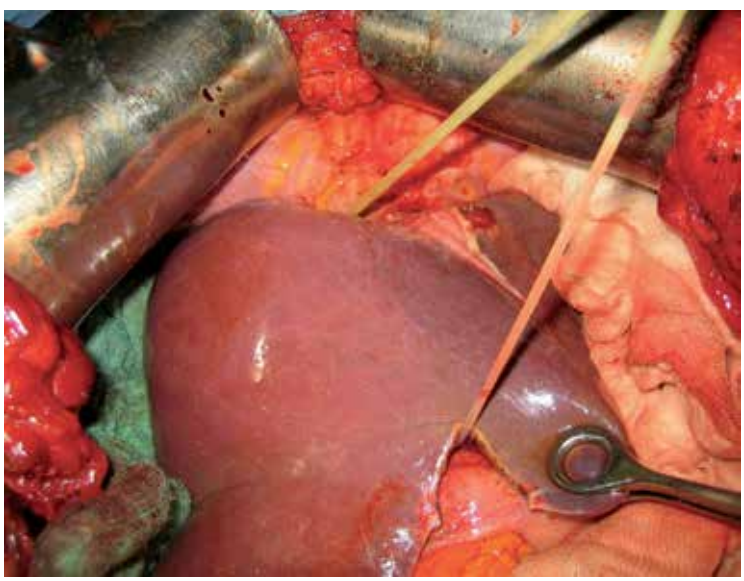

Fig. 3. Single 'hanging'-maneuver

Clamping the three hepatic veins enables a complete vascular exclusion with blood flow preservation through *vena cava inferior*. With single 'hanging'-maneuver, a rubber tape passes cranially between the right and middle hepatic veins but caudally - between hilar vessels for the right and left hemiliver (Fig. 3).
This method is applied in right/left hemihepatectomy or right segmentectomy. With double 'hanging'-maneuver, a second rubber tape is additionally used which passes cranially between the middle and left hepatic veins but caudally - between hilar vessels for the right and left hemiliver (Fig. 4). This method is applied in mesohepatectomy or proximal segmentectomy. With complete vascular exclusion and blood flow interruption through *vena cava inferior* the latter is clamped over the three hepatic veins and over the inflow of renal veins. For that purpose, *vena cava inferior* is mobilized at two sites - below the diaphragm and over the inflow of renal veins (Fig. 5). Right suprarenal vein is obligatorily interrupted.

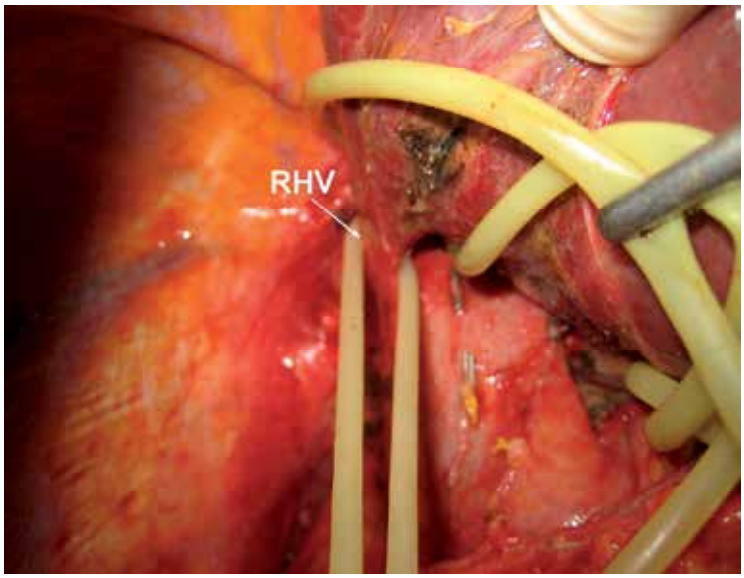

Fig. 4. Double 'hanging'-maneuver

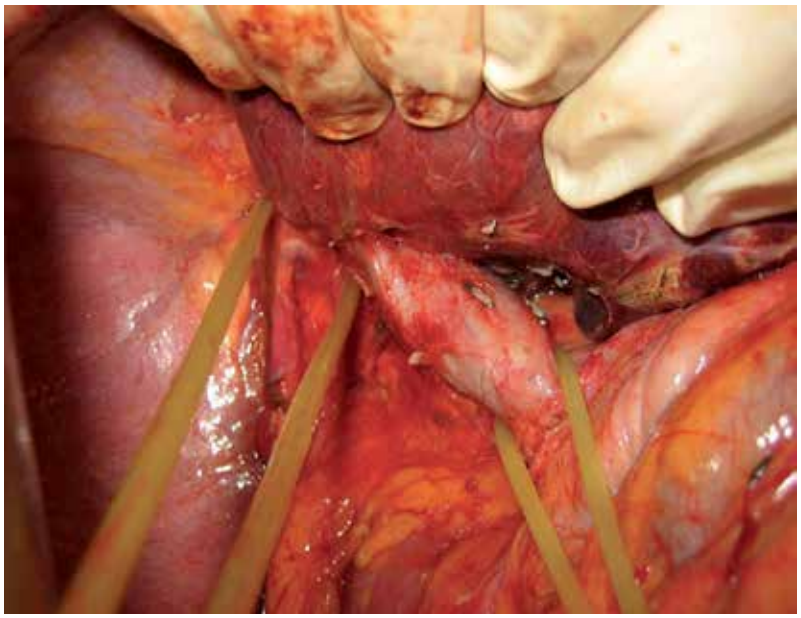

Fig. 5. Preparation for complete vascular exclusion and blood flow interruption through vena cava inferior. Cranial rubber tape passes circularly over the three hepatic veins but the caudal one does over renal veins. Right suprarenal vein is interrupted

5.2 Operative approaches

The following operative approaches can be made use of (Kostov & Kobakov, 2010):

i. extrahepatic approach to the hepatic inflow pedicles for ligation of a portal triad to the Sg 1 and 4, right anterior section, right posterior section, left hemiliver and right hemiliver,
ii. intrahepatic anterior approach to the hepatic inflow pedicles for ligation of a portal triad to an individual Sg (2, 3, 5, 6, 7 and 8),
iii. intrahepatic posterior approach to the hepatic pedicles by using Glissonian sheaths, and
iv. combined extrahepatic and intrahepatic approaches for ligation of a portal triad were used in some bisegmentectomies, right trisectionectomies, and left trisectionectomies.

5.3 Operative volumes

According to the localization and expansion of the pathologic process, one of the following operative volumes should be selected by liver surgeons (Kostov & Kobakov, 2010):

5.3.1 Segmentectomies

Stages of the following monosegmentectomies - segmentectomy 1 (Sg 1), Sg 2, Sg 3, Sg 4, Sg 5, Sg 6, Sg 7, Sg 8, and wedge resection:

Segmentectomy 1 passes through five stages: i) devascularization of *proc. caudatus*, ii) devascularization of Spiegel's lobe, iii) interruption of short retrohepatic veins which enter directly *vena cava inferior*, iv) mobilization of right, middle and left hepatic veins and v) parenchymal transection at the borderline between Sg1 and Sg4. Resection to the right ends at the borderline to Sg7.

The extrahepatic approach requires interruption of the afferent and efferent blood supply to the hepatic part outside the liver which is subject to removal. Among monosegmentectomies, only Sg1 devascularization can be entirely done through such an approach. With isolated segmentectomy 1, the line of parenchymal transsection passing behind the three hepatic veins is of interest (Fig. 6 through Fig. 8).

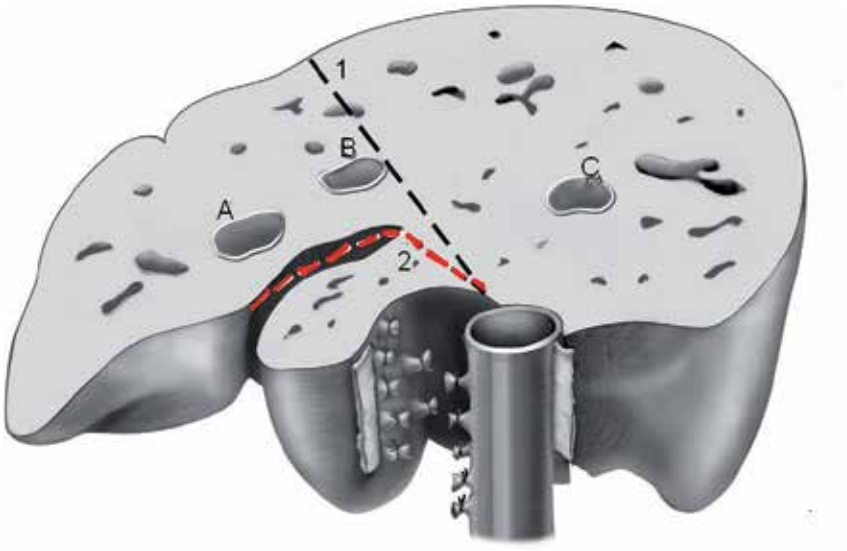

Fig. 6. Line of parenchymal transsection (2) when removing Sg 1. Line of dividing the liver into left and right hemiliver (1); left (A), middle (B), and right veins (Liau et al., 2004)

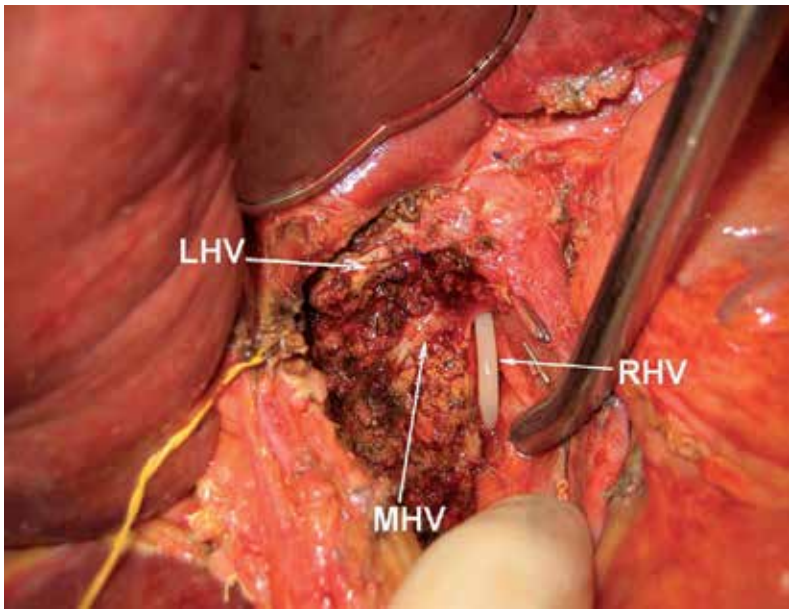

Fig. 7. Removed Sg1 - view from the left. LHV - left hepatic vein; MHV - middle hepatic vein; RHV- right hepatic vein

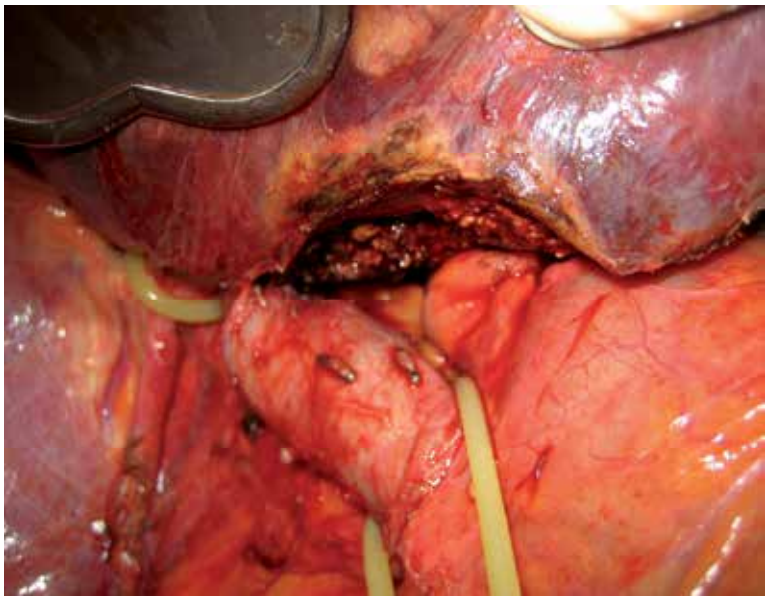

Fig. 8. Removed Sg1 - view from the right.

Both segmentectomy 2 and segmentectomy 3 pass through three stages each: i) definition of borderlines of Sg2 and Sg3 ii) parenchymal transection with intraparenchymal interruption of portal triad vessels for Sg2 and Sg3 (Fig. 9) and iii) interruption of branches of left hepatic vein for Sg2 and Sg3 through anterior intrahepatic access (Fig. 10).

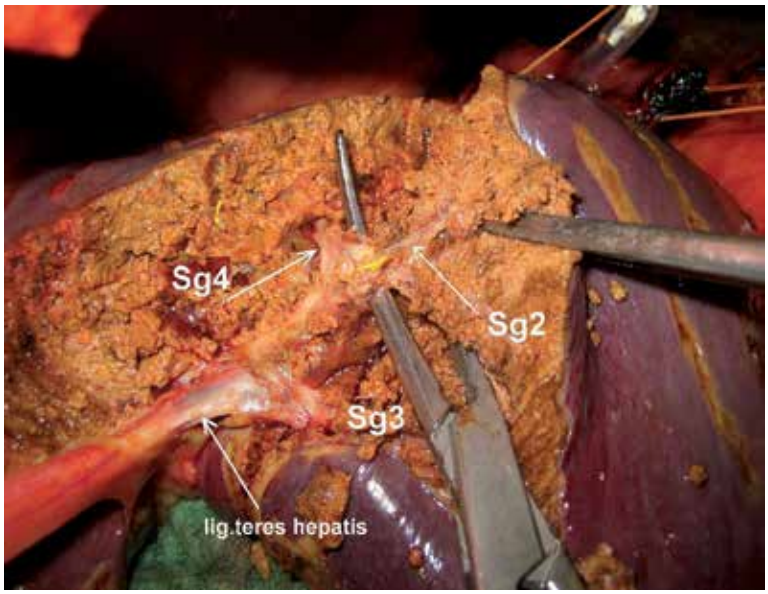

Fig. 9. Sites for interruption of portal triad vessels for Sg2 and Sg3

Segmentectomy 4 passes through five stages: i) extrahepatic interruption of the artery for *lobus quadratus* which, normally, is left hepatic artery branch, ii) ligation of some ascendent

portal veins through extrahepatic access, iii) interruption of descendent portal veins during parenchymal transection along *lig. falciforme hepatis* (Fig. 11), iv) opening and interruption of bile ducts for Sg4 in Rex recessus and v) parenchymal transection along Rex-Cantlie line as middle hepatic vein can be either interrupted, or preserved.

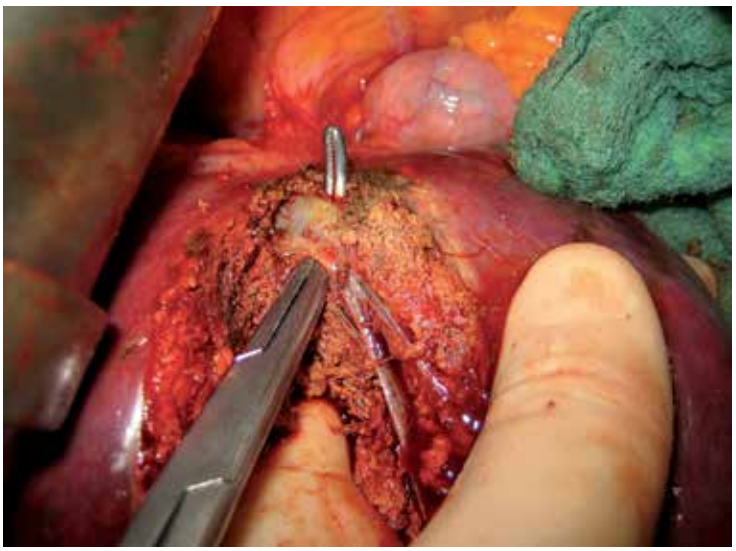

Fig. 10. Anterior intrahepatic access to left hepatic vein

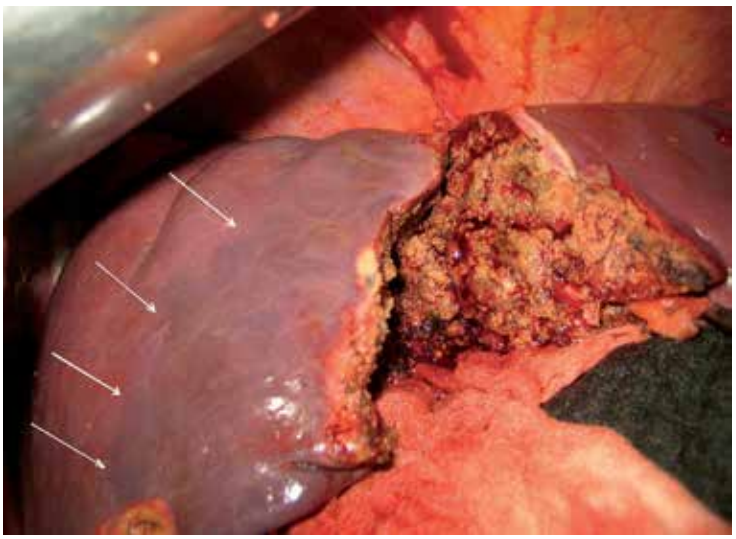

Fig. 11. Ischaemic demarcation of Sg4

Segmentectomy 5 passes through three stages: i) definition of resection borderlines of Sg5. Clamping the vessels for Sg5,8 causes their ischaemic demarcation and visualizes the left and right resection borderlines. The complete Sg5 volume is visualized after injection into segmental portal vein of 5 mL of methylene blue under echographic control, ii) parenchymal

transection along Rex-Cantlie line with intraparenchymal interruption of the vessels for Sg5 and middle hepatic vein preservation. Sg5 devascularization induces ischaemic demarcation of its borderlines and iii) parenchymal transection at the borderline with Sg8 and Sg6. Resection line should pass over Ganz furrow in which the vessels for Sg6 are located.
Segmentectomy 6 passes through three stages: i) definition of resection borderlines of Sg6. Clamping the artery and portal vein for Sg6,7 causes their ischaemic demarcation and visualizes the borderline to Sg5,8. The resection borderlines are visualized after injection into portal vein for Sg6,7 or into segmental portal vein for Sg6 of 5 mL of methylene blue under echographic control (Fig. 12). (ii) parenchymal transsection at the borderline between Sg5 and Sg6 with consecutive interruption of the vein and portal triad for Sg6. Sg6 ischaemia allows visualization of its borderline to Sg7 and iii) parenchymal transection along this bordeline.

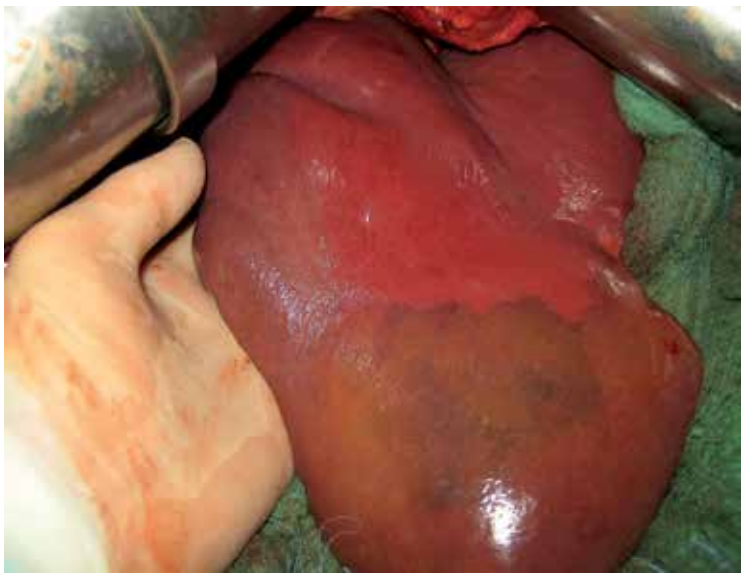

Fig. 12. Sg6 resection borderlines after metthylene-blue injection into segmental PV under echographic control

Segmentectomy 7 passes through three stages: i) definition of resection borderlines of Sg7. Clamping the artery and portal vein for Sg6,7 causes their ischaemic demarcation and visualizes the borderline to Sg5,8. The resection borderlines are visualized after injection into portal vein for Sg6,7 or into segmental portal vein for Sg7 of 5 mL of methylene blue under echographic control. Right hepatic vein mobilization prevents bleeding during resection, (ii) parenchymal transection at the borderline between Sg7 and Sg8 with consecutive interruption of right hepatic vein right branch and portal triad vessels for Sg7. Sg7 ischaemic demarcation visualizes its borderline to Sg6 and iii) parenchymal transection along this borderline.
Segmentectomy 8 can be performed through indirect and direct access.
Indirect access - segmentectomy 8 passes through three stages: i) definition of resection borderlines of Sg8. Clamping the artery and vein for Sg5,8 causes their ischaemic demarcation. Right hepatic vein mobilization prevents bleeding during resection, (ii) parenchymal transection along Rex-Cantlie line with intraparenchymal interruption of the

vessels for Sg5 and middle hepatic vein preservation as portal triad vessels for Sg8 is caudally identified and interrupted. Parenchymal transection continues cranially to the borderline with middle hepatic vein where the vein for Sg8 is interrupted. Sg8 devascularization visualizes its borderlines with Sg7 and Sg5 and iii) parenchymal transection along these bordelines in order to preserve right hepatic vein.
Direct access consists in immediate intervention on Sg8. Parenchymal transection passes through three stages: i) definition of resection borderlines of Sg8 visualized after injection into PV for Sg8 of 5 mL of methylene blue under echographic control (Fig. 13). Clamping the artery and vein for Sg5,8 causes ischaemic demarcation of left and right resection borderlines. Right hepatic vein is clamped, if necessary, ii) parenchymal transection at the borderline between Sg4a and Sg8 as, caudally, the vein draining blood from Sg8 into middle hepatic vein and segmental portal triad vessels are consecutively interrupted. Sg8 demarcation visualizes its borderlines with Sg5 and Sg7 and iii) parenchymal transection along these borderlines and obligatory preservation of right hepatic vein.
Wedge resection consists in removal of some part of a given liver segment only.

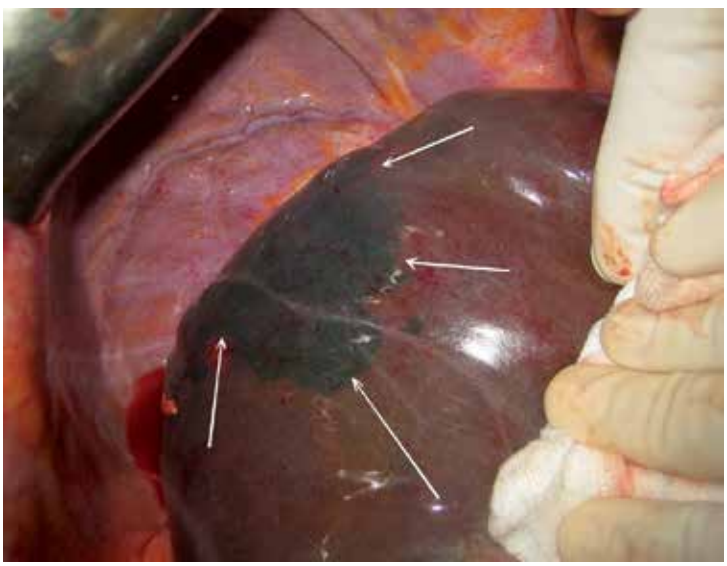

Fig. 13. Borderlines of Sg8 visualized after injection into PV for Sg8 of methylene blue under echographic control

5.3.2 Bisegmentectomies

Stages of the following bisegmentecomies - bisegmentectomy 2,3; 6,7; 5,8; 3,4b; 1,4; 4b,5; 5,6; 7,8, and 4a,8:
Bisegmentectomy 2,3 passes through three stages: i) mobilization of left hemiliver through consecutive interruption of *lig. triangulare sinistrum* and *lig. coronarium hepatis sinistrum*, ii) parenchymal transection along the left edge of *lig. falciforme hepatis* and caudal interruption of portal triad vessels for Sg2,3 and iii) left hepatic vein interruption either through extrahepatic access, or through anterior intrahepatic access at the end of parenchymal transection.
Bisegmentectomy 6,7 passes through four stages: i) mobilization of right hemiliver through interruption of *lig. triangulare dextrum* and *lig. coronarium hepatis dextrum*. Extrahepatic

portion of right hepatic vein is liberated and lifted on a rubber tape, ii) definition of resection borderline after interruption of the artery and portal vein for Sg6,7 or by injection of 5 mL of methylene blue into portal vein for Sg6,7, iii) parenchymal transection at the borderline between Sg6,7 and Sg5,8. Caudally, both the vein for Sg6 draining blood into right hepatic vein anterior branch and portal triad vessels for Sg6,7 under it are interrupted and iv) at the end of parenchymal transection, right hepatic vein posterior branch draining blood from Sg7 is interrupted as its anterior branch is preserved.

Bisegmentectomy 5,8 passes through four stages: i) mobilization of right hemiliver through interruption of lig. triangulare dextrum and lig. coronarium hepatis dextrum. Extrahepatic portion of right hepatic vein is liberated and lifted on a rubber tape, ii) definition of resection borderlines of Sg5,8. Extrahepatic interruption of the artery (Fig. 14) and portal vein (Fig. 15) for Sg5,8 causes their ischaemic demarcation (Fig. 16). Sg 5,8 visualization after injection of 5 mL of methylene blue into portal vein for Sg5,8, iii) parenchymal transection at the borderline to lobus quadratus through consecutive interruption of the vein for Sg5 draining blood into middle hepatic vein, of the portal triad vessels for Sg5,8 and the vein draining blood from Sg8 into middle hepatic vein. Sg5,8 devascularization results in ischaemic demarcation line at the borderline to Sg6,7 and iv) parenchymal transection along this borderline includes interruprion of the vein draining blood from Sg5 into right hepatic vein as both middle and right hepatic veins are obligatorily preserved.

Bisegmentectomy 3,4b passes through three stages: i) definition of resection borderlines by means of intraoperative echography. To the left, parenchymal transection passes along left hepatic vein but to the right it does along Rex-Cantlie line. Both left and middle hepatic veins are preserved, ii) parenchymal transection along *lig. falciforme hepatis* reaching caudally to the vessels for left segments. Only portal triad vessels for Sg3 and bile ducts for Sg4b are interrupted. Sg3 ischaemia causes demarcation of its borderline to Sg2 along which parenchymal transection is performed and iii) parenchymal transection along Rex-Cantlie line at the borderline between Sg4b and Sg5.

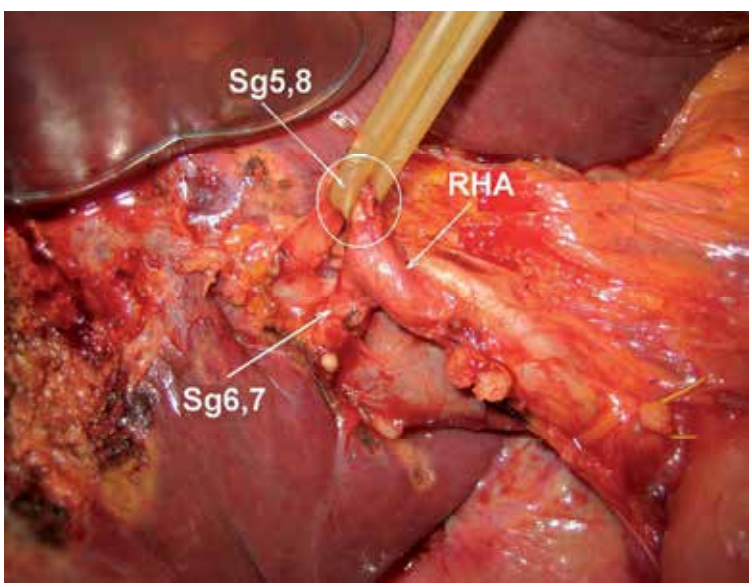

Fig. 14. Mobilization and preparation for interruption of the artery for Sg5,8. RHA - right hepatic artery

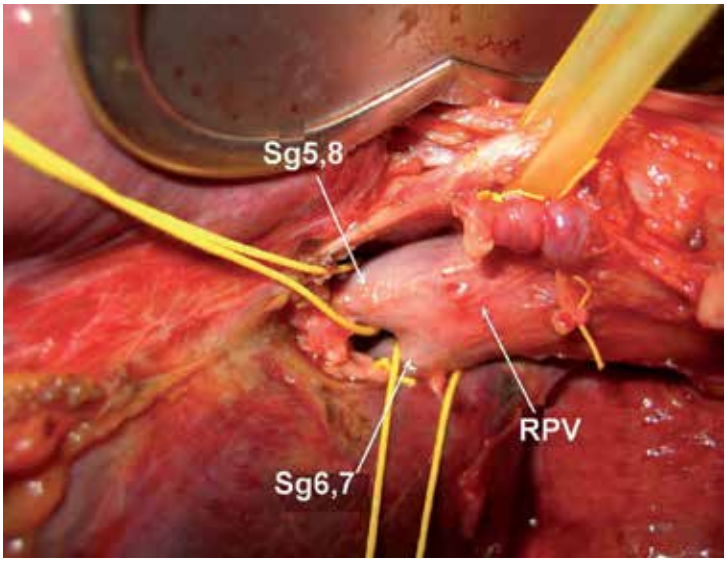

Fig. 15. Mobilization and preparation for interruption of portal vein for Sg5,8. RPV - right portal vein

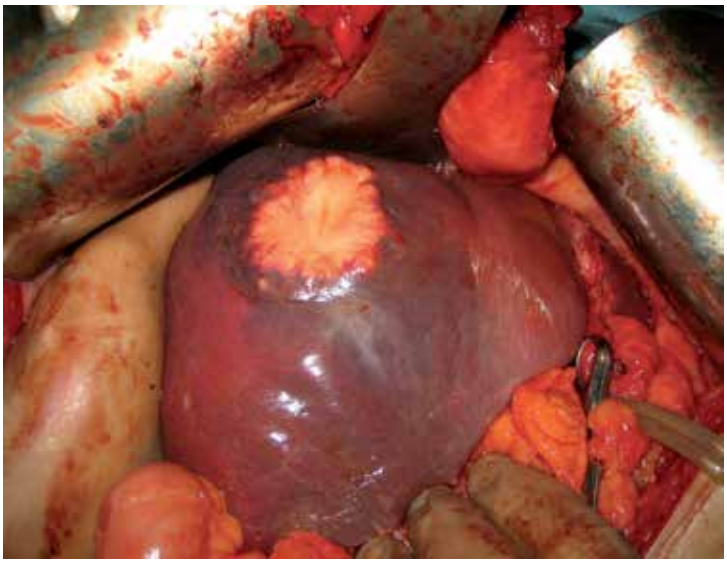

Fig. 16. Ischaemic demarcation of Sg5,8 after their devascularization

Bisegmentectomy 1,4 passes through five stages: i) mobilization of left and right hemiliver and definition of Sg4 resection borderlines. To the left, parenchymal transection passes along *lig. falciforme hepatis* but to the right it follows middle hepatic vein course as defined by means of intraoperative echography, ii) Sg4 devascularization by consecutive interruption of the artery and ascendant portal veins for Sg4 through extrahepatic access, iii) parenchymal transection along *lig. falciforme hepatis* and, caudally, interruption of descendent portal veins for Sg4. After interruption of portal veins for *lobus quadratus*, Rex recessus is reached where the bile duct for Sg4 is identified and interrupted, iv) Sg1

devascularization through extrahepatic access and v) parenchymal transection along Rex-Cantlie line. Cranially, middle hepatic vein is interrupted, if necessary. After bisegmentectomy 1,4, a large parenchymal defect is formed at which bottom the retrohepatic portion of *vena cava inferior* is visible.

Bisegmentectomy 4b,5 passes through four stages: i) definition of resection borderlines. To the left, parenchymal transection passes along *lig. falciforme hepatis* but to the right it does along Rex-Cantlie line. Hilar dissection with division of vessels for right and left hemiliver enables selective clamping the artery and portal vein for Sg2-8, if necessary, ii) parenchymal transection along *lig. falciforme hepatis* as the artery for Sg4 is provisorily clamped as well as ascendant portal veins and bile duct for Sg4b are interrupted, iii) parenchymal transection in a transversal plane at the borderline between Sg4b and Sg5, on the one hand, and between Sg4a and Sg8, on the other hand. Resection line is defined by means of intraoperative echography. Consecutively, distal portion of middle hepatic vein and portal triad vessels for Sg5 are interrupted and iv) Sg5 ischaemia results in demarcation line at the borderline to Sg6 along which parenchymal transection is performed. Caudally, the vein draining blood from Sg5 into right hepatic vein anterior branch is interrupted and portal triad vessels for Sg6-8 are obligatorily preserved.

Bisegmentectomy 5,6 passes through three stages: i) hilar dissection and isolation of the vessels for right hemiliver. Their clamping visualizes the parenchymal transection line between Sg5 and Sg6. Resection borderlines in bisegmentectomy 5,6 are defined by means of intraoperative echography, too, ii) parenchymal transection at the borderline between Sg4 and Sg5 and interruption of the vein draining blood from Sg5 into middle hepatic vein. Caudally, isolation of portal triad vessels for right hemiliver and consecutive interruption of blood supply for Sg5,6. Their ischaemia results in demarcation of their borderline to Sg7,8 and iii) parenchymal transection along this borderline. Caudally, right hepatic vein anterior branch draining blood from Sg5,6 is interrupted and portal triad vessels for Sg7,8 are obligatorily preserved.

Bisegmentectomy 7,8 is possible only in the presence of inferior right hepatic vein draining blood from Sg6. Bisegmentectomy 7,8 passes through four stages: i) mobilization of right hemiliver through interruption of *lig. triangulare dextrum* and *lig. coronarium hepatis dextrum.* Right hemiliver is luxated to the left and the retrohepatic portion of *vena cava inferior* is liberated. Identification of inferior right hepatic vein enables technical performance of bisegmentectomy 7,8, ii) definition of resection borderlines. Hilar clamping the vessels for right hemiliver results in ischaemic demarcation line at the borderline between Sg5,8 and Sg4. Parenchymal transection along this line between Sg8 and Sg4. 'Hanging'-maneuver facilitates hepatic resection. Resection borderline between proximal (Sg7,8) and transversal (Sg5,6) segments is defined by means of intraoperative echography, iii) parenchymal transection along resection borderlines with Sg7,8 devascularization starting at the borderline between Sg6 and Sg7. Initially, right hepatic vein anterior branch is identified and interrupted. Then, in a transversal plane, portal triad vessels for Sg7,8 are reached and interrupted and iv) parenchymal transection in a sagittal plane at the borderline between Sg8 and Sg4. Caudally, the vein draining blood from Sg8 into middle hepatic vein is ligated. Finally, right hepatic vein branch is interrupted.

Bisegmentectomy 4a,8 passes through four stages: i) mobilization of right hemiliver through interruption of *lig. triangulare dextrum* and *lig. coronarium hepatis dextrum.* Right hemiliver is luxated to the left and then right hepatic vein branch is extrahepatically mobilized, ii) definition of resection borderlines. Hilar dissection enables the isolation of the artery and portal vein for Sg5,8. Their clamping visualizes the borderline between Sg8 and Sg6, on the

one hand, and between left and right hemiliver, on the other hand. Transversal resection borderline between Sg8 and Sg5 is established by means of intraoperative echography while the left borderline passes along *lig. falciforme hepatis*, iii) parenchymal transection along Rex-Cantlie line and consecutive interruption of the veins draining blood from Sg5,8 into middle hepatic vein and portal triad vessels for Sg8. Sg8 devascularization results in ischaemic demarcation line at the borderline to Sg5 and iv) parenchymal transection along this line continuing in a sagittal plane between Sg7 and Sg8 and reaching cranially up to the borderline between the right and the middle hepatic vein. Next follows parenchymal transection along *lig. falciforme hepatis* in order to liberate Sg4a ending, cranially, at the borderline between the left and the middle hepatic vein. Finally, both Sg8 and Sg4a are entirely mobilized around middle hepatic vein which is interrupted at its basis.

5.3.3 Multisegmentectomies

i. Stages of the following multisegmentectomies - mesohepatectomy with preservation of Sg1; mesohepatectomy together with Sg1; resection of Sg4b,5,6 and Spiegel's lobe; resection of Sg3,4b,5; resection of Sg3,5-7; resection of parts of Sg3,4b,5,6,8; resection of Sg4b,6,7, and resection of Sg6-8:

Mesohepatectomy (Sg4,5,8) consists in removal of three segments both of which (Sg5 and Sg8) belong anatomically to the right hemiliver while Sg4 belongs to the left hemiliver. This operation is applied in centrally located liver metastases enabling R0 (Fig. 17). Mesohepatectomy with preservation of Sg1 passes through six stages: i) mobilization of left and right hemiliver as double 'hanging'-maneuver lifting on a holder of the right and left hepatic veins facilitates hepatic resection, ii) definition of right resection borderline by consecutive interruption of the artery and portal vein for Sg5,8 (Fig. 18). Parenchymal transection visualizes right resection borderline between Sg5,8 and Sg6,7, iii) interruption of the artery and ascendant portal veins for Sg4 through extrahepatic access. To the left, resection line passes along *lig. falciforme hepatis*. Sg4,5,8 devascularization enables mesohepatectomy at minimal blood loss, iv) parenchymal transection along *lig. falciforme*

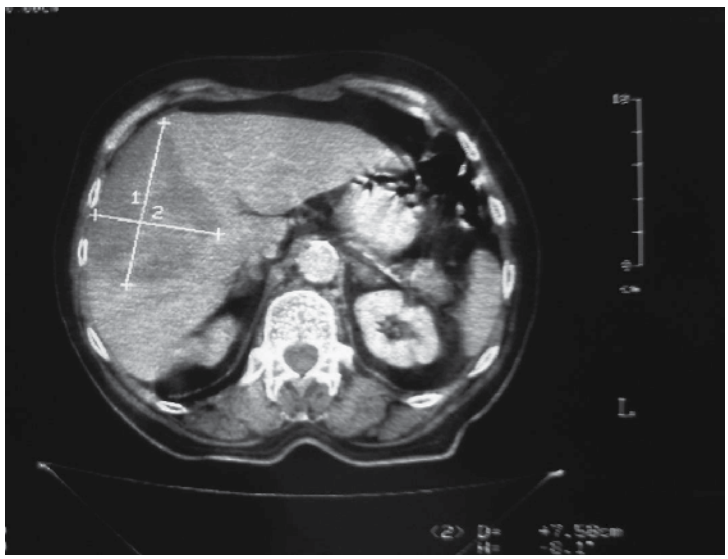

Fig. 17. CT image of liver metastasis in Sg4,5,8

hepatis ending at the borderline between the middle and the left hepatic vein. Consecutive interruption of descendent portal veins, the bile duct for Sg4 and the vein draining blood from *lobus quadratus* in left hepatic vein, v) parenchymal transection at the borderline between Sg5,8 and Sg6,7 and consecutive interruption of the vein draining blood from Sg5 into right hepatic vein anterior branch and portal triad vessels for Sg5,8. To the right, parenchymal transection ends at the borderline between the right and the middle hepatic vein and vi) interruption of middle hepatic vein around which these already liberated Sg5,8 and Sg4 are located (Fig. 19). Residual liver volume after removal of Sg4,5,8 is shown on Fig. 20.

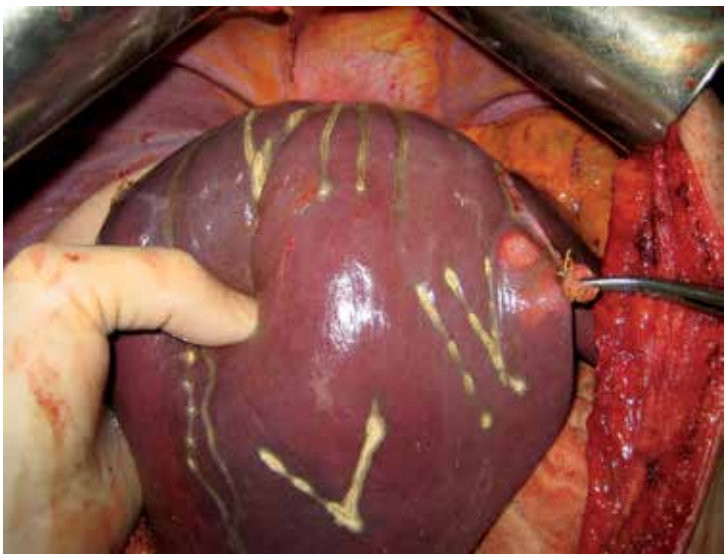

Fig. 18. Liver resection volume in mesohepatectomy (Sg4,5,8)

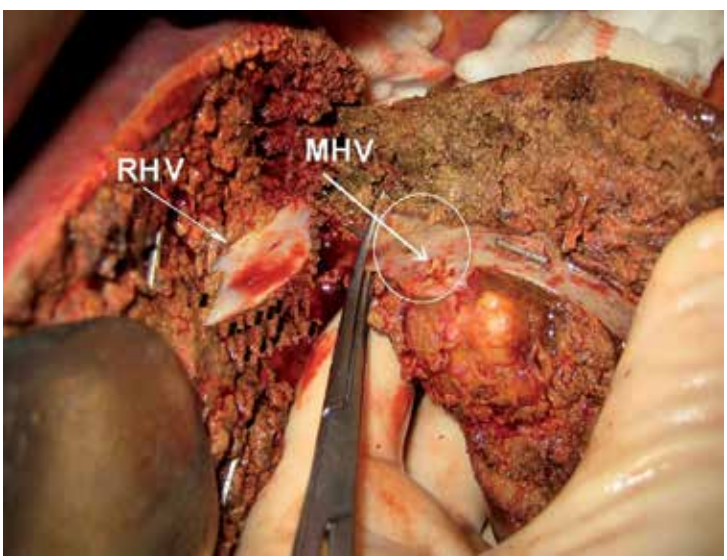

Fig. 19. The site for interruption of the middle hepatic vein is indicated by a circle. MHV - middle hepatic vein; RHV - right hepatic vein

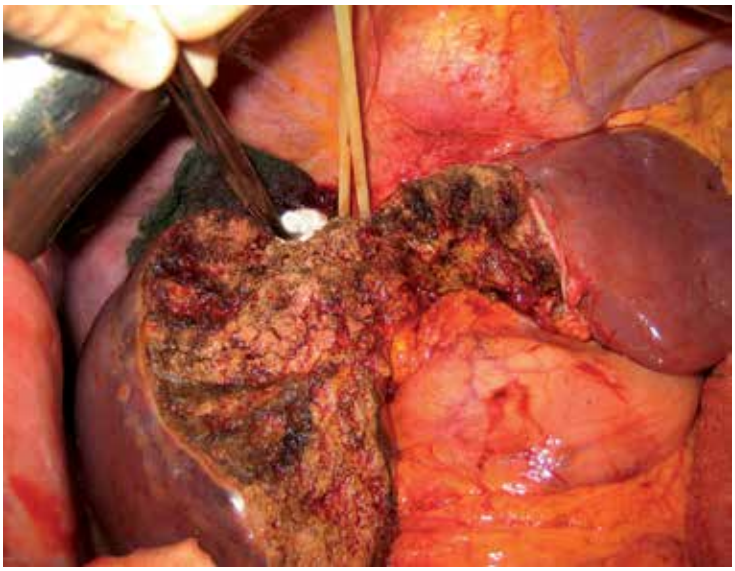

Fig. 20. Residual liver volume after mesohepatectomy (Sg4,5,8)

Mesohepatectomy with segmentectomy 1 is indicated in tumours of central location and passes through seven stages already described in detail in single segmentectomy chapters: i) mobilization of left and right hemiliver by means of double 'hanging'-maneuver, ii) Sg5,8 devascularization through extrahepatic access, iii) Sg4 devascularization through extrahepatic access for the artery and ascendant portal veins, iv) Sg1 devascularization with liberation of the retrohepatic part of *vena cava inferior*, v) parenchymal transection along *lig. falciforme hepatis* ending at the borderline between the middle and the left hepatic vein. Caudally, identification and interruption of descendent portal veins for Sg4 enabling the opening of Rex recessus. Interruption of bile ducts for Sg4 located in Rex recessus, vi) parenchymal transection at the borderline between Sg5,8 and Sg6,7 and vii) middle hepatic vein interruption at the end of parenchymal transection and obligatory intraoperative cholangiography after resection of Sg1,4,5,8 for control of bile drainage from the remnant liver parenchyma.

Resection of Sg4b,5,6 and Spiegel's lobe combines the already described stages in resections of Sg1,4-6. Portal triad vessels for Sg7,8 are obligatorily preserved.

Resection of Sg3,4b,5 combines the already described stages in resections of Sg3,4b,5. Portal triad vessels for Sg6,8 are obligatorily preserved.

Resection of Sg3,5-7 combines the already described stages in resections of Sg3,5-7. Portal triad vessels for Sg8 are obligatorily preserved.

Resection of parts of Sg3,4b,5,6,8 combines the already described stages in resections of Sg3,4b,5,6,8.

Resection of Sg4b,6,7 combines the already described stages in resections of Sg4b,6,7.

Resection of Sg6-8 combines the already described stages in resections of Sg6-8. Portal triad vessels for Sg5 are obligatorily preserved.

ii. Stages of the following hemihepatectomies - left hemihepatectomy (Sg2-4), and right hemihepatectomy (Sg5-8):

Left hemihepatectomy passes through three stages: i) mobilization of left hemiliver through consecutive interruption of *lig. triangulare dextrum* and *lig. coronarium hepatis sinistrum.* Devascularization of left hemiliver with ligation of left hepatic artery and portal vein left branch. If possible, left hepatic duct is liberated without its interruption. Extrahepatically, the trunk of the left and the middle hepatic vein is mobilized enabling the application of 'hanging'-maneuver, ii) parenchymal transection along the ischaemic demarcation line between left and right hemiliver. Resection borderline is defined after injection of 10 mL of methylene blue through portal vein left branch and iii) finally, consecutive interruption of left hepatic duct and left hepatic vein-middle hepatic vein branch. Middle hepatic vein is preserved, if indicated.

Right hemihepatectomy passes through three stages: i) mobilization of right hemiliver through consecutive interruption of *lig. triangulare dextrum* and *lig. coronarium hepatis dextrum*, ii) interruption of the right hepatic and the portal vein right branch. If possible, right hepatic duct is liberated without its interruption. Right hepatic vein mobilization by means of 'hanging'-maneuver facilitates liver resection. Right hemiliver devascularization results in ischaemic demarcation line at the borderline to left hemiliver, and iii) parenchymal transection at the borderline between left and right hemiliver and consecutive ligation of the veins draining blood from Sg5,8 into middle hepatic vein. Finally, interruption of right hepatic duct and right hepatic vein as well as of middle hepatic vein, if indicated.

iii. Stages of the following trisectionectomies - left trisectionectomy (Sg2-5,8), and right trisectionectomy (Sg4-8):

Left trisectionectomy is used in tumours affecting left hemiliver and Sg5,8. No preoperative embolization of portal vein left branch is needed as preserved Sg7 and Sg 6 amount to 30-35% of standard liver volume. Left trisectionectomy passes through five stages: i) mobilization of left hemiliver through consecutive interruption of *lig. triangulare sinistrum* and *lig. coronarium hepatis sinistrum*. 'Hanging'-maneuver application facilitates resection, ii) devascularization of left hemiliver with ligation of left hepatic artery and portal vein left branch, iii) Sg5,8 devascularization through extrahepatic access . Sg5,8 ischaemia causes demarcation line at the borderline to Sg6,7 along which parenchymal transection is performed, iv) consecutive interruption of the vein draining blood from Sg5 into middle hepatic vein and intraparenchymal portal triad vessels for Sg5,8. Parenchymal transection ends at the borderline between the right and the middle hepatic vein. Right hepatic vein is lifted on a rubber holder in order to prevent its injury and v) finally, the trunk of the middle and the left hepatic vein is interrupted. Portal triad vessels for Sg6,7 are obligatorily preserved.

Right trisectionectomy is used in tumours affecting right hemiliver and Sg4. In most cases, preoperative embolization of portal vein right branch is needed in order to achieve hypertrophy of the left hemiliver and, in particular, of Sg2 and Sg3. Right trisectionectomy passes through five stages: i) mobilization of right hemiliver through consecutive interruption of *lig. triangulare dextrum* and *lig. coronarium hepatis dextrum* and short retrohepatic veins; ii) devascularization of right hemiliver with ligation of the right and middle hepatic arteries and the right branch of the portal vein (Fig. 21); iii) devascularization of *lobus quadratus* by means of interruption of the artery and ascendent portal veins for Sg4 through extrahepatic access (Fig. 22).

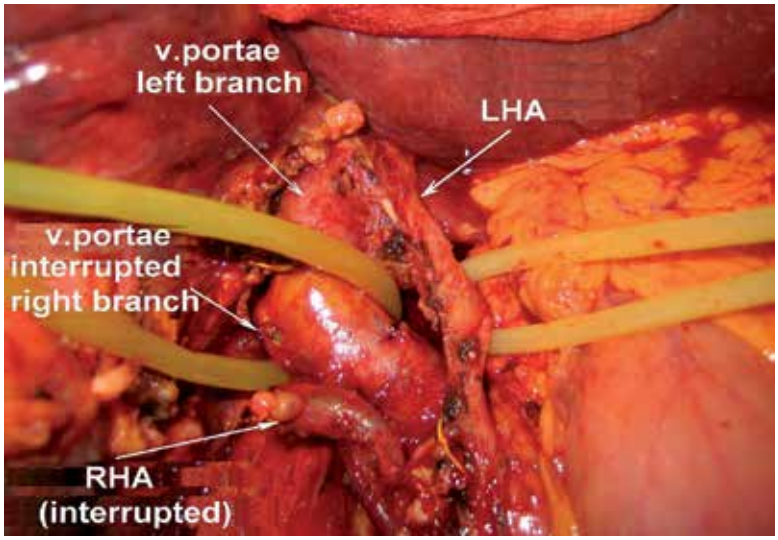

Fig. 21. Right and middle hepatic arteries as well as portal vein right branch are interrupted. LHA – left hepatic artery; RHA - right hepatic artery

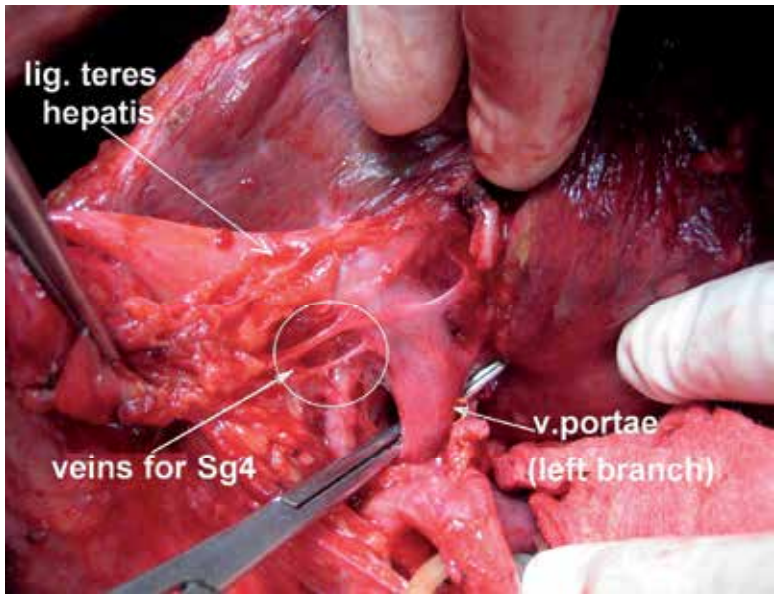

Fig. 22. Sites for interruption of the veins for Sg4 through a combined access.

The interruption of the right hepatic duct is presented on Fig. 23. A preserved left hepatic vein (single 'hanging'-maneuver) is indicated on Fig. 24, iv) parenchymal transection along *lig. falciforme hepatis* and consecutive interruption of descendent portal veins for Sg 4. Entering Rex *recessus* with interruption of the bile ducts for Sg4 (Fig. 25), and v) cranial ligation of the middle and the right hepatic veins (Fig. 26).

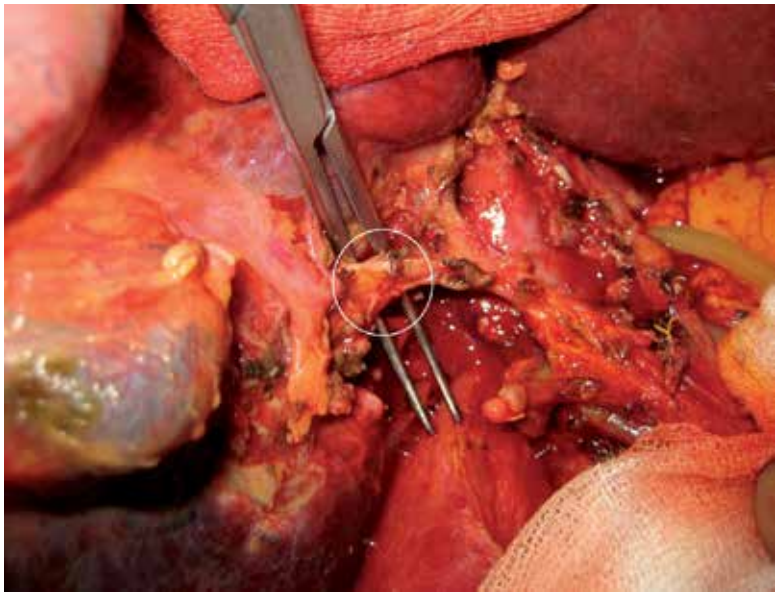

Fig. 23. Interruption of right hepatic duct

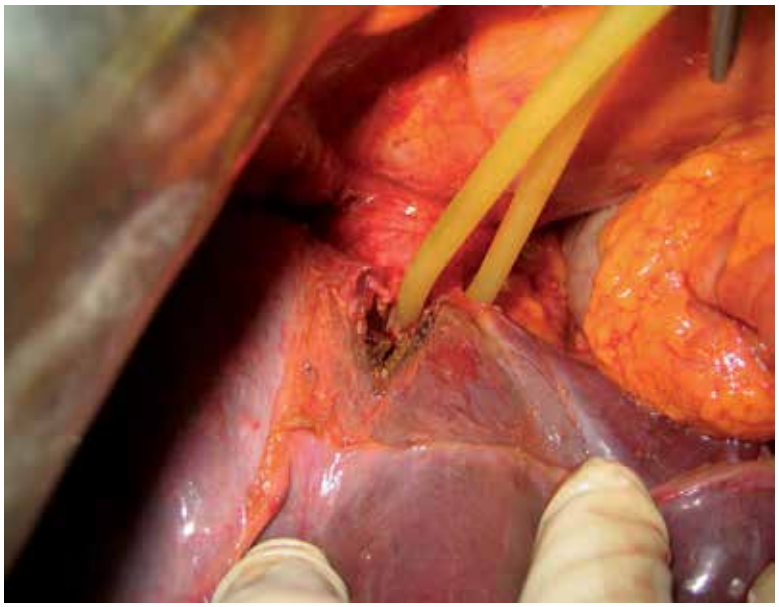

Fig. 24. Preservation of the left hepatic vein through a single 'hanging'-maneuver

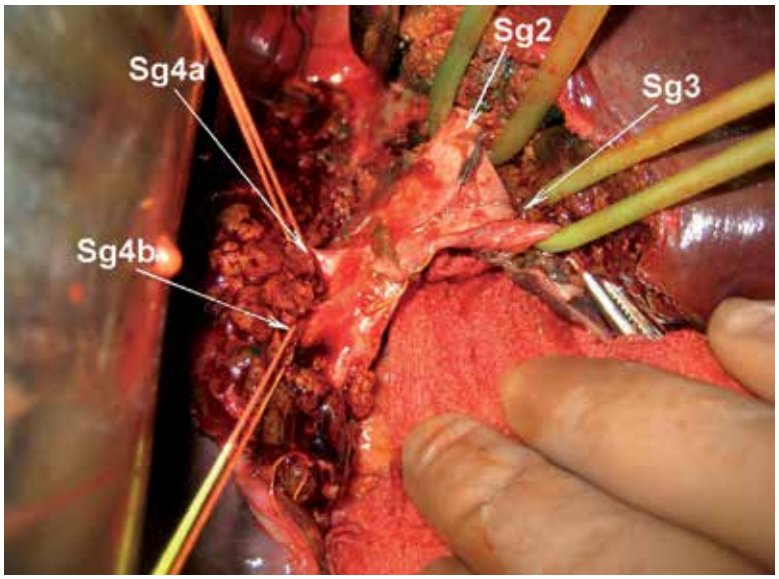

Fig. 25. Entering Rex *recessus* with interruption of the bile ducts for Sg4

In case of damaged blood supply to common hepatic duct, these vessels should be removed with subsequent biliodigestive anastomosis between left hepatic duct and intestinal loop isolated after Roux. Fig. 27 shows residual liver volume following right trisectionectomy.

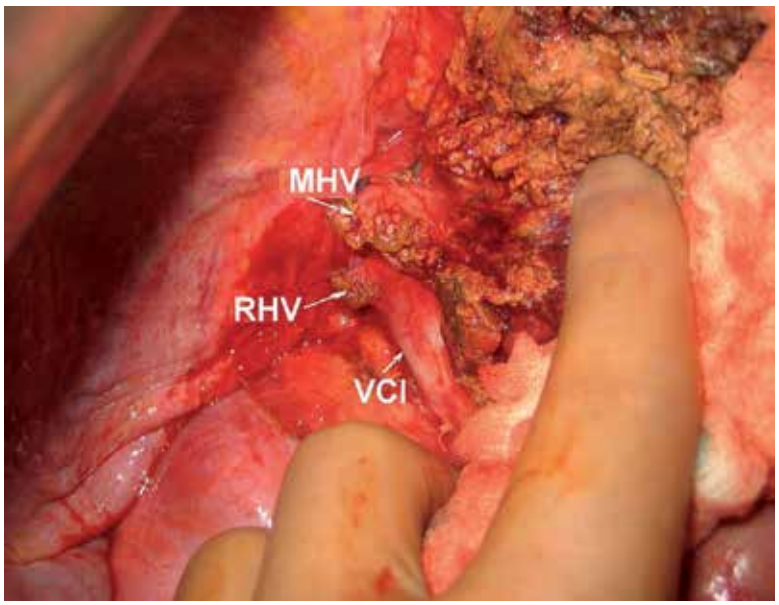

Fig. 26. Interruption of the middle and the right hepatic veins. MHV – middle hepatic vein; RHV – right hepatic vein, VCI - *vena cava inferior*

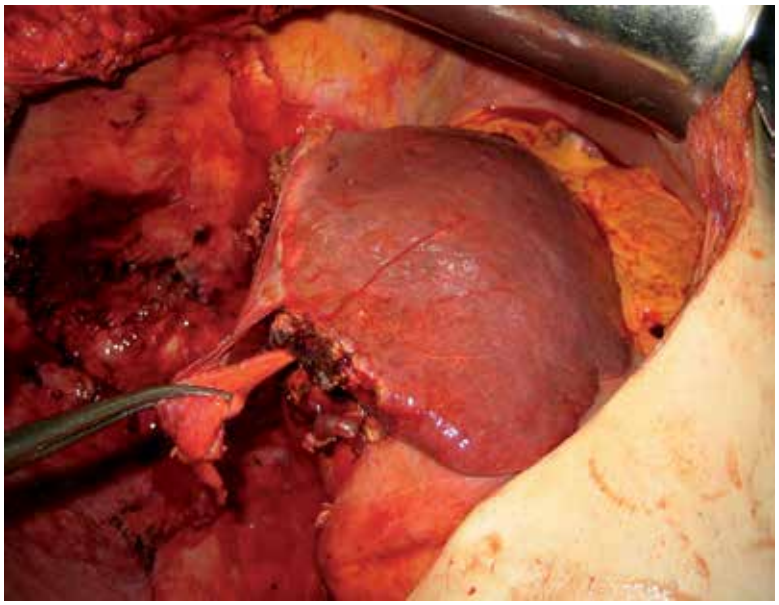

Fig. 27. Residual liver volume after right trisectionectomy

6. Results

The results concerning various clinical and laboratory characteristics of the patients having undergone different types of segmentectomies and major liver resections were comparatively demonstrated. The main attention was paid to the following basic parameters: number, diameter, and localization of colorectal liver metastases; postoperative mortality rate; complications; blood loss and required blood transfusions; operative duration; length of hospital stay; resection margins, one-, two and three-year disease-free and overall survival rates.

Some of them are shown in the present paper.

Postoperative complications following monosegmentectomies and bisegmentectomies, on the one hand, and multisegmentectomies, on the other hand, are comparatively presented on Table 5.

It is evident that, as a whole, liver damage caused by the surgical intervention itself occurs statistically significantly more commonly in the patients who have undergone multisegmentectomies.

Some surgical characteristics of monosegmentectomies and bisegmentectomies, on the one hand, and multisegmentectomies, on the other hand, are comparatively presented on Table 6.

Obviously, several surgical patterns of undoubted medical and socio-economic importance such as total blood loss, necessity of blood transfusions and of application of Pringle-maneuver are statistically significantly more unfavourable in the patients who have undergone multisegmentectomies.

Besides, these patients require statistically significantly more often the performance of repeated surgical interventions on the occasion of colorectal liver metastases than those who have undergone mono- or bisegmentectomies.

Complications	Number of removed segments		p
	one or two	≥ three	
number and percentage of patients with complications			
37 (34%)	11 (10%)	26 (24%)	0.092
surgical liver damage	8 (7.4%)	19 (18%)	0.009
hemorrhage	2 (1.8%)	4 (3.7%)	
liver failure	2 (1.8%)	14 (13%)	
bilirrhagia from an opened bile duct	3 (2.7%)	9 (8.3%)	
extrahepatic biliary tree necrosis	-	1 (1%)	
purulent perihepatic collection	2 (1.8%)	4 (3.7%)	
cholangitis	2 (1.8%)	3 (2.7%)	
mechanical jaundice	-	2 (1.8%)	
peritonitis	-	2 (1.8%)	
ascites	2 (1.8%)	14 (13%)	
respiratory tract damage	6 (5.5%)	11 (10%)	0.034
pneumothorax	2 (1.8%)	2 (1.8%)	
pulmonary thromboembolism	-	1 (1%)	
respiratory failure	2 (1.8%)	9 (8.3%)	
pleural effusion > 200 mL	4 (3.7%)	9 (8.3%)	
other complications	3 (2.7%)	8 (7.4%)	0.060
drug-resistant renal failure	2 (1.8%)	7 (6.5%)	
drug-resistant heart failure	2 (1.8%)	7 (6.5%)	
sepsis	2 (1.8%)	8 (7.4%)	
deep vein thrombosis	1 (1%)	1 (1%)	
complications of general nature	8 (7.4%)	15 (13.8%)	0.014
operative wound suppuration	8 (7.4%)	12 (11%)	
operative wound dehiscence	3 (2.7%)	6 (5.5%)	
postoperative herniation	8 (7.4%)	15 (13.8%)	

Table 5. Complications after liver resections

Parameters	Number of removed segments		p
	one or two	≥ three	
positive resection area	2 (1.8%)	4 (3.7%)	0.299
duration of surgery (min)	224±19	211±21	0.368
total blood loss (mL)	480±52	682±48	< 0.001
necessity of haemotransfusion (patients)	16 (14.8%)	45 (41.6%)	< 0.001
necessity of Pringle-maneuver (patients)	20 (18.5%)	52 (48.1%)	< 0.001
stay in reanimation ward (days)	2±1.2	1.5±0.5	0.459
hospital stay (days)	14.7±1.4	13.5±1.6	0.269
repeated operation	2 (1.8%)	9 (8.3%)	< 0.001

Table 6. Surgical patterns of patients with liver resections

Three-year patients' survival assessed by means of the variables of 22 prognostic criteria is presented on Table 7.

Variables of prognostic criteria	n	%	p
Males	32	47	
females	21	53	0.582
age < 65 years	41	51	
age ≥ 65 years	12	43	0.743
T2-T3 category	32	52	0.944
T4 category	21	46	0.934
G1-G2 tumour differentiation	35	50	
G3 tumour differentiation	18	47	0.677
negative lymph nodes during colorectal cancer surgery	33	53	
positive lymph nodes during colorectal cancer surgery	20	43	0.877
colonic primary tumour	38	51	
rectal primary tumour	15	45	0.983
CEA ≤ 200 ng/mL	42	71	
CEA > 200 ng/mL	11	26	< 0.001
synchronous metastases	13	59	
metachronous metastases	40	50	0.834
after < 12 months	35	51	
after ≥ 12 months	18	46	0.221
diameter < 50 mm	42	49	
diameter ≥ 50 mm	11	50	0.712
≤ 3 metastases	48	55	
> 3 metastases	5	30	< 0.001
unilobar metastases	41	48	
bilobar metastases	12	52	0.069
positive resection areas	-	-	
negative resection areas	53	52	< 0.001
positive lymph nodes in *lig. hepatogastroduodenale*	1	6	
negative lymph nodes in *lig. hepatogastroduodenale*	52	60	< 0.001
resection distance ≥ 10 mm	42	52	0.700
resection distance of 5-10 mm	5	56	0.790
resection distance ≤ 5 mm	6	50	
monosegmentectomy and bisegmentectomy	29	66	
multisegmentectomy	24	41	< 0.001
blood loss > 500 mL	29	48	
blood loss ≤ 500 mL	24	51	0.644
application of Pringle-maneuver	34	47	
no application of Pringle-maneuver	19	52	0.736
postoperative complications	29	46	
no postoperative complications	24	53	0.743
0-2 factors of MSKCC-CRS	41	64	
3-5 factors of MSKCC-CRS	12	27	< 0.001
extrahepatic dissemination	2	25	
no extrahepatic dissemination	51	54	< 0.001
neoadjuvant chemotherapy	13	52	
no neoadjuvant chemotherapy	40	48	0.628

Table 7. Prognostic criteria for three-year survival

We identify a small number of prognostic criteria which could be considered statistically significant in the patients with colorectal liver metastases. Here belong the increased levels of CEA, the higher number of colorectal liver metastases (more than three), the negative resection areas, the presence of negative lymph nodes in *lig. hepatogastroduodenale,* the implementation of multisegmentectomy as a less sparing surgical intervention, the presence of at least 3 factors of MSKCC-CRS and the absence of extrahepatic dissemination of the pathological process.
Thus our investigations should be enlarged in future in order to more comprehensively explain the dynamic interactions between the single risk factors for the relatively poor prognosis of this contingent of patients.

7. Discussion

Our own results demonstrate the substantial advantages of segmental resection for colorectal liver metastases over major liver resection (Kobakov & Kostov, 2006; Kostov & Kobakov, 2006b; Kostov & Kobakov, 2009). They are the following: conservation of a sufficient liver volume, achievement of lower perioperative morbidity and mortality rates as well as warranting the similar disease-free and overall survival rates. Liver conservation is essential in normal and damaged liver. It reduces the risk of postoperative liver insufficiency from a small liver remnant and in the patients at advanced age or with cirrhosis.
The following prognostic factors exert a statistically significant effect on short- and long-term survival rates after liver resections for colorectal liver metastases: CEA level, presence of metastatic nodes along *lig. hepatoduodenale*, number of metastases, extension of liver resection, resection volume, number of prognostic factors according to MSKCC, and extrahepatic dissemination of primary colorectal cancer.
The following therapeutic strategy should be recommended: i) liver resection for resectable colorectal metastases (at stages IVA and IVB); ii) neoadjuvant chemotherapy for primarily non-resectable colorectal metastases (at stage IVC) when downstaging is feasible to allow radical surgery, and iii) only chemotherapy for colorectal metastases in stage IVD patients.
Recent literature data convincingly indicate the uninterrupted progress in the interdisciplinary field of oncologic liver surgery. Along with original investigations, a lot of review papers, meta-analyses, multicentre reports and randomized controlled trials are currently published by authors from all over the world.
In this respect, multimodal therapy deserves a special attention. It increases the number of resections and improves long-term survival rate (currently more than 40% at 5 years) (Neumann et al., 2010). Advances in staging, surgical technique, perioperative care and systemic chemotherapy contribute to improvement in oncologic outcomes of stage IV colorectal cancer patients (Abdalla, 2011). The limits of resection expand to include cases with more, larger and bilateral colorectal liver metastases as 5-year overall survival exceeds 50% following resection. Tailored, patient-centered treatment includes a variety of liver resections, liver volumetry, and portal vein embolization for preoperative enhancement of the volume and function of the planned future remnant liver (Abdalla, 2011).
Multimodality approach of laparoscopic liver resection is feasible and safe in selected patients. It is associated with a low complications rate (Isoniemi et al., 2011, Lai et al., 2011). Intraoperative ablation extends the limits of hepatectomy in the patients not amenable to complete resection (Brown et al., 2011; Govindarajan et al., 2011; Hammill et al., 2011;

Hompes et al., 2011). Portal vein embolization, radiofrequency ablation, two-stage hepatectomy, conversion therapy and reverse treatment strategy along with hepatectomy are used in the presence of extrahepatic disease (Coimbra et al. 2011; Narita et al., 2011, Tsim et al., 2011). Resection of advanced colorectal liver metastases after a second-line chemotherapy regimen is safe and promising in certain cases. The addition of neoadjuvant chemotherapy should, however, be cost-effective.

Positron emission tomography/computed tomography have a higher accuracy for detection of extra-hepatic and colorectal liver metastases than computed tomography alone (Patel et al., 2011). In patients treated with neoadjuvant chemotherapy, magnetic resonance imaging measurements of steatosis show the highest correlation coefficient and the best diagnostic accuracy, as compared to computed tomography ones (Marsman et al., 2011). Intraoperative ultrasound and preoperative imaging significantly increase the diagnostic accuracy of patients undergoing liver resection for colorectal liver metastases (Lordan et al., 2011).

Metachronous resections have a better outcome than synchronous. Iterative resection is very encouraging and justifies an aggressive surgical approach (Tonelli et al., 2010). Simultaneous resection is safe and efficient in the treatment of patients with synchronous colorectal liver metastases while avoiding a second major operation (Chen et al., 2011). In patients with bilobar synchronous colorectal liver metastases who are candidates for two-stage hepatectomy, combined resection of the primary tumour and first-stage he patectomy reduces the number of procedures, optimizes chemotherapy administration and may improve outcome (Karoui et al., 2010). The two-stage strategy for colorectal liver metastases can be performed with acceptable morbidity and mortality. The second stage is not feasible in 20-25% of patients. Patients completing the two-stage approach may have long-term survival comparable to those treated with a planned single-stage hepatectomy (Tsai et al., 2010). Concomitant extrahepatic disease in a patient with colorectal liver metastases should not be a contraindication to their resection.

As there is no significant difference in morbidity, mortality, recurrence rate, or survival in anatomical and nonanatomical liver resections, the latter can be used as a save procedure to preserve liver parenchyma (Lalmahomed et al., 2011). The Pringle maneuver does not seem to affect the survival of patients with liver metastases (Ferrero et al., 2010). Ultrasound-guided finger compression of sectional portal pedicle feeding the right posterior section is a feasible, safe, and effective method for performing anatomical right posterior sectionectomy (Torzilli et al., 2011).

Prognostic factors and score systems occupy an important place in oncologic liver surgery (de Haas et al., 2011; Peng et al., 2011; Pulitanò et al., 2011). Although twelve prognostic scoring systems have been identified from 1996 to 2009, there is no 'ideal' system for the clinical management of patients with colorectal liver metastases (Gomez et al., 2010). A predicted positive surgical margin (R1 resection) is not any absolute contraindication to surgery for aggressive or advanced colorectal liver metastases (Tanaka et al., 2011). Liver resection has superior long-term survival which is, however, significantly reduced by the occurrence of post-surgical complications (Schepers et al., 2010). Superior overall health-related quality of life merits an aggressive surgical approach and intensive follow-up to detect recurrence early (Wiering et al., 2011).

8. Conclusion

Based on our own results and reliable scientific evidence available worldwide up-to-date, it can be concluded that the patient presenting with colorectal liver metastases deserves a

timely and individualized diagnostic and complex therapeutic approach by an interdisciplinary physician's team. Medical staff's behaviour should be maximally sparing, when possible.

New advances in image diagnostic modalities such as positron emission tomography/computer-aided tomography, steadily improved surgical and microsurgical techniques such as laparoscopic resections along with emerging opportunities for cost-effective chemotherapy and multimodal management promise better perspectives in this field of permanently rising social significance.

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Experimental Colorectal Cancer Liver Metastasis

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1. Introduction

With estimated 1 080 000 diagnosed cases each year, which account for 1.1% of all deaths, colorectal carcinoma (CRC) ranks fourth in cancer-related deaths in both sexes worldwide (WHOSIS, 2008). In Europe, CRC is the third most lethal malignancy after lung and stomach cancers in men, and it ranks second after breast cancer in women (WHOSIS, 2008).

CRC progression is characterized by increased growth of the primary carcinoma as well as lymphatic and haematogenic spread. The liver is often the first vascular bed in which disseminating colorectal cancer cells are trapped and therefore is affected in up to 10-20% of CRC patients at the time of presentation (Berney, et al., 1998). Another 40-50% of patients will eventually develop liver metastasis during the course of their illness, which is commonly the cause of death (Bentrem, et al., 2005, Stangl, et al., 1994, Sugarbaker, 1990). At present, liver resection is considered the treatment of choice for suited patients with colorectal liver metastases, offering a five-year survival rate of 25-44% (Choti, et al., 2002, Garden, et al., 2006, Zacharias, et al., 2004) to those 20-25% of patients with isolated liver metastasis (Adson, et al., 1984, Bismuth, et al., 1996, Fong, et al., 1999). Unfortunately, this procedure is feasible only in patients with no signs of irresectable extra-hepatic disease, whereas the median survival is only 9–19 months for patients with unresectable disease who receive systemic chemotherapy (de Gramont, et al., 2000, Giacchetti, et al., 2000, Meyerhardt and Mayer, 2005, Saltz, et al., 2000).

However, the fact that CRC malignancy develops over a long period and can only be efficiently controlled if detected early provokes many efforts to better understand the neoplastic progression of this cancer. It is well known, that there is a continuous shedding of tumor cells from a primary CRC (Chambers, et al., 2002), but not all disseminated CRC cells develop into macrometastases. It was hypothesized that sub-populations of malignant cells evolve a genetic advantage to become "highly metastatic". These clones are skilled to dissociate from the primary cancer, to intravasate into nearby blood and lymphatic vessels, to travel through the lymphatic and hematogenous systems, to survive the immune surveillance, to extravasate into distant tissues forming micrometastases, and to eventually colonize the target organ.

In this cascade, the epithelial-mesenchymal transition (EMT), characterized by the loss of cell-to-cell adhesion and cell polarity (Thiery, 2003), plays a crucial role in different stages;

namely the dissemination of tumor cells as well as their intra- and extra-vasation (Gupta and Massague, 2006). Several known transcription factors, such as Snail, Slug, and Twist, were found to induce EMT on one hand and were implicated with tumor progression and metastasis on the other hand. In line with this, some downstream genes of these regulatory factors are responsible for cell-to-cell adhesion, specially E-cadherin and claudins. For example, up-regulation of Snail induces EMT and down-regulates the transcription of different tight junctions proteins (TJs), such as claudins and occludin (Findley and Koval, 2009). Claudins (CLDNs) form the structural backbone of TJs, and comprise at least 27 members of integral transmembrane proteins (Mineta, et al., 2011) ranging in size between 20-27 kDa (Tsukita, et al., 2001). Recently, the altered expression of various claudins has been implicated in the progression of several human cancers (Cheung, et al., 2005, Hough, et al., 2000, Johnson, et al., 2005, Kominsky, et al., 2003, Long, et al., 2001, Michl, et al., 2001, Morin, 2005, Sanada, et al., 2006, Swisshelm, et al., 2005). In contrast to the published notion that claudin expression would decrease from tumorigenesis as tight junctions are lost during cellular transformation, the claudin status seems to change in a tissue-specific manner. For example, over-expression of *Cldn2* has been correlated to colorectal cancer (Kinugasa, et al., 2007), whereas decreased *Cldn7* expression has been reported in head and neck cancer (Usami, et al., 2006), invasive ductal breast carcinoma (Kominsky, et al., 2003), and metastatic breast cancer (Sauer, et al., 2005). In addition, *Cldn3* and *Cldn4* have been found repeatedly elevated in a variety of cancers including pancreatic ductal adenocarcinoma (Michl, et al., 2003) as well as ovarian, uterine, prostate, and breast cancers (Rangel, et al., 2003). In partial contrast, reduced expression of *Cldn4* and *Cldn5* was detected in hepatocellular and renal carcinomas (Soini, 2005). In CRC, both, up- and down-regulation of claudin4 expression have been described (de Oliveira, et al., 2005, Ueda, et al., 2007), as well as aberrant expression of *Cldn1.*

Another type of cell connection has been named cell-to-extracellular matrix (ECM) contacts. On their disruption, they are presumably also implicated in tumor initiation. It is well known from each stage of malignant progression that tumor cells communicate with their microenvironment and thereby elicit responses from it. This microenvironment is mainly composed of tumor cells, extracellular matrix (ECM), stromal cells, immune cells and micro-vessels (Farrow, et al., 2008, Jung, et al., 2002). The ECM is a scaffold of extracellular proteins that maintain tissue shape and provide the cellular compartment with structural support (Bosman and Stamenkovic, 2003). However, by influencing cell adhesion, migration, differentiation, proliferation and survival, the ECM is a remodeling network that contributes substantially to tumor progression and metastasis (Engbring and Kleinman, 2003, Ioachim, et al., 2002). Remodeling and deposition of the ECM is mostly regulated by a functional family of extracellular proteins known as matricellular proteins, which contribute to the structural integrity and composition of the ECM (Bornstein and Sage, 2002). One of the most important characteristics of matricellular proteins is their ability to manipulate the integration and turn-over of ECM (Bornstein and Sage, 2002, Kyriakides, et al., 2001). Furthermore, by playing a linker role between the ECM and the cell surface, matricellular proteins can also direct cell fate, survival, adhesion and motility (Bornstein and Sage, 2002, Brekken and Sage, 2000, Kyriakides, et al., 2001).

Osteopontin (OPN) is an acidic extracellular matrix phosphoprotein of ~298-amino acids secreted by a wide variety of cancers, which functionally favours tumor progression (Gao, et al., 2003, Weber, 2001). The secreted phosphoprotein binds to the integrins (e.g. ITG-αvβ3 or ITG-αvβ5) and CD44 families of receptors to propagate cellular signals (Agrawal, et al., 2002,

Yeatman and Chambers, 2003). In colorectal cancer, gene profiling studies have identified a positive correlation between advanced or metastatic colon tumors and abundant OPN expression (Wai and Kuo, 2004). Increased OPN expression is associated with tumor invasion or metastasis in cancers of the breast (Tuck, et al., 1999, Tuck, et al., 1998, Tuck, et al., 1997), stomach (Ue, et al., 1998), lung (Chambers, et al., 1996, Shijubo, et al., 1999), prostate (Thalmann, et al., 1999), liver (Gotoh, et al., 2002, Pan, et al., 2003), and colon (Agrawal, et al., 2002, Yeatman and Chambers, 2003). Analysis of the OPN promoter has uncovered multiple consensus binding sites for known transcription factors (Hijiya, et al., 1994).

The main aim of our experimental series was first to generate a model suited to be used for investigating the efficacy of new drugs (Eyol, et al., 2008, Seelig, et al., 2004, Wittmer, et al., 1999). One of the few well-characterized animal models for hepatic colorectal cancer makes use of the rat CC531 cell line. Following topical injection of CC531 cells, liver metastases develop and their growth has been frequently used for studying effects of various anti-cancer treatments (Marinelli, et al., 1991, Oldenburg, et al., 1994, Veenhuizen, et al., 1996).

A second aim was to identify metastasis-related changes in gene expression in tumor cells, which differ from those in the primary tumor and probably play a crucial role in metastasis formation. Therefore, temporal changes in gene expression of CRC cells homing to the liver have been investigated using the above *in vivo* model, which is characterized by a defined onset of metastatic proliferation in rat liver following intraportal inoculation of CC531 tumor cells. This, in turn, permits a close following of the time-dependent modulation of gene expression, as the tumor cells home into the liver and then grow to a lethal size. The technique of re-isolating these tumor cells from rat liver permitted to monitor for the first time the expression of several candidate genes in a time-dependent manner (Georges, et al., 2011).

2. Description of the CC531 rat model

2.1 Generation of the model

Initially, the CC531 cell line was induced by treatment of WAG rats with 1,2 dimethylhydrazine (DMH). Forty weeks after 6 weekly injections of 30 mg/kg DMH, a carcinoma originated in the ascending colon of injected WAG rats. After serial implantation to male rats, the resulting transplantable tumor was described to be moderately differentiated on histological examination (Marquet, et al., 1984).

The majority of animal liver metastasis models available at that time was based on the subcutaneous or intraperitoneal injection of tumor cells (Venditti, et al., 1984). To imitate as closely as possible the physiological metastatic spread of colon cancer, different orthotopic models were developed. These included intraportal (Griffini, et al., 1997, Griffini, et al., 1996, Thomas, et al., 1993) and spleen injections (Fukumura, et al., 1997) resulting in a diffuse outgrowth of tumor cells in the liver, as well as implantation of tumor tissue fragments or cells under the Glisson's liver capsule giving a local, limited, and nodal growth pattern (Aguiar, et al., 1987, Bartkowski, et al., 1986, Kamphorst, et al., 1999). Quantification of tumor growth in the latter models is often done by measuring tumor diameters (Aguiar, et al., 1987, Bartkowski, et al., 1986, Kamphorst, et al., 1999), whereas the diffuse models couldn't be quantified easily. To encounter this obstacle, many efforts have been made, including the 3-D reconstruction of metastases by consecutive serial sections (Griffini, et al., 1997), counting tumor nodules in the liver (Thomas, et al., 1993), or the use of tumor specific

antibodies for immunohistological examination (Thomas, et al., 1993). These assays are protracted and allow only a gross grading of tumor mass or cytostatic-induced loss of tumor load in the liver. The use of reporter genes such as green fluorescent protein (GFP), luciferase, or β-galactosidase (GLB1) for tracing tumor cells has greatly facilitated both, quantification and localization at the single cell level (Chishima, et al., 1997, Dooley, et al., 1993, Zhang, et al., 1994). In view of that, we aimed in our study to develop an orthotopic, diffusely growing liver metastasis model that can be used for diagnostic and therapeutic studies. For this purpose the CC531 rat colorectal cancer cell line with its natural homing into the liver was transfected by the *Glb1* gene.

The stable transfection of these cells with the *Glb1* gene (Fig. 1) allowed quantitation of the tumor cell load at any time after implantation and hence a quick evaluation of the efficacy of therapy that can be used for diagnostic and therapeutic studies (Wittmer, et al., 1999).

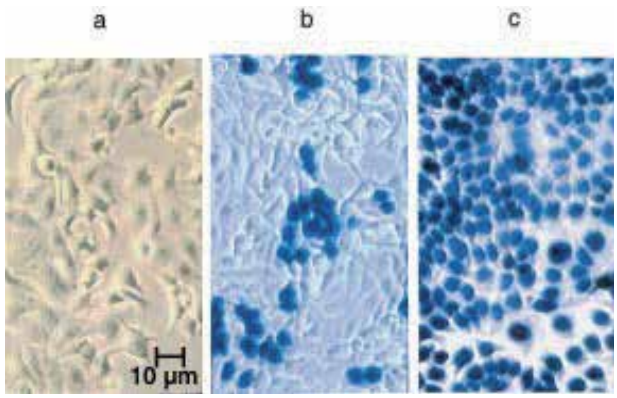

Fig. 1. CC531 cells growing in RPMI-1640 medium were stained by the activity of GLB1 converting X-gal, a chromogenic substrate for GLB1. Parental cells **(a)** are compared with transfected **(b)** and subcloned **(c)** CC531 cells. The magnification is identical for all three photographs (see scale bar)

2.2 Chemosensitivity of the model

2.2.1 Chemoembolization of rat liver metastasis with microspheres and gemcitabine or irinotecan followed by evaluation of tumor cell load by chemiluminescence

These experiments (Seelig, et al., 2004) were performed to determine the potential of hepatic artery chemoembolization (HACE) for reducing the tumor cell load. Seven days after the intraportal injection of CC531-lac-Z cells to male WAG/Rij rats, tumor positive animals were treated by intra-hepatic artery injection with solvent (n=17), degradable starch microspheres (DSM, 30 mg/kg; n=16), DSM plus 5-fluorouracil (5-FU; 90, 60, and 40 mg/kg; n_{total}=43) or DSM plus gemcitabine (Gem; 100, 80, 50, and 10 mg/kg; n_{total}=46). After 3 more weeks the experiment was terminated, the livers were weighed and the number of CC531-lac-Z cells per liver was determined. Injection of DSM reduced the tumor cell load by 21% (T/C%=79), whereas the combination with 5-FU reduced tumor cell number more intensively at 60 mg/kg (T/C%=86), and 90 mg/kg (T/C%=19). None of these effects was significantly different from controls. The combination of DSM plus Gem was well tolerated and significantly ($p<0.05$) effective at 80, 50 and 10 mg/kg (T/C%= 16, 9 and 26, respectively; Fig. 2).

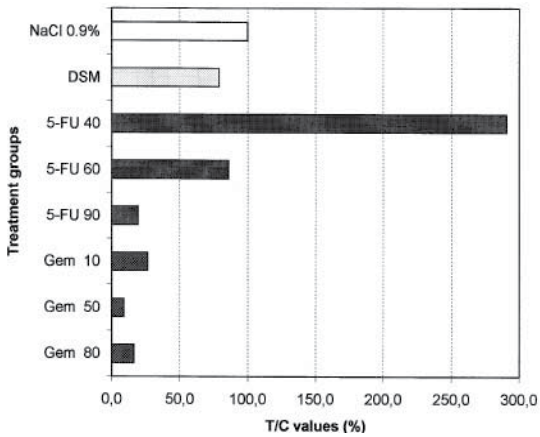

Fig. 2. Comparison of therapeutic efficacy: The mean tumor cell number of treated groups is given in percent of the respective control group (T/C* 100)

Thus, the comparison of HACE with 5-FU or Gem showed that the efficacy of Gem in reducing the hepatic tumor cell load was significantly higher and its therapeutic ratio was greater than that of 5-FU.

In a subsequent experiment, the effect of HACE with irinotecan was compared vs. 5-FU as a standard agent in rat liver metastasis (Saenger, et al., 2004). Briefly, 4×10^6 CC531-lac-Z cells were intraportally injected into male Wag/Rij rats. Irinotecan (10, 30 and 60 mg/kg) and 5-FU (40, 60 and 90 mg/kg) were administered concomitantly with DSM (30 mg/kg) for temporary embolization. The tumor cell load was determined quantitatively using the chemoluminescence assay mentioned above.

HACE with irinotecan induced a complete remission in 44% of the animals and the highest dose reduced the mean tumor cell load by 66% ($P<0.001$). In contrast, the highest dose of 5-FU caused a reduction of only 18% ($P = 0.026$) and altogether 23% complete remissions were observed in response to 5-FU (Table 1 and Fig. 3). Collectively, HACE with irinotecan had a greater effect than that of HACE with 5-FU, setting the basis for further investigation in clinical trials.

Group no.	Treatment	Mean tumor cell number per liver[a] ± SE[b]	O/E ratio[c]	Mean liver weight (g)[d] ± SE[b]	Increase in tumor cell number[e]	$aT_{1/2}$[f]	TCD[g]
1A	Untreated	4.1E+09±4.6E+08	–	34.2±2.7	1025	50.4	10.0
1B	Untreated	2.1E+09±3.3E+08	–	28.4±2.7	525	55.8	9.0
2	GEM 50 HACE	5.5E+08±2.3E+08	–	11.1±1.0	136	71.1	7.1
3	GEM 50 IV	1.2E+09±3.3E08	–	15.9±2.0	306	61.0	8.3
4	MTA 30 IV	2.8E+09±7.5E+08	–	22.9±3.6	706	53.3	9.5
5	MTA 60 IV	2.5E+09±4.3E+08	–	17.1±2.8	614	54.4	9.3
6	MTA 90 IV	2.8E+09±3.7E+08	–	21.1±2.1	690	53.4	9.4
7	MTA 30 (IV) + GEM 50 HACE	8.3E+08±1.3E+08	2.19	12.7±0.8	206	65.6	7.7
8	MTA 60 (IV) + GEM 50 (HACE)	3.3E+08±1.6E+08	1.02	8.3±0.6	83	79.0	6.4
9	MTA 90 (IV) + GEM 50 (HACE)	4.2E+08±1.7E+08	1.14	9.6±1.0	105	75.1	6.7
10	MTA 30 (PVCE)	3.3E+09±6.6E+08	–	25.9±3.3	819	52.1	9.7
11	MTA 60 (PVCE)	3.6E+09±6.6E+08	–	29.1±4.0	907	51.3	9.8
12	MTA 90 (PVCE)	3.7E+09±5.7E+08	–	30.8±3.4	918	51.2	9.8
13	MTA 30 (PVCE) + GEM 50 (HACE)	1.4E+09±2.5E+08	2.38	15.7±2.0	260	62.8	8.0
14	MTA 60 (PVCE) + GEM 50 (HACE)	3.1E+08±2.0E+08	0.64	8.4±1.0	77	80.4	6.3
15	MTA 90 (PVCE) + GEM 50 (HACE)	1.2E+09±3.6E+08	2.45	14.6±2.1	300	61.3	8.2

[a]Determined by ß-galactosidase assay
[b]Standard error of the mean
[c]O/E = ratio of observed versus expected treatment effect
[d]Wet liver weight
[e]Ratio of final and initial tumor cell numbers
[f]Apparent tumor cell doubling time (h)
[g]Number of tumor cell doublings

Table 1. Results of hepatic arterial chemoembolization on tumor cell reduction

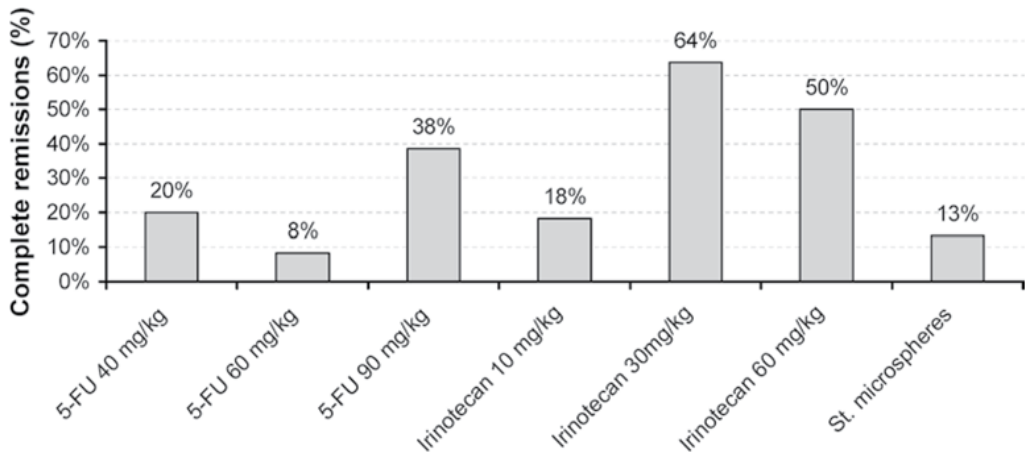

Fig. 3. The columns give the percentage of complete remissions (defined as chemoluminescence signal below that of a healthy control liver) in relation to the respective treatment group

2.2.2 Combination treatment of CC531-lac-Z rat liver metastases by chemoembolization with pemetrexed disodium and gemcitabine

The aim here was to evaluate the combination effect of pemetrexed disodium (MTA; Alimta; LY 231514) and gemcitabine (GEM) administered by hepatic artery and portal vein chemoembolization (HACE and PVCE) in our rat liver metastasis model (Rodenbach, et al., 2005). After implantation of CC531 cells, MTA (30, 60 and 90 mg/kg) was administered locoregionally by PVCE and compared with repeated systemic intravenous injection. GEM (50 mg/kg) was also given locoregionally by HACE as well as systemically. All routes of administration were examined alone as well as in combination. Efficacy of treatment in terms of liver metastases burden was determined with the chemoluminescence assay. Locoregional administration by HACE with GEM was significantly more effective than systemic intravenous bolus treatment (P=0.03). Repeated systemic treatment with MTA yielded a slight reduction in tumor cell load that was significant *vs.* control at the medium and high doses (60 mg/kg, P=0.009; 90 mg/kg, P=0.046) but not *vs.* PVCE. The combination treatment of systemic (60 and 90 mg/kg) or locoregional (60 mg/kg) MTA with HACE using GEM (50 mg/kg) resulted in >80% tumor growth inhibition; this antineoplastic combination effect was maximally additive (Fig. 4). HACE with GEM was superior to systemic intravenous bolus treatment, while PVCE with MTA was ineffective. The optimal *in vivo* regimen of MTA (intravenous or PVCE) preceding GEM (HACE) resulted in a maximally additive tumor growth inhibition indicating that MTA and GEM can successfully be combined and favor further evaluation in patients.

2.2.3 Chemoembolisation of rat colorectal liver metastases with drug eluting beads loaded with irinotecan or doxorubicin

Chemoembolisation with drug eluting beads (DEBs) designed to deliver drug at the target over a prolonged period was tested as a new strategy to reduce the tumor burden of liver metastases (Eyol, et al., 2008). Accordingly, DEBs possessing anionic groups capable of ionically complexing with cationic drugs were synthesized by a suspension polymerization

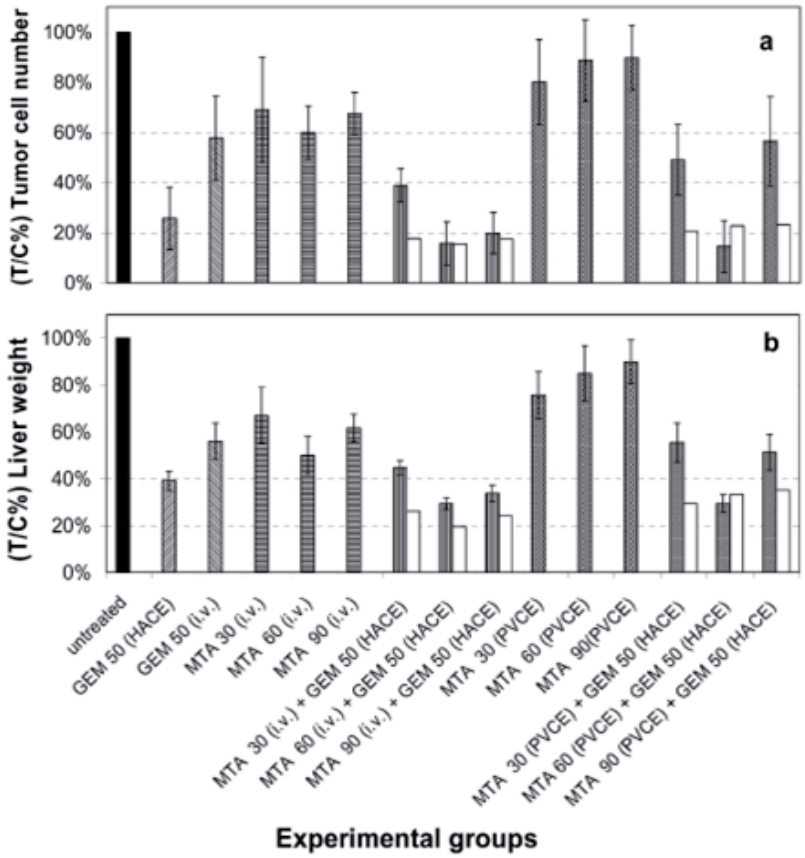

Fig. 4. Summary of treatment effects; columns denote the respective therapeutic efficacy as quotient of treated and control values (T/C%) for **(a)** tumor cell number determined by β-galactosidase assay and **(b)** wet liver weight. White columns indicate the expected combination effect. Bars symbolize standard error of the mean

method and were fractionated to produce an average size of 75 µm. The DEBs were loaded with the desired concentration of either doxorubicin hydrochloride or irinotecan hydrochloride prior to administration by immersion in the drug solution, yielding basically 100% loading efficiency (Fig. 5).

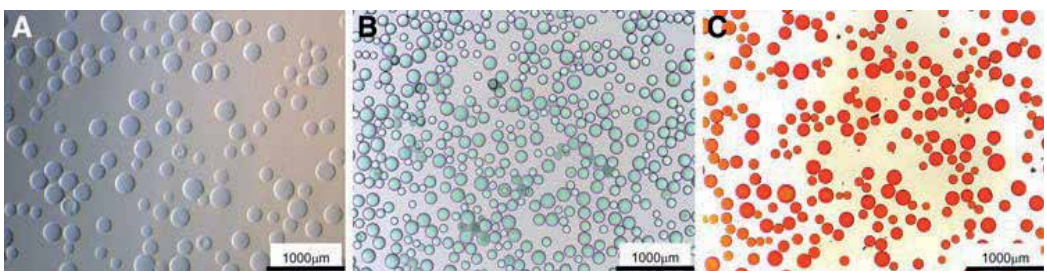

Fig. 5. Aspect of loaded and unloaded beads **(a)** Unloaded Beads; **(b)** Irinotecan DEB; **(c)** Doxorubicin DEB

After injection of CC531 cells as previously mentioned, DEBs loaded with irinotecan or doxorubicin were administered by single injection into the hepatic artery. The resulting reduction in liver tumor burden and the corresponding reduction in liver weight indicated significant anticancer activity (Fig. 6).
Comparing the two agents, irinotecan appeared more advantageous because of its significant activity and excellent tolerability following administration at 2 dosages of either 20 or 30 mg/kg. Doxorubicin showed a narrower activity window, being effective at 4 mg/kg but ineffective at the lower dose of 2 mg/kg. Therefore, HACE with DEBs with either agent may have potential for treating patients with colorectal liver metastasis.

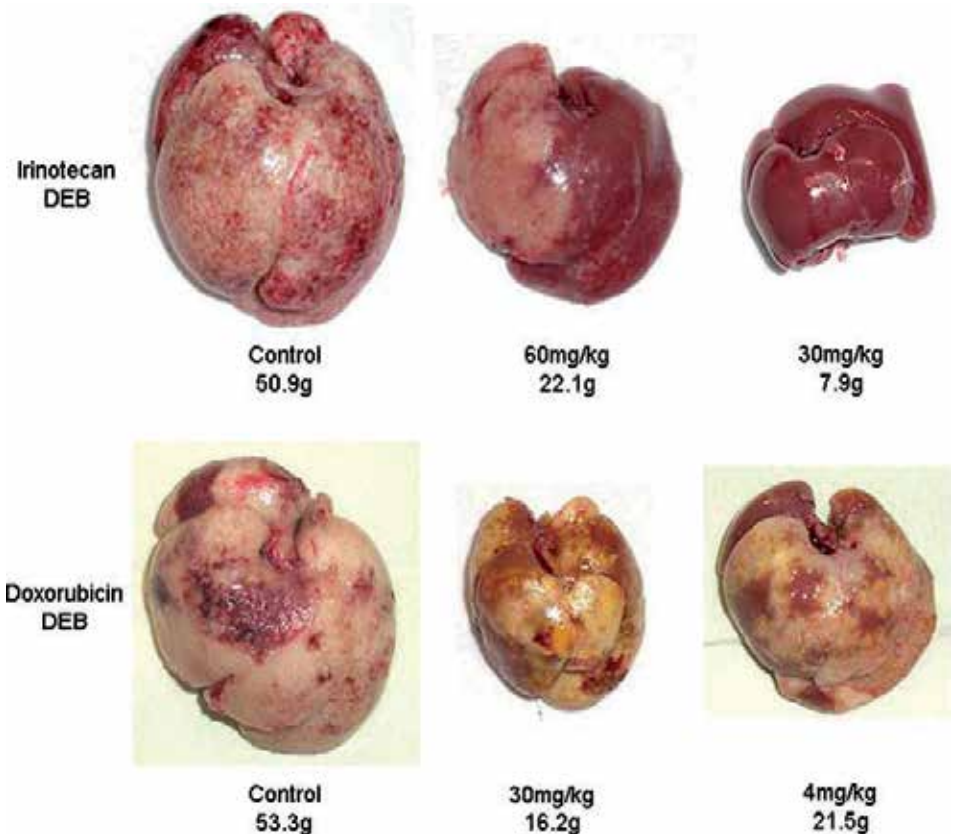

Fig. 6. Gross pathological aspects of implanted control and treated livers using irinotecan DEB (top line) and doxorubicin DEB (bottom line)

3. Search for genes that are involved in colorectal cancer liver metastasis

To identify genes that are involved in the metastatic phenotype of CC531 cells, cDNA microarrays were used to analyze mRNA expression profiles of these cells for changes related to their homing into the liver. Briefly, CC531 cells were intraportally implanted into the liver of Wag-Rij rats and re-isolated after 3, 6, 9, 14 and 21 days (Fig. 7 (A-E)). For the re-isolation purposes, the CC531 cells had been marked with stains for viable cells *i.e.* eGFP and RFP markers.

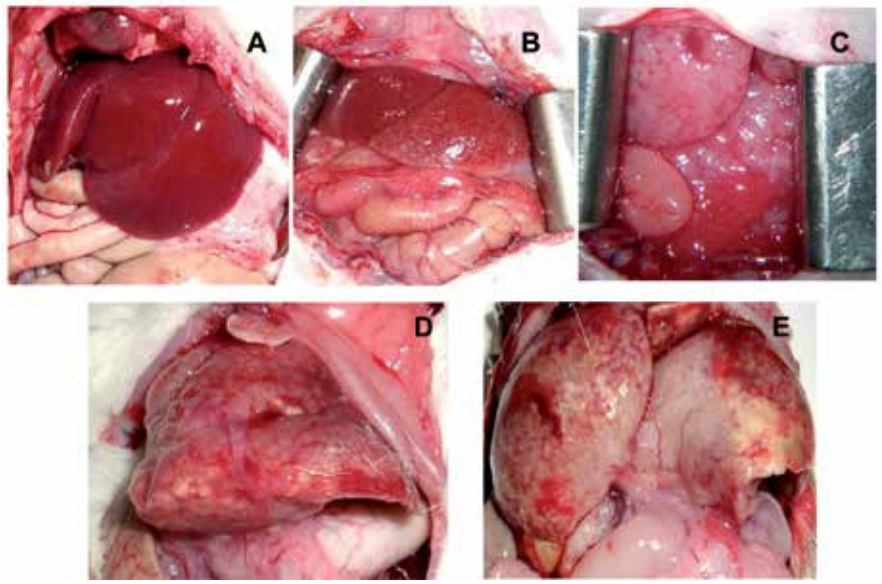

Fig. 7. **(A-E).** Photographs of rat liver taken at 3, 6, 9, 14 and 21 days after inoculation of CC531 cells, before re-isolation of the metastatic tumor cells

3.1 The re-isolation technique of tumor cells, hepatocytes and Kupffer cells

As mentioned before, the rats were kept for 3, 6, 9, 13 and 21 days after tumor cell implantation (Georges, et al., 2011). Then, the abdominal cavity was opened and a 22 G cannula was inserted into the portal vein, through which the liver was perfused with HBSS medium (20 ml/min, 37° C for 10 min). This medium was replaced with pre-warmed perfusion medium [125 ml HBSS containing $CaCl_2$ 1M, 0.1% pronase, 100 mg collagenase Type IV (Serva, Heidelberg, Germany), 37 ° C, for the following 10 min] to digest connective tissues. After getting the cells in suspension, they were filtered through a sterile filter (Cell strainer, 70 µm Nylon, BD, Germany) and centrifuged. The resulting cell suspension of liver and tumor cells was transferred into 50ml-tubes and layered carefully onto a Ficoll gradient medium (Amersham pharmacia Biotech AB, Uppsala, Sweden).

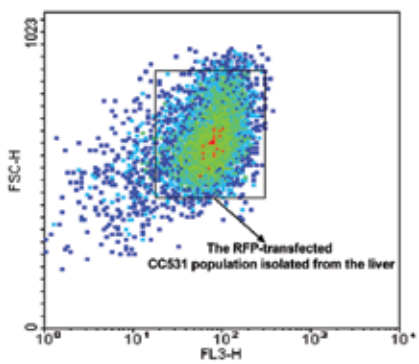

Fig. 8. The density plot illustrates the CC531 population which was obtained by FACS sorting. The marker protein RFP was used for isolating CC531 cells without contaminating liver cells

After centrifugation, the tumor cells were obtained from the top of the interface and resuspended in RPMI medium. To obtain a high purity of isolated tumor cells, CC531 cells were subsequently isolated by fluorescence-activated cell sorting technology using red fluorescent protein (RFP) as marker (Fig. 8).
Afterwards, the pure cells were pelleted and snap frozen at -80 °C. An aliquot of the cells, which were isolated on day 21, was used for re-culturing CC531 cells *in vitro*. These cells were propagated every 3 days, but two time points (14 and 22 days after tumor cell explantation) were chosen for subsequent microarray analysis, PCR, and Western blot.
For the isolation of rat hepatocytes (HCs) and Kupffer cells (KCs), the same perfusion method was performed as described above. However, to separate parenchymal (PCs) from non-parenchymal cells (NPCs), cell suspensions were gently pelleted and the resulting pellet, containing mainly hepatocytes, was taken up in Maintenance-Medium without FCS. Trypan blue exclusion (1 part trypan blue: 2 parts cell suspension) was used for cell counting and assessing their viability. 4×10^7 hepatocytes with 95% viability were usually obtained from one rat liver. Afterwards, the 25%/50% two-step Percoll gradient was used to isolate Kupffer cells as pure as possible.

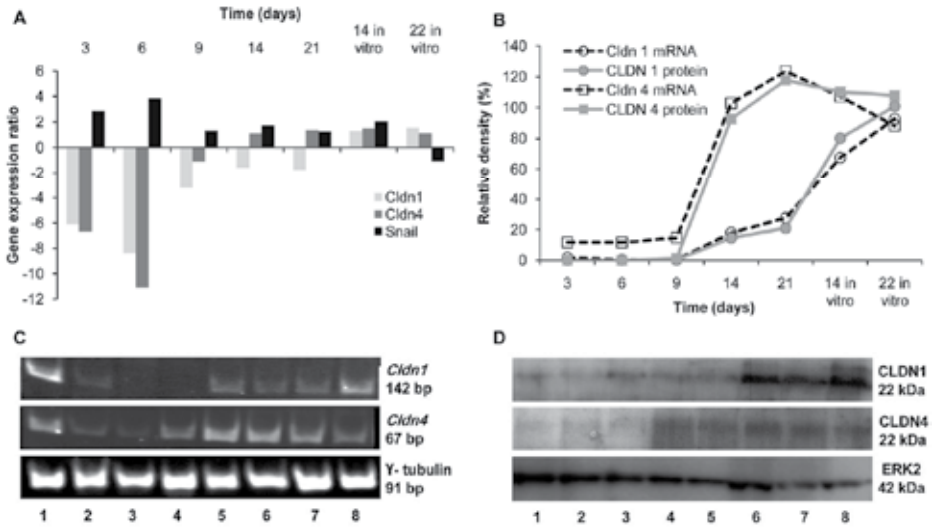

Fig. 9. Down-regulation of *Cldn1* and *Cldn4* in CC531 cells homing into the liver.
(A) Expression profile of claudins (1, 4) and Snail in CC531 cells as shown by microarray analysis. The values represent the gene expression in isolated metastasizing cells in comparison to the expression in cells growing *in vitro*. **(B)** The diagram represents the mRNA or protein expression levels in re-isolated CC531 cells in % of the expression detected in control cells (100%). Values were calculated using the pixel density of each PCR/or Western blot band normalized to the corresponding value of γ-tubulin (*Tubg1*) or ERK2, respectively. **(C)** Expression of claudins (1, 4) in CC531 cells as shown by RT-PCR. Lane 1: control CC531 cells, lanes 2-6: CC531 cells isolated from the liver after 3, 6, 9, 14 and 21 days, respectively, lanes 7, 8: CC531 cells re-isolated after 21 days and cultured *in vitro* for 14 and 22 days, respectively. **(D)** Expression of CLDNs (1, 4) in CC531 cells as shown by Western blot. Lanes 1-5: CC531 cells isolated from the liver after 3, 6, 9, 14 and 21 days, respectively, lanes 6, 7: CC531 cells isolated after 21 days and cultured *in vitro* for 14 and 22 days, respectively, lane 8: control CC531 cells

The cDNA microarray results showed that compared to control CC531 cells, claudin1 and claudin4 were among the ≥8-fold initially down-regulated genes (Fig. 9A).
Interestingly, both genes were at first down-regulated with a nadir (8 or 11 fold down-regulation) on day 6, followed by gradual up-regulation within the observation period. These results were confirmed with RT-PCR (maximum down-regulation of 80% on day 6 for both genes; Fig. 9B) and Western blot (specific bands below detection limit, >90% inhibition on day 6; Fig. 9C). It is noteworthy that the transcription repressor gene *Snail* showed an inverse modulation: an increased expression during the first week (up to 3.8 fold) with the peak of its expression corresponding to the nadir of *Cldn1* and *Cldn4* down-regulation (Fig. 9A).
Next, two experiments were performed to explain the initial down-regulation of *Cldn1* and *Cldn4*; these included co-culture of CC531 cells with isolated rat hepatocytes or Kupffer cells and the physical forces' effect experiment:

3.2 Co-culture/two compartment model

Briefly, this model is based on a two-compartment system in which hepatocytes or Kupffer cells, plated in the lower compartment, are co-cultured with CC531 tumor cells growing in the upper compartment, with the two cell types being separated by a porous membrane (0.4 μm pore size). This system, preventing a direct contact between the two compartments, allows the cells to be only indirectly influenced by molecules secreted from the cells in the other layer, respectively.
No down-regulation effect on claudin expression was noticed, whereas both genes were up-regulated after co-culture with hepatocytes (Fig. 10).

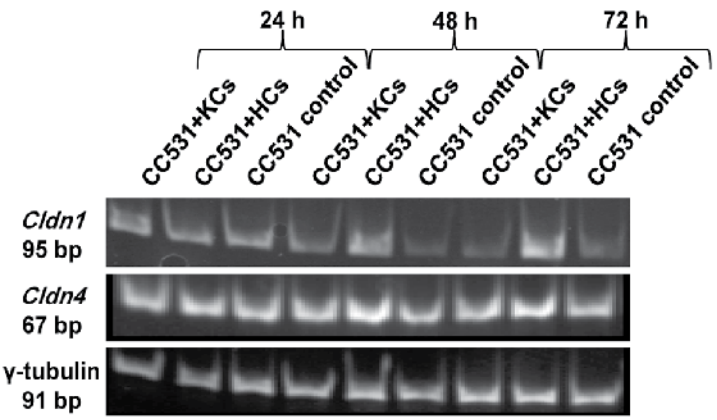

Fig. 10. Expression of claudins (1, 4) in CC531 cells co-cultured for 24 to 72 h with Kupffer cells (KCs) and hepatocytes (HCs) in comparison to the housekeeping gene γ-tubulin as shown by RT-PCR

3.3 Physical forces' effect experiment

$2x10^6$ CC531 cells were seeded in 25 cm^2 flat-bottom flasks or into round 50 ml glass bottles (Steiner GmbH, Siegen Eiserfeld, Germany), which were rotated on a roller (Stovall Life Science Incorporated, Greensboro, NC USA) at a speed of 1rpm, preventing the cells from adhesion to each other and onto the flask bottom. After 24 h, the cells in flat flasks and half

of the cells in round bottles were harvested for PCR analysis; the other half of cells was seeded in flat flasks till the next day to investigate the influence of adhesion status on claudin expression and then harvested after determining their viability under the microscope. This procedure was done daily for 3 days after seeding the cells (Fig. 11).

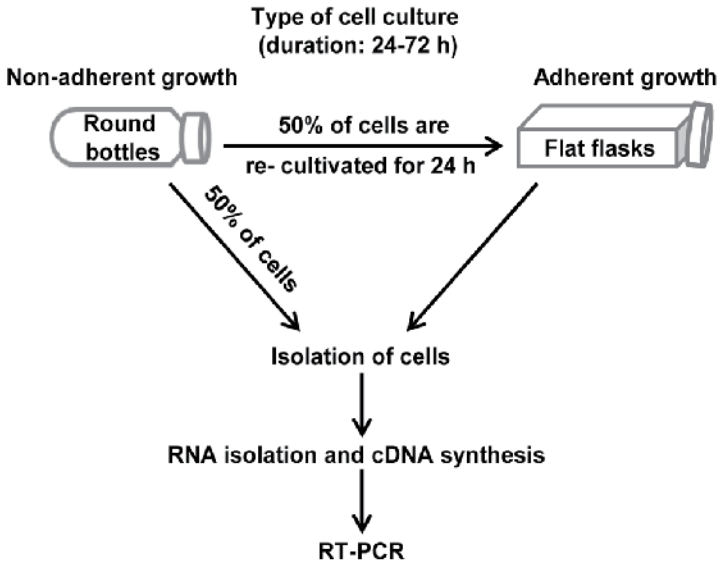

Fig. 11. Scheme indicating the experimental procedure for assessing the physical force's effects

As shown in Fig. 12, no change in *Cldn1* expression was noticed either in CC531 cells growing continuously in flat flasks or in round bottles. On the contrary, *Cldn4* mRNA expression was 2- and 1.8- fold higher in CC531 cells growing in flat flasks than their counterparts growing in round bottles at 48 and 72 h after seeding the cells, respectively.

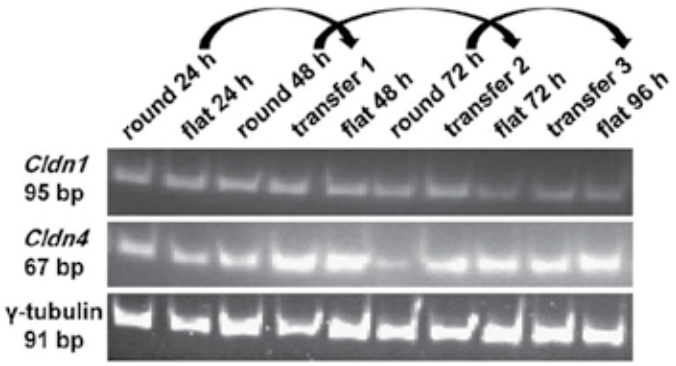

Fig. 12. Expression of claudins (1, 4) in CC531 cells harvested from round and flat flasks as shown by RT-PCR. Lanes 1, 3, 6: CC531 cells harvested from round bottles after 24, 48 and 72 h, respectively. Lanes 2, 5, 8, 10: CC531 cells harvested from flat flasks after 24, 48, 72 and 96 h, respectively. Lanes 4, 7, 9: CC531 cells harvested from flat flasks after being transferred from round bottles after 24, 48 and 72 h, respectively

Furthermore, transferring tumor cells from a non-adhesive state in round bottles to growing in flat bottom flasks for 24 h caused >2.5-fold increased expression of *Cldn4*, whereas no effect on *Cldn1* expression was noticed. Accordingly, the physical conditions and the adhesion status of the cells affected differently the expression of *Cldn1* and *Cldn4*, suggesting a direct relationship with the latter, but not with the former gene.

3.4 Small interfering RNA (siRNA) knockdown experiments

CC531 cells cultured in 6-well-plates were transfected with 100 nM siRNA or negative control using Lipofectamine 2000 (Invitrogen) following the manufacturer's instructions. The cells were harvested at 24, 48 and 72 h after treatment. As shown by RT-PCR in Fig. 13A, exposure to siRNA species directed against *Cldn1* and *Cldn4* caused reduced expression of mRNA to 24% and 15%, respectively.

To further investigate a possible interdependence of these two genes, the expression of *Cldn4* and *Cldn1* was investigated in CC531$^{si.Cldn1}$ cells and CC531$^{si.Cldn4}$ cells, respectively. Expression of *Cldn4* was down-regulated by 50% in tumor cells transfected with siRNA against *Cldn1* (Fig. 13B), whereas inhibition of *Cldn4* did not exert the same effect on *Cldn1* expression (data not shown).

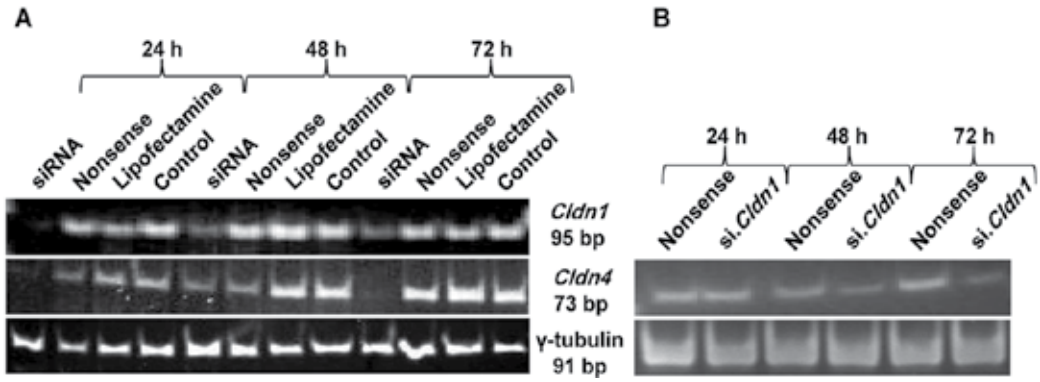

Fig. 13. **(A)** Down-regulation of claudins (1, 4) in CC531 cells after siRNA transfection as shown by RT-PCR. **(B)** Down-regulation of *Cldn4* after 24-72 h in CC531$^{si.Cldn1}$ cells (compared to CC531^{nonsense} cells) as shown by RT-PCR

The effect of *Cldn1* and *Cldn4* knockdown on cell growth (MTT assay), cell migration and colony formation (Adwan, et al., 2004, Georges, et al., 2011) was investigated as well. These *in vitro* experiments showed significantly increased migration and decreased clonogenic growth of tumor cells ($p<0.05$), but no effect on cell proliferation was noticed (Fig. 14).

3.5 Expression of CLDN1 and CLDN4 in neoplastic human CRC tissues

For the immunohistochemical (IHC) analyses of CLDN1 and CLDN4, 32 primary CRC tissue specimens with adjacent non-neoplastic tissue and 8 liver metastases were obtained from the Institute of Pathology, University of Heidelberg.

The patients had a median age of 65 years and were classified into UICC stages ll (n=24) and IV (n=8) and graded as G2 (n=25) and G3 (n=7). The histopathological analysis revealed that the expression of CLDN1 was high in 91% (n=30) and that of CLDN4 in 85% (n=28) of all tumor specimens. Comparing the CLDN expression related to UICC stages, CLDN1 and

CLDN4 had significantly lower expression in stage IV than in stage ll (p=0.01 and p=0.05, respectively). In line with this, liver metastases showed lower expression of CLDN1 and CLDN4 than in the corresponding primary carcinomas (Fig. 15). This difference was significant for CLDN1 (p<0.05) but not for CLDN4.

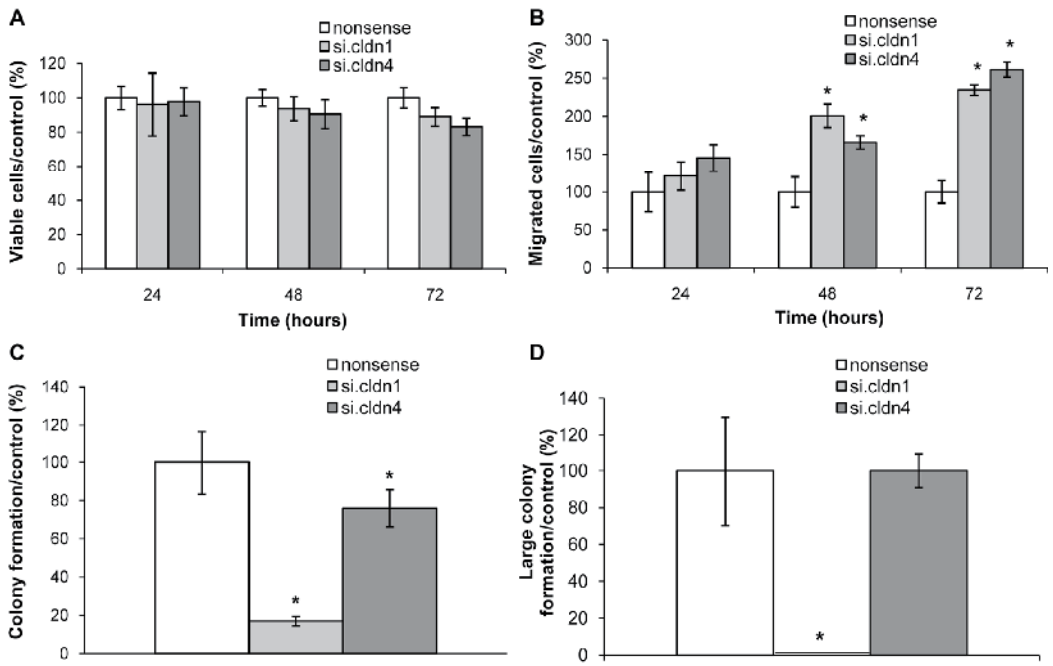

Fig. 14. Knockdown effects of *Cldn1* and *Cldn4* on cellular functions of CC531 cells .
(A) Proliferation of CC531 cells in response to si.*Cldn1* or si.*Cldn4*. **(B)** Increased migration of CC531 cells in response to si.*Cldn1* or si.*Cldn4*. **(C)** Inhibition of colony formation of CC531 cells in response to siRNA down-regulation of claudins (1, 4). **(D)** Inhibition of large colony formation of CC531 cells in response to siRNA down-regulation of *Cldn1* or *Cldn4*. Data (n=3) are shown as means ± S.D. in % of nonsense-transfected cells, an asterisk denotes a significant difference to control cells (p<0.05)

3.6 Correlation of CLDN4 or CLDN1 expression with prognosis in CRC patients

For real-time PCR analysis, 67 sporadic CRC patients, who were admitted and underwent surgery in the time between (01/98 - 07/01) at the Municipal Hospital in Nürnberg (Department of Abdominal-, Thorax-, and Endocrine Surgery) were selected.
The samples included in this study (for IHC and real-time PCR) were used based on the patients informed consent and approved by the Ethics Committee of the Universities of Heidelberg and Erlangen.

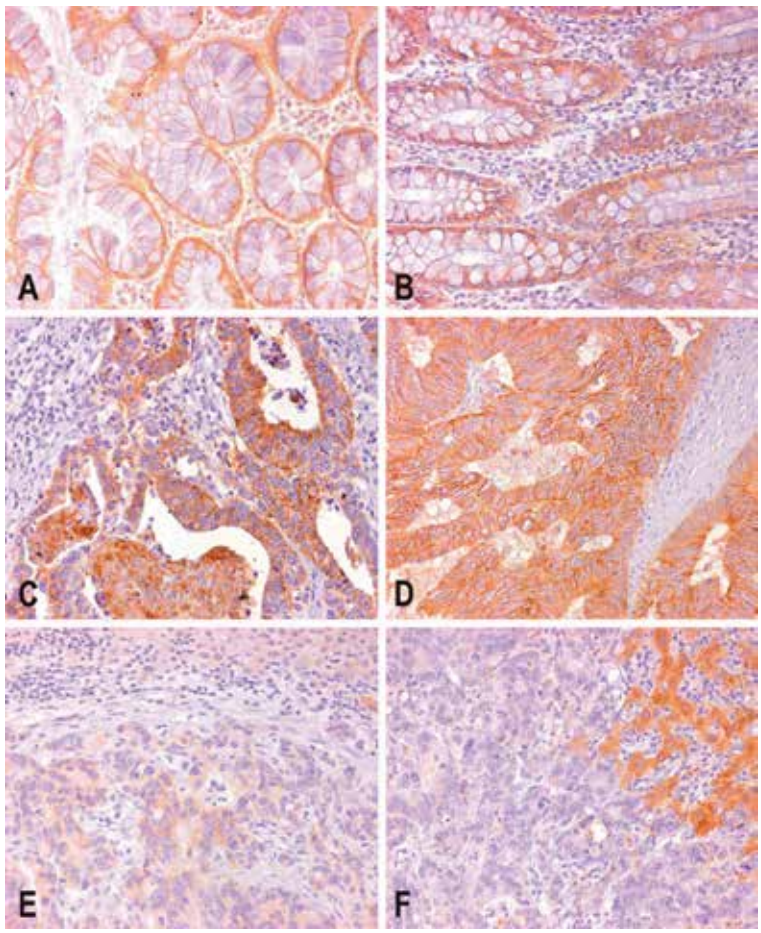

Fig. 15. Expression of CLDN1 and CLDN4 proteins in human CRC and liver metastasis tissues compared to normal mucosa as shown by immunohistochemistry. **(A)**, **(C)** and **(E)** Expression of CLDN1 in normal mucosa, cancerous tissue and liver metastasis, respectively. **(B)**, **(D)** and **(F)** expression of CLDN4 in normal mucosa, cancerous tissue and liver metastasis, respectively. Magnification x64

The 67 CRC patients (42 men and 25 women) had an average age of 67 years and were classified into 4 UICC stages (I, n=11; II, n=25; III, n=20; IV, n=11). The expression levels of *CLDN1* and *CLDN4* were significantly correlated ($P<0.05$; Fig. 16A). No correlation between *CLDN1* expression and age ($p=0.19$), tumor stage ($p=0.88$), or overall survival ($p=0.2$) was seen. With respect to *CLDN4*, also no correlation with age ($p=0.69$) or tumor stage ($p=0.38$) was noticed. However, the overall survival of CRC patients with high or low risk in relation to the median split of *CLDN4* expression levels differed significantly according to the log-rank test ($p=0.018$; Fig. 16B). Similarly, using a Cox model, there was not significant difference ($p=0.07$) between the low and high risk groups taking all stages together, whereas in patients with tumor stages l-lll, an elevated *CLDN4* level was clearly associated with a less favorable prognosis ($p=0.05$; Fig. 16C).

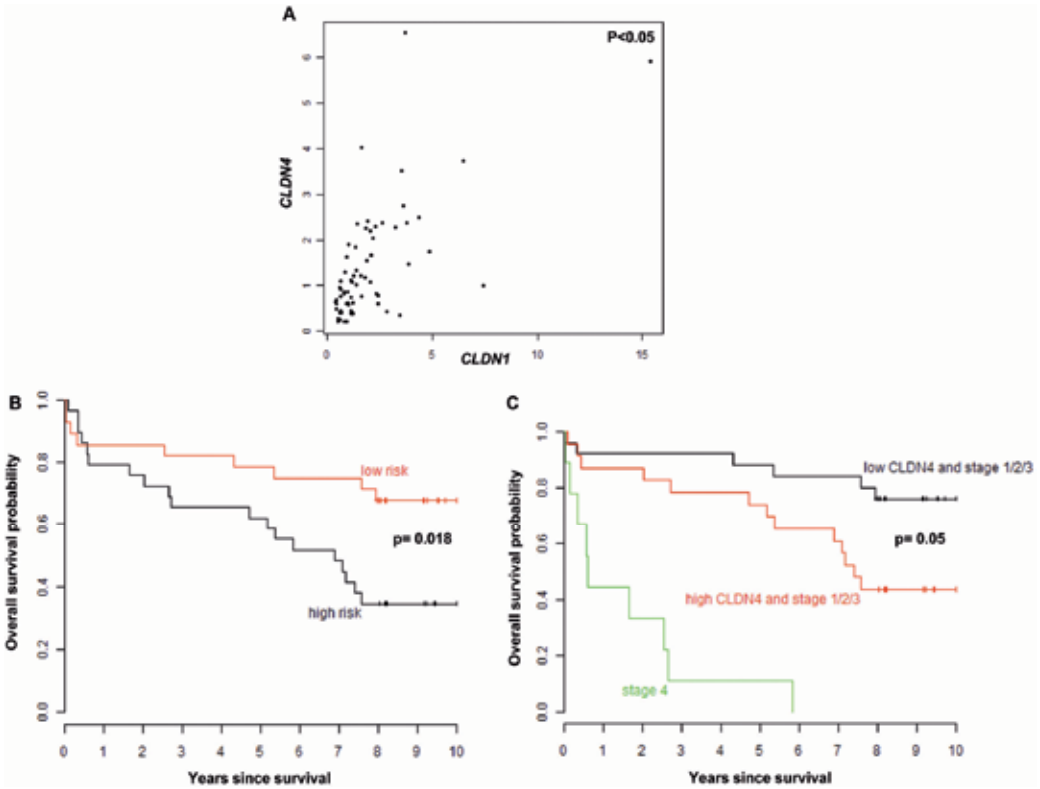

Fig. 16. Correlation of *CLDN1* or *CLDN4* expression levels with prognosis in 67 CRC patients and with each other. **(A)** Scatterplot of the correlation between *CLDN1* and *CLDN4* expression levels assessed with the non-parametric correlation coefficient from Spearman **(B)** The Kaplan-Meier plot represents the overall survival probability of CRC patients with high or low risk according to *CLDN4* median split for overall survival. The log-rank test shows a significant difference (p=0.018) between the two groups. **(C)** The Kaplan-Meier plot demonstrates the overall survival probability of CRC patients with high or low *CLDN4* expression, dichotomized into high and low risk groups by the *CLDN4* median split for overall survival and after separating the stages into I-III and IV stages. The Cox model shows a significant association between *CLDN4* elevated levels and less overall survival (p=0.05)

3.7 OPN expression profile in CC531 cells ex-vivo

After explanting the liver, a piece of liver containing tumor cells was resected and used for re-culturing CC531 cells (Georges, et al., 2010). These cells were cultured for three weeks and within this period passaged five times corresponding to 11, 13, 15, 18 and 21 days; respectively (passages 1-5). At these intervals the cells were investigated for their mRNA- and protein expression levels of OPN.

At the mRNA level, *Opn* was up-regulated in CC531 cells explanted from the tumor. Over time, this *Opn* mRNA level gradually decreased till it disappeared after the third passage; i.e. after two weeks (corresponding to a reduction by 88%; Fig. 17, left).

The OPN protein level, as shown by Western blot (Fig. 17, right), was first highly expressed but then down-regulated to minimally 5% within the next 15 days. A slight increase to 20% of the initial level was seen at the final passages 4 and 5.

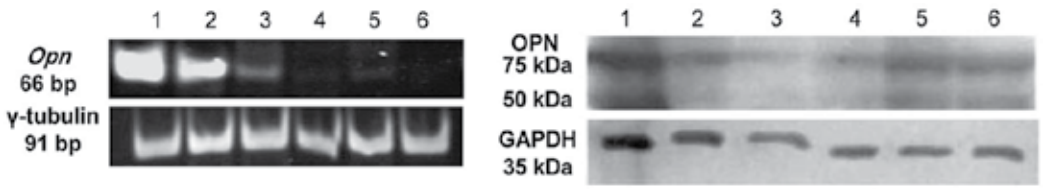

Fig. 17. Expression of OPN on mRNA (left; RT-PCR) and protein (right; Western blot) levels. Lane 1: CC531 cells explanted from the tumor, lanes (2-6): CC531 cells after 11, 13, 15, 18, and 21 days; respectively

4. Conclusion

In conclusion, the model described in this chapter evolved in two main steps. First, for quantitation of tumor cell load, the CC531 cells had been marked with GLB1. This marker proved useful for the purposes of therapeutic studies with various anti-cancer agents. Using the GLB1-based chemoluminescence assay, it could be shown that the efficacy of hepatic artery chemoembolization with gemcitabine-microspheres was significantly higher than with 5-FU in reducing the tumor cell load.

Also it was shown, that HACE with irinotecan-microspheres had a greater effect than that of HACE with 5-FU, setting the basis for further investigations in clinical trials.

The same assay allowed us to evaluate the combination effect of pemetrexed disodium and gemcitabine administered by hepatic artery and portal vein chemoembolization in our rat liver metastasis model. These experiments showed that HACE/gemcitabine was superior to systemic intravenous bolus treatment, while PVCE/pemetrexed disodium was ineffective. Interestingly, a maximum additive tumor growth inhibition *in vivo* was noticed using a regimen of (intravenous or PVCE)/pemetrexed disodium preceding HACE/gemcitabine indicating that these two agents can successfully be combined and favor further evaluation in patients.

The third set of therapeutic experiments, using HACE with drug eluting beads (DEBs) loaded with either doxorubicin hydrochloride or irinotecan hydrochloride showed that irinotecan is more advantageous because of its significant activity and excellent tolerability. In addition, HACE with DEBs with either agent may have potential for treating patients with colorectal liver metastasis.

The CC531 tumor cells marked with GLB1 allowed a determination of the tumor cell load only after termination of the experiment. To further develop the model for determining the tumor cell load in living animals, other markers had to be introduced. These were eGFP and RFP, which enabled the next series of experiments that were related to liver metastasis genes.

Realizing the fact that the most common cause of CRC-related death is the development of metastasis, especially into the liver, denotes the importance of identifying the metastasis-related changes in tumor cells, which probably differ from those related to the primary tumor. Therefore, temporal changes in gene expression of CRC cells homing into the liver have been investigated using our *in vivo* model, which had been improved by eGFP and RFP markers. The intraportal inoculation of CC531 cells defines the onset of metastatic proliferation in rat liver. This, in turn, permits a close following of the time-dependent modulation of gene expression, as the tumor cells home into the liver and then grow to a lethal size. The technique of re-isolating these tumor cells from rat liver permits monitoring for the first time the expression of several candidate genes in a time-dependent manner. The

following cDNA microarray analysis showed that the initial phase of rat CRC cells homing into the liver involves a transient down-regulation of *Cldn1* and in particular of *Cldn4*. The transcription repressor Snail, which regulates both claudins, was concomitantly up-regulated during the early stages of metastasis before returning to normal expression levels. Silencing of *Cldn1* and *Cldn4* by siRNA increased migration and reduced colony formation, with these phenotypes consistent with metastatic homing. These experimental results were paralleled in human CRC tumor samples, which show increased *CLDN1* and *CLDN4* expression in UICC stages I–III, and significantly reduced expression in stage IV and in liver metastasis. The results obtained with human specimens give first evidence of a modulated claudin expression similar to those in the rat model. However, a prospective study is needed to corroborate these results, taking into account separately the entities, colonic and rectal cancers. That research could be driven by our hypothesis that primary CRC tumors have an initial growth advantage from increased claudin expression, whereas metastasizing cells require a transient reduction in claudin expression to be liberated from the primary tumor and to then initiate metastatic growth in the liver.

Based on these experiments we conclude that the CC531 colorectal cancer liver metastasis model in rats is attractive for translational research: it is suited for therapeutic studies as well as for identifying genes involved in colorectal cancer liver metastasis.

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Part 6

Study Reports

23

Risk Factors for Wound Infection After Surgery for Colorectal Cancer: A Matched Case – Control Study

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1. Introduction

Elective surgery for colorectal cancer involves a semi-contaminated operation, with a 3% to 26% incidence of postoperative wound infection (1). Risk factors for postoperative wound infection include high body-mass index (BMI) (2, diabetes mellitus(3), body-weight loss(4), advanced age(5), smoking(6), blood transfusion(7), and high intraoperative blood loss (8). The development of wound infections can cause considerable discomfort and stress, as well as prolong the hospital stay, substantially increasing healthcare costs. Measures to prevent wound infections have been refined by adjusting the duration of antibiotic treatment and improving techniques for preoperative bowel preparation and drain placement. We performed a matched case-control study to clarify risk factors for perioperative wound infection in patients who underwent standard surgery for colorectal cancer, performed by the same operator at the same hospital. All patients received similar levels of perioperative care.

2. Subjects and methods

From January 2004 through December 2006, a total of 264 patients underwent surgery in our hospital for primary colorectal cancer with a solitary lesion. The same surgeon (T.N.) served as the operator or assistant. Preoperatively, all patients received mechanical bowel preparation. Two tablets of sennoside were given orally 2 days before surgery, and 1 packet of magnesium citrate and 1 bottle of sodium picosulfate were given the day before surgery. For wound closure, the peritoneum and fascia were sutured together with interrupted, polydioxanone absorbable sutures (0-PDS II™, Johnson and Johnson Co. Ltd). The same suture material was used to close the abdomen in patients who underwent laparoscopic surgery and those who underwent open surgery.

In patients who underwent high-pressure irrigation of their wounds, the muscle layer was sutured, and the subcutaneous tissue was washed with warm physiological saline solution applied under high pressure, using a 23-gauge ophthalmic washing catheter attached to a 20-mL syringe. The tip of the needle was positioned about 10 cm from the wound (Fig. 1). The applied volume of physiological saline solution was 400 mL for open surgery, and 200

mL for laparoscopic surgery. After irrigation, the skin was closed with polydioxanone absorbable sutures (4-0 PDS II™, Johnson and Johnson Co., Ltd.).

Fig. 1. Before closure of the abdomen during surgery, high-pressure irrigation of the subcutaneous tissue after muscle layer suturing

In patients who did not undergo high-pressure irrigation (non-high-pressure irrigation group), the peritoneum and fascia were sutured together with interrupted, polydioxanone absorbable sutures (0-PDS II™, Johnson and Johnson Co., Ltd.) Then, In the non-high-pressure lavage group, no syringe was used. The subcutaneous tissue was just washed with 500 mL of warm physiological saline solution.

The subcutaneous adipose tissue was sutured with polyglactin 910 absorbable sutures (3-0 Vicryl™, Johnson and Johnson Co., Ltd.), and the skin was closed with a skin stapler.

During the 19 months from January 2004 through July 2005, a total of 145 patients underwent surgery without high-pressure irrigation. During 16 months from August 2005 through December 2006, a total of 119 patients underwent surgery with high-pressure irrigation. The two groups were matched for the following 6 variables: sex, age (± 5 years), tumor location (right side of colon, transverse colon, left side of colon, rectum), surgical procedure (laparoscopic surgery, open surgery), tumor-node-metastasis (TNM) classification, and BMI (± 1). We studied a total of 100 patients: 50 in the high-pressure irrigation group and 50 in the non-high-pressure irrigation group (Table 1).

As for the demographic characteristics of the patients, the American Society of Anesthesiologists' physical status classification was class I in 37 patients (74%), class II in 10 (20%), and class III in 3 (6%) in the high-pressure irrigation group and class I in 37 patients (4%), class II in 11 (22%), and class III in 2 (4%) in the non-high-pressure irrigation group. The difference between the groups was not significant ($p = 0.884$). No patient had a preoperative hemoglobin level of ≤8.0 g/dL. Four patients received blood transfusions during surgery.

	High-pressur irrigation group (n=50)	Non High-high irrigation group (n=50)
No.of patients	50	50
Sex (male : femail)	36 : 14	36 : 14
Age(years)	66(42-87)	67(40-90)
Tumor location		
Cecum ,Ascending colon	13	13
Transverse colon	6	6
Descending colon ,Sigmoid colon	15	15
Rectum	16	16
Tumor diameter(cm)	4.1(0.8-8.0)	3.5(1.0-7.5)
Surgical procedure(laparoscopy : open surgery)	37 : 13	37 : 13
pTNM stage		
0	3	3
Ⅰ (pT1N0)	6	6
Ⅰ (pT2N0)	4	4
Ⅱ (pT3N0)	20	20
Ⅲ (pT3N1)	13	13
Ⅳ	4	4
Wound infection (presence : absence)	2 : 28	9 : 31
BMI(kg/m^2)	21.9(17.6-28.3)	22.8(16.6-26.8)
Median follow-up period (months) (range)	15(7-30)	30(21-39)

Table 1. Demographic characteristics of the patients

Laparoscopic surgery was performed if tumors invaded the lamina propria (Tis), the submucosa (T1), or the muscularis propria (T2). Open surgery was performed if tumors directly invaded other organs or structures and/or perforated the visceral peritoneum (T4, AI). If tumors invaded through the muscularis propria into the subserosa or into non-peritonealized pericolic or perirectal tissues (T3, A), the surgical procedure was decided in a randomized control trial after obtaining informed consent from the patient. Laparoscopic surgery was not switched to open surgery in any subject.

After skin closure, the wound was applied a polyurethane film dressing for 48 hours in all patients. Subsequently, the wound was uncovered and was not sterilized. To prevent infection during and after surgery, cefmetazole sodium (1 g/time) or flomoxef sodium (1 g/time) was given as a continuous intravenous infusion 1 hour before surgery and at 3-hour intervals thereafter. On the day after surgery, antibiotics were administered only one time.

After the operator confirmed the wound, wound infection was evaluated according to the 1999 Guidelines for the Prevention of Surgical Site Infection (SSI)(9). These guidelines do not require the results of culture studies to assess wound infection. In our study, however, a wound culture was performed if fluid or discharge was exuded from the wound. The median postoperative follow-up period was 15 months (range, 7 to 30) in the high-pressure irrigation group and 30 months (range, 21 to 39) in the non-high-pressure irrigation group. During follow-up, patients visited the outpatient clinic at 2- to 4-week intervals. Follow-up

examinations included examination of the surgical wound, administration of adjuvant chemotherapy, and computed tomography (CT) of the chest and abdomen. The preoperative and postoperative status of each patient was retrospectively examined on the basis of medical records.

A total of 10 potential risk factors for wound infection were compared between the groups: the presence or absence of high-pressure irrigation before wound closure, sex, age (<65 years, ≥65 years), BMI (≤25 kg/m^2, >25 kg/m^2), tumor location (colon, rectum), surgical procedure (laparoscopic surgery, open surgery), operation time (<180 minutes, ≥180 minutes), bleeding volume (<100 mL, ≥100 mL), tumor stage (I or II, III or IV), and antibiotic treatment (cefmetazole, flomoxef), (Table 1).

Chi-square tests with Yates' correction and multivariate logistic regression analysis were performed to identify variables with p values of less than 0.1. P values of less than 0.05 were considered to indicate statistical significance. SPSS version 8.0J (SPSS Inc., Chicago, USA) software was used for analysis.

3. Results

Wound infections developed after colorectal cancer surgery in 11 (11%) of the 100 patients. On univariate analysis, wound infection was significantly associated with tumors located in the rectum (p = 0.011), open surgery (p = 0.032), and non-high-pressure irrigation of wounds (Table 2). On multivariate analysis, independent risk factors for wound infection were wound treatment (non-high-pressure irrigation) (p = 0.034; odds ratio, 5.968) and surgical procedure (open surgery) (p = 0.039; odds ratio, 4.266) (Table 3).

	Wound infection(+) (n=11)	Wound infection (-)(n=89)	*p*-value
Sex (male:female)	6 : 5	66 : 23	0.189
Age (years)(≦65 : 65<)	6 : 5	39 : 50	0.710
Tumor location (Colon : Rectum)	3 : 8	60 : 29	0.011
BMI(kg/m2)(<25 : 25≦)	3 : 8	60 : 29	0.837
Surgical procedure (laparoscopy : open surgery)	5 : 6	69 : 20	0.032
Operation time (<180 : 180≦)	5 : 6	29 : 60	0.837
Intraoperative bleeding volume (ml)(<100 : 100≦)	8 : 3	65 : 24	0.983
High-pressure irrigation (presence : absence)	2 : 9	48 : 41	0.021
TNMstage(I-II : III, IV)	7 : 4	59 : 30	0.861
Antibiotics (CMZ : FMOX)	4 : 7	37 : 52	0.739

BMI:Boddy Mass Index,;TNM,Tumor node metastasis;CMZ:cefmetazole sodium, FMOX:flomoxef sodium

Table 2. Wound infection detected free of wound infection

Wound infection developed in 5 (7%) of the 74 patients who underwent laparoscopic surgery and 6 (23%) of the 26 patients who underwent open surgery. Wound infection occurred in 2 (4%) of the 50 patients in the high-pressure irrigation group and 9 (18%) of the 50 patients in the non-high-pressure irrigation group. The mean duration of the hospital

stay after surgery was 8 days (range, 5 to 31) in patients without wound infection, as compared with 15 (range, 7 to 40) in those with wound infection. This difference was significant (p = 0.041). During observation after discharge, there was no flare-up of wound infection, wound dehiscence, or adhesive ileus.

	Odds ratio	95%CI	*p*-valeue
Surgical procedure Open surgery : laparoscopy	4.266 : 1	1.079-16.866	0.039
High-pressure irrigation absence : presence	5.968 : 1	1.150-30.963	0.034

CI:confidence interval

Table 3. Results of multivariate analysis of risk for wound infection

The American Society of Anesthesiologists' Physical Status Classification was class I in 56 patients (76%), class II in 15 (20%), and class III in 3 (4%) in the laparoscopic surgery group and class I in 16 patients (67%), class II in 6 (23%), and class III in 2 (7%) in the open surgery group. The difference between the groups was not significant (p = 0.884).

Pus samples from infected wounds were cultured in 9 of the 11 patients with wound infection (2 in the high-pressure irrigation group and 7 in the non-high-pressure irrigation group). Pus cultures were positive in all patients in both the high-pressure irrigation group and the non-high-pressure irrigation group. The most common pathogen was bacteroides in 6 patients, followed by *Staphylococcus aureus* in 2 and *Pseudomonas aeruginosa* in 1. Pathogens did not differ between the high-pressure irrigation group and the non-high-pressure irrigation group. Strains isolated from the 6 patients with bacteroides infections and the 2 with *Staphylococcus aureus* infections were sensitive to the prophylactically administered antibiotics.

4. Discussion

Our study showed that that non-high-pressure irrigation and open surgery were independent risk factors for postoperative wound infection in patients with colorectal cancer. To minimize potential effects of confounding factors, our study was conducted under consistent conditions, i.e., the same operator performed surgery and perioperative management in the same hospital.

Carlos et al. performed a randomized control trial to evaluate whether syringe pressure irrigation of surgical wounds decreases wound infection after appendectomy (10). Patients were randomly assigned to receive prophylactic antibiotics alone before surgery or prophylactic antibiotics plus syringe pressure irrigation of their wounds. Irrigation was performed by applying 300 mL of physiological saline solution under high pressure with the use of a 20-mL syringe with a 19-gauge intravenous catheter. The tip of the catheter was placed about 2 cm from the wound. Among the 283 patients confirmed to have appendicitis, 95 (33.6%) had complications. Of these 95 patients, wound infection developed in 9 (16.3%) of the 55 patients who received prophylactic antibiotics plus wound irrigation, as compared with 29 (72.5%) of the 40 patients who received prophylactic antibiotics alone (p = 0.0006).

Johnson et al. prospectively studied the incidence of SSI in 715 patients who underwent a cesarean section procedure (11) · The use of subcuticular sutures for skin closure was associated with a significantly higher incidence of SSI than was the use of staples (7.9%, 20/252 vs. 13.0%, 59/459; p = 0.021).

To our knowledge, no previous study has evaluated high-pressure irrigation at the time of wound closure after muscle-layer suture in patients with colorectal cancer. This technique was originally developed in our hospital. A bacterial count of greater than 10^5 bacteria per gram of tissue is considered necessary for the development of wound infection(12). We believe that our method for high-pressure irrigation after muscle-layer suture effectively decreases the bacterial count in subcutaneous tissue.

Our study showed that the incidence of postoperative wound infection was significantly lower after laparoscopic surgery than after open surgery. Some previous studies have reported that the incidence of wound infection after laparoscopic surgery was similar to that after open surgery, whereas others have found that the incidence of wound infection was significantly lower after laparoscopic surgery(13). A meta-analysis performed by Abraham et al. showed that the incidence of postoperative wound infection was significantly lower after laparoscopic surgery (3.9%) than after open surgery (8.9%) ($p<0.005$). Our findings support the results of this meta-analysis(14).

The presence of suture material in a closed wound has been reported to increase the number of bacteria to 10^4 per gram of tissue (15) · Open surgery requires a longer skin incision and more stitches than laparoscopic surgery, increasing the bacterial count and potentially increasing the risk of wound infection. One study reported that the incidence of wound infection after laparoscopic surgery was 2.7% at the trocar site and 10.8% at the site of colorectal removal (16). In our hospital, no patient has had wound infection at the trocar site, and the incidence of wound infection at the site of colorectal removal was only 4%, which was lower than that reported previously(17). In general, wound infection has been reported to occur at an incidence of about 20% after open surgery, which is consistent with the incidence of 23% in our study.

The relation between surgical wound infection and diabetes mellitus in patients who undergo surgery for colorectal cancer remains unclear. The risk of SSI after cardiac surgery has been reported to be 2 to 3 times higher in patients with diabetes mellitus than in those without diabetes mellitus, even after adjustment for other risk factors (3). The higher risk of SSI may be related to long-term metabolic and microcirculatory disorders induced by diabetes mellitus, perioperative hyperglycemia, and other risk factors associated with diabetes mellitus, such as obesity. Because we strictly control blood levels before and after surgery in our hospital, diabetes mellitus was not a risk factor for wound infection.

As for the relation between BMI and wound infection, Smith et al. reported that the incidence of wound infection increases in parallel to BMI (2) · In their study, 53% of the patients had a BMI of ≥25 kg/m^2 or higher. In contrast, only 21% of our patients had a BMI of ≥25 kg/m^2. This lower proportion of patients with an increased BMI may have accounted for BMI not being a risk factor for wound infection.

In patients with a BMI of ≥25 kg/m^2, we close the wound with subcuticular sutures after inserting a closed subcutaneous drain to maintain fluid drainage. However, firm evidence supporting the use of a subcutaneous drain after colorectal cancer surgery has yet to be obtained. The insertion of a drain may increase healthcare costs and negatively affect patients' ability to walk after surgery. Therefore, further studies are needed.

Many surgical wound infections are caused by bacteroides and *Staphylococcus aureus*. Although these bacteria were sensitive to the antibiotics we administered prophylactically in our study, wound infection developed. The dose and duration of treatment with antibiotics should thus be reassessed. Further multicenter studies are needed to more clearly define risk factors and establish the most effective prophylactic treatment for wound infection.

5. References

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Modelling and Inference in Screening: Exemplification with the Faecal Occult Blood Test

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1. Introduction

Colorectal cancer (CRC) is the third most common form of cancer and the third leading cancer killer for both genders in the United States (American Cancer Society, 2011). In 2011, the American Cancer Society estimates 141,210 new cases in the U.S. and 49,380 deaths due to CRC (American Cancer Society, 2011). Similarly, the World Health Organization estimates over 940,000 new cases occurring annually worldwide (World Health Organization, 2003), and nearly 610,000 died from CRC in 2008 (World Health Organization, 2011).

Colorectal cancer is often found in people older than fifty, and its mortality rates are higher each year than HIV/AIDS and breast cancer combined (Colon Cancer Prevention Project, 2011). The age-specific colorectal cancer risk rises continuously with advancing age (National Cancer Institute, 2011). CRC is also one of the most preventable among cancers, studies show that regular screening could substantially reduce mortalities (Kronborg et al., 1996, Mandel et al., 1993, 1999). The reason is that colorectal cancer can take 5-15 years to develop, and screening exam, such as digital rectal exam (DRE), colonoscopy, flexible sigmoidoscopy (FSG) and faecal occult blood test (FOBT), can detect polyps before the cancer develops (Screen for Life: National Colorectal Cancer Action Campaign, 2009).

The current guidelines in the U.S. for CRC include several options among men and women aged 50 and older at average risk (American Cancer Society, 2011). Many considerations are needed when following the guidelines, including risk factors, type of test, and test interval including flexible sigmoidoscopy, colonoscopy, double-contrast barium enema, computed tomographic colonography, faecal occult blood test and stool DNA test (American Cancer Society, 2011). However, the acceptance of people about CRC screening has been low in the U.S. In most areas of the U.S., less than half of the population is in compliance with recommended CRC guidelines (American Cancer Society, 2011), and only one-third colorectal cancers are being diagnosed at an early and treatable stage, due to lack of screening or lack of disease symptoms (ARUP Laboratories, 2010).

The purpose of colorectal cancer screening is to detect early stage of the disease before clinical symptoms take place. CRC has a 90% treatable rate when detected early, therefore

screening saves lives (Colon Cancer Prevention Project, 2011). Prevention efforts in the population requires reliable estimates of the sensitivity of the test, the sojourn time of the disease, the transition probabilities from the disease-free state to the preclinical state, the lead time of the disease and the indirect effects in the screening per se in the estimates of rates of the disease.

The aim of this chapter is to introduce the concept of probability modelling in colorectal cancer screening, and the statistical methods developed by the authors in this area (Wu et al., 2005, 2007, 2009a, 2009b). We will estimate these essential components from a population based perspective. In section 2, we provide the definition, model, methods and application of essential parameters needed when estimating indicators of cancer screening. In section 3, we provide the methods and application for estimating the distribution of the lead time in cancer screening. In section 4, we provide the definition, methods and application when evaluating the long term screening outcomes in CRC. Finally, conclusions and recommendations for future research are provided in section 5. We will focus on one particular test, the faecal occult blood test (FOBT). FOBT has been used as a sign of colon cancer, given that tumours tend to bleed and blood in the stool can be detected using this test. We will apply our methods to the Minnesota Colorectal Cancer Control Study (MCCCS) (Mandel et al., 1999), to inform the readers about the benefits of probability modelling in colorectal cancer screening using FOBT, as well as reached recommendations for the test.

The Minnesota Colorectal Cancer Control Study (MCCCS) was carried out between 1976 and 1982 in Minnesota, U.S.A. (Mandel et al., 1999). Approximately 46,000 subjects were randomized to receive either: five annual FOBT screenings, three biennial FOBT screenings or no screening (usual care at the time of the study). Each screening cycle consisted of six hemoccult slides (Hemoccult®, Beckman Coulter, Palo Alto, California); about 83% of slides were re-hydrated. If any of the slides was positive, then the screen was considered positive and a definitive follow-up exam was done, including colonoscopy (Mandel et al., 1999). Due to a lower than expected death rate among the usual care group, the investigators resumed screening between 1986 and 1992. We restricted this analysis to the annual group and to the original five screenings.

2. Sensitivity, sojourn time and transition probability in colon cancer screening

We assume that the disease develops by progressing through three states, denoted by $S_0 \to S_p \to S_c$. S_0 represents the disease-free state. S_p represents the preclinical disease state, in which an asymptomatic individual unknowingly has disease that the screening exam can detect. Similarly, S_c represents the clinical state when the disease manifests itself in clinical symptoms.

Sensitivity is the probability that the screening exam is positive given that the individual is in the preclinical state S_p. The sensitivity cannot be easily estimated from data collected during screening, but can be estimated using probability modelling (Wu et al., 2005, 2009b). We exemplify the rationale for this issue using Table 1. Let us assume the data in Table 1 is generated from a single screening study. It is neither cost effective nor ethical to obtain a biopsy from each individual with a negative screening result. Therefore, the numbers n_{21} and n_{22} are not available; but n_{11}, n_{12} and $n_{21} + n_{22}$ are available information. Using the

definition, an estimator of sensitivity should be $n_{11} / (n_{11} + n_{21})$. However, since the number n_{21} is unknown, it cannot be obtained directly from data collected in the screening study.

Screening Result	Disease Status	
	Cancer	Non-Cancer
Positive (+)	True Positive (n_{11})	False Positive (n_{12})
Negative (-)	False Negative (n_{21})	True Negative (n_{22})

Table 1. Illustration of different kinds of counts in a screening study

Sojourn time refers to the time beginning when the disease first develops until the manifestation of clinical symptoms, which is the time length in the preclinical state. For individuals diagnosed with cancer by screening exams, they will be treated immediately; hence the onset of the clinical state S_c is not observable. For individuals diagnosed with cancer between screenings (the interval case), though the onset of the clinical state is available, the onset of the preclinical state is still unknown. Therefore, estimation of the sojourn time distribution is difficult from data collected in screening studies. However, the sojourn time duration can be estimated under model assumptions, the preclinical phase of colorectal cancer may last more than 5 years (American Cancer Society, 2011, Prevost et al., 1998).

The transition probability into the preclinical stage is the probability density function (PDF) of making a transition from the disease-free state to the preclinical state. It is continuously changing with one's age (Wu et al., 2009a) on CRC, and is difficult to estimate without proper modelling.

These three parameters are the key parameters for the estimation of other important indicators in cancer screening, and they cannot be easily estimated from data. We will briefly review the age-dependent likelihood method that we used in estimating these three parameters, and provide the key result using the MCCCS data (Wu et al., 2005, 2009b).

2.1 Model and method

Consider a cohort of initially asymptomatic individuals who enrolled in a screening program. The sensitivity is β(t), where t is the individual's age at the screening exam. The probability density function (PDF) of making a transition from S_0 to S_p at age t is $w(t)$. Let $q(x)$ be the probability density function of the sojourn time in S_p. Let $Q(z) = \int_z^\infty q(x)dx$ which is the survivor function of the sojourn time in the preclinical state S_p.

Consider a cohort of men or women in the study of interest who are all aged t_0 at study entry, and a protocol for K ordered screening exams occurring at ages $t_0 < t_1 < \cdots < t_{K-1}$, where $t_i = t_0 + i$ for annual screening exams. Define the i-th screening interval as the time interval between the i-th and the (i+1)-th screening exams (t_{i-1}, t_i), i=1, 2,…, K-1. We let $t_{-1} \equiv 0$. For each screening exam, let n_{i,t_0} be the total number of individuals in this cohort examined at the i-th screening, s_{i,t_0} is the number of diagnosed and confirmed cancer cases

at the i-th screening exam, and r_{i,t_0} is the number of cases diagnosed in the clinical state S_c within the interval (t_{i-1}, t_i), the interval cases.
The likelihood function for each gender cohort is:

$$L = \prod_{t_0} \prod_{k=1}^{K} D_{k,t_0}^{s_{k,t_0}} I_{k,t_0}^{r_{k,t_0}} (1 - D_{k,t_0} - I_{k,t_0})^{n_{k,t_0} - s_{k,t_0} - r_{k,t_0}} . \quad (1)$$

To facilitate the understanding of this likelihood function, we will describe it in terms of the MCCCS age groups. In the MCCCS, the initial age of participants varied from 28 to 90 years old, among men, and 36 to 93 years old, among women, so this is the range of t_0. Because the MCCCS required five annual FOBT screenings, K = 5. The D_{k,t_0} is the probability that an individual will be diagnosed at the k-th scheduled exam given that she/he is in S_p (see equation 2 and 3); and the I_{k,t_0} is the probability of being an incident case in the k-th screening interval (see equation 4).

$$D_{1,t_0} = \beta_0 \int_0^{t_0} w(x) Q(t_0 - x) dx, \quad (2)$$

$$D_{k,t_0} = \beta_{k-1} \left\{ \sum_{i=0}^{k-2} [1-\beta_i] \cdots [1-\beta_{k-2}] \int_{t_{i-1}}^{t_i} w(x) Q(t_{k-1} - x) dx + \int_{t_{k-2}}^{t_{k-1}} w(x) Q(t_{k-1} - x) dx \right\}, \quad (3)$$
$$k = 2, \ldots K.$$

$$I_{k,t_0} = \sum_{i=0}^{k-1} [1-\beta_i] \cdots [1-\beta_{k-1}] \int_{t_{i-1}}^{t_i} w(x) [Q(t_{k-1} - x) - Q(t_k - x)] dx + \int_{t_{k-1}}^{t_k} w(x) [1 - Q(t_k - x)] \, dx \ , \ k = 1, \ldots K. \quad (4)$$

Where $\beta = \beta_i = \beta(t_i)$ in the above formulae. We modelled the age effect t and the time duration x in the preclinical state very carefully using a parametric model stated in equation 5.

$$\beta(t) = \frac{1}{1 + \exp(-b_0 - b_1 * (t - m))},$$
$$w(t) = \frac{0.1}{\sqrt{2\pi}\sigma t} \exp\{-(\log t - \mu)^2 / (2\sigma^2)\}, \quad (5)$$
$$q(x) = \frac{\kappa x^{\kappa-1} \rho^{\kappa}}{(1 + (x\rho)^{\kappa})^2}.$$

Where m is the average age-at-entry in the study and $b_0, b_1, \mu, \sigma^2, \kappa, \rho$ are unknown parameters to be estimated. For simplicity, we will include these parameters in a vector form as $\theta = (b_0, b_1, \mu, \sigma^2, \kappa, \rho)$. If $b_1 > 0$, then $\beta(t)$ will be a monotone increasing function of age t. Usually, researchers can establish an upper bound for $w(t)$ from pilot studies or from health department data. For example, in the MCCCS, we picked 10% as a reasonable upper bound for $w(t)$ because the lifetime risk of being diagnosed with colorectal cancer reported by the National Cancer Institute (SEER Cancer Statistics Review 1975-2007, 2010) for years

2005-2007 was about 4.97% for females and 5.30% for males. Wu et al. 2005, 2009b shows the detailed justifications on how these age effect functions were chosen. Models in equation 1-5 were estimated using programs C/C++ (Silicon Graphics, I, 2003, Stroustrup, B, 2011) and we applied the likelihood separately for men and women in the MCCCS. Markov Chain Monte Carlo (MCMC) was used to generate random samples from the joint posterior distribution of the parameters in the likelihood for Bayesian inference (Wu et al., 2005, 2009b). The posterior distribution within the MCMC was partitioned into four sub-chains, e.g. sampling the posterior distribution for $(b_0, b_1), \mu, \sigma^2, (\kappa, \rho)$ separately. Non-informative priors were used for all parameters (Wu et al., 2009b). Each MCMC was run for 20,000 steps; after a burn-in of 15,000 iterations, then posterior samples were collected every 20 steps, which finally provided 250 samples from each chain (Wu et al., 2009b). Because four over-dispersed chains were simulated using MCMC, a pool of 1000 posterior samples were used for the analysis presented below. These Bayesian posterior samples are notated as θ_j^*. Bayes estimates of the highest posterior density (HPD) interval were also computed, which are similar to confidence interval from a frequentist perspective and also known as credible intervals from the Bayesian perspective.

2.2 Results

The original FOBT screening data from MCCCS for each age group, male and female, are published in table 1 and 2 in Wu et al. 2009b. The posterior estimates for parameters θ and the standard errors for each gender are listed in table 3 in Wu et al. 2009b. The age-dependent Bayesian estimates of the sensitivity β and the transition density $w(t)$ for males and females are listed in table 2 and 3 here.

Age	Sensitivity β			Transition probability[a] for $w(t)$		
	Median	Mean	S.E.	Median	Mean	S.E.
30	0.117	0.325	0.372	0.024	0.113	0.190
35	0.192	0.361	0.368	0.090	0.207	0.258
45	0.458	0.494	0.331	0.488	0.562	0.332
55	0.791	0.749	0.213	1.101	1.122	0.278
65	0.943	0.863	0.187	1.594	1.597	0.381
75	0.980	0.879	0.191	1.683	1.692	0.467
85	0.994	0.868	0.219	1.474	1.442	0.364

[a]The unit is 10^{-3}.

Table 2. Bayesian posterior estimates of β and $w(t)$ for male participants.

The sensitivity appears to increase with age for both male and female. A Bayes hypothesis test of $H_0 : b_1 \le 0$ versus $H_1 : b_1 > 0$ showed that for males, the posterior probability of a positive slope was $P(b_1 > 0 \mid Data) = 0.806$; for females, this posterior probability of a positive slope was 0.941.

The age-dependent transition probability was itself a sub-pdf from our model assumption. The posterior mean transition probability varied from 0.113×10^{-3} to 1.707×10^{-3} for males aged 30 to 90 and varied from 0.069×10^{-3} to 2.009×10^{-3} for females aged 40 to 90. The transition probability was not a monotone function of age, having a single maximum at age 72 for males and a single maximum at age 75 for female.

Age	Sensitivity β			Transition probability [a] for		
	Median	Mean	S.E.	Median	Mean	S.E.
45	0.333	0.418	0.315	0.113	0.162	0.161
55	0.802	0.769	0.191	0.600	0.624	0.228
65	0.976	0.920	0.122	1.413	1.427	0.273
75	0.996	0.955	0.093	1.968	2.009	0.468
85	0.999	0.964	0.098	1.931	1.946	0.392

[a]The unit is 10^{-3}.

Table 3. Bayesian posterior estimates of β and $w(t)$ for female participants.

The posterior mean sojourn time was 4.08 years for males and 2.41 years for females, with a posterior median of 1.66 years for males and 1.88 years for females. The 95% HPD interval was (0.97, 20.28) for males and (1.15, 5.96) for females. This shows that CRC may have a large variation in sojourn time, as oncologists believe that CRC usually has a long sojourn time more than 5 years.

3. Distribution of the lead time in colorectal cancer screening

The goal of screening is to catch the disease before clinical symptom appears. This means that the detection and removal of any precancerous growth is important as well as the diagnosis of cancer at an early stage. To understand this, several time events are essential to prevention efforts and they will be described briefly here. If a person enters the preclinical state (S_p) at age t_1, and his/her clinical symptoms present later at age t_2, then $(t_2 - t_1)$ is the sojourn time in the preclinical state. If a person is offered a screening exam at some time point t within the interval (t_1, t_2), and cancer is diagnosed, then the length of the time $(t_2 - t)$ is the lead time. We consider lead time as the time gained by screening for that particular person.

3.1 Methods

We will briefly review the probability distribution of the lead time derived under a progressive disease model (Wu et al., 2007, 2009a). Assume there are *K* ordered screenings that, for a specific individual, occur at ages $t_0 < t_1 < \cdots < t_{K-1}$. The lead time distribution is a conditional distribution given that someone will develop clinical disease before death. We let D represent a Bernoulli random variable, with $D=1$ indicating the development of clinical disease and $D=0$ indicating the absence of the clinical disease before death. We use L to denote the lead time. We consider the lead time to be zero for individuals whose disease

is not detected by the regular screening exam but who develops clinical symptoms between exams. The distribution of the lead time is a mixture of the conditional probability $P(L=0\,|\,D=1)$ and the conditional probability density function $f_L(z\,|\,D=1)$, for any $0<z\le T-t_0$. Here, T represents the span of the human life, which is a fixed upper bound, and t_0 is the individual's age at his/her initial screening exam. We define the same $\beta(t), w(t), q(x), Q(x)$ as in Section 2.1. The distribution for the lead time was derived and presented in equation 6 (Wu et al., 2007, 2009a).

$$P(L=0\,|\,D=1)=\frac{P(L=0,D=1)}{P(D=1)} \text{ and } f_L(z\,|\,D=1)=\frac{f_L(z,D=1)}{P(D=1)}. \tag{6}$$

Where $P(D=1)=\int_0^{t_0} w(x)[Q(t_0-x)-Q(T-x)]dx+\int_{t_0}^{T} w(x)[1-Q(T-x)]dx$, is the probability of developing colorectal cancer in one's life time after age t_0.

The lead time is zero if and only if the individual is an interval case, therefore the joint probability $P(L=0,D=1)=I_{K,1}+I_{K,2}+\cdots+I_{K,K}$, where $I_{K,j}$ is the probability of an interval case within the interval (t_{j-1},t_j), and it was derived as:

$$\begin{aligned} I_{K,j}=&\sum_{i=0}^{j-1}(1-\beta_i)\cdots(1-\beta_{j-1})\int_{t_{i-1}}^{t_i} w(x)[Q(t_{j-1}-x)-Q(t_j-x)]dx \\ &+\int_{t_{j-1}}^{t_j} w(x)[1-Q(t_j-x)]dx. \end{aligned} \tag{7}$$

for all j=1, 2… K, with $\beta_i=\beta(t_i)$ is the sensitivity at age t_i. The joint PDF $f_L(z,D=1)$ in equation 6 was derived and presented as:

$$\begin{aligned} f_L(z,D=1)=&\sum_{i=1}^{j-1}\beta_i\left\{\sum_{r=0}^{i-1}(1-\beta_r)\cdots(1-\beta_{i-1})\int_{t_{r-1}}^{t_r} w(x)q(t_i+z-x)dx+\int_{t_{i-1}}^{t_i} w(x)q(t_i+z-x)dx\right\} \\ &+\beta_0\int_0^{t_0} w(x)q(t_0+z-x)dx. \end{aligned} \tag{8}$$

Where $z\in(T-t_j,T-t_{j-1}), j=2,\cdots K$; and when j=1, it is simplified as:

$$f_L(z,D=1)=\beta_0\int_0^{t_0} w(x)q(t_0+z-x)dx \text{, if } z\in(T-t_1,T-t_0). \tag{9}$$

We used the posterior samples θ_j^* estimated from equation 5 to project the lead time distribution for the male and female participants. To do this, the posterior predictive distribution of the lead time can be estimated by MCMC (Wu et al., 2009a) as stated in equation 10.

$$f(z\,|\,Data)=\int f(z,\theta\,|\,Data)d\theta=\int f(z\,|\,\theta,Data)f(\theta\,|\,Data)d\theta\approx\frac{1}{n}\sum_j f(z\,|\,\theta_j^*). \tag{10}$$

Where $f(z\,|\,\theta_j^*)$ represents the mixture distribution in equation 6. The sample size n is 1000 in section 2 from the MCMC.

3.2 Results

We applied our method to make predictive inference of the lead time using FOBT for males and females. We assumed for this simulation that the initial age is 50, and an ending age of 80. It is clear that the lead time distribution is a function of the sensitivity, the sojourn time distribution, the transition density, the screening frequency, and the initial age and ending age. Accurate estimation of the sensitivity, the sojourn time distribution and transition density were acquired from MCCCS study in section 2. Now, we plugged the estimates obtained from Section 2 into the simulation equation 10 in Section 3.1, leading to the estimation of the lead time distribution under different screening scenarios. In other words, we estimated what the results would be if people were screened at different screening intervals. The results are summarized in table 2 in Wu et al. 2009a. The time interval between screens was 6, 9, 12, 18 and 24 months, within ages 50 (t_0) and 80 years (T). Also, the density curves for the lead time are shown in Figure 2 and 3 in Wu et al. 2009a for different screening intervals for both males and females. From those results, if a man begins annual screening (i.e. Δ = 12 months) when he is 50 years old and continues until he reaches 80, then there is a 18.87% chance that he will not benefit from early detection by the screening program if he develops colorectal cancer during those thirty years. His chance of no-early-detection from the screening program decreases to 6.45% for screening exams conducted 6 months apart. While for females, the chance of no-early-detection is 9.48% for annual screenings and 2.39% for screening every 6 months.

Also, Table 2 in Wu et al. 2009a showed that the mean lead time increases as the screening time interval decreases for both males and females. In other words, more screening exams will contribute to a longer lead time, which would translate to treatment of the disease at an earlier stage and, potentially improved prognosis. The increase in the mean lead time is partly due to the smaller point mass for zero lead time when screening exams are closer together. The standard error of the lead time decreases as the time between screening exams increases. Similarly, Table 2 in Wu et al. 2009a revealed that the standard deviation for the lead time was larger than the mean lead time (Wu et al., 2009a). In the same table, the mode of the lead time, which is the value that is most likely taken by the lead time when it is positive, was 0.68 years (or 8 months), corresponding to screening exams every 6 months for males, and 0.96 years (or 11.5 months) for females (Wu et al., 2009a). With annual exams, the mode value for the lead time is 0.60 years (6 months) for males and 0.78 years (9.4 months) for females.

4. Evaluating long term screening outcomes in colorectal cancer

Recently there have been heated arguments in the topic of over diagnosis, the diagnosis of ``disease'' that will not cause symptoms or death during a patient's lifetime (Lichtenfeld, J L, 2010). Some profound questions should be asked with regards to over diagnosis. How do we evaluate the long-term outcomes due to continuous regular screening? Will regular screening exams contribute to a greater chance of over diagnosis? What are the percentages of over diagnosis and true-early-detection among the screen-detected cancer patients? How should the probability of no-early-detection and the probability of disease-free-life be estimated?

Some research has been done in the area of over diagnosis. However, the majority of research in this area has been based on observational studies, and mainly in breast cancer

(Day, 2005, Duffy et al., 2008, Welch & Black, 2010, Zackrisson et al., 2006), there is little reference to this problem in colorectal cancer. The flaws of using observational studies are obvious: (a) the result based on one study cannot be extended to other scenarios. The reason is that for one particular study, with one particular screening interval, the result may be correct, however, one cannot use this result to make inference for studies with different screening intervals or different cohorts without probability modelling. On the other hand, it is clear that it is of great value to policy makers to know how the proportion of over diagnosis is changing with screening frequency, sensitivity of the screening modality, and other risk factors; (b) using observational studies usually needs a long follow-up period to collect cancer incidence data from both the screening group and the control group, because most of the observational studies compares the incidence rates in the two groups to estimate over diagnosis. This is not cost effective, and the inference maybe biased.

To overcome these flaws, we used probability modelling, and instead of dealing with over diagnosis alone, we will address the long-term outcomes for the whole cohort, with over diagnosis as one outcome. All initially superficially healthy participants will be classified into four mutually exclusive categories: true-early-detection, no-early-detection, over diagnosis and symptom-free-life (Wu & Rosner, 2010).

- Case 1 (Symptom-free-life or SympF): A person who took part in screening exams that never detected colorectal cancer, and ultimately the person died of other causes.
- Case 2 (No-early-detection or NoED): A person who took part in screening exams, but whose disease manifest itself clinically and was not detected by screening.
- Case 3 (True-early-detection or TrueED): A person whose colorectal cancer was diagnosed at a scheduled screening exam and whose clinical symptoms would have appeared before death.
- Case 4 (Over diagnosis or OverD): A person who was diagnosed with colorectal cancer at a scheduled screening exam but whose clinical symptoms would NOT have appeared before death.

Every participant who takes part in the screening will eventually fall into one of these four outcomes. It is hoped that this will provide a systematic approach and a frame work for the evaluation of long term outcomes in cancer screening.

4.1 Methods

For an initially asymptomatic individual taking K screenings at their ages $t_0 < t_1 < \cdots < t_{K-1}$, the conditional probability for each of the four cases was derived, given that their lifetime $T = t_K (> t_{K-1})$ and presented in equations 11-15.

$$P(Case \quad 1 : SympF \mid T = t_K) = 1 - \int_0^{t_K} \omega(x)dx + \int_{t_{K-1}}^{t_K} \omega(x)Q(t_K - x)dx + \sum_{j=0}^{K-1} (1-\beta_j)...(1-\beta_{K-1}) \int_{t_{j-1}}^{t_j} w(x)Q(t_k - x)dx \tag{11}$$

$$P(Case \quad 2 : NoED \mid T = t_k) = I_{K,1} + I_{K,2} + \cdots + I_{K,K}. \tag{12}$$

Where

$$I_{K,j} = \sum_{i=0}^{j-1}(1-\beta_i)...(1-\beta_{j-1})\int_{t_{i-1}}^{t_i}\omega(x)[Q(t_{j-1}-x)-Q(t_j-x)]dx + \int_{t_{j-1}}^{t_j}\omega(x)[1-Q(t_j-x)]dx, \text{ for all} j = 1, 2 \ldots, K. \quad (13)$$

$I_{K,j}$ is the probability of being an interval case in the interval (t_{j-1}, t_j) in a sequence of K screening exams.

$$P(Case \quad 3: TrueED \mid T = t_K) = \sum_{j=1}^{K-1}\beta_j\{\sum_{i=0}^{j-1}(1-\beta_i)...(1-\beta_{j-1})\int_{t_{i-1}}^{t_i}\omega(x)[Q(t_j-x)-Q(t_K-x)]dx + \int_{t_{j-1}}^{t_j}\omega(x)[Q(t_j-x)-Q(t_K-x)]dx\} + \beta_0\int_0^{t_0}\omega(x)[Q(t_0-x)-Q(t_K-x)]dx \quad (14)$$

$$P(Case \quad 4: OverD \mid T = t_K) = \sum_{j=1}^{K-1}\beta_j\{\sum_{i=0}^{j-1}(1-\beta_i)...(1-\beta_{j-1})\int_{t_{i-1}}^{t_i}\omega(x)Q(t_K-x)dx + \int_{t_{j-1}}^{t_j}\omega(x)Q(t_K-x)dx + \beta_0\int_0^{t_0}\omega(x)Q(t_K-x)dx\} \quad (15)$$

For an individual currently at age t_0, his/her lifetime is random, and it would not be practical to fix the number of screening exams to any fixed number K. If, however, he/she follows a pre-planned screening schedule, or, more simply, if he/she plans to be screened every 12, 18, or 24 months, then the probability of each outcome when his/her lifetime T is longer than t_0 can be obtained using equation 16.

$$P(Case \quad i \mid T \geq t_0) = \int_{t_0}^{\infty} P(Case \quad i \mid K = K(t), T = t) f_T(t \mid T \geq t_0)dt, \text{ for i = 1,2,3,4.} \quad (16)$$

Where the lifetime probability density function $f_T(t \mid T \geq t_0)$ is defined in equation 17.

$$f_T(t \mid T \geq t_0) = \frac{f_T(t)}{P(T > t_0)} = \frac{f_T(t)}{1 - F_T(t_0)}, \text{ if } t \geq t_0. \quad (17)$$

The probability $P(Case \quad i \mid K = K(t), T = t)$ was derived in equations 11-15, and the number of screening exams $K = K(t) = \lfloor (t - t_0)/\Delta \rfloor$, is the largest integer that is less than or equal to $(t - t_0)/\Delta$. Hence, K is a random variable as well, taking integer values and changing with one's lifetime T. It was proved that for any screening number K,

$\sum_{i=1}^{4} P(Case \quad i \mid A, T \geq t_0) = 1$. Where the event A = {an individual is asymptomatic of colorectal cancer before age t_0} and

$$P(A \mid T \geq t_0) = 1 - \int_0^{t_0} w(x)dx + \int_0^{t_0} w(x)Q(t_0 - x)dx. \tag{18}$$

The probability for each of the four cases is a function of the sensitivity $\beta(t)$, the transition probability density $w(t)$, the sojourn time distribution $q(x)$, a person's age at the first screening t_0 and his/her future screening interval Δ. The age-dependent sensitivity $\beta(t)$, the age-dependent transition probability, and the sojourn time distribution $q(x)$, were estimated from the MCCCS data (Wu et al., 2009a) and were given in Section 2.2.
Given the MCCCS data, the posterior predictive probability of each case can be estimated as:

$$P(Case \quad i \mid T > t_0, MCCCS) \approx \frac{1}{n} \sum_j P(Case \quad i \mid T > t_0, \theta_j^*). \tag{19}$$

Where θ_j^* is the MCMC random sample drawn from the posterior distribution and $n = 1000$ is the posterior sample size.
Furthermore, we defined a diagnosed case as when either an interval clinical incident case or a screen-detected case happens in a study. Researchers may be interested in the proportion of "no-early-detection", "true-early-detection" and "over diagnosis" given that it is a diagnosed case. For example, among females, what are the estimated probabilities of "no-early-detection", given that a woman has been diagnosed with colorectal cancer, either through scheduled screening exam or not. Last but not least, researchers are most interested in the probabilities of "true-early-detection" and "over diagnosis" given that it is a screen-detected case. All of these conditional probabilities were also estimated using equations 12-19 using the definition of conditional probability.

4.2 Results

In section 2.2 we estimated θ_j^* as a MCMC random sample drawn from the posterior distribution. A total of 1000 posterior samples were put into equation 19 to conduct the Bayesian inference. This Bayesian inference assumed that there is a program consisting of periodic screening exams from three hypothetical cohorts of asymptomatic individuals. Those cohorts have initial ages of 40, 50 and 60 at the first screening exam for males or females. For each group, we examined various screening frequencies, with screening interval Δ = 12, 18, and 24 months. For the lifetime distribution, we used the actuarial life table from the Social Security Administration, which was published online for year 2007 (Social Security Administration, 2011). The actuarial life table is based on mortality and provides the probability of death within one year from age 0 to age 119 years old.
Based on that life table, we derived the conditional lifetime distribution $f_T(t \mid T > t_0)$ (Wu and Rosner 2010) and estimated the probabilities of each of the four cases, i.e. $P(Case \quad i \mid A, T \geq t_0, MCCCS)$, using the estimations of sensitivity, sojourn time distribution,

and transition probability obtained from the MCCCS data for males and females. Overall, the proportion of over diagnosis was very small, less than 0.3% for any age and gender.

The probability of "true-early-detection" for males was between 1.91% (at 60 years old, with 24 months as screening interval) and 3.28% for 40 years old, with 12 months as screening interval. Correspondingly, the probability of "true-early-detection" for females was between 2.75% for 60 years old, with screening interval of 24 months and 3.76% for 40 years old, with screening interval of 12 months. Regardless of the age, the probability of "true-early-detection" slightly decreased as the screening time interval increased and overall, the probability of "true-early-detection" was consistently lower for males than for females.

The probability of "no-early-detection" for males was between 0.53% for 60 years old, with screening interval of 12 months to 1.95% for 40 years old, with screening interval of 24 months. The probability of "no-early-detection" for females was between 0.29% for 60 years old, with screening interval of 12 months to 1.34% for 40 years old, with screening interval of 12 months. In general, the probability of "no-early-detection" slightly increased as the screening interval increased, and slightly decreased as the age at initial screening was older.

The probability of "symptom-free-life" was very large (e.g. over 95%). Regardless of age or gender, the probability of "symptom-free-life" was almost constant for any number of months between two screenings. For example, among 50 years old males, the probability of "symptom-free-life" was 95.84% if 12 months was the screening time interval; 95.87% if 18 months was the screening time interval; and 95.90% if 24 months was the screening time interval. The sum of the probability of all four cases should add up to 1, and it was observed in the simulation, the total probability is above 0.998, due to simulation accuracy, this is clinically insignificant.

The box plot of the probability for each case when t_0 =60 is given in Figure 1. Within each box plot, the three left-hand-side boxes represent females and the three right-hand-side boxes represent males, and these probabilities are presented at different screening intervals. We decided to present the box plots when the initial screening age was 60 but similar box plots were observed for t_0 = 40 and 50. Again, we see in Figure 1 that the probability of "symptom-free-life" and the probability of "over diagnosis" are pretty stable over the screening time intervals, regardless of gender. The probability of "no-early-detection" increased monotonically with the screening time interval, while the probability of "true-early-detection" decreases monotonically with the length of the screening time interval.

The estimated conditional probabilities of "no-early-detection", "true-early-detection" and "over diagnosis", given that it is a diagnosed case, for females and males were estimated. Among the initial age of 40 years-old women group, the percentage of over diagnosis given that it was a diagnosed cancer case was 5.04%, if she was screened every 24 months apart; and 6.50%, if she was screened every 12 months apart. Similarly, the estimated conditional probabilities of "true-early-detection" and "over diagnosis" given that it is a screen-detected case, for females and males were also estimated. Among the 40 years-old women initial age group, the percentage of over diagnosis among the screen-detected cases was 6.75% (95% HPD: 2.56%-19.27%), if screened every 24 months apart, and 7.12% (95% HPD: 2.76%-19.91%), if screened every 12 months apart.

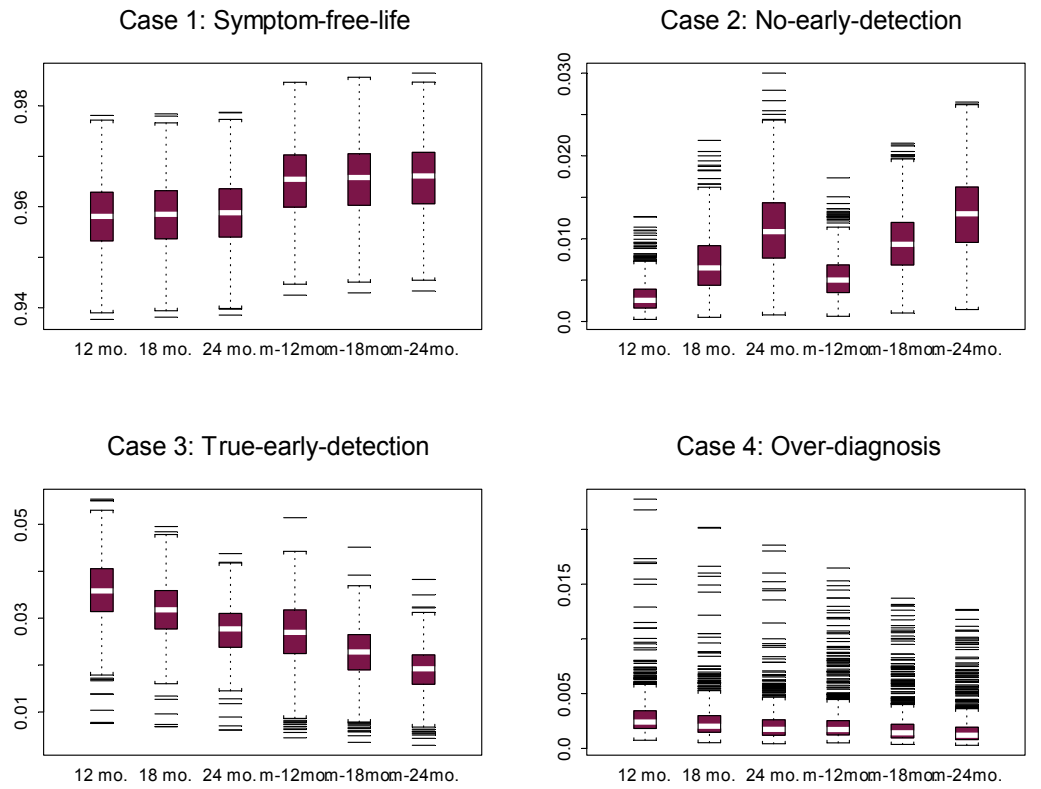

Fig. 1. The box plot of the estimated percentage for each outcomes, with t_0 =60.

Figure 2 shows the probabilities of "true-early-detection" and the probability of "over diagnosis" among those whose cancer would be diagnosed by regular screening exam for the initial-age-60 group of both genders. In Figure 2, the screening time interval for males and females are presented for 12, 18 and 24 months. The estimated mean percentage for "true-early-detection" and "over diagnosis" given that it is a screen-detected case was similar for males and females. However, the 95% C.I. for males were much larger than that for females; this indicates that there is more uncertainty of these probabilities for males.

5. Discussion

We presented some results in probability modelling and statistical inference in colorectal cancer screening, using FOBT as an example. As we have shown in section 2, the three key parameters are the sensitivity of the screening modality, the transition probability of the disease, and the sojourn time distribution. All other parameters of interests can be expressed as a function of these three key parameters, hence accurate estimation of them is very important. These three key parameters are the building blocks in the cancer screening model, many researchers are striving to improve the modelling and get more accurate estimates of these parameters.

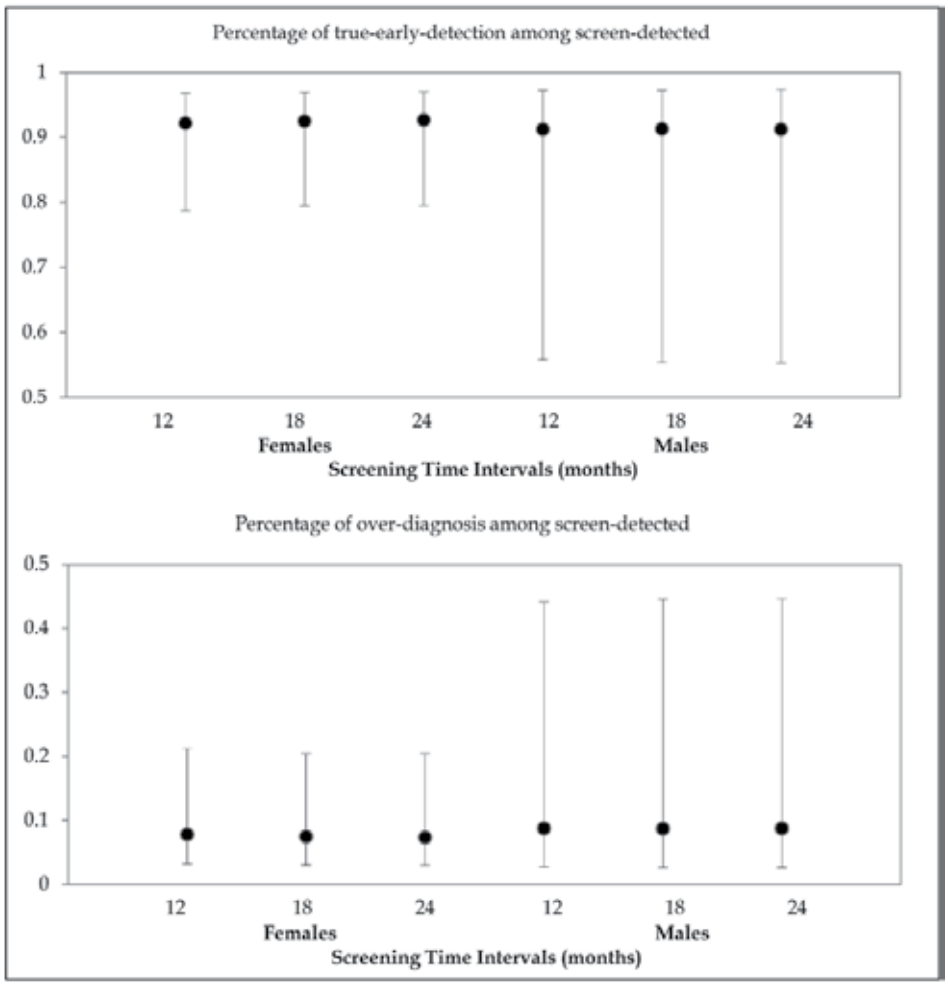

Fig. 2. Estimated percentage of true-early-detection and over diagnosis with its 95%HPD for t_0 =60.

Although, other researchers have also estimated the sensitivity and the mean sojourn time in fecal Hemoccult testing, using data from Calvados, France between 1991 and 1994 (Prevost et al., 1998), their models are different from the progressive model that we used here. Prevost et al. (1998) modelled the incidence of cancer as a Poisson random variable, with different parameter value for the mean of the Poisson distribution (Prevost et al., 1998). Another difference is that their sojourn time was assumed to follow an exponential distribution. They reported that the mean sojourn time increases with age, which is approximately two years among 45-54 years-old, 3 years among 55-64 years-old, and 6 years among 65-74 years-old (Prevost et al., 1998). Their estimation of sensitivity decreases with age, which is approximately 75% among 45-54 years-old, 50% among 55-64 years-old, and 40% among 65-74 years-old (Prevost et al., 1998).

Church et al (1997) used the same Minnesota study (MCCCS) to estimate the sensitivity. However, their estimate of program sensitivity is about 90%, regardless of age (Church et

al., 1997). Our estimates are more accurate for different age groups as reported in section 2. There are other data sets that were used to estimate the FOBT screening sensitivity and mean sojourn time. For example, French data reported by Launoy et al. (1997) estimated the FOBT mass-screening sensitivity to be about 50% (Launoy et al., 1997). Their estimated mean sojourn time was longer than our results, between 4.5 and 5 years for all combined cancer cases. Also, these researches showed that the estimation of the sensitivity and the sojourn time maybe negatively correlated (Launoy et al., 1997). Better modelling strategies are needed to handle this situation. We plan to explore solutions accounting for the negative correlation between the sensitivity and the sojourn time to solve this problem.

There is little research in the topic of lead time bias or the lead time distribution except in Wu et al. (2009) (Wu et al., 2009a). Since there is convincing evidence that FOBT and/or other colorectal screening modalities can significantly reduce mortality (Mandel et al., 1999), the U.S. Preventive Services Task Force has recommended screening people between 50-75 years-old since 2008 (U.S.Preventive Services Task Force, 2008). Unfortunately, the compliance to colorectal cancer screening is low in the U.S. and the world (Sarfaty & Wender, 2007). We hope the lead time results from our simulations and models (Section 3) can provide some helpful information to general audiences about the benefit of taking screening exams.

There is almost no research in the topic of over diagnosis or long-term outcomes in colorectal cancer, to our knowledge. As the first of the baby boomer generation turns age 65 this year, evaluating the long-term outcomes will provide useful information and great insights to policy makers. We hope our method will provide a frame work and a systematic approach for evaluation purposes. To explore this topic more, we will need to obtain more recent screening data. We are exploring if data from the Prostate, Lung Colorectal and Ovarian (PLCO) cancer screening trial can be released to us (National Cancer Institute Division of Cancer Prevention, 2011).

Our future research topic includes three areas: (1) exploring the relationship between sensitivity and the sojourn time distribution, and building up a better modelling strategy for these three parameters; (2) exploring the optimal screening interval based on an individual's screening history; and (3) exploring the survival benefit from screening after removing the lead time bias, hence we can have a better understanding of what we gained from screening. We hope the research will benefit the health of the general population.

6. Acknowledgment

The authors thank Laura Littler for her research assistant help and in proof-reading this manuscript. We thank the anonymous reviewers for their valuable suggestions and comments for improving this paper. The second author was supported by a research supplement (3R37CA057030-20S1) from the National Cancer Institute and by a grant from the Michael and Susan Dell Foundation during the writing of this chapter.

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Dietary Risks: Folate, Alcohol and Gene Polymorphisms

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1. Introduction

Folate, as a member of the water-soluble B-group vitamins, is found widely in foodstuffs. Folate cannot be synthesized by human therefore dietary intake is the only source for human to obtain folate. The pteropolyglumates, usually with 1~6 glutamic acid molecules, are the major forms of natural food folates (Lucock 2000) and the 5-methyl tetrahydrofolate (5-metTHF), derived from hydrolization of absorbed folate and folic acid (the synthetic form of folate) as well, is the primary form in circulation (Ulrich 2005; Pietrzik, Bailey et al. 2010). Through the transmembrane transportation, the 5-metTHF in cell can be reduced by dihydrafolate reductase to tetrahydrofolate (THF) that is directly involved in metabolic process (Lucock 2000; Pietrzik, Bailey et al. 2010), and then performs biological functions in several ways. THF can be metabolized to 5,10-methylene-THF and further be irreversibly reduced into 5-metTHF which is the key step in one-carbon unit metabolism that is catalyzed by the enzyme methylenetetrahydrofolate reductase (MTHFR). By using the methyl donated by 5-methyltetrahydrofolate, the enzyme methionine synthase (MS) converts homocystine to methionine and then the *de novo* synthesized methionine can be catalyzed by the methionine adenosyl transferase to yield S-adenosylmethionine which directly provides methyl for a variety of important *in vivo* methylation reactions (Lucock 2000; Sanderson, Stone et al. 2007). By using 5,10-methylene-THF as methyl donor, the enzyme thymidylate synthase converts deoxyuridylate (dUMP) to deoxythymidylate (dTMP), meanwhile the 5,10-formyltetrahydrofolate from 5,10-methylene-THF is involved in the production of both adenosine and guanosine, all are physiological building blocks of DNA replication (Bollheimer, Buettner et al. 2005; Duthie 2011). Thus, the most prominent function of folate is to transfer and process the one-carbon unit which is needed for methylation reactions and synthesis of thymine and purines. Consequently, folate deficiency

may biologically implicated in physiological processes including base misincorporation and DNA strand breaks, insufficient *de nove* nucleotide synthesis, as well as impaired DNA repair and methylation (Lucock 2000; Ames 2001; Kim 2003; Sanderson, Stone et al. 2007; Duthie 2011).

Therefore, folate has been implicated in colorectal cancer (CRC) because that the steps of folate metabolism may be involved in distinct biological process. A number of epidemiologic and experimental studies concluded that folate may have an inverse association with risk of CRC, however, the results are not consistent and it is argued that too much folate may be unfavourable for preventing the development of CRC especially in those with precursor lesions such as invisible minor adenoma (Giovannucci 2002; Sharp and Little 2004; Strohle, Wolters et al. 2005; Kim 2006; Sanderson, Stone et al. 2007; Sauer, Mason et al. 2009; Kennedy, Stern et al. 2011). The distinct effects of folate on the development of CRC in populations with diversely genetic background suggest that genetic factors, as well as the interaction with folate intake and other coenzymatical factors, may also play a role in the prevention or promotion of colorectal carcinogenesis (Giovannucci 2002; Sharp and Little 2004; Kim 2006; Arasaradnam, Commane et al. 2008; Hubner and Houlston 2009). Growing evidence revealed that the polymorphisms in key folate-metabolism genes may also modify CRC risk in relation to folate intake; but the results are not consistent. Studies suggested that several functional polymorphisms in key genes involved in folate metabolism, such as *MS* A2756G, *MTHFR* C677T and A1298C, may associate with risk of CRC (Giovannucci 2002; Sharp and Little 2004; Kim 2007; Sanderson, Stone et al. 2007; Yu, Zhang et al. 2010). For the *MS* gene, it is still debatable to what extent can the *MS* 2756G variant modulate enzyme activity and plasma homocysteine levels, though evidence from epidemiological studies suggests an association between *MS* A2756G polymorphism and risk of CRC (DeVos, Chanson et al. 2008; Yu, Zhang et al. 2010). To date, several published studies have investigated the role of the *MS* 2756G variant and its interaction with folate intake in the etiology of CRC, mainly in western but few in Chinese populations (Chen, Giovannucci et al. 1998; Ma, Stampfer et al. 1999; Goode, Potter et al. 2004; Ulvik, Vollset et al. 2004; Chen, Jiang et al. 2005; Matsuo, Ito et al. 2005; Ulrich, Curtin et al. 2005; Koushik, Kraft et al. 2006; Theodoratou, Farrington et al. 2008; Yamaji, Iwasaki et al. 2009; Yu, Zhang et al. 2010). For the *MTHFR* gene, the 677T variant encodes an enzyme that is thermolabile, and the heterozygous CT or homozygous TT genotype have nearly 35% or 70% reduction in normal function of the enzyme *in vitro*, respectively (Molloy, Daly et al. 1997). Similarly, the 1298A>C change leads to a decrease in the enzyme activity reportedly *in vitro*, but to a lesser extent, compared with the 677T variant (Weisberg, Jacques et al. 2001). Although the association between *MTHFR* C677T or A1298C polymorphism and CRC risk has also been extensively investigated, the results are not consistent (Chen, Giovannucci et al. 1996; Ma, Stampfer et al. 1997; Chen, Giovannucci et al. 1998; Chen, Ma et al. 2002; Sharp and Little 2004; Ulvik, Vollset et al. 2004; Matsuo, Ito et al. 2005; Huang, Han et al. 2007; Kim 2007).

In addition, alcohol drinking, as one of the known risk factors for CRC, can interfere with the metabolism of folate and one-carbon unit and thus may alter CRC risk in subjects carrying different genotypes. Some earlier studies on folate intake reported that the favourable effects of folate or some genotypes (such as the *MTHFR* 677TT genotype) can be conversely modified by alcohol drinking (Giovannucci 2004; Mason and Choi 2005; Matsuo, Ito et al. 2005). Many, but not all, epidemiological studies further suggested that the interactions among folate intake, alcohol drinking and polymorphisms in genes involved in

folate metabolism are likely presented in the etiology of CRC risk, but the published results were not consistent (Giovannucci 2004; Mason and Choi 2005; Matsuo, Ito et al. 2005; Kim 2007; Kim 2007). Several published studies have investigated the role of folate metabolizing gene polymorphisms and their interactions with folate intake or alcohol drinking in the etiology of CRC, but few in Chinese populations. Therefore, we performed a case-control study to assess the effect of folate intake and some reported functional polymorphisms in genes involved in folate metabolism in the etiology of CRC in Chinese populations, either for their individual effects or the joint effects with alcohol consumption and tobacco smoking.

2. Materials and methods

2.1 Subjects

All cases and controls were recruited from those who were registered into three hospitals of Chongqing City, Southwest China, between January 2001 and September 2004. All cases were newly diagnosed and histopathologically confirmed as having CRC, without any prior cancer history or any chemo-radiotherapies. Controls were cancer-free inpatients from those who had no other severe diseases (i.e., severe cardiovascular diseases, diabetes, severe hypertension, fatty liver and hepatocirrhosis), and without cancer history. Cases and controls were frequency matched by sex, age (±5 years), residence (the same city or county). All subjects were aged between 30-80 years and asked during personal interview to provide a one-time 2~5 ml peripheral blood sample and to complete a questionnaire that elicited information about lifestyles including alcohol drinking and tobacco smoking (1 year prior to the diagnosis for the cases and the time at recruitment for the controls). This study was approved by the Research Ethics Committee of The Third Military Medical University. All subjects provided a signed written informed consent or oral consent if illiterate. Finally, of a total of 1082 cases and 949 controls, we recruited 478 eligible cases (185 colon and 293 rectal cancer) and 838 eligible controls, who had consented to the present study, completed the questionnaires, and provided blood samples.

2.2 Assessment of folate intake and alcohol consumption

Information about folate intake and alcohol consumption one year prior to CRC diagnosis (for cases) or the reference date at recruitment (for controls) was obtained by using the 119-item semi-quantitative food frequency questionnaire developed specifically for Chongqing middle-aged population in our previous work as described elsewhere (Zhou, Takezaki et al. 2004). Briefly, according to the folate content listed in China Food Composition 2002 (Institute of Nutrition and Food Safety. China Center of Disease Control 2002), the frequencies of consumed portion of each food were converted into nutrients; for example, a crude mean of daily folate intake was calculated by multiplying the daily various food intake by its folate content (per 100 grams). The main sources of folate included in the questionnaire were cereals, beans, legumes, nuts, eggs, meats, fishes, bread, edible roots, melons, mushrooms, vegetables and fruits. Similarly, all subjects were also asked to provide detailed information about dietary supplements consumed in the period of one year before diagnosis or recruitment. For alcohol drinking, those who consumed alcohol more than 50 grams each week for more than 6 months were defined as “drinkers”. Consumption of all kinds of beverage (beer, alcohol, and wine) was

calculated as pure alcohol volume by their alcohol concentrations (%). For smoking, those who smoked more than four cigarettes each week in average for more than six months were defined as "smokers". Pack-years were calculated by multiplying the total years of smoking by the average packs smoked each day.

2.3 Genotyping

DNA was extracted for each subject from the buffy coat fraction with the Promega DNA Purification Wizard kit (Promega Co. Madison, WI). Genotyping was performed by the polymerase chain reaction restriction-fragments-length polymorphism analysis according to methods described previously for *MS* A2756G (De Marco, Calevo et al. 2002), *MTHFR* C677T (Chen, Giovannucci et al. 1998) and A1298C (Yi, Pogribny et al. 2002), respectively. For the *MS* A2756G polymorphism, a 285-bp PCR product was digested with HaeIII at 37°C and visualized after electrophoresis; the genotypes identified were: 265bp for AA, 265bp, 185bp, 80bp for AG, and 185bp, 80bp for GG. For *MTHFR* C677T polymorphism, a 198-bp PCR product was digested with Hinf I at 37°C and visualized after electrophoresis; the genotypes identified as follows: 198bp for CC, 198bp, 175bp, 23bp for CT, and 175bp, 23bp for TT. For *MTHFR* A1298C polymorphism, a 128-bp PCR product was digested with MboII at 37°C and visualized after electrophoresis; the genotypes identified were: 72bp, 28bp, 28bp for AA, 100bp, 72bp, 28bp for AC, and 100bp, 28bp for CC. The 23bp and 28bp fragments had been electrophoresed out of the gel and cannot be seen. Two cases and four controls failed to be amplified for *MTHFR* and *MS* polymorphisms, possibly due to poor quality of DNA. For genotyping quality control, electrophoresis results of genotypes were identified by a double-blind check and tested for Hardy-Weinberg equilibrium. Furthermore, randomly selected 92 cases and 136 controls were re-genotyped, and there were no discrepancies between the original and repeated genotyping results.

2.4 Statistical analysis

Energy-adjusted daily folate intake was categorized into quartiles based on the distribution in the controls, odds ratios (ORs) and their 95% confidence intervals (CI) were calculated by unconditional logistic regression models to estimate the strength of association between CRC risk and folate intake, the lowest quartile was used as the reference in OR calculation and was further adjusted for sex, age (in years), family cancer history of first degree relatives (yes vs. no) and second degree relatives (yes vs. no). Alcohol consumption status was divided into four categories based on alcohol (g/d) consumed: non-drinker (0), <30 g/d, 30~100 g/d, and >100 g/d for estimation of ORs (95% CI), and the drinking status was only classified into non-drinker and drinker when exploring the interaction with genotypes. Similarly, smoking status was also divided into four categories based on pack years smoked: non-smoker (0), ~10, 10~20, and >20 pack years for estimation of ORs (95% CI), and the tobacco exposure status was only classified into non-smoker and smoker when exploring the interaction with genotypes.

For evaluating the association between CRC risk and polymorphisms of *MS* (A2756G) and *MTHFR* (C677T and A1298C) genes, both ORs (95% CI) with and without adjustment for sex, age, family cancer history of first and second degree relatives, BMI of 10 years ago (divided into 4 subgroups: <20, 20~22.5, 22.5~25.0, >25.0), alcohol drinking and smoking status (yes vs. no) were calculated by using unconditional logistic regression models. ORs (95% CIs) for gene-gene or gene-environment interactions were assessed on a multiplicative

scale in the unconditional logistic regression model with and without adjustment for sex, age, family cancer history of first and second degree relatives. When analyzing the joint effects of two polymorphisms, we used the combined common genotypes as the reference. Hardy-Weinberg equilibrium in the controls was checked for all genotyping data with the χ test, and the exact *P* value was used to assess any departure of genotypes.
All statistical analyses were performed by using SAS (version 8.0, SAS institute, Cary, NC), with two-sided tests and a significance level of 0.05.

3. Results

3.1 Baseline characteristics

Table 1 shows the characteristics of 478 cases and 838 controls included in the final analysis. Overall, the cases were slightly older than the controls with a mean age of 54.3 years for cases and 52.0 years for controls. There was no difference in the distributions of sex, education, and BMI. However, these differences were further adjusted for their residual effects in the later analyses.

Variables	Cases (%) N=478	Controls (%) N=838
Age		
Mean	54.3±12.4	52.0±11.3
≤50 Years	171 (35.8)	358 (42.7)
51-60 Years	137 (28.7)	287 (34.3)
.≥61 Years	170 (35.4)	193 (23.0)
Gender		
Male	273 (57.1)	462 (55.1)
Female	205 (42.9)	376 (44.9)
Educations		
Illiterate	51 (10.7)	75 (8.9)
Primary, middle school	287 (60.0)	526 (62.8)
High school	98 (20.5)	151 (18.0)
College or upper	42 (8.8)	86 (10.3)
BMI (10 years ago)		
Mean	21.6±2.4	21.6±2.5
< 20.0	121 (25.3)	216 (25.8)
20-22.5	200 (41.8)	345 (41.2)
22.5-25.0	113 (23.6)	207 (24.7)
> 25.0	44 (9.2)	70 (8.4)

Table 1. Characteristics of cases and controls in the present study

3.2 Folate intake, alcohol consumption, genotypes, and CRC risk

The results of multiple logistic regression analyses of risk of CRC and folate intake, alcohol consumption, and tobacco smoking are summarized in Table 2.

Environmental Factor	Total Cases/ Controls	Total OR[a] (95%CI)	Male Cases/ Controls	Male OR[a] (95%CI)	Female Cases/ Controls	Female OR[a] (95%CI)
Folate intake (mean, μg/d)						
Lowest. (324.0)	188/209	1.00	85/92	1.00	103/117	1.00
Lower (442.3)	103/210	0.55 (0.40-0.76)	56/112	0.47 (0.30-0.76)	47/98	0.60 (0.38-0.95)
Middle (522.8)	100/210	0.53 (0.38-0.75)	64/116	0.52 (0.32-0.84)	36/94	0.52 (0.31-0.86)
Highest (673.7)	87/209	0.46 (0.31-0.67)	68/142	0.45 (0.28-0.74)	19/67	0.41 (0.21-0.82)
P_{trend}=		0.004		0.005		0.002
Alcohol drinking						
Non-Drinkers	305/612	1.00	116/249	1.00	189/363	1.00
Light ~30 g/d	75/90	1.99 (1.38-2.88)	60/78	1.93 (1.26-2.95)	15/12	2.69 (1.20-6.04)
Moderate ~100 g/d	62/89	1.64 (1.10-2.44)	62/88	1.62 (1.07-2.45)	0/1	NA
Heavy >100 g/d	36/47	1.98 (1.21-3.25)	35/47	1.95 (1.17-3.24)	1/0	NA
P_{trend}=		0.001		0.002		0.011
Years of alcohol drinking						
~5 years	314/624	1.00	123/258	1.00	191/366	1.00
6~20 years	48/55	2.35 (1.50-3.69)	41/49	2.30 (1.38-3.81)	7/6	3.04 (0.96-9.57)
20~ years	116/159	1.67 (1.20-2.31)	109/155	1.58 (1.11-2.23)	7/4	3.85 (1.07-13.81)
Ptrend=		0.001		0.005		0.009
Smoking						
Non-Smoker	280/521	1.00	77/156	1.00	203/365	1.00
Light ~10PY	42/78	1.20 (0.75-1.91)	41/71	1.45 (0.88-2.41)	1/7	0.28 (0.03-2.34)
Moderate 10~20PY	52/75	1.37 (0.87-2.15)	51/73	1.54 (0.96-2.47)	1/2	0.77 (0.07-8.91)
Heavy >20PY	104/164	1.26 (0.87-1.83)	104/162	1.40 (0.95-2.07)	0/2	NA
P_{trend}=		0.187		0.087		0.169

[a] Adjusted for age, gender, cancer history of first and second degree relatives and total energy intake.

Table 2. Odds Ratios (95% CI) of CRC associated with folate intake, alcohol consumption and smoking

In the four groups categorized by the quartile of folate intake, the CRC risk decreased significantly with the increasing folate intake in a dose-response manner (the same trend kept significance in either males or females). Compared with the lowest quartile (a mean intake of 324.1 μg/d), the ORs (95% CI) for the lower (442.3 μg/d), middle (522.8 μg/d) and

highest (673.7 μg/d) folate intake levels were 0.55 (0.40-0.76), 0.53 (0.38-0.75) and 0.46 (0.31-0.67), respectively (P_{trend} = 0.004), and the same trend kept significance in either males or females as described in table 2. Alcohol users had significantly increased CRC risks by nearly 1.6-2.0 folds higher than those who never drank. The ORs (95% CI) for those who consumed alcohol <30g/d, 30-100g/d, and >100g/d were 1.99 (1.38-2.88), 1.64 (1.10-2.44) and 1.98 (1.21-3.25), respectively (P_{trend}=0.001). A longer drinking time was associated with significant increased CRC risk (P_{trend}=0.001). Compared with those who drank less than five years, those who drank 6-20 years or more than 20 years had higher CRC risks with ORs (95% CI) of 2.35 (1.50-3.69) and 1.67 (1.20-2.31), respectively. However, we did not observe statistical evidence of an association between smoking and CRC risk in this study population.

Table 3 describes the distributions of *MS* A2756G, *MTHFR* C677T and A1298C polymorphisms and their associations with CRC risk. Those rare *MS* 2756G genotypes carriers had a 1.5 fold increased risk (OR=1.49; 95% CI: 1.11-1.96; P_{trend}=0.017), and the same trend was observed in the rare homozygous GG carriers though without significant difference. However, Both the C677T and A1298C polymorphisms of the *MTHFR* gene were not associated with CRC risk in this study population. We also tested the joint effect or locus-locus interaction. By using the combination of any two common genotypes as the reference, there were no meaningful interactions between *MS* A2756G and *MTHFR* C677T / A1298C polymorphisms.

Genotypes	Cases (%)	Controls (%)	OR[a] (95%CI)	OR[b] (95%CI)
MS A2756G				
AA	359 (75.4)	688 (82.4)	1.00	1.00
AG	113 (23.8)	141 (16.9)	1.52 (1.15-2.01)	1.50 (1.13-1.99)
GG	4 (0.8)	6 (0.7)	1.06 (0.29-3.88)	1.12 (0.30-4.11)
AG+GG	117(24.6)	147 (17.6)	1.50 (1.13-1.99)	1.49 (1.11-1.96)
P_{trend}=			0.009	0.017
MTHFR C677T				
CC	202 (42.3)	349 (41.9)	1.00	1.00
CT	210 (43.9)	362 (43.4)	0.97 (0.76-1.25)	0.99 (0.77-1.27)
TT	66 (13.8)	123 (14.7)	0.90 (0.63-1.28)	0.87 (0.61-1.24)
CT+TT	276 (57.7)	485 (58.2)	0.96 (0.76-1.21)	0.96 (0.76-1.22)
P_{trend}=			0.684	0.609
MTHFR A1298C				
AA	295 (62.0)	512 (61.3)	1.00	1.00
AC	158 (33.2)	282 (33.8)	0.96 (0.75-1.22)	0.96 (0.75-1.22)
CC	23 (4.8)	41 (4.9)	0.95 (0.55-1.62)	0.96 (0.56-1.65)
AC+CC	181 (38.0)	323 (38.7)	0.95 (0.75-1.21)	0.96 (0.76-1.21)
P_{trend}=			0.634	0.766

[a] Adjusted for age, gender, cancer history of first and second degree relatives and total energy intake; [b] Adjusted for age, gender, cancer history of first and second degree relatives, total energy intake, BMI of 10 years ago, alcohol drinking and smoking

Table 3. CRC risk associated with genotypes of *MS* and *MTHFR* gene

We further evaluated whether the impaction of folate intake on CRC risk was modulated by *MS* and *MTFHR* genes, alcohol drinking or tobacco smoking as summarized in Table 4.

Interactions	Cases/ Controls	OR[a] (95% CI)	Cases/ Controls	OR[a] (95% CI)	$P_{interaction}$
Folate × *MS* 2756	2756AA		AG+GG		
Lowest	145/179	Ref.	42/28	1.84 (1.07-3.16)	
Lower	74/168	0.55 (0.38-0.79)	29/42	0.84 (0.49-1.45)	
Higher	78/177	0.54 (0.37-0.79)	21/32	0.83 (0.45-1.53)	
Highest	62/164	0.46 (0.30-0.71)	25/45	0.65 (0.37-1.16)	0.556
Folate × *MTHFR 677*	677CC		CT+TT		
Lowest	77/80	Ref.	111/127	0.93 (0.62-1.41)	
Lower	44/88	0.55 (0.33-0.90)	59/122	0.50 (0.32-0.79)	
Higher	36/91	0.43 (0.25-0.72)	64/117	0.58 (0.36-0.92)	
Highest	45/90	0.54 (0.32-1.02)	42/119	0.35 (0.21-0.60)	0.629
Folate × *MTHFR 1298*	1298AA		AC+CC		
Lowest	111/138	Ref.	76/69	1.34 (0.88-2.05)	
Lower	66/120	0.69 (0.46-1.04)	37/90	0.50 (0.31-0.81)	
Higher	61/124	0.63 (0.41-0.97)	38/85	0.53 (0.32-0.87)	
Highest	57/130	0.54 (0.34-0.85)	30/79	0.47 (0.27-0.80)	0.205
Folate × Drinking	Non-Drinkers		Drinkers		
Lowest	134/163	Ref.	54/46	1.63 (1.00-2.65)	
Lower	72/154	0.58 (0.40-0.84)	31/56	0.80 (0.46-1.38)	
Higher	58/159	0.46 (0.31-0.69)	42/51	1.17 (0.69-1.99)	
Highest	41/136	0.39 (0.24-0.62)	46/73	0.86 (0.52-1.44)	0.188

Interactions	Cases/ Controls	OR[a] (95% CI)	Cases/ Controls	OR[a] (95% CI)	$P_{interaction}$
Folate × Smoking	Non-Smokers		Smokers		
Lowest	124/144	Ref.	64/65	1.06 (0.65-1.73)	
Lower	67/134	0.57 (0.38-0.85)	36/76	0.55 (0.32-0.96)	
Higher	52/131	0.45 (0.30-0.70)	48/79	0.72 (0.42-1.21)	
Highest	37/112	0.39 (0.24-0.65)	50/97	0.59 (0.35-1.01)	0.208

[a] Adjusted for age, gender, cancer history of first and second degree relatives, total energy intake and also adjusted for smoking status or drinking status, whenever appropriate.

Table 4. CRC risk associated with interactions between folate intake and genotypes (*MS* A2756G and *MTHFR* C677T and A1298C) or environmental factors (alcohol and smoke)

Foalte intake was inversely associated in a dose-dependent manner with CRC risk independent of the three genotypes of *MS* A2756G, *MTHFR* C677T and A1298C. Results in groups stratified by each genotype (common or rare) were similar, though the protective effect of folate intake almost disappeared in those carrying rare *MS* 2756 AG or GG genotype; there was no evidence of an interaction between folate intake and each polymorphism in a multiplicative model. When non-drinkers having the lowest level of folate intake was used as the reference, however, folate intake (from lower to highest) was associated with significantly decreasing CRC risk among non-drinkers (OR=0.39, 95% CI: 0.24-0.62 for the highest level), whereas the significance of protective effect of folate intake shown in Table 2 almost disappeared among drinkers who even had the highest level of folate intake (OR=0.86, 95% CI: 0.52-1.44); similarly, the protective effect of folate intake varied in smokers. Though alcohol drinking or tobacco smoking appeared to have an attenuated protective effect of folate intake, we failed to observe a statistically significant interaction with either drinking ($P_{interaction}$=0.188) or smoking ($P_{interaction}$=0.208) (Table 4).

3.3 Gene-environment interaction

Results of further analyses stratified by alcohol and smoking status are shown in Table 5. Here, we did observe a statistically significant interaction between the *MS* A2756G polymorphism and alcohol intake. An increased risk of CRC was observed in those alcohol drinkers carrying AG or GG genotype, whereas no significant association with alcohol drinking was observed among those carrying the AA genotype ($P_{interaction}$=0.04); Compared with non-drinkers carrying *MS* 2756 AA, the ORs (95% CI) for AG or GG genotype carriers who were drinkers of light (~30 g/d), moderate (30~100 g/d) and highest (~100 g/d) level were 2.84 (1.44-5.60), 3.14 (1.44-6.83) and 4.40 (1.88-10.32), respectively. We also observed a borderline significant interaction between *MTHFR* A1298C polymorphism and alcohol intake ($P_{interaction}$=0.07). For the 1298 AA genotype carriers, the CRC risk of drinkers was 2.0-

Interactions	Case/ Control	OR[a] (95% CI)	Case/ Control	OR[a] (95% CI)	$P_{interaction}$
MS 2756	2756AA		AG+GG		
MS 2756×Drinking					
Non-Drinker	242/502	1.00	62/107	1.19 (0.83-1.71)	
Light ~30 g/d	52/73	1.83 (1.20-2.78)	22/17	2.84 (1.44-5.60)	
Moderate ~100 g/d	44/76	1.43 (0.92-2.24)	18/13	3.14 (1.44-6.83)	
Heavy >100 g/d	21/37	1.46 (0.81-2.63)	15/10	4.40 (1.88-10.32)	0.041
MS 2756×Smoking					
Non-Smoker	224/427	1.00	55/91	1.13 (0.77-1.65)	
Light ~10PY	32/62	1.16 (0.70-1.95)	10/16	1.40 (0.60-3.28)	
Moderate 10~20PY	37/61	1.25 (0.76-2.06)	15/14	1.95 (0.87-4.35)	
Heavy >20PY	66/138	0.96 (0.64-1.45)	37/26	2.90 (1.61-5.22)	0.006
MTHFR 677	677CC		CT+TT		
MTHFR 677×Drinking					
Non-Drinker	133/250	1.00	172/358	0.89 (0.671.18)	
Light ~30 g/d	29/47	1.39 (0.81-2.39)	46/43	2.32 (1.42-3.82)	
Moderate ~100 g/d	25/32	1.80 (0.97-3.31)	37/57	1.36 (0.82-2.25)	
Heavy >100 g/d	15/20	1.83 (0.87-3.82)	21/27	1.82 (0.95-3.47)	0.780
MTHFR 677×Smoking					
Non-Smoker	130/214	1.00	150/304	0.81 (0.60-1.09)	
Light ~10PY	18/36	0.96 (0.50-1.83)	24/42	1.14 (0.62-2.09)	
Moderate 10~20PY	16/39	0.78 (0.39-1.54)	36/36	1.63 (0.92-2.89)	
Heavy >20PY	38/60	1.17 (0.69-1.98)	66/103	1.10 (0.70-1.72)	0.268
MTHFR 1298	1298AA		AC+CC		
MTHFR 1298×Drinking					
Non-Drinker	179/375	1.00	125/234	1.14 (0.85-1.52)	
Light ~30 g/d	46/50	2.40 (1.51-3.81)	28/40	1.66 (0.85-2.88)	
Moderate ~100 g/d	46/59	1.97 (1.23-3.14)	16/30	1.22 (0.61-2.44)	
Heavy >100 g/d	24/28	2.39 (1.30-4.42)	12/19	1.64 (0.75-3.57)	0.069
MTHFR 1298×Smoking					
Non-Smoker	165/323	1.00	114/195	1.09 (0.81-1.49)	
Light ~10PY	27/42	1.50 (0.85-2.66)	15/36	0.92 (0.46-1.82)	
Moderate 10~20PY	34/45	1.46 (0.84-2.51)	18/30	1.32 (0.67-2.60)	
Heavy >20PY	39/102	1.38 (0.89-2.13)	34/62	1.12 (0.66-1.89)	0.377

[a] Adjusted for age, cancer history of first and second degree relatives and total energy intake, smoking status or drinking status, whenever appropriate.

Table 5. CRC risk associated with interactions between genotypes (*MS* A2756G and *MTHFR* C677T and A1298C) and environmental factors (drinking and smoking)

2.4 folds of non-drinkers; however, for those carrying 1298 AC or CC genotype, alcohol drinking was non-significantly associated with CRC risk, compared with non-drinkers carrying the 1298 AA genotype (Table 5).

We also tested the gene-smoking interaction. The patterns of risk associated with *MS* A2756G genotypes seemed to vary by smoking status. For example, smoking was found to be associated with significantly increased CRC risk in *MS* 2756G carriers but not in 2756AA carriers, and there was evidence of an interaction ($P_{interaction}$=0.006). Compared with non-smokers carrying the AA genotype, an OR of 2.90 (1.61-5.22) was observed for those with AG or GG genotype and the highest smoking level (>20 pack-years). However, there were no evidence of an interaction between the genotypes of *MTHFR* C677T or A1298C and smoking (Table 5).

4. Discussion

4.1 Folate intake, alcohol drinking, *MS* A2756G polymorphism and CRC risk

Folate is traditionally regarded as a protective factor for CRC, and many studies have reported a beneficial role in reducing CRC risk, especially in some large-scale case-control or cohort studies (Giovannucci 2002; Terry, Jain et al. 2002; Sanjoaquin, Allen et al. 2005; Strohle, Wolters et al. 2005; Kennedy, Stern et al. 2011), but in recent years some clinical intervention trials have raised the controversy that an increased CRC risk may be produced when folate, especially fortified or supplemental folic acid (synthetic), was administered in an excessive dose and was inopportunely administered when there has some existing lesions (such as undetectable small cancer or precursors) (Strohle, Wolters et al. 2005; Hubner and Houlston 2009; Sauer, Mason et al. 2009). Nonetheless, there is no confirmative evidence against the hypothesis that the loss of homeostasis of folate-mediated one-carbon metabolism can cause abnormal DNA methylation or DNA misincorporation, thus resulting in colorectal neoplasia, but some studies argued that the folate (natural or synthetic) per se can indeed contribute to the reduction of CRC risk (Bollheimer, Buettner et al. 2005; Strohle, Wolters et al. 2005; Kim 2007; Sauer, Mason et al. 2009). The present study investigated the association between folate intake and the risk of CRC in a Chinese population, in which no one had the habit of daily use of any vitamin supplement; therefore, the "folate intake" evaluated in this study means only from natural food, and our results showed a significant association between higher folate intake and lower CRC risk (Table 2). Such a protective effect did not change substantially before and after multivariate adjustment, even in subgroups of colon or rectal cancer (data not shown). Therefore, the present study, generally consistent with many previously published reports (Giovannucci 2002; Terry, Jain et al. 2002; Sanjoaquin, Allen et al. 2005; Kennedy, Stern et al. 2011), provides a further insight and a support for an inverse association between folate (from food) intake and CRC risk in Chinese populations.

Although folate intake alone showed a significant protection against CRC risk, the variation in *MS* and *MTHFR* genes may also play important roles in the folate-mediated methyl cycles, and both alcohol drinking and cigarette smoking are known to impair the absorption and biological actions of folate. Although there was no evidence for an interaction in the present study, there was a trend that the protective effect of the folate appeared to be more obvious in those who were not exposed to the known risk factors drinking or smoking) compared with those who were exposed; in fact, the significant inverse association between folate intake and CRC risk was observed in 2756AA carriers or non-drinkers (non-smokers)

but not in either 2756G carriers or drinkers (or smokers). It seems that, to some extent, the favourable effect of folate may be impaired by the *MS* 2756G allele or drinking (or smoking), a finding consistent with other published studies (Kim 2007; Kim 2007). However, because our study was relatively small, larger studies, especially in Chinese populations, are needed to validate such an interaction between folate intake and *MS* 2756 AG+GG genotypes or drinking (smoking).

4.2 Gene polymorphisms and CRC risk

Several studies have investigated the association between the *MS* 2756 A>G polymorphism and CRC risk but generated conflicting results (Chen, Giovannucci et al. 1998; Ma, Stampfer et al. 1999; Goode, Potter et al. 2004; Ulvik, Vollset et al. 2004; Matsuo, Ito et al. 2005; Ulrich, Curtin et al. 2005; Koushik, Kraft et al. 2006; Theodoratou, Farrington et al. 2008; Yamaji, Iwasaki et al. 2009; Yu, Zhang et al. 2010). Using the common AA genotype as the reference, four studies reported no overall effect but a non-significantly association between CRC risk and the 2756G genotypes (Koushik, Kraft et al. 2006; Theodoratou, Farrington et al. 2008; Yamaji, Iwasaki et al. 2009). Recently, a Japanese study and an American study with 257/771 and 513/609 cases/controls, respectively, also supported the trend that the *MS* 2756G genotypes can elevate the risk of colorectal cancer or adenomas (Goode, Potter et al. 2004; Matsuo, Ito et al. 2005). One population-based case-control study of colon, but not rectal, cancer found no association (Ulrich, Curtin et al. 2005); only two earlier studies (one cancer and one adenomas) found significantly reduced risk among AG or GG carriers (Chen, Giovannucci et al. 1998; Ma, Stampfer et al. 1999), and one large-scale nested case-control study reported an inverse association between the G allele and CRC risk in Norwegians (Ulvik, Vollset et al. 2004). The present study was the first to explore the effect of the *MS* polymorphism on CRC risk in a Chinese population, and we found that the *MS* 2756 AG or GG genotypes were significantly associated with increased risk of CRC, further supporting a positive association between rare 2756 AG or GG genotypes and CRC risk.

We also observed another interesting finding that the frequency of the *MS* 2756G allele (9.2%) among controls was materially different from those (15%~20%) among different ethnic populations reported by other studies, and the frequency of 2756GG genotype in our study was less than 1% in both cases and controls, much lower than 3%-5% in other populations, such as Americans, Europeans and other Asia populations of Japanese or Hindoo (Goode, Potter et al. 2004; Ulvik, Vollset et al. 2004; Chen, Jiang et al. 2005; Matsuo, Ito et al. 2005; Ulrich, Curtin et al. 2005; Koushik, Kraft et al. 2006; Diwakar, Rudresh Kumar et al. 2008; Theodoratou, Farrington et al. 2008; Yamaji, Iwasaki et al. 2009). However, our results, especially for the frequencies of AG and GG genotypes among controls, were very similar to other studies in Chinese populations that investigated the association between the *MS* A2756G polymorphism and Alzheimer disease or lung cancer, in which the G allele frequency for controls was 8.5% and 9.8%, respectively (Liu, Jin et al. 2008; Zhao, Li et al. 2008). Therefore, it is likely that the allele frequency of the 2756G in Chinese is quite different from that of western or other Asia populations such as Japanese or Hindoo. It is still unclear whether the *MS* 2756A>G polymorphism has any functional consequences in its enzyme activity, but it was suggested that this polymorphism may probably decrease the enzyme activity, since the polymorphic site lies in a region connecting the vitamin B_{12} binding domain and the activation domain (Matthews, Sheppard et al. 1998). Considering epidemiologic evidence and the rare 2756G allele frequency in Chinese populations, our

findings suggested this variant may play a role in the etiology of CRC in Chinese populations, possibly a risk factor of CRC for Asia populations, in contrast to a protective effect in other ethnic populations. However, this finding needs to be further validated in larger studies of Asia populations.

In the present study we found that *MTHFR* 677 or 1298 variants were non-significantly associated with decreased CRC risk which is consistent in trend with other earlier epidemiological studies (Chen, Giovannucci et al. 1996; Ulvik, Vollset et al. 2004; Matsuo, Ito et al. 2005; Huang, Han et al. 2007; Kim 2007). The frequencies of 677T or 1298C alleles in our controls were very close to those of other Chinese populations and different Asia populations, such as Japanese or Korean, although the 1298C allele frequency was a slightly lower compared with western white populations (Chen, Jiang et al. 2005; Matsuo, Ito et al. 2005; Kim 2007). Laboratory evidence suggested that the rare 677T or 1298C allele can result in decreased enzyme activity *in vitro* (Molloy, Daly et al. 1997; Weisberg, Jacques et al. 2001) which seemed to favor an increased CRC risk, but on the contrary, most reported studies have not found an significant association between these *MTHFR* polymorphisms and CRC risk, and some earlier studies even reported an inverse association especially in white populations that were likely to have a relatively higher average total folate intake, partly due to use of vitamin supplements (Chen, Giovannucci et al. 1996; Ma, Stampfer et al. 1997). One-carbon unit metabolism may depend on a series of enzymatic steps forming a complex biochemical network, in which multiple dietary or environmental factors (e.g., vitamin B_2, B_{12}, and alcohol) may interact with folate, therefore the genetic variations of the *MTHFR* gene alone might not be sufficient to influence colorectal tumorigenesis during the one-carbon unit metabolism. Larger studies are required to further evaluate gene-gene and gene-environment interactions in the association between *MTHFR* C677T or A1298C polymorphisms and CRC risk in Chinese populations.

4.3 Gene-environment interactions and CRC risk

Though our study was relatively smaller, we did find some evidence of interactions between the *MS* A2756G polymorphism and three environmental factors (folate intake, alcohol use, and tobacco smoking) in the CRC etiology. Our study provided the first report of an effect of the *MS* 2756 A>G polymorphism and its interactions with dietary folate intake, alcohol consumption or cigarette smoking on CRC risk in a Chinese population.

Epidemiological studies have linked heavy alcohol use to increased risk of CRC, (Giovannucci 2002; Cho, Smith-Warner et al. 2004). Because alcohol can break the folate or disturb the one-carbon unit metabolism and thus may cause abnormal DNA methylation, DNA repair, or increase the activation of precarcinogen in liver by inducing cytochrome p-450 (Giovannucci 2004; Sharp and Little 2004), drinkers carrying rare *MS* 2756G, MTHFR *677T* or 1298C alleles may have additional CRC risk caused by abnormal folate metabolism (Giovannucci 2002; Sharp and Little 2004; Matsuo, Ito et al. 2005; Yamaji, Iwasaki et al. 2009). Our results supported such an association as well as a possible interaction between these polymorphisms and alcohol use in CRC risk.

Cigarette smoking may play a role in CRC but is not a major recognized risk factor, even after a long period of exposure (Giovannucci 2001; Anderson, Attam et al. 2003), and this was also true in the present study. However, we found an interaction between *MS A2756G* genotypes and smoking; compared with non-smokers carrying the *MS* 2756AA genotype, the AG or GG carriers of heavy smokers (>20 pack-years) had a 3-fold increased CRC risk. It

was reported that *MTHFR* 677T allele caused an increased plasm homocysteine concentration in heavy smokers than in moderate or non-smokers (Brown, Kluijtmans et al. 2004), and other studies found that an interaction between smoking and *MTHFR C677T* genotypes can be determinants of adenomatous and hyperplastic polyps of colorectum (Ulvik, Evensen et al. 2001). In the present study, however, we did not find any evidence of an interaction between smoking and *MTHFR* C677T or A1298C genotypes.

Overall, as Hubner mentioned very recently (Hubner and Houlston 2009), the existing evidence is still insufficient to confirm a protective effect of folate intake, and variants of the key metabolic-enzyme genes add the complexity to the unresolved problem of how and when the folate can have an effect on CRC risk. Our results suggested that gene-environment interactions may affect CRC risk more profoundly than the individual effect of folate intake or any of other known risk factors in this study population.

There are some potential limitations in the present study. First of all, because we did not have serum levels of folate or homocysteine, there may be biases in categorizing actual folate intake levels that were solely based on questionnaire data. Second, this hospital-based case-control study may have introduced some unknown selection biases. However, we reasonably believe that our cases and controls came from the same population base served by the hospitals, because all cases were newly diagnosed and most of controls were also registered at hospitals for the first time. Third, there was inherent recall bias in case-control studies; however, our interviewers did not know case-control status of the subjects. Lastly, our relatively smaller sample size may not have sufficient study power to detect interactions among folate, alcohol, smoking and the studied genotypes on CRC risk.

5. Conclusion

The present study suggested that sufficient folate intake may reduce the risk of CRC, and alcohol use can significantly increase CRC risk in the study population. The *MS* 2756 AG+GG genotypes may be associated with an increased CRC risk; our data further suggested that the interaction between *MS* 2756 A>G polymorphism and alcohol use may result in further increased CRC risk in this Chinese populations. How and to what extent can these joint effects modify the CRC risk need additional larger epidemiological studies especially in other Chinese populations.

6. Acknowledgment

The authors would like to thank the research staff from the Preventive Medicine College, The Third Military Medical University (TMMU) and the staff from the Department of General Surgery, Orthopaedics, and Clinical Trauma of Southwest hospital, Xin-qiao hospital and Da-ping hospital. We are also grateful to Dr. Man-tian Mi from Food and Nutrition Department, TMMU for supporting the data of folate content, to Mrs. Xiao-li Shen and Qing Liu for interviewing, and to Mrs. Ya-jing Li and Xue-zheng Li for their help for preparing for laboratory experiments in this study. We thank Dr. Ana Neumann and Dr. Qingyi Wei of The University of Texas M. D. Anderson Cancer Center for reviewing and scientifically editing the manuscript. This work was supported in part by a Major International (Regional) Joint Research Projects (30320140461) and General Programs (30771841 and 30700676) from the National Natural Science Foundation of China (NSFC), and by a Grant-in Aid for Scientific Research on Special Priority Areas of

Cancer from the Ministry of Education, Culture, Sports, Science and Technology of Japan (12670383), and also supported in part by the Doctoral Innovation Foundation of TMMU (200403).

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The Prognostic Significance of Number of Lymph Node Metastasis in Colon Cancer – Based on Japanese Techniques of Resection and Handling of Resected Specimens

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1. Introduction

Staging systems for cancer reflects the prognosis of the disease and it is used to choose the modality of treatment. The TNM classification has mainly been used in the west. In Japan, Japanese classification according to General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus(JGR)（Japanese Society for cancer of colon and rectum, 2009) is used. The degree of the lymph node metastasis in each staging system has some variations.

In 2009, 7th edition of (JGR, 2009) was revised to make it uniform with the 6th edition of TNM classification (Sobin & Wittekond, 2002). However, the 7th edition of TNM classification (Sobin et al., 2009) was further revised where the category of nodal status was subdivided (Table 1) on the basis of number of positive lymph nodes. The validity of which is based on the pooled SEER database of 109,953 cases of colorectal cancer lymph node metastases (Gunderson et al., 2010). Japanese classification of nodal status takes into account not only the number of positive lymph nodes but also the site from where they are retrieved according to the location of the tumor. Our study showed recategorization of lymph nodes such as 1, 2 to 6 and 7 or more lymph nodes with metastasis reflected the prognosis of the disease (Akagi et al., 2010). Thus, the number of lymph nodes retrieved plays a vital role in the staging system and is one of the main prognostic indicators of the disease. The various techniques of resection and handling of resected specimens may also vary according to different institutions and countries. The number of lymph nodes retrieved can depend on different factors like the surgical technique, length of resection, mesocolic excision, lymph node dissection, handling of resected specimen and criteria for pathological diagnosis which has some differences in Japan as compared to the west. Moreover, chemotherapy protocols and treatment of recurrence also may vary in different places. This can alter the stage, recurrence rate, as well as the outcome of the disease. Therefore, here we have elaborated our technique of resection, specimen handling and nodal dissection which is uniformly practiced in all centers of Japan and present data from our center where these techniques have been carried out consecutively.

	6th				7th		
	LN category		stage		LN category		stage
TNM	N1	1- 3 regional LNM	IIIA～IIIB	⇨	N1a	1 regional LNM	IIIA～IIIC
					N1b	2-3 regional LNM	
					N1c	Satellite without regional nodes	
	N2	4 or more regional LNM	IIIC		N2a	4-6 regional LNM	
					N2b	7 or more regional LNM	IIIB～IIIC
JGR	N1	Metastasis in pericolic LN	IIIa	⇨	N1	1-3 pericolic/perirectal, intermediate LNM	IIIa
	N2	Metastasis in intermediate LN	IIIb		N2	4 or more pericolic/perirectal, intermediate LNM	IIIb
	N3	Metastasis in main LN			N3	Main LNM (include Lateral LN)	
	N4	Metastasis in paraaortic LN	IV		M1	Metastasis beyond regional LN	IV

LN; Lymph node, LNM; Lymph node metastasis, JGR - Japanese classification according to General Rules for Clinical and Pathological Studies on Cancer of the Colon, Rectum and Anus

Table 1. The changes of lymph nodes category between former and current system

2. Methods

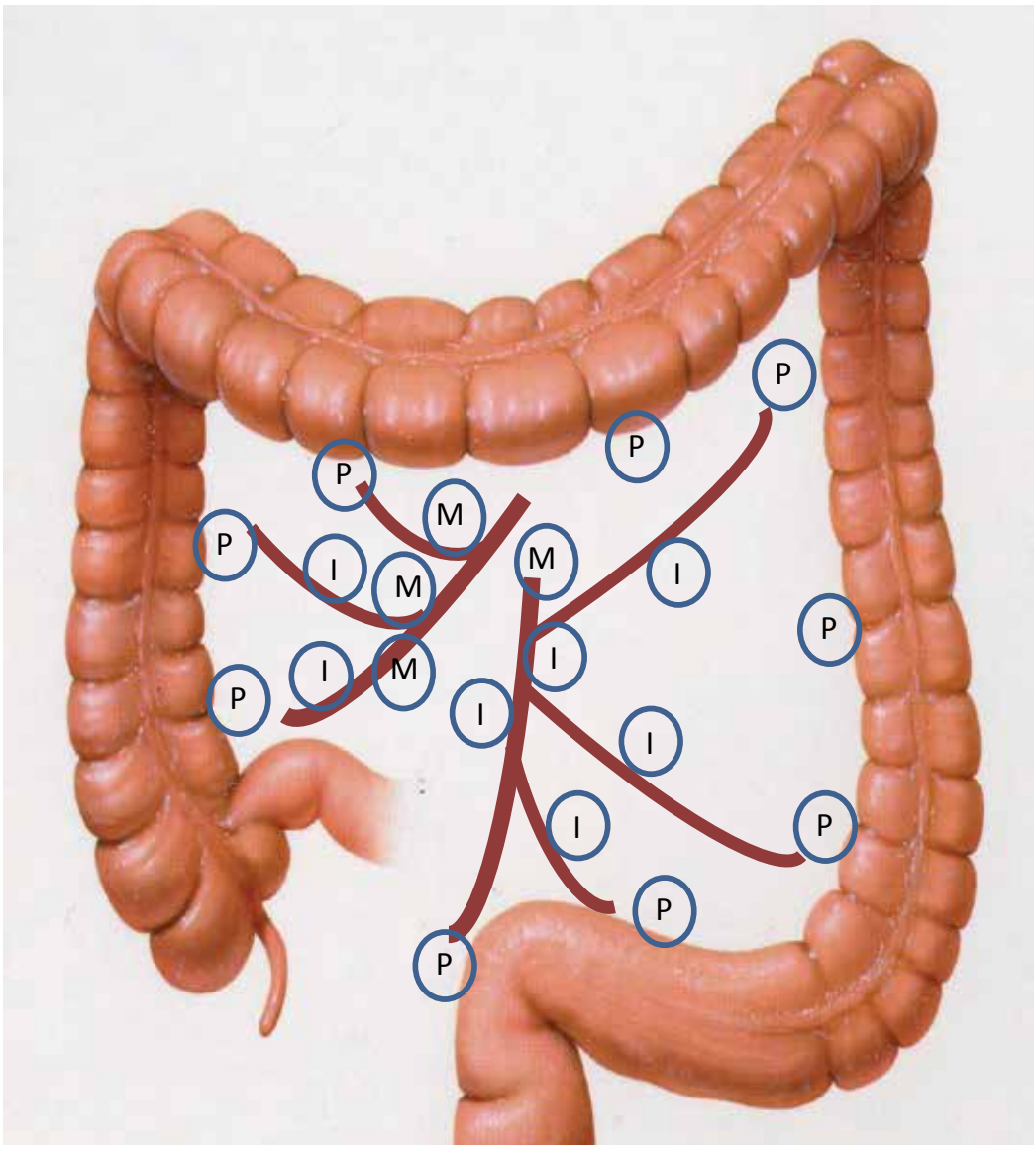
2.1 Patients

A total of 1107 patients with primary colon cancer treated by curative resection from January 1, 1985 to December 31, 2006, were identified from the colorectal cancer database of Kurume University, Fukuoka, Japan. Of these patients, 361 patients with Dukes C colon cancer located from caecum to recto sigmoid junction were included in this study. Patients who underwent neoadjuvavnt chemo-radiotherapy, patients with familial adenomatous polyposis (FAP) or inflammatory bowel disease (IBD) and patients with rectal cancer were excluded from the study. The median age of patients was 66 years (64.9±12.6) and 213 (59%) patients were male. Almost all patients were administered oral prodrug of 5-fluorouracil as postoperative adjuvant chemotherapy. The median number of nodes examined was 28 (range, 5-108, average, 30.7 ±16.5) and the median duration of follow-up was 68 months (24-186 months, average, 60.5±22.3) from the date of their initial surgery.

2.2 Surgical technique for resection of colon and staging of lymph node status

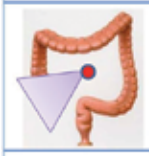
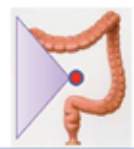
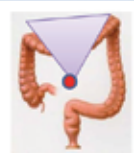
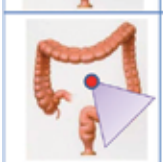
Surgery for colorectal cancer was performed by only certified colorectal surgeons. A similar protocol for length of resection and lymph node dissection for colon cancer was followed by all surgeons. The extent of the resection was determined by the location of cancer, its feeding arteries, cancer staging and the pattern of potential lymphatic spread. The feeding arteries were superior and inferior mesenteric artery and its branches such as ileocolic, right colic, middle colic, left colic and sigmoid arteries. The regional lymph nodes consisted of three groups; main, intermediate and pericolic lymph nodes (Figure 1.) Complete mesocolic

excision by sharp dissection of the entire mesocolon with intact facial layers and ligation of the supplying vessels at its origin was performed (Hohenberger et al. 2009). The pedicle of artery of the main lymph nodes was ligated and cut. In principle, the extent of mesocolon supplied by the feeding artery and all regional lymph nodes were removed en block for advanced cancer (Table 2.). For early cancer, the intermediate and pericolic lymph nodes of feeding artery were removed. Length of bowel resection was 10cm proximally and distally from location of the feeding arteries where the tumor was located.



M : Main lymph nodes, I : Intermediate lymph nodes P : Pericolic lymph nodes

Fig. 1.

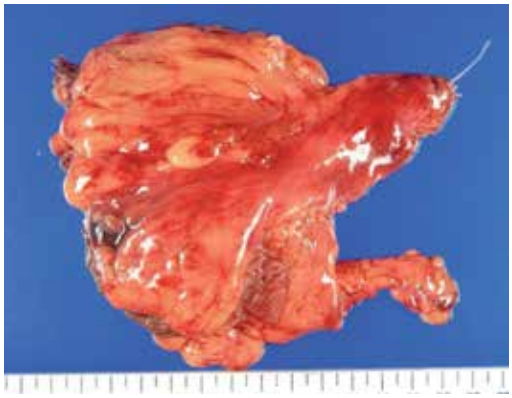
	Surgical procedures	Ligation arteries and (lymphadenectomy)
	Ileocecal resection	Ileocolic artery (Ileocolic root nodes)
	Right hemicolectomy	Rt.colic artery (Rt.colic root nodes)
	Transverse colectomy	Middle colic artery (Mid.colic root nodes)
	Left colectomy	Lt. colic artery (Inferior mesenteric nodes)
	Sigmoidectomy	Inferior mesenteric artery (Inferior mesenteric nodes)
	Anterior resection	Inferior mesenteric artery (Inferior mesenteric trunk nodes)

The intestine with tumor and adjacent mesocolon (: Lymph nodes are included in this) removed after ligation of pedicle ( ).

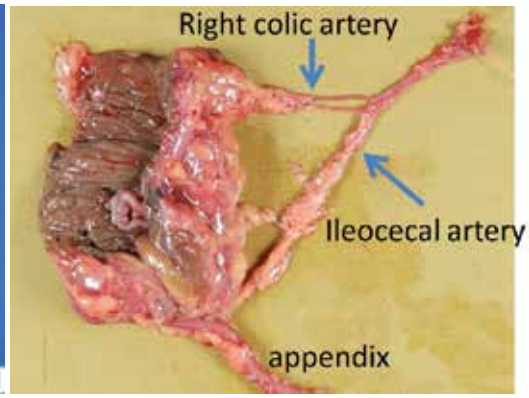
Table 2. Operation for advanced colon cancer

2.3 Handling of resected specimen

Lymph node dissection was carried out by the surgeon prior to formalin fixation in fresh resected specimens. The lymph nodes along the feeding vessels were picked up from the mesocolon and kept separately according to the lymph node stations and fixed in formalin (Fig. 2a,b,c). The pericolic nodes in the fat tissue beside the tumor were left intact for the correct judgment of depth of invasion. The opened intestine was placed on a board with the mucosal side up and the edge stretched and pinned to reproduce its original appearance. After formalin fixation for several days the tumor was sectioned at 5 mm intervals (Fig. 2d). One of the deepest invasive specimens was examined by expert pathologist (Fig. 2e). The final decision of histological examination of specimen and lymph node metastasis was made by the surgical colorectal pathologist (K. Shirouzu; co-author).



a)



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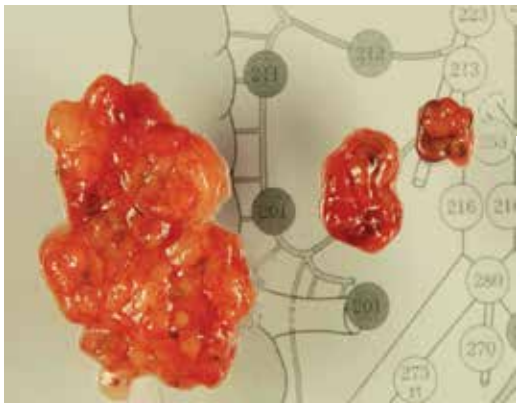

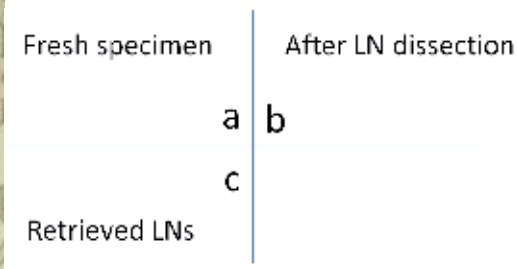

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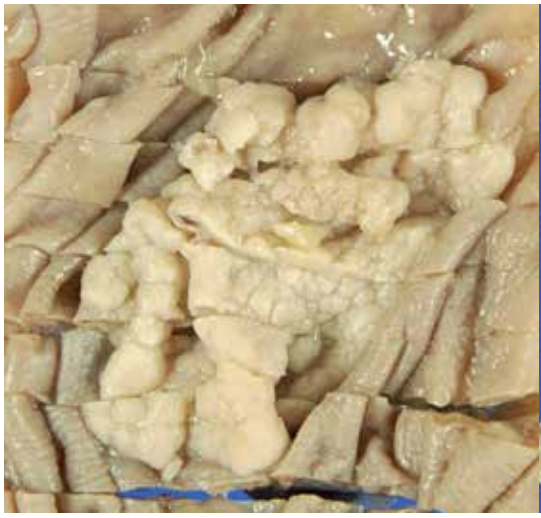
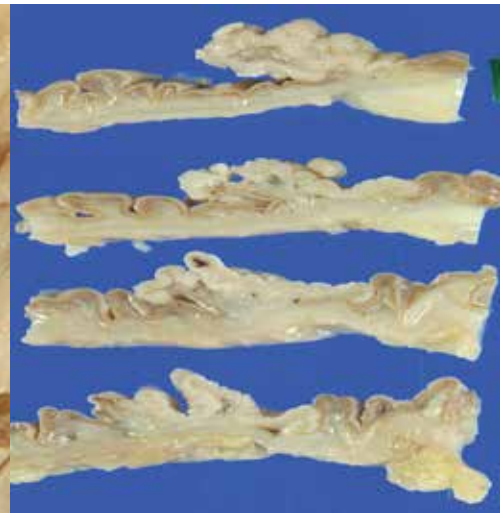


d) The tumor sectioned at 5mm intervals



e) Pathological specimen for examination.

Fig. 2. Handling of fresh specimen after ileocecal resection

2.4 Statistical analysis

The cases were classified according to the number of metastatic lymph nodes. Survival rate for each group was assessed. A new classification was then considered to recategorize the lymph nodes from cases with similar survival rates. In the new classification, survival rate was assessed with prognostic-relate factors inferred statistically. Analysis of variance or a t-test was used to analyze continuous variables. $\chi 2$ test was used for categorical variables. Five-year survival rates and prognostic factors were estimated using Kaplan-Meier survival method and Cox proportional hazard regression model, respectively. Log-rank test was used to assess whether survival differences were significant. Values of $p<0.05$ was considered statistically significant. Statistical analysis was performed using JMP software ver. 8.0 (SAS Institute, Cary, NC, USA).

3. Results

3.1 Number of lymph nodes retrieved and Lymph Node Metastasis (LNM)

Table 3. shows the number of cases, average number of lymph nodes retrieved, five year overall survival and recurrence rate in each group divided by number of LNM. The most common was the group which had one LNM. The median number of LNM was three (range, 1-14). The average number of lymph nodes retrieved in each group was from 26 to 47, and there is significant relation between number of LNM and lymph nodes retrieved. The more the number of lymph nodes retrieved increased, the number of LNM also increased. Similarly the recurrence rate was higher when the number of lymph nodes retrieved was more. However, it did not show statistical significance. The 5 year survival rate for each group classified by the number of LNM is shown in Table 3. 5-year survival rate of the group with 1 LNM was significantly better than that of other groups and the group with ≥7 LNM showed significantly worst survival than groups with ≤6 LNM.

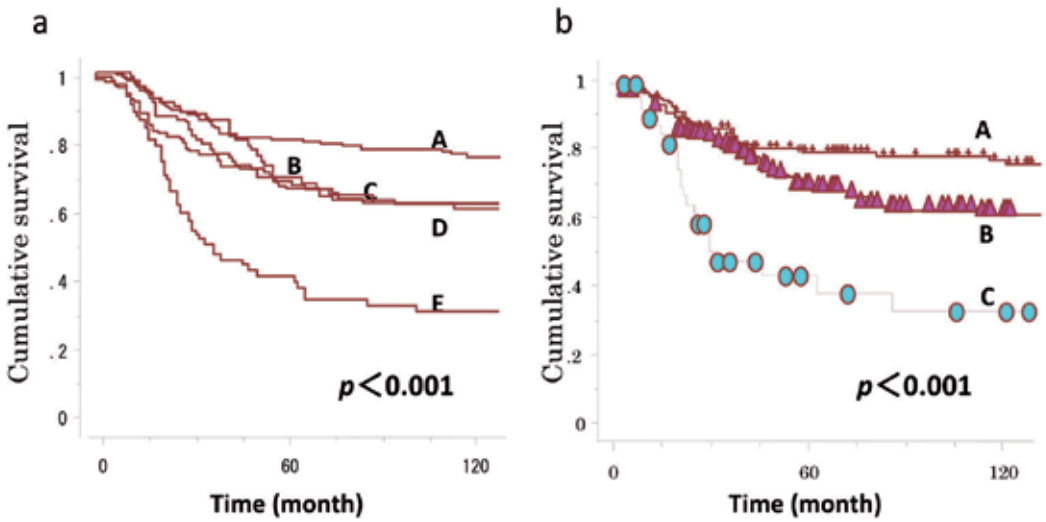
Number of LNM*	Case	Ave. of LN s retrieved*/**	recurrence rate**	5 y r OS		5 y s .OS
1	143	26 (5-108)	21.7	81.6%	⇒	81.6%
2	68	26 (5-66)	23.9	72.2%		
3	53	28 (5-70)	28.3	71.4%		
4	33	31 (6-78)	24.2	68.9%	⇒	70.9%
5	19	29 (19-71)	31.6	68.1%		
6	11	40 (17-71)	18.2	70.0%		
7	8	33 (12-52)	37.5	46.9%		
8	6	38 (32-68)	50	42.6%		
9	10	45 (17-86)	50	48.9%	⇒	44.1%
10 or more	14	47 (21-70)	57.1	29.9%		

LNM: Lymph nodes metastasis, Ave.: average (range), 5 yr OS: 5 year overall survival
*: $p<0.001$ (number of LNM vs Ave. of LNs retrieved,
**: $p= 0.763$ (Ave. of LNs retrieved vs recurrence rate)

Table 3. Recurrence and Survival on the number of lymph nodes metastasis

3.2 Recategorization and its association with survival

The survival curves of five groups based on the former method (Akagi et al., 2010) were compared. Group A consisted of cases of 1 LNM, group B 2 LNM, group C 3 LNM, group D 4-6 LNM, and group E 7 or more LNM. Survival curves for group B, C and D were similar (Fig. 3a). Based on the above-mentioned results, the survival curve of each group, survival rate, patient number and current classification was considered and integrated and reorganized into three groups (Table 3, Figure 3b). The new classification used 1 LNM for group A (143 cases), 2-6 LNM for group B (184 cases), and ≥7LNM for group C (38 cases). In brief, N category seemed the most appropriate when the number of LNM was classified as 1, 2-6, and ≥7. The 5-year survival rate for each group was A; 81.6%, B; 70.9%, and C; 44.1%, respectively (Table 3). This classification more accurately reflected the prognosis as compared to the conventional categories for LNM. Factors influencing prognosis were extracted from clinicopathological factors of every group by univariate analysis. Then, correlative prognostic factors were included in the new classification of LNM degree was estimated with multivariate analysis (Table 4). As for independent prognostic factors, degree of venous invasion and seven and more LNM were considered to be poor prognostic factors.



(a) According to number of lymph node metastasis (LNM).
A: 1 LNM, B: 2 LNM, C: 3 LNM, D: 2-6 LNM, E: 7 and more LNM
(b) According to recategorized group
A: 1 LNM, B: 2-6 LNM, C: 7 or more LNM

Fig. 3. Cumulative survival curves

Variable	Univariate	Multivariate		
	p-value(logrank test)	HR (95%CI:L-U)	*p*-value	Chi-square
Number of LN metastasis				
≥ 7 vs. ≤ 6	0.0001	2.11 (0.137−1.310)	0.0174	5.66
Depth of invasion				
se vs. ss	0.0076	1.76 (-0.014−1.224)	0.0562	3.65
Histologic type				
poor,muci vs. well,mod.	0.0952	1.13 (-0.539−0.731)	0.7019	0.15
Lymphatic invasion				
ly2-3 vs. ly0-1	0.0025	1.46(-0.205−0.935)	0.1972	1.67
Venous invasion				
v2-3 vs. v0-1	0.0013	2.63 (0.187−1.628)	0.0175	5.65
Preoperative CEA value				
≥ 5 vs. <5	0.1751	1.15 (-0.349−0.554)	0.0673	0.22

LN: lymph node, se: serosa, ss: sub serosa, poor: poorly differentiated adenocarcinoma, muci: mucinous carcinoma, well: well differentiated adenocarcinoma, mod: moderately differentiated adenocarcinoma, ly0-1, v0-1: negative to minimal invasion, ly2-3, v2-3: moderate to severe invasion, CEA:carcinoembryonic antigen, CI; confidence interval

Table 4. Independent Prognostic Factor for Desease Specific Survival using Cox Regression Analysis

4. Discussion

Depth of invasion and number of regional lymph node metastasis(LNM) are known to be important prognostic factors for colorectal cancer, and these factors are used to determine the stage of the disease (Chapuis et al.,1985; Vaccaro et al., 2004; Choen et al., 1991). In Japan, on the basis of clinical studies on colorectal cancer the general rules for clinical and pathological studies of cancer of the colon, rectum and anus which has been modified continuously (JGR, 1977). Based on these data the surgical procedure has been standardized with en bloc resection of tumor, distal and proximal normal colon, mesocolon along with apical vessels of feeding artery.

stage*	recurrence rate	5 year OS		
		colon	rectum	total
I	3.70%	90.60%	89.30%	90.60%
II	12.50%	83.60%	76.40%	81.20%
IIIa	24.10%	76.10%	64.70%	71.40%
IIIb	40.80%	62.10%	47.10%	56%

Japanese Society for cancer of colon and rectum, 1991-1996

Table 5. Recurrence rate of each stage of colorectal cancer after curative resection

A large difference has been found in the recurrence rate and prognosis in stage II and III colorectal cancer according to the report of 2004 in Japan. (Table 5.) (Japanese Society for Cancer of the Colon and Rectum, [JSCCR] Guidelines 2010 for the Treatment of Colorectal Cancer. (In Japanese). Similar data has also been published from other countries (Andre et

al., 2009). Only in stage III colon cancer does the prognosis depend upon the number of lymph node metastasis.

Lymph node (LN) involvement is an important prognostic indicator of carcinomas arising in the colon and the rectum. It also influences treatment decisions, as patients with node-positive colorectal carcinoma (CRC) are generally advised for systemic adjuvant chemotherapy. Thus, the accuracy of number of lymph node metastasis becomes a very important factor. However, when the precision of diagnosis of metastasis to lymph nodes is concerned factors such as length of dissection of bowel, lymph node dissection techniques and treatment of the specimen needs to be considered. In addition, the retrieval method may also depend on the facility, institutional protocol or the individual surgeon.

If the degree of LNM is inadequately assessed this changes the stage of the disease which in turn reflects on the further inappropriate treatment protocol and prognosis. Then the question of stage migration arises. However, inappropriate retrieval of lymph node and thus incorrect staging cannot be blamed on stage migration. Rectal cancer was excluded from this study as most of our patients did not undergo neoadjuvant chemo radiotherapy as patients in the west but underwent pelvic lymphadenectomy which increased the number of lymph nodes retrieved that may alter the surgical procedure preoperative stage and thus the prognosis.

There have been several papers regarding relationship between degree of LNM and its prognosis like the number of LNM, the site of metastatic lymph nodes, number of lymph nodes retrieved, and lymph node ratio (LNR). Our data showed colon cancer with only 1 LNM had significantly better prognosis than 2 or more LNM and patients with 7 or more LNM had the worst prognosis. Our recategorization considered here with both number and level is similar to the LN category in the 7th edition of TNM classification.

There are some reports that the prognosis of colon cancer is associated with number of LNM (Vaccaro et al., 2004) or classification by the number of LNM predicts prognosis better than classification by level of LNM (Carlos et al., 2004). On the other hand, Newland et al showed the level of LNM rather than the number of LNM is the most important variable associated with prognosis. (Newland et al., 1994) Tapper and Nelson et al. mentioned that staging for colorectal cancer required retrieval of 12-17 lymph nodes. (Tapper et al., 2001; Nelson et al., 2001) Kim et al. mentioned that retrieval of >10 lymph nodes offered almost certain identification of metastasis to lymph nodes, and tumor differentiation and T stage seemed to correlate with higher nodal metastasis rate. (Kim et al., 2006)

The 7th ed. TNM classification mentions that histological examination of a regional lymphadenectomy specimen will ordinarily include 12 or more lymph nodes. The most accepted limit for accurate staging seems to be at least 12 nodes, as also suggested by other current node metastasis related publications (Wittekend et al., 2003; Greene et al., 2002). Cserni suggested not only the minimum number of LNs should be considered in terms of staging but some qualitative features may also influence the accurate staging. The question arises whether accurate staging can be reached with fewer than 12 LNs or not (Cserni et al.,1999). However, the number of lymph nodes obtained in specimens of colorectal cancer is significantly associated with the length of resected bowel, patient age and tumor location (Shen et al., 2009). Recent study by Cserni et al have mentioned that nodal status of CRCs may be adequately assessed by examining the lymph nodes from the close fraction around the tumor and the 3 cm side long bowel segment in both directions (Cserni et al., 2011). Thus, the retrievable number of lymph nodes depends on different factors like stage, technique of dissection and the treatment of specimen.

In recent years, it has been reported that lymph node ratio (LNR) i.e., the number of tumor infiltrated nodes divided by the total number of resected nodes, is associated with prognosis (Schumacher et al., 2007; Vaccaro et al., 2009). LNR is a more accurate prognostic parameter than just the presence of lymph nodes metastasis (Rosenberg et al., 2008). However, this idea needs more verification as LNR changes according to the extracted lymph nodes number.
The size of the LNs is one such possible qualifier in the study about diagnosis for lymph node metastasis. Cserni et al found that the evaluation of the seven largest LNs gives a correct qualitative (negative vs positive) nodal status in 97% of the cases. (Cserni G, 2002). Thus, the diagnosis and staging the degree of lymph node metastasis seems to be still controversial.

5. Conclusion

LNM staging reflects the prognosis of the disease. The method of evaluation of LNM varies according to surgical treatment, the handling of specimens, pre and post operative chemo-radiotherapy protocols etc. which varies between different institutions and countries. Therefore, it is difficult to compare every data in detail. Since, staging systems are based on depth of invasion and lymph node metastasis which influences the management and prognosis of the disease thus a standard protocol reflecting the best method for dissection of nodes and handling of specimens is necessary which reflects the accurate stage and thus the prognosis of the disease. Further studies for lymph node staging are thus necessary to find a universally accepted technique and staging system with maximum validity and reliability.

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Minimally Invasive Robot – Assisted Colorectal Resections

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1. Introduction

Minimally invasive techniques have revolutionized general surgery, especially in the field of gastrointestinal surgery.
Many authors argue that the era of laparoscopic technique had begun in 1987, when Mouret performed the first laparoscopic cholecystectomy (Koopmann et al., 2008; Law et al., 2007). Since that point, laparoscopic technique has become the first choice for a multitude of surgical procedures: cholecystectomy, gastric bypass, fundoplication and its variants are some examples of procedures which are currently performed laparoscopically (Stage et al., 1997; Lacy et al., 2002). This spread has been fostered by the advantages of laparoscopic technique: reduced postoperative pain, decreased hospital stay and faster postoperative recovery, reduced incidence of postoperative complications, improved cosmetic outcome, decreased incidence of incisional hernias (Stage et al., 1997; Lacy et al., 2002; Guillou et al., 2005; Jayne et al., 2007; Fleshman et al., 2007; Nelson et al., 2004; Veldkamp et al., 2005; Ballantyne et al., 2001).
The first hemicolectomy was performed laparoscopically in 1990 (Weber et al., 2002; Delaney et al., 2003). Since then, the introduction of this technique for colorectal disease was gradual, especially in malignancy, because of early skepticism towards this technique. The major questions have arisen about the treatment of malignant disease. The oncological adequacy was analysed in terms of lymph node dissection, resection margins and intraoperative tumor dissemination.
Since 2002 a series of randomized clinical trials compared the laparoscopic and the open technique, the results of which definitely eliminated any doubts concerning the oncological adequacy of laparoscopic technique (Lacy et al., 2002; Jayne et al., 2007; Poulin et al., 1999; Hasegawa et al., 2003; Kaiser et al., 2004; Milsom et al., Tang et al., 2001; Champault et al., 2002). The advantages of minimally invasive approach in colorectal cancer surgery have been demonstrated in both pathophysiological (decreased inflammatory response → decreased immune response → decreased postoperative morbidity / comorbidity) and oncological aspects (similar results in terms of survival and outcome) (Leung et al., 2000; Delgado et al., 2001; Hu et al., 2003; Poulin et al., 1999; Hasegawa et al., 2003; Kaiser et al., 2004; Milsom et al., 1998; Tang et al., 2001; Champault et al., 2002).
The laparoscopic approach for colorectal disease, however, has both technical and "anatomical" disadvantages: the need of a long learning curve, the presence of a large

surgical field that requires a dynamic view and consequently, a skilled camera assistant surgeon, a constant dialogue between the operator and the assistant, the loss of three-dimensional vision, reduced ergonomics during specific phases of the procedure (need to take preternatural positions), poor dexterity of the laparoscopic instruments and decreased range of motion due to the rigidity of the insertion of the trocarts site, amplification of physiological tremor and the fulcrum effect. The so-called "surgical robots" or otherwise called "computer-assisted telemanipulators" have been introduced to overcome these "limits" in the practice of surgery (Mirnezami et al., 2009; Weber et al., 2002; Cadiere et al., 2001; Garcıa-Ruiz et al., 1998; Lanfranco et al., 2004; Morino et al., 2006; Horgan et al., 2001; Ballantyne et al., 2002; Hanly et al., 2004; Moorthy et al., 2005; Baik et al., 2008; Ballantyne et al., 2001).

2. Criteria in selecting patient for minimally invasive surgery

Patient's selection is based on pathophysiological and pathological conditions. Absolute contraindications in colorectal cancer robotic surgery, reflect those for whole minimally invasive surgery. Nevertheless, indications to minimally invasive approach are expanding rapidly suggesting the establishment of international guidelines in patient's selection.
There are several physiological parameters, which have been analyzed for patient's selection:

- Age. Age does not influence intraoperative or postoperative outcome as shown by several studies in which postoperative morbidity was lower than that in open surgery. Moreover, conversion rates were not statistically significant if different ages were considered (younger, middle-aged, elderly). Age is not a contraindication.
- Cardiopulmonary condition. Generally, altered cardiopulmonary functions are not considered a contraindication to minimally invasive approach. Nevertheless, minimally invasive approach needs continuous monitoring of patient's physiological parameters. The effect of the pneumoperitoneum on hemodynamics in patients ASA (American society of anesthesiologists score) I-II are not clinically relevant, while an invasive monitoring system of the blood pressure or circulating volume is advocated in patient ASA III-IV. In addition, gasless or low-pressure regimen should be maintained during the whole intervention in patients with limited cardiac function. Monitoring of end-tidal carbon-dioxide levels is mandatory, since pneumoperitoneum may causes a CO2-retention and head position and intrabdominal hypertension lead to ventilation-perfusion mismatch.
- Obesity. Obese patients' ventilation is often problematic because of pulmonary compliance reduced about 30% than in normal-weight patients. Although obesity is not considered an absolute contraindication, complications and conversion rate are higher at BMI (body mass index) greater than 30. Recently, similar postoperative short term outcomes have been demonstrated between obese and non-obese patients (Merkow et al., 2009; Blumberg et al., 2009). Complication rates are comparable with those expected after open surgery.

Pathological criteria:

Characteristics of tumor may influence the surgical approach. A tumor invading adjacent structures (T4) is considered an "absolute" contraindication to minimally invasive approach, as the principles of atraumatic manipulation and wide resection margins could be not

matched. Adhesions, localization and peritoneal carcinomatosis are not considered a contraindication by the majority of experts.

3. Patient preparation

The patient undergoes bowel preparation with Polyethylene glycol one day before the procedure and a slag-free diet three days before the procedure. The banding technique of the inferior arms associated to administration of a low-molecular-weight heparin 12 hours before the procedure is performed for thromboembolism prophylaxis.

3.1 Venous access

Before procedure starts, a venous access is needed to maintain correct blood volume and hydration of the patient and to infuse anesthetic agents during the whole procedure. The preferred choices of venous access are: right superior arm (18G catheter) for right colectomy and left superior arm for left colectomy and rectal resection (16/18 gauge catheter). If peripheral venous access is unavailable, a central catheter should be inserted before the day of the procedure. A heating system for venous access is needed in order to maintain the thermal homeostasis. Keeping patients warm has been associated with a threefold decrease in the rate of wound infection, reduction in operative blood loss, decrease in untoward cardiac events and patient discomfort. Maintenance of a normal temperature during surgery is important to reduce the stress of the surgical procedure and organ dysfunction.

3.2 Types of anesthesia

General anesthesia with orotracheal intubation and mechanical dynamic ventilation is the preferred technique among anesthesiologists. Currently, both intravenous and gas-intravenous (Desforane, Sevorane) techniques are used by anesthesiologists as there is no evidence of better results by the one or the other procedure. The usual scheme provides an association with epidural access in order to better control postoperative pain. N_2O (nitrous oxide) is contraindicated as it may increase the risk of pulmonary embolism. An optimal anesthesia may provide an acceptable muscular relaxation, better ventilation, thus minimizing the risk of pulmonary embolism and hemodynamics alteration, granting a wider operating field thus reducing the operative time.

3.3 Intraoperative monitoring

Intraoperative monitoring differs between ASA I-III or III+ patients:
In ASA I-III:
ECG+HR, O_2 saturation, Airways Pressure (PAW – Pressure plateau), capnography ($EtCO_2$)
In ASA III+ in addition to previous tests:
Neuromuscular function (TOF), Diuresis monitoring (bladder catheterization), Swan-Ganz catheter, PeakPressure Monitoring, arterial catheterization, haemo-gas analysis.
A peripheral arterial access is mandatory for monitoring blood pressure in high-risk patients.

3.4 Surgical instrumentation

Stereoscopic endoscope (da Vinci Surgical System)
CO_2 insufflator

Irrigation/suction system device
Video processor
1 Hasson-type trocart
1 10/12mm trocart
3 8mm robotic trocart
2 Robotic Cadiere's graspers
2 Robotic needle-holders
1 Laparoscopic needle-holder
1 Laparoscopic dissector
3 Laparoscopic forceps type Johann
1 Laparoscopic clip applier
1 45-mm laparoscopic stapler device with 2-3 cartridges (intestinal and vascular type)
1 Robotic ultrasound device
1 Robotic electrocautery hook
1 Robotic bipolar forceps
1 Wound protector for specimen extraction

4. Robotic right colectomy

4.1 Patient position and operating room setup

Patient is placed in supine reverse Trendelenburg position (15° to 20°), with 10° to 15° left lateral rotation and shoulder supports. The legs are secured at the thigh and calf with straps. The table is tilted to the left to allow the small intestine to fall off from the midline. The assistant surgeon stands on the patient's left side. The robotic cart is approached from the patient's right side. The operating room scheme is shown in fig. 1.

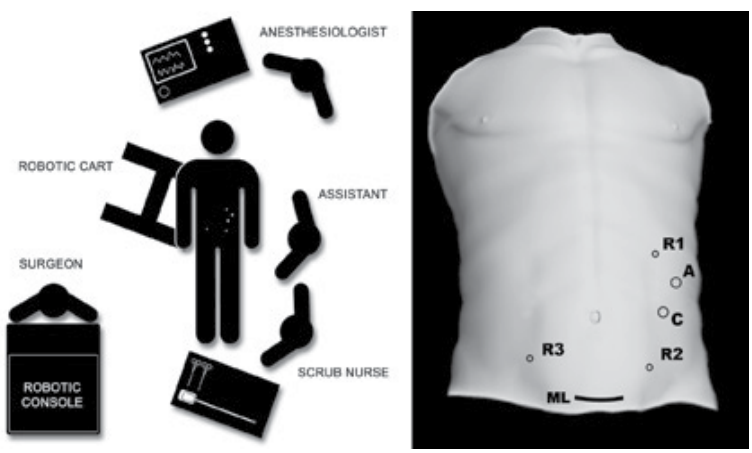

Fig. 1. Operating room setup and trocarts position

4.2 Trocarts position

A conventional 12mm port is placed by open technique on the lateral margin of the left rectal muscle, 1-2 cm above the transverse umbilical line, and pneumoperitoneum is induced until reaching a 12mmHg endoabdominal pressure. Then the 30° robotic stereo endoscope is inserted. Two daVinci 8-mm ports are inserted respectively in the left

hypochondrium for electrocautery/ultrasonic instruments and in the left iliac fossa for Cadiére grasper under direct vision. An additional 12-mm port is inserted in the left flank. This port is used by the assistant to help the surgeon during some steps of the procedure and to introduce the linear stapler for vascular, transverse colon and ileum resection. An additional robotic port is inserted in the right iliac fossa for the fourth robotic arm. It may be useful to provide effective and stable retraction during several steps of the procedure (i.e. to grasp the ileocecal valve and place the ileocolic vascular pedicle under tension, to lift up the hepatic flexure during the dissection of transverse mesocolon, etc.).

4.3 Description of the procedure

The procedure is carried out with a full robotic technique. The robotic cart approaches from the right side of the patient, and the three operative arms are connected to the ports.
The procedure begins by grasping upward and laterally the mesentery of the last ileal loop. This maneuver, performed with the forceps mounted on the fourth arm, enhances the prominence of the ileocolic vessels and provides stable and durable retraction. The peritoneal layer of the mesentery is incised just below this salience, and an accurate lymphadenectomy is performed along the superior mesenteric axis. Then, the ileocolic vessels are isolated and separately ligated and sectioned (fig. 2).

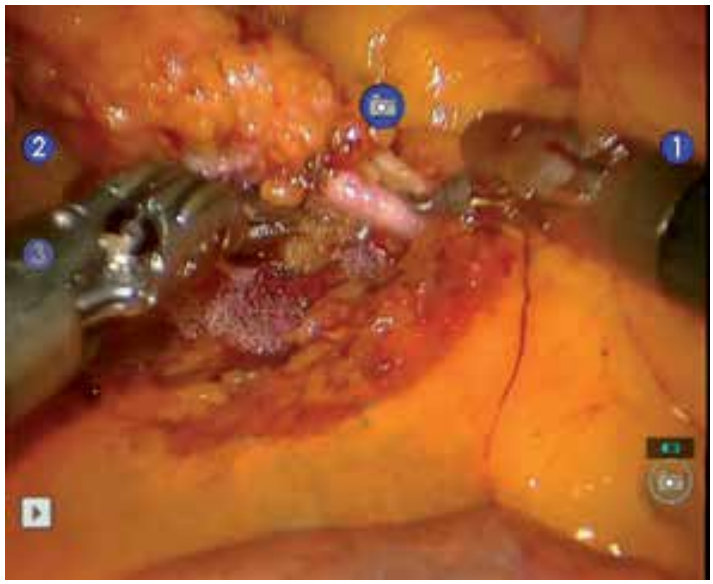

Fig. 2. Intraoperative view: Ileocolic vessels

Dissection of the right mesocolon follows a caudal-cranial pathway, along the right side of the superior mesenteric axis. Following this path, it is possible to remove the lymphatic tissue completely, safely identifying the inconstant right colic vessels, which may be sectioned at their origin, until reaching the root of the transverse mesocolon. Dissection along the lateral margin of the middle colic vessels allows the right branch of the middle colic vessels to be reached more easily, which is then treated as in the standard right colectomy (R1). Resection of the whole pedicle of the middle colic vessels is performed only for localization at the right colic flexure, for which extended right colectomy (R2) is needed.

Mobilization of the colon is performed in a medial-to-lateral direction in the avascular plane between Gerota's and Toldt's fasciae. During this step, the knee of the duodenum constitutes an important landmark to drive the dissection upward, over the duodenal third portion and the pancreatic head, along Fredet's fascia. The right ureter and the gonadic vessels are left below the plane of dissection. The hepatic flexure is then mobilized, sectioning the lateral portion of the gastrocolic and the hepatocolic ligaments. This step enables the resection to join the previously dissected plane and to complete the lymphadenectomy around the gastroepiploic vessels.
The transverse colon and the last ileal loop are finally sectioned by linear stapler. The ileum and the transverse colon are joined with a running suture, and an intracorporeal isoperistaltic double layer side-to-side ileocolic anastomosis is fashioned using a 3-0 absorbable monofilament suture (fig.3).
We performed an extracorporeal anastomosis in the first five cases: the daVinci system is disengaged from the patient, then a median supraumbilical minilaparotomy is performed, through which an isoperistaltic side-to-side ileocolic anastomosis is fashioned.
The specimen is retrieved at the end of the procedure through a small muscle-splitting Pfannenstiel minilaparotomy. This incision is protected from potential contamination by a wound protector.
A 10F Jackson-Pratt drain is placed laterally to the anastomosis through one of the lower trocart access.

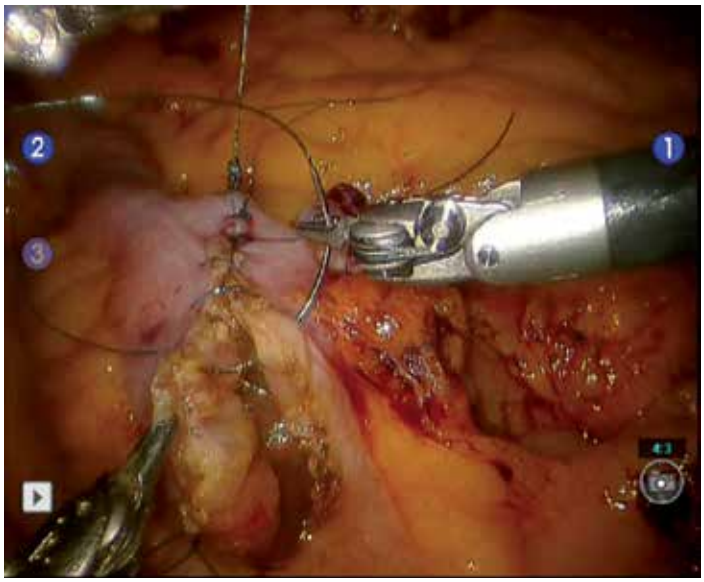

Fig. 3. Intraoperative view: intracorporeal ileocolic robot-assisted anastomosis

5. Left colectomy

5.1 Patient position and operating room setup

Patient is placed in supine Trendelenburg position (15° to 20°), with 10° to 15° right lateral rotation and shoulder supports. The legs are secured at the thigh and calf with straps. The table is tilted to the right to allow the small intestine to fall off from the midline. The

assistant surgeon stands on the patient's right side. The robotic cart is approached from the patient's right side.

5.2 Trocarts position

A conventional 12mm port is placed by open technique 2-cm right from the umbilicus along the umbilical transverse line, and pneumoperitoneum is induced until reaching a 12mmHg intrabdominal pressure. Then the 30° robotic stereo endoscope is inserted. Two da Vinci 8-mm ports are inserted under direct vision respectively in the epigastrium just 2 cm right from the midline and in the right flank. An additional 12-mm port is inserted between the two robotic trocarts. This port is used by the assistant to help the surgeon during some steps of the procedure and to introduce the linear stapler for vascular and left colon resection.

5.3 Description of the procedure

The procedure is carried out with a full robotic technique. The robotic cart approaches from the left side of the patient, and the two operative arms are connected to the ports.

The dissection begins with the identification of the inferior mesenteric vein at the level of the inferior margin of the pancreas and the incision of the peritoneum at the origin of the mesocolon under the salience of the vein, on the right side. Then, the inferior mesenteric vein is exposed and sectioned by linear stapler or between clips.

A sharp dissection is performed in a cranial-caudal and medial-to-lateral direction between the anterior and the posterior layer of Toldt's fascia. An incision of the peritoneum is performed from the promontory up to the origin of the inferior mesenteric artery, identifying and preserving the preaortic nerves. The dissection is carried out up to join the previous plane of dissection identifying and preserving the left ureter and the gonadal vessels. Then, the inferior mesenteric artery is exposed and sectioned at its origin by linear stapler or between clips.

Afterwards, the colon is freed laterally by the incision along Monk's line, from the sigmoid colon upward: the splenic flexure is taken down if necessary. The colon is divided distally to the tumor at the level of the promontory. Then, the specimen is usually extracted through a mini-Pfannenstiel incision, after the insertion of a wound protector. Before closing the minilaparotomy, the anvil of the circular stapler is inserted at the distal margin of the proximal colon.

Re-induction of the pneumoperitoneum is performed. The proximal colon is joined to the rectum by circular stapler, and a mechanical termino-terminal colorectal anastomosis is fashioned by Knight & Griffen technique.

Two 10F Jackson-Pratt drainages are placed anteriorly and posteriorly to the anastomosis through one of the lower trocart access.

6. Rectal surgery

6.1 Patient position and operating room setup

Patient is placed in a lithotomy position with his legs apart and no modification of position will occur during the whole procedure. The legs are secured at the thigh and calf with straps. The table is tilted to the right (15° – 20°) to allow the small intestine to fall off from the midline. The assistant stands on the patient's right side. The robotic cart then approaches to the operative bed by patient's left side, with a 60 degrees angle, following the imaginary line passing through the umbilicus and the left anterosuperior iliac spine.

6.2 Trocarts position

We usually perform a 5-ports technique. The first 12-mm periumbilical port (C) is placed by "open-laparoscopy" technique, for the stereoscopic endoscope. Then a 12-mmHg pneumoperitoneum is gained and other three 8-mm robotic ports are added: the first port (R1) is inserted in the right iliac fossa on an imaginary line between the anterosuperior iliac spine and the umbilicus. The second port (R2) is inserted in the right hypochondrium, and the third port (R3) is placed in patient's left flank. Last, a second 12-mm port is inserted in patient's right hip between R1 and R2 for the assistant surgeon (A). Trocarts' position is performed laparoscopically, and an exploration of the abdominal cavity precedes the robotic technique, with an adhesiolysis in case of visceral adhesions. Hence, the small bowel is displaced right in order to expose the Treitz ligament and the plane of the inferior mesenteric vein (IMV).

6.3 Description of the procedure

The procedure starts with the "vascular phase": the inferior mesenteric vein is identified at the level of the inferior margin of the pancreas. The peritoneum under the inferior mesenteric vein is then incised, and a smooth dissection is performed in a medial-to-lateral direction along the avascular plane between the Toldt's, above, and the Gerota's fascia, below, up to the left abdominal wall. The inferior mesenteric vein is subsequently divided between clips or by linear stapler. The dissection is prolonged up downward. The left ureter and the gonadic vessels are previously identified and preserved. The incision of the peritoneum at the level of the mesosigmoid is performed in order to reach the deeper plane of the inferior mesenteric artery. The iliac vessels and the left ureter are covered by the prerenal fascia. The dissection of the plane covering the inferior mesenteric artery is then performed with an accurate regional lymphadenectomy, preserving the preaortic nerves and the superior hypogastric plexus. The inferior mesenteric artery is then sectioned by stapler or between clips. A complete lateral dissection of the colon is carried out from the sigmoid to the splenic flexure. Flexure takedown is performed if the descending colon is needed to be used for the anastomosis.

Then, arm #2 is switched from R2 to R3. The assistant surgeon could use R2 and A trocarts in helping the surgeon during the total mesorectal excision (TME). Dissection starts posteriorly, at the level of the promontory, along the plane between the fascia recti propria, anteriorly, (peritoneum) and the presacral fascia (Waldeyer's fascia), posteriorly. Rectum is lifted up and laterally by a Cadiere on the arm #2 and dissection is carried out by the electrocautery hook or ultrasound device on the arm #1: the retrorectal plane has been opened. Care should be taken to preserve the inferior hypogastric nerves lying laterally along this plane. Pneumoperitoneum also helps the dissection between these two layers. At the level of the fourth sacral vertebra, the rectosacral fascia is incised in order to better mobilize the rectum and to access to the inferior part of the retrorectal space, in case of lower tumor localization. The mesorectal dissection has been completed behind the tip of the coccyx as the pelvic floor curves upward anteriorly toward the anorectal junction. Anteriorly, the rectum is retracted cranially and posteriorly by a Cadiere on the arm #2. The anterior peritoneal brim is incised by the electrocautery hook on the arm #1, and the dissection continues along the plane between the Denonvillier's fascia (or the rectovaginal fascia) and the fascia propria recti. At the level of the base of the prostate, Denonvillier's fascia is sectioned in order to preserve rectoprostatic (or rectovaginal) blood vessels and branches of the cavernous nerves (fig. 5).

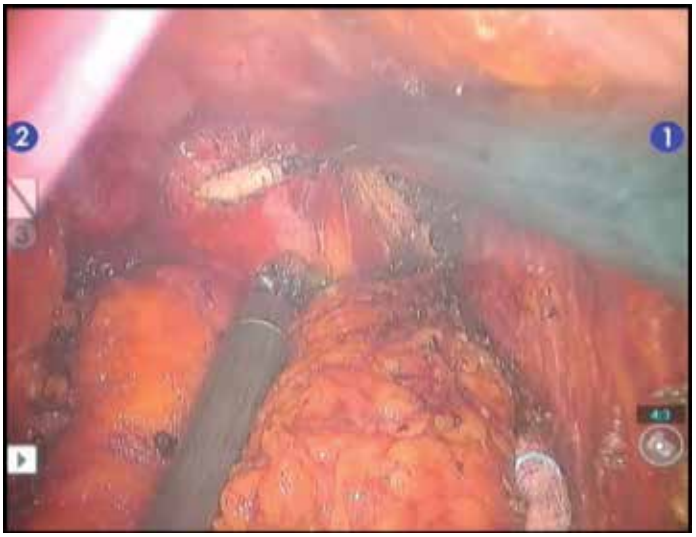

Fig. 5. Intraoperative view: Incision of Denonvillier's Fascia.

The dissection thus continues distal to the rectoprostatic (or rectovaginal) septum: the anterior mesorectal excision is completed, and the rectum is exposed anteriorly to the anorectal junction. A gentle traction of the rectum takes place laterally by a Cadiere on the arm #1, on either side, and the dissection is performed by the electrocautery hook until reaching the lateral ligaments of the rectum (LLR). At this level, the dissection is carried out close to the rectum, in order to avoid the injury of the inferior hypogastric plexus (IHP). The dissection must include only the rectal branches from the IHP and the small rectal branches from the middle rectal artery (MRA) (fig. 6).
The total mesorectal excision (TME) is finally completed and the rectum is sectioned at its distal end by linear stapler.

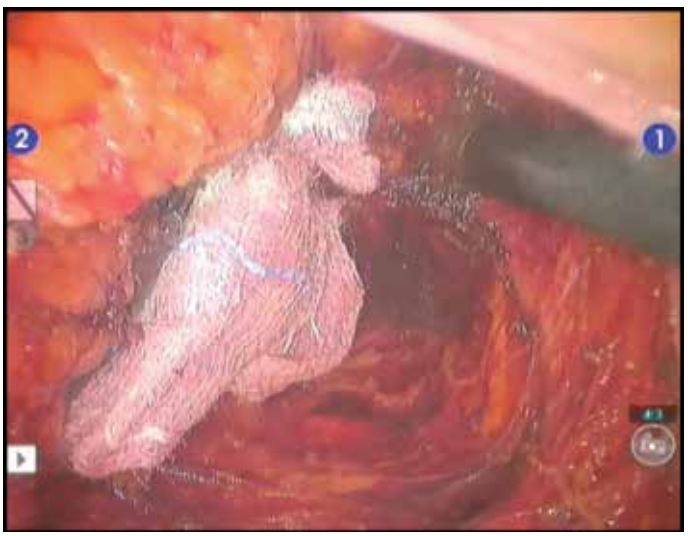

Fig. 6. Intraoperative view: lateral dissection of the rectum

Once the distal rectal transection ended, the robotic cart is disengaged, and a suprapubic minilaparotomy (Pfannenstiel) is performed. The specimen is then extracted, after protecting the minilaparotomy by a wound protector. The descending colon is transected, and the anvil of the stapler is inserted at the end of the colon.
The bowel reconstruction is conducted laparoscopically, and an intracorporeal mechanical colorectal termino-terminal anastomosis is performed by Knight & Griffen technique with a circular stapler. In case of coloanal anastomosis, the specimen is retrieved through the anal canal by a pull-trough technique and a manual colo-anal anastomosis is performed.
In case of ultra-low anterior resections a diverting loop-ileostomy is fashioned enlarging the R3 trocart site.

7. Postoperative patients' care, outcome and technical results

7.1 Technical results and outcome

Our study is based on about 300 consecutive robotic colorectal procedures structured as follows: 140 colic resections for cancer (84 right colectomies, 43 left colectomies, 13 others), 48 rectal resections and 110 colorectal resections for benign disease.

7.1.1 Right colectomy

Forty-three male and forty-one female patients underwent robotic right colectomy. Mean age was 73.34 ± 11 years. Median operative time was 213.50 (180–250) min. No conversion occurred. Specimen length was 28 ± 5 cm (range 21–50 cm). Number of harvested lymph nodes was 19.70 ± 7.2 (range 12–44), and mean number of positive lymph nodes was 1.65 ± 3 (range 0–17). Surgery-related morbidity was 2/84 (2,3%): one twisting of the mesentery in one of the first cases with extracorporeal anastomosis and a dehiscence of the colic stump in a patient with Crohn disease. All patients were included in a follow-up regimen. Neither conversions nor 30-day mortality occurred. Oral re-intake was on day 3.47 ± 0.6 (range 2–4) and length of stay was 7 ± 1.2 days (range 5–9 days). All patients were treated with curative-intent surgery and adjuvant chemotherapy (CHT) according to current international guidelines for colorectal cancer. At median follow-up of 36 months (range 6–96 months), disease-free survival was 90% (76/84), overall survival was 92% (78/84), and disease-related mortality was 4% (3/84) (Table 1). Stages included in the survival analyses were II, III, and IV. Disease-free survival was 90% (72/80), overall survival was 92% (73/80) and cancer-related mortality was 13% (11/80) at a median 3-year follow-up. Overall survival for stage II, III, and IV was 94.1%, 92.3%, and 66.7%, respectively. Disease-free survival for stage II and III was 100% and 84.6%, respectively (table 1).

Disease free survival	76 (90%)
Alive w/recurrence	5 (6%)
Drop-out	0
Deceased	3 (4% - 3 cancer-related)

Table 1. Robotic right colectomy Follow-up

7.1.2 Left colectomy, hartmann procedure, sigmoidectomy

Twenty-three male and twenty female patients underwent surgery for left-sided colon cancer. Mean age was 60 ± 12 years. Median operative time was 220 (215 – 230) min. One conversion occurred for a splenic injury at the beginning of the experience. Specimen length was 24 ± 7.7 (range 10 – 49) cm. Number of harvested lymph nodes was 12 ± 7 (6 – 24). Surgery-related morbidity was 1/43 (2,3%): an anastomotic dehiscence in one of the first cases. All patients were included in a follow-up regimen. No 30-day mortality occurred. Median hospital stay was 7 d (range, 6 – 11), oral diet resumption was 3 d (range, 2 – 10). All patients were treated with curative-intent surgery and adjuvant chemotherapy (CHT) according to current international guidelines for colorectal cancer. At median follow-up of 36 months, disease-free survival was 88.3% (38/43), overall survival was 90% (38/43), and disease-related mortality was 7% (3/43) (Table 1). One patient was lost during follow-up.

7.1.3 Rectal surgery

Thirty male and eighteen female patients underwent rectal surgery: 45 rectal anterior resections (RAR) with TME and 3 abdominalperineal resections (APA). Mean age was 67 ± 12 years. Median operative time was 270 (240 – 315) min. Specimen length was 23 (19 – 27,5) cm. Number of harvested lymph nodes was 15 (12 – 20). Circumferential margins are shown in table 2.

Cm	Upper rectum	Middle rectum	Lower rectum
< 0.2	0	0	0
0.2 – 0.6	1		7
0.6 – 1.0	0		12
> 1.0	11		17

Table 2. Circumferential margins

Median longitudinal distal margin was 3 (2 – 4) cm. Surgery-related morbidity was 8% (4/48): there were four anastomotic leakages: two were treated laparoscopically only by peritoneal washing and drainage. Two were treated conservatively. No 30-day mortality occurred. Median hospital stay was 8 d (range, 8 – 11), oral diet resumption was 3 d (range, 2 – 13). All patients were treated with curative-intent surgery and neoadjuvant chemoradiotherapy (CHT) according to current international guidelines for colorectal cancer. At median follow-up of 36 months, disease-free survival was 67.8% (38/48), overall survival was 87.5% (42/48), and disease-related mortality was 8.3% (4/48) (Table 3). One patient was lost during follow-up.

Disease free survival	38 (67.8%)
Alive w/recurrence	3 (13.0%)
Drop-out	1 (4.3%)
Deceased	6 (24.7% - 5 Cancer-related; 1 others)

Table 3. Robotic rectal surgery Follow-up

8. Discussion

Colorectal cancer is still the third leading cause of death in the US, even though death rates have also been declining by 2,2% per year since 1998.
The Medical Research Council Conventional versus Laparoscopic-Assisted Surgery In Colorectal Cancer (MRC CLASICC) trial was set up in 1996 to evaluate the technical and oncological safety and efficacy of laparoscopically assisted surgery in comparison with conventional open surgery for the treatment of colorectal cancer.
The last update of the CLASICC Trial showed the oncological adequacy of the laparoscopic technique compared to the open one. Moreover, minimally invasive surgery has general benefits such as less blood loss, postoperative pain, and use of anesthetics, as well as fewer early and late wound complications, a shorter hospital stay, and better aesthetic outcomes. However, there are some limitations of the laparoscopic surgery: tremor, unstable two-dimensional view, and limited degree of freedom of the instruments.
Robotic surgery is spreading all over the world for many surgical procedures ranging from cardiac to general and urologic surgery thanks to its potential advantages overcoming the negative aspects of laparoscopic approach. It provides the surgeon with a 3-dimension display which enhances depth perception, allows the surgeon to operate in a comfortable, seated position with eyes, hands and operative field in line. Furthermore, the robotic instruments contain articulation which recall human wrist movements with 7 degrees of freedom to improve dexterity.
These characteristics of the robotic system may improve dissection and consequently oncological outcome. It is acquired that presence of nodal metastasis and mainly its distribution are key factors in predicting disease-free and long-term survival and for deciding on postoperative adjuvant therapy. The American Joint Committee on Cancer (AJCC) and College of American Pathologists (CAP) recommend evaluation of a minimum of 12 lymph nodes. In right colon cancer, we were able to perform a correct right colectomy easily identifying the major colic vessels and carrying out accurate lymphadenectomy, taking advantage of the steady, 3-D image view and of the articulation of the robotic instruments, which allowed us to manage organs such as the pancreas or the duodenum gently. The average length of the resected specimen in this series was 28 ± 5 cm, and the mean number of harvested lymph nodes was 19.70 ± 7.2, above the minimum recommended by the AJCC. To our knowledge our experience on robotic right colon resection is the largest published in the literature. We report a median follow-up of 36 months. Disease-free and overall survival were 90% and 92%, respectively; survival rates for stage II and III was 94.1% and 92.3%, and disease-free survival was 100% and 93%, respectively. Recent studies have shown 3-year overall survival varying from 68% to 100% for stage II and from 68% to 97% for stage III, and 5-year survival rates for stage II and III of about 72–90% and 44–72%, respectively (Gattaj et al., 2003; Roxburgh, 2209; Japan National Cancer Center, 2010). A comparison of our results with the literature shows that robotic right colic resection is able to offer the same short-term outcome as right colic resection performed by conventional laparoscopy or laparotomy. Moreover, we assert that the da Vinci System allows better standardization of the surgical technique of right colectomy, positively increasing the percentage of correct lymphatic resections.
We agree with other authors (deSouza et al., 2010) that among all robotic colorectal resections, right colectomy may be also considered the ideal procedure for the surgeon at the beginning of the learning curve as the robotic left colectomy.

We consider the rectal anterior resection the procedure in which robotic system better expresses its potential advantages.
The current technique of TME was developed to reduce local recurrences and improve overall survival while maintaining an adequate quality of life. The concept of TME is founded on the anatomical dissection along the embryologic avascular areolar plane between the fascia propria recti and the parietal endopelvic fascia. The integrity of the mesorectum as well as clear circumferential and distal margins are important oncological and surgical end-points. Moreover, the complexity of the regional anatomy requires a precise and a sharp dissection under direct vision following anatomical pathways in order to preserve the autonomic innervation. All the advantages may contribute to improve oncological adequacy and nerve preservation during this procedure. The first step of TME starts with the incision of the posterior peritoneum at the level of the promontory on the bifurcation of the aorta into the common iliac arteries. At this level, the 3-D view allows the surgeon to better identify and preserve the preaortic nerves and the superior hypogastric plexus (SHP). The use of the articulated monopolar cautery hook helps to obtain a better energy delivery control, avoiding inopportune cauterization of the nervous bundle. Moreover, the steady image and the view magnification allow a correct identification of both fasciae and a sharp dissection of the "holy plane". Any dissection strayed to the presacral fascia may lead to injuries to the ureters, autonomic nerves and presacral veins.
The second step of the TME consists in the anterior dissection, following the plane between the Denonvilliers' fascia above and the fascia recti below. Denonvillier's fascia can be easily identified by robotic view, helping the surgeon to carry out a precise incision of this fascia at the level of the seminal vesicles, avoiding gross manipulation of the tissue and unintentional injuries to the posterior capsule of the prostate (male) or posterior vaginal wall (female). The third step of TME includes the lateral mobilization of the rectum by incision of the lateral ligaments. A dissection close to the rectal wall avoids injuries to this nervous bundle. Moreover, a gentle counter traction of the rectum may help opening the dissection plane: this maneuver seems to be improved by robotic assistance thanks to the stability and motion scaling of the robotic arms. Excessive traction may lead to risk of injury to the pelvic splanchnic nerve. Robotic stereoscopic view, in addition, makes these structures more clearly visible. Middle rectal artery or its branches may be easily identified and cauterized without any peculiar difficulty. The tip articulation of the instruments facilitates the TME also, allowing a fine and precise dissection even in a narrow space, where dissection may result difficult by conventional laparoscopy or open surgery. This aspect is important as the reduction of the local recurrence rate is directly related to the optimization of the free surgical margins with recognition of the importance of clear radial (CRM) and distal mesorectal margins and of the distance from the tumor rim. The extent of circumferential tumor clearance after rectal cancer excision impacts long-term oncologic outcomes. In our experience, robotic assistance allowed us to achieve a 0% CRM < 2 mm rate and a correct mesorectal excision in all cases. Moreover, median lymph node number was 15.60 (12 – 21) and median specimen length was 23 (19 – 27,50). Length of stay was similar to laparoscopic series and shorter than open experiences. Operative time was 270 (240 – 315) min. A comparison of our results to main robotic experiences in Literature reveals similar trends in terms of length of stay, pathological findings and short-term outcome (table 4).

Author Year	Procedures	Operative Time	LN	Positive RM	Complications	LOS
Ashwin 2011	36	337.9 (81.8)	15 (7.8)	0	11 (30.6)	7.0 (5.8)
Baek 2010	41	296	13.1	1	9	6.5
Bianchi 2010	25	240*	18*	0	4	6.5*
Park 2010	41	231.9 (61.4)	17.3 (7.7)	2	12	9.9 (4.2)
deSouza 2010	44	347*0	14 (5–45)	0	11	5
Koh 2010	21	292.3 ± 32.6	17.8 ± 7.1	1	5	6.4 ± 4.1
Luca 2009	28	290 ± 69	18.5 ±8.3	0	12	7.5 ± 2.8
D'Annibal e 2011	48	270*	15	0	4	8*

Table 4. Robotic rectal surgery experiences. LN: lymph nodes. LOS: length of stay

9. Conclusions

Laparoscopic colorectal surgery has become a mainstay in the treatment of benign and malignant colorectal diseases. Recently, a new update of the CLASICC trial has confirmed the oncological adequacy and the safety of laparoscopic colorectal surgery (Jayne et al., 2010).

There are some drawbacks, however, of the laparoscopic technique such as unstable video camera platform, limited motion of straight instruments, two-dimensional imaging, and poor ergonomics for the surgeon. Robotic surgery is spreading all over the world for many surgical procedures ranging from cardiac to general and urologic surgery thanks to its potential advantages overcoming the negative aspects of laparoscopic approach (Piazza et al., 1999; Reichenspurner et al., 2000; Kappert et al., 2000; Gill et al., 2000; Chen et al., 2009).

The da Vinci surgical system (Intuitive Surgical Inc., Sunnyvale, CA, USA) was the first telerobotic system approved for intra-abdominal surgery in the USA by the Food and Drug Administration (FDA, 2000). The first robot-assisted colectomy was reported by Ballantyne et al. in 2001 (Ballantyne et al., 2001). Since then, several surgeons have performed robotic colorectal surgery. The advantageous features of the robotic system are the physical separation of the surgeon from the patient, six degrees of freedom plus grasping of the robotic arms, hand-like motions of the instruments offering the surgeon the impression of an open access, elimination of tremor, optional motion downscaling (2:1 to 5:1), and three dimensional stereoscopic image (Ballantyne et al., 2002) The surgeons console and the projected three-dimensional virtual operative field offer an ergonomically comfortable position with minimum fatigue (Braumann et al., 2005).

In right colectomy procedures, we were able to perform correct R1 and R2 right colectomy, easily identifying the major colic vessels and carrying out accurate lymphadenectomy over the plane of the superior mesenteric axis, taking advantage of the steady, 3-D image view and of the articulation (Endowrist) of the robotic instruments, which allowed us to manage organs such as the pancreas or the duodenum gently. Our observation is that robotic technique could allow better standardization, leading to improved performance of minimally invasive right colic resection, especially in terms of achieving correct lymphatic

resection in a high fraction of cases. Moreover, if we compare our oncological results in terms of overall and disease-free survival to those in literature, it is clear how robotic right colic resection is able to offer the same short-term outcome as right colic resection performed by conventional laparoscopy or laparotomy not only by an oncological point of view but also by recovery time duration. In our experience, indeed, hospital stay was shorter than open one and comparable to laparoscopy.

In left colectomy procedures, the sole advantage of the robotic system consists in IMA dissection: the 3-D view and the Endowrist articulation allow the surgeon to better identify the preaortic parasympathetic fibers which may be incorrectly manipulated and injured, increasing the risk of sexual or urinary dysfunctions. Moreover, thanks to a stable and tridimensional view, it is possible to decrease the risk of vascular injuries. Besides this aspect, we believe that robotic left colectomy is to be considered as an initial step in the learning curve of robotic surgery for a surgeon.

In our opinion, the predominant procedure which best enhances the advantages of the robot is TME, and several other authors have reported their experience with the robot in TME. The main concerns about laparoscopic techniques relate to the poor dexterity and the rigidity of the instruments, the 2-dimensional view and the camera stability depending by the assistant skillness. The robot overcomes these limitations and allows for more precise oncologic dissection. In our experience, circumferential margins were acceptable and none of the analyzed specimens presented an infiltrated circumferential margin or less than 2 mm from the tumor bed. Moreover, the magnified, stable, 3-D view and the articulation of the tip of the robotic instruments allowed us to better identify the planes of dissection, so performing a correct nerve sparing resection and a correct TME as showed by the pathological reports. The advantages of the robotic system are emphasized especially in men, in which the narrow structure of the pelvis makes the dissection difficult by laparoscopy approach and "blinded" by open approach. Operative time is longer than the laparoscopic one, but we believe it may be reduced by experience. Moreover, the robot setup we adopted allows to reduce operative time by switching only one robotic arm from one trocart to another one, avoiding disengagement and re-engagement of the robotic system, as described by initial experiences. Recovery time was shorter than in open surgery, and morbidity was acceptable, confirming the safety and feasibility of robotic assistance in TME.

In conclusion, robotic assistance may help the surgeon in performing colorectal procedures and improve the patient outcomes and provides acceptable oncological results.

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Edited by Rajunor Ettarh

The projections for future growth in the number of new patients with colorectal cancer in most parts of the world remain unfavorable. When we consider the substantial morbidity and mortality that accompanies the disease, the acute need for improvements and better solutions in patient care becomes evident. This volume, organized in five sections, represents a synopsis of the significant efforts from scientists, clinicians and investigators towards finding improvements in different patient care aspects including nutrition, diagnostic approaches, treatment strategies with the addition of some novel therapeutic approaches, and prevention. For scientists involved in investigations that explore fundamental cellular events in colorectal cancer, this volume provides a framework for translational integration of cell biological and clinical information. Clinicians as well as other healthcare professionals involved in patient management for colorectal cancer will find this volume useful.

ISBN 978-953-51-6808-9

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