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Genetics and Etiology of Down Syndrome

Edited by Subrata Dey

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Meet the editor

Subrata Dey was born in India and grew up in the state of Assam. He received his Ph.D. from the University of Kalyani, West Bengal. He joined the faculty of West Bengal University of Technology as Professor of Biotechnology in 2005. In 2006 he became the Director of the School of Biotechnology and Biological Sciences. His laboratory has long been involved in research on Genetics of Down syndrome & other congenital disorders, Genetics of Alzheimer's disease, radiation induced genomic instability, radioprotection by antioxidants & stem cell biology. He has been teaching courses in Genetics, Molecular Biology, Evolution & Developmental Biology for more than thirty years. Prof. Dey received golden jubilee award of excellence from Presidency College (Presidency University), Kolkata, in recognition of his contributions to undergraduate and post graduate teaching. He is also the founder Director of the Centre for Genetic Counselling. Prof. Dey is the author of many scientific papers resulting from research funded by University Grants Commission, Council of Scientific & Industrial Research, Inter-University Accelerator Centre, National Tea Research Foundation & Department of Biotechnology.

Contents

Preface

This book provides the recent developments and advances in research on Down syndrome. It also covers a wide range of topics, including investigations on neurologic, urologic, dental and allergic disorders in Down syndrome. Chromosomal aneuploidy is the leading cause of fetal death in our species and the information about chromosomal nondisjunction in man largely comes from studies in trisomy 21 or Down syndrome, the most frequent of the autosomal trisomies in liveborns. The cause of nondisjunction of chromosome 21 remains largely unknown. Accurate investigations on meiotic nondisjunction have been made possible in recent years by the development and utilization of microsatellite markers. Although several hypotheses have been put forward, it is still unclear as to whether particular gene loci on chromosome 21 are sufficient to cause Down syndrome and its associated features. For over two decades trisomy 21 has represented a prototype disorder for the study of human aneuploidy and copy-number variation, but the genes responsible for most Down syndrome phenotypes are still unknown. The genetic mechanism by which wide variability in the phenotypes arise is not understood, additional complexity may exist due to possible epigenetic changes that may act differently on Down syndrome. Consequently, gene-disease links have often been based on indirect evidence from cellular or animal models. Numerous mouse models with features reminiscent of those seen in individuals with Down syndrome have been produced and studied in some depth, and these have added considerable insight into possible genetic mechanisms by which trisomy 21 leads to Down syndrome.

The book is organized into four sections. All sections include chapters on recent advances in Down syndrome research.

Section I deals with our present knowledge on the genetics and etiology of Down syndrome.

Section II discusses the utility of using mouse model for in depth study of Down syndrome. Down syndrome could be used as model for understanding the genetics of Alzheimer's disease.

Section III describes the etiology and clinical aspects of some common disorders of Down syndrome patients such as neurologic, urologic, dental and allergic disorders.

Section IV focuses on prenatal diagnosis and screening of Down syndrome.

This book provides a concise yet comprehensive source of current information on Down syndrome. Research workers, scientists, medical graduates and paediatricians will find it an excellent source for reference and review.

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The editor wants to acknowledge the superb assistance of staff members and management of InTech Publisher. In particular, Ms. Romina Krebel for her co-ordination and editorial assistance. We are grateful to all contributing authors and scientists who made this book possible by providing valuable research and review papers.

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Part 1

Genetics and Etiology

1

Genetics of Down Syndrome

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1. Introduction

1.1 Morphology

According to the International System for Human Cytogenetic Nomenclature (ISCN) human chromosomes (2n=46) are divided into two groups (Shaffer et al., 2009). These are the two sex chromosomes or gonosomes (X,Y) and the 44 non-sex chromosomes or autosomes, respectively. Chromosomes of the latter group are numbered as 1 to 22, according to their decreasing size. Autosomes in somatic cells are comprised of two homologous, genetically identical chromosomes.

The time of the first conference for nomenclature in 1959 is called the pre-banding area. Individual chromosomes could not yet be ascertained beyond reasonable doubt. Thus it happened that the second smallest chromosome, chromosome 21, which had been analysed three times in the patient's karyotype, was believed to cause Down Syndrome (DS). Later studies showed that DS is trisomic in the smallest chromosome. To avoid conflict between previous and subsequent publications, the position of the two smallest chromosomes (21 and 22) was switched, resulting in the definition of DS as trisomy 21.

The relative length of chromosome 21 is 1.9 ± 0.17 % of the total length of the human genome, and its size is approximately 60 Mb. Chromosome 21 belongs to the acrocentric chromosomes, i.e. the centromere is localised closer to the end of the short arm (p). The short arm 21p is heterochromatic but consists of different types of repetitive DNA (Figure 1)(Wyandt and Tonk, 2004).

The relative length of the short arm of chromosome 21 comprises 30 % of its total length (Figure 1). Variants in brilliant fluorescence after QFQ-staining are diagnosed in 2.0 % of band p11.2 and 10.0 % of band p13. Duplications in p12 show a frequency of 0.7-1.3 % and 0.1 % in the satellites of p13. Deletions in all three regions (p11.2, p12, p13) are rare (Kalz et al., 2004).

These frequencies are derived from population studies based on Europeans. Significant differences in comparison to other ethnic groups have been observed (Kalz et al., 2005). The polymorphic regions in the short arm of chromosome 21 allowed the first studies on the parental origin of trisomy 21 (Mikkelsen et al., 1980).

The long arm (q) of chromosome 21 is euchromatic, with the exception of the peri-centromeric region q11.1 and the distal telomere.

Chromosomes are usually presented and analysed in the metaphase of mitosis after *in vitro* cultivation, which is not identical to their appearance *in vivo*. Among the differentiated cells,

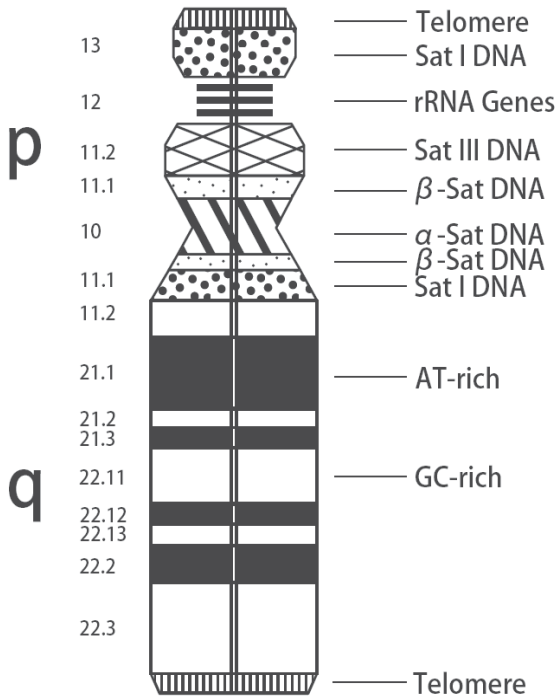


Fig. 1. Structure and morphology of chromosome 21. Ideogram according to the ISCN (Shaffer et al., 2009).

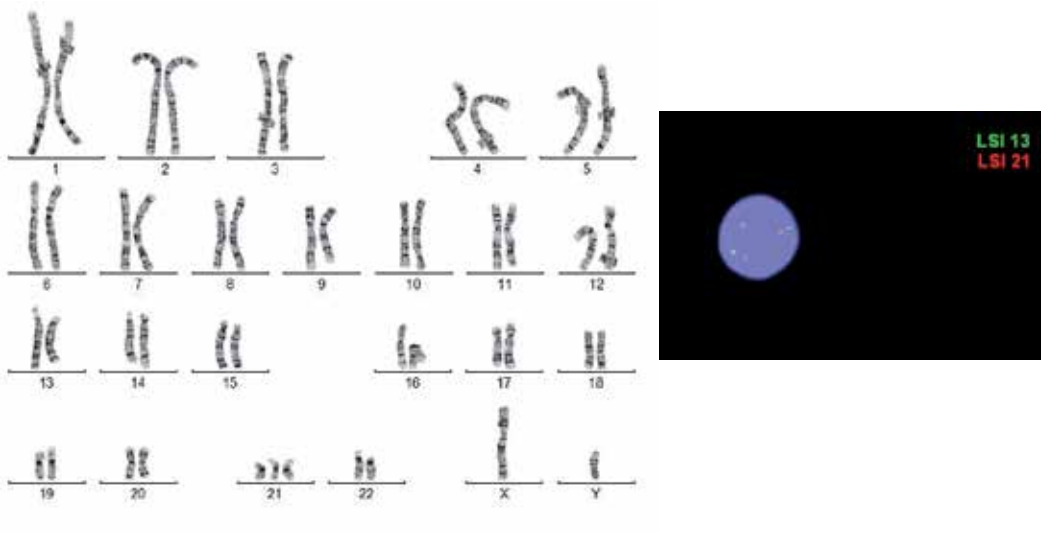
only some (i.e. T-lymphocytes in a blood sample) can be stimulated *in vitro* to enter the cell cycle again and thus represent a selected cell population. In addition, cells are treated with colcemid. This substance arrests the chromosomes in the c-metaphase of mitosis and, at the same time, increases the contraction of chromosomes, rendering the centromeres and the fissure between the two chromatids visible (Figure 2a).

1.2 Structure

The central part of the centromere of chromosome 21 consists of α-satellite DNA that is almost identical to the centromere of chromosome 13 (homology 99.7%)(Figure 1). On both sides α-satellite DNA is flanked by β-satellite DNA. These two non-coding regions can vary significantly in size through duplication or deletion. They are irrelevant for the carrier, unless their length is less than 20 % of the average length of the region and thus prevents the normal development of the kinetochores. This would result in the failure of exact separation of the chromatids in the anaphase of mitosis (Waye et al., 1989; Mitchell et al., 1992).

Distal of the β-satellite DNA, satellite DNA class III is situated on the short arm (p11.2). Significantly varying in size, this band shows a specific absorption of DNA-dyes. Therefore, it is defined as a polymorphic region. It is followed in the short arm by the band p12, which is also named the nucleolus organising region (NOR) and contains the ribosomal RNA-genes. It is characterised by its slightly lateral expansion (satellite stalks). It is polymorphic and can be deleted or amplified (Tagarro et al., 1994a). The most distal regions of the short arms are the satellites (p13 or s), consisting of Sat I DNA with the telomeres at the ends

(Tagarro et al., 1994b). Satellites are also polymorphic varying in size and staining characteristics. They also have the ability to duplicate.
In describing the structure of the short arm of chromosome 21, only the main components of the different bands are mentioned. Especially p11.1, p11.2, and p13 contain further subgroups of repetitive DNA.



a) b)

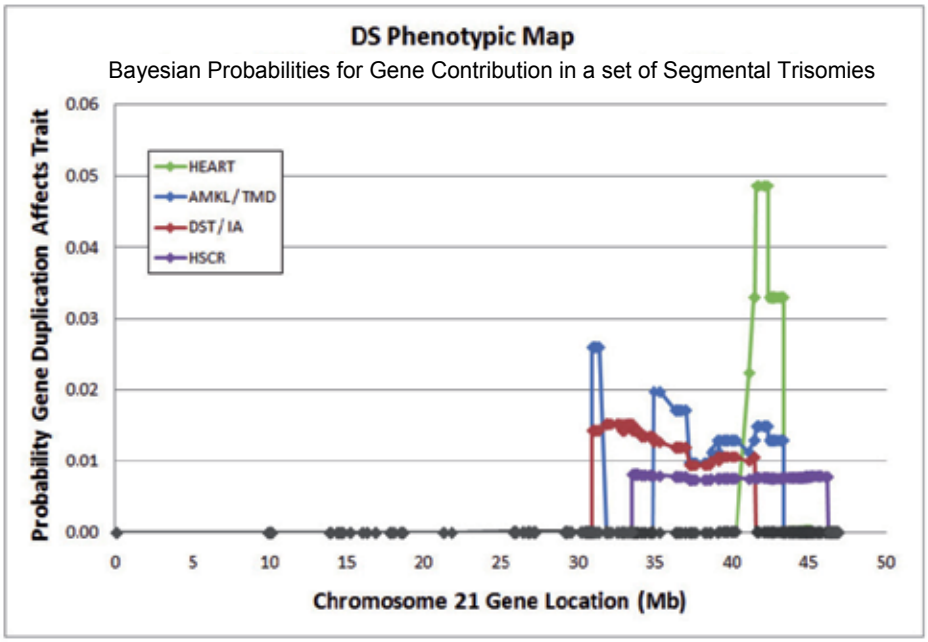
Fig. 2. Routine diagnostic workup for identification of trisomy 21. **a)** Standard karyotype (47,XY,+21) by GTG banding (by kind courtesy of U. Mau-Holzmann, Tübingen). **b)** Interphase FISH showing three signals of chromosome 21 and two of chromosome 13 (LSI21:21q22.13q22.2; AneuVysion multicolor DNA probe Kit, Vysis).

According to the literature, the proximal heterochromatic region of the long arm (q11.1) of chromosome 21 consists of β-satellite DNA boarding the central α-satellite DNA and followed distal by Sat I DNA (Waye et al., 1989; Mitchell et al., 1992; Tagarro et al., 1994a, 1994b). The main part of the long arm is euchromatic. AT- and GC-rich bands have characteristic sequences and can be differentiated by their typical staining features (figure 1). These bands of single copy DNA are interspersed by non-coding repetitive DNA (SINEs and LINEs).

1.3 Aneuploidy and gene content

A complete or partially aneuploid chromosome is associated with a pathologic phenotype in the carrier, the expression of which depends on the type and amount of the aberrant genetic material.
In contrast to chromosome 22, chromosome 21 consists of a high number of AT sequences which contain a smaller amount of vitality-determining genes than the GC-rich ones.
GC-rich or housekeeping genes are expressed in most cell types. They lead to proteins that carry out various metabolic and structural functions. In contrast, the AT-rich genes are tissue-specific and are only active in certain cell types while being inactivated in others by methylation. This gene inactivation is accompanied by a more condensed structure of the

chromatin and, consequently, the DNA of these genes is not accessible to the transcription factors. These AT-rich DNA regions show a higher staining intensity and can thus be localised by chromosome analysis.
Because of its high content of AT-rich regions, trisomy 21 is compatible with life, and in the majority of cases, leads only to retardation in the development of the carrier and not, as in trisomy 22, to lethality.
The gene map of chromosome 21 was initially constructed by combining the analyses of small structural aberrations with the results of different gene product analyses (dosage effect). Chromosome 21 was sequenced in 2000 (Hattori et al., 2000), and 225 loci (genes) were identified, which was less than expected. This might explain the relatively mild phenotype of the carriers.
In the following years, a high number of small regions in 21q has been analysed in order to localise the DS critical region (Figure 3)(Wong, 2011), but recent investigations revealed in contrast to the first assumptions that a direct genotype-phenotype correlation does not exist, since a large number of gene products from chromosome 21 also influences gene products and their function on heterologous chromosomes (Gardner and Sutherland, 2004; Weinhaeusel et al., 2011).



(AMKL acute megakaryocytic leukemia; TMD transient myeloproliferative disorder; DST duodenal stenosis; IA inperforate anus; HSCR Hirschsprung disease)

Fig. 3. Genotype-phenotype correlation in trisomy 21 based on partial trisomy 21 cases (from Korbel et al., 2009). (with kind permission of J.R. Korenberg)

2. Historic development of the cytogenetics of DS

DS was the first malformation complex that could be delineated as a chromosome abnormality in 1959. This was enabled by the new technology to prepare chromosomes in

the metaphase of mitosis. In the early years, mitoses were analysed after direct preparations of bone marrow cells and long term cell cultures of tissue biopsies. Starting in 1960, the lymphocyte culture of peripheral blood was established. Thereby screening of handicapped persons on a large scale became possible. At the beginning of the 1980s, prenatal diagnoses were started for high-risk groups.
Any extension of the spectrum of investigations, any more precise definition of the localisation of the aberration and characterisation of the patients´ symptoms were combined with an improvement of the investigation methods.
The direct preparation of meristematic somatic cells was followed by long-term and short-term cell cultures of differentiated somatic cells removed postpartum, by the culture of amniotic fluid specimen and biopsy of chorionic villi, as well as the analysis of germ cells and their precursor stages in certain special cases, of polar bodies and early postzygotic stages like morula and gastrula in preimplantation diagnostics.
Initially, the presentation of chromosomes was only possible through homogeneous staining, which was succeeded by application of radioactive markers and subsequently of the differentiated characterisation of the chromosomal banding patterns (GTG, GAG, QFQ, RBG, RBA, CBG, and others).
Today, a necessary requirement in diagnostic investigations is a high differentiation of the euchromatin (usually 550 bands per genome). By that way, structural aberrations of chromosome 21 can be safely detected microscopically, starting with a minimal length of 5 Mb. In the 1980s, fluorescence-in-situ hybridisation (FISH) as a new technique was introduced (Figure 2b). By FISH the characterisation of either the entire euchromatin or the centromeric areas or selected euchromatic bands with specific DNA-probes became possible. Simultaneously, this allowed the analysis of cells in interphase and the rapid investigation of larger amounts of cells without cell culture. This so-called rapid aneuploidy testing is especially important for prenatal diagnostics.
Further improvement of the investigation spectrum provided the development of the comparative genomic hybridisation (CGH) with the advancement to the microarray, which has been depicting an improvement and specification of diagnostics on the molecular-genetic level through development of specific tiling arrays.
The newest development is next-generation sequencing. This method is still known to be in trial tests, and its establishment in diagnostics is to be expected.
The various methods of investigation are often combined to improve diagnostics.

3. Types and frequencies of chromosome 21 aberrations

In addition to the predominance of standard trisomy 21 as the cause of DS, further types of aberrations exist. They differ in relation to the type of abnormality, and they lead to different prognoses as to the chances of development of the carrier and recurrence risks for the relatives of a carrier.
Therefore, the indication for a chromosome analysis is always given in the presence of the distinct phenotype of DS.

3.1 Standard trisomy 21

In this type of aberration, the carrier has 47 chromosomes, including three chromosomes 21. It accounts for nearly 90% of DS cases. Standard trisomy 21 typically occurs sporadically, therefore the recurrence risk is low.

The majority of free trisomy 21 cases (85-90%) originates from errors in maternal meiosis. In particular, maternal meiosis I is the most frequently affected stage of nondisjunction (>75%), whereas maternal meiosis II errors account for >20%. In 5% of free trisomy 21, paternal meiotic errors can be observed, here meiosis II nondisjunction is more frequent than meiosis I errors. In addition, postzygotic mitotic errors have also been reported (5%). The predominant influence of disturbed maternal meiosis is reflected by the decreased number of of chiasmata in meiosis I increasing with maternal age. Indeed, the reason for this association is unknown, however numerous hypotheses have been proposed (for review: Hultén et al., 2010).

3.2 Robertsonian translocations

Trisomy 21 due to an unbalanced translocation of chromosome 21 with a hetero- or homologous acrocentric, satellite-bearing chromosome (13, 14, 15, 21, 22) is called a Robertsonian translocation. However, the frequency of translocation partners varies and is a result of homologies in the heterochromatin of the short arm, thus leading to failures in the pairing of meisosis I. The resulting fusion products can be monocentric or dicentric with an inactive centromere.

In monocentric translocation chromosomes, the centromere can derive from each of the two partners or can be a hybrid structure originating from both of them.

Robertsonian translocations involve about 5% of the cases of trisomy 21. Approximately 75% are formed de-novo in the carrier , and 25% are familial (for review: Gardner and Sutherland, 2004). Among these, translocation 21/21 is an unusual rearrangement, but in the majority of cases, it is not a fusion of homologous but the formation of an isochromosome.

In general rule, carriers of the balanced Robertsonian translocation display only 45 chromosomes, the unbalanced ones show 46, as in the majority of carriers, and two short-arm regions are lost. The loss of two NOR-regions does not lead to clinical symptoms in the carriers of balanced translocations.

Among the different heterologous translocations of the acrocentric chromosomes with chromosome 21, the combination with chromosome 14 (rob(14q21q)) is the most frequent one with about 60%. This is followed by the translocation rob(21q21q) or by the formation of isochromosome i(21q21q), respectively, in 35% of the cases. The other translocations are rare and do not exceed 5%. (for review: Gardner and Sutherland, 2004)

Current studies of meiosis are leading to new insights on frequency of formation and postzygotic selection of Robertsonian translocations in familial cases. These studies are largely based on analyses of translocation rob(14q21q) as the most common subgroup. According to these, women with a balanced translocation have an aberrant karyotype in about 20 % of their polar bodies as well as in the oocytes and men in 10-15% of their sperms. These frequencies decrease postzygotically in the course of the development of the embryo, therefore the risk of a child with heterologous translocation trisomy 21 amounts to only 8% if the mother is the carrier and to 4 % with the father (for review: Gardner and Sutherland, 2004).

It is noticeable that in families with translocations, children with a normal phenotype carry a balanced translocation more often than the normal karyotype if the origin is maternal (60:40), while the ratio is equal with paternal origin (50:50).

3.3 Reciprocal translocations

Reciprocal translocations are caused by the exchange of euchromatic regions of chromosome 21 with the euchromatin of different autosomes or gonosomes. In addition to trisomic

regions in various length and location of chromosome 21, unbalanced forms at the same time show partial monosomy for the exchanged regions of the second translocation chromosome. As a result, the phenotype in the carriers of unbalanced translocations is not consistent.
Caused by the rare occurrence of these translocations, there are no reliable data for their incidence, a frequency of less than 1:1000 standard trisomies can be assumed. According to the literature, the most common partners for a reciprocal translocation seem to be the chromosomes 18 and 22 (Schinzel, 2001).

3.4 Duplications

This type of aberration is always formed de-novo in carriers with a noticeable pathologic phenotype. If the duplicated segment is only of small size or originated from a postzygotic mosaic, the impairment of the carrier may be mild, and he might have an almost unrestricted life opening the possibility of inheriting the duplication to his offsprings.
A duplication in the cells of the carrier can be caused by an unequal pairing of homologous chromosomes in the pachytene of meiosis I and an aberrant crossing-over as the consequence.
A paracentric inversion in the long arm of a parental chromosome 21 may present an increased risk for the formation of a duplication. According to published cases, the size of the duplicated region can vary significantly, and so far, no preferential sites for the exchange have been documented. With the few existing case reports, no data concerning the frequency of duplications can be given.

3.5 Submicroscopic aberrations

Investigations with FISH probes and applications of molecular-genetic methods for small euchromatic regions enabled the detection of structural aberrations in chromosome 21 in a size of less than 5 Mb. The amount of disorders that can be attributed to small duplications and deletions of chromosome 21 therefore has risen significantly within the last years. The phenotype of the carriers is predominantly not characteristic for the DS.

3.6 Gene mutations

Meanwhile, extensive genotype-phenotype correlations on the basis of structural aberrations of chromosome 21 have been reported, which help to narrow down the DS critical regions (Figures 3, 4)(for review: Korbel et al., 2009). A small number of genes has been proposed to cause the specific DS features, and among them are: DSCR1, DYRK1A or APP (for further details see other chapters of this book).

3.7 Mosaicism

The frequency of trisomy 21 mosaics after chromosomal analysis is about 3-5 %. This number is most likely too small since tissue-specific mosaics cannot always be detected.
Mosaics always originate from mitotic aberrations during the early postzygotic development of the conceptus. Their formation may be caused by an aberrant zygote loosing one of the three chromosomes 21 of a standard trisomy in a portion of the cells. Alternatively, the zygote can have a normal karyotype, but in a postzygotic mitosis, nondisjunction of chromosomes 21 takes place.

Structural aberrations of chromosome 21 as mosaics are always caused by postzygotic rearrangements. Carriers of mosaics with the aberrant cells occuring only in the gonads have an increased risk compared to the general population of the same age for the birth of a child with the aberration in all cells.
The risk of mosaic carriers for a retardation or affected offspring can not be specified because of the different types of aberrant karyotypes and their unequal distribution in the organism.
If the percentage of trisomic cells in all somatic tissues is small (0.5 – 5 %), the phenotype of the carrier can be normal.
Carriers with mosaics have a better prognosis than carriers of non-mosaic trisomies, but there is always a risk of uneven distribution of pathologic cells in the various tissues. Therefore, only the analyses of cells type stemming from different germ layers can lead to a reliable prognosis concerning the development of the patient.

3.8 Tetrasomy 21

A specific type of hyperploidy 21 is the tetrasomy where the chromosome 21 is present for four times. In the literature, only single cases have been published (Gardner and Sutherland, 2004). This abnormality can either consist of four free chromosomes 21 or of two normal chromosomes and in addition an isochromosome 21. The aberration is usually lethal in the conceptus in early pregnancy, but a mosaic constitution has been diagnosed in patients postnatally.

4. Techniques for DS testing

Many different cytogenetic as well as molecular-genetic techniques have been developed in the past to detect standard trisomy 21 and structural aberrations of chromosome 21. Whereas the "simple" detection of genomic imbalances can be performed with numerous molecular techniques (short tandem repeat typing, MLPA, molecular karyotyping), information on structural rearrangements is usually up till now achieved by classical microscopic methods, e.g. chromosome analysis and FISH. In future the development of high resolution next generation sequencing techniques will allow a nearly complete overview on all numerical and unbalanced structural rearrangements in one molecular assay.

4.1 Chromosome analyses

Chromosome investigation is the conventional cytogenetic method based on cells undergoing mitosis to obtain metaphase spreads. The chromosomes can be pre-treated and stained according to different protocols to induce specific banding patterns. By karyotyping, the specifically banded chromosomes can be arranged into seven groups (A to G) based on descending order of size and of the position of the centromere. According to the type of induced banding pattern, two subtypes can be defined: a) those resulting in bands distributed along the length of the whole chromosome, such as G-, Q- and R-bands, and b) those that stain specific chromosome structures (e.g. C-bands, nucleolus organizing regions, telomeric bands)(Shaffer et al., 2009).
The advantage of classical cytogenetics is that both unbalanced as well as balanced chromosomal aberrations are detectable. However, the technique is limited by the microscopic solution. Therefore, imbalances <5 Mb are not analysable by routine cytogenetics. Another disadvantage is that the majority of cell types has to be cultured in vitro either as a short-term

or long-term culture. The long culture times bear the risk of *in-vitro* chromosomal changes. The direct preparation of mitoses is only possible when analysing meristematic cells (bone marrow biopsies, trophoblast cells, germ cells and their precursors). This mehtod has the advantage that the chromosomal analysis reflects the situation in-vivo.
Chromosome analyses are mainly done after lymphocyte culture from peripheral blood samples. The cells are easily obtained and stimulated to mitosis, and the time of cultivation is only 48-72h.
The chromosomes are arranged in a formal karyotype, according to their size, centromere position, and banding pattern (Figure 2a) following the International System for Human Cytogenetic Nomenclature (ISCN; Shaffer et al. 2009).

4.2 Fluorescence-in-situ-hybridization (FISH)

FISH is a widely used method to analyse different target DNA sequences by supplying specific DNA - probes. It combines cytogenetic and molecular genetic techniques. The principle of FISH is the interaction of a labelled single-stranded DNA with a denatured metaphase or interphase.
Since a much higher resolution than chromosome analysis, FISH is used to identify and characterise small structural chromosome aberrations in clinical cytogenetics, including microdeletions and microduplications. A precise and detailed breakpoint analysis is possible. However, a FISH investigation is only applicable if the aberration in question is suspected.
At first this technique was restricted to metaphase analysis, meanwhile, it has gained importance in interphase diagnostics as well. In particular the latter procedure is extremely helpful to detect low-level mosaicism. Furthermore, it has enabled rapid prenatal testing for the frequent aneuploidies in the fetus, including trisomy 21 (for review: Caine et al., 2005).

4.3 Microsatellite typing

Short tandem repeat markers (STRs, microsatellites) are highly informative molecular markers which are easy to handle. STRs have been described as an abundant class of DNA - polymorphisms in the human genome, consisting of highly repetitive short DNA - sequences. They can be typed by using PCR and single-copy primers flanking the repeats, followed by denaturing on a high resolution gel or by capillary electrophoresis. In 1991, Petersen and co-workers were the first to describe the application of these markers in order to determine the parental origin of the extra chromosome in families with a trisomy 21 patient. Meanwhile, numerous studies on the origin of unbalanced chromosomal aberrations have been published.
It is the advantage of microsatellite analysis that it needs only minimal amounts of genomic DNA. Furthermore, it is a fast and simple method which is widely established. It is therefore not amazing that this technique is one of the most frequently used methods for rapid prenatal aneuploidy testing (Mann et al., 2004). It circumvents time-consuming cell cultivation and needs approximately 6-8 h from taking the sample to final report. In addition, the comparison of the allelic distribution of a fetus and his parents allows the exclusion of a maternal contamination in fetal DNA - samples.
However, the technique does not allow the detection of balanced rearrangements. Furthermore, a reduced informativity of microsatellite markers might hamper the interpretation. In particular in case of parental consanguinity, the informativity might be reduced.

4.4 Multiplex Ligation-dependent Probe Amplification (MLPA)

Multiplex Ligation-dependent Probe Amplification (MLPA) is a simple, high throughput method that allows detection of DNA copy number changes of up to 40 sequences in a single reaction. It is based on the semi-quantitative polymerase chain reaction principle and can be applied for detecting copy number changes and has been developed by MRC Holland (http://www.mlpa.com/). MLPA has rapidly gained acceptance in genetic diagnostic laboratories due to its simplicity compared to other methods, its relatively low costs, the capacity for high throughput, and its robustness. Typical for the MLPA is that not target sequences are amplified but MLPA probes that hybridise to the target sequence. In contrast to a standard multiplex PCR, a single pair of PCR primers is used for MLPA amplification. The resulting amplification products of an MLPA assay range between 130 and 480 nucleotides in length and can be analysed by capillary electrophoresis. By comparing the peak pattern obtained to that of reference samples it can be delineated which sequences have aberrant copy numbers. The MLPA reaction can be divided into five major steps: 1) DNA denaturation and hybridisation of MLPA probes; 2) ligation reaction; 3) PCR reaction; 4) separation of amplification products by electrophoresis; and 5) data analysis. For trisomy 21 and further frequent aneuploidies (trisomies 13 and 18), an aneuploidy MLPA kit is commercially available.

The advantage of MLPA is indeed the low amount of genomic DNA needed for genotyping and the fact that that parental samples are not necessary for comparison. However, the procedure is time-consuming and needs at least two days.

4.5 Array analysis

Molecular karyotyping is meanwhile a well established method to identify genomic imbalances. In particular the resolution is much better than that of conventional cytogenetics. While chromosome analysis detects imbalances (deletions and duplications) >5 Mb, array typing has a resolution of <100 kb (figure 4). Microarray analysis allows the identification of any type of segmental imbalance by virtue of its design, but it does not allow the identification of balanced rearrangements or small mosaics.

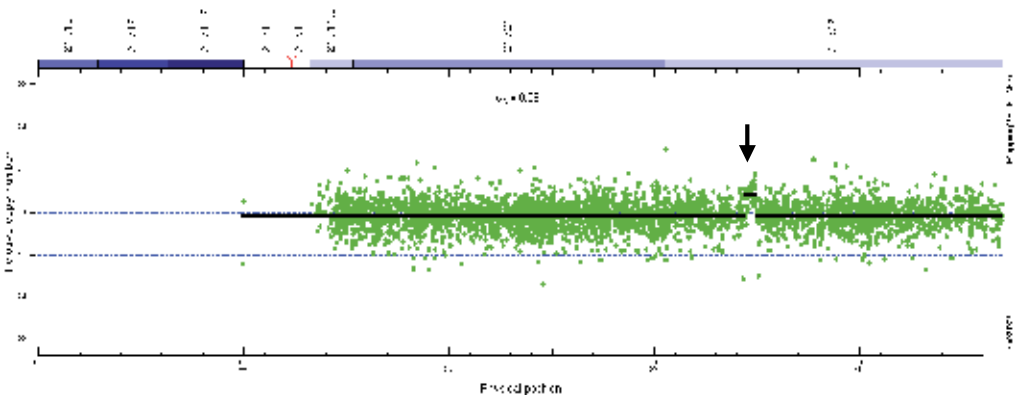


Fig. 4. Example of Micro-array based characterisation of a partial trisomy 21 in a patient affected by Silver-Russell syndrome (Eggermann et al., 2010). The patient did not exhibit any symptoms of DS, thus the trisomic region could be excluded to be involved in the specific phenotypic expression of DS.

As a large number of strategies and platforms are commercially available and cannot be covered here, we strongly emphasize checking the updated literature. Dependent on the array type, molecular karyotyping allows the detection of imbalances with a size of a few kb. And thus the detection rate for chromosomal aberrations in patients with mental retardation could be increased impressively (for review: Shaffer and Bejjani, 2010). However, the interpretation of microarray data is currently hampered by the large genomic instability mainly in non-coding regions, resulting in the analysis of a growing number of copy number variations (CNVs).
Nevertheless, first guidelines for molecular karyotyping have recently been published (Vermeesch et al., 2010).

4.6 Next generation sequencing

Due to the development of genome-wide sequencing (next generation sequencing) the cheap and accurate characterisation of whole genomes in a short time has become possible. Indeed, the methods and applications are extremely manifold and can be merely covered in this short paragraph (for review: Metzker, 2010). Of course, it is too early to apply this complex technology for characterisation of whole chromosome aberrations like trisomy 21. However, target assays will be developed, and it has to be considered that next generation sequencing will allow to rapidly identify and reliably characterise genomic disturbances – balanced as well as unbalanced – and breakpoints in cases of structural rearrangements.

5. Probability for the birth of a child with DS

As the clinical symptoms of a child with the phenotype of DS cannot be considered as a reliable classification, the diagnosis must always be accompanied by a chromosome analysis.
The occurrence of trisomy 21 in young mothers lead to screening investigations to ascertain whether exogeneous factors might play a role in the aetiology of trisomy 21 besides the well-known genetic risk factors.

5.1 Subjects for discussion on exogeneous risk factors

Different substances known as mutagens, co-mutagens, and teratogens have been investigated in detail.
The atomic bomb blasts in Japan at the end of the World War Two and the explosion of the atomic reactor in Chernobyl in 1986 are well-known examples of exposures and have been thoroughly explored. However, they did not lead to an increased birth of children with DS (Dean et al., 2000).
Furthermore, neither contact with different organic substances or heavy metals nor an increased consumption of caffeine, alcohol, or nicotine could be shown to increase the number of births of children with trisomy 21 or other chromosome aberrations.

5.2 Endogeneous risk factors

This group encloses carriers of balanced and unbalanced chromosome 21 aberrations and includes the risk of advanced maternal age at pregnancy as well.

5.2.1 Carriers of standard trisomy 21

Adequate therapy has increased the life expectancy of carriers of trisomy 21 significantly within the last decades. Furthermore, a largerly normal life by a specific support of motor and

mental abilities has become possible for many DS individuals. This leads to pregnancies in DS women and makes adequate genetic counselling necessary. The theoretical risk for a woman with trisomy 21 to have a child with trisomy 21 is 50%, but it is reduced by the high amount of abortions in early pregnancy. An empirical risk of 30-40% can therefore be delineated.
However, genetic counselling has also to take into account the risks of a pregnancy in a DS woman suffering from malformations such as heart defects or kidney anomalies. Males with a trisomy 21 are usually sterile.

5.2.2 Recurrence risk in carriers of mosaic trisomy 21

In mosaic cases, the risk of a female or male in rare cases carrier to have a child with full trisomy 21 cannot be estimated precisely, because only the amount of trisomic cells in the somatic tissues can be analysed but not the one in the gonads. The ratio of trisomic cells may even be 100% in the ovaries and testes, leading to the same risk factor as in carriers with a full trisomy.
A special situation is given if the carrier has a normal karyotype in all somatic cells investigated, and the trisomic cells are restricted to the gonads (parental germline mosaicism). In these cases the empiric recurrence risk is delineated as 1-2%, but in two cases of two or more pregnancies with trisomy 21 it is estimated to be much higher (empirical value of more than 10%) (Warburton et al., 2001).
Mosaics are usually found in free trisomy 21 but mosaicism of translocation trisomy 21 has also been observed.

5.2.3 Carriers of a balanced heterologous Robertsonian translocation

Significant differences concerning risk factors have been observed for female and male carriers of heterologous translocations. In female carriers, the empiric risk of having a child with an unbalanced translocation is about 10%, and in males it is only 1-2% (Ferguson-Smith, 1983; Daniel et al., 1989). In both sexes, the translocations rob(14q21q) and rob(15q21q) harbour the additional risk of uniparental disomy 14 or 15 as a consequence of trisomic rescue (Kotzot and Utermann, 2005) which is associated with specific clinical syndromes.

5.2.4 Robertsonian translocation rob(21q21q) and isochromosome 21

Balanced carriers of these two types of rearrangements have a risk of 100% that the offspring will inherit a translocation trisomy 21, regardless of the sex.

5.2.5 Parents with a balanced reciprocal translocation

In the rare group of parents carrying a balanced chromosome rearrangement affecting whole or partial 21q, the risk of having a child with a complete or partial trisomy 21 is relatively high, with about 20% in females and 10% in males. If the translocation chromosomes and their normal homologous show pairing difficulties in meiosis I, the additional risk of a 3:1 segregation has to be taken into account, leading to a recurrence risk of up to 30%. Offspring with an unbalanced translocation show monosomy of the heterologous chromosomal segment, in addition to the complete or partial trisomy 21. As a result, the phenotype will be complex and heterogeneous, depending on the origin of the second translocation chromosome.

5.2.6 Parents with normal karyotype having a child with DS

This is the main group of consultants. The maternal age at pregnancy is significantly elevated in the majority of cases, and therefore, an increased risk for elder women can be

delineated (risk of 1:1667 at 20 years and 1:32 at 45 years (Morris et al., 2002). There is a second slight increase of risk in very young mothers (1:1000 at 15 years). The probability for a child with DS is not correlated with paternal age.
However, in a small cohort of families an age independent high risk for pregnancies with different trisomies has been observed (Munné et al., 2004; Baart et al., 2006).
Molecular investigations made it probable that in these cases the increased aneuploidy rate is caused by an autosomal recessive mutation. To identify this group of carriers a thorough pedigree analysis is necessary, cytogenetic karyotyping is indicated for the consultants and their offspring – children and miscarriages. Failures from pregnancy induction by IVF (in-vitro fertilisation) have to be included in the risk estimation. These couples are usually recommended to a special IVF program for a further pregnancy, and preimplantation-testing procedures might be considered (Stumm et al., 2006).

5.2.7 Siblings and 3rd degree relatives of a patient with DS

Healthy relatives of a patient with a free trisomy 21 have no increased risk for the birth of a child with trisomy 21 when compared to the average population of the same age.
Relatives of a proband with DS caused by an unbalanced structural rearrangement have an increased risk if they are balanced carriers of the translocation (see above). The risk factor depends on the type of rearrangement and on the sex of the carrier.

5.2.8 Prenatal findings of symptoms characteristic for DS detectable by ultrasound

At the end of the first trimester of pregnancy (10th to 12th week,) ultrasound investigations are routinely recommended. At that age, the majority of fetuses with trisomy 21 show a number of characteristic features. These include nuchal translucency, absence of nasal bone, heart defect and growth retardation. In the second trimester, the main symptoms detectable by ultrasonography are a flat occiput, a flat profile of the face, small nose, dysmorphic and deep seated ears, receding chin and short neck, malformations of the internal organs, and growth retardation.
These observations by ultrasound are an indication for prenatal chromosome analysis either by chorionic villi sampling (CVS) or by amniocentesis (AC).

5.2.9 Comparison of invasive and non-invasive prenatal investigations

Non-invasive methods

The main technique in prenatal diagnostics is ultrasound. In Germany, it is usually applied three times during pregnancy. Ultrasound can meanwhile be regarded as a prerequisite for CVS, AC, or fetal blood sampling (FBS) since morphologic abnormalities are caused by a pathologic karyotype in 20-50% of the cases (personal observation).
A second group of non-invasive parameters are specific biochemical factors (AFP, β-HCG, estriol) analysed from maternal blood. They are combined with the risk delineated from the given maternal age. If the result differs from expectation and corresponds to an increased risk for DS, invasive methods to determine the karyotype are applied.
A third possibility for non-invasive investigations in pregnancy is the analysis of fetal cells and fetal DNA in the maternal blood. By special procedures, fetal DNA can be isolated and enriched from maternal blood, however, this method is still under development and is currently not applied in routine prenatal testing.

Invasive prenatal investigations

Three different methods are usually applied: CVS in the first trimester, AC in the second, and AC, FBS, and placenta biopsy in the third.
All three methods generally include an investigation risk of about 1%. Therefore, these methods should only be applied if the genetic risk is higher than the risk of investigation.
Sometimes different methods have to be combined to receive a diagnosis. However, this increases the time of investigation and prolongs the psychological stress of the parents (Geskas et al., 2011).
Usually, a reliable, final diagnosis can only be achieved by invasive investigations but the non-invasive methods enable the investigator to get information of an increased or decreased extent of the pre-existing risk in the individual pregnancy.

6. Comparison of type and contretype of DS

As a full monosomy 21 is lethal in life-born children and also exceedingly rare in spontaneous abortions, the phenotype of carriers of this aberration is delineated from mosaic cases and partial deletions. The clinical findings revealed a number of symptoms, especially facial dysmorphic features that can be defined as opposite type or "contretype" to patients with trisomy 21 (Table 1).

Trisomy 21	**Partial monosomy 21**
Muscular hypotonia	Muscular hypertonia
Overextension of joints	Spasticity
Hyperflexible fingers	Camptodactyly
Flat occiput	Protuberant occiput
Upslanting palpebral fissures	Downslanting palpebral fissures
Small round ears	Large ears
Aplastic nasal bridge	Protuberant nasal bridge

Table 1. Phenotypes of trisomy and (partial or mosaic) monosomy 21. (Schinzel, 2001)

7. DS and uniparental disomy

As trisomic rescue is the most frequent way of uniparental disomy formation, this type of cytogenetic aberration has to be discussed in context with trisomy 21. Uniparental disomy (UPD) is the inheritance of both homologous of a chromosome pair from only one parent. The concept of UPD was first postulated to cause specific phenotypes in the eighties (Engel, 1980). This hypothesis was then confirmed by UPD of different chromosomes in association with typical syndromes. The best examples of this type of aberration are maternal UPD15 (upd(15)mat) in Prader-Willi and paternal UPD15 (upd(15)pat) in Angelman syndrome.
Different mechanisms may lead to UPD (Spence et al., 1988): each of them includes at least two errors either during meiosis or in postzygotic mitoses. During meiosis, a nondisjunction of two homologous chromosomes occurs, which leads to a trisomy in the zygote. In most cases, this zygote will not be viable, unless a correction or a "trisomic rescue" happens. Statistically, in one third of cases, the chromosome of the parent who did not contribute to the trisomy is lost, thus resulting in UPD.

A cytogenetic hint for a trisomy rescue is the confined placental mosaicism (CPM), which describes the presence of a partial chromosomal aberration (usually a trisomy mosaic) in the placenta but not in the fetus. This constitution can be diagnosed in approximately 1-2% of chorionic villous samples (Kalousek et al., 1989). CPM can have relevant clinical consequences since it may lead to placental insufficiency and then induces intrauterine growth retardation.

Clinical features in context with UPD can be caused by:

a. hidden chromosomal mosaicism originating by the UPD formation via trisomy rescue;
b. by the imbalanced expression of imprinted genes in the respective chromosomal region resulting in a specific imprinting disorder;
c. homozygosity for recessive mutations. Interestingly, this phenomenon led to the detection of the first case of UPD. Spence et al. (1988) reported a patient suffering from cystic fibrosis who was homozygous for the mutation F508del in the CFTR gene. Only his mother was a heterozygous carrier for F508del transmitting the mutant gene copy twice to her child. Therefore, UPD always involves the usually unpredictable risk for homozygosity of mutant genes in addition to imprinted gene effects.

Due to the frequency of trisomy 21, UPD of this chromosome should be a well-known aberration. Indeed, maternal as well as paternal UPD21 (upd(21)mat, upd(21)pat) have been reported for several times (for review: Kotzot and Utermann, 2005), including healthy carriers. Based on the latter finding, it can be decided that the clinical course of upd(21)mat or upd(21)pat carriers is rather caused by a hidden chromosomal mosaicism which can be delineated from the UPD formation mechanism or by homozygosity of a recessive allele than by the UPD itself. Imprinted genes involved in the aetiology of imprinting disorders are not localised on chromosome 21. A different epigenetic effect was shown by recent investigations.

It could be demonstrated that trisomy 21 affects the methylation pattern of different heterologous chromosomes by decreasing their extent of methylation (Weinhaeusel et al., 2011) and thus leas to clinical abnormalitiesof the carrier.

8. The simultaneous occurrence of trisomy 21 and other chromosomal aberrations

This group of combined aberrations comprises numerical and structural abnormalities which present as pathologic karyotype in all cells of an organism or as different types of mosaicism.

Investigations usually rely on conventional chromosome analyses of spontaneous abortions as in this group the frequency among conceptus with pathological karyotype is about 25% compared to life-born children with 0.2% (own investigations and findings from the literature).

8.1 Double aneuploidies

Trisomy 21 can be combined with aneuploidies of different heterologous chromosomes (Micale et al., 2010). The maternal age of a pregnancy with a conceptus showing a double aneuploidy is on average higher than that with a single trisomy. The life expectance of the carrier is lower in prenatal and postnatal period than in single trisomies. Double trisomies of autosomes show a higher lethality than combinations of autosomes and gonosomes.

8.1.1 Combinations with gonosome aberrations

Gonosomal aneuploidies in patients with an additional trisomy 21 were diagnosed in the four possible combinations with monosomy X, XXY, XYY and XXX. The most frequent gonosome aberration in this context is XXY. Monosomy X often presents as mosaic.
The phenotype of the patients usually corresponds to that of trisomy 21. The reason might be the inactivation of more than one X chromsome and the low number of genes on the Y chromosome. The combinations of these double aneuploidies are more frequent than the total amount of the two single trisomies, or the trisomy combined with that of monosomy X.

8.1.2 Combinations with heterologous autosome aberrations

In life-born children, only three combinations of trisomy 21 with additional heterologous chromosomes have been observed: +8, +13, +18, and trisomy 8 is always occurring as mosaic. The double trisomy 21 and 18 seems to be the most frequent combination with an average of 1:1000 among trisomy 21 carriers. The frequency of this combination is much higher in spontaneous abortions with about 2.5 in 100 trisomies. This is explained by the high and early lethality of conceptus with a double autosomal trisomy.
Clinical investigations reveal a heterogenous picture: The carrier can show the phenotype of one of the 2 trisomies or a combination of both. The majority of double trisomies presents as a mosaic with one or two trisomic cell lines.

8.2 Trisomy 21 in combination with structural aberrations

The most frequent aberration is a trisomy 21 in combination with a balanced Robertsonian translocation rob(13q14q). It has been hypothesized that a familial translocation rob(13q14q) induces frequently errors of pairing of heterologous chromosomes in the prophase of meiosis I leading to an aneuploid gamete (interchromosomal effect). Recent investigations could not prove this hypothesis. It is nowadays assumed that the reduced fertility of the translocation carriers is the reason for pregnancies at increased maternal age, which leads to an elevated risk for pregnancies with trisomy 21.
Further single cases of trisomy 21 combined with structural aberrations comprise unbalanced and balanced reciprocal translocations, deletions, and sub-microscopic aberrations.

9. Differential diagnoses

Carriers of trisomy 21 are characterised by a number of clinical symptoms caused by disturbances during early embryonic development. This abnormal course of differentiation can also occur in other syndromes based on an aberrant embryogenesis.
These syndromes can be caused by other chromosome abnormalities, by monogenic mutations, and by exogeneous factors or teratogenic agents.
Chromosome syndromes with a phenotype that, especially in early childhood, resembles that of children with trisomy 21 are the two poly-X syndromes, penta-X syndrome in the female, and XXXXY in the male.
A monogenic disease with a phenotype similar to that of trisomy 21 is hypothyroidism.
The best-known differential diagnosis to trisomy 21 though, is fetal alcohol syndrome.
The similarity between them is so high and the facial dysmorphic features are so characteristic for both that even in the general population the typical phenotype is well-

known but often misinterpreted. Therefore, it may happen that a child with DS is wrongly mistaken as one with fetal alcohol syndrome and which can lead to discrimination of the family.
The possibility that the phenotype of DS may be caused by other diseases than trisomy 21 makes it necessary for all patients with the clinical diagnosis of DS to be investigated cytogenetically because otherwise prognosis, therapy, and estimation of recurrence risk might not be correct.

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2

Etiology of Down Syndrome: Risk of Advanced Maternal Age and Altered Meiotic Recombination for Chromosome 21 Nondisjunction

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1. Introduction

Down Syndrome (DS) is the most frequent live born aneuploidy and recognizable form of mental retardation among all the ethnic groups of human population across the globe. The overwhelming majority of this birth defect is caused by trisomy 21 due to nondisjunction (NDJ), i.e., failure of chromosomes to separate properly during meiosis at parental gametogenesis and the fact was initially reported by Lejeune et al. (1959). Since that time attempts were made to explore the etiologic factors that are associated with the underlying mechanism of NDJ of chromosome 21(Ch21). Like that of other autosomal aneuploidy, the errors during maternal oogenesis accounts for about 90% of DS births (Antonarakis, 1991; Freeman et al. 2007), of which majority occurs at first meiotic division(MI) (Antonarakis et al. 1992; Yoon et al. 1996). In searching the maternal risk factors for DS birth, researchers have identified advanced maternal age (Hassold and Chiu, 1985) and altered meiotic recombination (Warren at al. 1987; Sherman et al. 1991) as two strong correlates associated with underlying mechanism of Ch21 NDJ in oocyte and the risk factors are preferentially present in oocyte due to its mode of development in the lifetime of women.

The meiosis in fetal ovary initiates at about 11-12 weeks of gestation (Gondos et al. 1986) and becomes arrested at late prophase I following pairing, synapsis and recombination. The process resumes at the onset of puberty after the follicle receives proper hormonal signal and immediately completes the MI and progress through metaphase of meiosis II (MII) where it pauses until it is fertilized and the meiosis is then completed. Thus the individual oocyte remains arrested in prophase I for 10 to 50 years, depending on the time of ovulation in reproductive life. This protracted event of oocyte growth includes three distinct error prone phases (Hassold et al., 2007). First, the prophase event in fetal ovary, at which change in usual pattern of recombination might lead to subsequent aneuploid oocyte formation. The second risk prone phase is the follicular growth during which the meiosis remains arrested and the genetic and environmental challenges get chance to accumulate in ovarian milieu. The third and the final risk phase is the maturation of oocyte which is associated with the adverse effect of advancing maternal age on protein components involved in

chromosome separation system and rapidly deteriorating endocrine environments. In contrast, spermatogenesis begins at puberty and spermatogonial cells complete both MI and MII without any delay (Sherman et al. 2007).

As mentioned earlier, the overwhelming majority of Ch21 NDJ is maternal in origin among all the ethnic varieties of human population studied to date. Based on results from the US (Allen et al. 2009) and other population-based studies (Mikkelsen et al., 1995; Gomez et al., 2000), it has now been estimated that over 90% of NDJ errors leading to trisomy 21 arise in the oocyte and the majority of those occur at MI.

We carried out similar study on Indian trisomy 21 samples, particularly from eastern part of the country and obtained strong replication of those observations (Table 1). This study was started from the year 2001 and till date we included about 400 families having free trisomy21 child. Our STR(short tandem repeat)-PCR analyses estimated over 88% maternal errors with majority of cases (~77%) having NDJ events at MI. The paternal errors account for about 10 % with almost equal distribution of MI and MII NDJ events. The post zygotic mitotic error was estimated about 2%. Very concordant results were also reported for Ukraine and Russian cohorts (Machatkova et al. 2005), and Spanish cohort (Gomez et al. 2000). Little difference among these datasets that does exist is probably due to sampling variation. In this article we discuss the maternal stress factors responsible for the origin of nondisjunction.

2. Effect of advanced maternal age

The effect of 'maternal age' remains as 'black-box' for DS birth. Initially Penrose identified that advanced maternal age as risk for DS birth (Penrose 1933, 1934) and postulated that the maternal age dependent increase in birth rate of DS is in some way associated with the NDJ mechanism. But this effect is restricted only to NDJ that occur in the oocyte (Antonarakis et al., 1992; Ballesta et al., 1999; Muller et al., 2000; Sherman et al., 2005). That is, adverse effect of advanced maternal age is not evident among mothers whose offspring received an extra copy of chromosome 21 as a result of: (1) a NDJ error in spermatogenesis i.e., paternal errors (Yoon et al., 1996; Sherman et al., 2005), (2) a post zygotic mitotic error (Antonarakis et al., 1993; Sherman et al., 2005), or (3) a translocation (inherited or de novo) (Hook, 1983).

The results of earlier studies (Antonarakis et al. 1992; Ballesta et al. 1999; Muller et al. 2000), revealed that the average age of mother at the time of conception of a fetus with DS is significantly higher than that of mothers with normal euploid baby. This observation was confirmed further in the population based study in the Atlanta Down syndrome project (Allen et al. 2009) for US population and recently by us (Ghosh et al. 2010a) for Indian population. All these reports suggest that the advanced maternal age is risk factor for both the MI and MII errors and both types of error are potentially related in respect to their association with risk factors. Further, Atlanta Down syndrome project suggests (Allen et al. 2009) that maternal age specific incidence rate for live birth with free trisomy 21 may differ between MI and MII errors: the increasing risk for MII errors is shifted to the older maternal ages compared with MI errors. Interestingly, the women with MII errors are in average older than mothers with MI errors, as evident in both US (Allen et al. 2009) and Indian cohorts (Table 1). All these observations led the workers to propose several hypotheses to explain the intriguing association between advanced maternal age and an increasing chance of Ch21 nondisjunction.

Parental Origin	Meiotic Stage of Nondis-junction	Sample size	Proportion	Frequency	Maternal Age at Conception (Years±SD)	Paternal Age at Conception (Years±SD)
Maternal	Meiosis I (MI)	242	MI/(MI+MII)=242/314	77.07%	29.91±6.12	33.95±2.04
	Meiosis II (MII)	72	MII/(MI+MII)=72/314	22.9%	31.01± 3.44	34.01±4.66
	Stage Unknown	17				
	Subtotal	331	Maternal/All=331/373	88.73%		
Paternal	Meiosis I (PI)	11	PI/(PI+PII)=11/27	40.74%	24.55±3.02	31.85±5.6
	Meiosis II (PII)	16	PII/(PI+PII)=16/27	59.25%	26.92±4.91	33.98±4.4
	Stage Unknown	7				
	Subtotal	34	Paternal/All=34/314	10.82%		
Post Zygotic Mitotic Error		8	8/373	2.14%	25.66±3.26	31.76±5.21
Origin Unknown		19	19/392	4.8%		
Total Informative Cases		373				
Total Cases		392				
Control		206			24.82±3.9	32.01±4.04

Table 1. Origin of Trisomy 21 in Indian Cohort and Parental Age at conception of Trisomy Foetus

3. Biological aging hypothesis

The hypothesis was originally proposed by Brook et al (1984). The central idea of this hypothesis is that the increasing rate of meiotic errors and subsequent aneuploid birth is related to 'biological aging' of ovary not to the chronological age of women. Two different views do exist about how the biological aging is implicated for increased incidence of trisomic birth. The first view relates the suboptimal level of hormonal signal with higher rate of meiotic errors in aging ovary. The number of antral follicle at various stages of development also declines with increasing maternal age as the fact has been confirmed in

several studies (Reuss et al. 1996; Gougeon 1998; Scheffer et al. 1999; Kline et al. 2004). This decline in antral follicle count, together with the accompanying decrease in total oocyte pool generates an imbalance in the hormonal environment in ovary (Warburton, 2005) which predisposes the women for aneuploid conception. Support to this postulate came from the studies on human and mouse (Freeman et al., 2000; Roberts et al. 2005). Alternate to this concept has been proposed by Warburton (1989) in her "limited oocyte pool" hypothesis which suggests a more direct effect of antral oocyte pool size on the risk of aneuploidy. Among older women available antral follicles are limited and ovary has to compromise in selecting a suboptimal or erroneous oocyte for ovulation.

The 'biological aging' can also be interpreted in term of senescence associated degradation of ovarian protein components that are implicated in chromosome separation system in oocyte (Sherman 2005). Interestingly, level of hundred of transcripts, including cell cycle genes have been reported to decrease with increased maternal age in mice and women (Hamatani et al., 2004; Steuerwald et al., 2007).

4. Genetic aging hypothesis

We proposed 'genetic aging' hypothesis (Ghosh et al. 2010b), which states that some of the mothers who have DS baby are genetically older than the mothers of same chronological age who have euploid baby (Ghosh et al. 2010b) and this genetic aging is the underlying cause of biological aging in ovary. In this analyses we estimated the telomere length (TL) of age matched controls and cases to get insight into the state of molecular aging, stratifying the mothers by stage of NDJ and their age of conception (young ,<29 years; middle ,29-35 years; and old ,>35 years). Our results showed that all three groups(M1,MII & control) have similar TL on average for younger mothers. As age increases, all groups show telomere loss, but that loss is largest in the meiosis II mother group and smallest in the euploid mother group with the meiosis I mother group in the middle(Figure 1). Our results do not support the theory that younger women who have babies with Down syndrome do so because they are '*genetically older*' than their chronological age, but we proposed that older mothers who have DS baby are "*genetically older*" than controls, who have euploid babies at the same age. This finding, however, is consistent with the previous result (Dorland et al. 1998), showing no difference in genetic age among young DS mothers and young controls.

The fact of telomere shortening among women with DS child can be explained in several ways. Apparently, the result suggests a possible functional link between telomere maintenance system and chromosome segregating apparatus at molecular level. Degradation of this possible 'molecular link' with age may affect the both system simultaneously. In this regard BubR1 is most promising candidate as mutation in this gene causes rapid senescence and high rate of aneuploidy in mouse (Baker et al., 2004) and the protein shows rapid fall with age. Alternatively, the environmental factor that induces rapid telomere loss at advanced reproductive age might simultaneously affect the chromosome separation system in oocyte. (Chen et al.,2007; Sebastián et al., 2009; Eichenlaub-Ritter et al,. 2007; Susiarjo et al., 2007).

5. Reduced meiotic recombination and its interaction with maternal age

Aside from maternal age, only single factor that has been identified unambiguously to be associated with maternal NDJ is altered pattern of meiotic recombination. The first evidence

for association of reduced recombination with the events of NDJ of Ch21 was provided by Warren et al. (1987). Chiasmata are physical connections between homologous chromosomes at the site of recombination and they function to stabilize the paired homologues or tetrad at MI along with sister chromatids and centromere cohesion. It aids in proper chromosome orientation on the meiotic spindle (Carpenter 1994) and ensure their proper segregation to opposite poles. Absence of chiasma formation left the homologous pair free to drift randomly to the poles and if they move together to same pole aneuploidy results. As far as chromosome 21 NDJ is concerned, achiasmate meiosis is the major cause of reduction in recombination frequency (Lamb et al., 2005a, 2005b), although fall in double exchange frequency was reported too (Hawley et al. 1994).

In our analysis of etiology of DS birth in Indian cohort, we recorded only ~22% detectable crossover on MI nondisjoined chromosome in maternal meiosis (Ghosh et al., 2009). This observation was very consistent with the previous observation by Sherman et al. (2007), who reported 45% achiasmate meiosis associated with MI NDJ of Ch 21 in US population. Sherman and her co-workers constructed the linkage map of nondisjoined Ch21 (1994) and estimated 55% reduction in map length than the control CEPH map (39.4cM in contrast to 72.1cM). With similar approach for Indian DS population (Ghosh et al. 2010a), we scored 30.8cM map length of maternal MI nondisjoined Ch 21, which further confirmed the fact that reduced recombination due to absence of chiasma or less recombination frequency in some way increases the risk of NDJ.

In elucidation of the relationship between reduced recombination and maternal age, Sherman et al. (1994) hypothesized that the trisomy 21 conception at advanced maternal age is strongly associated with reduction in recombination frequency. The authors estimated shorter map length of Ch21 for mothers of >35 years with their linkage analysis approach. Very recently, Oliver et al. (2008) also reported a highest occurrence of non-exchange Ch21 pair among the old age (>34 years) women in compare to young (<29 yrs) and middle (29-35 yrs.), although the frequency of non-exchange tetrads remain most frequent among all the risk factors when only young mothers (<29 years) were considered. The authors proposed a model for explaining the risk of Ch21 NDJ in relation to maternal age categories. Among the young mothers risks related to aging is minimum and therefore absence of recombination becomes the predominant cause of NDJ in total risk scenario. If this remains true, then lack of recombination is an age-independent risk factor for Ch21 NDJ. This hypothesis was supported by our previous studies (Ghosh et al. 2009; 2010a) in which we estimated about 80% of younger mothers with achiasmate Ch21 who had NDJ at MI.

The highest frequency of non-exchange Ch 21 among older mothers is difficult to explain as the events of chiasma formation and recombination take place in foetal ovary. The fact led workers (Oliver et al. 2008; Ghosh et al. 2009) to speculate presence of maternal age dependent NDJ mechanism which gains support from the studies on model organisms. Mutation in the gene *nod* (no distributive disjunction) in *Drosphila* causes high frequency of NDJ of non-exchange chromosome (Knowles and Hawley, 1991) and it suggests existence of the genetic component that acts as surveillance system to ensure proper segregation of non-exchange meiotic chromosomes. Presence of such 'back-up system' is also evident in yeast in which, the gene *Mad3* performs the same function (Gillett et al. 2004). Interestingly, proteins with similar function in human have been shown to be down regulated with increasing ovarian age (Baker et al. 2004; Steuerwald et al. 2001). Thus, age-dependent down-regulation of these essential proteins may lead to the decreased ability to segregate properly the non-

exchange chromosomes in aging oocyte. However, more direct evidence is needed to establish this speculation as fact.

6. Susceptible chiasma formation and its interaction with maternal age

Aside reduced recombination, unusual chiasma placement is another risk for Ch21 NDJ. Chiasma formation usually takes place at the middle of normally disjoining chromosomes (Lynn et al. 2000). This medially placed chiasma probably maintains the proper balance by counteracting the pull from opposite poles which is needed for proper segregation of chromosomes. But a chiasma close to centromere or close to telomere seems to confer instability and makes the Ch21 susceptible for random segregation and subsequent NDJ (Lamb et al. 1996; 2005a, 2005b). The increased risk of NDJ due to sub-optimally placed chiasma on the chromosome is also evident in model organisms such as *Drosophila* (Rasooly et al.1991; Moore et al. 1994; Koehler et al. 1996a), yeast (Sears et al. 1995;Krawchuk and Wahls,1999) and *Caenorhabditis elegans* (Zetka and Rose, 1995). The study of Lamb et al. (1996), suggested for the first time that a single telomeric chiasma is a risk for malsegregation of Ch21 at MI in oocyte in contrast to single pericentromeric chiasma which increases risk of MII NDJ.

Very recently, Oliver et al.(2008) and we (Ghosh et al.2009) independently conducted population based studies on US and Indian DS populations respectively to get an insight into the interaction between susceptible chiasma configuration on Ch21 in oocyte and maternal age. In doing so we used family linkage approach to detect exchange pattern on nondisjoined Ch21, using set of microsatellite markers and all the analyses were done by stratifying the participating mothers into three age groups:young (>29 yrs.), middle (29-34 yrs) and old (>34 yrs). Surprisingly, the two sets (US set and Indian set) of results were very concordant and revealed that single telomeric exchange is prevalent among younger mothers whose Ch21 nondisjoined at MI. In contrary, single centromeric chiasma is risk for MII NDJ, particularly at older age. For Indian DS sample, we recorded susceptible single chiasma within the 3.1Mb peri-telomeric and 4Mb peri-centromeric segment of 21q for MI younger and MII older categories, respectively (unpublished data). These observations led us (Oliver et al. 2008; Ghosh et al. 2009) to propose a hypothesis which states that maternal age independent risk factor is one which affects all the age groups equally and be detected in highest frequency among younger mothers for whom aging related risk factors are minimum. Alternately, age-dependent risk factors usually intensify with advancing age and so one would expect highest frequency of such factors among older age group (Figure 2). If our prediction is true, the telomeric single chiasma is maternal age independent risk, whereas, the single peri-centromeric chiasma is maternal age dependent factor.

The relationship between centromeric exchange and advancing maternal age can be interpreted in two different ways: 1) pericentromeric exchange set up a sub-optimal configuration that initiates or exacerbates the susceptibility to maternal age-related risk factors, or 2) a pericentromeric exchange protect the bivalent against age related risk factor allowing proper segregation of homologues, but not the sister chromatids at MII (Oliver et al., 2008). A chiasma very close to centromere may cause 'chromosomal entanglement' at MI, with the bivalent being unable to separate, passing intact to MII metaphase plate (Lamb et al.1996). Upon MII division, the bivalent divides reductionally, resulting in disomic gamete with identical centromeres. In this manner, proximal pericentromeric exchange, which occurred during MI, is resolved and visualized as MII error. According to an

alternate model, studied in *Drosophila* (Koehler et al. 1996b), proximal chiasma lead to premature sister chromatid separation just prior to anaphase I. Resolution of chiasma requires the release of sister chromatid cohesion distal to the site of exchange (Hawley et al., 1994). Attempt to resolve chiasmata that are very near to centromere could result in premature separation of chromatids. If the sister chromatids migrate to a common pole at MI, they have 50% probability to move randomly into the same pole at MII, resulting in an apparent MII NDJ. Similar observation is evident in yeast in which centromere-proximal crossover promotes local loss of sister-chromatid cohesion (Rockmill et al., 2006). One of the members of centromeric cohesion complex *shugoshin*, when down regulated due to aging shows high frequency of MII NDJ of bivalent with peri-centromeric exchange (Marston et al. 2004). Alternatively, a pericentromeric exchange may protect the bivalent from maternal age related risk factors. The effect of degradation of centromere or sister chromatid cohesion complexes or of spindle proteins with age of oocyte may lead to premature sister chromatid separation. Perhaps the pericentromeric exchanges help to stabilize the compromised tetrad through MI. This would lead to an enrichment of MII errors among the older oocytes. Although there is no specific model system in favor of this mechanism, but some findings in model organisms can be interpreted in this way.

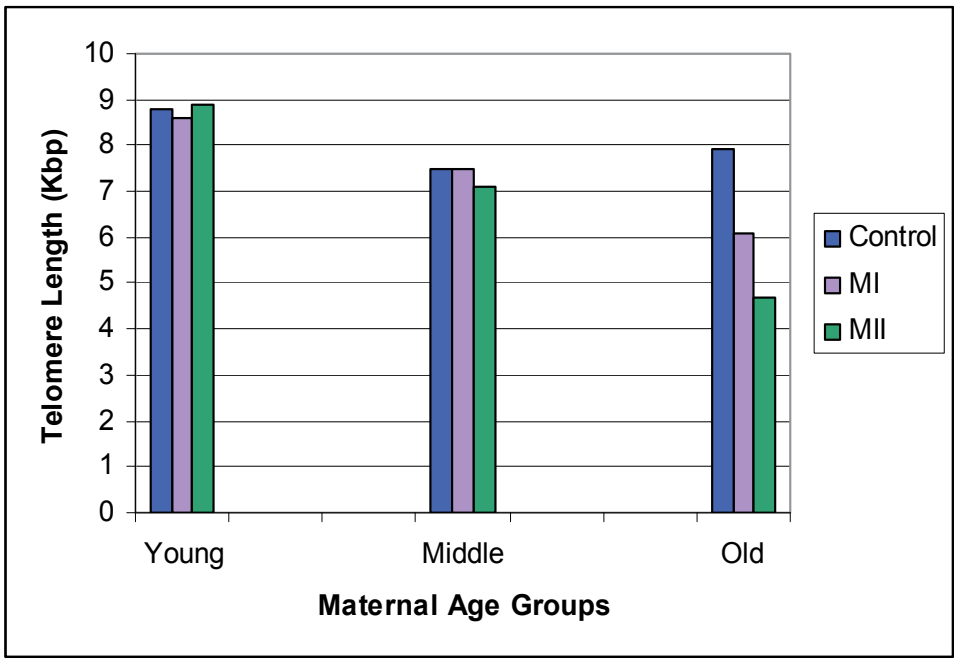

Fig. 1. Telomere length (Kbp) among control and meiotic outcome groups stratified by age categories.

A telomeric chiasma imparts its susceptibility for MI NDJ probably due to recruitment of minimal amount of sister chromatid cohesion complex remaining distal to the exchange event (Orr-Weaver, 1996). Specifically, when the exchange is too far from kinetochore, this could prevent the bi-orientation of the homologues on the meiotic spindle (Nicklas 1974; Hawley et al.1994; Koehler et al.1996b). Alternatively, the integrity of chiasma may be

compromised when a minimum amount of cohesin remains to hold homologue together. Thus bivalent may act as pair of functional univalents during MI, as has been evident in human oocyte (Angell 1994, 1995).

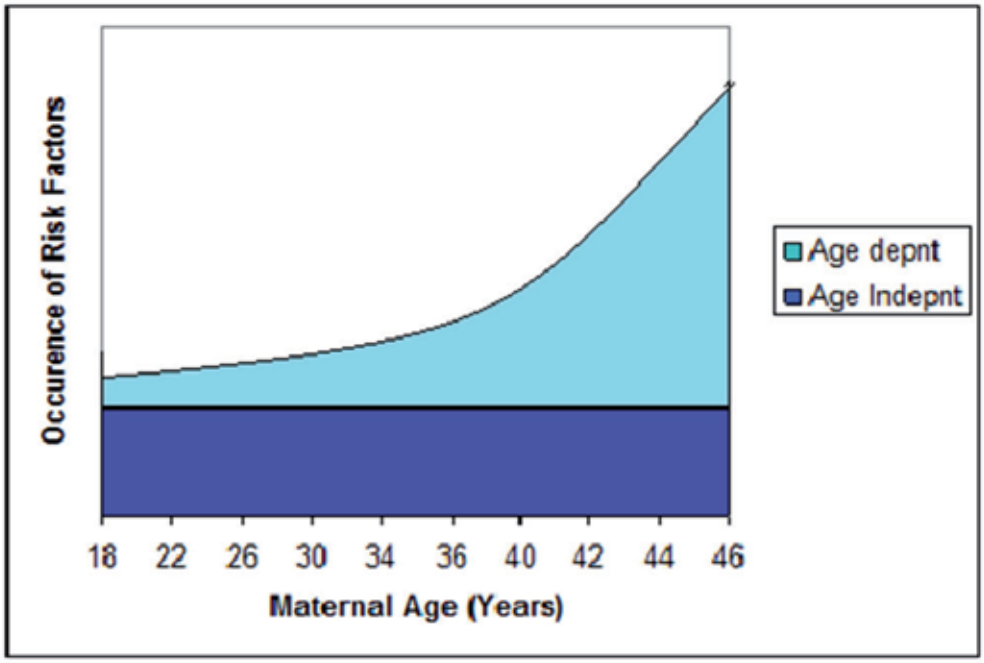

Fig. 2. Risk factor model for Down syndrome birth showing consistent presence of maternal age independent risk factors (Age Indepnt) among all the ages and gradual increased incidence of age dependent risk factors (Age Depnt) with increasing age.

Further, we evaluated the interaction between maternal age and multiple chiasmata on MI nondisjoined Ch21 and found that there is a linear increase in multiple chiasama frequency with advancing age (Ghosh et al.2010a). Interestingly, similar trend is also evident for chromosome 15, 18 and X chromosome (Robinson et al. 1998; Thomas et al. 2001; Bugge et al.1998). This finding suggests two important possibilities. The first one is that the multiple chiasmata might be protective and chromosomes with multiple recombinants probably more resistant to NDJ at least at MI because of an increase in bivalent stability. Secondly, instead of enjoying multiple recombinations some bivalents segregate improperly, particularly in older oocyte, which suggests presence of some aging associated factors that impart risk to these otherwise recombination perfect chromosomes.

7. Summary and conclusion

We have paved half of a century after the initial discovery of cause of DS, but we are still in dark regarding etiology of DS. Although advanced maternal age has been identified unambiguously as risk, its molecular relation with chromosome separation system is enigmatic. It is still elusive whether or not some women are genetically predisposed to altered meiotic recombination and subsequent chromosomal NDJ. Very recently, the gene

PRDM9 has drawn the interest. The gene controls the recombination hotspot of meiotic homologues (Parvanov et al. 2010). The variant of this gene has been reported to make the women susceptible for recurrent miscarriages, infertility and aneuploid pregnancy (Cheung et al. 2010). So PRDM9 is prospective candidate gene whose altered functional state might increase susceptibility of Ch21 NDJ. Similarly, genetic variant of any component of meiotic chromosome separation system could increase the risk for chromosome missegregation in oocyte. Intuitively, the gene BubR1 is of special interest as it is a member of centromere cohesion complex and also known for its role in cellular aging (Baker et al. 2004). In *Drosophila* hypomorphic bubR1 causes high rate of NDJ at MII due to premature sister chromatid separation and these nondisjoined chromosome exhibited centromeric exchange. All these findings suggest the possibility of BubR1 to be a 'missing link' between the molecular mechanism of cellular aging and higher incidence of chromosomal NDJ at advanced age.

The effect of environmental agents on chromosome segregation, particularly in connection with maternal age and recombination remains unexplored. As environmental aneugens have great opportunity to become accumulated within the ovarian microenvironment during protracted oocyte growth phase, their probable effects cannot be underestimated. Although epidemiologic association of some environmental agents with DS birth have been identified, their influence on meiotic recombination and aging is intriguing. The periconceptional smoking and contraceptive use have been identified as potential risk for Ch21 NDJ (Yang et al. 1999), but this observation needs further confirmation. We conducted an epidemiological study on the risk of chewing tobacco and contraceptive use among mothers having DS baby and found some association of chewing tobacco with MI NDJ and contraceptive for both MII and MI (unpublished data). Moreover, the fetal incidence of chiasma formation and recombination make us curious to the probable 'grand maternal' influence on DS birth. Presently we are in position to realize at least that the risk factors associated with DS birth is multidimensional and several mechanisms are involved for chromosome 21 NDJ in women. At this point, it is worth mentioning that the etiology of maternal Ch21 NDJ and subsequent DS birth may be similar across the human population divides irrespective of ethnic and socio-cultural differences. The future investigations should be focused to resolve all these pending issues so that we could move towards complete understanding of risk factors associated with DS birth.

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3

Combinatorial Gene Effects on the Neural Progenitor Pool in Down Syndrome

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1. Introduction

Down syndrome (DS) arises from triplication of genes on chromosome 21 (HSA21) and is characterized by neurological complications including cognitive dysfunction, epilepsy, and early onset Alzheimer's disease (AD). At the histological and cellular level, DS brains show prolongation in the cell cycle length of neural progenitors, increased oxidative stress, and mitochondrial dysfunction. These pathological processes likely contribute to the observed decrease in neurogenesis, increased neuronal cell death, enhanced gliosis in the cortex, and a corresponding decrease in both neuronal glutamatergic and GABAergic expression.

With recent advances in molecular biology and human genetics, many HSA21 genes have been identified, putative functions assigned to these genes and network analyses used to predict interconnecting, functional gene pathways. Interactions at this level are further complicated by gene regulatory mechanisms such as DNA methylation, histone acetylation and microRNA. In this respect, the neurological phenotypes seen in DS are not merely due to genomic imbalance from triplication of HSA21 genes, but also additive effects due to influences on associated genes within a given network or pathway and modification of gene expression due to epigenetic factors.

The development of stem cell technologies has provided the opportunity to isolate human neural progenitors (HNPs) and induced pluripotent stem cells (iPS) from patients with known neurological disorders. These reagents are also used as tools to study molecular mechanisms and pharmacological therapies in various human diseases. They provide a means by which an understanding is established for interactions between various genes causal for a human disorder, especially in contiguous gene syndromes that are difficult to faithfully replicate in animal models. We have focused on the study of DS HNPs in relation to neurological phenotypes in the early onset of DS in these individuals.

In the current review, we summarize the approaches taken, recent progress and insights gained from studies of DS during neural development, incorporating insights gained from the use of human DS neural progenitors.

2. Down syndrome phenotype and genotype during neurodevelopment

The most prominent neurological phenotypes in DS include mental retardation (MR), epilepsy, and dementia (early onset AD). Individuals with DS generally have mild to moderate MR with average intelligence quotient (IQ) among children at 50, as compared

with normal children at an IQ of 100. Children with DS generally exhibit better visual motor skills (such as drawing) than auditory and linguistic skills, which typically lead to a delay in language skill development. MR can also be accompanied by behavioural issues such as attention-deficit disorder (sometimes with hyperactivity) and autistic behaviour, especially in those with severe intellectual disability. The onset of seizures tends to occur in a bimodal distribution. At early ages, individuals with DS are susceptible to infantile spasms and tonic clonic seizures with myoclonus. These epilepsies are associated with MR and likely due to early impairments in brain development. Individuals with DS in the third decade of life are more prone to developing simple partial or complex partial seizures in addition to tonic-clonic seizures. These focal epilepsies are likely associated with the increasing burden of parenchymal damage sustained from the progressive neuronal degeneration. It is estimated that 46% of Down's patients over the age of 50 have a diagnosis of epilepsy. Finally, AD is generally considered a neurodegenerative disorder that affects the elderly at 60 years of age and over. Premature AD is seen in DS in the early 40s (Arya et al., 2011; Contestabile et al., 2010; Lott and Dierssen, 2010; Menendez, 2005)

The clinical phenotypes in DS correlate with functional changes seen at the histological and cellular levels in DS brain. DS cortex shows increased simplified gyral patterning and delayed myelination (Banik et al., 1975; Cragg, 1975; Koo et al., 1992; Wisniewski, 1990; Wisniewski and Schmidt-Sidor, 1989). Neuronal loss has been seen in DS brain both during development, following differentiation and aging (Becker et al., 1991; Benda, 1947; Colon, 1972; Davidoff, 1928; Golden and Hyman, 1994; Guidi et al., 2008; Ross et al., 1984; Wisniewski et al., 1984). Histological evidence of neurofibrillary plaques and tangles can be seen in the DS brain, consistent with the premature onset of AD (Motte and Williams, 1989). Changes in dendritic branching as well as neurotransmitter levels have also been reported in the DS brain tissue and likely correlate with the behavioural and cognitive difficulties seen in these individuals (Reynolds and Warner, 1988; Ross et al., 1984). At the cellular level, DS progenitors have prolonged cell cycle lengths, thereby impairing neurogenesis (Chakrabarti et al., 2007; Contestabile et al., 2007; Guidi et al., 2008). Neuronal numbers further decline presumably through increased levels of reactive oxygen species (ROS), mitochondrial dysfunction and increased cell death in mature neurons (Busciglio et al., 2002; Guidi et al., 2008; Seidl et al., 2001). Following differentiation, neurons also show an imbalance of excitatory-inhibitory neurotransmission (Bhattacharyya et al., 2009), with reduced dendritic branching and spine density (Benavides-Piccione et al., 2004). Overall, these changes could all contribute to the MR, epilepsy and AD phenotypes seen in DS.

Various neurological features in DS have been attributed to over-expression of individual genes on HSA21, and several of these genes might contribute to a final common pathway. For example, APP has been shown to promote neuronal degeneration and oxidative stress. Similar functions have been implicated in other HSA21 genes including BACH1, SOD1, and S100B, raising a possible role in AD. Other HSA21 genes (TIAM1, SYNJ1, ITSN1, DSCR1) have been associated with synaptic dysfunction (suggestive of a pathological role in MR and epilepsy). Individual HSA21 genes with proposed neurological functions are summarized in Table 1.

3. Identification of genes and pathways responsible for AD or MR

Although HSA21 is a relatively small chromosome and single candidate genes have been shown to mimic certain DS features, the combinatorial effects of contiguous genes on

HSA21 genes	Downstream targets	Endophenotype	Exophenotype	Ref
NRIP1		Inhibit estrogen and glucocorticoid receptors		(Cavailles et al., 1995; Gardiner and Costa, 2006; Subramaniam et al., 1999; Teyssier et al., 2003)
APP	Ptch1/smo Tau RAGE Tau IL6/gp130 Notch MAP kinases	Abeta deposition, astocytosis, microgliosis, neuronal loss, hyperphosphorylation Tau, decreased proliferation, increased cell death, mitochondrial dysfunction	Dementia, learning and memory deficits	(Abramov et al., 2004; Arancio et al., 2004; Harris-Cerruti et al., 2004; Howlett and Richardson, 2009; Korbel et al., 2009; Kwak et al., 2010; Kwak et al., 2011; Mori et al., 2010; Prasher et al., 1998; Rovelet-Lecrux et al., 2006; Takuma et al., 2009; Trazzi et al.)
BACH1		Increase ROS		(Ferrando-Miguel et al., 2003; Shim et al., 2003)
TIAM1	RAC, NMDAR EphBR	Altered neurite formation, abnormal dendrite spines		(Matsuo et al., 2002; Siddiqui et al., 2008; Tolias et al., 2005; Tolias et al., 2007)
SOD1	APP	Increase ROS, mitochondrial dysfunction	Learning and memory deficits	(Furuta et al., 1995; Harris-Cerruti et al., 2004; Iannello et al., 1999; Lott et al., 2006)
SYNJ1	PtdIns(4,5)P(2)	Synaptic dysfunction, abnormal vesicle trafficking Astrogliagenesis	Learning and memory deficits	(Arai et al., 2002; Chang and Min, 2009; Herrera et al., 2009; Voronov et al., 2008)
Olig2	S100B	Increased inhibitory neurons Increased gliosis Decreased IPSC		(Chakrabarti et al.; Chen et al., 2008; Lu et al., 2002; Tatsumi et al., 2008; Zhou and Anderson, 2002)
Olig1		Increased inhibitory neurons Decreased IPSC		(Chakrabarti et al.; Zhou and Anderson, 2002)
ITSN1		Synaptic dysfunction, abnormal vesicle trafficking		(Chang and Min, 2009; Keating et al., 2006; Yu et al., 2008)
DSCR1	NFAT Calcineurin Tollip ILR1 Caspase9/3	Synaptic dysfunction; abnormal vesicle trafficking, increased neuronal susceptibility to oxidative stress, neuronal apoptosis, mitochondrial dysfunction		(Arron et al., 2006; Chang and Min, 2005; Keating et al., 2008; Lee et al., 2009; Porta et al., 2007; Sun et al.)

HSA21 genes	Downstream targets	Endophenotype	Exophenotype	Ref
SIM2	Drebrin		Learning and memory impairment, reduced exploratory behavior and social interactions, increased tolerance to pain	(Chrast et al., 2000; Ema et al., 1999; Vialard et al., 2000)
DYRK1A	NRSF/REST Calcineurin CREB NFAT Tau Notch P53 APP	↓neuroectodermal progenitor, less neuron with abnormal branched neurites, LTP deficits, hyperphosphorylation Tau and APP, premature neuronal differentiation	Learning and memory deficits, motor defects	(Altafaj et al., 2001; Arron et al., 2006; Canzonetta et al., 2008; Fernandez-Martinez et al., 2009; Guimera et al., 1999; O'Doherty et al., 2005; Olson et al., 2004b; Park et al., 2010; Park et al., 2007; Ryoo et al., 2007; Sago et al., 1998; Siarey et al., 2005; Smith et al., 1997; Woods et al., 2001; Yabut et al., 2010; Yang et al., 2001)
KCNJ6 (GIRK2)	GABAB	Imbalance of excitatory-inhibitory neuronal transmission		(Best et al., 2007; Cramer et al., 2010; Harashima et al., 2006)
ETS2	APP	Increased neuronal apoptosis, increased APP production, mitochondrial dysfunction		(Helguera et al., 2005; Wolvetang et al., 2003a; Wolvetang et al., 2003b)
PCP4	Calmodulin			(Kleerekoper and Putkey, 2009; Thomas et al., 2003; Utal et al., 1998)
DSCAM		Overexpression in Purkinje cells, cortical neurons and senile plaques		(Hattori et al., 2007; Saito et al., 2000; Yamakawa et al., 1998)
PKNOX1	FABP7			(Sanchez-Font et al., 2003)
DNMT3L	DNMT3A/3B HDAC	Transcription repression		(Deplus et al., 2002; Holz-Schietinger and Reich, 2010)
SUMO3	APP NRIP1 ELK	Inhibit glucocorticoid receptors		(Dorval et al., 2007; Gardiner, 2006; Holmstrom et al., 2003)

HSA21 genes	Downstream targets	Endophenotype	Exophenotype	Ref
S100B	RAGE AQP4 JNK GSK3ß Tau P53 ATAD3A	Increased ROS, mitochondrial dysfunction and cell death, GSK3ß and Tau hyperphosphorylation, astrocytosis and neurite proliferation	Premature neurological aging	(Esposito et al., 2008a; Esposito et al., 2008b; Gilquin et al., 2010; Lin et al., 2001; Mori et al., 2010; Reeves et al., 1994)

Table 1. HSA21 genes implicated in neurodevelopment

particular DS phenotypes are not known. Several approaches have been used to ascertain HSA21 genotype phenotype contributions: analyses of gene clusters based on their physical linkage, common function/ interactions and/or temporal-spatial distribution of expression.

3.1 Structure-based clusters

Genes that are physically linked based on their location within the same region are usually active together and often contribute to the same phenotype. Based on these structure-based clusters, comparison of full trisomy 21 and partial trisomy 21 genotypes provide some insight between contiguous genes and DS phenotypes. One such region includes APP, which is positioned between 27.25 to 27.54Mb on the long arm of HSA21. Five families with various segmental trisomies that range from 0.58 to 6.37Mb in length and contain APP were all shown to have abundant parenchymal and vascular deposits of beta amyloid without MR on brain autopsy, strongly linking APP to AD (Rovelet-Lecrux et al., 2006).

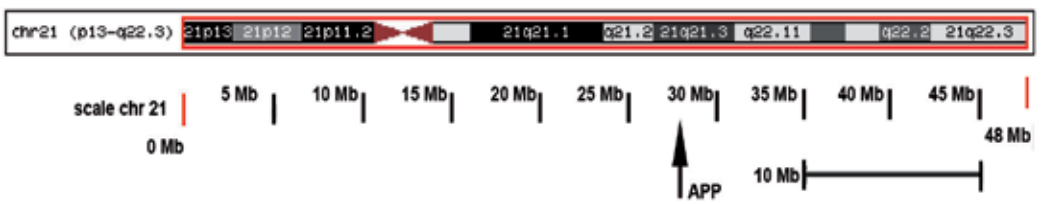

Fig. 1. APP is positioned between 27.25 to 27.54Mb on the long arm of HSA21.

Furthermore, a DS patient with duplication of the distal last 12Mb of HSA21 (a fragment that does not include APP) did not develop typical AD pathology (Prasher et al., 1998). Recently, Korbel et al. analyzed 30 patients carrying rare segmental trisomies of various regions of HSA21 and identified discrete regions between 1.8-16.3Mb in length that were likely to be involved in 8 DS CNS and non-CNS phenotypes including MR and AD (Korbel et al., 2009). They described a patient with a duplication of 18.8 Mb (28.12-telo) without APP, who did not have dementia and had no amyloid accumulation by fMRI. Conversely, several patients with segmental trisomies containing APP did show AD characteristics in the absence of severe MR, which supports the involvement of APP in AD but not MR. These authors were able to narrow down a 1.95Mb interval including APP responsible for the AD phenotype, that excludes genes located distal to 28.12 Mb on HSA21q, including BACH1 and SOD1. These genes were previously suggested to play important roles in the development of AD and MR in DS (Ferrando-Miguel et al., 2003; Lott et al., 2006).

Collectively, these studies conclude a small-segment containing APP as the basis for AD phenotype, with multiple focal regions contributing to MR.
Through segmental trisomy analyses, the primary features of DS have previously been ascribed to the Down syndrome critical region (DSCR). This interval spans about 5Mb around 21q22.12-q22.2 and contains about 33 conserved genes, including DSCR1 (35.88-35.99 Mb), SIM2 (38.07-38.13 Mb), DYRK1A (38.74-38.89), KCNJ6 (38.99-39.29Mb), ETS2 (40.18-40.2Mb), PCP4 (41.24-41.3Mb) and DSCAM (41.38-42.22Mb). The contiguous genes are thought to be sufficient to replicate the DS features including craniofacial abnormalities, short stature, joint hyperlaxity, hypotonia and mental retardation (Delabar et al., 1993; Korenberg, 1990; Korenberg, 1993; McCormick et al., 1989; Olson et al., 2004a; Rahmani et al., 1989). Further studies suggest that DSCR1 and DYRA1A cooperatively destabilize NFATC and induce DS features (Arron et al., 2006; de la Luna and Estivill, 2006). However, several genotype-phenotype correlation studies argue against this hypothesis, given that other patients with partial trisomy 21 that do not include DSCR1, DSCAM or genes within the DSCR have severe DS features including MR (Korbel et al., 2009; Lyle et al., 2009; Ronan et al., 2007).

3.2 Function-based clusters

Microarray expression profiling and sequencing of the human genome have allowed for data analyses and interconnection of functional pathways responsible for various human disorders. By comparing differentially expressed genes between normal and pathological samples, function-based clusters can be generated and attributed to certain disease pathways. We have used this approach in the study of DS human neural progenitors (HNPs) at 18 weeks gestation age (Esposito et al., 2008a). Through pairwise comparison of mRNA expression levels for approximately 54,000 probes in the HU133 plus 2.0 Affymetrix microarray, we observed that some 1900 transcripts (3.8%) were significantly different between WT and DS samples, and that these transcripts were distributed across every chromosome – not just HSA21. Moreover, of these 1900 transcripts, only 330 transcripts (0.07%) were at least 1.5 to 2 fold in excess/deficit on pairwise comparison. These transcripts could then be grouped into specific functional networks, including gene clusters involved in cell cycle (proliferation), cell compromise (cell death, oxidative stress), cell signalling (stress kinases) and cell function and maintenance (differentiation and survival). For example, HSA21 genes such as S100B and amyloid precursor protein (APP) were constitutively over-expressed in DS HNPs along with various stress related kinases and the water channel aquaporin 4 (AQP4). These dysregulated genes comprised a cell signaling functional cluster, whereby HSA21 associated S100B and APP led to increased ROS formation, activation of stress response kinases, and compensatory AQP4 expression. siRNA inhibition of AQP4 resulted in elevated levels of ROS following S100B exposure whereas loss of AQP4 expression led to increased programmed cell death. Results from these studies support the hypothesis that HSA21 gene overdose lead to profound disruption of the entire transcriptome, but presumably in fairly specific functional groups (FitzPatrick et al., 2002; Saran et al., 2003; Tang et al., 2004).
Using a similar strategy, Bhattacharyya et al. compared the differential expression of genes in DS versus WT HNPs at 13 weeks gestation age. Within these earlier aged progenitors, they observed similar functional clusters involved in cell death, cell cycle/ proliferation, and cell fate/ neuronal development. These studies focused on impairments in interneuron neurogenesis, perhaps related to increased expression of gliocentric genes such as Olig1, Olig2, OMG and COUP-TF1/NR2F1 and downregulation of the interneuron related genes DLX1, DLX2 and DLX5.

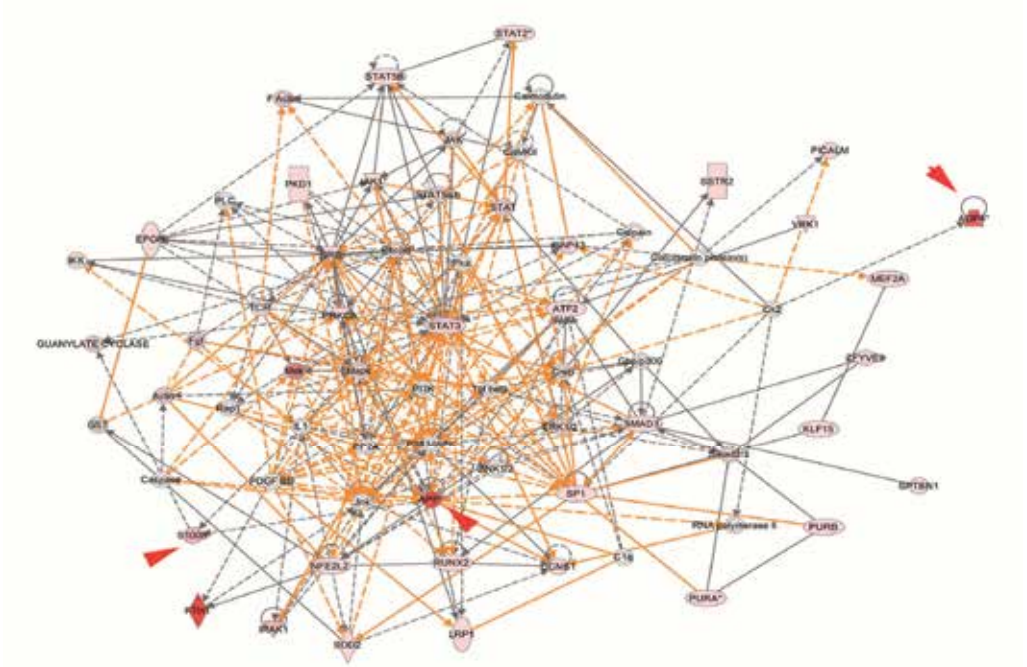

Fig. 2. Constitutively overexpressed genes on HSA21 (S100B & APP) and AQP4 (shown by red arrows) are predicted to form tight interrelationships within known cell signaling pathways shown in the networks pooled out from many differentially expressed genes in DS HNPs with microarray.

In another report, Canzonetta used differential expression analyses following microarray profiling of normal and trsisomy 21 mouse ES cells and pooled the differentially expressed genes into endodermal, mesodermal or ectodermal subgroups. They found reduced expression of stem cell genes controlling pluripotency such as Nanog and Sox2; increased expression of endodermal genes such as Gata4, Gata6 and Foxa2; increased expression of mesodermal genes such as Snail1 and Pitx2 and reduced expression of neuroectodermal genes, such as Nestin, Tubb3, Map2 in trisomy 21 ES cells (Canzonetta et al., 2008). More pervasively, they observed a decrease in Nrsf/Rest, a key regulator of neuronal differentiation and pluripotency. Quantitative linkage analysis (QTL) mapped the REST level change to a 3Mb region of HSA21 containing DYRK1A, suggesting that dysregulation of this HSA21 gene was an early regulator of various embryonic lineages.

3.3 Temporal-spatial mapping-based clusters

Although structure and function cluster approaches provide correlation between DS genotype-phenotypes, temporal and spatial distribution of gene expression further refines the contribution of individual HSA21 genes in DS neural development and later maturation. Previous reports analyzing human trisomy 21 tissue or cells have shown that overexpression of different chromosome 21 genes is cell type and developmental time specific (Ait Yahya-Graison et al., 2007; Chung et al., 2005; Giannone et al., 2004; Gross et al., 2002; Li et al., 2006; Lubec and Sohn, 2003; Mao et al., 2005; Mao et al., 2003; Rozovski et al., 2007). For example, by RNA whole-mount in situ hybridization, gene expression maps of

HSA21 orthologues in various aged embryonic or newborn mouse brain have been reported (Gitton et al., 2002; Reymond et al., 2002). Of the 158 orthologous genes examined in postnatal day 2 mouse brain, 41% were expressed in new cortex, 25% in hippocampus and 25% in cerebellum. The transcripts expressed in neocortex represent a pool of candidate genes for MR of DS, and include DSCAM, SYNJ1 and TIAM1. These genes are implicated in synaptic function, axonal guidance, cell migration and neurite outgrowth (Leeuwen et al., 1997; Saito et al., 2000; Yamakawa et al., 1998).

While several studies have used human cortical progenitors to identify changes in expression profiling, the findings of gene expression levels vary between studies. The temporal-spatial variations in DS HNPs may account for these differences. For example, the REST/NRSF transcription factor was previously reported to be selectively repressed in the DS HNPs (Bahn et al., 2002). These changes were not seen in our studies or those of others (Bhattacharyya et al., 2009; Esposito et al., 2008a). However, this discrepancy could be due to the fact that the gestational ages of the euploid and DS fetal tissues used in these differential expression studies were not the same. Similarly, our observations of increased S100B and aquaporin 4 levels in 19 week gestational age DS HNPs were not appreciated in 13 week gestational age HNPs. This difference would be consistent with the previous notion that HNPs mimic normal developmental patterns such that later generated or propagated HNPs generate fewer neurons than HNPs harvested at an earlier age. Given that S100B and AQP4 are associated with more gliocentric phenotypes, upregulation of these genes would be expected in later gestational aged HNPs. Similar differences appear to exist with other HSA21 genes such as Olig1/2 and DNMT3L (unpublished observations).

4. Characterization of endophenotypes for AD or MR

Endophenotypes are pathological changes observed at the histological, cellular or molecular levels, which provide an association between candidate genes and the clinical symptoms. Clinical neurological symptoms of DS include early onset AD, mental retardation and seizures. Histological changes such as increased cell death, gliosis, demyelination, loss in neuronal numbers, and gyral simplification are thought to contribute to AD and/or MR in DS brain. At the cellular level, DS cells show impairments in mitochondrial function, increased levels of oxidative stress, and changes in proliferative rates. An imbalance in excitatory-inhibitory neurotransmission, expression changes of genes that affect cell fate/differentiation, proliferation/cell cycle and cell injury have been observed at the molecular level.

4.1 Mitochondrial dysfunction and apoptosis

Oxidative stress is increased in DS brain and the imbalance of reactive oxygen species (ROS) metabolism may be the key component giving rise to DS pathogenesis- namely neuronal degeneration (Brooksbank and Balazs, 1984; Busciglio and Yankner, 1995). The mitochondrial respiratory system is the primary source of ROS, and dysregulation of the redox states may induce sequential apoptosis. Several lines of evidence implicate multiple HSA21 genes in mitochondrial dysfunction and DS/AD neuropathology. APP is a membrane protein associated with neuronal survival and overexpression of APP and its products Abeta has been attributed to neurodegeneration in DS and AD (Conti et al., 2010; Isacson et al., 2002; Salehi et al., 2006). APP and Abeta are located in mitochondria (Devi and Anandatheerthavarada, 2010; Manczak et al., 2006). Abeta peptides induce mitochondrial

dysfunction and oxidative stress in astrocytes leading to cell death in neurons (Abramov et al., 2004). Impairments in mitochondrial function in astrocytes also alter metabolism and secretion of neuroprotective APP and increase neuronal vulnerability (Busciglio et al., 2002). Mitochondrial dysfunction, however, does not require aberrant APP and Abeta processing specifically. The Ts1Cje DS mouse model, whose partial trisomy 16 does not include the APP gene, also exhibits diminished mitochondrial activity (Shukkur et al., 2006). These mice show decreased mitochondrial membrane potential and ATP production, increased ROS, and increased GSK3ß and JNK/SAPK activities. GSK3ß preserves mitochondrial function, and inhibits Ca(2+)-induced mitochondrial permeability (Mio et al., 2009). In addition to GSK3ß, several other genes within the Ts1Cje trisomic segment are thought to be involved in mitochondrial function. DSCR1 has been reported to be located in the mitochondria and to play a critical role in mitochondrial function (Chang and Min, 2005). DYRK1A can phosphorylate tau and APP (Park et al., 2007; Woods et al., 2001), and the transcription factor ETS-2 increases APP production and promotes the activation of a mitochondrial death pathway in DS neurons (Helguera et al., 2005; Wolvetang et al., 2003a; Wolvetang et al., 2003b). S100B is another HSA21 gene that can increase ROS, it is a calcium binding protein that affects calcium-dependent signals, targeting mitochondrial proteins such as p53 and ATPase ATAD3A, thereby assisting the cytoplasmic processing of proteins for proper folding and subcellular localization. S100B overexpression can also lead to p53 inactivation (Donato, 2003; Gilquin et al., 2010; Leclerc et al., 2010; Lin et al., 2001; Mihara et al., 2003). Finally, similar mechanisms involving mitochondrial dysfunction have been proposed with other HSA21 genes including SOD1 and BATCH1 (Arbuzova et al., 2002; Ferrando-Miguel et al., 2003; Furuta et al., 1995; Harris-Cerruti et al., 2004; Iannello et al., 1999; Lott et al., 2006; Shim et al., 2003). The added, combinatorial effects of these various HSA21 genes in oxidative stress, apoptosis in neurons and neuronal progenitors are not known.

While mitochondrial dysfunction, oxidative stress, and subsequent apoptosis have been well appreciated in neurons, only recently changes have been reported in DS HNPs. We have shown that upregulation of glial-associated proteins such as S100B lead to tau and GSK3ß hyperphosphorylation, suggesting loss of mitochondrial function (Esposito et al., 2008a; Esposito et al., 2008b). HNPs over-express the HSA21-localized S100B, leading to increased ROS formation such as hydrogen peroxide and nitric oxide, activation of stress response kinase pathways (JNK/STAT), and the upregulation of the glial-associated water channel AQP4 (Esposito et al., 2008a). This same functional network also predicts an added contribution through HSA21 localized APP. Both APP and S100B are upregulated in DS HNPs, they reciprocally enhance the expression of one another, have an additive effect on increasing hydrogen peroxide, decreasing mitochondrial membrane potential and increasing apoptosis (unpublished data). The co-localization of APP and S100B within the same HNPs along VZ of cortex may make progenitors even more vulnerable to oxidative stress, which is supported by the fact that increased progenitor cell death is seen at nano or pico level of S100B or Abeta stimulation (unpublished observations). These same doses are neuroprotective in more mature neurons (Lambert et al., 1998; Van Eldik and Wainwright, 2003; Yankner and Lu, 2009). The additive effects from S100B and APP may depend upon their common activation through the extracellular RAGE receptor which has been shown to regulate intracellular mitochondrial function (Devi and Anandatheerthavarada, 2010; Donato, 2003; Leclerc et al., 2010; Manczak et al., 2006). The *in vitro* observations from DS HNPs are consistent with *in vivo* observations in polytransgenic mice. S100B/APP double transgenic (Tg2576/APP-huS100B) mice display augmented reactive astrocytosis and

microgliosis, increased levels of pro-inflammatory cytokines and enhanced apoptosis (Mori et al., 2010). The double transgenic (mutant APP (mAPP)/RAGE) mice demonstrate increased activation of stress pathways (phosphorylation of p38 and JNK) and altered expression of markers of synaptic plasticity (MAP kinases), leading to early abnormalities in spatial learning/memory (Arancio et al., 2004).

4.2 Gliosis and inflammatory changes

Increased astrocytes and gliosis have previously been reported in DS brain (Guidi et al., 2008). Altered redox states and mitochondrial compromise leading to cell death are thought to promote these inflammatory changes in mature DS brain (Griffin et al., 1989). In DS the increase in the glial marker S100B could be explained, in part, by a gene dosage effect and in part by reactive gliosis (Selinfreund et al., 1991).

A shift in the DS progenitor pool may contribute to the neuronal reduction and increase in glial cells within the mature trisomy 21 brain. Some studies have suggested impairments in interneuron neurogenesis within the DS HNP pool, possibly due to over-expression of the transcription factor COUP-TF1/NR2F1 (Bhattacharyya et al., 2009). Increased levels of COUP-TF1/NR2F1 cause increases in the generation of earlier born neurons and depletion of later born interneurons. Alternatively, other reports suggest that HSA21 associated DYRK1A causes a skewed ratio of primitive endoderm at the expense of neuroectodermal progenitors, leading to a reduction in neurogenesis (Canzonetta et al., 2008). Mensah also reported an inhibition of neuroectodermal differentiation in a teratoma model transplanted with mouse ES cells injected with HSA21 (Mensah et al., 2007). Our prior studies of DS HNPs had demonstrated a constitutive increase in the glial and HSA21 associated S100B protein, which in turn, enhanced expression of another glial-associated protein, the water channel AQP4. Aquaporins can partially mitigate damage due to ROS, such that glial progenitors are more resistant to oxidative damage (Esposito et al., 2008a). In this setting endogenous overexpression of a glial-associated gene S100B might drive gliogenic progenitors, while the inflammatory changes from oxidative stressors would further promote a gliocentric progenitor pool. Other candidate genes such as HSA21-localized Olig1 and Olig2 are basic helix-loop-helix (bHLH) transcription factors essential for development of oligodendrocytes (Jakovcevski and Zecevic, 2005; Lu et al., 2002; Takebayashi et al., 2002; Zhou and Anderson, 2002) or astrocytes (Cai et al., 2007; Marshall et al., 2005; Ono et al., 2008); and SYNJ1 could increase astrogliosis (Herrera et al., 2009). The number of Olig1/Olig2(+) progenitors increases in the injured CNS (Arnett et al., 2004), and Olig2(+) cells preferentially differentiate into GFAP-expressing astrocytes, the main contributors to glial scars which further secrete S100B (Chen et al., 2008; Tatsumi et al., 2008). S100B also contributes to oligodendrocyte progenitor cells (OPC) differentiation in response to demyelinating insults (Deloulme et al., 2004). In addition to transcription factors, epigenetic regulation can also control the cell fate changes within progenitors. One possible candidate gene in DS is HSA21 associated DNMT3L. DNMT3L is a DNA methyltransferase like gene playing a role in DNA methylation by activating DNMT3A or DNMT3B (Jia et al., 2007; Ooi et al., 2007; Suetake et al., 2004). It also represses transcription by recruiting histone deacetylase (HDAC) (Aapola et al., 2002; Deplus et al., 2002). Hypomethylation in neural progenitors leads to precocious astroglial differentiation and perturbed neuronal maturation in DNMT1 or DNMT3A knockout mice (Fan et al., 2005; Hutnick et al., 2009), whereas HDAC activation increases oligodendrocytes differentiation (Hsieh et al., 2004; Liu et al.,

2009; Liu and Casaccia, 2010; Shen et al., 2005; Ye et al., 2009). Again the combinatorial effects from these various HSA21 genes associated with cell fate changes are not known.

4.3 Prolonged cell cycle and reduced proliferation

Proliferation changes during development have been thought to contribute to the decreased neuronal numbers and reduced cortical volume in DS brains. Recent studies demonstrated impairments in proliferation within both hippocampus and neocortical germinal matrix of 17 weeks to 21 weeks gestation age fetal DS brains (Contestabile et al., 2007; Guidi et al., 2008). The DS progenitor cell cycle is prolonged with more cells remaining in G2 phase, thereby causing a reduction in neurogenesis and presumptive increase in astrocytosis. Similar endophenotypes are also found in animal models, which show a reduced progenitor pool and neurogenesis in ventricular zone or dentate gyrus (Contestabile et al., 2009; Haydar et al., 2000; Lorenzi and Reeves, 2006; Moldrich et al., 2009). The molecular mechanisms behind G2 phase prolongation are not known although many genes on HSA21 can cause proliferation changes. Overexpression of APP would antagonistically compete with the APP-BP1, which is required for the cell cycle progression from G1 to S phase. This change would lead to a predicted slowing in proliferation but at the G1 to S phase (Joo et al., 2010). Overexpression of S100B could promote p53 nuclear accumulation and inhibit proliferation (Scotto et al., 1999). Olig2 overexpression has been shown to increase CDK inhibitor p27, leading to decreased proliferation (Tabu et al., 2006). Presumably, various HSA21 genes may directly act in a combinatorial fashion to affect progenitor proliferation.

Studies from human DS HNPs raise another potential explanation for altered progenitor proliferative rates. Consistent with previous reports, we have also found reduced proliferation in HNPs, localized along the ventricular zone of DS cortex, and within DS HNPs cultured *in vitro*. However, characterization of these progenitors have shown that these progenitors, while retaining neural progenitor markers, are more gliocentric in their expression profile. Some preliminary studies suggest that the DS HNPs have reduced voltage gated, outward rectifying potassium channel activity, which correlated to decreased proliferation. Increased potassium channel activity has been correlated with increased proliferation, whereas decreased channel activity coincides with oligodendrocyte differentiation (Chittajallu et al., 2002). Taken in this context, changes in progenitor cell fates (due to such HSA21 genes as S100B, APP, and Olig2) may in part explain the reduction in cell proliferation.

4.4 Imbalance of excitatory-inhibitory neurotransmission

The balance of neurotransmission is important for maintaining normal physiology and behavior. Imbalance of excitatory-inhibitory neurotransmission could contribute to the MR phenotypes seen in DS. Prior studies have shown decreased excitatory and increased inhibitory activities in DS neurons or Trisomy 16 mice. The excitatory neurotransmitter glutamate and inhibitory neurotransmitter GABA are both decreased in DS fetal brain (Reynolds and Warner, 1988; Risser et al., 1997; Smigielska-Kuzia et al., 2010; Whittle et al., 2007). Ts65Dn mice show increased inhibitory synapses and decreased excitatory synapses in the hippocampus (Belichenko et al., 2009; Belichenko et al., 2004; Kurt et al., 2000; Perez-Cremades et al., 2010). Excessive GABA inhibition also alters LTP in the hippocampus (Costa and Grybko, 2005; Kleschevnikov et al., 2004; Siarey et al., 1997). Likewise, Ts1Cje mice show a shortened long term potentiation and an increase in long term depression, as

well as a decrease in the evoked field excitatory postsynaptic potentials- all consistent with impairments in short and long term plasticity (Siarey et al., 2005).
Various HSA21 genes could theoretically affect synaptic function and thereby alter memory and learning. TINM1, SYNJ1 and ITSN1 are thought to play some role in synaptic plasticity, KCNJ6, KCNJ15, KCNE1 and KCNE2 would alter channel activity, whereas NRIP1, ETS2, PCP4, DSCR1, DYRK1A, S100B and APP have some role in affecting neurotransmitter receptors or intracellular signaling. Another more closely scrutinized HSA21 gene GIRK2 (KCNJ6), an inwardly rectifying K channel, could contribute to synaptic inhibition. GIRK2 channels are overexpressed in Ts65Dn hippocampus and frontal cortex (Harashima et al., 2006). The overexpression leads to a significant increase in GABAB-mediated GIRK current in primary cultured hippocampal neurons, and likely affects the balance between excitatory and inhibitory neuronal transmission (Best et al., 2007). Pharmacological treatment of the trisomy 16 mice with GABAA antagonists at non-epileptic doses causes a persistent post-drug recovery of cognition and LTP (Fernandez et al., 2007). Interestingly, treatment of these same mice with the uncompetitive NMDAR antagonist memantine rescues the performance deficits on a fear-conditioning test (Costa et al., 2008). Memantine mimics calcineurin action and the HSA21 DSCR1 gene inhibits calcineurin activity. This raises the hypothesis that DSCR1 overexpression produces a pathological increase in NMDAR activity.
Earlier developmental changes in DS HNPs could also contribute to the imbalance of excitatory and inhibitory neurotransmission. Alterations in cell fate through genes such as Olig1 and Olig2 could lead to an abnormal ratio or distribution of excitatory and inhibitory neurons. Overexpression of HSA21 located Olig1 and Olig2 have been shown to increase GABAergic interneuron neurogenesis in Ts65Dn mice, and the dysregulation of excitatory-inhibitory imbalance could be rescued by knocking down the extra copy of these two genes (Chakrabarti et al., 2010). This phenotype contrasts with our observations and those from other groups, who have observed a reduction of interneurons in DS HNPs at the expense of increasing oligodendrocytes (Bhattacharyya et al., 2009). We see a shift from neuronal to gliocentric progenitors with overexpression of S100B (Esposito et al., 2008a) and APP (unpublished observations), which would potentially further compromise the interneuron population. This decline in interneuron neuronal production is consistent with the loss in GABA-ergic interneurons, as opposed to total neuronal numbers, reported in the DS cortex, further reiterating differences between the mouse and human disorders (Golden and Hyman, 1994; Ross et al., 1984; Weitzdoerfer et al., 2001).

5. Combinatorial gene effects in DS HNPs contribute to the DS phenotype

HSA21 associated genes which are implicated in cell death and oxidative stress could contribute to the later endophenotypes seen in DS. To identify functional modules of gene expression and interacting partners relevant to DS HNPs, we had applied network-based analyses through the Ingenuity Pathways Knowledge Base (IPA) (Calvano et al., 2005). Among 1902 genes that were significantly different on pairwise comparison between WT-DS expression profiling of HNPs, 334 genes were shown to interact within 46 functional networks (Esposito et al., 2008a). The top four interactive networks incorporated 93 dysregulated genes involved in cell cycle, cell death, oxidative stress and several canonical signal transduction pathways. These findings would suggest that genes involved in these primary functional clusters might later contribute to the observed DS endophenotypes.

To understand the role of HSA21 genes in progenitor cell death and changes in redox state, we first focused on HSA21 genes APP and S100B. These molecules were included in one of the top functional clusters and were associated with other cell signaling molecules, including JAK/STAT and MAPK, which have been implicated in the stress response pathway, inflammation and potentially gliosis. Using this potential pathway, we found that upregulation of S100B activated the JAK/STAT MAPK pathway through the RAGE receptor. RAGE receptor activation induced mitochondrial disruption and oxidative stress. Activation of this pathway enhanced progenitor cell death and likely gliosis, as suggested by increased expression of glial associated water channel AQP4 (Esposito et al., 2008a). We have also been able to observe a S100B-mediated induction of tau protein hyperphosphorylation via Dickopff-1 up-regulation and disruption of the Wnt pathway, suggesting a shared common final pathway with APP (Esposito et al., 2008b).

Although APP and S100B have previously been implicated in oxidative stress and cell death, constitutive over-expression of these and other HSA21 genes would also predict upregulation of inflammatory responses (astrocytosis), leading to a skewed gliocentric progenitor phenotype. The ongoing cell death and inflammation from APP and S100B promote reactive astrocytosis and astrocytes secrete S100B, causing further cell injury and death (Li et al., 2011). This same mechanism of injury in the mature brain could also take place within the progenitor population during development. Additionally, the transcription factors Olig1/2 increase oligodendroglial progenitor numbers and would likely augment any gliocentric shift. Similarly, the HSA21 DNA methylthansferase like DNMT3L gene recruits HDACs, which compete with beta catenin to regulate TCF4 dependent transcriptional inhibition of neuronal progenitor proliferation and activation of oligoprogenitor phenotypes (Aapola et al., 2002; Deplus et al., 2002; Liu et al., 2009; Ye et al., 2009).

A gliocentric shift in the progenitor pool could alter rates of proliferation and create an imbalance in excitatory-inhibitory transmission. Gliocentric progenitors would be predicted to demonstrate reduced voltage gated, outward rectifying potassium channel activity, and consequent slower rates of proliferation. Moreover, the ongoing neural progenitor cell death from oxidative stress enhances gliocentric progenitor characteristics at the expense of neuronal progenitor phenotypes. This shift becomes more prominent later in cortical development when the cyclical and synergistic roles played by such inflammatory mediators such as S100B and APP become more pronounced. Under this paradigm, the loss in neuronogenic progenitors would manifest later in development and thereby affect interneuron production.

These observations raise a hypothetical paradigm whereby several specific HSA21-localized genes promote a deleterious, cyclical pathway involving ROS, hypersecretion of S100B, APP overproduction and proliferation of gliocentric progenitors. A gliocentric shift in the progenitor pool would provide a potential explanation for the predominant endophenotypes seen in the DS brain.

6. Pharmacological approach using DS HNPs

From a translational standpoint, human neurally-derived reagents will be useful to identify pathological mechanisms and address the efficacy of pharmacotherapeutics. Given that DS is a contiguous gene syndrome, multiple genes on HSA21 will interact, share substrates, and/or influence the same processes or pathways relevant to neural function. Our ongoing work demonstrates that some genes such as S100B and APP are over-expressed from

development into adulthood and would therefore have potentially cumulative effects over time. In this respect, treatment directed toward correcting aberrant pathways regulated by these genes may require early and ongoing management to achieve efficacy. Other genes which might also contribute to gliocentric phenotypes and which reside on HSA21 such as Olig1/2 appear to be more temporally restricted (personal observations, Lu and Sheen) and therefore might be more amenable to a finite pharmacological approach. It remains to be seen to what extent the observations made from various treatment modalities for trisomy mouse models and/or DS HNPs are faithfully replicated in actual individuals with DS. That said, the use of DS HNPs would provide another measure of validation for pharmocotherapies devised in treatment of these disorders.

7. Conclusion

DS is a contiguous gene syndrome giving rise to MR, dementia, and seizures. These clinical outcomes are mirrored by endophenotypes including mitochondrial dysfunction, oxidative stress, cell death, gliosis, inflammation, prolonged cell cycle, reduction in proliferation and imbalances in excitatory and inhibitory neurotransmission. While these characteristics have largely been observed in neurons in the mature brain, function based cluster analyses of pair-wise comparisons between normal and DS human neural progenitors suggest that similar changes are ongoing during development. Moreover, these endophenotypes likely arise from the integration of various genetic and epigenetic factors on chromosome 21 such as APP, S100B, Olig1/2 and DNMT3L. The ongoing disruption of mitochondrial redux states and inflammation promote early gliocentric phenotypes in the progenitor pool, which would alter progenitor proliferative rates and contribute to a decline in interneuron production. Overall, early developmental changes in the progenitor population could promote many of the deleterious changes seen later in the mature DS brain.

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4

Down Syndrome: A Complex and Interactive Genetic Disorder

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1. Introduction

1.1 Trisomy 21 causes phenotypes associated with Down syndrome

Down syndrome (DS) occurs in approximately 1 out of 700 live births and most commonly results from three copies of human chromosome 21 (Hsa21) (Christianson, 2006). DS is a multifaceted disorder with over 80 clinically defined phenotypes including those affecting the central nervous system, heart, gastrointestinal tract, skeleton and immune system (Epstein, 2001; Van Cleve and Cohen, 2006). Phenotypes associated with individuals with DS vary in both incidence and severity, leading to a vast array of phenotypic combinations among those with Trisomy 21 (Ts21) (Deutsch et al., 2005; Epstein, 2001; Van Cleve et al., 2006; Van Cleve and Cohen, 2006). For example, cognitive impairment, hypotonia and craniofacial features are universal phenotypes, whereas cardiac abnormalities only affect approximately half of individuals with DS. Individual phenotypes similar to those seen in DS have been documented in individuals without Ts21, but a general higher incidence and severity of these phenotypes in individuals with DS suggests that trisomy plays an important role in initiating or modifying these features (Epstein, 2001; Roper and Reeves, 2006). Although it has been suggested that common mechanisms may be involved in similar phenotypes seen in individuals with and without Ts21, common pathophysiology must be proven for each individual phenotype.

A chromosomal basis for DS was postulated as early as 1932 (Patterson and Costa, 2005). After Jerome Lejeune established that DS was caused by an extra Hsa21, he studied the metabolic pathways associated with Ts21 phenotypes in an attempt to cure DS (Neri and Opitz, 2009). Alternate causes of DS, including translocations and mosaicism of extra material from Hsa21, were established shortly after the chromosomal basis of DS had been discovered (Patterson and Costa, 2005). These early genetic findings set the stage for current paradigms and research surrounding the gene-phenotype relationships in DS.

Although ~95% of the incidence of DS is due to trisomy of the entire Hsa21, DS also results from extra genetic material from Hsa21 translocated to other chromosomes. Additionally, some individuals with DS phenotypes have Ts21 in only a portion of their cells (mosaicism). Differences in genetic and cellular composition of the trisomy may lead to the observed differences in DS phenotypic incidence and severity. Genotypic and phenotypic variations have also been used to hypothesize about trisomic genes or chromosomal regions that may cause or significantly alter DS phenotypes. Such genotype-phenotype correlations are important in defining the etiology of traits associated with DS as well as suggesting possible therapeutic mechanisms to overcome deficits seen in individuals with DS.

1.2 Hypotheses regarding genotype-phenotype correlation in DS

Early investigations into the genotype-phenotype relationship drew upon information from individuals with partial Ts21. By examining these individuals, determining the extra chromosomal material and corresponding phenotypes, an early causal hypothesis linked DS to trisomy of the distal end of Hsa21 (Patterson and Costa, 2005). A reductionist variation of this hypothesis later stated that individual phenotypes and features could be mapped to specific regions of the genome, and the addition of these regions with their respective phenotypes would cause the multitude of traits associated with DS (Neri and Opitz, 2009). Others had a more global or genomic view of the genotype-phenotype relationship and hypothesized that DS phenotypes were due to a general genomic imbalance initiated by Ts21. Many new ideas have extended or combined previous hypotheses as well as proposed novel views about the correlation between genes and phenotypes in DS, especially with an increased ability to precisely define the genotypes and phenotypes associated with DS. Yet, the exact mechanisms of how the triplication of genes on Hsa21, the smallest and least gene dense of the autosomes, causes one of the myriad phenotypes associated with DS have still largely not been established. One or more of the hypothesized mechanisms may be correct for a single phenotype associated with DS, yet it is unlikely that a single overriding mechanism would describe the etiology of all Ts21 phenotypes (Epstein, 2001).

1.2.1 Gene dosage imbalance hypothesis

The "gene dosage imbalance" hypothesis suggests that an increased dosage or copy number of genes on Hsa21 would lead to an increase in gene expression and protein product in the individual (Antonarakis et al., 2004; Epstein, 2001; Pritchard and Kola, 1999). In essence, overexpression of the products of dosage sensitive genes on Hsa21 would lead to DS phenotypes. This hypothesis has been extended to include the possibility that specific genes or subsets of genes may control specific DS phenotypes (Patterson, 2007). Most studies have concluded that three copies of Hsa21 genes leads to an average 50% higher expression of trisomic genes as compared to euploid (normal chromosomal complement) individuals (Gardiner et al., 2010). As additional high throughput analyses are performed, however it is becoming increasingly clear that the gene expression changes may be specific to a group of genes in a particular tissue at a precise developmental or mature stage. Additionally, some trisomic gene expression changes may overlap with normal expression of these genes in the euploid chromosomal component (Ait Yahya-Graison et al., 2007; Prandini et al., 2007; Wiseman et al., 2009).

1.2.2 Amplified developmental instability hypothesis

The "amplified developmental instability" hypothesis states that a non-specific dosage of a number of trisomic genes leads to a genetic imbalance that causes a large impact on the expression and regulation of many genes throughout the genome (Pritchard and Kola, 1999; Shapiro, 1983). This disruption in genetic homeostasis or more generalized change in gene expression throughout the genome would affect signaling pathways and lead to phenotypes associated with DS. Therefore, it has been hypothesized that traits in individuals with DS are not caused by the altered expression of a particular gene, but rather the general changes in expression throughout the genome caused by trisomy that lead to DS traits (Patterson, 2009). An additional premise of this hypothesis is that the larger the number of trisomic genes, the higher the incidence and severity of DS phenotypes due to increased genetic instability. An extension of this hypothesis is that some phenotypic changes associated with

DS may not be attributable to specific genetic changes but rather to changes in the expression of the entire chromosomal domain (Gardiner et al., 2010).

1.2.3 Critical region(s) hypothesis

Phenotypic analyses conducted on individuals with partial trisomy for Hsa21 led to a hypothesis that only one or a few small chromosomal regions, termed "Down syndrome critical regions" (DSCR), contain genes responsible for the majority of DS phenotypes (Delabar et al., 1993; Korenberg et al., 1990). It has been suggested that DS (including many of the major, most well defined phenotypes) was caused by one specific DSCR (Epstein, 2001). Even before the identification of specific genes on Hsa21, a region of 1.6-2.5 Mb on Hsa21was thought to be responsible and sufficient to cause most DS phenotypes (Dahmane et al., 1998; Ohira et al., 1996). The sequencing of Hsa21 proved to be an important factor in the progression of DS research (Hattori et al., 2000) and led to further insight into genotype-phenotype correlations associated with DS and precise characterizations of DSCR regions (Patterson, 2009). A region of 3.8-6.5 Mb on 21q21.22 containing approximately 30 genes has been traditionally identified as the DSCR, although recent studies have demonstrated that this DSCR is not sufficient for all major DS phenotypes, though its inclusive genes may be relevant for some individual phenotypes (Delabar et al., 1993; Lyle et al., 2009). A current hypothesis states that certain chromosomal regions may contain a significant gene or genes necessary for the development or maintenance of specific but not all DS phenotypes (Korbel et al., 2009; Lyle et al., 2009; Olson et al., 2007).

1.2.4 Other genetic, epigenetic and environmental considerations

The hypotheses of how genetic mechanisms control DS phenotypes are not mutually exclusive, may include a combination of different mechanisms and may be unique for each particular phenotype. It is now believed that contributions to phenotypes associated with DS are likely to come from a number of genes. Variability in genomes may also come from allelic heterogeneity or other differences in the genetic architecture (Antonarakis et al., 2004). There may be genes that have a larger effect on certain phenotypes (Epstein, 2001; Patterson, 2009), or genetic contributions may be additive, subtractive, or epistatic (Gardiner et al., 2010). Recently it has been shown that epigenetic modifications and allelic differences also play a significant role in the development of these phenotypes, not just the simple presence of the chromosomal imbalance (Belichenko et al., 2009; Elton et al., 2010). Other factors including environmental influences and stochastic events also add to the differential manifestation of phenotypes.

1.2.5 Dosage compensation

Extra copies of genes may be found as a result of a number of duplication events, yet many of the resultant trisomies may be difficult to detect or have subtle phenotypes. Though there may be an extra copy of a gene, and even alterations in gene expression with the resultant protein product, dosage imbalance for a specific gene may have no effect on a phenotype. Such genes have been "dosage compensated" by some mechanism (Antonarakis et al., 2004). Gene expression experiments of cells with Ts21 have shown that a high percentage of three copy genes may undergo dosage compensation (Ait Yahya-Graison et al., 2007). This compensation could be accomplished by gene regulatory networks or negative feedback

loops. Dosage compensation may be tissue or time dependent and may play an important role in the development and progression of DS phenotypes.

1.3 Mouse models of DS to understand genotype-phenotype correlation

In addition to being valuable tools for better understanding Ts21 and DS phenotypes, DS mouse models have also been utilized as predictive models of novel DS phenotypes (Baxter et al., 2000). Mice provide access to all tissues at all developmental stages, the ability to understand genetic and cellular mechanisms caused by trisomy, and a resource to understand potential treatments for phenotypes. Genes found on Hsa21 are highly conserved in order and homology on three different mouse chromosomes: mouse chromosome (Mmu)16, 17, and 10. Several DS mouse models have been created with segmental trisomy for different regions of the distal portion Mmu16, that contains nearly half the gene homologs found on Hsa21 (Hattori et al., 2000; Pletcher et al., 2001). The Ts65Dn mouse is the most commonly used DS model with segmental trisomy for the distal portion of Mmu16. Three copies of the region between *Mrpl39* and *Znf295* spanning 13.6 Mb on Mmu16 and containing over 100 Hsa21 gene homologs results in several DS-like phenotypes (Reeves et al., 1995). The Ts1Cje mouse contains segmental trisomy for approximately 78% (*Sod1-Znf295* excluding *Sod1*) of the triplicated genes in the Ts65Dn model (Olson et al., 2004b; Sago et al., 1998). Both Ts65Dn and Ts1Cje mice contain three copies of the putative DSCR region and exhibit a significant amount of phenotypic similarity to humans with DS. The Ts1Rhr/Ms1Rhr mouse models were created by generating a 3.9 Mb reciprocal duplication/deletion containing 33 genes (*Cbr1-Orf9*) homologous to those found in the most commonly defined human DSCR (Olson et al., 2004a). The transchromosomal Tc1 mouse model of DS has a copy of Hsa21 in its cells, though there are some deletions of Hsa21 regions and significant mosaicism in this model (O'Doherty et al., 2005). The Ts1Yey model contains the entire region of Hsa21 homology on Mmu16 from *D930038D03Rik-Znf295* duplicated on one Mmu16 and results in trisomy for this region in the mouse model (Li et al., 2007) (See Table 1).

More recently, mouse models of DS have been made that contain small regions of homology from chromosomes other than Mmu16. The Ts1Yah model contains a duplication of Mmu17 from *Abcg1-U2af1* with homology to Hsa21 (Pereira et al., 2009). The Ts2Yey model has a duplication of the entire region of Mmu10 homologous to Hsa21 (*Prmt2-Pdxk*). The Ts3Yey mouse has a duplication of the entire Mmu17 chromosomal region that corresponds to Hsa21 (*Abcg1-Rrp1b*) (Yu et al., 2010). Mice containing entire regions of homology to Hsa21 are being generated to examine DS phenotypes. Mouse models of DS may exhibit certain DS-like phenotypes with differing severity, but phenotypes may not be conserved across models, thus adding to the usefulness of the models for understanding the genotype-phenotype relationships associated with DS.

1.4 Using modern genetic and genomic tools to understand genotype-phenotype correlation in DS

Because DS and its resultant phenotypes are caused by trisomy, the gene-phenotype correlation (the genes responsible for the cellular or developmental changes) is of great importance in understanding DS. DS may be viewed as a chromosomal disorder due to three copies of Hsa21, a genetic disorder resulting from the altered expression of trisomic genes, or a disorder that results from alterations in gene expression and pathways

Common Name	Genetic triplication	Number of triplicated genes
Ts65Dn	Ts(17^{16})65Dn	104 (Mmu 16)
Ts1Rhr	Dp(16*Cbr1-Orf9*)1Rhr	33 (Mmu 16)
Ts1Cje	Ts(12^{16}C-tel)1Cje	81 (Mmu16)
Tc1	Tc(Hsa21)1TybEmcf	131 (~92% of Hsa 21)
Ts1Yey	*Dp(*16 *D930038D03Rik-Znf295)1Yey*	Mmu 16
Ts2Yey	*Dp(10Prmt2-Pdxk)2Yey*	Mmu 10
Ts3Yey	*Dp(17Abcg1-Rrp1b)3Yey*	Mmu 17

Table 1. Common DS mouse models with defined trisomic regions.

throughout the entire genome. Several studies have documented differential gene expression in tissues originating from varying spatial and developmental environments in attempts to determine genetic mechanisms affecting DS phenotypes (Conti et al., 2007; Lintas et al., 2010; Lyle et al., 2004; Moldrich et al., 2007). High throughput analysis utilizing tools such as microarray analysis, qPCR (quantitative polymerase chain reaction) and SAGE (serial analysis of gene expression) not only allow for a better understanding of gene expression aberrations in a spatiotemporal sense, but also permit for a better understanding of gene interactions within the context of natural physiological conditions. Though disparities exist between tissues and developmental time points, such analyses have provided insight into genes that appear to be critical to the development of specific DS phenotypes. New genetic information, coupled with precisely defined cellular and molecular phenotypes, (often using mouse models), has allowed the testing of multiple hypotheses concerning the gene-phenotype relationship. Functional groupings of dysregulated genes may add additional mechanistic insight into the origin of DS phenotypes, and such groupings may transcend both time and tissue type. The intersection of genetic and functional information may lead to new insights into the gene-phenotype relationship and the mechanisms leading to the development of DS phenotypes. With increasing ability to assess large scale gene expression, the importance of both trisomic and non-trisomic genes and genetic pathways utilizing gene products from the entire genome has been investigated. Instead of a simple notion of trisomy for Hsa21 causing a disorder or trisomic expression of specific genes on Hsa21 causing a disorder, current technologies and techniques now suggest that DS, with its myriad phenotypes, is a complex disorder with many interactions on genetic and mechanistic levels.

2. Trisomic and non-trisomic genes

2.1 The importance of trisomic and non-trisomic genes

Numerous gene expression studies have been performed on an assortment of tissues from individuals with DS as well as mouse models of DS (Tables 2 and 3). Although these assays vary widely in technique and scope, they have generally focused on the altered expression of trisomic genes. A strict interpretation of the gene dosage hypothesis suggests that all trisomic genes theoretically exhibit a 1.5 fold upregulation in every tissue and cell when compared to normal subjects, and nearly all studies use this expected fold change as a standard for expression analysis (Giannone et al., 2004; Mao et al., 2003). Interestingly, in studies that have analyzed both trisomic and disomic (non-trisomic) gene expression in

trisomic compared to normal individuals, a significant number of non-trisomic genes have been found to be dysregulated in association with DS phenotypes (Table 4) (Lockstone et al., 2007; Rozovski et al., 2007; Slonim et al., 2009).

Study	Strain	Age	Tissue	Platform
Chrast et al. 2000	Ts65Dn	Adult	Brain	SAGE
Saran et al. 2003	Ts65Dn	3-4 months	Cerebellum	Microarray
Amano et al. 2004	Ts1Cje	Postnatal 0	Brain	Microarray
Lyle et al. 2004	Ts65Dn	Adult	Brain, Heart, Kidney, Liver, Lung, Muscle	qPCR
Dauphinot et al. 2005	Ts1Cje	Postnatal 0, 15, 30	Cerebellum	Microarray
Kahlem et al 2007	Ts65Dn	3-4 months	Lung, Skeletal Muscle, Midbrain, Cerebellum, Cortex, Testis, Liver, Heart, Kidney	Microarray
Sultan et al. 2007	Ts65Dn	13-26 weeks	Cerebellum, Midbrain, Cortex	qPCR
Laffaire et al. 2009	Ts1Cje	Postnatal 0, 3, 7, 10	Cerebellum, Granule Cell Layer	Microarray
Moldrich et al. 2009	Ts1Cje	E14	Neural Progenitor Cells	Microarray
Hewitt et al. 2010	Ts1Cje	Adult	Neural Stem Cells	Microarray, qPCR

Table 2. Features of gene expression assays performed in mice.

Study	Age	Tissue	Platform
Gross et al. 2002	Gest. 16-24 weeks	Placenta	Microarray
FitzPatrick et al. 2002	Fetal	Amniocytes	Microarray, qPCR
Mao et al. 2003	Gest. 17-21 weeks	Cerebral cortex, astrocytes	Microarray
Giannone et al. 2004	Adult	T lymphocytes	Microarray
Tang et al. 2004	Adult	Blood	Microarray
Chung et al. 2005	Fetal	Amniocytes	Microarray
Deutsch et al. 2005	Adult	Lymphoblastoids	qPCR
Mao et al. 2005	Fetal	Cerebellum, heart	Microarray
Li et al. 2006	Gest. 15-23 weeks	Heart, fibroblasts	Microarray, qPCR
Ait Yahya-Graison et al. 2007	Adult	Lymphoblasts	Microarray
Altug-Teber et al. 2007	Fetal	Amniocytes, Chorionic Villus Cells	Microarray
Conti et al. 2007	Gest. 18-22 weeks	Heart	Microarray
Lockstone et al. 2007	47-76 years	Brain	Microarray
Prandini et al. 2007	25 years	Lymphoblasts, Fibroblasts	qPCR
Rozovski et al. 2007	Fetal	Placenta	Microarray, qPCR
Chou et al. 2008	Gest. 16-21 weeks	Amniocytes	Microarray
Esposito et al. 2008	Gest. 19-21 weeks	Frontal cortex	Microarray
Sommer et al. 2008	1-4 years	Lymphocytes	SAGE, qPCR
Slonim et al. 2009	Fetal	Aminocytes	Microarray

Table 3. Features of gene expression assays from human derived tissues.

The number and variety of genes found to be dysregulated in response to trisomy seems to vary greatly in a spatiotemporal dependent manner, and provide evidence against the idea of a specific 1.5 fold dysregulation of trisomic genes in all tissue types (Mao et al., 2005; Potier et al., 2006; Sultan et al., 2007). Additionally, much of the variation in both trisomic and disomic gene expression in subjects with trisomy overlaps with the variation observed among normal individuals, which may limit the number of trisomic genes found to be differentially expressed (Prandini et al., 2007), and contribute to the variable phenotypes associated with DS (Chou et al., 2008). We therefore hypothesize that both trisomic and non-trisomic genes are important for the origin and development of DS phenotypes.

2.2 Gene expression studies in humans and mouse models of DS

2.2.1 Altered expression of trisomic genes and the gene dosage hypothesis

The gene dosage hypothesis and the proposed 1.5 fold increase in all trisomic gene expression have motivated much of the gene expression research of DS phenotypes. Assessment of gene expression in human fetal brain tissue and astrocyte cell lines taken at 17-20 weeks gestation revealed a general upregulation of trisomic gene expression, and seemed to agree with the 1.5 fold change hypothesis (Mao et al., 2003). Analysis of brain tissue isolated from 13-16 week old Ts65Dn mice also identified a global upregulation of trisomic genes in the cerebellum, cortex, and midbrain, and the average fold changes of trisomic gene expression were 1.44, 1.37, and 1.39, respectively, in Ts65Dn mice (Sultan et al., 2007).

Yet, other studies suggest the global upregulation of trisomic genes in agreement with the gene-dosage hypothesis is likely not occurring in DS. Many studies indicate that not all trisomic genes are dysregulated in all trisomic tissues. Furthermore, several studies on trisomic tissues have demonstrated expression levels significantly different from the expected 1.5 fold increase, indicating that having three copies of a gene does not necessarily confer a 1.5 fold upregulation. For example, expression analysis of 99 genes in 6 different tissues (brain, heart, lung, kidney, liver, and muscle) in P30 and 11 month old Ts65Dn mice revealed that 37% of the trisomic genes were expressed at a 1.5 fold increase, 45% were <1.5 increase, 18% >1.5 increase, and 9% were not significantly different from normal mice (Lyle et al., 2004). Additionally, only 15% and 29% of tested trisomic genes were found to be overexpressed in DS fetal hearts and cultured fibroblasts, respectively (Li et al., 2006). Of 134 differentially expressed genes assessed in lymphoblastoid cell lines (LCLs), 58/134 had overexpression greater than 1, (1.25-2.27), and 86/134 exhibited fold changes significantly different from 1.5 (9 were greater than 1.5 fold (1.64-2.27) and 77 were less than 1.5 (0.74-1.4)) (Ait Yahya-Graison et al., 2007). Of 117 trisomic genes in LCLs and 114 trisomic genes in fibroblasts, only 39% and 62% respectively were found to have significant expression differences from euploid cells (Prandini et al., 2007). Based on these data, four classes of genes in trisomic tissues have been suggested: those that exhibit expression significantly different from 1 but not different from 1.5 fold, amplified genes (expression significantly greater than 1.5 fold), compensated genes (expression significantly less than 1.5 fold), and the genes with expression differences not different from 1 or 1.5 fold (Ait Yahya-Graison et al., 2007; Prandini et al., 2007). These extensive studies indicate that a global 1.5 fold upregulation of all trisomic genes in DS tissues is unlikely and suggest that mechanisms in addition to trisomy are also affecting gene regulation. Although the expression levels of some trisomic genes fit within the theoretical 50% upregulation, trisomic genes in several tissues at different time points exhibit expression levels much different from the expected 1.5 fold increase.

Study	Trisomic Genes	Disomic Genes	# Significant Trisomic Genes	# Significant Disomic Genes	# Genes Examined
Gross et al. 2002	*C21ORF3*	*KRT8, ALDH7, KISS1*	2	5	8976
FitzPatrick et al. 2002	*N/A*	*IGFBP3, RGS5, IGFBP5*	39	1934	8020
Mao et al. 2003	*DSCR2, SOD1*	N/A	Global upregulation	N/A	~15,000
Giannone et al. 2004	*SOD1,CSTB*	*HLA-DRB3, GABRG2, ACAT2*	2	15	334
Tang et al. 2004	*DSCR2, CSTB*	*FHL1, ALOX12, RGS10*	~20%	6%	~12,000
Chung et al. 2005	*COL6A1, PRSS7*	*AKT, CASP5, JUN*	2	8	102
Deutsch et al. 2005	*APP*	N/A	25	7	41
Mao et al. 2005	*SOD1, SON, DSCR3*	*ADAMTS1*	26	14	~12,500
Li et al. 2006	*SH3BGR, MX1, GART*	*IFI27*	17	41	~10,000
Ait Yahya-Graison et al. 2007	*SAMSN1, DYRK1A SNF1LK, MX1*	N/A	29%	N/A	359
Altug-Teber et al. 2007	*DSCR1*	N/A	16	N/A	~12,500
Conti et al. 2007	*DSCR1, DYRK1A*	*NFATc*	32	441	~6300
Lockstone et al. 2007	*APP, RCAN, BACE2*	*APOE, NOTCH2*	40 (25%)	4.4% dysregulated	~12,500
Prandini et al. 2007	*GABAPA, PFKL, U2AF1*		39%, 62%	N/A	123, 132
Rozovski et al. 2007	*APP*	*LOX, MEST, MAT2A*	41 overexpressed	709 overexpressed	~12,500
Chou et al. 2008	N/A	N/A	~1.28 fold	17 with high variation	~18,000
Esposito et al. 2008	*APP, S100B*	*AQP4*	~1.5 fold in DSCR	Modest pathway-specific changes	~12,500
Sommer et al. 2008	*N/A*	*2M, CD74, CD52*	7	~250	N/A
Slonim et al. 2009	*CLIC6, ITGB2, RUNX1*	N/A	5	409	~12,500
Chrast et al. 2000	*Ifnar2, Ifngr2, Cbr*	N/A	3	330	N/A
Saran et al. 2003	N/A	N/A	~1.45 fold average	~1.1 fold average	~12,500
Amano et al. 2004	N/A	N/A	~1.5 fold average	~1.0 fold average	~11,300
Lyle et al. 2004	*Adamts1, Mx1*	N/A	~1.5 fold average	N/A	99
Dauphinot et al. 2005	*Dscr3, Hmgp14, Donson*	*HoxA5, Dlx1*	~1.5 fold average	~1.0 fold average	~12,500
Kahlem et al 2007	*Bace2, App, Mx1*	N/A	~1.5 fold average	N/A	136
Sultan et al. 2007	*Bace2, Kcne2, App, Cbr1*	N/A	~1.5 fold average	N/A	50
Laffaire et al. 2009	*Olig1, Dscam, Girk2, Son*	N/A	11-13 in cerebellum 23 in GCL	372-1164 in cerebellum 9 in GCL	~15,600
Moldrich et al. 2009	*Olig1, Olig2, Dscam, Dscr3*	*Sox21, Calcoco1*	29 of 54	1847	~25,000
Hewitt et al. 2010	*Itsn1*	*Mcm7, Brca2,Prim1*	~1.5 fold average	6.5% of probes	~14,000

Table 4. Significant trisomic and disomic genes highlighted by gene expression studies. Common genes of interest were selected from studies that stated more than three genes. N/A= not applicable or available.

2.2.2 Altered expression of non-trisomic genes

While many gene expression studies have focused on expression levels of genes in three copies, increasingly the expression of non-trisomic genes has also been analyzed. Two early microarray studies using amniotic fluid and placenta found extreme variation associated with the genes on Hsa21, however both of these studies showed that disomic genes also contained high variation in expression in trisomic tissue (Chung et al., 2005; Deutsch et al., 2005). It was observed that two-copy genes were downregulated implying that Ts21 was affecting genes on other chromosomes (Chung et al., 2005). An additional microarray analysis on cells derived from DS fetal placenta found 750 genes were overexpressed with the majority located on chromosomes other than 21, implicating non-trisomic genes as significant contributors to DS phenotypes (Rozovski et al., 2007). Adult human DS brains exhibited 400 differentially expressed genes when compared to normal individuals and approximately 350 of these were not found on Hsa21 (Lockstone et al., 2007). Expression analyses conducted on uncultured DS amniotic fluid cells identified a total of 414 dysregulated genes with only five located on Hsa21 (Slonim et al., 2009). Two recent analyses of cells from developing Ts1Cje brains have also indicated a large number of dysregulated non-trisomic genes in addition to significantly dysregulated trisomic genes (Laffaire et al., 2009; Moldrich et al., 2009). Additionally, 6.5% of all disomic genes were found to be dysregulated in the primary neural progenitor cells isolated from the brains of adult DS mice (Hewitt et al., 2010). Based on these findings, it has been suggested that trisomy causes a disruption in gene regulation throughout the entire genome and not just of the genes in three copies. Despite these findings, the phenotypic effects of altered disomic gene expression in DS is highly debated and not well understood (Lyle et al., 2004; Mao et al., 2005; Saran et al., 2003).

2.3 Measuring gene regulation with tissue and age specificity

The most tissue-specific developmental microarray research in mouse models of DS has focused on the developing cerebellum, which continues to develop after birth. Extensive microarray analyses of the hypocellular cerebellum in adult and early postnatal trisomic mice have revealed dysregulation of both trisomic and non-trisomic genes. For example, microarray analyses were performed on the entire cerebellum during postnatal days 0, 15, and 30 (P0, P15, P30) in the Ts1Cje model of DS. Though genes present in three copies had an average relative expression of about 1.5 fold, only five (P0), nine (P15), and seven (P30) three copy genes of the 63 trisomic genes tested were expressed at levels >2 or <1.2. Additionally, 406 of 8250 two copy (non-trisomic) genes examined had ratios comparing trisomic and euploid expression that were significantly different from 1 (Dauphinot et al., 2005). Three trisomic genes were dysregulated at all developmental time points. Interestingly, changes in development influenced gene expression differences between stages more than between trisomy and euploid animals (Dauphinot et al., 2005; Potier et al., 2006). As these studies showed, gene expression can vary greatly in one tissue from early in development to adulthood. Because individual genes are shown to have variation in expression throughout development, certain genes may be relevant to a particular phenotype at one time point and unrelated at another.

2.4 Gene expression variation in response to Trisomy 21

In addition to the importance of understanding the temporal and spatial relationships associated with trisomic gene expression, variation in gene expression of both disomic and

trisomic genes may contribute to DS phenotypes. Expression studies conducted on fibroblasts and LCLs identified large gene expression variation between cell lines as well as between individual genes (Ait Yahya-Graison et al., 2007; Deutsch et al., 2005; Prandini et al., 2007). It was suggested that high levels of gene expression variation could explain why the majority of Hsa21 genes were not significantly upregulated and the variability in DS phenotypes. The latter is explained by the level of overlap between gene expression in trisomic and euploid individuals. Genes with distinct expression profiles may be candidates for the constant features of DS, whereas the genes that exhibit overlapping expression profiles may be responsible for the variable DS phenotypes (Prandini et al., 2007).

Studies conducted in Ts65Dn mice also exhibited differing levels of variable gene expression. Sultan et al. showed that 31 out of 33 trisomic genes were upregulated in the Ts65Dn brain. Of these 31 genes, 24 genes exhibited a small amount of variation (coefficient of variation of <0.2) and seven genes exhibited high variation. These authors suggested that the level of variation in gene expression indicated how tightly the genes were being regulated in the brain tissue of Ts65Dn animals.

Understanding gene expression variation in trisomic tissues is a key component in the study of DS gene-phenotype relationships. This expression variation may influence the results of microarray and qPCR studies and lead to the false identification/exclusion of candidate genes for specific phenotypes. Additionally the phenotypic variability observed in individuals with DS may be attributed to trisomic gene expression variation in different tissues, and it is likely that the level of variation within an individual gene is directly related to how tightly that particular gene is regulated. Further research is needed to identify the exact role expression variation plays in the development of DS phenotypes.

2.5 Summary

Gene expression assays in DS are an essential tool for understanding the mechanisms behind Ts21. While the gene dosage hypothesis may be applicable to specific genes with spatiotemporal specific expression, gene expression analyses suggest that not all trisomic genes are dysregulated at a 1.5 fold ratio, the expression of some trisomic genes is similar to euploid, and variation in gene expression plays a significant role in influencing phenotypic development. Differences in developmental stages as well as particular tissue types may contribute to some of the variable findings across studies. Additionally, the level of gene expression variation differs between individuals of the same genotype, adding to the complexity of understanding expression analyses. Though trisomic genes are important and are the likely initiators of DS phenotypes, disomic genes are also dysregulated suggesting that both trisomic and disomic genes can influence the DS phenotype.

3. Complex genotype-phenotype relationships

Characterization of certain DS phenotypes along with the genetic analysis of individuals with partial trisomies led to a hypothesis of the "Down syndrome critical or chromosomal region" (DSCR) believed to contain the genes necessary for the development of the most common phenotypes observed in individuals with DS (Delabar et al., 1993; Korenberg et al., 1990). The sequencing of Hsa21 and mapping of specific genes in the putative DSCR further defined the genetic content of the region and allowed for a more detailed trisomic gene-phenotype correlation. More recent analyses of critical regions using mouse models of DS as well as samples from individuals with partial trisomies have questioned the validity of the

original DSCR hypothesis of a single region or gene important for most of the common DS traits (Korbel et al., 2009; Lyle et al., 2009; Olson et al., 2004a). Current data suggest a single critical region or critical gene is not sufficient to cause multiple characteristic DS phenotypes. Rather, specific genes within susceptibility regions may play important roles in the establishment and maintenance of specific, but not all, DS phenotypes.

3.1 Origins of the Down syndrome critical region hypothesis

The DSCR was originally hypothesized based on the assessment of a family with partial Hsa21 trisomy and the variable expression of several of the characteristic physical features of DS. A "critical region" within 21q22 was believed to be responsible for several DS phenotypes including craniofacial abnormalities, congenital heart defects of the endocardial cushions, clinodactyly of the fifth finger and mental retardation (Niebuhr, 1974). Soon after the DSCR hypothesis, genetic analyses of the Hsa21 DNA revealed several gene-rich segments located within 21q22 and placed further emphasis on this region as critical to the development of DS phenotypes (Brahe et al., 1990).

The hypothesis of a DSCR shifted the focus of many in the DS research community toward understanding how these genomic regions could correlate with the development of DS phenotypes. Regions linked to congenital heart defects (CHD), duodenal stenosis and craniofacial abnormalities in individuals with DS were identified through further analyses (Korenberg et al., 1992). An analysis of ten patients with partial trisomy for different segments of Hsa21 led to the mapping of 24 DS phenotypes to six chromosomal regions. Of these 24 phenotypes, 13 were mapped to 21g22.2 – proximal 21q22.3 region and six were linked to the *D21S55-MX1* region (Delabar et al., 1993). CHD was not included in the phenotypes associated with the D21S55-*MX1* region, and a later assessment of 19 individuals led to the mapping of CHD to a region outside of the D21S55-*MX1* region on 21q22.3 including *DSCAM,* a gene known to be involved in cell adhesion and expressed in the heart during development (Barlow et al., 2001a). A similar experiment comparing gene overlap regions with the phenotypic traits of 16 individuals with partial Ts21 along with the phenotypic variability observed in DS led to the hypothesis that factors in addition to trisomic genes both within and outside the DSCR likely contribute to most DS phenotypes, as opposed to a single critical region (Korenberg et al., 1994). Several additional genetic aspects including allelic heterogeneity, epistatic interactions, imprinting effects, uncharacterized epigenetic modifications and environmental events as well as the general variability observed in non-affected individuals were also suggested to have an impact on phenotypic variability (Korenberg et al., 1994).

The DSCR hypothesis, derived from analyses of the shortest region of overlap in individuals with partial Ts21, paved the way for the establishment of gene-phenotype relationships associated with DS. However, the high variability in phenotypic expression caused by an array of trisomic genetic consequences severely restricts the resolution of the gene-phenotype relationships associated with DS. The sequencing of Hsa21 as well as the development of several mouse models of DS provided further insight into the DSCR hypothesis.

3.2 Disproving the DSCR with mouse models

Unlike the analyses of partial trisomy in humans, comparisons of DS mouse models with differing segmental aneuploidies (both on their own and crossed between each other) has allowed for a systematic and meticulous analysis of the relationship between certain

chromosomal regions and DS phenotypes. The Ts1Rhr/Ms1Rhr mouse model was created by generating a 3.9 Mb reciprocal duplication/deletion containing 33 genes (*Cbr1-Orf9*) homologous to those found in the most commonly recognized putative human DSCR (Olson et al., 2004a). Human DS-like phenotypes documented in Ts65Dn and Ts1Cje mice, which contain larger segmental trisomies, and the creation of Ts1Rhr and Ms1Rhr mice, established a system suitable to test the DSCR hypothesis in mice. Ts65Dn mice are smaller in size with shorter femurs and exhibit craniofacial abnormalities including smaller skulls and mandible bones, as well as brachycephaly when compared to euploid littermates—defects also found in humans with DS and previously attributed to the DSCR (Delabar et al., 1993; Richtsmeier et al., 2000). Analysis of Ts1Rhr mice revealed significant increases in overall size, length of femur, and mandible size when compared to euploid littermates and an absence of brachycephaly, indicating major differences between Ts1Rhr and Ts65Dn or Ts1Cje phenotypes (Olson et al., 2004a). Mice generated from a cross between Ts65Dn and Ms1Rhr mice (removing triplication of the DSCR in the Ts65Dn mouse) exhibited craniofacial phenotypes similar to those observed in Ts65Dn mice (Olson et al., 2004a). Comparison of the craniofacial phenotypes observed in Ts1Rhr and Ts65Dn/Ms1Rhr mice indicated that the DSCR is not sufficient to produce these phenotypes in DS mouse models, but may still contribute in the genetic context of the phenotypes observed in Ts65Dn mice through genetic interactions.

In addition to the studies conducted on craniofacial skeletal abnormalities, DS-associated brain phenotypes have also been compared in DS mouse models. Ts65Dn mice exhibit a similar sized brain and hippocampus but smaller cerebellum and reductions in granule and Purkinje cell density when compared to euploid littermates (Baxter et al., 2000). Ts1Cje and Ms1Cje/Ts65Dn mice exhibit similar but less severe defects to those observed in Ts65Dn mice (Olson et al., 2004b). Analysis of Ts1Rhr mice revealed a similar sized brain and hippocampus when compared to euploid littermates, analogous to what was observed in Ts65Dn and Ts1Cje mouse models. The cerebellum of Ts1Rhr mice exhibited a small but significant reduction in size compared to euploid mice, but no differences were found in granule or Purkinje cell density, suggesting that trisomy for the DSCR is not sufficient to produce some brain phenotypes associated with DS (Olson et al., 2007).

Furthermore, studies have shown that Ts65Dn mice exhibit impairment of hippocampal function similar to that observed in individuals with DS (Holtzman et al., 1996; Reeves et al., 1995). Based on the DSCR hypothesis, genes within this region control the cognitive impairment phenotype associated with DS. If this were true, Ts1Rhr mice should exhibit similar deficits to Ts65Dn mice in hippocampal function. In contrast to Ts65Dn mice, Ts1Rhr exhibited normal hippocampal function in the Morris water maze test and normal induction of long term potentiation (LTP) in the CA1 suggesting the DSCR is not sufficient to cause the cognitive impairment associated with DS (Aldridge et al., 2007; Olson et al., 2007). Interestingly, when three copies of the DSCR were reduced to two, Ts65Dn/Ms1Rhr mice performed similarly to euploid animals in the water maze test suggesting that although the DSCR is not sufficient on its own to cause cognitive impairment, it is necessary for the phenotype to occur (Olson et al., 2007).

It is important to note that there are background differences between Ts65Dn, Ts1Cje, Ms1Rhr, and Ts1Rhr mice utilized in the studies described above. Inbreeding strategies for Ts65Dn and Ms1Rhr mice have not been successful, and these mice are maintained on an ~50% B6 and 50% C3H advanced intercross genetic background. The majority of studies previously

documented in Ts1Rhr mice used a mixed background of approximately 50% B6, 25% 129 and 25% C3H (except for the Morris water maze and LTP study where Ts1Rhr were on a B6 background) and it is possible that background differences may cloud direct comparisons between these mouse models. Behavioral and neurophysiologic phenotypes were assessed in Ts1Rhr mice maintained on a ~50% B6 and 50% C3H background to mimic the same genetic background as Ts65Dn mice. In contrast to what was observed in Ts1Rhr mice on other backgrounds, these mice exhibited several (20 of 48) similar neurologic phenotypes as Ts65Dn mice suggesting that the DSCR may be sufficient to confer some characteristic DS cognitive and brain abnormalities (Belichenko et al., 2009). The differences in neurological phenotypes in Ts1Rhr mice on different genetic backgrounds suggest the importance of understanding the complex genetic interactions associated with DS. Although evidence in mouse models implies that there is no single region critical to the majority of DS phenotypes, it is apparent that a gene or genes within this region are important in certain phenotypes and these phenotypes may be dependent on genetic background. Either allelic differences associated with trisomic genes or other two-copy genes in the genome may be responsible for the differences observed between trisomies with different genetic backgrounds.

3.3 New views on the DSCR and additional analyses in humans

Advancements in DNA technology as well as additional individuals identified with partial trisomy have led to more information on the hypothesized DSCR and gene-phenotype relationships in individuals with DS. A genotype-phenotype correlation analysis conducted on 30 individuals with either partial trisomy or partial monosomy for Hsa21 using array comparative genome hybridization found that four individuals with partial trisomy for the proximal portion of Hsa21 (not including the putative DSCR) exhibited several DS phenotypes (Lyle et al., 2009). Additionally, it was shown that there are multiple regions necessary to produce the cognitive impairment and hypotonia associated with DS, as well as a region previously identified as a candidate for CHD (*D21S3-PFKL*) (Barlow et al., 2001a). These new results suggest that the concept of one chromosomal region important for the majority of DS phenotypes does not exist due to multiple genetic regions critical for DS phenotypes. Due to the identification of multiple regions contributing to the same phenotypes, these regions may be more aptly termed "susceptibility" regions and may correspond to one or a limited number of phenotypes (Lyle et al., 2009). Individuals with triplication of these genes are more likely to exhibit the DS phenotypes associated with these regions.

High-resolution genetic mapping to determine gene involvement of eight specific DS phenotypes using 30 individuals with segmental trisomy for Hsa21 also concluded that specific genetic regions may be important for certain DS phenotypes. Genomic analysis revealed that the interaction of *DYRK1A* and *RCAN1*, thought to be involved in many DS phenotypes (Arron et al., 2006), were not essential in the development of CHD and mental retardation because individuals without trisomy for these genes exhibited severe abnormalities (Korbel et al., 2009). Additionally, several DS phenotypes, including transient myeloproliferative disorder, AMKL, and cognitive impairment, require triplication of multiple Hsa21 regions and genes (Korbel et al., 2009). A combination of data from this work and that in mouse models led to the specification of a CHD-causing region smaller than but included in a previously defined region for CHD (Barlow et al., 2001a). The newly defined region contains only 10 genes including *DSCAM*, a cell adhesion molecule highly expressed in the developing heart, also believed to contribute to the high levels of Hirschprung disease associated with DS (Korbel et al., 2009; Korbel et al., 2007).

Additional evidence against the synergy of *DYRK1A* and *RCAN1* in causing craniofacial and cardiac abnormalities associated with DS (Arron et al., 2006; Richtsmeier et al., 2000) came from a family with a 4.3 Mb duplication of chromosome 21q22 (including *DYRK1A* but not *RCAN1* or *DSCAM*). Individuals with the duplication presented with severe DS-like craniofacial abnormalities, but other malformations including cardiac defects were not observed. Based on these observations, the authors suggested two distinct regions important for these DS phenotypes with the distal region containing the genes including *DYRK1A* associated with craniofacial abnormalities and the proximal region (including *RCAN1* and *DSCAM*) associated with cardiac abnormalities (Ronan et al., 2007).

Interestingly, studies of individuals without DS provide evidence against the theory of only these distal and proximal specific regions associated with DS phenotypes. A patient with Silver-Russell syndrome, which has little similarity with DS, and his healthy father displayed a 0.46 Mb duplication of 21q22 including the *RCAN1* gene. No DS-associated phenotypes were documented in either individual suggesting that *RCAN1* alone is not sufficient to produce DS phenotypes (Eggermann et al., 2010). Furthermore, a child with a non-mosaic ring chromosome 21 duplication containing most of the long arm of Hsa21 including the hypothesized DSCR was found to have several characteristic DS phenotypes including cardiac and gastrointestinal defects but lacked the usual facial features associated with DS (Crombez et al., 2005).

Taken together, these studies provide evidence against the original DSCR hypothesis. It has been shown that genes and regions of genes located both within and outside of the hypothesized DSCR are critical to the initiation and severity of specific DS phenotypes. Studies conducted on humans with DS, DS mouse models, and other transgenic models have uncovered that several genes found within the putative DSCR are known to have a major effect on DS phenotypes. Thus, although the DSCR concept as traditionally defined does not seem to be correct, there are critical genes within this region that have a major impact on specific DS phenotypes.

3.4 Individual genes associated with the DSCR

Although it is evident the DSCR does not contribute to all of the phenotypes associated with DS, because the DSCR has been heavily studied, several genes found within this region have been implicated as candidate genes for individual DS phenotypes. The most extensively studied genes found within this region are Regulator of calcineurin1 (*RCAN1/DSCR1*) and dual specificity tyrosine-phosphorylation kinase 1a (*DYRK1A*), which are hypothesized to play important roles in several developmental pathways, including CNS, craniofacial skeletal and cardiac (Arron et al., 2006; Park et al., 2009; Richtsmeier et al., 2000). *DYRK1A* and *RCAN1* are both involved in the regulation of NFAT, a critical transcription factor necessary for the processes of vertebrate development and organogenesis (Graef et al., 2001). Transgenic *Nfatc* mutant mice exhibit several characteristics similar to DS mouse models as well as humans with DS including cognitive impairment and craniofacial and cardiac abnormalities. Mice with overexpression of *Dyrk1a* and *Rcan1* exhibited similar phenotypes to *Nfatc* mutants, suggesting that these genes are likely playing a part in the development of DS phenotypes (Arron et al., 2006).

In addition to *DYRK1A* and *RCAN1*, Down syndrome cell adhesion molecule (*DSCAM*) has been suggested to play a critical role in the developing brain and has also been identified as a candidate gene for the increased levels of CHD observed in DS individuals (Alves-Sampaio et al., 2010; Barlow et al., 2001a; Barlow et al., 2002). DSCAM is a critical factor in

neural differentiation, axon guidance, and the establishment of neural networks and it has been suggested that the disruption of these processes contributes to the DS neurocognitive phenotype (Agarwala et al., 2001; Barlow et al., 2001b; Yamakawa et al., 1998). A recent study found that *Dscam* was overexpressed in hippocampal neurons of the Ts1Cje mouse model. Overexpression of *Dscam* impaired dendritic branching leading to an inhibitory effect on synaptogenesis and neurite outgrowth, further implicating the role of *Dscam* in the development of the DS brain phenotype (Alves-Sampaio et al., 2010).
Also found within the DSCR, *ETS2* is a transcription factor known to be involved in the regulation of cellular proliferation, differentiation, transformation and apoptosis (Seth and Watson, 2005). Extensive studies on *Ets2* in transgenic and DS mouse models have revealed that the gene may play a role in the neuronal, tumor suppressive, and craniofacial phenotypes associated with DS (Hill et al., 2009; Sussan et al., 2008; Wolvetang et al., 2003). Additionally, *KCNJ6/GIRK2* overexpression in the hippocampus of Ts65Dn mice has been shown to cause an abnormal balance between inhibitory and excitatory synapses, implicating the gene in the DS-brain phenotype (Best et al., 2007). Additionally, this overexpression was also implicated in the reduced cerebellar size and alterations in granule cell neuron differentiation observed in the weaver mouse (Patil et al., 1995).

3.5 Gene(s)-phenotype relationships

The establishment of a specific phenotype requires the successful coordination of a number of genetic interactions. In many human disorders a single gene or gene network is responsible for causing the associated phenotypes. However, evidence from both humans and mouse models of DS suggests that specific DS phenotypes are influenced by genetic aberrations in multiple genes as opposed to a single gene. The most glaring case of multiple genes affecting a phenotype comes from the study of cognitive impairment through the analysis of human and mouse DS brains. *DYRK1A, RCAN1,* and *DSCAM* have all been shown to regulate the stages of neuronal cell maturation (proliferation, differentiation, and apoptosis) in the developing brain (Agarwala et al., 2001; Barlow et al., 2001b; Park et al., 2010; Sun et al., 2011). Triplication of *Olig1*, *Olig2*, and *Kcnj6,* leads to significant changes in the ratio of inhibitory to excitatory neurons in the Ts65Dn forebrain (Best et al., 2007; Chakrabarti et al., 2010) and *DSCAM* regulates dendritic branching and neuronal network establishment (Alves-Sampaio et al., 2010), suggesting that increased inhibitory activity and the inability to form neuronal networks are also contributing to the cognitive impairment phenotype. In addition to cognitive impairment, an AD-like phenotype is apparent in most adult individuals with DS. Dysregulation of *DYRK1A, RCAN1,* and/or *Ets2* lead to an increase in the number of neurofibrillary tangles and β-amyloid plaques in the brains of humans and mice (Ermak et al., 2001; Ryoo et al., 2008; Sun et al., 2011), respectively, indicating that multiple genes are contributing to the DS-AD phenotype.

3.6 Summary

Based on thorough analyses of studies on humans and DS mouse models, it is evident that there is not a single critical region of genes sufficient to cause all DS phenotypes. Alternatively, it is likely that there are multiple critical regions or critical genes contributing to a respective phenotype or group of phenotypes associated with DS (Lyle et al., 2009). Although studies conducted regarding the DSCR have provided a wealth of evidence refuting the idea, it is important to understand that several genes within this region have

been identified as key contributors to more specific DS phenotypes. Furthermore, both non-trisomic genes and trisomic genes located outside of the hypothesized DSCR have also been implicated in the development of specific DS phenotypes and in some cases may be linked with DSCR associated genes. It is evident that DS phenotypes are influenced by a multitude of complex genetic interactions and it seems likely that multiple genes and gene networks will be involved in the development of most DS phenotypes. Important genes or regions of genes contributing to specific DS phenotypes should be defined as susceptibility genes or regions, as opposed to defining a single Down syndrome critical region or single gene-phenotype relationship (Lyle et al., 2009).

4. Functional analysis of genes

4.1 Introduction

Although hypotheses have been developed concerning cellular and developmental mechanisms relating to DS phenotypes, no conclusive evidence exists for a single mechanism likely responsible for the majority of DS phenotypes. Instead, multiple common mechanisms may be responsible for individual phenotypes in specific tissues or at precise developmental stages and groups of genes with related functions may also be dysregulated in DS pathology. Functional databases provide the foundation for elucidating gene-phenotype relationships by clustering dysregulated genes with common functions. These clusters identify potential cellular, developmental and biological functions, as well as the number of genes in each cluster and significance of those categories. Depending on the functional annotation tool, relevant pathways, molecules for potential pharmacological interventions or insight into genetic mechanisms may be suggested. The use of functional databases extends the value of high throughput arrays and tests hypotheses that specific groups of genes with related function may be dysregulated in DS. Rather than a simple dosage increase of Hsa21 genes, it has been hypothesized that trisomy has a more global dysregulational effect on the genome, though developmental changes and tissue type do maintain a significant role (Altug-Teber et al., 2007; Dauphinot et al., 2005; Sommer et al., 2008). We hypothesize that the interaction between trisomic and disomic genes have a significant effect on the way DS phenotypes arise, manifest and progress.

Several studies have documented differential gene expression in unique spatial and developmental environments to determine mechanisms affecting DS phenotypes (Conti et al., 2007; Lyle et al., 2004; Moldrich et al., 2007). Other studies often concentrate on the cellular or developmental mechanisms as a causative factor of DS phenotypes (Chakrabarti et al., 2007; Contestabile et al., 2009; Cooper et al., 2001; Roper et al., 2006; Roper et al., 2009). High throughput analyses are important for investigating gene-phenotype relationships in analyses of various tissue and cell types from individuals with DS, mouse models of DS, and cell lines previously derived from individuals with DS or engineered to contain this extra genetic material. We hypothesize that a single generalized pathway or mechanism does not underlie the phenotypes of DS, but rather several pathways and mechanisms contribute to the phenotypes of DS, though some functional groups may cluster together in certain tissues or within phenotypes. Assessment of the gene-phenotype relationships in high throughput meta-analysis provides novel information regarding the importance of developmental processes in the DS pathophysiology.

4.2 Previously established functional analyses

Studies of DS have utilized high throughput analysis on several tissues including cultured DS neural progenitor cells and tissue samples from DS amniocytes, hearts, cerebra and cerebella and cultured Ts1Cje neural progenitor cells (NPCs). Though differences are present between developmental time points and tissues, genes involved with cellular cycling, cell adhesion, signal transduction, DNA and RNA metabolism and binding, gene expression regulation and transcription, mitochondrial function and oxidative phosphorylation, kinase activity, and ECM production and maintenance were the most highly dysregulated and common categories observed, as well as maintain the ability to transcend time and tissue differences (Table 5). More specifically, certain cell types of representative models of DS or from individuals with DS share multiple common annotation results. We suggest a similar phenomenon may regulate multiple DS phenotypes in which dysregulated mechanisms affect the same tissue in order to produce a phenotype (Figure 1).

4.2.1 Cell cycle alterations contribute to neurological phenotypes

NPCs display altered gene expression related to cell cycling, proliferation, signaling, transcription and metabolism of chromosomal material. It is well established that deficits in proliferation and mitotic activity of specific cellular populations in the DS brain exist, including areas of the cerebellum and multiple areas of the cerebrum (Baxter et al., 2000; Chakrabarti et al., 2007; Gardiner et al., 2010; Roper et al., 2006). Additionally, impairment of proliferation in the cerebellum of fetuses with DS has also been reported (Guidi et al., 2010) as well as in the Ts65Dn neonate peripheral tissues and fibroblasts, suggesting a general deficit in proliferation as a mechanism for multiple DS abnormalities (Contestabile et al., 2009). Individuals born with DS have reduced brain weights coupled with a smaller, dysmorphic skull and multiple cellular abnormalities within the brain including reduction in the number of neurons in the cerebral cortex as well as cellular deficiencies in multiple other structures (Aylward et al., 1997; Fink et al., 1975; Wisniewski, 1990). Therefore, these phenotypic deficits may be caused by a similar mechanism. However, downregulation of genes with proliferation-promoting function or upregulation of genes involved in the arrest of proliferation could equally, if not synergistically, contribute to the general proliferation deficit hypothesized to occur in several DS phenotypes. Overexpression of *Dyrk1a* in mouse NPCs was recently found to inhibit proliferation and stimulate precocious neuronal differentiation (Park et al., 2010; Yabut et al., 2010). *DYRK1A* has previously been implicated in the physiopathology of the cognitive impairment observed in individuals with DS (Altafaj et al., 2001; Smith et al., 1997) and is overexpressed approximately 1.5 fold in the DS and Ts65Dn brain (Dowjat et al., 2007; Guimera et al., 1999), suggesting upregulation of *DYRK1A* in concert with other Hsa21 and disomic genes in the DS brain may lead to deficits that underlie both cognitive phenotypes and other cellular phenotypes of DS.

4.2.2 Changes in cell adhesion contribute to DS phenotypes

In addition to brain, other tissues appear to be affected by alterations in cell homeostasis. Because cell cycling, cell adhesion, signal transduction, and ECM production all display dysregulation in DS fetal amniocytes, heart, cerebra and cerebella, one cannot conclude that a simple deficit in proliferation, such as that observed in DS skin fibroblasts (Kimura et al., 2005), is sufficient to result in the DS phenotypes observed. While the DS brain is reduced in

size overall, it is also dysmorphic, pointing to both a decrease in proliferation, but perhaps also altered cell cycling, changes in the migratory pattern of progenitor cells and even the decreased founder population of these cells due to decreases in neurogenesis, implicating a complex interaction of mechanisms in the DS neurological phenotype (Bhattacharyya et al., 2009; Guidi et al., 2010).

Study	Tissue	Time point	Analysis Tool	RNA/DNA metabolism	Cell cycling	Cell adhesion	Cell fate	Transcription	Oxidative phosphorylation	Mitochondria	Cell Proliferation	Apoptosis	Regulation of gene expression	RNA/DNA binding	Signal Transduction	Cytoskeleton organization	Monovalent cation transport	Collagen	Integral to plasma membrane	Other annotations of interest
(Mao et al., 2005)	DS cerebrum		G					+		+				+			+			Monovalent cation transport
(Mao et al., 2005)	DS cerebellum		G	+										+						Nerve impulses
(Mao et al., 2005)	DS astrocytes		G	+		+												+	+	
(Mao et al., 2005)	DS heart		G	+						+							+		+	Cell-cell signalling
(Conti et al., 2007)	DS heart	18-22 wks	G			22			9	48								8		ATP synthesis, ECM, phosphate transport
(Lockstone et al., 2007)	DS dorsolateral prefrontal cortex		O		16	3	3	5			9	7	6			6				Immune system, Notch and tryrosine signalling, cell migration, endocytosis
(Rozovski et al., 2007)	DS trophoblasts	Fetal	N	4					6											Ubiquitine cycle, purine biosynthesis
(Chou et al., 2008)	DS amniocytes	16-22 wks	G			+		+							+					
(Esposito et al., 2008)	DS NPCs	19-21 wks	I		25								20		25					Molecular transport
(Sommer et al., 2008)	DS lymphocytes	1-4 yrs	D	12	5				14							12				Protein synthesis, kinase binding, antigen presentation
(Bhattacharyya et al., 2009)	DS NPCs		G		131		131				131	81								Neurogenesis
(Moldrich et al., 2009)	Ts1Cje NPCs	E14.5	G				75													Development, homeostasis

Table 5. Published functional analysis data for phenotypes of DS. Functional annotations were selected by lowest p-values for the categories established by the investigators and of specific interest to the authors. Studies are listed by tissue type and annotations are listed by most common to least common categories horizontally. Analysis tool abbreviations: G: GeneOntology (www.geneontology.org), I: Ingenuity Pathway Analysis (www.ingenuity.com), O: OntoExpress (vortex.cs.wayne.edu/projects.htm), N: NetAffex (www.affymetrix.com/analysis/index.affx), D: DAVID (david.abcc.ncifcrf.gov).

In addition, approximately 50% of newborns with DS display a congenital heart defect, most of which involve septal and canal defects (Pediatrics, 2001). Previous studies have

established a link between DS atrioventricular septal defects (AVSDs) and the importance of collagen VI in developing endocardial cushions (Baptista et al., 2000; Davies et al., 1995). Characterization of the involvement of collagen VI in cardiac development was later established when investigators observed altered collagen VI expression in AV endocardial cushions in the DS heart (Gittenberger-de Groot et al., 2003). Interestingly, genes encoding collagen VI are found on Hsa21 and its role as an extracellular matrix (ECM) component makes its presence ubiquitous throughout the body (Aumailley et al., 1991). A similar idea has recently been proposed in which atrioventricular canal (AVC) defects, which comprise the majority of congenital heart defects observed in DS, are a result of increased cell adhesion. Deficits in cell adhesion may lead to decreased cell migration to the ventricular canal because of changes in the ECM, leading to alterations in epithelial-mesenchymal transformations (Delom et al., 2009). Using a transchromosomic model of DS, investigators found both decreased cell migration and an increased affinity for adhesion to collagen VI (Delom et al., 2009). Importantly, differences in Hsa21 genes or groups of genes causing aberrations in both cellular properties may act in concert to lead to the development of AVC defects. It is therefore plausible that cell adhesion complexes coupled with ECM component alterations significantly contribute to congenital heart abnormalities in DS.

In addition to congenital heart defects, individuals with DS are at an increased risk for dermatological disorders such as atopic dermatitis, causing susceptibility toward opportunistic infections (Madan et al., 2006). Studies utilizing DS skin fibroblasts have shown that these cells display an increased adhesion to collagen VI, likely leading to aberrant migration of these cells to target areas of the body (Jongewaard et al., 2002). This decreased migration would leave a smaller population of cells to make up an epidermal barrier and thus would compromise the integrity of the skin. Dysregulation of genes involved in cell adhesion may also lead to changes in junctional complexes that are found to be altered in functional analyses, and these changes may contribute to atypical migration of the cells with which they interact.

4.2.3 Changes in DNA and RNA metabolism may lead to transcription and translation dysregulation

Genes involved with the metabolism and expression of nucleic acids are highly dysregulated in multiple DS tissue types (brain, heart, trophoblasts) and time points in multiple functional analyses. The observed dysregulation of the genome due to the presence of the extra chromosomal material and its interaction with the rest of the genome is likely directed by multiple types of regulation. It may be that fewer transcripts are formed given a higher rate of metabolism of genetic material. The reduced transcripts directing cellular proliferation, migration or an anti-apoptotic state could lead to the smaller or altered structures observed in DS.

In particular, changes in metabolism of genetic transcripts have been documented in the expression of multiple genes in both DS-related and unrelated tissues. Several studies sampling multiple DS-derived tissues have described the transcriptional alterations of Hsa21 genes, including two genes involved in nucleic acid metabolism: GART (purine metabolism) and ZNF294 (transcriptional regulation) (Lintas et al., 2010). DNA and RNA binding and metabolism may also be related to transcription factor activity, dysregulating genes downstream of these alterations. Alterations of transcription factors such as NFAT, as a result of dysregulation of *DYRK1A* and *DSCR1*, have the potential to lead to DS developmental phenotypes (Arron et al., 2006). The dysregulation of NFAT may initiate a

cascade of events affecting multiple downstream targets. Many of these genes may also be involved with cell cycling and maintenance as well as DNA and RNA binding and metabolism. Such studies, however, remain to be performed.

4.2.4 Mitochondrial changes penetrate neurological and metabolic phenotypes

The high incidence of mitochondria-related dysregulation in DS hearts, lymphocytes, and trophoblasts coupled with the increased prevalence of biomarkers of reactive oxygen species (ROS) in individuals with DS (Jovanovic et al., 1998) is an interesting and well-studied paradigm. Mitochondrial impairment in fetal hearts suggested by the downregulation of genes from five mitochondrial complexes, as well as the cerebellum and other brain regions, platelets, astrocytes and cultured fibroblasts suggests pervasive impairment of mitochondria in individuals with DS (Arbuzova et al., 2002; Busciglio et al., 2002; Conti et al., 2007; Kim et al., 2001; Kim et al., 2000; Prince et al., 1994). Interestingly, this mitochondrial impairment may affect other DS pathophysiology. Dysregulation of the mitochondria has the ability to cause alterations in ATP synthesis, electron transport, monovalent cation transporter activity, oxidative phosphorylation, and general changes in physical components (Conti et al., 2007) (all found as dysregulated functional groups in Table 5). Furthermore, changes in membrane potential caused by this dysregulation may lead to increased mitochondrial susceptibility to other insults, leading to a constant feedback in the balance of genetic regulation (Roat et al., 2007). Two genes in particular located on Hsa21, *SOD1* and *BACH1*, have been implicated the generation of ROS due to elevated H_2O_2 levels and decreased transcriptional activity, respectively. Elevated levels of *SOD1* have been implicated in deposition of β amyloid and *BACH1* downregulation has been proposed as a contributing mechanism to the development of Alzheimer disease neuropathology (Percy et al., 1990; Shim et al., 2003). High levels of oxidative stress in the brains of individuals with DS may indicate that dysregulation of normal processes in the mitochondria lead to anomalies including Alzheimer pathology (de Haan et al., 1997; Lockstone et al., 2007). A disproportionate number of ROS, caused by mitochondrial and other changes, combined with altered metabolism and feedback mechanisms suggest the complexity of the interacting mechanisms involved in DS phenotypes affecting the brain, heart and skin (Busciglio and Yankner, 1995; Li et al., 2006; Sinha, 2005).

4.3 DAVID analysis of trisomic genes in humans with DS and DS mouse models

DS mouse models have the ability to predict phenotypes not previously observed in individuals with DS (Baxter et al., 2000; Pennington et al., 2003). We hypothesized that by analyzing the genes trisomic genes on Hsa21 as well as their homologs in two DS mouse models, we would observe common mechanisms as found by functional analysis of altered gene expression. Using DAVID (http://david.abcc.ncifcrf.gov/) we analyzed trisomic genes from Hsa21 and the Ts1Rhr and Ts65Dn mouse models (http://chr21.molgen.mpg.de/HSA21db.html). Functional annotations were obtained using the protocol described by Huang et al. 2009.

Our analyses of Hsa21 genes revealed dysregulation of functional annotations including keratin, intermediate filament/cytoskeleton digestion, protein dimerization, interferon receptor activity, cytokine receptor activity, multiple junctions, cell adhesion, ionic channels and ECM (Table 6). Analysis of the genes triplicated in Ts65Dn mice revealed dysregulation of genes involved in ion transport, voltage-gated channels, ionic channels, monovalent inorganic cation transport, tight junctions, calcium dependent cell-cell adhesion, regulation

of transcription, DNA binding, behavior, neuron development, neuron differentiation and synaptic transmission and cell-cell signaling. Analysis of genes in three copies from the Ts1Rhr mouse model identified functions integral to binding and related to regulation of transcription factor activity, transcription and DNA binding as the most enriched categories with the lowest p-values.

As predicted, some, but not all functional groups found in previous analyses were also seen in our DAVID analysis. Data compiled using DAVID analysis of Hsa21 and two DS mouse models provide insight into what mechanisms appear to be generally dysregulated when trisomy for these regions occurs. Ts65Dn mice display many similarities to individuals with DS with regard to general brain, behavior, stature, heart and craniofacial phenotypes. Interestingly, a high level of overlap in functional categories also exists between the two analyses. For example, our analysis of Hsa21 and Ts65Dn revealed dysregulation of genes involved in cell adhesion, tight junctions and ionic transport. Ts65Dn mice replicate some of the heart defects observed in DS, and aberrant cell adhesion and migration have been implicated in these defects (Delom et al., 2009; Moore, 2006; Williams et al., 2008). Ts1Rhr and Ts65Dn triplicated genes led to functional hits in transcription, transcription factor activity, and RNA/DNA binding. While Ts1Rhr and Ts65Dn mice display some similar phenotypes, Ts1Rhr mice exhibit a number of phenotypes contrary to those seen in DS. It is noteworthy, though, that though these functional groups are dysregulated, they may be regulated in a manner divergent from one another, such as higher levels of transcription and RNA/DNA binding in Ts1Rhr than Ts65Dn, leading to the exaggerated phenotypes which Ts1Rhr mice display. Interestingly, no functional groups overlapped between the Hsa21 and Ts1Rhr analyses and this result provides additional evidence for the different phenotypes observed between humans with DS and this mouse model. Clearly, the involvement of other trisomic genes as well as disomic genes plays an important role in the identification of functional categories. Given that our analyses only included trisomic genes, it seems plausible that significant overlap between the past and current studies was not found.

Study	RNA/DNA binding	Cell adhesion	Transcription	Transcription factor activity	Tight Junctions	Ionic transport	Voltage-gated channel	Cell junctions	Cell signaling	Apical Junction Complex	Intermediate Filaments	Protein dimerization activity	ECM	
Hsa21 Genes		13			6	8		7		6	27	11	6	Keratin, digestion, interferon receptor activity, cytokine receptor activity
Ts65Dn mouse model	7	4	7	6	5	8	5		3					Behavior, neuron development, neuron differentiation, synaptic transmission
Ts1Rhr mouse model	3		4	3										Integral to membrane

Table 6. DAVID analysis of trisomic genes from Hsa21 and Ts65Dn and Ts1Rhr mice. Functional annotations are listed in columns by most common dysregulated annotations, then by highest to lowest enrichment score and smallest to largest p-value in succession for each category.

4.4 Summary

Functional analyses of genetic information for DS have the potential to revolutionize our understanding about the gene-phenotype relationships in DS (Ait Yahya-Graison et al., 2007; Gardiner, 2010). Our meta-analysis of current functional analyses has indicated that cell cycling, maintenance, function, and adhesion, as well as DNA and RNA metabolism and binding and mitochondrial function are leading common mechanisms underlying DS phenotypes. The combination of multiple genes to produce specific disorder-related phenotypes appears to be a common theme in DS phenotypes. Groups of genes may act in concert to produce one or more phenotypes, or contribute a key factor in the development or maintenance of a phenotype. In many cases, functional groups and mechanisms overlap between tissues of different origins, but no general mechanism appears to be ubiquitously dysregulated amongst the tissues studied (Figure 1).

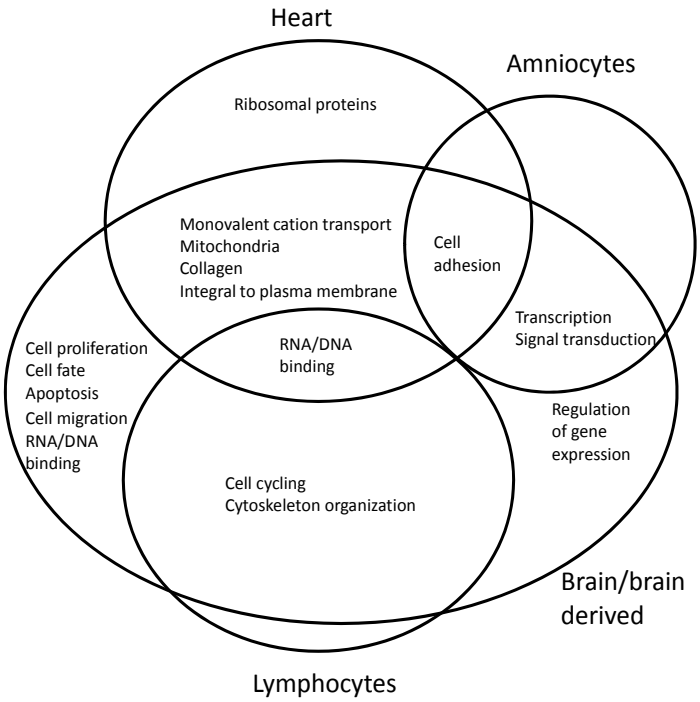

Fig. 1. Overlap of dysregulated functional groups occurs between multiple tissues, but no single mechanism appears to be dysregulated among all tissues studied.

5. Conclusions

Although the initial genetic insult is known and the phenotypes related to DS have been characterized, the relationship between genes and phenotypes has not been well distinguished for most DS phenotypes. Though phenotypes associated with DS must be caused by Ts21, high throughput gene expression analyses demonstrate the dysregulation of both trisomic and non-trisomic genes in tissues that are important for traits associated with Ts21. Hypotheses concerning critical regions and genes, thought to be important in most of

the major phenotypes associated with DS, have been disproved. Instead, it is postulated that a region of Hsa21 may be critical for a specific phenotype and a genomic region may contain a gene that is important (but not exclusive) for the causation of the trait. Moreover, there may be singular genes that are important in many, but not all, phenotypes associated with DS. Functional analyses of differentially expressed genes and genes in three copies may be used to further understand the relationships between genes and phenotypes. A number of differentially expressed genes may be tied into a mechanism and there may be mechanisms that are critical to a number of phenotypes. Thus, more accurate genotyping, large scale gene expression meta-analysis and functional mechanism investigations are helping to define gene-phenotype relationships in this complex and interactive disorder.

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5

Abnormal Folate Metabolism and Maternal Risk for Down Syndrome

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1. Introduction

Down syndrome (DS) or trisomy 21 (MIM 190685) is the most common genetic disorder with a prevalence of 1 in 660 live births (Jones, 2006). DS is the leading cause of genetically-defined intellectual disability (Contestabile et al., 2010) and its phenotype is complex and variable among individuals, who may present with a combination of dysmorphic features (Ahmed et al., 2005; Pavarino-Bertelli et al., 2009), congenital heart disease (Abbag, 2006), neurological abnormalities such as early manifestations of Alzheimer's disease (Lott & Head, 2005), immunological impairments (Ram & Chinen, 2011), elevated risk of specific types of leukemia (Hasle et al., 2000), and other clinical complications (Venail et al., 2004).
Trisomy 21 can be caused by three types of chromosomal abnormalities: free trisomy, translocation, or mosaicism. Mosaicism accounts for the minority of DS cases (about 1%) and is characterized by some cells containing 46 chromosomes and others, 47 chromosomes. Translocations are attributed to 3-4% of the cases, with Robertsonian translocation involving chromosomes 14 and 21 being the most common type. Finally, free trisomy occurs in about 95% of cases (Ahmed et al., 2005; J.M. Biselli et al., 2008b) and is characterized by the presence of three complete copies of chromosome 21.
Free trisomy, the main chromosomal abnormality leading to DS, is caused by the failure of normal chromosome 21 segregation during meiosis (meiotic nondisjunction) (Hassold & Hunt, 2000). The parental origin of the extra chromosome 21 is maternal in about 80% of cases (Jyothy et al., 2001), and most (about 77%) occur during the first maternal meiotic division in the maturing oocyte, before conception (Antonarakis et al., 1992).

2. Meiosis and chromosomal segregation

Faithful transmission of a genome from one generation to another depends on the mechanism of cell division in which each pair of replicated chromosomes is separated and equally distributed to mother and daughter cells. Meiosis generates haploid gametes through a specialized cell division process that consists of one round of DNA replication followed by two cell divisions. The first division, meiosis I (MI), involves the segregation of

homologous chromosomes from each other, whereas meiosis II (MII) involves the segregation of the sister chromatids (Hassold & Hunt, 2000).

Timing of chromosome attachment and loss of cohesion is essential to faithful chromosome segregation. During MI, the cohesion between sister chromatid arms assures physical attachment by the chiasmata of homologous chromosomes, ensuring their alignment on the meiosis-I spindle, and maintains them at the site of recombination. Chiasmata are resolved at anaphase I by the loss of cohesion between the arms of sister chromatids in the homologous chromosomes; the chromosomes then segregate to opposite poles of the cell. Cohesion, however, must be maintained at centromeres between sister chromatids beyond meiosis I to prevent premature chromatid separation (predivision) and ensure proper attachment of the sister chromatids to opposite spindle poles in meiosis II (Barbero, 2011; Sakuno & Watanabe, 2009; Vogt et al., 2008).

The centromeric cohesion during meiosis I results from the attachment of kinetochores of sister chromatids to only one spindle pole (Sakuno & Watanabe, 2009). Kinetochores are situated on opposite sides of the centromeric heterochromatin at the centromeres of each sister chromatid and they capture and stabilize microtubules for the formation of kinetochore fibers, only then they are capable of chromosome bi-orientation during the metaphase and chromosome segregation during the anaphase of meiosis (Vogt et al., 2008).

During cell division, several chromosomal mal-segregation mechanisms can occur. Classical nondisjunction is due to the failure to resolve chiasmata between homologous chromosomes, whereby both homologues segregate together. In addition, premature resolution of chiasmata or the failure to establish a chiasma between a pair of homologues results in the independent segregation of homologues at MI, which leads to an error if both segregate to the same pole of the MI spindle. A MI error can also involve the segregation of sister chromatids, rather than homologous chromosomes, whereby the premature separation of sister chromatids at MI can result in the segregation of a whole chromosome and a single chromatid to one of the poles. At MII, errors result from the failure of sister chromatid separation (Hassold & Hunt, 2000).

3. The origin of maternal chromosome 21 nondisjunction

The molecular mechanisms involved in meiotic nondisjunction leading to trisomy 21 are still poorly understood and the only well-established risk factor for DS is advanced maternal age at conception (35 years or older) (Allen et al., 2009; Jyothy et al., 2001; Lamb et al., 2005). Studies have suggested many explanations for the maternal age-associated increase in aneuploidy. One model attributes the effect of advanced maternal age to the uterine environment, indicating that there might be an age-related decline in the ability to recognize and then abort trisomic fetuses (Aymé & Lippman-Hand, 1982; Stein et al., 1986). However, the observation that the advanced maternal age effect is restricted to chromosome 21 nondisjunction of maternal origin, but not associated with cases resulting from sperm or post-zygotic mitotic errors, suggests that the uterus is the source of the age effect (Allen et al., 2009).

On the other hand, Zheng & Byers (1993) proposed that age-dependent trisomy 21 results primarily from a mechanism that favors maturation and utilization of euploid oocytes over the pre-existing aneuploid products of mitotic (premeiotic) nondisjunction at an early stage of the reproductive lifespan. In addition, decreased expression of checkpoint proteins in aging oocytes (Vogt et al., 2008) and failure to effectively replace cohesion proteins that are lost from chromosomes during aging (Chiang et al., 2010) also are pointed out as risk factors for predisposing oocytes to errors in chromosome segregation.

A link between altered recombination and maternal age-related nondisjunction has been described. It was observed that recombination is reduced among nondisjoined chromosomes 21 at MI, and this reduction seems to be age-related (Sherman et al., 1994). Lamb et al. (1996) proposed that at least two "hits" are required for chromosome 21 nondisjuntion: (1) the establishment in the fetal ovary of a susceptible pattern of meiotic recombination, and (2) the abnormal processing of susceptible chromosomes in the adult ovary. The second "hit" would involve degradation of a meiotic process (e.g., a spindle component, a sister chromatid cohesion protein, a meiotic motor protein, a checkpoint control protein) that increases the risk of improper segregation for these susceptible bivalents (Hassold & Sherman, 2000). Further studies have shown susceptible patterns of chromosome 21 meiotic recombination, including pericentromeric and telomeric exchanges, described as maternal risk factors for DS even in young DS mothers (Gosh et al., 2009; Lamb et al., 2005).

Besides advanced maternal age, the age of the maternal grandmother at the time of birth of the mother has also been pointed out as a risk factor for the occurrence of DS. At an advanced age, the grandmother's reproductive system may fail to make the essential proteins needed for proper meiotic segregation in the germ cells of her daughter, leading to nondisjunction of chromosome 21 during the embryogenesis of DS child's mother when she was in the grandmother's womb (Malini & Ramachandra, 2006). However, more recent studies failed to support the suggestion that advanced age of the DS grandmother is responsible for meiotic disturbances in her daughter (Allen et al., 2009; Kovaleva et al., 2010).

Although the risk of bearing a child with DS increases substantially with increasing maternal age, many DS children are born to mothers aged less than 35 years-old, suggesting other risk factors influencing DS etiology. In 1999, James et al. produced the first evidence that the occurrence of DS independent of maternal age is associated with DNA hypomethylation due to impairments in folate metabolism.

4. Folate metabolism

Folate represents an essential nutrition component in the human diet, and is involved in many metabolic pathways, mainly the folate metabolism, i.e., a single-carbon transfer from one molecule to another through a series of interconnected biochemical reactions. Folate is a generic term for a family of compounds present in most foods, e.g., legumes, leafy greens, some fruits, vegetables (e.g., spinach, broccoli, asparagus, and lettuce), liver, milk, and dairy products (Lin & Young, 2000). Humans, as all mammals, are unable to synthesize folate, thus its ingestion, either from normal diet or nutritional supplements, is very important. After intestinal absorption, natural folate, known as polyglutamate, requires reduction into monoglutamate by conjugases in the small intestine before it can be absorbed. On the other hand, in its synthetic form, folic acid exists as monoglutamate and does not need to be reduced for release into the blood and cellular uptake (Bailey & Gregory, 1999; Hall & Solehdin, 1998). Another disadvantage of natural food folate is its poor stability especially under typical cooking conditions, which can substantially reduce the vitamin content before it is even ingested, a significant additional factor limiting the ability of natural food folates to enhance folate status (McNulty & Pentieva, 2004; McNulty & Scott, 2008).

Folate metabolism is a complex metabolic pathway that involves multiple enzymes and water-soluble B vitamins such as folate, vitamin B_6 and vitamin B_{12}, that play key roles

as enzyme cofactors or substrates in this metabolism. It includes two main cycles: purine and pyrimidine synthesis, necessary for synthesis and repair of DNA, and DNA methylation, an epigenetic process that acts on the control associated with gene expression and genomic stability essential for normal cellular methylation reactions (Figure 1).

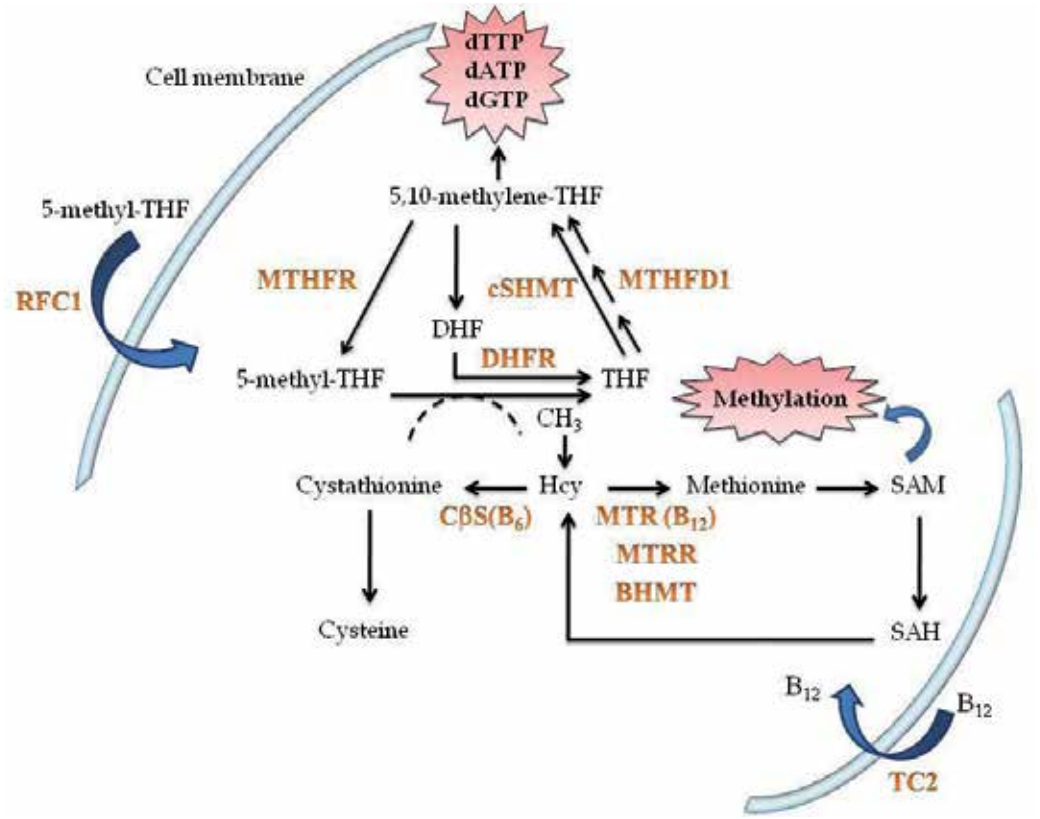

Fig. 1. Folate metabolism. BHMT = Betaine-homocysteine methyltransferase; B_6 = vitamin B6; B_{12} = vitamin B12; CβS = Cystathionine β- synthase; CH_3 = Methyl; dATP = Deoxyadenosine 5'-triphosphate; dGTP = Deoxyguanosine 5'-triphosphate; dTTP = Deoxythymidine 5'-triphosphate; DHF = Dihydrofolate; DHFR = Dihydrofolate reductase; Hcy = Homocysteine; MTHFD1 = Methylenetetrahydrofolate dehydrogenase 1; MTHFR = Methylenetetrahydrofolate reductase; MTR = Methionine synthase; MTRR = Methionine synthase reductase; RFC1 = Reduced folate carrier 1; SAH = S-adenosylhomocysteine; SAM = S- adenosylmethionine; cSHMT= Serine hydroxymethyltransferase; TC2 = Transcobalamin 2; THF = Tetrahydrofolate.

Folate requires several transport systems to enter the cells and the one best characterized is the reduced folate carrier (RFC1), an enzyme located on intestinal cell membranes that carries out the transport of 5-methyltetrahydrofolate (5-methyl-THF) to the interior of a variety of cells, representing an important determinant of folate concentration in the interior of cells (Nguyen et al., 1997). In addition to the folate transport system, several genes and their respective enzymes play important roles in folate metabolism. The D*ihydrofolate reductase* (*DHFR*) gene encodes an enzyme that catalyzes the conversion of dihydrofolate

(DHF) into tetrahydrofolate (THF) (Stanisiawska-Sachadyn et al., 2008), which is then converted into the corresponding 10-formyl, 5,10-methenyl, and 5,10-methylene derivatives by Methylenetetrahydrofolate dehydrogenase 1 (MTHFD1), a trifunctional nicotinamide adenine dinucleotide phosphate-dependent cytoplasmic enzyme. The donor cofactors for *de novo* purine and pyrimidine biosynthesis and, thus, the biosynthesis of DNA (Hum, 1988) are 10-formyl-THF and 5,10-methylene-THF. By an alternative route, THF is converted into 5,10-methylene-THF and glycine by the cytosolic form of the enzyme Serine hydroxymethyltransferase (cSHMT) (Steck et al., 2008).

Methylenetetrahydrofolate reductase (MTHFR) is responsible for the conversion of 5,10-methylene-THF to 5-methyl-THF, the main circulating form of folate that donates methyl groups for homocysteine (Hcy) remethylation into methionine. This latter reaction is catalyzed by the enzyme Methionine synthase (MTR), which requires vitamin B_{12} or cobalamin (Cbl) as a cofactor, and results in the formation of S-adenosylmethionine (SAM), the primary methyl (CH_3) donor for DNA methylation reactions (Finkelstein & Martin, 2000). SAM is demethylated to form S-adenosylhomocysteine (SAH) and then hydrolyzed to form adenine and Hcy. The DNA methyltransferase (DNMTs) enzymes catalyze the transfer of the methyl group, obtained from conversion of SAM into SAH, to position 5′ of cytosine residues located mainly in dinucleotide cytosine-guanine (CpG) (Bestor, 2000; DeAngelis et al., 2008).

Methionine synthase reductase (MTRR), an enzyme codified by the *MTRR* gene, is responsible for the maintenance of the active form of the enzyme MTR. During remethylation of Hcy to methionine, a reaction catalyzed by MTR, methylcob(III)alamine acts as a methyl donor. In this reaction, the transfer of a methyl group from methylcob(III)alamine results in the formation of highly reactive cob(I)alamine, which is oxidized into cob(II)alamine, resulting in MTR inactivation (Yamada et al., 2006). In this inactivation process, a complex is formed between the enzymes MTR and MTRR, and derivative electrons from the oxidation of nicotinamide adenine dinucleotide phosphate (NADPH), catalyzed by MTR, are transferred to the inactive form of MTR. This process favors the transfer of methyl from the SAM to the MTR enzyme, resulting in methylcob(III)alamine, thus reestablishing MTR activity (Leclerc et al., 1999; Olteanu et al., 2001, 2002).

Betaine-homocysteine methyltransferase (BHMT) catalyses the conversion of Hcy to methionine by an alternative pathway of remethylation using the amino acid bethaine as methyl donor. When the Hcy folate-dependent remethylation catalyzed by the MTR enzyme is impaired by genetics or environmental factors, the BHMT enzyme plays an important role maintaining the homeostasis of Hcy (Pajares & Pérez-Salab, 2006).

In the transsulfuration cycle, Hcy is converted into cystathionine by Cystathionine β-synthase (CβS), a vitamin B_6-dependent enzyme, and then into cysteine (Kraus et al., 1998). Under normal physical conditions, all Hcy is remethylated into methionine or catalyzed into cystathionine. The increase of Hcy concentration represents impairment in folate metabolism and thus in methylation reactions (Fenech, 2002).

Besides the enzymes that act directly on folate metabolism, cobalamin-transporting proteins also play an important role in this metabolic pathway, since the MTR enzyme is cobalamin-dependent. The enzyme Transcobalamin 2 (TC2) is synthesized in the intestinal villi and binds itself to Cbl in the interstitial fluid. This formed complex goes into the intestinal villi microcirculation and then reaches the systemic circulation. This circulation distributes the vitamin to all tissues where specific receptors on cell membranes bind and internalize the TC2-Cbl complex by endocytosis (Quadros et al., 1999; Seetharam & Li, 2000).

5. Folate metabolism, genomic stability, and maternal risk for chromosome 21 nondisjunction

Based on evidence that stable centromeric DNA chromatin may depend on the epigenetic inheritance of specific centromeric methylation patterns and on the binding of specific methyl-sensitive proteins to maintain the higher order DNA architecture necessary for kinetochore assembly (Karpen & Allshire, 1997), James et al. (1999) hypothesized that pericentromeric hypomethylation, resulting from impaired folate metabolism secondary to polymorphism of the *MTHFR* gene, could impair chromosomal segregation and increase the risk for chromosome 21 nondisjunction in young mothers. They observed that the risk of having a child with DS was 2.6-fold higher in mothers with 677 C→T substitution in one or both alleles of the *MTHFR* gene than in mothers without the 677 C→T substitution. In addition, DS mothers displayed a significant increase in plasma Hcy concentrations and lymphocyte methotrexate cytotoxicity, consistent with abnormal folate and methyl metabolism.

As described above, the MTHFR enzyme plays an important role in regulating DNA methylation through the reduction of 5,10-methylene-THF to 5-methyl-THF (Figure 1). The 677 C→T polymorphism is known to decrease the affinity of the enzyme for the flavin-adenine-dinucleotide (FAD) cofactor, decreasing enzyme activity (Guenther et al., 1999; Yamada et al., 2001). The *MTHFR* 677 CT genotype seems to reduce enzyme activity by about 35% and the homozygous TT genotype by 70% (Frosst et al., 1995). Since the study by James et al. (1999), polymorphisms in the *MTHFR* gene are the most frequently investigated in attempt to clarify the role of folate and methyl metabolism in the maternal risk for DS (Martínez-Frías et al., 2008). Several studies have associated the MTHFR 677C→T polymorphism and the risk of bearing a child with DS (da Silva et al., 2005; Meguid et al., 2008; Sadiq et al., 2011; Wang et al., 2008) as well as with increasing plasma Hcy concentration (P.M. Biselli et al., 2007; da Silva et al., 2005; Narayanan et al., 2004; Ulvik et al., 2007).

Another common polymorphism in the *MTHFR* gene, the substitution of alanine for cytosine at the 1298 position, was already associated with DS risk and increased plasma Hcy concentration (Martínez-Frías et al., 2006; Meguid et al., 2008; Narayanan et al., 2004, Rai et al., 2006; Scala et al., 2006; Weisberg et al., 2001). This polymorphism proved to have an impact on enzyme activity resulting in an even more pronounced decrease in its activity in homozygous 1298 CC compared to the heterozygous individuals (van der Putt et al., 1998).

In addition to the *MTHFR* gene, other genetic polymorphisms involved in the folate pathway seem to modulate the maternal risk for bearing a child with DS (Bosco et al., 2003; J.M. Biselli et al., 2008a; Meguid et al., 2008; Pozzi et al., 2009; Sadiq et al., 2011; Scala et al., 2006; Wang et al., 2008) as well as the concentrations of metabolites involved in the folate pathway (Ananth et al. 2007; Barbosa et al., 2008; Cheng et al., 2010; Devos et al., 2008). The *MTR* 2756 A→G polymorphism has been associated with increased maternal risk for DS in the presence of AG or GG genotypes, as well as when combined with polymorphisms *MTRR* 66 A→G (*MTR* 2756AG/*MTRR* 66AG) (Bosco et al., 2003) and *MTHFR* 677 C→T (*MTHFR* 677TT/*MTR* 2756AA). In addition, the allele *MTR* 2756 G proved to be more frequent, both in homozygosis and heterozygosis, in DS mothers as compared to mothers of individuals without the syndrome (Pozzi et al., 2009). Concerning its influence on Hcy concentrations, studies have shown conflicting results, since some have associated the *MTR* 2756 A allele to increased Hcy concentration (Fredriksen et al., 2007; Harmon et al., 1999), while others found the same association, but with the polymorphic 2756 G alelle (Feix et al., 2001; Fillon-Emery et al., 2004).

As to the *MTRR* 66 A→G polymorphism, some studies have supported an independent role for this polymorphism in the maternal risk for DS in the presence of the homozygous *MTRR* 66 GG genotype (Hobbs et al., 2000; Pozzi et al., 2009; Wang et al., 2008). Most of the studies have associated this polymorphism with the risk of DS and increased Hcy concentration when combined to other polymorphisms, such as *MTHFR* 677 C→T (Hobbs et al., 2000; Martínez-Frías et al., 2006; O'Leary et al, 2002; Yang et al., 2008). Additionally, a steady state kinetic analysis showed a significantly decreased affinity of *MTRR* for *MTR* accompanying substitution 66 A→G, revealing a significant difference in the relative efficacies of the MTRR enzyme (Olteanu et al., 2002). However, several studies have failed to find association between DS risk and the *MTRR* 66 A→G polymorphism, whether alone or combined with other genetic variants (Coppedè et al., 2009; Chango et al., 2005; Scala et al., 2006).

The *RFC1* gene is polymorphic at nucleotide 80 (A→G), and investigation of the impact of this polymorphism on protein function have demonstrated a difference in its affinity for subtracts and/or efficiency in transport in comparison with the wild type enzyme (Whetstine et al., 2001). Few studies have evaluated the influence of the *RFC1* 80 A→G polymorphism on DS risk (J.M. Biselli, 2008a, 2008c; Chango et al., 2005; Coppedè et al., 2006). Some studies have found no association between this polymorphism and DS (Chango et al. 2005; Fintelman-Rodrigues et al., 2009); however, Coppedè et al. (2006) and J.M. Biselli et al. (2008a) suggest a role for this polymorphism when combined with other polymorphisms in genes involved in folate metabolism. Supporting this hypothesis, the combined *RFC1* 80 GG/*MTHFR* 677 TT genotype has been associated with increased Hcy concentration and the *RFC1* 80 AA/*MTHFR* 677 CT combined genotype with higher plasma folate concentration (Chango et al., 2000).

A common polymorphism in the *CβS* gene, 68-base pair (bp) insertion at nucleotide position 844 (844ins68), is also investigated in the risk for DS, but there is no evidence that this variant plays an independent role on this risk (da Silva et al., 2005; Chango et al., 2005; Scala et al., 2006). The *CβS* 844ins68 polymorphism has been associated with reduction of Hcy concentration in the presence of the insertion (Tsai et al., 1996; Tsai et al., 1999; Tsai et al., 2000), and it is believed that this insertion is related to increased enzyme activity (Tsai et al., 1996, Tsai et al., 1999). This variant is always found to be associated in *cis* with an additional polymorphism in the *CβS* gene, a thymine-to-cytosine transition at nucleotide position 833, which causes a threonine-to-isoleucine amino acid substitution, and is reported, together with *CβS* 844ins68, as a 833 T→C/844ins68 *in cis* double mutation (Pepe et al., 1999; Vyletal et al., 2007). Da Silva et al., (2005) observed that the 844ins68 polymorphism, in association with other polymorphisms of the folate pathway, is related to increased risk for DS. Concerning its influence on folate metabolite concentrations, such as folate, Hcy, and vitamin B_{12}, the *CβS* 844ins68 polymorphism showed no significant association with any of the biochemical variables involved in folate metabolism (Bowron et al., 2005; Kumar et al., 2010; Summers et al., 2008).

The *MTHFD1* gene presents a functional polymorphism, a guanine-to-adenine substitution at position 1958 (1958 G→A), that has been shown to reduce the activity and stability of the variant enzyme (Christensen et al., 2008). There are only two studies to date on the influence of this polymorphism on maternal risk for DS. Scala et al. (2006) showed an association of the *MTHFD1* 1958 AA genotype with DS risk, but only when combined with the *RFC1* 80

GG genotype; however, more recently, Neagos et al. (2010) failed to find association. Thus, further investigations are necessary to clarify the role of *MTHFD*1 1958 G→A in the chromosome 21 nondisjunction.

Johnson et al. (2004) described a 19-base pair (bp) deletion polymorphism in intron-1 of the *DHFR* gene and hypothesized that this polymorphism could be functional since the deletion removes a possible transcription factor binding site that affects gene regulation. A study with mothers of individuals with spina bifida showed that the expression of the messenger ribonucleic acid (mRNA) from the *DHFR* gene was 50% higher in the presence of del/del genotype than in the ins/ins genotype (Parle-McDermott et al., 2007). This polymorphism has been associated with the modulation of metabolites' concentrations involved in the folate pathway. Gellekink et al. (2007) reported association between the del/del genotype and reduction of plasma Hcy concentration, but found no association between this genotype and concentrations of serum and erythrocyte folate. Another study found no effect on Hcy concentration, but found increased plasma and erythrocyte folate levels in del/del individuals (Stanislawska-Sachadyn et al., 2008). The results of the only study that investigated the 19-bp deletion polymorphism of *DHFR* gene in DS mothers did not support an association between this variant and the maternal risk for DS. In addition, the polymorphism was not associated with variations in serum folate and plasma Hcy and methylmalonic acid (MMA) concentrations in the study population (Mendes et al., 2010).

The *TC2* gene, which codifies a transporting protein required for the cellular uptake of vitamin B_{12} (Seetharam &Li, 2000), is polymorphic at nucleotide position 776 (C→G). There is evidence that the presence of the *TC2* 776 CC genotype may be more efficient in delivering vitamin B_{12} to tissues, resulting in enhanced B_{12} functional status (Miller et al., 2002; Namour et al., 1998). In other studies, the presence of the *TC2* 776 GG genotype was shown to affect negatively the serum concentration of the TC2 protein-vitamin B_{12} complex (von Castel-Dunwoody et al., 2005) and was associated with low concentrations of SAM in childbearing-age women (Barbosa et al., 2008). Considering that SAM is the major methyl donor for DNA methylation reactions, it was hypothesized that the variant *TC2* 776 C→G could influence the maternal risk for DS by modifying the DNA methylation pattern. This polymorphism has only been investigated in DS risk by two groups to date (J.M. Biselli et al., 2008c; Fintelman-Rodrigues et al., 2009), but no association has been found.

The conflicting results shown by literature have raised the suggestion that the presence of individual polymorphisms in genes involved in folate metabolism might not increase the risk of having a child with DS, although the effect of combined risk genotypes might modify their individual effect and increase DS risk (J.M., Biselli et al., 2008a; Brandalize et al., 2010; Coppedè et al., 2006; Coppedè et al., 2009; da Silva et al., 2005; Martínez-Frías, et al., 2006; Scala et al., 2006; Wang et al., 2008). Moreover, there is evidence that the significance of genetic polymorphisms seems to depend on interactions with nutritional factors (Papoutsakis et al., 2010; Stover & Caudill, 2008).

6. Folate metabolism, genomic stability, and genetic polymorphisms

Both *in vitro* and *in vivo* studies have shown that DNA methylation is an important mechanism for the maintenance of genomic stability. Literature provides several examples that genome-wide DNA hypomethylation enhances the occurrence of aneuploidy and chromosomal rearrangements (Herrera et al., 2008), loss of heterozygosity (Matsuzaki et al., 2005), and chromosome malsegregation (Fenech et al., 2011). Folate and vitamin B_{12} are

among the most important minerals and vitamins required for DNA maintenance and prevention of DNA damage that could be induced by inadequate intake of these antimutagenic vitamins (Fenech, 2002). In human cells, folate deficiency is associated with DNA hypomethylation (Chang et al., 2011; Linhart et al., 2009), DNA instability (strand breakage, uracil misincorporation) (Linhart et al., 2009; Williams & Jacobson, 2010), aneuploidy of chromosomes 17 and 21 (Beetstra et al., 2005; Wang et al., 2004), apoptosis (Li et al., 2003), and necrosis (Beetstra et al., 2005). Low vitamin B_{12} status is also associated with DNA hypomethylation (Brunaud et al., 2003) and genetic instability (Andreassi et al., 2003; Botto et al., 2003).

There is increasing evidence of association between polymorphisms in folate and Hcy metabolizing genes and levels of chromosome damage. The *MTHFR* 677 C→T polymorphism is associated with diminished levels of 5-methylcytosine and DNA hypomethylation (Chen et al., 2010; Friso et al., 2002; Paz et al., 2002), micronucleus formation (Andreassi et al., 2003; Botto et al., 2003), and microsatellite instability (Naghibalhossaini et al., 2010) in the presence of the variant T allele. The homozygous variant genotype of another polymorphism of the *MTHFR* gene, 1298 A→C, was more frequent in patients with Turner syndrome (de Oliveira et al., 2008), and a higher frequency of the C allele was observed in spontaneous abortions with fetal chromosomal aneuploidy as compared to those with normal fetal karyotypes (Kim et al., 2011), suggesting its involvement in the origin of chromosomal imbalances. The *MTR* 2756 A→G polymorphism was associated with reduced number of hypermethylated CpG islands of suppressor tumor genes and with higher micronucleus rates in the presence of the *MTRR* 66 GG variant genotype (Botto et al., 2003; Paz et al., 2002; Zijno et al., 2003).

The polymorphism *RFC1* 80 A→G has been associated with reduced percentage of 5-methylcytosine in the DNA of mothers of children with autism in the presence of homozygous and heterozygous genotypes for the G allele as compared to AA genotype (James et al., 2010); however, the presence of the A allele was recently associated with increased oxidative DNA damage, while the *cSHMT* 1420 C→T polymorphism was associated with reduced oxidative DNA damage (CC>CT>TT) (Mohammad et al., 2011).

Moreover, Piskac-Collier et al. (2011) recently demonstrated that lymphocytes from lung cancer patients showed a considerably increased frequency of cytogenetic damage in the presence of *MTHFR* 677 C→T, *MTHFR* 1298 A→C, and *cSHMT* 435 C→T allelic variants, suggesting that interactions between genetic polymorphisms may also have a significant impact on genetic instability.

7. Predisposition to chromosome malsegregation in young DS mothers and its association with folate-metabolizing gene polymorphisms

Studies with women who have a DS child at a young age have suggested that they present genetic predispositions to chromosome malsegregation in both somatic and germ line cells. Migliore et al. (2006) observed increased frequency of binucleated-micronucleated lymphocytes in women who had a DS child before 35 years of age, and fluorescence in situ hybridization analysis revealed that micronuclei were mainly originating from chromosomal malsegregation events, including chromosome 21 malsegregation. Further studies from their group confirmed increased chromosome damage in blood cells of young DS mothers and showed a significant correlation between micronucleated cells and both

MTHFR 677C→T and 1298A→C polymorphisms. The mean frequency of binucleated-micronucleated cells increased significantly with the increasing number of *MTHFR* 677 T alleles, and *MTHFR* 1298 AA women have significantly higher binucleated-micronucleated cells frequency than do *MTHFR* 1298 AC + CC carriers (Coppedè et al., 2007; Coppedè, 2009). In addition, mothers who had a DS child at a young age showed increased frequency (of about 5-fold) of Alzheimer's disease (AD) (Schupf, et al., 2001). A unifying hypothesis trying to relate DS, trisomy 21, and AD has proposed that trisomy 21 mosaicism at the germ cell level or in brain cells could account for the familial aggregation of AD and DS (Potter, 1991). Together, these results suggest that young DS mothers are more prone to chromosome malsegregation, which could be true both for somatic (peripheral blood lymphocytes, brain) and for germ cells and, importantly, folate-metabolizing gene polymorphisms seem to play an important role on this susceptibility to aneuploidy.

8. Folate supplementation and DS prevention

Two important emerging areas of nutrition science are nutrigenomics, which refers to the effect of diet on DNA stability, and nutrigenetics, which refers to the impact of genetic differences between individuals on their response to a specific dietary pattern, functional food, or supplement for a specific health outcome. On these terms, two premises are important: (a) inappropriate nutrient supply can cause considerable levels of genome mutation and alter the expression of genes required for genome maintenance, and (b) common genetic polymorphisms may alter the activity of genes that affect the bioavailability of micronutrients and/or the affinity for micronutrient cofactors in key enzymes involved in DNA metabolism or repair, resulting in a lower or higher reaction rate (Bull & Fenech, 2008; Fenech, 2005).

As mentioned before, the folate-dependent biosynthesis of nucleotide precursors for DNA synthesis and genome methylation is dependent on the availability of many vitamins, including B_{12}, B_6, niacin, riboflavin, and minerals (zinc, cobalt), and is subject to regulation by other nutrients, such as iron and vitamin A, not directly involved in DNA or SAM biosynthesis (Stover, & Caudill 2008). Therefore, impairments in one-carbon metabolism, and the SAM cycle in particular, induced by nutritional deficiencies and/or genetic polymorphisms that encode folate-dependent enzymes, alter genome methylation patterns and gene expression levels (Stover, 2004; Stover, & Caudill 2008).

Since 1992, supplementation with 0.4 mg/daily of folic acid is recommended for women of childbearing age for the prevention of neural tube defects (Centers for Disease Control, 1992). Barkai et al. (2003) observed that families at risk for neural tube defects present with a higher frequency of DS cases and vice-versa, suggesting that both disorders are influenced by the same folate-related risk factors. However, two issues ought to be considered in the prevention of DS by folic acid: the dose and the timing of folic acid intake (Scala et al., 2006). It has been proposed that genomic instability is reduced at plasma folate concentrations above 34 nmol/L and Hcy concentrations below 7.5 μmol/L; these concentrations can only be reached with the ingestion of more than 0.4 mg/day of folic acid (Fenech, 2002). A report of a decreased occurrence of DS offspring in mothers supplemented with high doses of folic acid (6 mg/day) (Czeizel & Puho, 2005) supports the hypothesis of an involvement of folate in the etiology of DS. Concerning the timing of folate intake, it should be remembered that maternal MI errors in the primary oocyte may occur in a process that begins during fetal life and ends at the time of ovulation, whereas MII errors occur at the time of fertilization (Yoon

et al., 1996). Therefore, it is likely that only MII errors would be immediately affected by folic acid intake in adult women (Ray et al., 2003).

9. Conclusion

Currently available literature suggests that abnormal folate metabolism is associated with increased maternal risk for DS, with a complex interaction between genetic polymorphisms, environmental factors (i.e., nutritional factors), and epigenetic processes. However, given the complexity of the folate pathway, these complex interactions cannot be easily understood and none of the polymorphisms studied so far can be used in genetic counseling to predict the maternal risk for having a DS child (Coppedè et al., 2009). However, nutrigenetics and nutrigenomics are promising areas for evaluating the possibility of DS prevention with folic acid supplementation associated with susceptible genotypes. Thus, further large-scale studies are necessary to better understand the complex association between chromosomal 21 nondisjunction and folate metabolism.

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6

Down Syndrome Expressed Protein; DSCR-1 Deters Cancer and Septic Inflammation

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1. Introduction

Down syndrome is the most common genetic cause of mental retardation in humans, occurring in one out of 700 live births. Epidemiological studies suggest that although individuals with Down syndrome have an increased risk of infant cardiovascular malformation, muscle hypotonia, lymphatic edema, and leukemia, noteworthy they have a considerably reduced incidence of most solid tumor, atherosclerosis, and pathological angiogenesis-mediated diabetic retinopathy and kidney dysfunction.

Such data indicate that one or more of the 231 trisomic genes on chromosome 21 are responsible for protecting these individuals against cancer and vascular disease. We and others recently have identified the candidate genes are Down syndrome critical region (DSCR)-1, and A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)-1. In primary cultured endothelial cells, vascular endothelial cell growth factor (VEGF) resulted in rapid and profound upregulation of both genes, which in turn negatively feeds back to attenuate VEGF-mediated signaling and following the endothelial cell activation. In genome-wide screening, important regulatory transcription factor for many pathological features of Down syndrome, NFAT, bound more than 10,000 independent regions in VEGF-treated activated endothelial cells. Down syndrome trisomy model mice or endothelium-specific modest DSCR-1 increases in mice resulted in significant suppression of the vascular density in matrigel-plugs, inflammatory leukocyte infiltration, and tumor growth. In contrast, DSCR-1 null mice demonstrated markedly decreased vascular integrity and increased susceptibility to tumor metastasis. In a mouse model of endotoxemia, DSCR-1 null mice showed greater morbidity and mortality compared with wild-type littermate. Conversely, adenovirus-mediated overexpression of DSCR-1 resulted in marked attenuation of lipopolysaccharide (LPS) or VEGF-mediated inflammation. Collectively, these data provide that Down syndrome overexpressed protein; DSCR-1 serves to dampen the host response to infection and the tumor growth. The molecular research for Down syndrome with patients or model mice unexpectedly provide us a great hint for therapeutic targets in solid tumor and vasculopathic disease against all individuals.

2. Down Syndrome Critical Region (DSCR)-1 expression in activated endothelium

2.1 Foundation of the DSCR-1 from endothelial cell research

The endothelium is highly malleable cell layer, constantly responding to changes within the extracellular environment and responding in ways that are usually beneficial, but at times harmful to the organism. Several mediators, including growth factors (e.g. vascular endothelial growth factor, VEGF), inflammatory cytokines (e.g. tumor necrosis factor-α, TNF-α), and thrombosis mediator (e.g. thrombin), activate gene transcription in endothelial cells, resulting in changes in hemostatic balance, increased leukocyte adhesion, loss of barrier function, increased permeability, migration, proliferation and successive angiogenesis (Minami and Aird, 2005). The tight control of these processes is essential for homeostasis - endothelial cell activation, if excessive, sustained or spatially and temporally misplaced, may result in vasculopathic disease. Indeed, different extra-cellular mediators engage the endothelium in ways that differ from one signal to the next. A major important point is to survey the temporal and spatial dynamics of endothelial cell activation. Using DNA microarrays, I carried out a global survey of mRNA in human umbilical vein endothelial cells (HUVEC) treated in the VEGF, thrombin, or TNF-α. Clustering analyses of the data revealed a far closer relationship between VEGF and thrombin, than between other pairings (Fig. 1A). Of the various transcripts that were responsive both to VEGF and thrombin, DSCR-1 was the most highly induced at the earliest time point (1 h). Compared with VEGF and thrombin, TNF-α treatment of HUVEC resulted in far less induction of DSCR-1 (3.2-fold at 1 h) (not shown). The rest of the VEGF-mediated induced gene was early growth response (Egr)-3, nerve growth factor inducible (NGFI)-Bβ, cyclooxigenase (COX)-2, and ADAMTS-1 (**Fig. 1B**).

2.2 Molecular information of the DSCR-1

The DSCR-1 gene consists of 7 exons, of which exons 1-4 can be alternatively spliced, resulting in a number of different mRNA isoforms, each of which exhibit different expression patterns. In adult, there are two major isoforms, DSCR-1 long variant (DSCR-1L) and DSCR-1 short variant (DSCR-1s), expressed in organs (Fuentes et al., 1997). DSCR-1L, encoded by exons 1, 5, 6, and 7, is highly expressed in brain. Exon 1 was originally thought to encode a 29 amino acid region, but later studies revealed a start site further upstream, resulting in a larger 84 amino acid region (Genesca et al., 2003). In contrast, DSCR-1s is encoded by exons 4-7 and is under the control of a different promoter located in intron 3 (intergenic promoter) (**Fig. 1C**). Each promoter contains different regulatory transcriptional subunits. For example, DSCR-1s is mainly regulated by the calcineurin-NFAT pathway, which is highly induced by angiogenic and inflammatory stimuli in endothelial cells (Minami et al., 2004; Minami et al., 2006).

While, the DSCR-1L isoform is under the control of a Notch and Hes-1-dependent pathway (Mammucari et al., 2005) or TEF-1 dependent pathway (Liu et al., 2008). DSCR-1s inhibits calcineurin phosphatase activity, and the C-terminal 57 residues are sufficient for this activity. DSCR-1s strongly inhibits the calcineurin mediated NFAT signaling via two ways; its ability to disrupt binding of calcineurin to NFAT, and to disrupt calcineurin enzymatic activities (**Fig. 1D**).

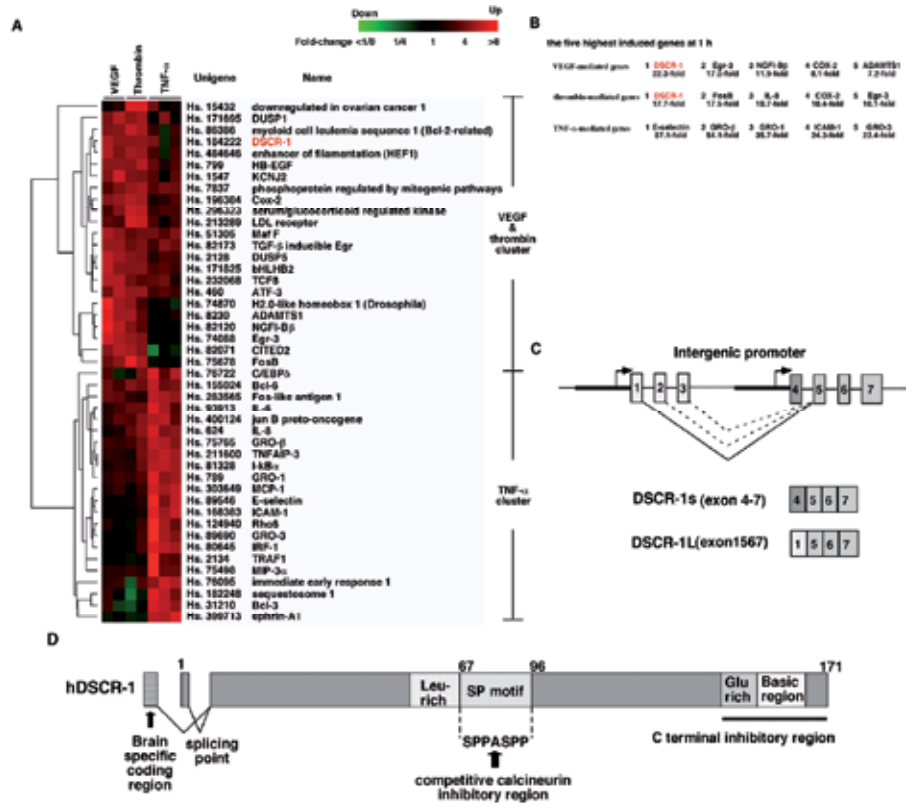

Fig. 1. VEGF / thrombin-mediated DSCR-1 induction *A,* heat map representation of induced genes in HUVEC. *B,* Top 5 genes induced via VEGF, thrombin, or TNF-α. *C,* major two variants of DSCR-1. *D,* structure information of DSCR-1

2.3 DSCR-1 expression in cultured cells

VEGF or thrombin induces the DSCR-1s expression in endothelial cells, through the coordinate binding of NFATc and GATA to closely positioned NFAT and GATA motifs in the intergenic promoter (Minami et al., 2004). VEGF/thrombin induces NFATc nuclear localization, and overexpression of the nuclear NFATc1 greatly induces the targeted DSCR-1s expression (Hesser et al., 2004; Minami et al., 2004; Minami et al., 2006). In addition, endothelial cells from the Down syndrome model mice (Ts65Dn) increased DSCR-1 mRNA by 1.7-2.0 fold (Baek et al., 2009). NFATc is an important factor for regulating the vertebrate development (Graef et al., 2001). In endothelial cells, NFATc1, c2, and c3 are expressed (Minami et al., 2009). To survey the NFATc1 binding in genome-widely, we carried out the chromatin immunoprecipitation using the antibody against NFATc1 following the comprehensive sequencing (ChIP-seq) in endothelial cells. We found totally 10,938 regions (*P* value >20) were identified as NFATc1 enrichment area from the ChIP-seq. DSCR-1 revealed the profound NFATc1 binding after the VEGF treatment within the proximal DSCR-1s promoter (**Fig. 2**). The area overlapped with positive signals from acetylated histone H4 (transcriptional active chromatin) and tri-methylated lysine of histone H3 (H3K4me3; active promoter marking) (**Fig. 2**).

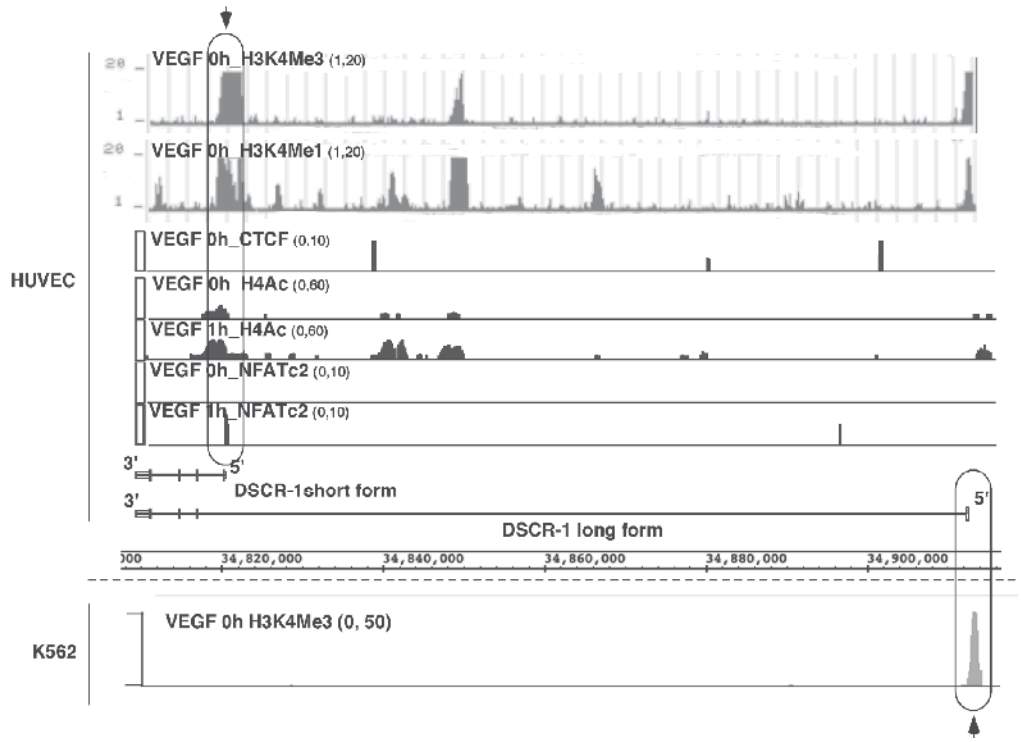

Fig. 2. Epigenetic information of the DSCR-1 locus Arrow indicates the significant enrichment from the ChIP-seq results

In contrast, erythroid lineage cells isolated from leukemia; K562 indicated the H3K4me3 positive signals within the proximal DSCR-1L promoter region, but not proximal DSCR-1s promoter region (**Fig. 2**). DSCR-1L reported the proceeding the pathological function in neurons (Cook et al., 2005). Moreover, Down syndrome patients have an increased risk of leukemia (Lott, 1982). Collectively, DSCR-1s and DSCR-1L obtained separate transcriptional machinery. VEGF mediated NFATc activation selectively transactivates the DSCR-1s via the profound binding within the promoter.

2.4 Characterization of the NFAT dependent genes overexpressed in Down syndrome

Besides DSCR-1, other genes encoded in chromosome 21 also reported as a candidate for pathogenesis on the Down syndrome. By using the combination of several NFATc knockout mice, dysfunction of NFAT was shown as a key point for the onset of Down syndrome (Arron et al., 2006). Around 1.5-fold increasing of both DSCR-1 and DYRK1A caused complete NFAT dysfunction. Thus, we test whether many Down syndrome genes obtain the NFATc1 binding on the each proximal promoter, by using the whole-genome NFATc1 ChIP-seq data (**Table 1**). Interestingly, DYRK1A obtained positive NFATc1 binding. VEGF inducible ADAMTS-1 (see **Fig. 1B**) also showed the NFATc1 positive binding. Ets family, Ets2, ERG, and GABPα, were highly expressed in endothelial cells, which was shown the regulation for the endothelial cell-specific expression or-essential function. All of them have a possibility as a NFATc1 direct target downstream gene.

	Gene	Description	NFATc1 bound	Bound peak area
From Nature (Reynolds et al.2010)				
	ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs, type 1	+	5′UTR
	ERG	v-ets erythroblastosis virus E26 oncogene homolog	+	+5367 & 5th intron
	Ets2	v-ets erythroblastosis virus E26 oncogene homolog 2	+	+55
	JAM2	junction adhesion molecule 2	-	
	PTTG1IP	pituitary tumor-transforming 1 interacting protein	+	+883
From Lancet (Roizen and Patterson, 2003)				
Energy and reactive oxygen species metabolism				
	BTG3	B-cell translocation gene 3	+, weak	1st intron
	MRPL39	mitochondrial ribosomal protein L39	+, weak	1st exon
	ATP5J	ATP synthase, H+ transporting, subunit F6	+, weak	1st exon
	GABPA	GA binding protein transcription factor, alpha	+, weak	+80
	BACH1	BTB and CNC homology 1, basic leucine zipper transcription factor 1	+, weak	1st intron
	SOD1	superoxide dismutase 1	+	5′UTR
	CRYZL1	crystallin, zeta-like 1	+, weak	1st Exon & +520
	ATP5O	ATP synthase, H+ transporting, O subunit	+	5′UTR
	MRPS6	mitochondrial ribosomal protein S6	+, weak	5'-UTR
	DSCR-1	Down syndrome critical region gene 1	+	Indicated in Fig. 2
	CBR1	carbonyl reductase 1	+, weak	1st exon
	CBR3	carbonyl reductase 3	+	1st exon
	SH3BGR	SH3 domain binding glutamic acid-rich protein	+	5′UTR
	NDUFV3	NADH dehydrogenase flavoprotein 3, 10kDa	+	+139
	SNF1LK	salt-inducible kinase 1	-	
	C21orf2	chromosome 21 open reading frame 2	+, weak	+140
Brain development, neuronal loss, and Alzheimer's type neuropathology				
	SIM2	single-minded homolog 2	-	
	DYRK1A	dual-specificity tyrosine-phosphorylation regulated kinase 1A	+	+1680

	GART	phosphoribosylglycinamide formyltransferase	+	+513 & 5'-UTR
	PCP4	Purkinje cell protein 4	-	
	DSCAM	Down syndrome cell adhesion molecule	-	
	GRIK1	glutamate receptor, ionotropic, kainate 1	-	
	APP	amyloid beta (A4) precursor protein	+, weak	1st intron
	S100B	S100 Ca-binding protein B	-	
Folate methyl group metabolism				
	N6AMT1	N-6 adenine-specific DNA methyltransferase 1	+, weak	1st exon
	CBS	cystathionine-beta-synthase	-	
	DNMT3L	DNA methyltransferase 3-like	-	
	SLC19A1	Solute carrier family 19 , member 1	-	
	FTCD	formiminotransferase cyclodeaminase	-	
	HRMT1L1 (PRMT2)	Protein arginine methyltransferase 2	+	5'UTR

Table 1. Candidate genes for Down syndrome, where NFATc1 occupancy on the promoter. 'Weak 'means *P* value < 20

2.5 DSCR-1 expression in organ

Increased DSCR-1 expression was observed in human fetal Down syndrome kidney versus age-matched control kidney (**Fig. 3A**). To determine whether the DSCR-1s promoter region directed inducible expression *in vivo*, the -1664/+83 DSCR-1s promoter was coupled to the *lac*Z reporter gene and targeted the resulting transgenic cassette (DSCR-1-*lac*Z) to the *Hprt* locus of mice using homologous recombination. The *Hprt*-locus *in vivo* promoter analysis system has been previously shown to be beneficial in controlling and avoiding the undesirable and undetectable effects of copy number and integration site on promoter activity (Cvetkovic et al., 2000; Ryan and Sigmund, 2003). We have used this system successfully to show the vascular bed specific expression patterns of endothelial cell specific promoters, Flt-1, vWF, ROBO4, and Tie-2 (Minami et al., 2002; Minami et al., 2003; Okada et al., 2007). At embryonic day 11, whole-mount *lac*Z staining revealed widespread expression of the transgene in the vasculature. In cryosections, strong staining was observed in the dorsal aorta, intersomitic vessels, carotid arteries, caudal veins, the primary head vein branch, and the endocardium (**Fig. 3B**). *Lac*Z colocalized with endothelial PECAM-1 (**Fig. 3B**). However, after the embryonic day 14, profound DSCR-1s promoter activation in vascular endothelium was markedly downregulated correlated with the decline of embryonic VEGF levels after the critical steps for angiogenesis and vascular remodeling. In adult mice, DSCR-1s-*lac*Z activity was detected in only a subset of endothelial cells in the brain, heart, lung and kidney. Expression was also observed in occasional neurons, vascular smooth muscle cells, cardiomyocytes, and renal epithelial cells. In contrast, DSCR-1s-*lac*Z activity was undetectable in the liver, spleen, thigh skeletal muscle, and thymus. These findings suggest that the DSCR-1s promoter, though widely expressed in the endothelium of embryonic days 11, is downregulated in the later stages of development and in adults.

Subsequently, to determine whether the DSCR-1s promoter confers response to inflammatory or angiogenic stimuli *in vivo*, DSCR-1-*lacZ* mice were systemically administrated VEGF or LPS. In whole mount preparations, the X-gal reaction product was detectable in the brain and heart of untreated mice and was further upregulated by VEGF and LPS. In contrast, *lacZ* staining was not observed in skeletal muscle, liver, and spleen vasculature even after the stimulus (data not shown).

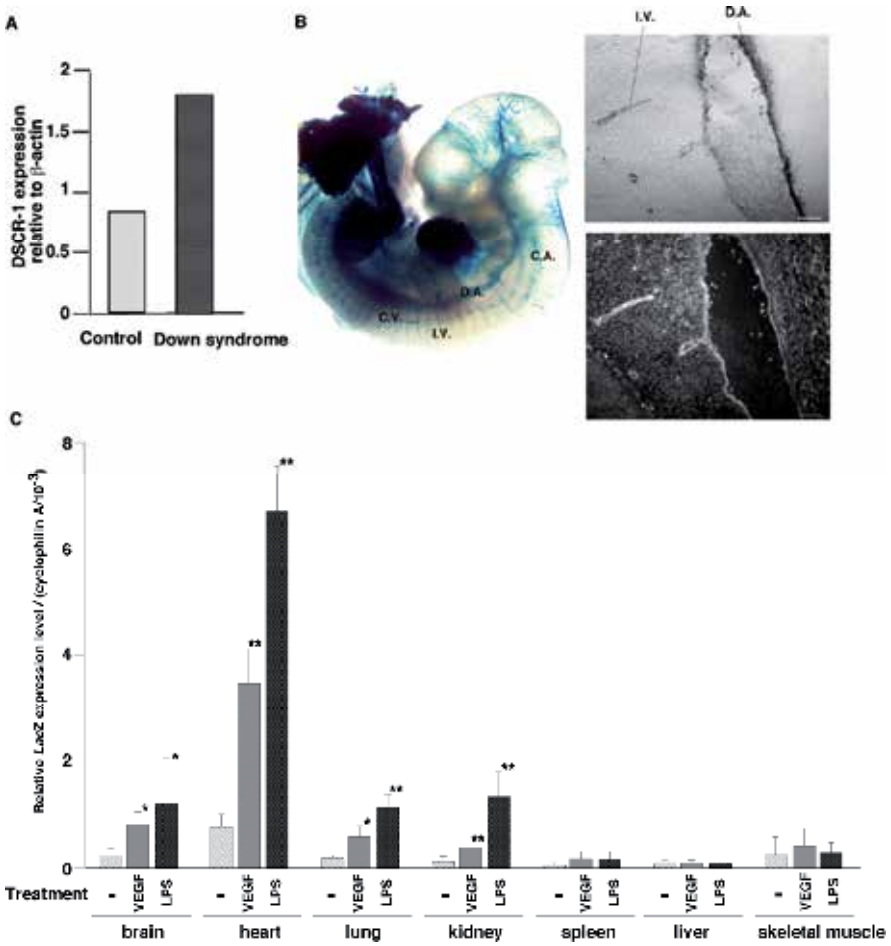

Fig. 3. DSCR-1 promoter activation *in vivo A,* highly DSCR-1 expression in kidney from Down syndrome individuals. *B, left*, whole-mount *lacZ* staining of embryonic day 11 Hprt-targeted embryos. C.A., carotid artery; D.A., dorsal aorta; I.V., intersomite vessel. *Right,* serial tissue sections from Hprt-targeted embryo. *lacZ* (*up*) and PECAM-1 (*down*) immuno-stainings were shown. *C,* real-time PCR quantification of *lacZ* epression in various organs. *P<0.04, **P<0.01 compared mock treatment in each organ.

Real-time PCR analysis was used to quantify changes in transgene expression. Under basal conditions, *lacZ* mRNA expression was highest in the heart, followed by the brain, lung, and kidney (**Fig. 3C**). Expression in skeletal muscle, the spleen and liver was below the level of detection. VEGF and LPS resulted in significant induction of *lacZ* transcripts in the heart,

brain, lung, and kidney, but not in spleen, liver or skeletal muscle (**Fig. 3C**). LPS-mediated induction of the endogenous DSCR-1s gene was similarly restricted to the heart (25.3-fold), brain (7.0-fold), lung (10.3-fold), and kidney (9.3-fold) (not shown). Agonist treatment failed to alter DSCR-1L transcript levels. Thus, VEGF and LPS promote vascular bed-specific expression of both the DSCR-1s promoter and the endogenous DSCR-1s gene.

2.6 DSCR-1 expression in tumor

Solid tumors produce a variety of pro-angiogenic molecules and inflammatory cytokines, which have important paracrine effects on surrounding endothelial cells. To investigated whether the DSCR-1s transgene is activated in tumor blood vessels, B16-F1 melanoma and Lewis lung carcinoma (LLC) cells were implanted subcutaneously into the flank of DSCR-1s-*lacZ Hprt* mice. When tumors reached ≈2.5 cm^3 in volume, the xenografts were harvested, sectioned and stained for *lacZ*. As shown in **Fig. 4A**, there was widespread reporter gene activity within both B16-F1 melanoma and LLC tumor neovessels. In double immunofluorescence studies, *lacZ* co-localized with endothelial PECAM-1 (**Fig. 4B**). Consistent with these findings, endogenous DSCR-1 also co-localized with PECAM-1 in tumor vessels of both B16-F1 melanoma and LLC xenografts (not shown).

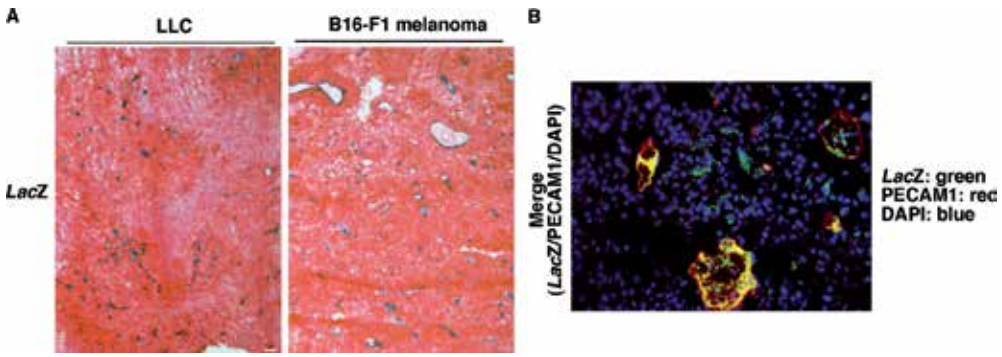

Fig. 4. DSCR-1s promoter activation in tumor vasculature. *A, lacZ* stainings were performed of LLC and B16-F1 melanoma xenografts. *B,* merged image of immunofluorescence staining with antibodies against *lacZ*, PECAM-1 or DAPI.

3. Biological function of DSCR-1

3.1 DSCR-1 inhibits nuclear localization of NFATc

Adenovirus mediated overexpression of DSCR-1, but not control, inhibited VEGF mediated nuclear localization of NFATc1 and NFATc2 (Minami et al., 2004). DYRK1A is another potential NFAT regulators, which encodes a nuclear serine/threonine kinase that primes substrates for phsphorylation by Glycogen synthase kinase (GSK) 3(Gwack et al., 2006). GSK3 phosphorylates NFATc proteins in the nucleus, resulting in their inactivation and export (Beals et al., 1997). DYRK1A is expressed at elevated levels in some human Down syndrome fetal tissues (Arron et al., 2006). In neuronal cells, DYRK1A inhibits FGF8-mediated induction of NFAT activity. Moreover, it has been shown that a 1.5-fold increase in the dosage of DSCR-1 and DYRK1A, both of which lie within the critical region of human chromosome 21, cooperatively destabilized the calcineurin-NFAT regulatory circuit (Fig. 5), leading to many of the features of Down syndrome (Arron et al., 2006).

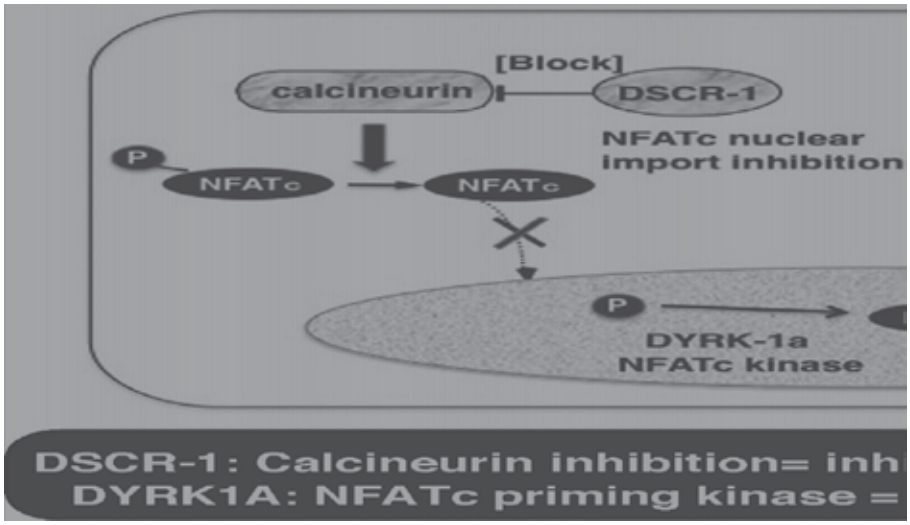

Fig. 5. schematic model of NFAT inhibition with DSCR-1 and DYRK1A.

3.2 DSCR-1s auto-inhibits the VEGF-mediated vascular activation

VEGF is an endothelial cell specific mitogen, and chemotactic agent, which is involved in wound repair, angiogenesis of ischemic tissue, tumor growth, microvascular permeability, hemostasis and endothelial cell survival (Isner and Losordo, 1999). DSCR-1 overexpression inhibits VEGF-mediated vessel growth, and monocyte cell adhesion (Minami et al., 2006). DSCR-1 overexpression did not lead in increased apoptosis (Minami et al., 2009). Taken together, these findings suggest that DSCR-1 constitutive expression lead the endothelial cells to quiescent status form the VEGF-mediated activated status.

3.3 DSCR-1s attenuates septic inflammation

As shown above, DSCR-1s attenuates VEGF-mediated activation of cultured endothelial cells. These data led us to hypothesize that VEGF- and LPS-inducible expression of DSCR-1s in mice may serve as a negative feedback inhibitor of endothelial activation *in vivo*. To test this hypothesis, I examined the effect of DSCR-1 deficiency or overexpression on endotoxemia phenotype. The generation of DSCR-1$^{-/-}$ mice, which carry a targeted deletion of both DSCR-1s and DSCR-1L. To overexpress DSCR-1s, I have chosen an adenoviral delivery system in which the endothelial-specific Flt-1 promoter is coupled to DSCR-1s cassette (Ad-Flt1-DSCR-1s). *In vivo* delivery of Ad-Flt1-DSCR-1s results in overexpression of DSCR-1s in the intact endothelium of mice. Endotoxemia in mice is associated with a reduction in heart rate, blood pressure, and body temperature, and an increase in circulating interleukin (IL)-6 levels. This effect was accentuated in DSCR-1$^{-/-}$ mice, and attenuated in DSCR-1s overexpressing animals. Recently, Yano et.al. reported that endotoxemia in mice is associated with increased circulating levels of VEGF (Yano et al., 2006). Further, VEGF plays a pathogenic role in sepsis (Yano et al., 2008). Interestingly, resting levels of plasma VEGF were 5.0-fold higher in DSCR-1$^{-/-}$ mice compared with wild-type littermates (**Fig. 6A**), which is a parallel correlation with the report that lower VEGF expression in stem cell culture derived from amniotic fluid in Down Syndrome (Salvolini et al., 2010). In response to septic treatment, DSCR-1$^{-/-}$ mice demonstrated super-induction of circulating VEGF levels (2.2-

fold higher vs. wild-type mice). Ad-Flt1-DSCR-1s-injected mice had no change in resting VEGF levels. However, in response to endotoxemia, DSCR-1s-overexpressing mice demonstrated a 61% reduction in circulating VEGF levels compared with septic Ad-Flt1-control (**Fig. 6A**).

To assay for endothelial activation, real-time PCR was performed to measure mRNA expression of E-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 in tissues from mice 6 h following injection of PBS (control) or LPS. Compared with wild-type littermate controls, LPS-treated DSCR-1$^{-/-}$ mice demonstrated super-induction of E-selectin in the heart and lung, ICAM-1 in heart, and VCAM-1 in lung. In contrast, LPS-mediated induction of cell adhesion molecules was attenuated by overexpression of DSCR-1s (data not shown).

I have recently shown that hyper-activation of the VEGF-calcineurin-NFAT pathway triggers apoptosis in DSCR-1-deficient tumor endothelial cells (Minami et al., 2009). Given that DSCR-1$^{-/-}$ mice have elevated circulating levels of VEGF level (see **Fig. 6A**), I hypothesized that endotoxemia may result in increased endothelial cell apoptosis in DSCR-1$^{-/-}$ mice. To test this hypothesis, TUNEL assay was carried out in tissue sections from the heart and lung of LPS-treated DSCR-1-null mice and their wild-type littermates. Endotoxemic wild-type mice demonstrated a small number of TUNEL-positive endothelial cells in the heart, and even fewer in the lung. However, in DSCR-1$^{-/-}$ mice, LPS administration resulted in a significant increase in the number of TUNEL-positive cells in both organs (Minami et al., 2009).

Finally in survival studies, LPS-treated DSCR-1$^{-/-}$ mice demonstrated markedly increased mortality compared with endotoxemic wild-type littermates (**Fig.6B**, *left*). In contrast, Ad-mediated overexpression of DSCR-1s conferred a survival advantage compared with Ad-Flt1-control (**Fig. 6B**, *right*). Taken together, these findings suggest that inflammatory induced DSCR-1s obtains a critical role in the host response.

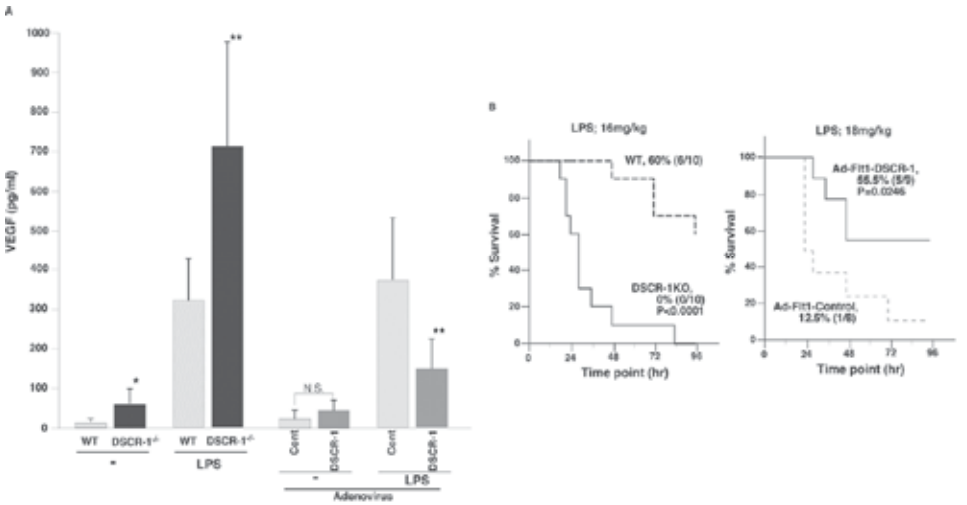

Fig. 6. DSCR-1 modulates inflammatory VEGF expression and sepsis mortality. *A,* Blood was harvested for plasma, and VEGF levels were measured using ELISA. *P<0.001, **P<0.01 compared with wild type or Ad-Flt1-control (cont), n=10. *B,* Survival studies in mice administrated LPS. Percentages of mice (surviving/total) are indicated.

3.4 DSCR-1s attenuates tumor progression

Having established an inhibitory role for DSCR-1 on inflammation *in vivo*, I next study the functional relevance of angiogenesis, tumor growth, and tumor metastasis *in vivo*. Adenovirus mediated locally DSCR-1 expression in vascular demonstrated significant reduction of the blood vessel formation in a matrigel-plug, compared to the Ad-control treated-plug vascularity. In a xenograft model with B16 melanoma and Lewis lung carcinoma revealed that DSCR-1 overexpression statistically significant (more than 70%) reduction of the tumor growth, in the parallel for the reduction of the vessel density from DSCR-1 treated tumor. Subsequently, to test whether DSCR-1 stable expression in the vascular endothelium inhibit the tumor growth, the transgenic mice was generated containing the endothelial cell specific Tie 2 promoter-linked DSCR-1s cDNA constract. Two independent transgenic lines indicate the vascular-specific DSCR-1s expression, both of which delayed the tumor growth at the early step, up to the tumor mass ≈1,500 cm^3. Cryosection of the xenografted tumor and immunostained with anti-PECAM1 antibody revealed the reduction of the vascular density in vascular specific DSCR-1s transgenic mice compared with wild type control mice. Taken together, DSCR-1s expressed in endothelial cells *in vivo* would function as an anti-angiogenic molecule. Stable expression of the DSCR-1s would lead the endogenous anti-tumor activities.

3.5 DSCR-1s attenuates tumor metastasis

During the study for the DSCR-1s promoter activity *in vivo*, by using the tumor metastasis model, we found the DSCR-1s promoter was already active in the lung microvascular endothelium, before the tumor metastasis colony had not yet observed. This DSCR-1s-*lacZ* activity in endothelial lining of lung was clearly abolished by systemic treatment with cyclosporine A. In addition, we have previously shown that stimuli induced DSCR-1 auto-inhibited inflammation in HUVEC (Minami et al., 2004; Minami et al., 2006). Thus, I hypothesized two things; DSCR-1s promoter would be useful for the marker for the pre-metastatic condition, and the DSCR-1s stable expression would overcome the tumor metastasis. At first, to test the latter thing, mice were injected intravenously with $2x10^5$ B16 melanoma cells. Three days later, mice were administrated with adenovirus containing Flt-1 promoter-DSCR-1s, or the control into the lung via the airway with vapor infection. Twenty days after the B16-F10 injection, lungs were harvested and photographed. There was no difference in body weight between the experimental and control groups (data not shown). The Ad-Flt1-Control group exhibited significant endothelial cell surface invaded-melanoma metastasis and the lungs showed significant swelling (**Fig. 7A**). In contrast, the Ad-Flt1-DSCR-1s group exhibited little B16 melanoma metastasis and showed no significant lung swelling. To semi-quantify the metastasis rate, the melanoma-growing area per whole surface was calculated. Compared with control, Ad-Flt1-DSCR-1s treatment resulted in statistically significant reduction (54%) of the B16 melanoma metastasis (**Fig. 7B**). In addition to DSCR-1s's ability to inhibit B16 melanoma metastasis to lung, DSCR-1s treated lung showed a significant reduction in the expression of the inflammatory adhesion molecules, VCAM-1 and E-selectin. It has been reported that endothelial cells expressed VCAM-1 and E-selectin positively influence cancer cell adhesion and migration to lung (Biancone et al., 1996; Fukuda et al., 2000; Futakuchi et al., 2004). Our performed metastasis assays, however, could not distinguish whether reduction in these adhesion molecules attenuated tumor metastasis, or reduced tumor cell migration to lung inhibited

inflammation and adhesion molecule induction. We have reported DSCR-1s strongly attenuates adhesion molecule expression in cultured endothelial cells (Minami et al., 2006). Moreover, DSCR-1 stably expressed in cultured endothelial cells significantly blocked B16 melanoma attachment to the endothelial cell surface (data not shown), suggesting that the blunting of adhesion molecule expression by DSCR-1s might be a critical factor in the inhibition of tumor metastasis. Collectively, These data suggest that DSCR-1s stable expression inhibits the inflammatory coordinated pre-metastatic niche formation, resulting the strong interfering of the onset of tumor metastasis to lung.

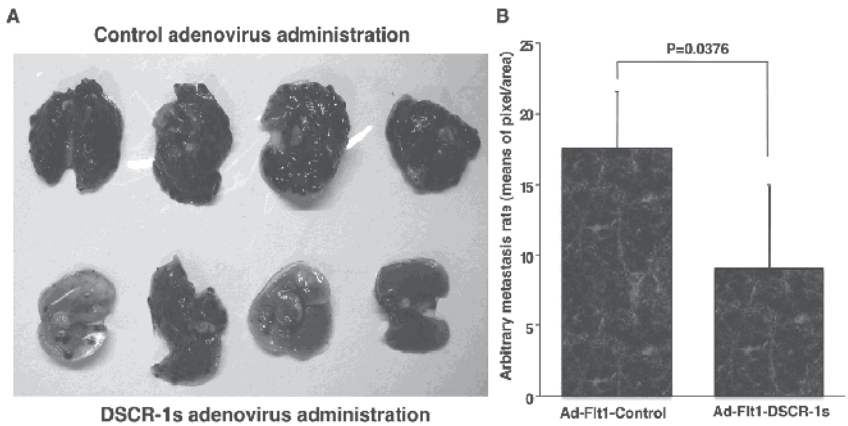

Fig. 7. Endothelial specific DSCR-1s overexpression downregulates the tumor metastasis to lung. *A,* after 20 days for B16-F10 injection administrated either Ad-Flt1-control or Ad-Flt1-DSCR-1s, lungs were harvested, washed and photographed. *B,* to quantify the metastasis rate, metastasis area from the whole surface lung was calculated by using Image J software. Data are expressed as means and standard deviations; n=10.

3.6 Lacking DSCR-1 results with controversy

While our findings reported here lend further evidence toward DSCR-1s as a negative regulator of NFAT-calcineurin signaling *in vivo*, the exact function of DSCR-1 is not without controversy. It has been reported that in CHO cells cultured *in vitro*, a portion of the phosphorylated form of DSCR-1 associated with the 14-3-3 protein, which competitively activated calcineurin activity (Abbasi et al., 2006). However, in a separate report it was shown that phosphorylation of DSCR-1 markedly decreased its stability (Genesca et al., 2003), likely leading to degradation, and thus an increase in calcineurin activity. In endothelial cells, we observed that the non-phosphorylated form of DSCR-1, which we consider to be the pre-active form, was the dominant form during the early phase response to VEGF or thrombin. The phenotype exhibited by the DSCR-1 whole gene knockout mice shows exacerbated constitutively active calcineurin-dependent cardiac hypertrophy, whereas cardiac hypertrophy in response to pressure overload and chronic adrenergic stimulation was blunted in these mice (Vega et al., 2003). In addition, double knockout mice of DSCR-1 and modulator of calcineurin interacting protein (MCIP) 2 also resulted in calcineurin facilitation, although it is difficult to distinguish the relative contribution between DSCR-1 and MCIP2 in these events (Sanna et al., 2006). It light of previous results and the results presented here suggesting differing expression patterns and functions of the DSCR-1s and 1L isoforms, it is plausible that the phenotype of the DSCR-1 null mice results

from the complex deletion of both DSCR-1L and DSCR-1s isoforms. Qin et.al., recently reported DSCR-1s inhibited vascular growth and capillary tube formation, consistent with our findings, whereas DSCR-1L induced NFATc transcriptional activity and endothelial cell growth (Qin et al., 2006). Using siRNA or adenoviral miRNA in endothelial cells or in mice *in vivo*, respectively, I recently showed that the DSCR-1s promoter is specifically activated through NFATc1, c2, c3 and GATA-2 (Minami et al., 2009), whereas DSCR-1L, which lacks the NFAT consensus region, is regulated by Notch and glucocorticoid signaling (Mammucari et al., 2005). The lack of such elements in the DSCR-1L promoter, suggest that once DSCR-1L is expressed, the tightly regulated calcineurin-NFATc-DSCR-1s feedback loop may be broken, owing to a lack of NFATc regulation of the DSCR-1L promoter. I also cannot rule out the possibility that the DSCR-1 exon 1 has an as yet 'undetermined' function. Interestingly, in an attempt to understand this regulatory system, an attractive computationally simulated threshold model was shown by Shin, et. al (Shin et al., 2006), in which low-level stimulus causes weak NFAT activation, resulting in only minor DSCR-1s upregulation, and at a level not sufficient to block the target calcineurin (under the threshold). Continuing stimulus facilitates the dissociation of the calcineurin-DSCR-1 complex, so that DSCR-1 appears as an activator of calcineurin.

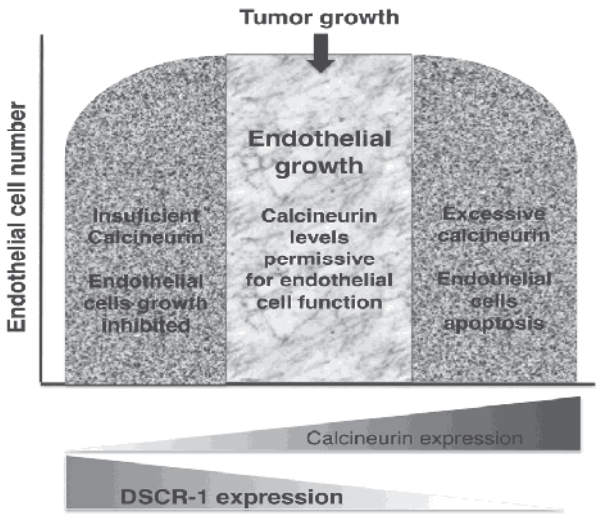

Fig. 8. Schmatic model of the balance of the endothelial cell growth

Interestingly in the endothelial cells, Sandra.et.al., indicated that DSCR-1$^{-/-}$ mice demonstrated reduced blood vessel formation in Matrigel, cornel micropocket, and tumor xenograft assays (Ryeom et al., 2008). DSCR-1$^{-/-}$ endothelial cells displayed hyper-activation of the calcineurin/NFAT pathway and increased sensitivity to VEGF signaling. However, rather than inducing cell proliferation, VEGF-mediated activation of calcineurin/NFAT in DSCR-1$^{-/-}$ endothelial cells 're-routed' downstream signaling, resulting in increased apoptosis, which thus explains the paradoxical reduction in neovascularization. Collectively, considered with the data from DSCR-1 stable expression and null mutation, calcineurin/NFAT activity and DSCR-1s expression level was tightly regulated, resulting the balance would define the endothelial cell growth, viability and tumor angiogenesis (**Fig. 8**). Future animal studies of DSCR-1 function should be performed by endothelial cell-specific knockout mice targeting either DSCR-1s or DSCR-1L separately.

4. Conclusion

DSCR-1 was identified by the study with vascular activation. DSCR-1 was highest induced by VEGF treatment in primary cultured endothelial cells. Previously, DSCR-1 was simply termed by the localization of the human chromosome 21. However, DSCR-1 indeed highly expressed in Down syndrome individuals, and clearly upregulated with NFAT activation in cells. Moreover, combined with same 21st chromosome encoded protein; 'DYRK1A', DSCR-1 strongly feedback attenuated the NFAT activation, resulting the pathogenesis of Down syndrome. I show here that DSCR-1s is highly expressed during embryonic vascular development, and then largely downregulated in adult, yet was highly activated predominantly in endothelium in response to the administration of VEGF or LPS. Stimulated DSCR-1s worked in the auto-inhibition of endothelial cell activation and inflammation. It has still unanswered problems with understanding the phenotypes from DSCR-1 lacking condition, and pathogenesis from DSCR-1L overexpression in neuron. However, based on this knowledge, I believe that DSCR-1s stable expression or the way of DSCR-1s stabilization may lend itself to therapeutic manipulation in vasculopathic disease states, including tumor angiogenesis, metastasis, and inflammation.

5. Acknowledgement

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7

Down Syndrome and Vascular Disease: DSCR1 and NFAT Signaling

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1. Introduction

Down Syndrome (DS) is one of the most common genetic disorders to date, occurring in 1 out of every 800-1000 live births (Egan *et al.* 2004; Roizen & Patterson, 2003; Stoll *et al.* 1990). DS patients often display several developmental and cognitive deficiencies. Common phenotypes of DS patients include congenital heart disease, dysmorphic physical features, and early-onset Alzheimer's disease (AD). Since the discovery in 1959 that DS occurs from an extra copy of human chromosome 21 (hChr21) (Lejeune J, Gautier & Turpin, 1959), questions arose whether a 1.5-fold increase in a gene or set of genes were responsible for the phenotypes associated with DS. Sequence analysis of hChr21 indentified over 225 genes and/or predicted genes (Hattori *et al.* 2000). With the recent advances in sequencing, the gene content of hChr21 is now estimated to exceed 300 genes (Roizen & Patterson, 2003). While typically characterized by complete trisomy of hChr21, several DS cases, however, demonstrate that partial trisomy of hChr21 is enough to elicit the phenotypes associated with DS (Stoll *et al.* 1990), hence arising the concept of a Down Syndrome Critical Region (DSCR). The DSCR theory suggests that enhanced expression of a few genes located in this critical chromosomal region (between markers D21S17 and D21S55 (Delabar *et al.* 1993; Korenberg *et al.* 1994)) are responsible for some, if not all, of the features of DS. Olson LE *et al* show, however, that triplication of this region alone is not enough to fully manifest the phenotypes associated with DS (Olson *et al.* 2004; Olson *et al.* 2007). These findings contradict the longstanding DSCR theory, indicating that the genetic instability seen with hChr21 triplication is not merely from excess gene expression. Rather, the functional interactions occurring within a set of overexpressed genes may explain the various phenotypes observed in DS.

Many of the triplicate genes on hChr21 are implicated in several diseases. Cohorts of genes, interestingly, have been associated with pathologies that appear to be more common for DS individuals. A group of at least 16 genes on hChr21 have been correlated with a role in energy and reactive oxygen species (ROS) metabolism (Roizen & Patterson, 2003). Several studies have linked mitochondrial dysfunction and metabolic disorders with DS (Arbuzova, Hutchin & Cuckle, 2002). *In vitro,* DS neurons were observed to have 3- to 4-fold increases in reactive oxygen species compared to control cells, with elevated rates of consequent cell apoptosis (Busciglio & Yankner, 1995). Degeneration of DS neurons was rescued by administration of free-radical scavengers, suggesting DS neurons are unable to efficiently metabolize ROS. DS patients are also evidenced to have increased mitochondrial superoxide

production compared to control groups (Capone *et al.* 2002). In addition, Busciglio J *et al.* show altered processing of the amyloid precursor protein (APP) in DS astrocytes and neurons (Busciglio *et al.* 2002). Elevated levels of APP, a gene also located on hChr21, have been associated with AD progression. Given that DS patients invariably display the characteristic plaques and tangles suggestive of AD (Hardy & Selkoe, 2002), this 1.5-fold increase in particular hChr21-located genes may explain the metabolic-related diseases associated with DS patients.

Another cohort of at least 10 genes located on hChr21 have been demonstrated to influence the central nervous system (CNS), and may hence play a role in the neuropathogenesis of DS (Capone, 2001). Considering the major phenotypes of DS patients include altered brain development, neuronal loss, and Alzheimer's-like neuropathology, the identification of CNS-related genes is not surprising. As demonstrated by these two gene clusters, phenotypic outcome is dependent not only on genotype but also on successive protein interactions. It is therefore possible that additional sets of genes, when in excess as a result of trisomy hChr21, lead to the manifestation of other DS-related pathologies, such as vascular-related pathologies.

2. Clinical observations and chapter overview

While developmental and behavioral disorders have been extensively studied in adults with DS, relatively little is known regarding their propensity to vascular-related diseases. Several intriguing clinical observations relating to the vasculature have been reported amongst DS patients over the last several decades. Unlike the aforementioned developmental deficits of DS individuals, DS patients appear to have a unique advantage in the context of several vascular pathologies.

2.1 Cancer incidence in DS patients

While adults with DS have been shown to have a higher incidence of leukemia compared to the general public, the risk of solid tumors appear to be strikingly low in individuals with DS (Hasle, Clemmensen & Mikkelsen, 2000). A population-based study on causes of mortality in over 17,000 DS patients showed a significant lack of malignant neoplasms as a cause of death (Yang, Rasmussen & Friedman, 2002). These findings suggest that individuals with DS express possible tumor-suppressor genes on hChr21, such that trisomy of a set of genes results in an antineoplastic effect on solid tumors. It is possible that these set of genes modulate a common biological mechanism that is critical for tumorigenesis and/or cancer progression.

2.2 Atherosclerosis in DS patients

A post-mortem, observational study published in 1977 found that 40-66 year old patients with DS displayed an almost complete absence of atherosclerotic plaque formation in arteries, compared to those without DS (Murdoch *et al.* 1977). Additional population studies in DS patients have found consistent results (Draheim, Geijer & Dengel, 2010; Yla-Herttuala *et al.* 1989), leading one to speculate that an unidentified factor(s) specific to DS patients explains the near absence of atherosclerotic plaque formation. Considering the vascular similarities between tumorigenesis and atherosclerosis, it is possible these events are driven by a shared molecular mechanism.

2.3 Other vascular pathologies in DS patients

Studies focusing on vascular tone show that DS patients have extremely low percentages of hypertension (Kerins *et al.* 2008). Significant decreases in both systolic and diastolic pressures were observed in DS patients when compared to reference populations (Morrison *et al.* 1996; Richards & Enver, 1979). Low blood pressures have also been observed in Alzheimer's disease (AD) (Burke *et al.* 1994; Landin *et al.* 1993), suggesting the mechanisms driving AD contribute to the observed hypotensive tendencies of DS patients. The already mentioned propensity for DS patients to develop AD, in addition to blood pressure levels similar to that of AD patients, strongly suggests a shared genetic contribution(s) of hChr21-located genes.

The observed decrease in solid tumor growth, lack of atherosclerotic plaque formation, and hypotensive nature of DS adults indicates onset of these vascular disorders may share an underlying molecular mechanism. This chapter will aim to discuss the observed differences in the vasculature of DS patients based on current literature to date. As of now, there is no concrete evidence distinguishing DS patients from non-DS patients on a genetic level concerning vascular anomalies. Correlative studies, however, suggest DS individuals harness a genetic advantage in regard to vascular health and disease.

3. Tumorigenesis in Down Syndrome

The molecular mechanisms underlying oncogenesis are undoubtedly one of the most intricate processes to date. Accumulation of multiple genomic mutations leads to the dysregulation of cell signaling pathways that are critical for controlling basic cellular functions including cell growth, survival, and cell fate. The extensive cross-talk between various signaling cascades highlights the sophisticated communication that transpires during cancer development and progression.

3.1 Calcium/calcineurin/NFAT signaling

Calcium (Ca^{2+}) fluxes are involved in multiple cell processes such as cell metabolism, membrane transport, cell permeability, and apoptosis. Changes in intracellular Ca^{2+} levels affect downstream gene expression and cell function through various mechanisms. In particular, intracellular Ca^{2+} concentrations often regulate kinase and phosphatase activity through the Ca^{2+}-binding protein, calmodulin (CaM). Increases in intracellular Ca^{2+} have been demonstrated in various cell types to activate the Ca^{2+}/CaM-dependent serine-threonine protein phosphatase, calcineurin (Cn). The Cn pathway is critical for the transduction of many extracellular, adaptive stimuli. Activated Cn subsequently dephosphorylates target proteins, including the nuclear factor of activated T-cells (NFATc1-c4) family of transcription factors (**Figure 1**). The NFAT family was originally identified in lymphocytes for its role in cytokine gene expression. While first identified in immune cells, recent findings show NFAT proteins are ubiquitously expressed. NFAT at the basal state are localized to the cytoplasm in an inactive form, with more than 20 identified phosphorylation sites (Okamura *et al.* 2000). CaM/Cn-mediated dephosphorylation of NFAT promotes nuclear translocation and target gene transcription (**Figure 1**). As downstream effectors of the Cn signaling pathway, NFAT-dependent gene transcription influences a range of cellular functions, including endothelial cell migration (Minami *et al.* 2004), skeletal muscle differentiation (Rothermel *et al.* 2000), cardiac valve development (Lange, Molkentin & Yutzey, 2004), and vascular smooth muscle cell adaptation (Crabtree & Olson, 2002; Horsley& Pavlath, 2002; Lee *et al.* 2010).

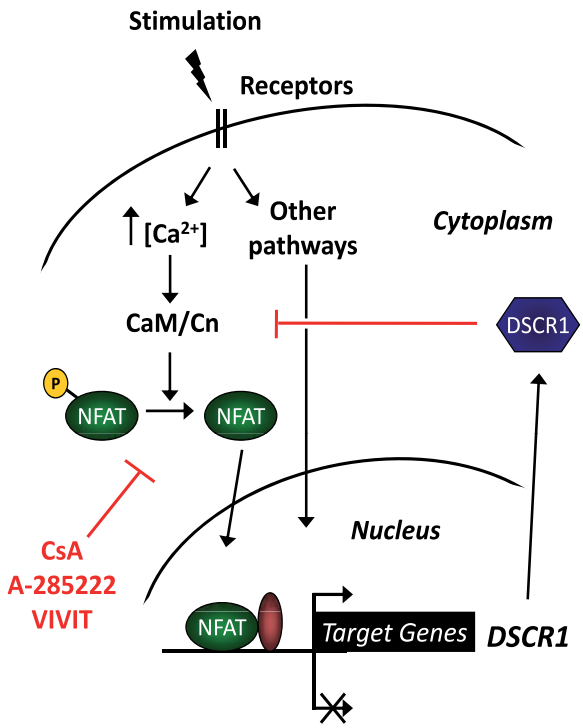

Fig. 1. The Cn/NFAT signaling pathway. calmodulin (CaM), calcineurin (Cn), cyclosporin A (CsA) (adapted from Viola, J.B.P., 2005)

Given the versatility of NFAT activation, it is not surprising that solid tumors and haematological malignances are correlated with overexpression of NFATc isoforms. For example, pancreatic carcinomas display elevated levels of NFATc1 for transcriptional activation of the c-myc oncogene (Buchholz *et al.* 2006). Studies using human breast carcinoma cell lines also show NFATc2 expression promotes carcinoma migration and invasion (Jauliac *et al.* 2002). The increased NFATc activity in malignant cells suggests activation of downstream NFATc targets is critical for tumor progression. Cyclooxygenase-2 (COX2/PTGS2) has been reported by several groups as an NFAT-dependent gene in non-lymphoid tissues, including endothelial cells (Hernandez *et al.* 2001), mesangial cells (Sugimoto *et al.* 2001), and vascular smooth muscle cells (Pritchard, Jr. *et al.* 1994). As an enzyme involved in prostaglandin formation, COX2 expression plays an important role in cell survival and angiogenesis. A gain-of-function approach shows that forced overexpression of COX2 alone is enough to induce mammary gland tumorigenesis (Liu *et al.* 2001). On the converse, inhibition of COX2 significantly reduces colon tumor incidence and multiplicity in a dose-dependent manner (Reddy *et al.* 2000). These findings strongly support the notion that tumor growth/invasion necessitates an abundance of COX2 levels. The increased expression of COX2 in a multitude of cancer lines (*i.e.* gastric, esophageal, breast, bladder, cervical, colorectal, endometrial (Brown & DuBois, 2005; Duque, Fresno & Iniguez, 2005b)) have thus launched the production of several COX2 inhibitors with therapeutic potential. It is, however, important to note the importance of COX2 in other cell types. Unlike the constitutively expressed COX1 isoform, COX2 expression occurs in a

limited number of cell types, primarily in response to cytokines, inflammatory mediators, and tissue damage (Lipsky *et al.* 2000). The inducible nature of COX2 activation results in prostaglandin formation, including prostacyclin, a vasodilator and a potent inhibitor of platelet aggregation. Prostacylin formation in vascular endothelial cells thus provides a mechanism by which the thrombotic response in platelet activation can be regulated (Rudic *et al.* 2005). Hence, COX2 expression is necessary to maintain vascular health. While COX2-selective inhibitors may attenuate tumor angiogenesis, COX2 antagonists will undoubtedly shift eicosanoid balance toward a prothrombotic state, promoting the risk for cardiovascular events (Mukherjee, Nissen & Topol, 2001). Targeting other proteins involved in Cn/NFAT signaling aside from COX2 may yield similar anti-neoplastic results.

Tumor survival and growth is highly-dependent on a vascular system for delivery of essential nutrients and oxygen. A tumor's vasculature also provides a mechanism by which aberrant cells can disseminate and metastasize to other organs. Without a constant blood supply, tumor volumes tend to remain 1-2mm^3 in size (Brown & DuBois, 2005). Angiogenesis is a multi-step process that involves migration, proliferation, and differentiation of both endothelial and vascular smooth muscle cells for generation of mature vessels. Tumor cells can facilitate an 'angiogeneic switch' by altering gene transcription and the surrounding microenvironment. Somatic mutations of various proto-oncogenes and/or tumor suppressor genes offset the balance between activators and inhibitors of angiogenesis, ultimately favoring angiogenic activity (Hanahan & Weinberg, 2000). Pro-angiogeneic gene expression is induced by a number of physiological stimuli, such as hypoxia. Cells in hypoxic conditions produce the hypoxia-inducible factor (HIF) transcription factor, where expression of HIF-1 results in Vascular Endothelial Growth Factor (VEGF) transcription (Forsythe *et al.* 1996). VEGF, a major stimulator of angiogenesis, is a potent endothelial mitogen involved in both physiological and pathological blood vessel formation. VEGF is critical for proper embryonic vasculogenesis, as even heterozygous VEGF-deficient mice display abnormal blood vessel formation and consequent embryonic lethality (Carmeliet *et al.* 1996). While necessary for proper blood vessel development, excess VEGF can result in pathological angiogenesis. HIF-1-mediated VEGF expression and activation of other angiogenesis-related genes exemplifies the adaptive responses seen in tumor survival and growth. VEGF has also been demonstrated to induce COX2 expression in a Cn/NFAT-dependent manner (Duque, Fresno & Iniguez, 2005c). As previously mentioned, COX2 expression is critical for tumor cell migration and tube formation. The emerging role of NFAT signaling in oncogenesis is further highlighted, as pharmacological inhibition of NFAT blocks VEGF-stimulated COX2 expression and subsequent cell proliferation and/or angiogenesis both *in vitro* and *in vivo (*Hernandez *et al. 2001)*. These results imply activation of Cn/NFAT signaling is required for the formation of new blood vessels, where regulation of angiogenesis is dependent on a local balance between angiogenic factors and inhibitors. The identification of Cn/NFAT activators and downstream NFAT target genes in the last few decades has therefore helped elucidate the biological mechanisms underlying oncogenesis.

3.2 DSCR1 in cancer progression

Cancer incidence for all solid tumors and malignancies have been shown in multiple DS population studies to be significantly lower in DS patients compared to control groups (Hasle *et al.* 2000; Yang *et al.* 2002). Interestingly, a large focus has been placed on the role of the Down Syndrome Candidate Region 1 (DSCR1/RCAN1/MCIP1) gene in cancer

progression. Located on hChr21, DSCR1 has been demonstrated as a negative feedback regulator of Cn/NFAT signaling through physical binding to the catalytic subunit of Cn (Fuentes *et al.* 2000) (**Figure 1**). In endothelial cells, VEGF and thrombin stimulation dramatically upregulate DSCR1 expression in a Cn/NFAT dependent manner (Iizuka *et al.* 2004; Minami *et al.* 2004). Several studies in endothelial cells have shown that DSCR1 overexpression inhibits NFAT transcriptional activity, suppresses NFAT nuclear accumulation, and attenuates NFAT-dependent inflammatory marker gene expression, such as COX2 (Hesser *et al.* 2004; Yao & Duh, 2004). Studies also show DSCR1 overexpression in endothelial cells reduces vascular density in matrigel plugs *in vitro* and in tumor growth in mice (Minami *et al.* 2004). Lastly, functional data indicate constitutive expression of DSCR1 impairs endothelial cell proliferation and tube formation (Minami *et al.* 2004). Taken together, VEGF and thrombin-mediated endothelial cell proliferation/vascularization induces expression of DSCR1 as a mechanism to negatively regulate the angiogenic processes.

Researchers have attempted to explain the lack of tumorigenesis in the DS population through use of genetically modified mice. Comparative mapping between mice and humans identify that hChr21 is genetically homologous to mouse chromosome 16 (mChr16) (Reeves & Citron, 1994). Thus, mice exhibiting trisomy of mChr16 (Ts16) were originally developed to study DS. Ts16 mice, however, die *in utero* and therefore pose as a limitation. In addition, several genes located on mChr16 are found on human chromosomes other than hChr21. The observed embryonic lethality as well as gene dosage imbalances resulted in generation of the Ts65Dn mouse (Reeves *et al.* 1995). The Ts65Dn mouse model reflects partial trisomy of hChr21, where mice have been reported to display a variety of phenotypes, including behavioral abnormalities consistent with DS (Reeves *et al.* 1995). Ts65Dn mice are now widely used to study DS at all stages of development.

A recent publication highlights the suppression of tumor growth seen in DS individuals using two different models: 1) the Ts65Dn mouse and 2) a DSCR1-transgenic mouse. Ts65Dn mice possess extra genetic information including the DSCR1 gene – Ts65Dn mice exhibit a 1.7-fold increase in DSCR1 protein expression compared to diploid controls (Baek *et al.* 2009). Baek KH *et al* demonstrate that both lung carcinoma and melanoma cell growth was significantly suppressed in Ts65Dn mice, when compared to control groups (Baek *et al.* 2009). Under the transplantable tumor model, Ts65Dn mice also display significant decreases in tumor growth and microvessel density (Baek *et al.* 2009). Similar results were seen when using DSCR1-transgenic mice, suggesting that one extra copy of DSCR1 is sufficient for tumor growth suppression. Given that hChr21 contains over 200 genes, however, it is likely that other genes act in concert with DSCR1 to facilitate tumor suppressive effects. Another gene of interest includes Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase (DYRK1A), a nuclear serine/threonine kinase located on hChr21. DYRK1A phosphorylates several transcription factors including NFAT. DYRK1A can therefore reduce Cn/NFAT-dependent transcription through its ability to export nuclear NFAT. Baek KH *et al* also show that endothelial cells overexpressing DSCR1 together with DYRK1A demonstrate an even greater suppression of VEGF-mediated cell proliferation (Baek *et al.* 2009). Future work by Baek KH *et al* will focus on additional hChr21-located genes known to suppress tumor angiogenesis through alternate mechanisms (Ryeom, Baek & Zaslavsky, 2009), such as COLLAGEN XVIII (COL18A1), a precursor to the angiogenesis inhibitor endostatin, and ADAMTS1, a metalloproteinase known to regulate the anti-angiogeneic function of thrombospondin-1 (Iruela-Arispe, Carpizo & Luque, 2003;

Zaslavsky *et al.* 2010). In summary, the tumorigenic protective effects seen in DS adults may be due to increased expression of select hChr21-located genes, including DSCR1.

4. Atherosclerosis in Down Syndrome

Atherosclerosis progression is a complex process involving several cell types, multiple biological mechanisms, and environmental factors. The pathogenesis of atherosclerotic plaque formation is highly dependent on hemodynamic flow patterns (DeBakey, Lawrie & Glaeser, 1985; Vanderlaan, Reardon & Getz, 2004), thrombotic processes, and inflammatory signaling cascades (Lusis, 2000). Atherosclerosis is one of the leading causes of mortality in Westernized societies, and thus several cardiovascular risk factors have been identified over the last few decades in an effort to predict cardiovascular events. Given that atherosclerosis is characterized by accumulation of lipids in the large arteries, it is not surprising that elevated plasma triglyceride levels have been demonstrated as a direct propagator of cardiovascular disease (Austin, Hokanson & Edwards, 1998; Hokanson & Austin, 1996). Other plasma measurements indicative of cardiovascular risk include reduced levels of high-density lipoprotein (HDL) (Assmann *et al.* 1996; Gordon *et al.* 1977), and elevated serum levels of C-reactive protein (CRP), an indicator of inflammation (Mendall *et al.* 1996). High values for body mass index have also been demonstrated as a measure of cardiovascular risk (Berenson *et al.* 1998).

Atherosclerotic cardiovascular disease has been associated with metabolic syndrome, which includes several clinical disorders such as obesity, insulin resistance, glucose intolerance, hypertension, and dyslipidemia (Moller & Kaufman, 2005). Consistent with the aforementioned metabolic deficiencies associated with DS patients, individuals with DS also exhibit impaired lipid metabolism (Ishihara *et al.* 2009). In addition, DS patients have lower levels of low-density lipoprotein receptor (LDLR) expression compared to adults without DS (Corsi *et al.* 2005). Interestingly, lack of or dysfunctional LDLR expression results in familial hypercholesterolemia, a genetic disease characterized by elevated cholesterol levels and premature coronary artery disease (Brown & Goldstein, 1976). LDLR-null mice have since been generated and is now a widely used atherosclerotic mouse model (Ishibashi *et al.* 1993; Wouters *et al.* 2005). Lastly, DS patients exhibit significantly elevated levels of plasma triglycerides, low levels of HDL, high levels of CRP, and high levels of total body fat (Corsi *et al.* 2005; Draheim *et al.* 2010; Nishida *et al.* 1977). Taken together, these findings imply DS adults are surely at risk for cardiovascular events. DS patients, however, are significantly less susceptible to cardiovascular disease. A comparison of intimal-to-medial thickness (IMT) of the common carotid artery in DS adults show significantly lower IMT ratios when compared to control groups (Draheim *et al.* 2010). These findings corroborate the observational findings in 1977 where DS patients were proposed to be an "an atheroma-free model" (Murdoch *et al.* 1977). One can speculate that DS patients thus express an altered gene expression profile where an additional copy of hChr21-located genes results in protection against atheroma formation, despite the many validated plasma indicators of cardiovascular risk. Similar to the identified gene clusters responsible for the metabolic and developmental deficiencies in DS patients, it is highly likely that a cohort of genes located on hChr21 is responsible for the protective effects against cardiovascular disease. Identification of these genes will therefore provide a therapeutic direction as we continue to understand the etiology behind atherosclerotic plaque formation.

4.1 Vascular smooth muscle and atherogenesis

At the cellular level, atherosclerosis progression is characterized by lipid accumulation and fibrous lesion formation in large arteries, resulting in chronic arterial wall inflammation. The release of cytokines and growth factors induce vascular smooth muscle cells (SMC) to undergo a process known as phenotypic modulation, whereby an adult, contractile SMC alters its expression profile to facilitate cell proliferation, migration, and/or inflammatory cell recruitment (Shin *et al.* 2008) (**Figure 2**). Phenotypic modulation is a key event underlying atherogenesis and in-stent restenosis that involves several signaling cascades, including the Cn/NFAT pathway. Activation of Cn/NFAT signaling has been shown to induce vascular SMC proliferation and migration in response to receptor tyrosine kinase and G-protein-coupled receptor agonists, respectively (Liu, Dronadula & Rao, 2004; Yellaturu *et al.* 2002). Cn/NFAT activity is also critical for vessel wall assembly (Graef *et al.* 2001). Cyclosporin A (CsA) and the VIVIT peptide are commonly used Cn and NFAT inhibitors, respectively (**Figure 1**). A third pharmacological compound, A-285222, also inhibits Cn/NFAT signaling (Lee *et al.* 2010; Nilsson *et al.* 2007). Inhibition of Cn/NFAT signaling through use of either CsA or A-285222 attenuates Cn/NFAT-mediated SMC migration and proliferation *in vitro* (unpublished, **Figure 1**). Previous literature also shows that blocking Cn/NFAT signaling *in vivo* significantly inhibits vascular-injury-induced neointima formation (Liu *et al.* 2005), supporting the notion that Cn/NFAT signaling is intimately involved in the smooth muscle response to vascular injury. While NFAT-dependent gene regulation has been widely studied in lymphocytes, cardiac and skeletal muscle, the lack of known NFAT target genes in SMCs limits our understanding of Cn/NFAT signaling in vascular remodeling.

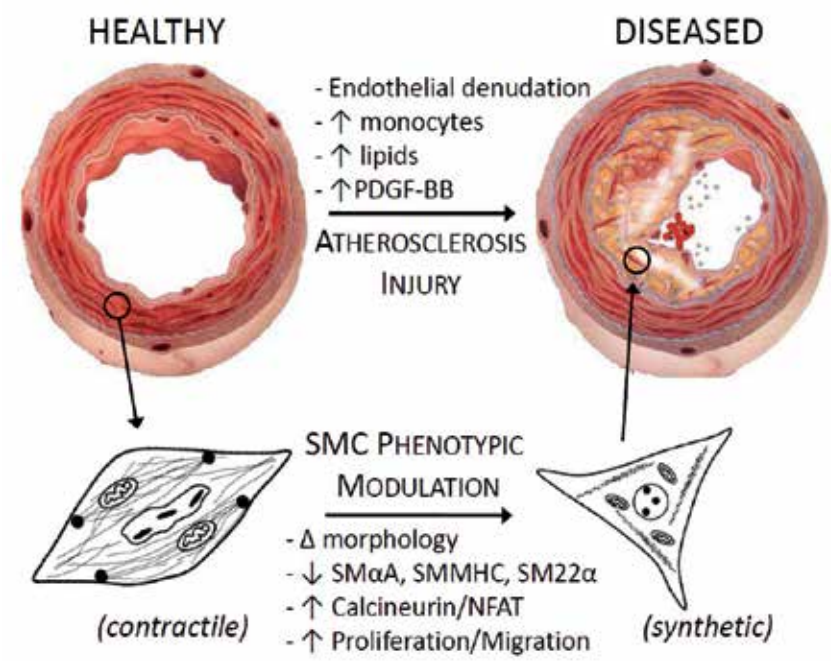

Fig. 2. The interplay between SMC phenotypic modulation and Cn/NFAT signaling in vascular disease.

4.2 Identification of DSCR1 using integrative genomics

We recently developed an unbiased, top-down, integrative genomics approach to determine downstream targets of Cn/NFAT activation in vascular SMCs (Lee *et al.* 2010). In brief, whole-genome expression data sets were analyzed to identify differentially upregulated genes in human, mouse, and rat SMCs. Comparison between control and phenotypic modulatory stimuli identified 63 species-conserved, upregulated genes. Differentially upregulated genes were then compared against an *in silico* NFAT-ome (a list of gene promoters containing at least one species-conserved physical NFAT binding site), yielding 18 potential Cn/NFAT-dependent genes. Use of pharmacological Cn/NFAT inhibitors substantiated the NFAT-dependent role for COX2, a previously identified Cn/NFAT dependent gene, and revealed the Cn/NFAT-dependent property of DSCR1 in vascular SMCs (Lee *et al.* 2010). DSCR1 has been reported in other cell types to function as a negative feedback regulator of Cn/NFAT signaling (**Figure 1**). Both gain- and loss-of-function studies were conducted to determine the role of DSCR1 in vascular SMCs. Overexpression of DSCR1 resulted in decreased NFAT transcriptional activity and reduced expression of other Cn/NFAT-dependent genes, such as COX2. Conversely, knockdown of endogenous DSCR1 enhanced NFAT transcriptional activity suggesting DSCR1 also functions as a negative regulator in vascular SMCs (Lee *et al.* 2010). Lastly, analysis of *in vivo* whole-genome expression arrays found significant DSCR1 upregulation with acute vascular injury in mouse carotid arteries, regardless of strain type (Lee *et al.* 2010). The significant induction of DSCR1 exemplifies its functional importance and may be critical for modulating the vascular injury response. Similarly, an extra copy of the DSCR1 gene in DS patients may function in an atheroprotective manner through its ability to negatively regulate Cn/NFAT signaling. The recent study highlighting significantly less tumor growth in DSCR1-transgenic mice (Baek *et al.* 2009) fosters the regulatory role of DSCR1 in Cn/NFAT-mediated cell growth. It is possible DSCR1 functions in both endothelial cells and vascular SMCs as a modulator of cell proliferation, thereby providing a shared mechanism underlying tumorigenesis and atheroma formation.

4.3 Ongoing research

Several studies are currently underway in an effort to understand the functional role of DSCR1 in SMC phenotypic modulation and atherosclerotic plaque formation. DSCR1-deficient mice have since been obtained and are proving to be instrumental as we continue to elucidate the role of DSCR1 in atherogenesis. DSCR1-related family members, however, have been identified in most eukaryotic organisms. In humans, DSCR1 family members include DSCR1-like 1 (DSCR1L1/ZAKI-4/RCAN2) and DSCR1-like 2 (DSCR1L2/RCAN3) (Strippoli *et al.* 2000). Interestingly, DSCR1L1 mimics the inhibitory effects of DSCR1 on Cn signaling in endothelial cells and inhibits angiogenesis (Gollogly, Ryeom & Yoon, 2007). In lymphocytes, DSCR1L2 blocks nuclear NFAT translocation and inhibits NFAT-dependent cytokine gene expression (Mulero *et al.* 2007). Given the functional overlap between the DSCR1 family members, global DSCR1-/- mouse models may possibly exhibit compensation by DSCR1L1 and/or DSCR1L2. In addition, DSCR1 plays a critical role in each of the major cell types seen with atherogenesis and will therefore be difficult to attribute our findings as a vascular SMC-specific response. Until a SMC-specific conditional knock-out mouse study is carried out, a conservative approach must be taken when interpreting results gathered from DSCR1-null mice given the complexities surrounding

DSCR1 expression and regulation in other cell types. Nonetheless, the data gathered will provide a better understanding of DSCR1 function.

Apolipoprotein E (ApoE) is critical for lipoprotein metabolism. Defects in ApoE expression can consequently increase plasma cholesterol and triglyceride levels. Hence, targeted mutagenesis of the ApoE gene in mice promotes elevated serum cholesterol levels, where combination with a high fat/cholesterol diet results in massive accumulation of cholesterol and causative atherosclerotic lesion formation. DSCR1$^{-/-}$; ApoE$^{-/-}$-double knockout mice have been generated in an effort to study how the absence of DSCR1 affects atherosclerotic plaque formation. These double-null mice have been placed on a 20-week, high fat/cholesterol diet to ensure plaque formation, where atherosclerotic plaque size will be quantified and compared against proper control groups. Given the literature to date, we predict atherosclerotic lesion formation and size to significantly increase in DSCR1$^{-/-}$; ApoE$^{-/-}$ double-null mice. The absence of DSCR1, a negative regulator of Cn/NFAT activity, should in theory promote SMC proliferation and infiltration into the neointima. A recent publication, however, emphasizes the dual role of DSCR1, suggesting DSCR1 is necessary for both Cn/NFAT activation and self-regulation (Liu, Busby & Molkentin, 2009). This effect has been demonstrated in cardiomyocytes and hypertrophy, an adaptive response driven by Cn and NFATc4 signaling (De Windt *et al.* 2001; Molkentin *et al.* 1998). Several groups have shown an attenuation of cardiac hypertrophy with overexpression of DSCR1 (Rothermel *et al.* 2001). DSCR1$^{-/-}$; DSCR1L1$^{-/-}$ double-null mice were thus hypothesized to demonstrate significantly greater cardiac hypertrophy. DSCR1$^{-/-}$; DSCR1L1$^{-/-}$ double-null mice, however, exhibit the reverse response – cardiac hypertrophy is impaired in response to adrenergic stimulation or exercise, similar to responses seen in calcineurin Aβ-/- mice (Sanna *et al.* 2006). These results suggest a basal level of DSCR1 is necessary to properly activate Cn/NFAT signaling. The DSCR1 yeast homolog, RCN1/CBP1, has also been functionally characterized as a facilitator of Cn/NFAT signaling (Kingsbury & Cunningham, 2000). The notion that DSCR1 may also activate Cn/NFAT signaling highlights the elaborate nature of this protein, suggesting that the paradoxical function of DSCR1 depends on initial protein concentrations (Shin *et al.* 2006). It is thus possible we may instead observe a decrease in atherosclerotic lesion formation in the DSCR1$^{-/-}$; ApoE$^{-/-}$ double-null mice. With this in mind, we have also placed DSCR1$^{+/-}$; ApoE$^{-/-}$ mice to observe how DSCR1 haploinsufficiency affects plaque formation. The presence of one DSCR1 allele will allow for minimal DSCR1 expression, yet also test the effects of reduced DSCR1 expression on lesion formation.

We are also performing several *in vitro* assays on primary aortic SMC cultures from DSCR1$^{-/-}$ mice to determine the consequences of DSCR1 absence on cell functionality. With our recent findings demonstrating DSCR1 as a negative regulator of Cn/NFAT signaling in vascular SMCs (Lee *et al.* 2010), one would hypothesize that DSCR1-null vascular SMCs to exhibit increased cell proliferation in response to phenotypic modulatory stimuli. Preliminary data, however, show a reduced proliferative response in DSCR1-null vascular SMCs when stimulated with either platelet-derived growth factor (PDGF-BB) or serum (unpublished). These initial results support the emerging dual role of DSCR1, suggesting basal levels of DSCR1 is necessary for Cn/NFAT activation. While our model cannot fully explain the almost complete absence of atheroma formation in DS patients, our results will not only help elucidate the functional role of DSCR1 in atherogenesis, but also provide insight on the effects of trisomy 21 through a single gene approach.

5. Vascular tone and Down Syndrome

Enzyme activity of endothelial nitric oxide synthase (eNOS) plays a critical role in determining vascular tone through its ability to generate nitric oxide (NO) and consequently affect vascular smooth muscle contraction. Regulation of eNOS enzymatic activity is a highly dynamic system involving several kinases and phosphatases with significant downstream effects on NO-signaling (Duran, Breslin & Sanchez, 2010). While phosphorylation of eNOS is typically associated with enzyme activation, several groups demonstrate that dephosphorylation of eNOS can also trigger eNOS activation and subsequent NO production (Harris *et al.* 2001; Kou, Greif & Michel, 2002; Thomas, Chen & Keaney, Jr., 2002). The Cn phosphatase has been shown to target several proteins aside from NFAT, including eNOS (Kou *et al.* 2002). While the absolute role of DSCR1 is unknown in the vasculature, recent literature suggests DSCR1 plays a vital role in vessel contraction. DSCR1$^{-/-}$ mice have been reported to exhibit an attenuated vasocontriction response with phenylephrine treatment compared to appropriate controls (Riper *et al.* 2008). Riper DV *et al* speculate that the altered vascular constriction in DSCR1$^{-/-}$ mice may be due to Cn dysregulation, thereby facilitating excess endothelial NO production. Based on the proposed mechanism, overexpression of DSCR1 should then limit NO formation and promote vasoconstriction. As stated earlier, however, adults with DS are significantly hypotensive when compared to control groups (Morrison *et al.* 1996; Richards & Enver, 1979). Although the attenuated vasoconstriction response in DSCR1$^{-/-}$ mice may contradict the hypertensive nature of DS patients, the account by Riper DV *et al* is the first publication to date revealing a potential role of DSCR1 in vascular tone.

It is also important to note that these observations of a weakened vasoconstriction response in DSCR1$^{-/-}$ mice reflect those of mesenteric rather than carotid arteries. While the underlying effects of NO on smooth muscle and vascular tone may be synonymous between the differing vascular beds, vasoconstriction could be regulated through different mechanisms. The effect of vascular bed type is further exemplified by the fact that while DS patients may be significantly hypotensive, they are also at an increased risk of developing pulmonary arterial hypertension (PAH) (Cua *et al.* 2007). The observed increase in PAH incidence amongst DS individuals may be due to several factors such as chronic upper airway obstruction (Jacobs, Gray & Todd, 1996) and abnormal pulmonary vasculature growth (Chi, 1975). Vascular tone therefore appears to depend not only on the vascular bed of interest but also on peripheral influences. In addition, basal levels of DSCR1 may be required for proper Cn/NFAT function, as previously described. Future contractility studies in haploinsufficient DSCR1$^{+/-}$ mice may thus provide clarity on the true role of DSCR1 in vascular tone. This study clearly demonstrates the widespread effect of DSCR1 loss, where dysregulation of Cn/NFAT activity in the endothelium alters paracrine signaling molecule production, creating invariable effects on neighboring cell types. The prevalent nature of DSCR1 prompts consideration for cell-cell interaction effects as we continue to decipher DSCR1 function in vascular disease.

6. NFAT regulation and other considerations

The importance of calcium signaling, NFATc transcriptional regulation, and ensuing downstream gene expression is increasingly evident. Adequate and controlled Cn/NFAT signaling is necessary, as dysregulation results in several vascular pathologies. Both the

versatility in DNA binding and the presence of multiple NFAT family members suggest NFAT proteins have a more extensive role than originally anticipated. A few notable features of NFAT are brought to attention below.

NFAT transcription factors bind to DNA in a rather versatile manner. The promiscuous nature of NFAT-DNA binding suggests the presence of other binding partners. The most common and well-documented binding partner of NFAT is AP-1, a transcription factor comprised of Fos- and Jun- family proteins (Rao, Luo & Hogan, 1997). Several groups have demonstrated the need for cooperative NFAT-AP1 binding for optimal target gene transcription (Hogan *et al.* 2003). In addition to AP-1, NFAT has been shown to interact with other transcription factors involved in cell growth and differentiation, including GATA4, MEF2, and FOXP3 (Crabtree & Olson, 2002; Wu *et al.* 2006). Several groups have also noted the presence of multiple NFAT binding sites in validated NFAT-target gene promoters, such as interleukin-2 (Randak *et al.* 1990), interleukin-4 (Chuvpilo *et al.* 1993), and DSCR1 (Harris, Ermak & Davies, 2005). These multiple NFAT binding domains most likely confer a synergistic effect between NFAT-containing complexes and thereby promote efficient gene transcription (Rao *et al.* 1997). Thus, NFAT proteins may serve as 'coincidence detectors' (Crabtree & Olson, 2002) that can integrate information from multiple signaling pathways to coordinate gene expression.

The ubiquitous nature of NFAT amongst various cell types and processes demonstrates the broad-range effects of NFAT activity. While functional redundancy has been noted between the different NFAT isoforms, differences in tissue distribution (Rao *et al.* 1997), evidence of multiple splice variants, and distinguishing phenotypes of NFAT-deficient mice suggest NFAT proteins may in fact possess unique isoform-specific functions. For example, while NFATc1 and NFATc2 share 72% sequence agreement and demonstrate functional similarities, genetic deletion for either NFATc isoform results in divergent mouse phenotypes. Whereas NFATc2$^{-/-}$ mice are viable and demonstrate an enhanced immune response (Duque, Fresno & Iniguez, 2005a), NFATc1$^{-/-}$ mice display abnormal cardiac valve development and consequent embryonic lethality (de la Pompa *et al.* 1998). Variations between NFAT proteins is further seen where targeted disruption of NFATc3, but not NFATc4, significantly reduces Cn transgene-induced cardiac hypertrophy (Wilkins *et al.* 2002). The disparate outcomes from NFATc genetic deletions as well as the ability for NFATc isoforms to compensate one another limits the use of NFATc-specific knock-out mice.

Recent data have also challenged the longstanding premise viewing NFAT as a transcriptional activator. Several groups have in fact demonstrated the dual role of NFAT as both a gene activator and silencer (Robbs *et al.* 2008). NFATc1 and NFATc2 have been shown to repress cyclin-dependent kinase 4 (Horsley *et al.* 2008) and cyclinA2 (Carvalho *et al.* 2007) expression, respectively. NFATc2 has also been demonstrated as a negative regulator of cyclin B1 and cyclin E in lymphocytes (Caetano *et al.* 2002). The nonoverlapping functions and apparent gene silencing effects of individual NFATc proteins further complicates our understanding of Cn/NFAT signaling. NFAT is clearly involved in maintaining a balance between cell quiescence and cell proliferation – the two fundamental biological mechanisms underlying tumorigenesis and atherosclerosis.

7. Conclusions

The Cn/NFAT signaling cascade is undoubtedly a major determinant of cell growth and differentiation. Regulation of Cn/NFAT signaling is a critical determinant of neoplastic and

cardiovascular risk. With DSCR1 now known as an endogenous regulator of Cn/NFAT signaling, several groups have successfully manipulated downstream gene expression and cell function by altering DSCR1 levels. The observation that DS patients exhibit significantly less tumor growth and arterial plaque formation cannot be mere coincidence, but rather evidence supporting the notion that these large-scale, vascular-related pathologies share an underlying mechanism. This is further reinforced by the fact that DSCR1 and other candidate antiangiogenic genes found on hChr21 are of growing attention. A more comprehensive analysis of hChr21-located genes may therefore provide direction as we continue to elucidate the mechanisms underlying tumorigenesis and atherosclerotic plaque formation.

8. References

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Part 2

Down Syndrome Models

8

Down Syndrome Model of Alzheimer's Disease: Beyond Trisomy 21Nondisjunction

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1. Introduction

Alzheimer's disease (AD), the most common type of dementia in old age, is a complex, multifactorial neurodegenerative disorder currently affecting 35,6 million people in either familial (genetic) or sporadic form, whose prevalence is expected to quadruple worldwide by the year 2050 (Alzheimer's Disease International, 2010; Ferri et al., 2005). While genetic or early-onset AD accounts for only 5% of all cases (Cummings, 2004), 95% of sporadic or late-onset AD is attributed to the interaction between advancing age, environmental factors (Grant, et al., 2002; Tanzi & Bertram, 2001), and to the few risk-enhancing genetic polymorphisms discovered so far (Bertram & Tanzi, 2004). Autosomal dominant mutations in the amyloid precursor protein (APP), presenilin 1 (PS1), and presenilin 2 (PS2) are linked to early-onset (familial) AD (FAD, Hardy, 2009). A combination of bio-psycho-social factors, especially if experienced at midlife, such as abnormal glucose and cholesterol metabolism, cerebral hypoperfusion, hypertension, physical and mental inactivity, diet, head injury, depression and small social networks are thought to contribute to sporadic AD initiation and development (reviewed in Flicker, 2010; Bendlin et al., 2010; Hughes & Ganguli, 2009). There is a temporal gap between clinical and pathological manifestation of Alzheimer's dementia; AD has a long prodromal phase and many clinically silent older adults are experiencing substantial pathological burden (Elias et al., 2000; Small et al., 2000). Common clinical manifestation of AD include memory impairment and dysfunctions in several cognitive domains such as language, problem solving, executive function, visuospatial skills, and others along with changes in personality and behavior (Maurer & Hoyer, 2006).

1.1 Neuropathology of Alzheimer's disease

The brain pathology of AD is characterized by extracellular deposits of the amyloid-beta peptide (Aβ), a main constituent of senile plaques, and intracellular accumulation of the hyperphosphorylated protein tau (p-tau) (Selkoe, 1999; 2001). The central hypothesis of AD, the amyloid cascade hypothesis, posits that an imbalance between Aβ production, aggregation and clearance from the brain is the initiating event in the disease process leading to synaptic loss, neuronal degeneration, inflammation, senile plaque formation and dementia (Blennow, de Leon, & Zetterberg, 2006; Hardy, 2006; Hardy & Selkoe, 2002; Lee et al., 2004; Potter et al., 2001).

The main constituent of the extracellular senile plaques is a highly neurotoxic Aβ42 isoform of Aβ protein, produced by consecutive action of two cleaving proteases, β- (BACE-1) and γ-

secretase, from a larger transmembrane protein, the Amyloid Precursor Protein (APP) (Hardy, 2009; Haass & Selkoe, 1993; Wolfe, 2003) within cholesterol rich membrane domains (Wahrle et al., 2002). A number of biochemical and genetic studies have indicated that oligomerization or polymerization of Aβ peptide driven by inflammation-induced proteins is a crucial step in AD pathogenesis (Potter et al., 2001; Hardy, 2006). Aβ42 self-aggregates and forms insoluble plaque deposits that include dystrophic neurites, activated microglia and reactive astrocytes (Itagati et al., 1989) which express a number of proinflammatory proteins (Akiyama et al., 2000; Potter et al., 2001). Furthermore, Aβ induces increased phosphorylation of the intracellular protein tau, resulting in destabilization of microtubules (MT) (Small & Duff, 2008). The evidence from transgenic mice and human studies indicates that not only extracellular, but also intracellular Aβ accumulates in diseased neurons and contributes to dementia progression by affecting the function of mitochondria, calcium ion channels and synapses (La Ferla, Green, and Oddo, 2007; Li et al., 2007). It is still unclear which toxic species of the Aβ peptide are most damaging to the neurons or whether the process of aggregation itself is detrimental to neuronal membranes.

Neurofibrillary tangles (NFTs) are the second major pathological hallmark in AD, which result from abnormally aggregated tau protein filling the intracellular space of the neurons. In AD brain, tau gets hyperphosphorylated, detaches from microtubules and forms paired helical filaments, which cause disruption in neuronal signaling, synaptic failure, impaired nutrient trafficking and neuronal death (Alonso et al., 2006; Grundke-Iqbal et al., 1986). Furthermore, oxidative damage as a consequence of free radical and reactive oxygen species (ROS) attacks has been observed in post-mortem AD brains in the form of oxidized lipids and proteins, mutated DNA, and mitochondrial damage (Floyd & Hensley, 2002). Formation of amyloid plaques and neurofibrillary tangles is not only associated with neuronal and synaptic loss, and depletion of neurotransmitters (Scheff, et al., 2006; Selkoe, 1999; 2004), but with abnormal axonal transport of key molecules and organelles important for neural cells survival and communication early in the disease process (Stokin et al., 2005; Stokin & Goldstein, 2006). Neurons, with their long axons, branched dendrites and large cell surface are especially vulnerable to energy and/or oxygen deprivation, impaired movement of molecules, and neurotoxins observed in AD pathology (Mattson & Magnus, 2006). The development of early diagnosis and successful treatments of AD will be greatly aided by a complete understanding of the pathological pathway that leads to formation of misfolded proteins, inflammation and neurodegeneration.

1.2 The Down syndrome model of Alzheimer's disease

The discovery that Down syndrome (DS) patients who live beyond the age of 30 or 40 develop neuropathology indistinguishable from the one observed in classic AD (Glenner & Wang, 1984; Olson & Shaw, 1969; Wisniewski et al., 1988) provided an important insight into AD pathogenesis, shifting the focus on nondisjunction of human chromosome 21 (HSA21) where the APP gene resides (Goldgaber et al. 1987; Neve et al., 1988; Petterson et al., 1988; Tanzi et al., 1987), and on the consequence of the gene overexpression in DS (Epstein, 1990). The fact that an extra copy of APP and subsequent 50% increase in gene dosage due to trisomy 21 in every cell of the body in DS individuals is sufficient to cause AD later in life instigated research on common biological links between AD and DS (Potter, 1991, 2008; Geller & Potter, 1999). Trisomy 21 results in altered APP processing and in an increased ratio of more amyloidogenic Aβ42 over Aβ40 (Teller et al., 1996), similar to the process observed in animal models of AD and in patients harboring mutations in FAD

genes, PS1, PS2 and APP (Haas & De Stooper, 1999; Hardy & Selkoe, 2002; Suzuki et al., 1994; Wolfe, 2003).

Several biochemical and genetic studies have shown that both sporadic and familial AD patients, including those carrying FAD mutations, are abnormal in one or more aspects of the cell cycle (Arendt et al., 1996; Geller & Potter, 1999; Potter, 1991; Varvel et al., 2008; Yang et al., 2001; 2006; Yang & Herrup, 2007; reviewed in Nagy, 2005; Obrenovic et al., 2003; Potter, 2004, 2008). The universal presence of AD pathology in DS individuals and the occurrence of an aberrant cell cycle in the brains of FAD mouse models and AD patients, led us to hypothesize that a slow accumulation of aneuploid, including trisomy 21 cells through defective mitosis and chromosome mis-segregation in central and peripheral tissues over the course of life of an individual could cause or at least help promote late-onset Alzheimer's (Potter, 1991; 2008). The extra copy of chromsome 21 that in full human trisomy of DS leads to neurodegeneration and dementia, could account for both genetic and sporadic AD, depending upon whether the chromosomal instability and mosaic aneuploidy was induced by a genetic (familial) mutation or by environmental insults. We further postulated that the microtubule disfunction likely responsible for the mitotic defects and genomic instability in AD could be linked to altered APP production and increased Aβ levels, probably affecting other aspects of neuronal physiology and function (Borysov et al., 2011; Granic et al., 2010; Potter, 2008).

The Down syndrome model and chromosome mis-segregation/microtubule disfunction hypothesis of AD made several predictions (Geller & Potter, 1999; Potter, 2008):

1. Alzheimer's patients should harbor a small number of aneuploid, including trisomy 21 cells in their somatic tissues. Altered genomic stability and development of trisomy 21 mosaicism would contribute to dementia onset and neurodegeneration but at slower pace than in DS due to the modulating effect of mostly disomic cells in the body.
2. Mutations that cause familial AD should occur in genes that encode proteins directly or indirectly involved in the cell cycle and chromosome segregation.
3. There should be alternations in microtubules, mitotic spindle apparatus and mitosis-related proteins in AD cells that could lead to aneuploidy, including trisomy 21 mosaicism.

In the past twenty years, compelling epidemiological and molecular evidence from our and other laboratories has been accumulated to test all three predictions. Together, the evidence suggests a link between pathological changes observed in the brains of DS and AD individuals and chromosomal instability and mosaic aneuploidy, including nondisjunction of HSA21 which likely contributes to dementia initiation and/or progression, with important implications for AD diagnosis and therapy.

2. The epidemiology of trisomy 21 mosaicism in Alzheimer's disease

Early epidemiological evidence indicating that chromosome mis-segregation and trisomy 21 mosaicism might be implicated in AD pathogenesis came for the studies showing a significantly higher number of Down syndrome offspring born in some families with FAD mutations (Heston et al., 1981; Heyman et al., 1983). The studies that failed to confirm this association reported to have too small sample sizes to observe statistically significant results (Amaducci et al., 1986; Chandra et al., 1987) suggesting that larger scale studies are needed to establish a connection between a higher frequency of DS children in families with genetic forms of AD. An important result that provided support for the trisomy 21 model of AD

came from a retrospective study of young mothers (aged <35) showing a five-fold greater risk of developing AD later in life compared to either older DS mothers or the general population (Schupf et al., 1994; 2001). Schupf and her colleagues interpreted this phenomenon as a novel form of 'accelerated aging'. In the light of our trisomy 21 model of AD, we postulated that the young DS mothers were most likely mosaic for chromosome 21 and had a predisposition for genomic instability, which resulted in DS progeny and their own increased risk of AD later in life. Indeed, a recent study by Migliore et al. (2006; 2009) confirmed the susceptibility to aneuploidy and trisomy 21 nondisjunction in young mothers of DS children. Case studies of patients with trisomy 21 mosaicism and no intellectual impairments of the DS type who developed AD by the age of 40 demonstrated that a small percentage of chromosomal instability is sufficient to result in early-onset AD (Hardy, et al., 1989; Ringman et al. 2008; Schapiro et al., 1989). Similarly, a slow accumulation of a low number of trisomy 21 cells over the life span may lead and/or contribute to the pathogenesis of both genetic and sporadic form of AD.

In order to directly test if Alzheimer's patients harboured mis-segregated including trisomy 21 cells, we and others have used fluorescence *in situ* hybridization (FISH), a cytogenetic technique that allows one to detect the number of copies of a particular chromosome in both metaphase and interphase cells (Ried, 1998) with great sensitivity and specificity. This method is especially suitable for poorly and non-dividing cells, or for the cells with a very low level of aneuploidy (e.g., lymphocytes). In our early study of chromosome mis-segregation in AD, we found more than twice the frequency of trisomy 21 in skin fibroblasts of AD patients ($p=0.007$) compared to age-matched controls, which was not related to the age of affected individuals (Figure 1, Geller & Potter, 1999). A small parallel study of chromosome 18 nondisjunction showed a similar increase in aneuploidy, indicating that the mitotic defect likely affected all chromosomes. The AD fibroblasts in our study included those with sporadic (late-onset) AD and those carrying a familial AD mutation in PS1, PS2, or APP which are now known to cause early AD onset and altered APP processing (e.g., Li et al., 1995; Schellenberg et al., 1993; Rogaev et al. 1995).

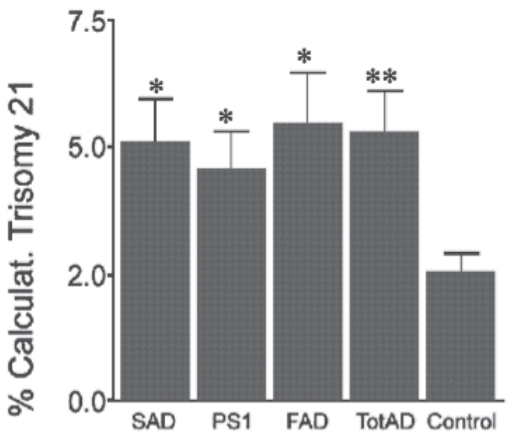

Fig. 1. AD fibroblasts, including those with PS1 or PS2 mutation (FAD) and those of sporadic origin (SAD) showed increased trisomy 21, *$p < 0.01$; **$p < 0.001$. (Used with permission from Geller & Potter, 1999, *Neurobiology of Disease).*

Trisomy 21 mosaicism and chromosome mis-segregation have also been observed among peripheral blood lymphocytes (Migliore 1997; 1999; Trippi et al., 2001), buccal cells (Thomas & Fenech, 2008), and neurons from sporadic AD patients (Arendt et al., 2010; Iourov et al., 2009; Mosch et al., 2007; Yang et al., 2001). Specifically, AD brains harbor up to 35% of hyperploid, including trisomy 21 and other tetrasomic neurons which are detectable at mild stages of the disease before any evident neuronal loss (Arendt et al., 2010; Iourov et al. 2009). The presence of tetrasomic neurons as explained by some reseachers, may indicate re-entry into an incomplete cell cycle and selective vulnerability to cell death of post-mitotic, mosaic neurons as an important pathogenic event in AD (Arendt et al., 2010; Obrenovich et al., 2003; Varvel et al., 2008; Vincent et al., 1996; Yang & Herrup, 2007). In our studies of AD mosaicism (Boeras et al., 2008; Geller & Potter, 1999; Granic et al., 2010), we failed to observe statistically significant induction of tetrasomic cells in sporadic and familial models of AD, but rather pronounced accumulation of trisomy cells in both peripheral and brain tissues. A larger scale cytogenetic autopsy and biopsy study, preferably at the single cell level is needed to further elucitate the type and extent of pathologic genetic instability in AD and other neurodegenerative diseases.

In summary, several laboratories confirmed that trisomy 21 mosaicism is commonly associated with AD. Moreover, there is an indication of a dose response effect in which full trisomy 21 in DS individuals elicits AD-like pathology by age 20 and by middle age in familial AD and even later in sporadic Alzheimer's patients, who also belonged to the trisomy 21 mosaic group found to have an increased frequency of DS children before the age of 35. The recent discovery of families that develop early-onset inherited AD only because the APP gene on one chromosome 21 is duplicated (McNaughton et al., 2010; Rovelet-Lecrux et al., 2006; Sleegers et al., 2006) indicates that the extra copy of the APP gene and consequent overproduction of Aβ peptide is the likely cause of AD in both Down syndrome and trisomy 21 mosaic individuals.

2.1 Mitotic defects in Alzheimer's disease

Along with the studies described above, separate lines of investigations provided independent evidence that other mitotic defects and mitosis-specific proteins may be present in the cells of AD patients (reviewed in Potter, 2004). For instance, the mitotic spindles in dividing AD cells exhibit abnormalities and susceptibility to premature centromere division (PCD) and micronucleation upon chemically induced (e.g., colchicines) microtubule damage (e.g., Fitzgerald et al., 1986; Potter et al., 1995; Trippi et al., 2001). The event of PCD, in which individual sister chromatids are separated by a clear gap and not connected at the centromeres, has been observed in patients prone to genomic instability and chromosome mis-segregation, and recently confirmed in neurons of individuals with sporadic AD (Spremo-Potparević et al., 2008).

Further evidence linking cell cycle defects with AD pathogenesis came for the finding that both APP and the microtubule-stabilizing tau protein get increasingly phosphorylated during mitosis (Suzuki et al., 1994; Pope et al., 1994; Padmanabhan et al., 2011). Also, phospho-tau and other mitosis-specific phospho-proteins are overexpressed in AD but not in normal brains (e.g., Arendt et al., 1996; Vincent et al., 1996; Nagy et al., 1997), indicating a cycling stage of AD neurons. The idea of unscheduled cycle in fully differentiated neurons challenged the dogma of their post-mitotic nature and the inability to replicate (Rakic, 1985). To date, the evidence drawn from animal models of AD and autopsy studies of human brains indicates that reactivation of the cell cycle and DNA duplication (e.g., Yang et al.,

2001) in the vulnerable population of AD neurons may present a fundamental initiator of AD pathogenesis (Nagy, 2005; Vincent et al., 1996; Varvel et al., 2008; Yang & Herrup, 2007; Yang et al., 2006) present before deposition of fibrilar Aβ and neuronal death, and could also lead to chromosome mis-segregation and aneuploidy.

3. Presenilin and APP mutations induced aneuploidy

The finding that fibroblast cultures from patients carrying autosomal dominant mutations in the PS1, PS2 or APP gene harbour chromosomal instability and trisomy 21 mosaicism provided the first indication that FAD genes are likely to be involved in mitosis and chromosome mis-segregation, or to be associated with structures and proteins of the cell cycle (Geller & Potter, 1999).

Indeed, we and others have confirmed a major location of PS1 and PS2 proteins in dividing cells in the centromeres, the nuclear envelope, and the kinetochores during interphase (Li et al., 1997; Honda et al., 2000). Further support for a mitotic function of presenilins comes from studies showing inhibition of the cell cycle (Janicki & Montero, 1999) and increased sensitivity to apoptosis (Vito et al., 1996; Wolozin et al., 1996) in the cells carrying a mutated PS gene. Furthermore, polymorphisms in the PS1 gene have been associated with an increased risk of AD (Wragg et al., 1996; Higuchi et al., 1996; Scott et al., 1996) and with an increase of DS offspring via a meiosis II defect (Petersen et al., 2000; Lucarelli et al., 2004) as a more direct confirmation of PS1 involvement in the cell cycle.

Further research should be directed at discovering the mechanism by which the mutant presenilins influence chromosome segregation. One possibility supported by the data collected so far points to inability of altered presenilin proteins to properly link the chromosomes to the nuclear envelope and to release them at the appropriate time during mitosis, which may lead to chromosome mis-segregation and other cell cycle abnormalities. Another possibility discussed further below links PS mutations to altered processing of APP and increased production of neurotoxic Aβ42 as a likely effector molecule responsible for cell cycle defects, including mitotic spindle abnormalities and chromosome mis-segregation (Boeras et al., 2008; Borysov et al., 2011, Granic et al., 2010). Similarly, APP is also found to localize to the centrosomes and nuclear membrane in dividing cells (Nizzari et al., 2007; Zitnik et al., 2006), and to get increasingly phosphorylated during the cell cycle (Padmanabhan et al., 2011).

In a series of *in vivo* and *in vitro* experiments we investigated the role of mutated PS1 and APP genes and their proteolytic product, the Aβ peptide in chromosome mis-segregation and trisomy 21 mosaicism. All assays, tissues and cells from transgenic mice carrying AD mutations and the cells transfected with FAD genes or treated with Aβ peptide yielded comparable results: overexpression of FAD genes *in vivo* and *in vitro* and exposure to Aβ peptide induced chromosome instability and trisomy 21 mosaicism through several defects in mitotic spindle apparatii and dysfunction of microtubule assembly (Boeras et al., 2008; Borysov et al., 2011; Granic et al., 2010; Potter et al., 2008).

3.1 Presenilin and APP mutations induced aneuploidy in transgenic mice

We asked whether chromosome mis-segregation and trisomy 21 mosaicism observed in human fibroblasts with PS1 or APP mutations could be mimicked in peripheral and brain tissues of FAD-transgenic mice. For example, whole brains from PS1 (M146l and M146V) and APP (V717) mutant mice and non-transgenic littermates were processed to yield

primary cultures. The isolated neurons were hybridized with a mouse chromosome 16 BAC probe (Kulnane et al., 2002), followed by immunocytochemistry to stain for neurons. Most cells were disomic with two copies of chromosome 16; while the neurons from PS1 mutant and PS1 knock in mice exhibited up to 4% of trisomy 16 (data not shown, Boeras et al, 2008), the APP-transgenic mice had about 6.5% of trisomies (Figure 2, Granic et al., 2010).

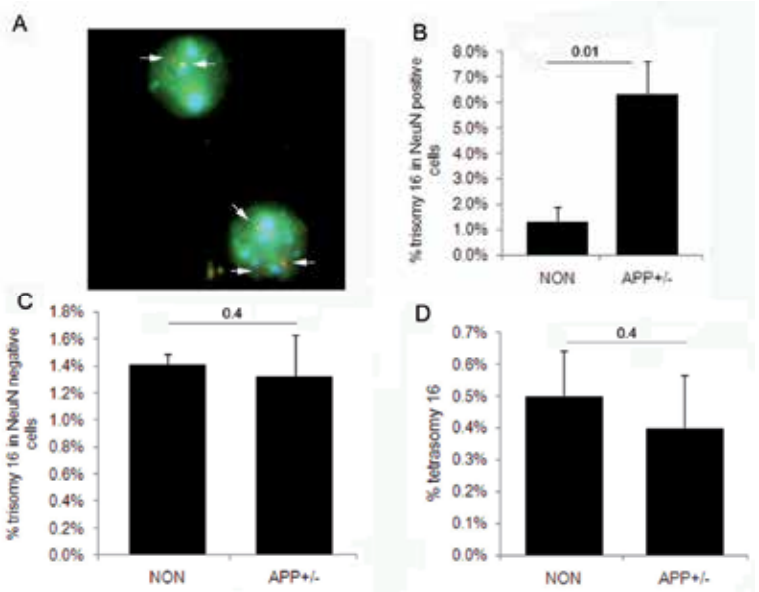

Fig. 2. Quantitative FISH analysis revealed significantly higher levels of trisomy 16 in APP mice (A & B) compared to controls but no tetrasomy (D) and no induction of aneuploidy in non-neuronal cells (C). (Used with permission from Granic et al., 2010, *Molecular Biology of the Cell*).

3.2 Presenilin and APP mutations induced aneuploidy in transfected cells

To determine whether the aneuploidy observed in FAD-transgenic and knock-in mice was caused directly by mutated genes and not by some other factors, parallel cultures of the hTERT-HME1, an immortalized primary mammary epithelial cell line with a stable karyotype (Clontech) were transiently transfected with WT-PS1, mutant PS1 (M146L), mutant APP (K595N/M596L and V642I or V717) and control empty vector (pcDNA3 and paG3). FISH was used to assess the levels of aneuploidy for chromosome 21 and 12. Overexpression of FAD-genes induced between 2-3% of trisomy 21 and/or trisomy 12 (Boeras et al., 2008; Granic et al., 2010), and about 30% of total aneuploidy in metaphase cells within 48 hours (Boeras et al., 2008). These results indicated that an aneugenic effect of FAD-mutations likely affected all chromosomes with random gains and losses of whole chromosomes, and that chromosome mis-segregation was not restricted only to the cells expressing mutated genes, but extended to nearby, non-transfected cells. We hypothesized that Aβ peptide itself found at increased levels in both familial and sporadic AD might be the probable effector molecule interfering with mitosis and chromosome segregation (Boeras et al., 2008; Potter, 2008). Lastly, immunocytochemistry of PS1-transfected cells revealed several abnormalities in the mitotic spindles, with disarrayed microtubules, multiple

centosomes and lagging chromosomes as the most prominent spindle malformations (Boeras et al., 2008).

3.3 Aβ induced aneuploidy and the role of tau

Sequential cleavage of the APP protein with β- and presenilin/γ-secretase enzymes yields more amyloidogenic Aβ42 peptide as a central event in AD pathogenesis. We proposed to test the role of Aβ in genomic instability and trisomy 21 induction. Led by our initial observation that more cells became aneuploid than are transfected and express FAD genes, we examined the aneugenic effect of Aβ peptide in culture. hTERT-HME cells treated with 1μM Aβ40 and Aβ42 develop more than 20% aneuploid metaphases and about 2% trisomy 21 and 12 cells within the 48 hours of exposure compared to 6% and less than 1% of the cells treated with various control peptides (Figure 3, Granic et al., 2010). These results indicated that AD might be a self-propagating disorder in which the product of FAD mutations and trisomy 21, the Aβ peptide, further induces chromosome segregation and generation of trisomy 21 cells.

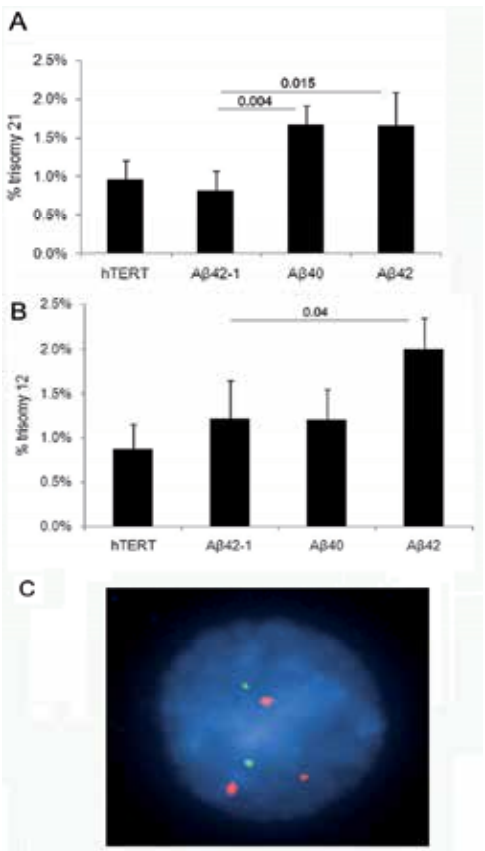

Fig. 3. Quantitative FISH analysis with a dual color probe revealed an increased induction of trisomy 21 (SpectrumOrange, Vysis) and trisomy 12 (SpectrumGreen) in Aβ treated hTERT-HME cells (A-C). (Used with permission from Granic et al., 2010, *Molecular Biology of the Cell*).

To investigate the mechanism by which Aβ exerts its aneugenic effect on dividing cells, we analyzed the peptide's other toxic activities especially those related to microtubule function. Several lines of investigation have indicated that Aβ induces and requires downstream changes/defects in microtubules (MT) to exert its neurodegenerative activity. Numerous *in vitro* and *in vivo* studies have shown that Aβ induces phosphorylation of tau (e.g., Small & Duff, 2008), and that Aβ toxicity depends on the presence of tau (Rappaport et al., 2002). Therefore, we investigated the role of tau in Aβ induced aneuploidy. Splenocytes prepared from normal, Tau+/-, Tau -/- mice were treated with Aβ peptide and analyzed for aneuploidy 48 hours later. Knocking out one or even more effectively both copies of Tau induced up to 5% aneuploidy (trisomy 16). However, the Aβ aneugenic effect was greatly attenuated in the cells lacking Tau but not in normal cells, indicating that Aβ induced chromosome mis-segregation requires tau protein and disrupts normal tau-stabilizing microtubule function. In a series of studies, Borysov et al. (2011) have shown that Aβ42 peptide added to *Xenopus* egg extracts impairs the structure and stability of mitotic spindles, and inhibits three motor kinesins, Eg5, KIF4A, and MCAK, required for normal mitotic spindle function and proper chromosome segregation.

Recently, we have shown that overexperssion of APP prevents the localization of low density lipoprotein receptor (LDLR) from the Golgi to the cell membrane (Abisambra et al., 2010). This latest finding led us to hypothetize that neurons exposed to Aβ in AD brains may also fail to localize other key receptors to the cell membrane, including those for neurotrophins and neurotransmitters, causing neuronal disfunction and dementia. Future studies are under way to confirm if the interference with microtubule function by Aβ will cause defects in neuroplasticity through mis-localization of the receptors away from the plasma membrane, as well as contributing to defective neurogenesis leading to dysfunctional, aneuploid, including trisomy 21 neurons prone to Aβ overproduction and neurodegeneration.

4. How trisomy 21 mosaicism may lead to Alzheimer's disease

Several potential mechanisms could explain how trisomy 21 mosaicism could lead to AD (Potter, 1991; 2004; 2008). For instance, aneuploidy cells might be prone to cell death and neurodegeneration (Arendt et al., 2010), similar to cortical neurons in DS brains that undergo spontaneous apoptosis (Busciglio & Yankner, 1997). Apoptosis could also indirectly affect APP processing and Aβ levels in mosaic AD brain. The support for the latter hypothesis comes from the finding that embryonic DS brains and adult sera contain a higher ratio of neurotoxic Aβ42 over Aβ40 peptide (Teller et al., 1996). Also, trisomy 21 microglia overexpress inflammatory proteins and begin an inflammatory cascade that promotes Aβ fibrilization (Potter et al., 2001). Finally, aneuploidy in AD may arise from a defect in microtubule function which may lead to poor protein, neurotransmitters and nutrient trafficking (Cash et al., 2003). Trisomy 21 may be both a cause and an effect of microtubule dysfunction generating a feed-forward loop further promoting AD progression.

5. Implications of the trisomy 21 model of Alzheimer's disease for diagnosis and therapy

The mechanistic implication of the results discussed in this review is that an early step in Alzheimer's disease pathogenesis may be the development of genomic instability and

trisomy 21, contributing to progression of dementia. The search for more effective diagnoses should take into account the events of mitotic defects in peripheral and central tissues of individuals at risk. Chromosome analysis and detection of low levels of trisomy 21 in skin fibroblasts or buccal cells in patients during the pre-clinical phase of dementia could be a potential diagnostic test. Another implication of the data presented above that trisomy 21 in AD may be the initiating event in disease pathogenesis also suggests new approaches to treatments (Potter, 2004). For example, drugs that would repair the mitotic defects and strengthen the fidelity of the chromosome segregation could be searched for and used prophylactically. Further, aneugenic environmental agents that cause chromosome instability could be identified and counteracted with drugs that restore genomic homeostasis. Another more difficult but equally effective approach to therapy would be to detect and remove mis-segregated cells from the body by exploiting their unique cell biology and/or gene expression.

To summarize, the results from several laboratories over the past twenty years have shown that Alzheimer's patients are prone to genomic instability accumulating about 2-3 fold more trisomy 21 cells throughout the body compared to age matched healthy controls. The precise mechanism by which these abnormal cells arise during the life span of an individual and how or whether they contribute to disease initiation and progression are subjects of active investigation. The better understanding of these novel findings has the potential to contribute to the development of future diagnoses and therapies for Alzheimer's disease.

6. Summary

Convincing epidemiological and molecular evidence has been accumulating that link pathological changes observed in the brains of both Down syndrome individuals and neurodegeneration in Alzheimer's disease to chromosomal instability and trisomy 21 mosaicism. The results from several laboratories indicate that errors in mitosis, specifically mis-segregation of somatic chromosomes in peripheral and brain tissues of AD patients may play an important role in both early (familial) and late (sporadic) onset of disease. Here, we proposed a unifying hypothesis for Alzheimer's and Down syndrome neurodegeneration— development of a mosaic population of aneuploid, including trisomy 21 cells and alternation in genomic stability may lead to classic AD neuropathology observed in both diseases. The evidence for this hypothesis include: a) cells from familial and sporadic AD patients exhibit mis-segregated, including trisomy 21 cells in brain, blood, mucosa and skin, and harbour abnormalities in several aspects of the cell cycle that may contribute to aneuploidy and neurodegeneration; b) overexpression of mutated Alzheimer's genes, presenilin 1 and APP, in cellular and transgenic mouse models induce aneuploidy, including trisomy 21; and c) Aβ peptide is the likely effector molecule responsible for disruption of proper functioning of microtubules and mitotic spindle integrity leading to mitotic defects and apoptosis. The possibility that many cases of Alzheimer's disease are mosaic for trisomy 21 opens new approaches for diagnosis and therapy.

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9

Deficiency of Adult Neurogenesis in the Ts65Dn Mouse Model of Down Syndrome

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1. Introduction

Neurogenesis in the mammalian adult brain is a well established phenomenon (for the recent reviews see Carpentier & Palmer, 2009; Kaneko & Sawamoto, 2009; Rodriguez & Verkhratsky, 2011) that is important in both young and aging brain (Galvan & Jin, 2007; Rao et al., 2006). The subventricular zone, subgranular layer of the dentate gyrus (DG), and cortex are the main sites of adult neurogenesis (Gould et al., 1999; Luskin & Boone, 1994; Palmer et al., 2000; Seki et al., 2007; Yoneyama et al., 2011). Newly-born neurons during adult neurogenesis have the ability to integrate into previously established neuronal networks (Kee et al., 2007; Markakis & Gage, 1999; Sandoval et al., 2011). Alteration of neurogenesis under different experimental and pathological conditions has been described to a great extent (Rodriguez & Verkhratsky, 2011; Sandoval et al., 2011; Winner et al., 2011; Yoneyama et al., 2011; Yu et al., 2009). Significant decreases of neurogenesis have been found in neurodevelopmental (Contestabile et al., 2007; Guidi et al., 2008, 2010) and in neurodegenerative diseases (Rodriguez & Verkhratsky, 2011). Numerous studies provide evidence that a lack of neurogenesis significantly diminishes plasticity in the adult brain and interferes with learning and memory (reviewed in Koehl & Abrous, 2011; Mongiat & Schinder, 2011). Different mechanisms have been proposed to regulate neurogenesis in the adult brain, including brain injury (Kernie & Parent, 2010; Moriyama et al., 2011), ischemia (Kernie & Parent, 2010; Kreuzberg et al., 2010), and inflammation (Volobouéva et al., 2010).
One of the least studied factors that affects neurogenesis are chromosomal aberrations. Down syndrome (DS) results from the extra copy of chromosome 21 occurring with a prevalence of 1 in 733 live births (Canfield et al., 2006). Subjects with DS show developmental regression, diminished cognitive ability, and autonomic dysfunction (Antonarakis & Epstein, 2006; Chapman & Hesketh, 2000). The DS brain is severely affected showing a reduction in both overall size and of particular areas (frontal cortex, hippocampus, cerebellum, and brainstem) due to a reduced number of neurons (Aylward et al., 1997, 1999; Kesslak et al., 1994; Pinter et al., 2001; Raz et al., 1995; Wisniewski et al., 1984).

1.1 Neurogenesis in subjects with DS

Very little data is available describing neurognesis in subjects with DS. In hippocampal DG and neocortex of fetuses with DS the number of proliferating cells was found to be significantly reduced (Contestabile et al., 2007). These reductions were mainly due to a

reduced number of cycling cells in S phase (Contestabile et al., 2007). Both a reduction in neurogenesis and significantly higher incidence of apoptotic cell death in the hippocampal region of fetuses with DS has been noted (Guidi et al., 2008). Also, trisomy 21 significantly modify cell phenotypes producing higher numbers of cycling cells going to a glial phenotype and less cells expressing neuronal markers (Guidi et al., 2008). Detailed analysis of the consequences of both reduced neurogenesis and increased apoptosis in fetuses with DS, revealed a severe reduction in the volume and cell number of investigated brain areas (Guidi et al., 2008). More recently, significant neurogenesis impairments were also revealed in the cerebellum of fetuses with DS (Guidi et al., 2010). However, apoptotic cell death was similar in the cerebellum in fetuses with DS vs. controls (Guidi et al., 2010). The differences between the levels of apoptosis in hippocampus vs. cerebellum in fetuses with DS points to different mechanisms for the reduced cell number in hippocampus and cerebellum in DS. For the hippocampus, a reduction of neurogenesis, increased apoptosis and changing cell phenotypes in favor of glia may underlie reduced cell number in fetuses with DS. As for the cerebellum, only a reduction in neurogenesis may be the main factor in decreased cerebellar volume in fetuses with DS.

1.2 Neurogenesis in mouse models of DS

Taking to account that this subject is very important for the neurobiology of DS, only a few papers addressed neurogenesis in mouse models of DS (Bianchi et al., 2010; Chakrabarti et al., 2007; Clark et al., 2006; Contestabile et al., 2007; Lorenzi & Reeves, 2006; Rueda et al., 2005). It was noted that in prenatal and newborn Ts65Dn mice neurogenesis was significantly reduced (Chakrabarti et al., 2007; Contestabile et al., 2007; Lorenzi & Reeves, 2006). At postnatal day 2 cell proliferation in Ts65Dn mice, assessed 2 hours after single injection of bromodeoxyuridine (BrdU) was significantly reduced (Contestabile et al., 2007); similar results were revealed at postnatal day 15 (Bianchi et al., 2010). However in young adult Ts65Dn mice the results are conflicting (Clark et al., 2006; Rueda et al., 2005). Similar procedures in young (3-5 months) Ts65Dn mice shows no difference in hippocampal cell proliferation and survival (Rueda et al., 2005). However, in aged (13-15 months) Ts65Dn mice, cell proliferation in the hippocampus (Rueda et al., 2005) and in the subventricular zone (Bianchi et al., 2010) were significantly reduced. Finally, Clark et al. (2006) found a difference in DG neurogenesis in young (2-5 months) Ts65Dn mice.

It seems that genetic dissections of mouse partial trisomy 16 (Ts65Dn model) did not restore reduced neurogenesis. Indeed, Ts1Cje and Ts2Cje mouse models of DS showed a similar degree of impairment in adult neurogenesis (Hewitt et al., 2010; Ishihara et al., 2010).

To solve these conflicting results in DS mouse models a recent study was initiated to examine young adult neurogenesis, and we also expanded this study to include DG, cortex and corpus callosum (CC) at three different levels. We chose to investigate whether or not trisomy has any affect on neurogenesis in Ts65Dn mice. To carry out this work we injected BrdU in 2N and Ts65Dn mice. Combining BrdU immunoreactivity and high resolution confocal microscopy, we examined the number of BrdU-positive (BrdU+) neurons in DG, cortex and CC at three different levels: rostral, middle and caudal. Comparing 2N and Ts65Dn mice, we discovered significant decreases in the density of BrdU+ cells in trisomic mice in the cortex and DG. We found that the number of cells per cluster was significantly reduced in both the rostral and the caudal parts of DG in the Ts65Dn vs. 2N. This change resulted in reduced cell density in the caudal DG, while the density of clusters was not

altered. Our findings are evidence for decreased neurogenesis in young adult cortex and DG of Ts65Dn mice and indicate neurogenesis as a potential target for pharmacological intervention to avert neuronal plasticity in individuals with DS.

2. Materials and methods

All experiments were conducted in accordance with the National Institutes of Health guidelines for the care and use of animals and with an approved animal protocol from the Stanford University Institutional Animal Care and Use Committee. All efforts were made to minimize animal stress and discomfort.

2.1 Mice husbandry

Details of maintenance and using Ts65Dn mouse colony were described previously (Belichenko et al., 2004). The Ts65Dn mouse colony was maintained for more than 10 generations by crossing B6EiC3Sn-Ts(17^{16})65Dn females (Jackson Laboratory, Bar Harbor, ME) with B6EiC3Sn F1/J A/a males (Jackson Laboratory). This breeding scheme was used because trisomic mice breed very poorly or not at all when inbred; the B6C3 background has been the most successful. To distinguish 2N from Ts65Dn mice, genomic DNA was extracted from tail samples. A quantitative PCR protocol (provided by The Jackson laboratory) was used to measure Mx1 gene expression, which is present in three copies in Ts65Dn. Each mouse was genotyped twice. Male littermates mice were used in all studies at ages 2-3 months.

2.2 BrdU injections and immunofluorescent staining

In the beginning of experiment we weighed mice. Each animal was injected once a day for 6 consecutive days with 5-bromo-2′-deoxyuridine (BrdU, 10 mg/ml in saline, i.p.) (Cat. # B9285, Sigma-Aldrich Corp., St. Louis, MO, USA) to a final dose of 50 mg/kg body weight. Twenty four hours after the last injection, animals were deeply anesthetized with sodium pentobarbital (200 mg/kg i.p.) (Abbott Laboratories, North Chicago, IL, USA), again weighed, and transcardially perfused for 1 minute with 0.9% sodium chloride (10 ml) and then for 20 minutes with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4 (200 ml). After perfusion, the brain was immediately removed and postfixed for another 24 hours. The weight of the entire brain (including the olfactory bulbs, cerebellum, and brainstem along with the cervical spinal cord through C1–C2) was recorded. The brain was then sectioned coronally at 100-μm with a Vibratome (series 1000, TPI Inc., St. Louis, MO, USA), and sections were placed in 0.9% NaCl solution. Immunofluorescence method with anti-BrdU antibody was used to reveal BrdU+ cells as follows. Free-floating sections were rinsed twice in saline (0.9 % NaCl) for 10 min at RT, and then pretreated with 1M HCl for 30 minutes at 37°C. Sections were neutralized by rinsing 3 times in PBS and then pre-incubated in a solution of 0.1 M PBS containing 5% non-fat milk and 0.3% Triton-X 100 (PBS+), for 1 hour at room temperature. Then sections were incubated overnight at 4°C with primary rat anti-BrdU antibody (Cat. #MCA2060, Serotec, Raleigh, NC, USA) at a dilution 1:100 in PBS+. Sections were rinsed in PBS (20 minutes, three changes) and incubated for 1 hour at room temperature with biotinylated donkey anti-rat IgG (1:200; Jackson ImmunoResearch Labs, West Grove, PA). After being rinsed with PBS (20 minutes, three changes), sections were incubated with FITC-conjugated streptavidin (1:500; Jackson

ImmunoResearch Labs, West Grove, PA) for 1 hour at room temperature. Following further careful rinsing, the sections were mounted on microscope glass slides and coverslipped with 90% glycerol in PB.

2.3 Confocal microscopy imaging, cell quantification, cluster and statistical analyses

To study cell proliferation and clustering in 2N and Ts65Dn mice we used confocal microscopy imaging of BrdU+ cells in the cortex, hippocampus and CC. Confocal imaging of brain slices labeled with one fluorophores was performed as previously described (Belichenko et al., 2004). In brief, slices with BrdU+ cells were examined and scanned in a Radiance 2000 confocal microscope (BioRad, Hertfordshire, United Kingdom) attached to a Nikon Eclipse E800 fluorescence microscope. LaserSharp software (Bio Rad) was used to establish optimal conditions for collecting images. The laser was an argon/krypton mixed gas laser with exciting wavelengths for FITC (488 λ). The emission was registered with HQ515/30 "green" filter. Sections with FITC staining of BrdU immunoreactivity were studied under the following optimal conditions: the lens was a x10 objective (Nikon; Plan Fluor x10/0.30); laser power was 10%; the zoom factor was 1; scan speed was 500 lines per second; each optical section was the result of 3 scans followed by Kalman filtering; the size of the image was 512 x 512 pixels (i.e., 1208 x 1208 μm).

LazerPix software (Bio Rad) analysis of confocal images was used to measure the area or length of investigated brain structures, and for quantitative analysis of BrdU+ cells. Three slices per mouse (500 μm apart) were used. The location of each coronal section was designated based on its relative position to bregma in mm (Hof et al., 2000). Rostral section was corresponded to bregma -1.60 mm level, middle section – to bregma -2.10 mm level, and caudal section to bregma -2.60 mm level. Entire DG at these three different locations was imaged. CC was imaged from midline and then for 1200 μm to the left or right extension at the same three different locations. Based on mouse brain atlas (Hof et al., 2000), cortex were correspondent to motor cortex at rostral and middle sections and to visual cortex at caudal section). Images from the both the left and right side for each mouse brain were analyzed. To count number of BrdU+ cells, we first outlined the area of interest and measure area or length (in a case of DG). Next, for each image, the intensity thresholds were estimated by analyzing the distribution of pixel intensities in the image areas that did not contain immunoreactivity. This value, the background threshold, was then subtracted and number of BrdU+ cells was counted. In addition each BrdU+ cells were traced manually and their XY coordinates were exported to Excel. The number of BrdU+ cell in each hemisphere was expressed for DG as per 100-μm length of neurogenic subgranule zone, and for cortex and CC as per 1 mm^2. We also counted the occurrence of BrdU+ cell clusters. To quantify the number of clusters we used previously established criteria (Palmer et al., 2000), where cluster was defined as a contiguous group of cells separated by less than 25 μm. Special macro was design in Excel program for cluster counting. For every section, X and Y coordinates of individual BrdU+ cell were recorded using LaserPix software (BioRad). Distance between all pairs of the BrdU+ cells within one section were calculated using an Excel Microsoft macro, and the cells with distances < 25 μm were regarded as belonging to a cluster. All analyses were performed by the same investigator blinded to mice genotype.

Digital images were imported, enhanced for brightness and contrast, assembled and labeled in Adobe Photoshop CS2 9.0.2, and archived. Figures were printed with a Phaser 7300 color printer (Tektronix by Xerox, Norwalk, CT).

The data for body and brain weights, total number of BrdU+ cells, their coordinates and cluster evaluations were exported to Excel (Microsoft, Redmond, WA) and statistical comparisons were performed using two-way analysis of variance (ANOVA) and for two samples using two-tailed Student's *t*-tests. All results are expressed as mean ± SEM, and *P* values < 0.05 were considered to be significant.

3. Results

3.1 Body and brain weights

The body weight of 2N and Ts65Dn mice at 2-3 months was similar (2N: 31.1 ± 1.6 g, n = 6; Ts65Dn = 26.7 ± 2.3 g, n = 5; *P* = 0.14). The brain weight was also unchanged (2N: 0.43 ± 0.01 g, n = 6; Ts65Dn = 0.43 ± 0.01 g, n = 5; *P* =0.60). Of note, there was no effect of the BrdU injection on changes in body weight during 6 day injections since no significant differences were found between 2N and Ts65Dn mice (2N: -0.60 ± 0.29 g, n = 6; Ts65Dn = -0.20 ± 0.51 g, n = 5; *P* =0.49). These mice also showed no aversive or any other neurological symptoms during injection.

3.2 Changes in BrdU+ cells number and in cell cluster density in Ts65Dn mice

We examined the density of BrdU+ cells in DG of hippocampus, cortex and CC in 2N and Ts65Dn male mice (Table 1, Figs. 1, 2). The BrdU+ cells were bright fluorescent, allowing for a detailed quantitative analysis by confocal microscopy (Figs. 1a, 2a). For this analysis, the number of BrdU+ cells per 100 μm length of subgranule zone for DG or per 1 mm^2 area of cortex or CC was counted to define cell density (Table 1). In addition we measured the number of cell cluster, the number of BrdU+ cells per cluster and the frequency distribution of BrdU+ cells per cluster (Table 1; Figs. 1 d,e; 2 d,e).

Comparing BrdU+ cell densities in 2N and Ts65Dn mice, there was a statistically significant decrease in the caudal part of DG (by 27%) and in the rostral part of motor cortex (by 36%; *P* < 0. 01; Table 1). Interestingly, BrdU+ neurons in the inferior blade were on average 30% fewer than in the superior blade for 2N, and 23% fewer for Ts65Dn mice (*P* < 0.01; Table 1). BrdU+ cell density were similar for CC in 2N and Ts65Dn mice, and 33% more in caudal then rostral CC for 2N mice (*P* < 0.01; Table 1).

Comparing BrdU+ cell cluster densities in 2N and Ts65Dn mice, there was a statistically significant decrease in the rostral part of motor cortex (by 29%; *P* < 0. 01; Table 1). In the inferior blade there were on average 9% fewer than in superior blade for 2N, and 25% fewer for Ts65Dn mice (*P* < 0.05; Table 1). Interestingly, in the DG the BrdU+ cell cluster density was not different in 2N and Ts65Dn mice (Table 1).

We next quantified the number of cells per cluster (Table 1). As compared to 2N mice, significantly lower numbers of BrdU+ cells per cluster were present in rostral (mainly due to superior blade, *P* < 0.003; Table 1) and in the caudal part of DG in Ts65Dn mice (*P* = 0.03; Table 1), and in the rostral part of motor cortex in Ts65Dn mice (*P* < 0.01; Table 1). Comparing the superior and inferior blades in 2N mice, numbers of cells per cluster were ~1.5 times higher in the rostral and middle parts of the superior blade of the DG in 2N mice (*P* < 0.02; Table 1).

In conclusion, significant differences in the number of BrdU+ cells, cell cluster densities, and in number of BrdU+ cells per cluster were evident for Ts65Dn mice as compared to their 2N cohorts. These findings are evidence for reduced neurogenesis in both DG and cortex due to mouse chromosome 16 trisomy.

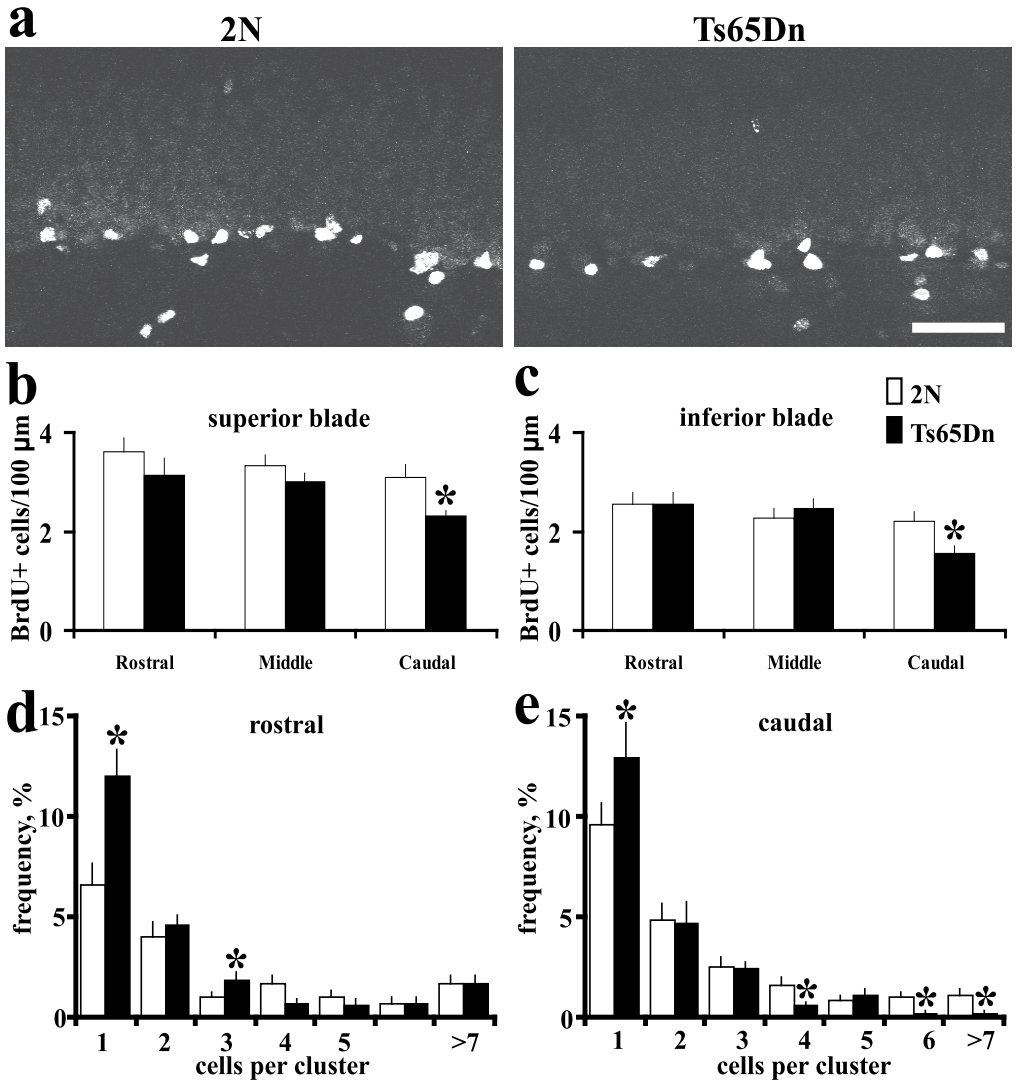

Fig. 1. Morphometry of BrdU+ cells in dentate gyrus in 2N and Ts65Dn mice. (a) Confocal image of BrdU+ cells in caudal part of dentate gyrus of 2N and Ts65Dn mice. BrdU+ cell density in superior (**b**) and inferior blades (**c**) of dentate gyrus. Note a significant reduction of BrdU+ cell in caudal DG in Ts65Dn mice. The number of cells per cluster in rostral (**d**) and caudal (**e**) part of DG. Scale bar = 50 µm.

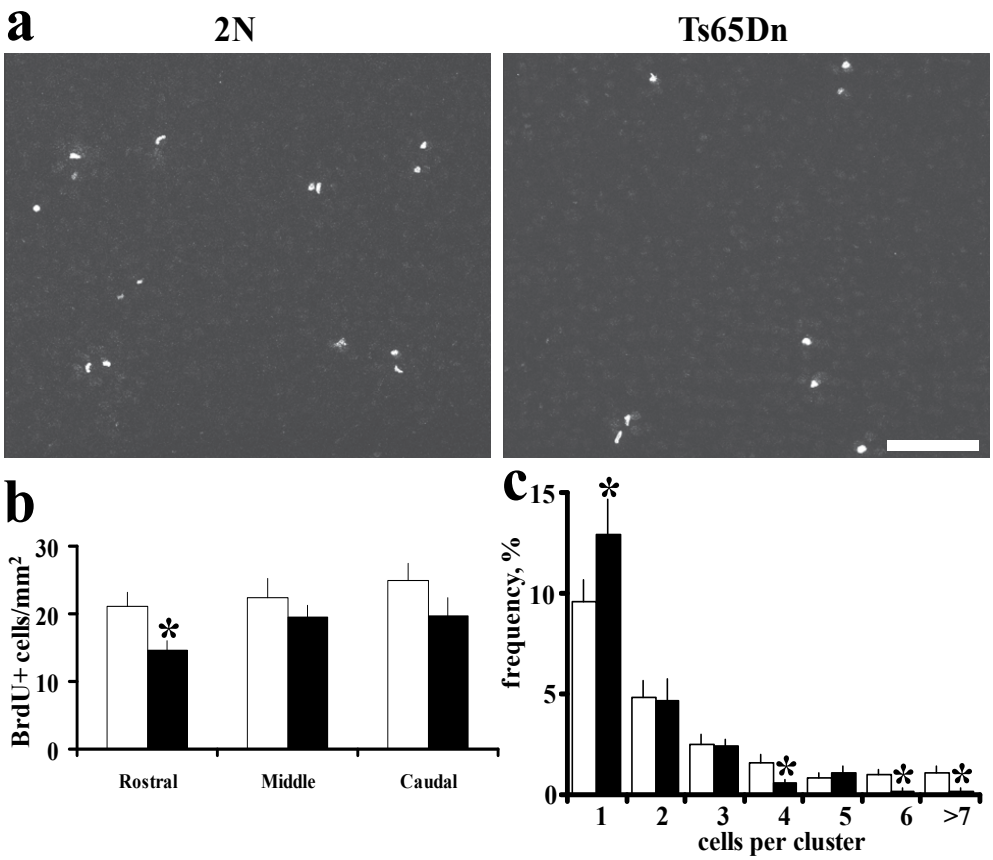

Fig. 2. Neurogenesis in cortex in 2N and Ts65Dn mice. (a) Confocal image of BrdU+ cells in the rostral part of cortex of 2N and Ts65Dn mice. (**b**) BrdU+ cell density in cortex. Note significant reduction of BrdU+ cell in rostral cortex in Ts65Dn mice. (**c**) The number of cells per cluster in rostral part of cortex. Scale bar = 100 μm.

	cell density		cluster density		# cells per cluster	
	2N	Ts65Dn	2N	Ts65Dn	2N	Ts65Dn
Dentate gyrus						
Rostral						
Total	3.06 ± 0.27	2.87 ± 0.25	1.01 ± 0.08	1.22 ± 0.09	3.05 ± 0.22	2.39 ± 0.16a
Superior blade	3.61 ± 0.29	3.13 ± 0.36	1.02 ± 0.11	1.25 ± 0.09	3.69 ± 0.29	2.47 ± 0.21a
Inferior blade	2.55 ± 0.25b	2.55 ± 0.25	0.99 ± 0.08	1.19 ± 0.13	2.65 ± 0.29b	2.42 ± 0.37
Middle						
Total	2.88 ± 0.17	2.77 ± 0.15	1.21 ± 0.05	1.17 ± 0.08	2.56 ± 0.20	2.52 ± 0.12
Superior blade	3.33 ± 0.21	3.01 ± 0.17	1.23 ± 0.05	1.24 ± 0.09	3.01 ± 0.26	2.66 ± 0.12
Inferior blade	2.28 ± 0.18b	2.46 ± 0.20c	1.19 ± 0.08	1.08 ± 0.14	1.96 ± 0.17b	2.51 ± 0.34
Caudal						
Total	2.73 ± 0.19	2.00 ± 0.08a	1.13 ± 0.04	1.10 ± 0.08	2.59 ± 0.31	2.01 ± 0.12a
Superior blade	3.09 ± 0.27	2.31 ± 0.12a	1.21 ± 0.07	1.22 ± 0.10	2.84 ± 0.27	1.69 ± 0.16a
Inferior blade	2.20 ± 0.19b	1.55 ± 0.15a,c	1.10 ± 0.06b	0.92 ± 0.07c	2.21 ± 0.22	1.69 ± 0.16a
Cortex						
Rostral	21.1 ± 2.2	14.6 ± 1.4a	14.4 ± 1.4	10.2 ± 1.2a	1.46 ± 0.04	1.24 ± 0.05a
Middle	22.3 ± 2.9	19.5 ± 1.8	15.9 ± 1.5	14.9 ± 1.7	1.25 ± 0.03	1.22 ± 0.02
Caudal	25.0 ± 2.5	19.7 ± 2.7	14.9 ± 1.7	13.9 ± 1.7	1.30 ± 0.03	1.26 ± 0.04
Corpus callosum						
Rostral	74.7 ± 11.3	87.2 ± 12.6	56.7 ± 3.1	53.0 ± 5.6	1.15 ± 0.05	1.16 ± 0.03
Middle	96.1 ± 15.8	77.3 ± 13.4	64.1 ± 7.2	62.6 ± 7.6	1.28 ± 0.07	1.22 ± 0.03
Caudal	111.2 ± 11.8b	105.1 ± 16.1	73.8 ± 7.3	73.2 ± 9.4	1.36 ± 0.06	1.32 ± 0.04

The number of mice used was as follows: 2N/Ts65Dn = 6/5.

Each value represents the mean ± SEM.

BrdU+ cells and clusters densities are expressed as: for FD per 100 μm length of subgranule zone, for cortex and CC per 1 mm^2.

a, $P < 0.05$, significantly different from 2N mice.

b, $P < 0.05$, significantly different between 2N mice.

c, $P < 0.05$, significantly different between Ts65Dn mice.

Table 1. Morphometry of BrdU+ cells in 2N and Ts65Dn mice

4. Discussion

Numerous studies have shown that adult neurogenesis is affected by various neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's diseases (reviewed in Curtis et al., 2011; Kaneko & Sawamoto, 2009; Rodriguez & Verkhratsky, 2011; Winner et al., 2011). Alteration of neurogenesis in neurodevelopmental disorders were studied to a lesser extent in DS (Contestabile et al., 2007; Guidi et al., 2008, 2010), autism spectrum disorders (Greco et al., 2011; Wegiel et al., 2010), including Rett syndrome (Ronnett et al., 2003). Studies to understand the neurobiology of DS have benefited from the ability to examine mouse models of the disorder (Belichenko et al., 2004; 2007; 2009; Kleschevnikov et al., 2004; Popov et al., 2011). Previous studies demonstrated that trisomy in Ts65Dn mouse model of DS resulted in alteration of hippocampal long-term potentiation (LTP) (Kleschevnikov et al., 2004; Siarey et al., 1997). Significant alteration of neurogenesis could be one of the contributing factors to diminished plasticity in DS. Interestingly, studies involving mice to different enrichments or pharmacotherapies enhanced both neurogenesis and learning (Deng et al., 2010; Mongiat & Schinder, 2011; Saxe et al., 2006; Shors et al., 2002; Singer at al., 2011), including Ts65Dn mice (Bianchi et al., 2010). Moreover, manipulations that stimulate adult neurogenesis in mice also increase LTP (van Praag et al., 1999).

Our study in the Ts65Dn mouse model of DS presents novel evidence of altered neurogenesis in adult young mice (2-3 months of age). Significant increases in the number of clusters containing single BrdU+ cells in Ts65Dn mice point to severely affected cell proliferation. Our present data are in agreement with previous data (Clark et al., 2006) and conflicting with Rueda et al. (2005) data on young adult Ts65Dn mice. The discrepancy with the Rueda et al. (2005) results likely result from several factors including the different gender of mice (we used only male mice and no gender of studied mice were mentioned in Rueda et al., 2005), the different brain areas investigated (we studied rostral, middle, and caudal parts of hippocampus, cortex and CC separately, while in Rueda et al. (2005) the entire hippocampus was studied). The BrdU injection protocol was also different. We used standard a procedure with BrdU injection during 6 days, while Rueda et al., (2005) used a 12 days injection approach.

The majority of BrdU+ cells in adult brain during the proliferation phase formed clusters (Palmer et al., 2000; Seki et al., 2007). Data on neurogenesis in adult rat hippocampus suggests that newly-born cells appeared to stay in clusters for a few days and then migrate from clusters (Seki et al., 2007). Currently, little is known about the molecular mechanisms of cluster formation. Previous reports suggested that cell clustering supports intercellular contacts and/or signals that encourage neurogenesis (Seki et al., 2007). It is possible that the initial formation of a cluster is triggered by a certain factor, which is not affected in Ts65Dn mice. Thus, we have similar cluster density in Ts65Dn vs. 2N mice. The 'just-divided' cells could be divided further, thus resulting in a cluster (Seki et al., 2007). However, this could be only accomplished when the cell is going through all cycles. Indeed, as was shown previously (Contestabile et al., 2008) that the dividing in Ts65Dn cells are 'stuck' in S phase, thus decreasing the probability of cell cycle re-entry. This delay could suspend the second and the following divisions, thus resulting in a reduced number of the newly-born cells per cluster. Interestingly, we observed that the number of cells per cluster was reduced only in the rostral and caudal, but not in the middle part of the Ts65Dn hippocampus. The exact nature of this phenomenon is not clear yet, but may result from differences in the structure and function of hippocampus along the rostro-caudal axis. Indeed, neuronal network architecture is different in hippocampus (Witter et al., 2000).

Reduction in the number of cells per cluster leads to a reduction of total number of newly-born neurons in the Ts65Dn hippocampus. It was shown previously that LTP in the DG depends strongly on neurogenesis, and that a blockade of neurogenesis results in reduced synaptic plasticity (Snyder et al., 2001). Because we observed that LTP in the DG of Ts65Dn mice is significantly diminished (Kleschevnikov et al., 2004), it is tempting to assume that reduced neurogenesis is one of the factors contributing to this abnormality. It is clear that alteration in prenatal growth of the cortex and hippocampus in trisomic mice is due to a longer cell cycle and reduced neurogenesis (Chakrabarti et al., 2007; Contestabile et al., 2007).

We further propose that alteration of neurogenesis may also affect the balance between excitation and inhibition. Indeed, the putative young neurons are completely unaffected by GABA(A) inhibition (Wang et al., 2000). Reduced number of such disinhibited neurons in DG of Ts65Dn mice could thus result in elevation of the overall efficiency of the inhibitory system (Belichenko et al., 2009; Kleschevnikov et al., 2004). The excitatory/inhibitory neuron ratio could be affected also in other bran regions. The new granule cells of the DG, olfactory bulb, and cerebellum and the new cells in cortex are constantly produced during adult neurogenesis. However, there are many differences between these cells. For example, in the olfactory bulb and neocortex the newly-born cells are mainly inhibitory (GABAergic) (reviewed in Cameron & Dayer, 2008). Reduced neurogenesis in these regions could result in relative disinhibition and, thus, could be regarded as a factor contributing to epilepsy in DS (Goldberg-Stern et al., 2001; Menendez, 2005; Pueschel et al., 1991). In contrast, DG and cerebellar granule cells are excitatory (glutamatergic). However, this question needs to be studied in greater detail. The influence of chromosomal aberration on the transition from proliferating stage to differentiated stage into either glutamatergic or GABA-ergic neurons should be also studied.

Numerous factors can be implicated in the control of adult neurogenesis, including chromosomal aberrations. Although the exact mechanism(s) of influence of partial or complete trisomy on neurogenesis is not known, we can extract some data from the scattered information. Impaired neurogenesis has been described not only in Ts65Dn (Rueda et al., 2005; Clark et al., 2006; Lorenzi and Reeves, 2006; Constestabile et al., 2007; Chakrabarti et al., 2007; Bianchi et al., 2010) and Ts2Cje (Ishihara et al., 2010) mouse models of DS, but also in TsCje1 mice (Hewitt et al., 2010; Ishihara et al., 2010) with smaller triplicated chromosomal region. Genetic dissection approaches (Ts65Dn vs. Ts1Cje) point out to the same genes in smaller triplicated region, i.e. I*tsn1, Dyrk1A, Olig2* may involve in neurogenesis (Hewitt et al., 2010; Ishihara et al., 2010). It has been proposed that overexpressions of Dyrk1A in trisomic segment may be responsible for impaired neurogenesis in Ts65Dn, Ts2Cje, and Ts1Cje mouse models of DS (Canzonetta et al., 2008; Ishihara et al., 2010). These authors also suggest that altered neurogenesis in the Ts1Cje mouse is due to a reduction in neuronal progenitors and neuroblasts rather than neural stem cells (Hewitt et al., 2010). The further identification and characterization of triplicated genes in altered neurogenesis may significantly contribute to understanding the neurobiology of DS and may lead to the development of pharmacotherapy for DS.

5. Conclusion

Down syndrome (DS) is a neurodevelopmental disorder caused by the triplication of chromosome 21. Few studies have explored neurogenesis in mouse models of DS and these have produced conflicting results on the alteration of neurogenesis. Using confocal

microscopy analysis we have examined adult neurogenesis in the hippocampus (dentate gyrus, DG), motor cortex and corpus callosum (CC) of male wild-type (2N) and Ts65Dn mice at age 2-3 months. In Ts65D we found significant decreases in the BrdU+ cells density in DG and motor cortex. BrdU+ cell cluster density were higher in rostral motor cortex in Ts65Dn. Number of BrdU+ cells per cluster were significantly lower in DG and motor cortex of Ts65Dn mice, mainly due to the presence of single cell clusters. Density of BrdU+ cells was significantly lower in the inferior than in the superior blade of DG. Thus, we show for the first time a lower density of BrdU+ cells in Ts65Dn mice. Our findings are evidence on a deficiency of adult neurogenesis in Ts65Dn mouse model of DS. Moreover, we propose a correlation of the present data with our previously published data on alteration of LTP due to excessive inhibition in Ts65Dn mouse model of DS. The new findings can shed light on possible mechanisms underlying the changes in the Ts65Dn neurogenesis, synaptic structure and function that were described previously. Based on our data and recent studies we reasonable suggested that new complex therapies should improve neurogenesis, and as consequence also learning and memory.

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Part 3

Neurologic, Urologic, Dental and Allergic Disorders

10

Dermatological Manifestations of Down Syndrome

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1. Introduction

Down syndrome (DS) is increased frequency of some common dermatoses and associated with rare dermatological disorders. With the increasing life span and number of DS patients in the population, dermatologists are more likely to encounter skin manifestations associated with DS. Subjects with DS have high rates of infections and autoimmune phenomena. The immunological disturbances in DS (High IgG and low IgM levels, reduced total T-lymphocyte numbers, high CD8+ and low CD4+ count, decreased chemotaxis,…) could be implicated in some higher cutaneous manifestations.
In this chapter, it has been exposed the dermatological disorders associated with DS. Very few studies in the literature have studied dermatological manifestations of DS. Newer reports are mostly in the form of case reports highlighting the rare dermatological findings. Dermatologic manifestation in DS has been studied in 6 major surveys but the results are not concordant. These studies differ in some aspects (age range, living conditions of the study group,...). Daneshpazhooh *et al* studied 100 children with DS with a mean age of 11.2 years; Carter and Jegasothy examined 213 institutionalized patients with ages between 12 and 48 years old; Ercis *et al* studied 71 children who live with their families; Polenghi *et* al observed adults with ages between 14 and 53 years old; Schepis *et al* examined 203 patients from infancy till adulthood with a mean age of 11.7 years; and Sureshbabu *et al* studied 95 patients with a mean age of 11.9 years, ranging from 6 months to 40 years.
These dermatologic disorders can be separated in manifestations that could be, or not, associated to immunological alterations (Table 1).

Associated to immunological alterations	**Probably not associated to immunological alterations**
Atopic dermatitis *Seborrhoeic dermatitis* *Alopecia areata* *Vitiligo* *Infections* *- Onychomycosis* *- Scabies*	*Anetoderma* *Milia-like calcinosis cutis* *Acanthosis nigricans* *Syringomas elastosis perforans serpiginosa* *Leukaemia cutis* *Acrocyanosis* *Cutis marmorata* *Keratodermatoses*

Table 1. Dermatological manifestations of Down's syndrome associates or probably not associated to immunological alterations

2. Dermatological manifestations that could be associated to immunological alterations

2.1 Atopic dermatitis

Atopic dermatitis (AD) is a chronic inflammatory skin condition that appears to involve a genetic defect in the proteins supporting the epidermal barrier. AD affects approximately 5-20% of children worldwide. The incidence of AD appears to be increasing. Onset tends to occur during childhood and gradually diminishes with age, although it can persist or even appear in adults. The term AD was coined by Wise and Sulzberger in 1933 to define an entity characterized by dry skin, pruritus, erythematous lesions, and a chronic recurrent course. Currently, the terms AD and atopic eczema are used and both are acceptable.

Its prevalence has increased 3-fold or 4- fold in recent decades in some countries. According to the International Study of Asthma and Allergies in Childhood, during a minimum period of 1 year, the prevalence of symptoms of AD in 6- or 7-year-old children presented great variability between different geographic areas. Thus, prevalence was almost 20% in England or Australia but less than 2% in China or Iran. There is a higher prevalence in urban areas than in rural ones in developed countries, and higher social classes are more affected. Regarding the disease by sex, a study of 12- to 16-year-olds found a higher prevalence among girls (25.7%) than among boys (17%). The onset of AD occurred during the first 6 months of life in 45% of children, in the first year in 60%, and in the first 5 years in more than 85%.

One study diagnosed AD in 56.5% of DS, and another a prevalence of more than 50%. The high reported prevalence of AD, in some studies, could be an overestimate and isolated signs such as facial dermatitis and generalized xerosis could easily be misinterpreted as AD.

Pathogenesis of AD is complex; several factors are involved, many of which are still not well understood. Likewise, it has yet to be determined how these factors might interact with one another origin of atopic eczema seems to involve a feedback cycle: pruritus and mechanical damage caused by scratching leads to the production of proinflammatory cytokines (interleukin [IL]-1, IL-18, tumor necrosis factor, granulocyte-macrophage colony stimulating factor) that recruit leukocytes to the skin.

Different leukocyte populations are activated through different processes; on induction by dendritic cells, the lymphocytes differentiate via the Th2 pathway; these dendritic cells also show increased antigen-presenting capacity and bind to the IgE-antigen complex. The IgE antigen complex in turn induces mast cell accumulation and activation. The activated Th2 cells release IL-4 and IL-13, which suppress the production of antimicrobial peptides. Viruses, bacteria, and fungi take advantage of these reduced peptide levels, colonizing the skin and releasing proinflammatory products (superantigens, proteoglycans, and lipoteichoic acid) that amplify leukocyte activation. This activation increases the release of inflammatory mediators, such as proteases and IL-31, which perpetuate pruritus.

Increased prevalence of childhood AD in developed countries has led to the appearance of many theories on the possible involvement of environmental factors. Increases in the prevalence of allergic disease probably depend more on environmental factors than on other individual characteristics.

The diagnosis of AD is based on a constellation of signs and symptoms. There is no laboratory "gold standard" for the diagnosis of AD. In a majority of the cases, the diagnosis is quite easy. Establishing firm diagnostic criteria for all forms of AD is difficult due to the

clinical and pathophysiological heterogeneity. Atopic individuals can also suffer from other dermatitis or dermatoses, and because every dermatitis in an atopic individual need not be AD. Hanifin and Rajka for the first time proposed a systematic approach toward the standardization of the diagnosis of AD by incorporating three major/basic and 23 minor features. They suggested that a diagnosis of AD can be established if 3 of the major and 3 of the minor criteria are present. (Table 2).

Major	Minor
1. *Pruritus* 2. *Typcial morphology and distribution:* - *Flexural lichenification or linearity in adults* - *Facial and extensor involvement in infants and children* 3. *Chronic or chronically relapsing dermatitis* 4. *Personal or family history of atopy (asthma, AR, atopic dermatitis)*	1. *Xerosis* 2. *Ichthyosis/palmar hyperlinearity/keratosis pilaris* 3. *Immediate (type 1) skin test reactivity* 4. *Elevated serum IgE* 5. *Early age at onset* 6. *Tendency toward cutaneous infections (esp. Staph. aureus and Herpes simplex)/impaired cell-mediated immunity* 7. *Tendency toward nonspecific hand or foot dermatitis* 8. *Nipple eczema* 9. *Cheilitis* 10. *Recurrent conjuntivitis* 11. *Dennie-Morgan infraorbital folds* 12. *Keratoconus* 13. *Anterior subcapsular cataracts* 14. *Orbital darkening* 15. *Facial pallor/facial erythema* 16. *Pityriasis alba* 17. *Anterior neck folds* 18. *Itch when sweating* 19. *Intolerance to wool or lipid solvents* 20. *Perifollicular accentuation* 21. *Food intolerance* 22. *Course influenced by environmental/emotional factors* 23. *White dermographism/delayed blanch*

Table 2. Diagnostic criteria of Hanifin and Rajka Diagnosis of AD can be established if 3 of the major and 3 of the minor criteria are present

In DS, the skin in infancy is usually soft, but soon becomes dry, thick, and rough, with patchy lichenification. The reported frequency of xerosis differs from 9.8 percent to 85 percent in various studies. Sureshbabu *et al* observed disorders lichenification as the most common disorder, seen in 52.6% of DS patients, with a peak incidence in the 5-10 year age group. High incidence of AD reported by Carter *et al* (56.5%) and cited in some major review articles contrast with Sureshbabu *et al* study which none of subjects fulfilled the criteria of Hanifin and Rajka for AD. Two studies observed 3% and 4.9% for the prevalence of AD in

DS using the criteria of Rajka and Hanifin, These results are in favour of the opinion that AD is not as common as previously thought when using the acknowledged diagnostic criteria of Rajka and Hanifin.

In the treatment of AD, topical corticosteroids are considered the gold standard for assessment of other treatments. The potency and formulation employed depends on the area to be treated and the chronicity of the lesions. Areas with have undergone lichenification requiring stronger formulations. One application per day is sufficient, as treatment twice a day confers no advantage while increasing the likelihood of adverse reactions. Adverse reactions are well known and frequently overestimated by patients and their family members, even though the new formulations have a demonstrated lower risk of causing cutaneous atrophy than the older ones and that several studies have found a far lower incidence of local and systemic complications.

Application of topical antibiotics in combination with corticosteroid therapy has advantages compared to topical corticosteroids. Fusidic acid appears to be the topical antibacterial treatment of choice, due to its low minimum inhibitory concentration and its good penetration.

Topical calcineurin inhibitors have proven to be effective in the treatment of AD. Topical tacrolimus seems to have an efficacy similar to high-potency corticosteroids, whereas pimecrolimus is substantially weaker. Controlled pediatric studies have confirmed the superior efficacy of topical tacrolimus compared to pimecrolimus and hydrocortisone. There appear to be no significant differences between the response of children to concentrations of 0.03% and 0.1%. Neither tacrolimus nor pimecrolimus cause cutaneous atrophy, but they can cause other adverse reactions such as local itching-burning sensation when being applied, which is an added discomfort for the skin of children with AD.

Use of emollients is widely recognized as a basic measure in the treatment and prevention of flares of AD. It has been shown that their use in combination with topical corticosteroids accelerates healing and decreases the total dose of corticosteroids required to resolve the flare.

Dietary restrictions have proven effective in the case of children with egg-specific IgE, but not for other foods which have the same effect. It seems reasonable to establish diets that avoid food proven to cause an allergic response using the radioallergosorbent test, although the most relevant test would be the challenge test, which in many cases cannot be performed. Psychological care is recommended to help deal with the emotional needs of patients with atopic dermatitis, and should be based on providing education and information on the clinical and preventable aspects of the disease. One study found that this type of intervention reduced anxiety scores.

Short-course systemic corticosteroids are recommended to control acute flares of AD, taking into account that new flares are frequent after stopping treatment. Their long term use in children is not recommended. Little evidence exists to support using oral antihistamines in AD. Other systemic treatments to AD are: Cyclosporine A, azathioprine, interferon, light therapy or intravenous immunoglobulins. Biologic treatment has recently appeared in the field of dermatology and has shown some potential.

2.2 Seborrhoeic dermatitis

Seborrhoeic dermatitis (SD) is a common, chronic dermatoses of unknown aetiology, characterized by scaling and redness occurring primarily in the areas with the highest

concentration of sebaceous glands such as the scalp, face (mid-facial region), and certain areas of the trunk, such as the mid-thoracic and interscapular areas and the area around the buttocks. On the scalp, it generally appears first as small patches of scales, progressing to involve the entire scalp with exfoliation of excessive amounts of dry scales.

It affects around 1% to 3% of the immunocompetent adult population, with a higher prevalence in men than women. Although it can appear at any age, the highest prevalence is observed in individuals aged 30 to 60 years and in the first 3 months of life in the infantile form of the disease.

Clinically, the disease is characterized by erythema and flaking of the skin in the affected area. Lesions are well delimited, reddish, and covered with oily yellowish-white scales. On the scalp, in the milder form of the disease, the scales are small, dry, and whitish, and they detach easily and spontaneously in steady amounts. In the more severe form of the disease, plaques are observed that range in size from a few centimeters to areas covering a large part of the scalp; they are made up of thick dry scales. On the face they are found in the eyebrows, around the nose, at the edge of the scalp, and on the inner surface of the auricle. In the thorax, the lesions are rounded, well delimited, and reddish brown; they are located on the medial part of the chest and on the back, between the shoulder blades. All of these forms are associated with varying degrees of itching. In adults, the course of SD involves periods of remission and exacerbation, irrespective of the treatments administered. Outbreaks are common under conditions of emotional stress, fatigue, and depression.

While the disease rarely causes serious complications, it always leads to a marked esthetic deterioration that leads to emotional and social difficulties for the affected individual.

The etiology of seborrheic dermatitis is not fully understood but is known to involve various factors. Increased secretion by the sebaceous glands favours the development of microorganisms of the genus *Malassezia*, which are responsible for the symptoms.

It appears more frequently in patients with neurological disorders such as Parkinson disease, in those suffering from depression, and in patients with AIDS. One study, by Carter and Jegasothy, found a 36% prevalence of SD in DS patients and another was found a similar prevalence (Ercis *et* 31%; mostly during the first year of life). SD was seen in 4.2% of DS by Sureshbabu *et al*. *al*. SD is commonly seen in patients with immunodeficiency. High prevalence of *pityrosporum folliculitis* in DS patients could have a pathogenic role.

A diet rich in animal fats and lacking in vegetables, as well as alcohol consumption can also potentiate the appearance of lesions. Topical corticosteroids are the first choice treatment for SD. Others drugs can currently be used to minimize the effects of this dermatologic disease: antifungals, keratolytics, tar or pyrithione derivatives, and selenium sulfate. Many of these treatments have been tested as both monotherapy and in combination.

2.3 Alopecia areata

Alopecia areata (AA) is a recurrent nonscarring type of hair loss that can affect any hair-bearing area. Clinically, AA can manifest many different patterns. Although medically benign, AA can cause tremendous emotional and psychosocial distress in affected patients and their families.

AA is a genetically determined, immune-mediated disorder of the hair follicle with an estimated lifetime risk of approximately 2%, making it one of the most common autoimmune diseases

AA is characterized by patchy hair loss on the scalp, which can eventually involve the entire scalp (*alopecia totalis*) or the entire body (*alopecia universalis*). The onset of the disease can be sudden, its progression is unpredictable, and it can be recurrent throughout life. It is thought that AA represents a breakdown in immune privelege with the subsequent unable to function of the hair follicle by T lymphocytes. AA has a deeply disturbing psychologic impact on affected individuals. It shows a spectrum of severity that ranges from patchy localized hair loss on the scalp to the complete absence of hair everywhere on the body.
Treatment of AA may induce hair growth, but usually does not change the course of the disease. When treatment is stopped, hair loss recurs. Many patients with one or two small patches can be managed without treatment and with reassurance of the benign nature of the condition. Treatment with topical and oral steroids, topical minoxidil, topical cyclosporine, and photo-dynamic therapy has been found no long-term benefit of these interventions. In patients with persistent hair loss and less than 50% scalp involvement, intralesional corticosteroid therapy is the first-line treatment. Patients with more than 50% hair loss can be treated with topical immunotherapy using diphenyl-cyclopropenone or squaric acid. *Alopecia totalis* and *alopecia universalis* have the worst prognosis, with fewer than 10% of patients recovering.
AA occurs in approximately 0.1% of the general but a recognized association of AA with DS exists and it was reported between 20% to 1.4% prevalence. Sureshbabu *et al* saw in 9.4% of DS with AA. Female predilection was observed by Carter and Jegasothy but not in other studies. AA was observed in 3% of DS patients in the study of Schepis *et al*, with the usual age of onset in middle childhood (5-10 years). AA, in DS patients, is usually severe and refractory to the standard treatments.

2.4 Vitiligo

Vitiligo is a hypopigmentation disorder where the loss of functioning melanocytes causes the appearance of white patches on the skin. It occurs when melanocytes, the cells responsible for skin pigmentation, die or are unable to function. The cause of vitiligo is unknown, but research suggests that it may arise from autoimmune, genetic, oxidative stress, neural, or viral causes. Vitiligo is an autoimmune disease characterized by melanocyte loss, which results in patchy depigmentation of skin and hair, and is associated with an elevated risk of other autoimmune diseases. It is a genetically complex disorder involving multiple susceptibility genes and unknown environmental triggers. Recent data provide strong evidence supporting an autoimmune pathogenesis of vitiligo. Genetic factors also appear to play a role in the etiopathogenesis of vitiligo as 20% to 30% of patients have a family history of the disorder. Vitiligo is associated with other autoimmune diseases (Addison´s disease, hyperthyroidism and pernicious anemia).
Vitiligo affects 1% of the world population, but the prevalence has been reported as high as 4% in some South Asian, Mexican and American populations. Vitiligo can develop at any age, but several studies report that 50% of cases appear before the age of 20 years old. Only one study investigated the association between vitiligo and DS found a prevalence of 1.9%. 75% of this also had AA. However, Sureshbabu *et al* did not observed an incidence significantly higher in DS people.
Flat areas of normal-feeling skin without any pigment appear suddenly or gradually. These areas have a darker border. The edges are well defined but irregular. Vitiligo most often affects the face, elbows and knees, hands and feet, and genits. It affects both sides of the

body equally. Vitiligo is more noticeable in darker-skinned people because of the contrast of white patches against dark skin. No other skin changes occur. Sometimes, the use of Wood's light can improve to see lesions. In some cases, a skin biopsy may be needed to rule out other causes of pigment loss.
Vitiligo is difficult to treat. As vitiligo can have a major effect on quality of life, treatment can be considered and should preferably begin early when the disease is active. Current treatment modalities are directed towards stopping progression of the disease and achieving repigmentation. Therapies include corticosteroids, topical immunomodulators, phototherapy, surgery, combination therapies and depigmentation of normall pigmented skin. Topical corticosteroids can be used for localized vitiligo. The use of topical immunomodulators seems to be equally effective as topical steroids, especially when used in the face and neck region. Narrowband ultraviolet-B therapy seems to be superior to psoralen ultraviolet-A. Depigmentation therapy can be considered if vitiligo affects more than 60% to 80% of the body.

2.5 Onychomycosis

Onychomycosis is one of the most prevalent fungal infections in the population with a higher rate of treatment failures. Onychomycosis is a frequent nail disease caused by dermatophytes, yeasts, and nondermatophyte molds. *Trichophyton rubrum, T mentagrophytes*, and *Epidermophyton floccosum* are the most common etiologic agents worldwide.
Onychomycosis may be classified into several types: distal subungual, white superficial, proximal subungual, endonyx, and total dystrophic. Distal subungual onychomycosis, the most common type, involves the nail bed and, subsequently, the nail plate. White superficial onychomycosis usually manifests as superficial white patches with distinct edges on the surface of the nail plate. Proximal subungual onychomycosis results when the fungal organism enters via the cuticle and the ventral aspect of the proximal nail fold. In endonyx onychomycosis, fungal organisms invade the nail plate without resulting nail bed hyperkeratosis, onycholysis, or nail bed inflammatory changes. In total dystrophic onychomycosis, complete dystrophy of the nail plate occurs; these changes may be primary or secondary.
Diagnosis is corroborated by direct microscopic examination, culture, and histomycology. Treatment is based on oral antifungals. Systemic treatment for onychomycosis includes terbinafine, an allylamine that is primarily fungicidal, and itraconazole, a triazole that is primarily fungistatic. Both represent a major therapeutic advancement over griseofulvin in the treatment of this condition.
For toenail infection, terbinafine is usually taken continuously for 12 weeks, whereas itraconazole is taken either continuously or intermittently that is 1 week in 4 weeks for the same period. Because therapeutic concentration of itraconazole is believed to persist in the nail for a considerable time after systemic treatment is stopped, intermittent therapy with higher daily doses to achieve and maintain therapeutic concentration might be an effective alternative to continuous treatment.
Such intermittent treatment is widely used currently to treat onychomycosis and is claimed to be as effective for this condition as both continuous itraconazole and continuous terbinafine
Although both itraconazole and terbinafine are well tolerated and highly effective drugs, continuous terbinafine is more effective than intermittent itraconazole at achieving mycological cure of toenail onychomycosis.

There is a higher frequency of onychomycosis in DS patients. It has been reported prevalence of 67,8% to 4,4%. A low incidence of infection was seen in Sureshbabu *et al* study in contrast to Carter *et al* who reported a high prevalence of fungal infections. Schepis *et al* also reported a relatively low figure for onychomycosis and tinea corporis. Fungal infections may have been over diagnosed in the past or may be caused by poor hygienic conditions.

2.6 Scabies

Scabies (caused by *Sarcoptes scabiei*) is probably one of the most common parasitic infections, usually spread by bodily contact, though other contaminated objects can also infect. There is severe itching, usually worse at night, with lesions (mostly excoriated burrows) on the fingerwebs, volar wrists, buttocks, Scabies is an infestation caused by human itch mite, Sarcoptes scabiei, which infests some 300 million persons each year and is one of the most common causes of itching dermatoses throughout the world. Gravid female mite measuring 0.3 to 0.4 mm in length burrows superficially beneath the stratum corneum for a month, depositing two or three eggs a day. Nymphs that hatch from these eggs mature in about 2 weeks through a series of molts and then emerge as adults to the surface of the skin, where they mate and subsequently reinvade the skin of the same or another host. Transfer of newly fertilized female mites from person to person occurs by intimate personal contact and is facilitated by crowding, uncleanliness and sexual promiscuity.

The most commonly used to treatment scabies is permethrin 5% cream which applied all over the body. The whole family or sexual partners of infected people should be treated, even if they do not have symptoms. Creams are applied as a one-time treatment or they may be repeated in 1 week. Wash underwear, towels, and sleepwear in hot water. Vacuum the carpets and upholstered furniture. For difficult cases, oral Ivermectin 200-400 μgr/Kg may be used.

Other creams include benzyl benzoate, sulfur in petrolatum, and crotamiton. Lindane is rarely used because of its side effects.

Itching may continue for 2 weeks or more after treatment begins, but it will disappear if the infection is over.

Patients with DS seem predisposed to crusted scabies. Immunological dysfunction has been proposed as one factor for this propensity. However, Norwegian scabies can appear as isolated or epidemic conditions but no cases were recorded in some studies

3. Other dermatological manifestations that would not be associated to immunological alterations

3.1 Anetoderma

Anetoderma is a rare benign dermatosis caused by a loss of mid-dermal elastic tissue resulting in well-circumscribed areas of pouchlike herniations of flaccid skin. It has been hypothesized that a congenital malformation of elastic fibres in this population may be responsible for anetoderma.

Sureshbabu *et al* observed anetoderma in 3.2% of DS. Schepis *et al* reported 3.9% of patients having anetoderma in their study. It has been hypothesized that a congenital malformation of elastic fibers in this population may be responsible for anetoderma secondary to chronic folliculitis. Anetoderma is due to elastolysis probably induced by leukocytes or bacteria during the recurrent inflammatory events

3.2 Milia-like calcinosis cutis

Milia-like idiopathic calcinosis cutis (MICC) is a rare entity. Only few cases have been reported so far. Two-thirds of these have been associated with Down syndrome.

MICC is a micronodular, whitish, acral, calcified lesion. Laboratory tests are usually within normal range (including serum calcium and phosphate, urinary calcium and parathyroid hormone). The term MICC was introduced in the literature by Smith *et al* who described a 6-year-old child affected by DS. Some additional cases of MICC have been reported in children or adolescents affected by DS.

Milia-like idiopathic calcinosis cutis has long been regarded as a peculiar subtype of idiopathic calcinosis cutis. The pathogenesis of the disorder remains unclear. The pathogenesis of MICC in association with DS is unclear. Higher concentrations of calcium in sweat have been found in DS, which may lead to sweat-duct calcification

3.3 Acanthosis nigricans

Acanthosis nigricans (AN) is a skin disorder in which there is darker, thick, velvety skin in body folds and creases. Obesity can lead to AN, as can some endocrine disorders. It is often found in people with obesity-related diabetes Some drugs, particularly hormones such as human growth hormone or oral contraceptives, can also cause AN. Because AN usually only changes the skin's appearance, no treatment is needed. It is important, however, to treat any underlying medical problem that may be causing these skin changes. When AN is related to obesity, losing weight often improves the condition.

AN was seen by Sureshbabu *et al* in 8,4% of DS. The increased incidence of AN in adults is more than in children, but this fact may be attributed to the fact that all these patients were obese with a high BMI (> 30).

3.4 *Elastosis perforans serpiginosa*

*Elastosis perforans serpiginosa (*EPS) is characterized clinically by papules and keratotic plaques and histologically by focal elastosis of the dermis and transepidermal elimination of abnormal elastic fibers. It is a rare skin disease classified as a primary perforating dermatosis similar to reactive perforating collagenosis. It was given the current denomination of EPS by Dammert and Putkonen.

The incidence of EPS has yet to be established, although looks like higher in DS people. Around 90% of patients develop symptoms of the disease prior to 30 years of age, the majority between 6 and 20 years of age; however, age at onset may range from 5 to 89 years, as reported in the literature. Approximately 75% of those affected are male.

Etiopathogenesis is as yet unclear. It is believed that the focal inflammation in the dermis, which has a biochemical or mechanical origin, may induce the formation of epidermal and follicular channels to expulse abnormal elastic fibers considered irritants.

The disorder can present with papules and erythematous or normochromic, keratotic, asymptomatic or pruriginous plaques grouped in an annular, arciform or serpiginous pattern, surrounded by satellite lesions. They have umbilicated centers from which dermal material is eliminated. The lesions are characteristically symmetrical except in cases associated with DS.

Various forms of treatment have been indicated; however, management of the disease is difficult and there is no standard treatment. There have been reports of therapeutic success using cryotherapy with liquid nitrogen and oral isotretinoin.

3.5 Others

It also has been reported higher prevalence of other skin alterations I DS patients: syringomas, leukaemia cutis, acrocyanosis and cutis marmorata, keratodermatoses… However, none all studies have observed them; for example Sureshbabu *et al* did not see syringoma, milia-like calcinosis cutis, leukemia cutis, elastosis perforans serpiginosa, carotenemia, or vascular instability; they referred that may relate to the fact that their study was not hospital based, unlike other previous studies. Other miscellaneous cutaneous lesions were probably coincidentally seen in the studies, concordant with that seen in the general population.

4. Conclusions

DS is increased frequency of some common dermatoses. Some dermatologic manifestation in DS can been in relation with their immunological alterations. Skin alterations of DS have been observed by few studies. Their management is equal to rest patient, but they can occasionally be more resistant to conventional treatment, for example AA.

AD is one of the most frequent skin diseases associated a DS. However, perhaps this high association observed in first studies could be overestimated. Newer studies, which have used criteria of Hanifin and Rajka to diagnostic AD, does not show so higher incidence of AD in DS. SD can be more extensive than in other patients.

Some immunologic skin diseases, as vitiligo and AA, have been reported with high incidence in DS. Also, fungal infection and scabies has been observed with higher prevalence in DS than rest population. However, scabies in DS could be overestimated in some studies with patients under epidemic conditions.

MICC y EPS are rare skin alterations observed more frequently in DS. AN, frequently associated to overweight, can usually been observed in patients with IMC>30. Other miscellaneous cutaneous lesions were probably coincidentally seen in every study.

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11

Down Syndrome and Periodontal Disease

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1. Introduction

Periodontal diseases are inflammatory diseases of the supporting structures of the teeth (Cochran, 2008; Kornman, 2008). They are initiated by periodontopathic bacteria and result in progressive destruction and loss of the periodontium (Cochran, 2008; Kornman, 2008). Progression of periodontal disease eventually leads to tooth loss (Chambrone, et al., 2010). Periodontal diseases are multifactorial with complex pathogenesis (Cochran, 2008; Kornman, 2008).

Plaque bacteria trigger a host inflammatory response in the gingival tissues (Kornman, 2008). Neutrophils migrate from within the gingival tissues towards the gingival crevice and build a barrier wall against the bacteria (Kornman, 2008). Within the gingival connective tissue, the gingival macrophages and fibroblasts produce inflammatory cytokines (e.g. interleukin-1 and tumor necrosis factor- alpha) that activate collagenases and other degrading enzymes (Kornman, 2008). These enzymes when released and activated destroy the gingival collagen. Lymphocytes are recruited to the gingival lesion to initiate an adaptive immune response and help with containing the infection (Kornman, 2008). With persistence of the microbial infection, the inflammatory changes in the gingival tissues expand apically and reach the alveolar bone (Cochran, 2008). Inflammatory mediators such as interleukin-1, interleukin-6, tumor necrosis factor alpha and prostaglandins induce osteoclasogenesis (Cochran, 2008). Increased inflammatory activity disrupts the normal balance of bone formation/resorption and results in alveolar bone loss (Cochran, 2008).

Periodontal disease is a serious and morbid oral condition among Down-syndrome (DS) affected individuals (Morgan, 2007). Gingivitis and periodontitis start early in life and their severity increases with age (Reuland-Bosma, et al., 1988). Periodontal disease advances rapidly in DS individuals and is characterized by severe gingival inflammation, loss of periodontal attachment and radiographic alveolar bone loss (Agholme, et al., 1999; Saxen, et al., 1977). Heavy amounts of plaque and calculus are often present (Morgan, 2007). Periodontal disease is an important cause of tooth loss among DS individuals (Reuland-Bosma, et al., 2001).

The exact reason (or reasons) for this increased susceptibility to periodontitis is (are) not known. Understanding the pathogenesis of periodontitis in DS individuals would greatly help with the management and control of the destructive process associated with the disease and help DS affected individuals retain their teeth hopefully throughout their lifetime.

Previously researchers have investigated factors usually associated with periodontitis such as subgingival plaque microbial composition, immune and inflammatory responses *individually* in DS affected individuals. The individual factors investigated were never collectively evaluated together to provide an overall understanding of the pathogenesis of periodontitis in

individuals with DS. The objective of this chapter is to review in a systematic fashion all the involved factors previously reported *together* to generate a hypothetical collective model of the pathogenesis of periodontal disease in individuals with DS. Such a model would enhance our understanding of periodontal disease development/progression in this vulnerable group, would help with disease management, would identify gaps in knowledge, and would provide enlightenment for future research endeavors.
In this review I have searched the available dental/medical literature for studies investigating the main factors suspected in the increased susceptibility to periodontitis in DS individuals. I have summarized the main findings from these studies and used this information to generate a hypothetical model of the pathogenesis of periodontitis in DS individuals.

2. Methods

The methodology applied in this review covers the literature search strategy, inclusion and exclusion criteria for choosing articles, screening of articles and quality assessment of the selected articles.

2.1 Search strategy

Electronic searching was performed using the following databases: MEDLINE (1948-2011) and PubMed. The search terms that were used related to the subject groups of interest: Down syndrome and mental retardation; the oral condition: gingivitis, periodontitis, periodontal disease, gingiva, periodontium, tooth loss; etiological factors: microbiological (dental plaque, subgingival plaque, periodontopathic bacteria), immune factors (neutrophils, lymphocytes, antibody production) and inflammatory factors (cytokines, prostaglandins, metalloproteinases) and the type of study: comparative study, randomized controlled trial, or review. The search was limited to English language literature. The electronic search was supplemented by checking bibliographies of review papers. One hundred forty five articles were initially identified.

2.2 Inclusion criteria

Studies were included if they met the following minimum criteria:
1. Human subjects with Down syndrome.
2. Clinical measures of periodontal status.
3. Microbiological, immune or inflammatory measures.
4. Comparison of DS subjects to a control/comparison group(s).
5. Written in English.

2.3 Exclusion criteria

Editorial letters, historical reviews and descriptive studies such as case reports were excluded. In addition, studies that did not specifically address the main focus of the search or duplicated other studies were also excluded.

2.4 Screening and selection of papers

At first the articles were screened by title and abstract. A review of abstracts based on the criteria above led to initial consideration of 32 articles. Then full text was retrieved either

electronically or in printed format. Further review of full texts resulted in inclusion of 20 articles.

2.5 Quality assessment

Systematic reviews need to assess the methodological *quality* of the included studies (Pai, et al., 2004). Quality within the context of this chapter refers to the minimization of bias. An objective of this assessment was to judge the strength of the scientific evidence with higher score given to studies free of bias. To minimize bias, the comparison group needs to be matched as close as possible to the DS group. In addition, the evaluated parameters should be assessed in the same manner between the groups. Studies with an adequately matched control group were scored higher than studies that used population data or previously collected data for comparison.

The instrument used for judging articles for this chapter was based freely on the use of STROBE methodology (von Elm, et al., 2007) evaluating the following parameters:

- Down syndrome diagnosis: clearly stated and explained.
- Inclusion of a comparison group: well matched (age, gender, race) or not.
- Periodontal parameters: how periodontitis was defined and measured. Adequate if clinical attachment level was reported. Inadequate when only periodontal probing depth or radiographic measurements are reported.
- Laboratory methodology to assess microbial, immune or inflammatory factors: was the methodology clearly explained. Was the methodology appropriate or not.
- Examiner calibration: adequate if reported, inadequate if not reported.
- Descriptive information: characteristics of study participants (number of subjects, gender, mean and range of age) were clearly presented.
- Confounders identified: confounders that may affect periodontal status such as age, gender, and plaque levels were clearly defined.
- Data analysis: adequate when multivariate analysis adjusting for confounders was presented. Inadequate if only bivariate analysis was presented.

The studies included in the review were scored as follows:

- Score 1: when all the parameters presented above were reported in the study.
- Score 2: when only DS diagnosis, inclusion of a comparison group, clinical periodontal measures and laboratory measures were reported.
- Score 3: other than score 1 and 2.

3. Results

A total of 20 articles were selected, 5 articles related to microbiological evaluations, 3 articles related to neutrophil function, 3 articles related to gingival cellular immunity, 4 articles related to antibody production and 5 articles related to degrading enzymes and inflammatory mediators. All selected articles met the minimum inclusion criteria previously described. Two articles were scored as 1, fifteen articles were scored as 2 and three articles were scored as 3.

Nineteen out of the twenty selected studies were designed as cross-sectional case-controlled studies. Thus in addition to the DS group, they included a matched comparison group of either medically healthy and periodontally healthy individuals or a comparison group with mental disability other than DS. One study was longitudinal (Zaldivar-Chiapa, et al., 2005).

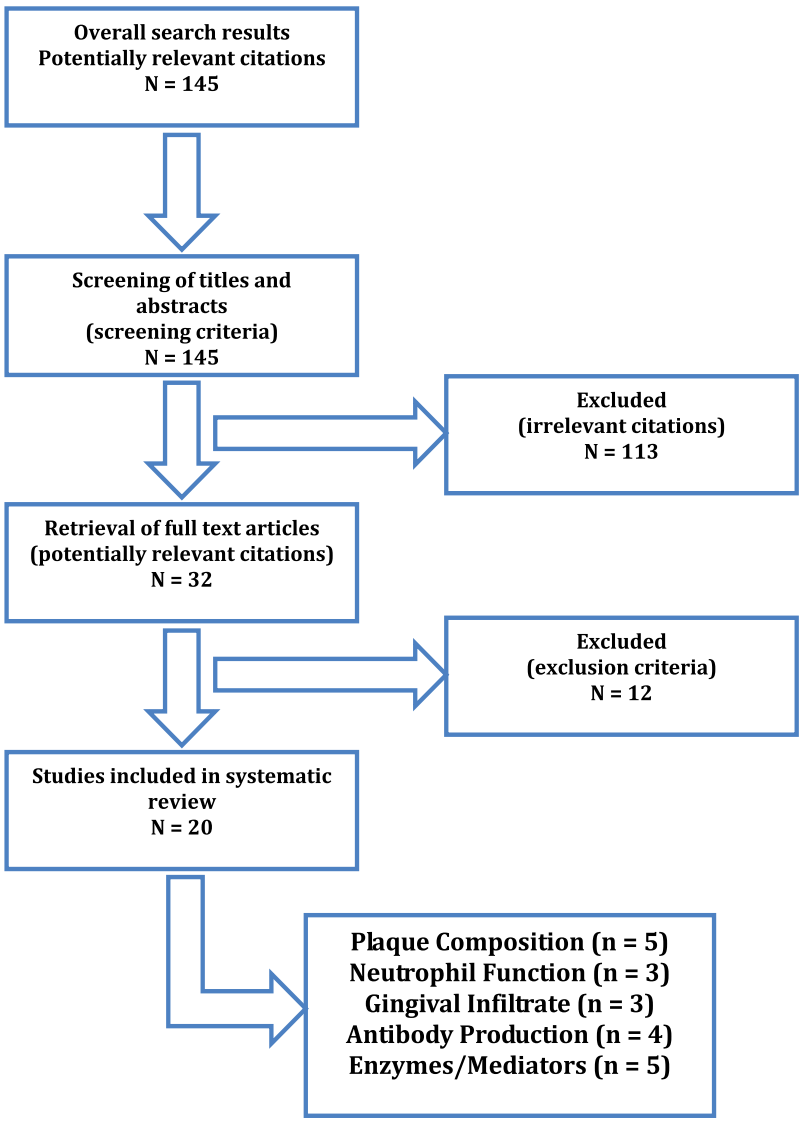

Fig. 1. Summary of search strategy.

Down subjects in most of the studies were either children or young adults. Few of the selected studies included DS adults (Amano, et al., 2001; Chaushu, et al., 2007; Komatsu, et al., 2001; Sakellari, et al., 2005; Sohoel, et al., 1995).

3.1 Summary of study methodologies

Microbiology studies employed a variety of techniques including culturing, polymerase chain reaction and checkerboard DND-DNA hybridization techniques to investigate the composition of subgingival plaque. All the studies attempted to compare the subgingival plaque composition of DS individuals to a matched comparison group. In addition some studies attempted to determine at what age important periodontopathic bacteria become

detectable in DS individuals. A few studies attempted to investigate the association between presence of periodontopathic bacteria and clinical periodontal parameters in DS individuals.
Neutrophil function studies mainly focused on neutrophil chemotaxis. Neutrophil chemotaxis was measured by the Boyden chamber method. All selected studies compared neutrophil chemotaxis of DS individuals to a matched comparison group. One of the studies investigated the relation between neutrophil chemotaxis measures with clinical and radiographic periodontal measures.
Gingival cellular immunity studies examined the immune cellular composition, expression of HLA Class II antigens on the surfaces of immune cells and gamma/delta T-cell receptor-bearing lymphocytes in discarded gingival tissues from DS individuals. Indirect immunofluorescent techniques were used to examine the gingival tissues. All the selected studies compared the gingival tissue cellular findings in DS individuals to medically and periodontally healthy individuals.
The selected antibody production studies focused on specific antibodies against some important periodontopathic bacteria. The studies examined either serum antibody titers or salivary antibody levels. All selected studies used an enzyme-linked immunosorbent assay (ELISA). Four of the selected studies compared the antibody serum titer or saliva levels in DS individuals to matched controls. One study made the comparison to a normal adult reference serum pool.
The selected studies investigating degrading enzymes and inflammatory mediators mainly focused on matrix metalloproteinases (MMP) activity in gingival fluid. One study examined cultured fibroblast MMP activity. Two studies examined prostaglandin E2 (PGE2) and one study examined interleukin-1 (IL-1) in gingival fluid. All selected studies compared their findings in DS individuals with non-DS controls. MMP activity was assessed with a variety of techniques including gel electrophoresis, Western blot analysis and ELISA. PGE2 was assessed with a radio-immuno-assay and IL-1 with ELISA.

3.2 Summary of study findings

A summary of all selected articles and their main findings is presented in the following tables:

3.2.1 Subgingival plaque composition studies

Author	Scr.	DS	CG	Measures	Findings
Barr-Agholme 1992	2	37	37	Levels of *Aggregatibacter (Actinobacillus) actinomycetemcomitans*, *Capnocytophaga* and *Porphyromonas gingivalis* were determined in subgingival plaque samples.	*A. actinomycetemcomitans* was detected in the subgingival plaque in 35% of the DS adolescents and in 5% of the controls. On site level, *A. actinomycetemcomitans* and *Capnocytophaga* were more frequent in the subgingival plaque samples of DS children than in those of controls.

Author	Scr.	DS	CG	Measures	Findings
Amano 2000	2	60 Yng.	60	Ten periodontal pathogens were surveyed in subgingival plaque samples using a polymerase chain reaction.	All of the pathogens were detected with greater frequency in the DS children. *Tannerella forsythia* (*Bacteroides forsythus*), *Treponema denticola, Prevotella nigrescens,* and *Campylobacter rectus* were significantly prevalent throughout all age brackets of the DS children. The occurrence of *Porphyromonas gingivalis* was also significant in the DS subjects over 5 years old.
Amano 2001	2	67 Adt.	41	Ten periodontal pathogens were surveyed in subgingival plaque samples using a polymerase chain reaction.	No significant differences were observed in the bacterial profiles between the two groups.
Sakellari 2005	1	70	121 NC 76 MR	Clinical parameters and microbiological analysis by "checkerboard" DNA-DNA hybridization.	Important periodontal pathogens including *Porphyromonas gingivalis*, *Tannerella forsythia* (*Bacteroides forsythus*) and *A. actinomycetemcomitans* colonize these subjects earlier and at higher levels.
Reuland-Bosma 2001	2	17	17	Prevalence and proportions of the putative periodontal pathogens *A. actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia, Tannerella forsythia (Bacteroides forsythus), Peptostreptococcus micros, Fusobacterium nucleatum* and *Campylobacter rectus* in the subgingival plaque were determined using anaerobic culture techniques.	No differences in the prevalence of distinct suspected periodontopathic bacteria and bacterial subgingival composition between the DS group and the comparison group could be established.

Scr = score, DS = number of DS group, CG = number of comparison group, Yng = young, Adt = adult, NC = normal controls, MR = mental retardation not Down.

Table 1. Summarizes the findings of the subgingival plaque composition studies.

3.2.2 Neutrophil function studies

Author	Scr.	DS	CG	Measures	Findings
Izumi 1989	2	14	14	Periodontal clinical measures, radiographic measures and neutrophil chemotaxis.	DS patients showed significantly lower chemotaxis than healthy volunteers. Bone loss in DS subjects was inversely proportional to the chemotactic index.
Yavuzyilm-az 1993	2	15	15	Clinical periodontal parameters, chemotaxis and random migration of neutrophils.	The random migration and chemotaxis of neutrophils from DS subjects were significantly decreased in comparison with the control group.
Zaldivar-Chiapa 2005 Longitudinal	2	14	9	Clinical parameters and neutrophil function.	Neutrophil chemotaxis, phagocytic activity, and production of super-oxide anion were significantly decreased in the DS patients.

Scr = score, DS = number of DS group, CG = number of comparison group.

Table 2. Summarizes the neutrophil function studies.

3.2.3 Gingival immune response studies

Author	Scr.	DS	CG	Measures	Findings
Sohoel 1992	3	16	12	Indirect immune-fluorescence used to study immune cellular composition of gingival tissues from DS individuals.	Denser inflammatory infiltrate in DS individuals. Counting showed different cell distribution and cell profiles between DS subjects and non-DS subjects.
Sohoel 1995	3	16	14	Expression of HLA Class II antigens on the surfaces of immune cells.	Increased frequency of HLA Class II (HLA-expression on inflammatory cells and on keratinocytes of the oral gingival epithelium) in chronic periodontitis of DS patients compared to sections from non-DS subjects.
Sohoel 1995	3	NR	NR	Assessment of Gamma/delta T-cell receptor-bearing lymphocytes in gingival tissues.	Gamma/delta T-cell receptor-bearing lymphocytes are decreased in inflamed gingival tissues of DS individuals.

Scr = score, DS = number of DS group, CG = number of comparison group, NR = not reported.

Table 3. Summarizes the gingival immune cellular response studies.

3.2.4 Antibody production studies

Author	Scr.	DS	CG	Measures	Findings
Santos 1996	2	16	10	Circulating antibody titers to *A. actinomycetemcomitans*.	Significant differences were noted between the control group and the DS subjects (p = 0.05), with the DS periodontitis group having the highest response, followed by the DS gingivitis group and normal controls, respectively. The DS groups were not significantly different.
Morinushi 1997	3	75	RSP	Antibody titers to *Porphyromonas gingivalis (Pg), Prevotella intermedia (Pi), Treponema denticola (Td), Fusobacterium nucleatum (Fn), Selenomonas sputigena (Sel), A. actinomycetemcomitans (Aa),* and *Streptococcus mitis (Mi).*	IgG antibody titers to *Pg, Aa, Sel*, and *Mi* increased significantly with increasing gingival inflammtion score. Furthermore, the IgG antibody titers to *Pg* were higher ($P < 0.05$) in the most extensive disease group compared to the DS no-disease group. The IgG antibody titers to *Pg* at early puberty were significantly higher when compared to preschool children.
Barr-Agholme 1998	2	20	19	Salivary levels of immunoglobulins sIgA, IgM, and IgG subclass distribution and albumin, quantified by enzyme-linked immunosorbent assay.	The immunoglobulin levels of sIgA, IgM, the sum of IgG subclasses, and the concentration of albumin did not differ significantly between the 2 groups. However, the proportion of IgG1 expressed as percentage of the sum of total IgG was significantly higher ($P < 0.01$) in the Down syndrome group compared to controls. On the contrary, the proportion of IgG2, IgG3, and IgG4 subclasses in saliva did not differ between the 2 groups.
Chaushu 2007	2	40	39	The levels of total IgA, and specific antibodies to three common oral pathogens (*Porphyromonas gingivalis, Actinobacillus (Aggregatibacter) actinomycetemcomitans* and *Streptococcus mutans*) were analyzed.	The median secretion rates of the specific antibodies in whole and parotid saliva were 70-77% and 34-60% (respectively) lower in young DS individuals as compared to young controls and farther 77-100% and 75-88% (respectively) lower in old DS compared to young DS.

Scr = score, DS = number of DS group, CG = number of comparison group, RSP = reference serum pool.

Table 4. Summarizes the antibody production studies.

3.2.5 Inflammatory mediator studies

Author	Scr.	DS	CG	Measures	Findings
Halinen 1996	2	9	9	Clinical periodontal measures and matrix metalloproteinase (MMP-8 and -9) activities in saliva and in gingival crevicular fluid.	The endogenously active collagenase and total collagenase activities were slightly higher in GCF of DS children compared to healthy controls. GCF collagenase of DS patients was human neutrophil collagenase (MMP-8 or collagenase-2), in DS patients, but not in controls. Salivary collagenase in DS was high when compared to controls but of the same MMP-8 type as in control saliva.
Barr-Agholme 1997	2	15	15	Levels of prostaglandin E2 (PGE2) and interleukin-1 beta (IL-1 beta) were determined in gingival crevicular fluid.	The mean level of PGE2 in GCF was significantly higher ($P < 0.05$) in the Down syndrome group than in the control group. In GCF samples collected from sites characterized as non-inflamed, the mean level of PGE2 was significantly higher ($P < 0.001$) in the Down syndrome group than in the controls. The mean level of PGE2 in samples from inflamed sites, on the other hand, did not differ between the two groups. The mean level of IL-1 beta was not significantly higher in the Down syndrome group than in the controls.
Komatsu 2001	2	9	9	Enzyme activity and the mRNA expression pattern of matrix metalloproteinases (MMPs) of cultured gingival fibroblasts (GF) and fresh gingival tissues.	The production of the active type of MMP-2 in GF from Down's syndrome patients (D-GF) was found to be significantly higher ($P < 0.05$) than that of the control GF (C-GF) at the protein level.
Tsilingaridis 2003	2	18	14	Levels of prostaglandin E2 (PGE2), leukotriene B4 (LTB4), and matrix metalloproteinase-9 (MMP-9) in gingival crevicular fluid.	The mean levels of PGE2, LTB4, and MMP-9 were significantly ($P<0.05$) higher in GCF from Down syndrome patients compared to controls.
Yamazaki-Kubota 2010	1	14	14	Matrix metalloproteinase (MMP-8 and MMP-2) activity in gingival crevicular fluid.	Levels of MMP-2 and MMP-8 in Down's syndrome patients were higher than those in healthy control subjects.

Scr = score, DS = number of DS group, CG = number of comparison group.

Table 5. Summarizes the inflammatory mediator studies.

4. Discussion

Periodontal disease is a common problem among DS individuals with an estimated prevalence between 58% and 96% for those under 35 years of age (Morgan, 2007). The disease starts early in life and progresses with age eventually leading to tooth loss (Reuland-Bosma, et al., 2001; Saxen, et al., 1977; Saxen & Aula, 1982). Periodontal disease in DS individuals adversely impacts on the quality of their life (Amaral Loureiro, et al., 2007). The increased prevalence and severity of periodontal disease in DS individuals inspired many researchers to investigate the various factors that might be involved.

Periodontal diseases are initiated by bacterial plaque build-up in the dentogingival region (Kornman, 2008). It is well documented that DS individuals have difficulty with maintaining adequate oral hygiene levels and thus tend to harbor high levels of bacterial plaque on their teeth (Cohen, et al., 1961; Khocht, et al., 2010; Sakellari, et al., 2005). In addition, DS individuals following oral hygiene instructions have reduced ability to master adequate plaque control (Sakellari, et al., 2001). It was often surmised that mental disability associated with DS is an important factor in their reduced ability to maintain adequate oral hygiene and consequently increases their susceptibility to periodontitis (Desai, 1997; Morgan, 2007). Our group (Khocht, et al., 2010) recently showed in a multivariate model including traditional risk factors for periodontitis combined with mental disability that loss of periodontal attachment in DS individuals was not associated with mental disability. Thus other factors associated with DS might be involved.

It is well documented that DS is associated with immune deficiencies and host response impairment (Kusters, et al., 2009; Reuland-Bosma, et al., 1988). Infections, in particular respiratory infections are an important cause of death in DS individuals (Thase, 1982). The most likely reason for this increased susceptibility to infection and reduced immunity in DS individuals is an increased dosage of a protein product or products encoded by chromosome 21. Several proteins important in immune function are encoded on chromosome 21. Examples include superoxide dismutase (SOD), carbonyl reductase (NADPH) (Lemieux, et al., 1993) and integrin beta-2 (CD18). Increased SOD and NADPH production is associated with increased oxidative stress and tissue injury in DS individuals (Akinci, et al., 2010; Strydom, et al., 2009). Aberrant expression of CD18 integrin on immune cell surfaces in DS individuals may be associated with altered lymphocyte function (Kusters, et al., 2009; Taylor, 1987). The IL10RB component of the IL-10 receptor (involved with resolution of inflammation) is encoded by chromosome 21 and its function may be altered in DS individuals (Glocker, et al., 2009). In addition, it seems that interleukin-1 (IL-1) is upregulated indirectly by some chromosome 21 based genes (Mrak & Griffin, 2004). IL-1 is an important immune/inflammatory mediator. Its increased production in DS individuals was associated with brain tissue damage (Mrak & Griffin, 2004).

Since periodontitis is initiated by bacterial infections, indeed it is conceivable that altered immunity in DS individuals may be the primary reason for their increased susceptibility to periodontal infections. Perhaps reduced immunity in DS individuals would make it easier for virulent periodontopathic microbial species to colonize their subgingival plaque. If true, such elevated microbial presence, unchallenged and unchecked, would induce an intense inflammatory reaction within the gingival tissues. Increased gingival inflammation within the gingival tissues would lead to elevated production of degrading enzymes and alter bone remodeling. The end result of these inflammatory induced changes would be the loss and destruction of the periodontium and eventually tooth loss. Several studies (microbiological, immune and inflammatory) attempted to investigate these hypotheses.

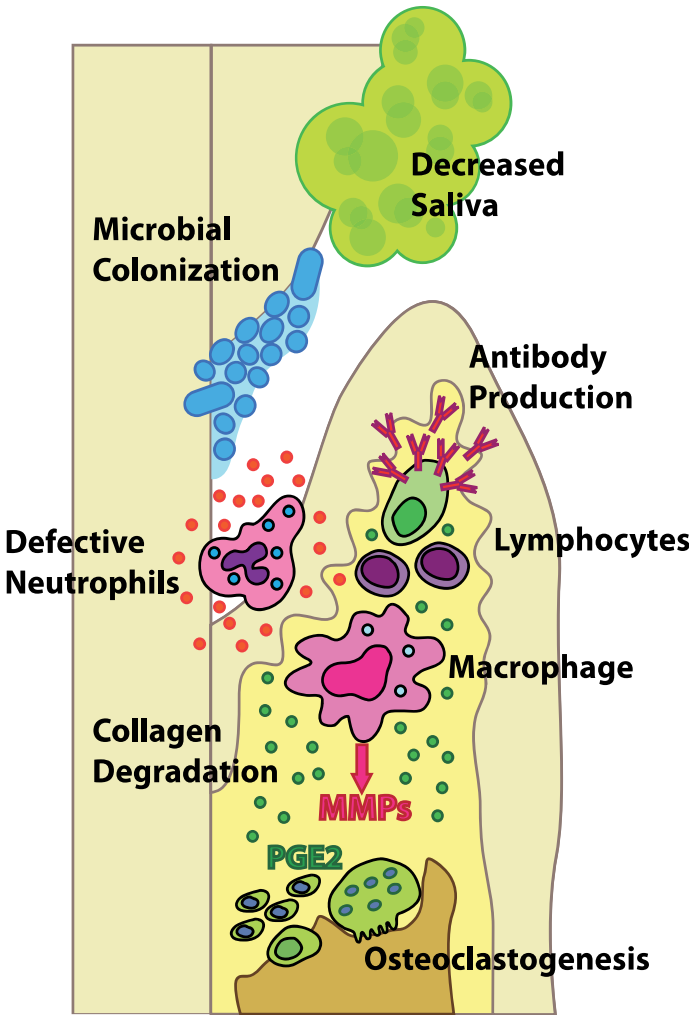

Fig. 2. The above schematic illustrates the main findings of the selected articles. Decreased salivary flow and associated decreased salivary antibody production. Neutrophils with defective chemotaxis incapable of reaching the target pathogens. Early colonization of the dentogingival region with periodntopathic bacteria. Gingival marginal tissues heavily infiltrated with immune cells such as macrophages and lymphocytes. Antigen presenting cells active in processing and presenting microbial antigens. Robust humoral antibody production. Macrophages and other gingival cells engaged in producing degrading enzymes (MMPs). Collagen degradation. Tissue injury and release of prostaglandins (PGE2). Increased osteoclastic activity and alveolar bone loss.

4.1 Microbiological studies

Barr-Agholme et al. (1992) reported increased presence of *Aggregatibacter* (*Actinobacillus*) *actinomycetemcomitans, Capnocytophaga and Porphyromonas gingivalis* in subgingival plaque of *adolescents* with DS. *A. Actinomycetemcomitans* was detected in 35% of patients with DS compared to 5% in the healthy, age and sex matched controls. The authors suggested that

this increased frequency of *A. actinomycetemcomitans* indicated an altered microbial composition in the subgingival plaque of DS patients as compared to healthy controls.
Amano et al. (2000) found various periodontal disease-causing bacteria present in *very young* DS patients. The authors reported that various periodontopathic bacteria could colonize the teeth in the very early childhood of DS patients. Pathogens in DS patient's subgingival plaque were detected with far greater frequency than in the age-matched controls. This may be the reason why these DS patients have such intense gingival inflammation. The authors concluded that periodontopathic pathogens establish a presence at a very early age, and that certain bacteria, like *P. gingivalis*, play a key role in the initiation of gingival inflammation.
Sakellari et al. (2005) evaluated seventy DS patients, 121 age-matched healthy individuals and 76 patients with cerebral palsy. Full-mouth recordings of clinical periodontal parameters were assessed and subgingival plaque samples were taken from the Ramfjord teeth and analysed for 14 species using "checkerboard" DNA-DNA hybridization. They reported that important periodontal pathogens colonize these subjects earlier and at higher levels compared with age-matched healthy individuals and patients with cerebral palsy.
Reuland-Bosma et al. (2001) compared subgingival microflora in DS adult patients to other mentally retarded individuals. Despite advanced periodontitis in DS patients, no differences in the prevalence of distinct suspected periodontopathic bacteria were established between the DS patients and the control group. The authors concluded that host factors are the most likely explanation for the advanced periodontal disease associated with DS patients.
Amano et al. (2001) took subgingival plaque specimens from 67 DS young adults and 41 age-matched systemically healthy individuals with mental disabilities (MD). The prevalence of *Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, Bacteroides forsythus, Treponema denticola, Prevotella intermedia, Prevotella nigrescens, Capnocytophaga ochracea, Capnocytophaga sputigena, Campylobacter rectus, and Eikenella corrodens,* were investigated in subgingival plaque samples using a polymerase chain reaction method. The authors found no significant differences in the bacterial profiles between the groups.
The cited microbiological studies indicate early colonization of important periodontal pathogens in children and adolescents with DS. However, the microbial subgingival profile of *adult* DS individuals is not different from matched non-DS individuals. Perhaps reduced immunity in young DS individuals facilitates early colonization in comparison to young non-DS individuals with normal immunity. With age, and a combination of long-term exposure to pathogens and changes in immune response, non-DS individuals may also be susceptible to colonization by periodontal pathogens. However, despite lack of differences in microbial profiles between adult DS and non-DS individuals, adult DS individuals still show greater loss of periodontal attachment (Amano, et al., 2001; Reuland-Bosma, et al., 2001). This suggests that the host response to the same bacteria is different between DS and non-DS individuals. It seems that the immune-inflammatory response in DS is more intense resulting in greater tissue damage. The following studies will investigate this claim.

4.2 Immune dysfunction studies

As we said earlier, DS is associated with immune dysfunction (Kusters, et al., 2009). Various studies investigated different components of the immune system in relation to periodontitis in DS patients. These studies mainly focused on neutrophil function, the gingival immune cellular response and antibody production against periodontopathic bacteria.

4.2.1 Neutrophil function studies

Izumi et al. (1989) found a faulty neutrophil chemotaxis in DS patients. The authors reported that DS patients had significantly lower chemotaxis compared to healthy controls. Since the neutrophils are the main cells involved in the first line of host defense in a bacterial invasion, having a defective neutrophil chemotaxis can lead to the progression of periodontitis. Significant correlations were identified between the amount of bone loss and the age and chemotactic index of the DS patients. The authors found that the rate of periodontal destruction was dependent on the degree of defective chemotaxis.

Yavuzyilmaz et al. (1993) evaluated clinical parameters, chemotaxis and random migration of neutrophils in 15 patients with DS and 15 healthy subjects. Signs of more severe gingival inflammation were present in the DS group. The random migration and chemotaxis of neutrophils were significantly decreased in comparison with the control group.

Zaldivar-Chiapa (2005) examined patients with DS to evaluate the effectiveness of surgical and non-surgical periodontal therapies and to assess their neutrophil immunological status. The population consisted of 14 DS patients, 14 to 30 years old. Surgical and non-surgical periodontal therapies were compared in a split-mouth design. Clinical periodontal parameters were recorded at baseline, post-treatment, 6 months, and 1 year. Neutrophil chemotaxis, phagocytic activity, and production of super-oxide anion were compared between DS patients and healthy controls. Both surgical and non-surgical therapies showed a significant improvement in all the clinical parameters compared to baseline. Neutrophil chemotaxis, phagocytic activity, and production of super-oxide anion were significantly decreased in the DS patients. The authors concluded that the neutrophil impairment does not seem to affect the clinical response to therapy.

All the presented studies showed deficient neutrophil chemotaxis in DS subjects. The reason for impaired neutrophil chemotaxis in DS individuals may be secondary to increased oxidative stress associated with trisomy of chromosome 21 (Akinci, et al., 2010). Oxidative stress may impair internal cell function and disrupt chemotaxis. One study positively correlated such reduced chemotaxis with measures of periodontitis (Izumi, et al., 1989). Another study gave hope that despite reduced neutrophil chemotaxis, periodontal therapy aiming at reducing plaque and correcting periodontal architecture is still helpful (Zaldivar-Chiapa, et al., 2005).

4.2.2 Gingival Immune cellular response

Sohoel et al. (1992) examined the composition of mononuclear cells in the gingival inflammatory infiltrate in DS patients with marginal periodontitis. The authors reported that DS patients had a higher number of cells in the cellular infiltrate of chronic marginal periodontitis (CMP) compared to normal patients. There were also an increased number of CD22+ cells (B lymphocytes), CD3+ cells, CD4+ cells, CD8+ cells, and CD11+ cells (macrophages). There was also a significantly higher CD4+/CD8+ ratio in DS patients when compared to the normal controls, which could indicate active tissue destruction. This study concluded that DS patients have a more pronounced and altered gingival cellular immune response when compare to controls.

Sohoel et al. (1995) investigated the expression of HLA class II antigens in chronic marginal periodontitis (CMP) in patients with DS. Variations in the expression of HLA class II antigens on antigen-presenting cells play an important role in immune regulation. The results of this study indicated an increased frequency of HLA class II antigens in the

gingival tissues of CMP DS patients when compared to controls. There were significantly higher numbers of CD1a+ cells and ratios of HLA-DR+/CD1a+ cells and HLA-DP+/CD1a+ cells in the DS group compared to the control group. The authors concluded that there is a highly activated immune response in DS patients.

The same investigators (Sohoel, et al., 1995) also investigated gamma/delta T lymphocytes in gingival tissues of DS individuals. The T-cell receptor (TCR) of gamma/delta T lymphocytes is different from the alpha/beta TCR. The gamma/delta TCR binds to antigens that are intact proteins and antigens that are not presented within class I or class II histocompatibility molecules. The gamma/delta T lymphocytes usually reside within epithelial tissues and encounter antigens on the surface of epithelial cells. The researchers reported that the percentage of gamma/delta T lymphocytes in the gingival tissues of DS subjects was less than 1%.

The presented studies showed an intense presence of a variety of immune cells within the gingival tissues of DS patients with periodontitis. Increased production of HLA class II antigens on the surfaces of antigen producing cells suggests that the cells are locally engaged in specific immune responses. The low presence of gamma/delta T lymphocytes may increase the vulnerability to microbial noxious agents.

4.2.3 Antibody/immunoglobulin production

These studies investigated specific antibodies against periodontopathic bacteria in serum and saliva.

Santos et al. (1996) determined the circulating antibody titers to *Aggregatibacter (Actinobacillus) actinomycetemcomitans (Aa)* in sera of DS and normal patients. Eleven DS patients with periodontitis (pocket depth > 4 mm), five DS patients with gingivitis (inflammation and pocket depth < or = 3 mm), and 10 non-DS healthy subjects had blood drawn and analyzed for antibody response to *Aa*. The authors noted significant differences between the control group and the DS groups ($p = 0.05$), with the DS periodontal group having the highest response, followed by the DS gingivitis and normal controls, respectively.

Morinushi et al. (1997) obtained sera from 75 DS subjects (aged 2 to 18 years) and their gingival health assessed using a modified gingival inflammation index (PMA Index). Antibody titers to *Porphyromonas gingivalis, Prevotella intermedia, Treponema denticola, Fusobacterium nucleatum, Selenomonas sputigena, Actinobacillus actinomycetemcomitans, and Streptococcus mitis* were determined using a micro-ELISA. The average antibody titers to *A. actinomycetemcomitans, S. mitis*, and *F. nucleatum* exceeded those of the normal adult reference serum pool. In addition, IgG antibody titers to *P. gingivalis , A actinomycetemcomitans, F. nucleatum, S. sputigena*, and *S. mitis* correlated significantly with the modified PMA scores.

Barr-Agholme et al. (1998) investigated the clinical periodontal conditions and salivary immunoglobulins in patients with DS. The results showed an altered distribution of IgG subclasses in saliva, with an increased amount of IgG1 in DS patients compared to controls. This is in agreement with other studies that show increased IgG1 in DS patients (Kusters, et al., 2009). On the contrary, the proportion of IgG2, IgG3, and IgG4 subclasses in saliva did not differ between the 2 groups. Also, in DS patients with bone loss, it was noted that they have an increased level of sIgA, compared to those DS patients without bone loss.

Chaushu et al. (2007) assessed age-related changes in the salivary-specific humoral immunity of DS subjects. Parotid and whole saliva were collected from a young group of DS, an older group of DS individuals and compared to two age-matched groups of healthy volunteers. The levels of total IgA, and specific antibodies to three common oral pathogens (*Porphyromonas gingivalis, Aggregatibacter (Actinobacillus) actinomycetemcomitans and Streptococcus mutans*) were analyzed. The median secretion rates of the specific antibodies in whole and parotid saliva were 70-77% and 34-60% (respectively) lower in young DS individuals as compared to young controls and farther 77-100% and 75-88% (respectively) lower in old DS compared to young DS.

The presented studies were somewhat controversial and indicated different antibody responses between saliva and serum in DS individuals. While in saliva the antibody response was low in DS individuals, in serum the antibody responses to several periodontopathic bacteria were elevated. The low antibody responses in saliva were associated with decreased salivary flow in DS individuals. The low salivary antibody activity may facilitate the colonization of periodontal pathogens in DS individuals. The elevated antibody titers in DS serum corroborate the gingival immune cellular activity described previously. It demonstrates that DS individuals despite known immune deficiencies are capable of mounting a humoral specific immune response. Such antibodies would find their way into the gingival tissues and fluid and help with containing the microbial damage. Increased antibody levels in gingival tissues may also accentuate the gingival inflammatory response through complement activation.

4.3 Inflammatory response studies

Inflammatory response studies focused on inflammatory mediators and degrading enzymes in gingival crevicular fluid.

4.3.1 Studies investigating inflammatory mediators

Barr-Agholme et al. (1997) investigated the levels of prostaglandin E2 (PGE2) and interleukin-1β (IL-1β) in gingival crevicular fluid (GCF). GCF was collected from both DS patients and healthy controls. They found the mean level of PGE2 in GCF was significantly higher in the DS patients as compared to the controls. This finding suggests an alteration in arachidonic acid metabolism in DS patients. The mean level of IL-1β in the GCF was not significantly higher in the DS patients as compared to the healthy controls. Perhaps the reason the IL-1β levels did not differ between the groups, despite the fact that the gingival inflammation was more severe in the DS group, may be because PGE2 has been reported to down-regulate the production of IL-1β (Kunkel, et al., 1987). This issue has not been revisited since and indeed is worthy of further investigation.

Tsilingaridis et al. (2003) determined the levels of PGE2, LTB4, and MMP-9 in GCF from 18 Down syndrome patients and from 14 controls matched with respect to age and degree of gingival inflammation. Clinical periodontal parameters were recorded including probing depth (PD) and bleeding on probing (BOP). The mean levels of PGE2, LTB4, and MMP-9 were significantly ($P<0.05$) higher in GCF from Down syndrome patients compared to controls. When comparing the two groups, the correlation coefficients for LTB4 to BOP and PD, respectively, as well as for MMP-9 to BOP significantly differed between Down syndrome and controls ($P<0.05$).

4.3.2 Studies investigating degrading enzymes

Halinen et al. (1996) characterized the periodontal status of 9 non-institutionalized DS children 9 to 17 years old relative to their age-matched systemically and periodontally healthy controls. Clinical periodontal parameters were recorded. They also assessed the collagenase and gelatinase activities in the gingival crevicular fluid (GCF) and saliva samples collected from DS patients and from the controls. The endogenously active collagenase and total collagenase activities were slightly higher in GCF of DS children compared to healthy controls. Western blot demonstrated that GCF collagenase of DS patients was human neutrophil collagenase (MMP-8 or collagenase-2). Salivary collagenase in DS was high when compared to controls but of the same MMP-8 type as in control saliva.

Komatsu et al. (2001) examined both the amount present and the enzyme activity of matrix metalloproteinases (MMP-2) in the gingival tissues of Down syndrome patients and controls. The authors reported that there was a significantly higher production of MMP-2 in the cultured gingival fibroblasts of the Down syndrome patients when compared with the controls. In addition, the mRNA expressions of membrane-type I metalloproteinases (MTI-MMP) and MMP-2 were markedly different when the cultured fibroblasts of the DS patients were compared to the controls. This would indicate that the increased amount of active MMP-2 produced in DS could be linked to the simultaneous expression of MTI-MMP, which could also be connected to the cause of periodontal disease that is seen in a majority of DS patients.

Yamazaki-Kubota et al. (2010) investigated levels of matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-8 (MMP-8) in gingival crevicular fluid (GCF) and detection of periodontopathic bacteria from subgingival plaque. Samples of GCF and plaque were isolated from central incisors. Levels of MMPs were evaluated by enzyme-linked immunosorbent assay, and periodontopathic bacteria were detected by polymerase chain reaction. Levels of MMP-2 and MMP-8 in DS patients were higher than those in healthy control subjects. In the DS group, increases in these MMPs were observed in GCF from patients with good oral hygiene and absence of bleeding on probing. The detection rate of periodontopathic bacteria in DS patients was higher than that in the control subjects. Surprisingly, MMP-2 levels in sites harbouring *Porphyromonas gingivalis* or *Aggregatibacter (Actinobacillus) actinomycetemcomitans* were lower than in those without these microorganisms.

The cited studies indicate increased matrix metalloproteinase activity in the gingival tissues of DS individuals. The presence of MMP-8 suggests that neutrophils in their frustration to reach their target pathogens release their enzymes extracellularly. Matrix metalloproteinases are involved in the breakdown of the extracellular matrix. Their increased activity in the gingival tissues of DS individuals explains the gingival tissue loss and associated clinical signs (increased probing depth and loss of attachment) described. In addition the increased levels of prostaglandin E2 in combination with increased activity of MMP-9 suggests increased osteoclastic activity and explains the increased alveolar bone loss described in DS individuals.

5. A hypothesized model of the pathogenesis of periodontitis in Down syndrome

The presented articles viewed collectively suggest the following sequence of events in the pathogenesis of periodontitis in DS. Decreased salivary flow accompanied by reduced

salivary antibody production and defective neutrophil chemotaxis facilitates early microbial colonization in the dentogingival region and makes it easier for periodontal pathogens to gain a foothold. The heavy microbial presence initiates a strong gingival immune/inflammatory response characterized by the presence of high numbers of macrophages and lymphocytes in the gingival tissues. Antigen presenting cells are active in initiating adaptive immunity (as evidenced by the increased expression of HLA Class II antigens on inflammatory cells) and eventually the production of a strong humoral antibody response. The specific antibodies may help with containing the microbial infection. In addition, macrophages and other gingival resident cells (fibroblasts) seem to be engaged in high production of degrading enzymes. Frustrated neutrophils may release their degrading enzymes extracellularly into the gingival tissues. Tissue injury releases arachidonic acid metabolites (prostaglandins). The degrading enzymes and prostaglandins are involved with periodontal tissue destruction (figure 3).

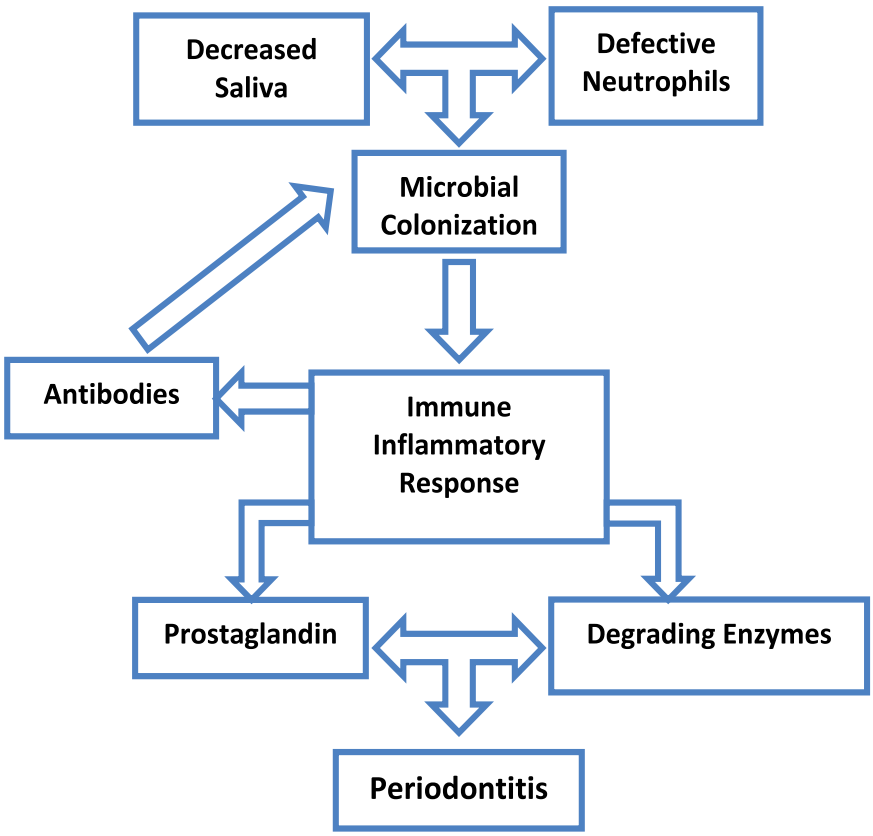

Fig. 3. Hypothesized model of periodontitis pathogenesis in Down syndrome.

6. Conclusions

In summary I have attempted to use the available periodontal literature on periodontitis in DS to construct a hypothesized model of the pathogenesis of the disease. Periodontitis in DS individuals is characterized by an intense and persistent immune/inflammatory response. Viewing this body of literature as a temporal sequence of events suggests that therapies aiming at reduction of microbial colonization could greatly benefit DS individuals by reducing the immune/inflammatory response and associated inflammatory mediators and degrading enzymes. The model also may suggest future therapies such as host response modulation. Strikingly absent from the model is information related to core immune/inflammatory abnormalities associated with DS such as increased oxidative stress, altered integrin expression (and associated altered lymphocyte function), altered IL-10 receptor expression (and possible impact on resolution of inflammation), and increased IL-1 production and their relation to periodontal disease. Further studies are needed to resolve these gaps in knowledge, and to access the implications for everyday periodontal care of this periodontally vulnerable population.

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12

Dysfunctional Voiding of Non-Neurogenic Neurogenic Bladder: A Urological Disorder Associated with Down Syndrome

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1. Introduction

Down syndrome (DS), the most common chromosomal abnormality with a prevalence of 1 case per 600 live births (Dennis & Marder,2001), has many manifestations that can affect multiple organ systems (American Academy of Pediatrics Comittee on Genetics, 2001; Kallen et al., 1996) including urological abnormalities which have been recognized throughout urinary tract (Ariel et al., 1991; Berg et al.,1960; Ahmed, 1990; Kravtzova et al., 1975; Handel et al., 2003; Kupferman et al., 1996; Bielek et al., 1996; Narashiman & Gupta, 2004; Koksal et al., 2003; Lang et al., 1987; Paulozzi et al., 1997). The extra copy of chromosome 21 has been suggested as an actor underlying the accompanied phenotypic abnormalities in the syndrome (Lejeune, 1959).

Berg et al. described the first cases of urological anomalies associated with DS in their study of 141 DS autopsy cases and found 1 patient (0.7%) with renal agenesis and 5 patients (3.6%) with horseshoe kidney (Berg et al., 1960). Glomerular microcysts, renal hypoplasia and obstructive uropathy have also been recognized as the most common upper urinary tract anomalies in DS and their incidences were reported by Ariel et al. who examined 124 autopsy cases of DS for abnormalities in urinary tract system to be as high as 23.7% (23 of 97 patients), 21.4% (18 of 84 patients), and 6.45% (8 of the 124 cases), respectively (Ariel et al., 1991). In addition, simple renal cysts were also found in 7 of 124 cases (5.6%).

Anomalies in ureteral structure has also been identified in DS (Ariel et al., 1991; Kravtzova et al., 1975; Handel et al., 2003). The incidence of obstructed megaureter or hydroureteronephrosis was reported in 2 of 124 cases (1.6%), while ureteral atresia was seen in 1 of 124 cases (0.8%) (Ariel et al., 1991). In addition, vesicoureteral reflux (Kravtzova et al., 1975), ureterovesical junction and ureteropelvic junction obstructions have also been described in patients with trisomy 21 (Handel et al., 2003).

Trisomy 21 is one of genetic disorders that can also be accompanied by some urological abnormalities in lower urinary tract. Non-neurogenic neurogenic bladder (NNB) has been reported as a functional lower urinary tract abnormality seen in DS. Handel et al. retrospectively reviewed 26 patients with trisomy 21 and found 4 patients (15.4%) with NNB (Handel et al., 2003). Posterior urethral valve has also been identified in patients with trisomy 21 (Bielek et al., 1996; Narashiman & Gupta, 2004; Koksal et al., 2003; Lang DJ et al., 1987). Bielek et al who reviewed 48 cases of posterior urethral valves found 4 patients with

DS (Bielek et al., 1996). Hypospadia has also a higher incidence in patients with the syndrome (Paulozzi et al., 1997).
There is a lack of awareness regarding DS related urological manifestations in daily clinical practice. The associated urological anomalies have received little attention from physicians especially for NNB which often cause the delay in referring the patients for further management. In this chapter we will focus on the NNB which represents voiding dysfunction due to functional bladder outlet obstruction recognized in DS. The discussion will include the evaluation and management of this particular disorder. Urodynamic assessment as the most important part in the diagnosis of NNB will also be described.

2. Dysfunctional voiding of non-neurogenic neurogenic bladder in Down Syndrome

2.1 Historical note

Non-neurogenic neurogenic bladder, the most severe form of dysfunctional voiding, is a pure functional bladder outlet obstruction without any neurological abnormalities which was described by Hinman (Hinman, 1986). However, it was first described by Beer who reported four patients with chronic urinary retention and upper urinary tract changes without any evidence of neurological pathology (Beer, 1915). He observed the disharmony between the detrusor and sphincter muscles in the patients.
Incoordination syndrome of voiding contraction reflex was also found by Laidley (Ladley, 1942). The syndrome was then called "achalasia of the urinary tract". Paquin et al. reported symptoms of dysfunctional voiding with large bladder in 27 children and the term "megacystis syndrome" was used to represent the syndrome (Paquin et al., 1960). Ambrose and Swanson reported the 13 children with hypertonic detrusor contraction without any neurologic lesion and labeled the syndrome as "hypertonic-type neurogenic bladder" (Ambrose & Swanson, 1960).
Hinman & Baumann reported voiding difficulties in children associated with upper urinary tract deterioration without any anatomical obstruction or neurologic lesion with the identified psychological disorder (Hinman & Baumann, 1972). The term "non-neurogenic neurogenic bladder" was introduced in order to emphasize the absence of neurologic lesions. The cases were followed for 15 years and then reported with new term "Hinman syndrome" according to the important of psychological factors which contribute to the syndrome and change the previous term which was not appropriate and cause confusion. However, the underlying psychological disturbances were not recognized in the syndrome as reported by Allan (Allan ,1977).
Non neurogenic-neurogenic syndrome in association with trisomy 21 was first identified by Handel et al. who reviewed 26 children with DS (all boys) and found higher incidence of NNB compare to other urological abnormalities in DS (Handel et al., 2003). Hicks et al reported the first women with NNB associated with DS and also found higher incidence of NNB cases in trisomy 21 (Hicks et al., 2007).

2.2 Epidemiology

The true prevalence of NNB has not been reported. The available prevalence is only based on data obtained from some centers. Groutz et al. reported that NNB was found in 2% of patients who underwent video-urodynamic studies but the real prevalence would be even

higher (Groutz et al., 2001). Jorgensen et al. reported lower prevalence of NNB (0.5%) among patients referred for urodynamic evaluation (Jorgensen et al., 1982).
Handel et al. reported a higher incidence of NNB in patients with trisomy 21. Among 26 patients with DS who were reviewed, 4 (15.4%) was reported to have NNB (Handel et al., 2003). Hicks et al also found higher incidence of NNB cases in DS (Hicks et al., 2007).[23] Non-neurogenic neurogenic bladder has been reported not only in infant and children but also in adult patients (Handel et al., 2003; Hicks et al., 2007; Jayanthi et al., 1997; Kai et al., 2007).

2.3 Etiology

The etiology of the disease is not clearly defined. Some evidences suggest overtraining of the pelvic floor to avoid urine loss due to not only various emotional conditions such as depression, phobias, stress, trauma associated with toilet training but also psychiatric diseases, the presence of detrusor overactivity and other associated voiding dysfunctions as an underlying factors which may contribute to cause NNB (Hoebeke et al., 1996; Koff et al., 1979; Goldston & Perlmutter, 1973; Schimtt, 1982; Ellsworth, 1995). The well known mental retardation and psychological problems in patients with DS may also explain the higher incidence of NNB in this trisomy 21.
In normal voiding function, bladder filling is accommodated at low bladder pressures with a bladder outlet that remains closed while bladder emptying requires a coordinated and sustained contraction of the bladder muscle with concomitant relaxation of the external urethral sphincter. The reflex of micturition is controlled by parasympathetic and somatic components of the sacral spinal cord and sympathetic innervation.
In NNB, functional bladder outlet obstruction is caused by active contractions of the voluntary external sphincters during voiding. The inability to inhibit the detrusor contraction reflex as well as overactivity of the external sphincter as compensation has become fundamental characteristics of patients with NNB (Hinman, 1986). The dyssynergistic voiding pattern created by the incoordination between the bladder and sphincter contraction reflex may result in impairment of renal function caused by functional obstruction (obstructive uropathy) due to unacceptable high voiding pressure with potential upper and lower urinary tract deterioration .

2.4 Diagnosis

General history-taking should include relevant questions for the screening of NNB. A thorough patient history will indicate incontinence associated with chronic urinary retention. History-taking in patients with DS is difficult especially in children due to the impairment of cognitive function and should be supplemented by information obtained from the parents. Because of the intelligence level, some of the existence as well as other underlying factors in patients with DS may be masked or neglected resulting in the delay of the diagnosis which often seen in clinical practice (Handel et al., 2003; Kai et al., 2007; Fernandez & Moore., 1986).
Although unerderlying factors could not be found in some cases, It is important to make sure that history taking have already included all the underlying factors of NNB which may guide to the diagnosis (Goldston & Perlmutter, 1973; Schmitt, 1982; Ellsworth et al., 1995). Neurological symptoms and abnormalities will not be present in NNB but should be confirmed by carefully clinical examination. Laboratory results may show elevated serum creatinine and reduce clearance or decreased renal function with or without signs of urinary tract infection in most of the cases.

Ultrasound is useful to evaluate bladder after voiding which can show residual urine volume as well as determine the bladder thickness. In addition, ultrasound is also important to detect the upper urinary tract dilatation with or without dilatation of the ureters as a complication of functional obstruction in NNB. In trisomy 21, this tool is also useful to identify other associated organ anomalies including abnormalities in the kidney.
To diagnose NNB in trisomy 21 patients, other associated anomalies such as Hirschsprung's disease, spinal cord defects, and posterior urethral valves must be excluded first before concluding that they have a functional rather than anatomical obstruction especially those who present with constipation, recurrent urinary tract infections, incontinence and voiding dysfunction (Handel et al., 2003). In order to rule out the neurological lesions, imaging studies of computed tomography (CT) scans and magnetic resonance imaging (MRI) should be performed (Johnson et al., 1992).
Non-neurogenic neurogenic bladder should be suspected if negative results have been confirmed by standard rectal biopsies and urological evaluation demonstrates the absence of anatomical obstruction and neurological evaluation including imaging studies confirms the absence of any neurologic lesions (Handel et al., 2003).

2.4.1 Voiding-cystourethrography

Voiding-cystourethrography is an important radiologic tool to show varying grade of vesico-ureter reflux in some cases of NNB. In addition, it also shows bladder appearance as a result of functional obstruction such as a trabeculated and enlarged bladder with a lot of residual urine still remains in the bladder after voiding without any evidence of mechanical obstruction (Fig. 1).

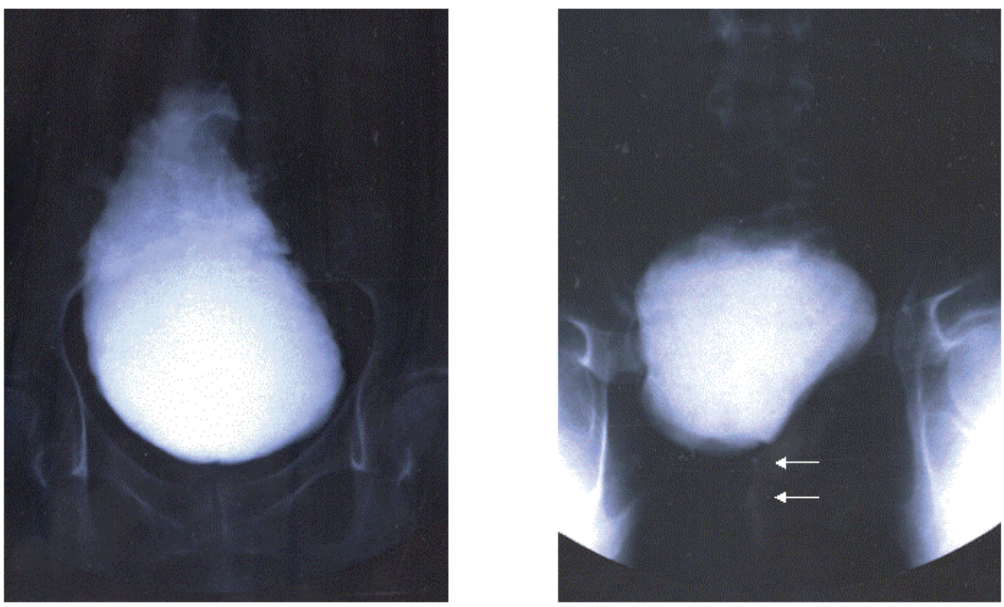

Fig. 1. Voiding cystourethrography shows a deformity of the bladder wall with trabeculation (left) and narrowing of the posterior urethra as pointed by white arrows with significant residual urine (right) without vesico-urethral reflux

In contrast, anatomical obstruction by posterior urethral valve can be identified by posterior urethral dilatation with poor visualization of the anterior urethra. However, it is important to note that the appearance of posterior urethra may entirely normal during early voiding but distended after contraction of the external sphincter as voiding progress which was found in 4 patients who previously diagnosed with NNB as reported by Johnson et al who reviewed the genitourinary images of six boys in whom NNB was diagnosed in the past 5 years (Johnson et al., 1992).

2.4.2 Uroflowmetry

Uroflowmetry is useful for the screening of NNB since it is able to measure the urinary stream during the voiding phase, providing information of bladder and outlet function (Allan & Bright, 1978; Stanton et al., 1983). In uroflowmetry measurement, it is important to pay attention on the the flow pattern beside other important parameters (Vijverberg et al., 1997). Normal urine flow is continuous bell-shaped curve. Continuous plateau shape curve (lower urinary flow rate with prolonged voiding time) is typical for anatomical obstruction.
In contrast, functional obstruction is typically reflected by a staccato pattern as interrupted flow of urine occurs due to uninhibited external sphincter contraction and usually demonstrates intermittency with reduced flow rates and prolonged voiding time (Fig. 2). The results also depend on the stage of the disease since the above pattern is hardly ever seen in advance cases with atonia of the bladder and poor detrusor contractility due to decompensated bladder. Uroflowmetry can also be used as a tool for follow-up of bladder training, and biofeedback training (Vijverberg et al., 1997; Hellstrom et al., 1987; Hjalmas, 1988; Griffiths & Scholtmeijer, 1984; Wear et al., 1979).

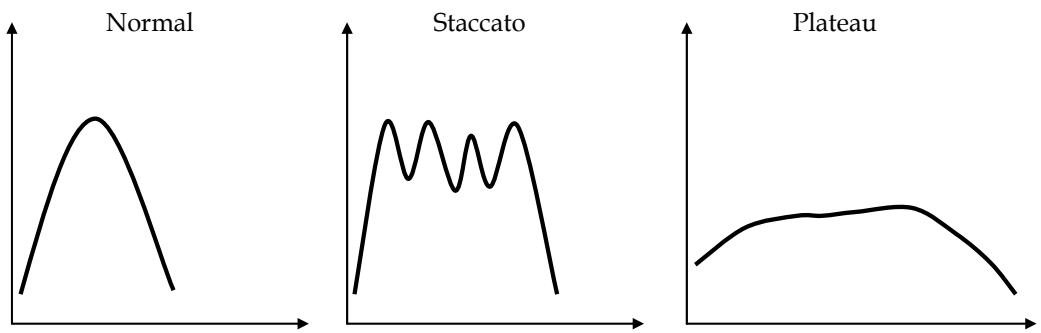

Fig. 2. The illustration of normal and abnormal uroflowmetry patterns.

2.4.3 Urodynamics

Urodynamics study is the gold standard for the diagnosis of patients with voiding dysfunction. (Nitti, 2005) Hypertonicity, instability with impaired external sphincter relaxation during micturation are typical characteristics in patients with NNB (Allan & Bright, 1978). Electromyography (EMG) shows a spastic activity with a high amplitude during voiding phase (Fig. 3).

True detrusor-external sphincter dyssynergia and NNB can be differentiated urodynamically (Rudy & Woodside, 1991). Detrusor-external sphincter dyssynergia is characterized urodynamically by increasing EMG activity during upslope and decreasing activity during down slope of the intravesical pressure tracing during a detrusor contraction. However, NNB is characterized urodynamically by quieting of the EMG during upslope and augmented activity during the down slope of the detrusor contraction.

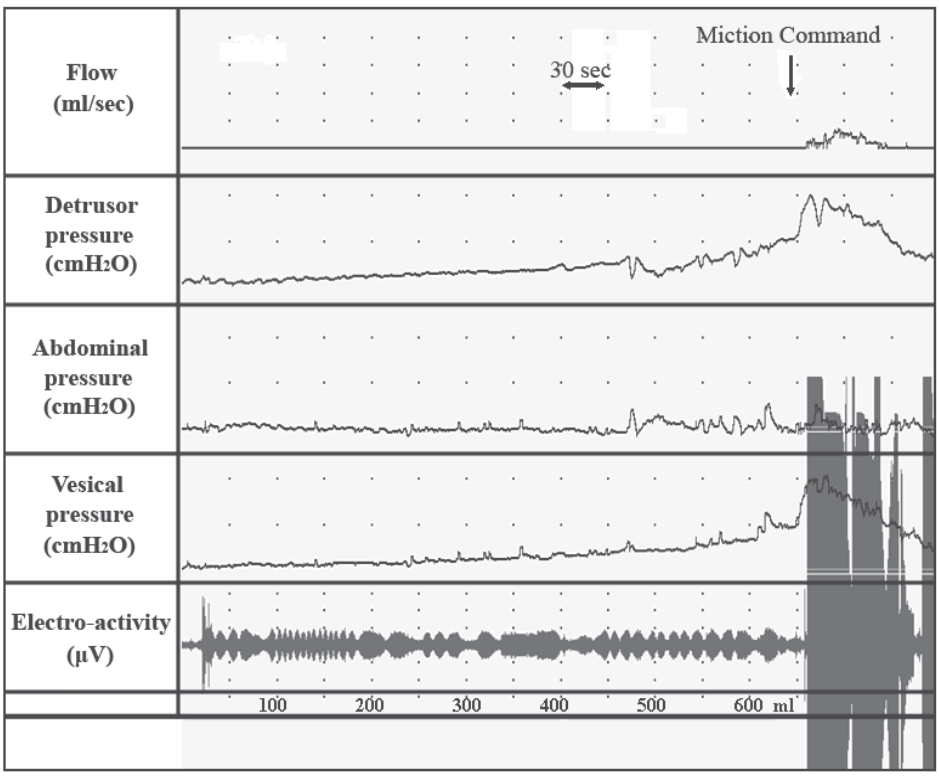

Fig. 3. Urodynamic results indcate a decrease in the bladder sensation without detrusor overactivity. A low urinary flow rate with a high detrusor pressure is shown in the voiding phase. Electromyography shows a spastic activity with a high amplitude during voiding. (Filling rate = 50 ml/min).

2.5 Management

Management of patients with NNB must be focused on the relaxation of external urethral sphincter muscle in order to obtain a more physiological micturation with relaxed sphincter muscle during voiding and to restore the balance of voiding and voluntary sphincter contraction reflex to prevent upper and lower urinary tract deterioration. The additional goals of treatment include prevention of urinary tract infections with proper antibiotics. The management should be addressed to all patients regardless of their intellectual capacity including those with trisomy 21. The intellectual Impairment of patients with DS may cause some difficulties in conducting the treatment.

Anti-cholinergics and alpha blocker therapy either alone or in combination may be useful in the treatment of NNB (Krane & Olsson, 1973; Paul et al., 1999; Bogaert et al., 2004) since significant improvement in emptying was shown in neurogenic cases treated with non selective alpha blocker (Krane & Olsson, 1973). If there is no response to conservative treatment, intermittent catheterization may be required. Trisomy 21 associated NNB patients with abnormal voiding mechanism against a closed sphincter may deal with the difficulty in conducting urethral clean intermittent catheterization due to their intellectual limitation (Handel et al, 2003).

Surgical treatment for renal preservation is also required for social continence. Self-catheterization protocols via the catheterizable stoma may allow trisomy 21 patients with a sufficient intellectual level master such techniques. Patients with NNB who are refractory to conservative treatment or present with an advanced pathological condition may require surgical intervention. Augmentation of the bladder in order to increase the bladder capacity may still be required in those in whom the upper tracts are threatened (Handel et al, 2003).

The use of Botulinum-A toxin injection into detrusor or the external sphincter may help cause striated muscle paralysis and may also restore voiding function especially in those who failed with drug therapy (Schurch et al., 1996). Patients with NNB in DS could also be performed such procedure. This particular treatment is dose dependent and also reversible. Injection may be necessary in every two months.

3. Summary

Children with trisomy 21 have many associated multi organ anomalies including urological abnormalities. Non-neurogenic neurogenic bladder is the most frequent DS associated lower urinary tract disorder. Early diagnosis and prompt management should be conducted in order to prevent upper urinary tract deterioration. According to the high incidence of NND in DS, early and regular sonographic and urodynamic evaluation of the bladder function is mandatory in patients with Down syndrome.

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13

Down Syndrome and Epilepsy

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1. Introduction

Down syndrome (DS) is associated with many neurological complications including cognitive deficits, early-onset dementia -which resembles Alzheimer's disease- and seizures. Although seizures and epilepsy were not mentioned in the original description of DS (Down, 1866) the prevalence of seizures in individuals with DS is now known to be higher than in the general population, but lower than in patients with some other types of mental retardation (Corvett et al., 1975). Reported rates of epilepsy in DS range from 1 to 13% (see table 1) (Tatsumo et al., 1984). Individual series are difficult to compare because of differences in inclusion criteria and study populations. The increased seizure susceptibility in DS has been attributed to inherent structural and molecular anomalies of the brain or to associated medical complications, such as cardiovascular abnormalities and recurrent infections.

Medical interventions in DS have resulted in increased longevity, with estimated life expectancy of people with DS in developed countries increasing from an average of 12 years in the 1940s to an average of 57,8 years for women and 61,1 years for men (Bittles et al., 2007). Epilepsy onset in people with DS is age-specific; therefore, because certain complications will arise in childhood and others in adulthood, their occurrence is relevant to paediatric and adult neurologists. This chapter will provide a critical overview of epilepsy in DS.

Authors	No. of patients	Percentage (%)
Romano et al.	113	13.00
Pueschel et al.	405	8.10
Stafstrom et al.	737	6.40

Table 1. Reported incidence of seizures in patients with Down syndrome in the 1990s

2. Epidemiology

Patients with DS show a higher incidence of febrile and non febrile seizures than non-DS individuals. Seizures occur in a bimodal distribution in DS, with 40% of individuals first developing seizures before 1 year of age and another 40% having an onset in their thirties or

later (Pueschel et al., 1991). Boys tend to have an earlier age onset, regardless of seizure type, although this may reflect the general male predominance in the infantile spasm group aged less than 1 year at onset. The prevalence of epilepsy increases with age and reaches 46% in those over 50. In general, about 8% of patients with DS have seizure disorders: 47% of them develop partial seizures, 32% infantile spasms and 21% generalized tonic-clonic seizures.

3. Pathophysiology

The mechanisms underlying the increased seizure susceptibility in DS have not yet been completely elucidated. Seizures in infancy have been linked to inherent structural brain abnormalities, such as fewer inhibitory neurons, abnormal cortical lamination, persistent fetal dendritic morphology, and underdeveloped synaptic profiles (Kemper et al., 1988). Concentrations of carbonic anhydrase II, which potentially increases seizure susceptibility, are upregulated in the brains of young children with DS and in a mouse model of the disorder. (Palminello et al., 2008) (Tatsuno et al., 1984).

Altered membrane potassium permeability, which may lead to a decreased voltage threshold for spike generation, smaller hyperpolarization following spikes, or increased action potential duration, has also been documented in patients with DS. Indeed, in the mouse model of trisomy 16, the experimental model of DS, a rapid spike rise and fall was recorded from the dorsal root ganglia neurons (Scott et al., 1981)

In DS there is an overexpression of the 21st chromosome. Many enzymes that are encoded on the extra 21st chromosome are known to be actively transcribed, which results in overexpression of the enzymes, overconsumption of enzymatic substrates and overproduction of metabolic end-products. For example, the superoxide dismutase-1 gene on the 21st chromosome is approximately 50% overexpressed, which decreases levels of superoxide (the enzyme's substrate) and increases levels of hydrogen peroxide (the enzyme's metabolite, end product or output). These primary consequences of genetic overexpression may then produce secondary metabolic adaptations as homeostatic systems attempt to compensate. Thus, decreased levels of superoxide might alter levels of nitric oxide, peroxynitrate, and nitric oxide synthetase, or they may impair aromatic hydroxylation enzymes and thereby impair neurotransmitter synthesis. For another example, increased levels of hydrogen peroxide might induce glutathione peroxidase production and thereby increase selenium requirements. Such genetically driven enzymatic and metabolic disturbances may help explain why individuals with DS appear to be more likely to develop various forms of epilepsy and intractable epilepsy (Smigielska-Kuzia et al. 2009).

Seizures can also be regarded as complications of congenital cardiovascular anomalies in children with DS (Marsh et al., 2009). Moyamoya's disease is a rare vascular complication which seems to occur with a higher frequency in children with DS than in those without it. Moyamoya's disease is characterised by a chronic occlusive cerebrovascular alteration of unknown pathogenesis in which there is progressive stenosis of the supraclinoid portions of the internal carotid arteries. Associated with this stenosis is the formation of convoluted arterial collaterals at the base of the brain. Although the presentation of Moyamoya's disease in adults is haemorrhagic, the presenting symptoms in children are typically ischaemic, with a fixed unilateral neurological deficit or alternating hemiplegia. Some children with Down's syndrome who have Moyamoya's disease also develop seizures or involuntary movements. (Nascimento et al. 2006).

Adult-onset seizures in the absence of dementia are rare in people with DS but might become more frequent in the future because of the extension of the lifespan of people with the disorder. Late-onset seizures in people with DS seem to be associated with a propensity to dementia resembling Alzheimer's disease. The cause of seizures in adults with DS who do not have dementia is not yet clear (Puri, 2001)

Once seizures occur in the course of dementia in patients with DS, functional decline is often rapid, to the point where floor effects preclude further cognitive testing. Seizures are common in people with early-onset Alzheimer's disease associated with genetic defects, including mutations that result in overexpression of amyloid-β, such as those involving presenilin 1 genes. Animals with overexpression of the APP gene have a lower threshold to induced seizures. High concentrations of amyloid-β caused by APP overexpression result in epileptiform activity in vivo, even in the absence of neurodegeneration, suggesting that these high concentrations are a direct cause of epilepsy. (T Llot 2010).

4. Clinical and electrophysiological features

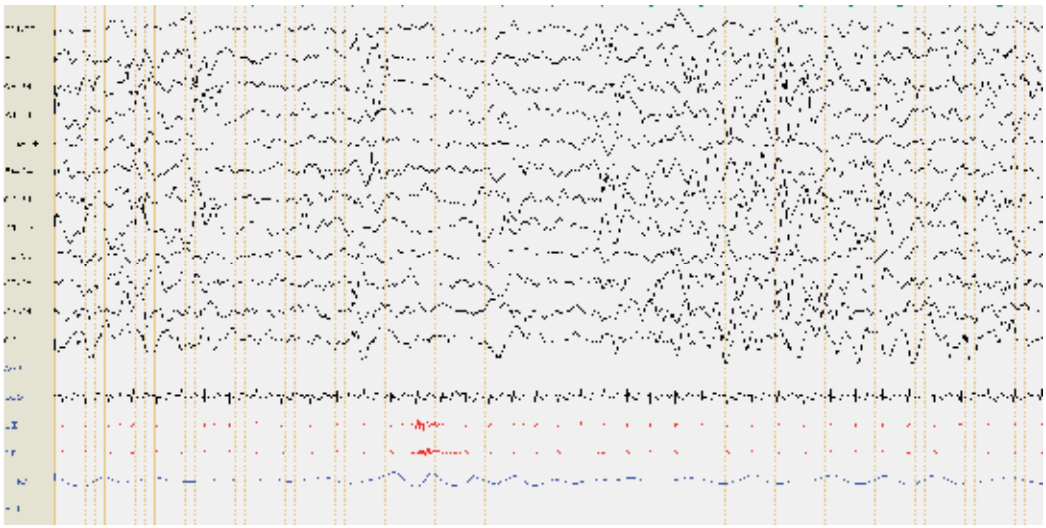
Epilepsy in DS showed a bimodal distribution; in the younger age group, infantile spasms and tonic-clonic seizures with myoclonus are the main finding, whereas older patients often have partial simplex or partial complex seizures as well as tonic-clonic seizures (Pueschel et al., 1991).

4.1 Seizures in infancy and childhood

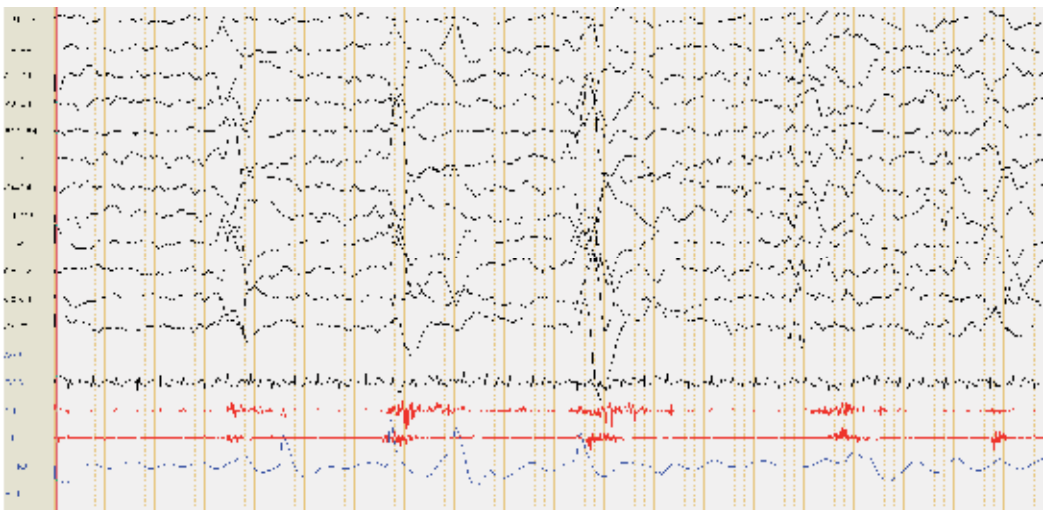
It is currently known that there is a higher incidence of seizures, particularly infantile spasms, in DS compared to the general population. Few children with DS have their first epileptic attacks after the age of 3. These patients often have partial simplex or partial complex seizures as well as tonic-clonic seizures.

Infantile spasms are an age-dependent epilepsy that most frequently appears in the first year of life, with ictal episodes consisting of spasms that usually occur in clusters (Dulac et al., 1994). There is a characteristic chaotic and high-voltage interictal electroencephalography (EEG) pattern, which, when typical, is called hypsarrhythmia. *West's syndrome* is the term employed when such spasms are concomitant with delayed psychomotor development and EEG hypsarrhythmia. It has been reported that 6.4% of 737 patients with DS had epilepsy, and 12.8% of epileptic patients with DS had West syndrome. In addition, it has been reported that 8.1% of 405 patients with DS had epilepsy in childhood, and 18% of the epileptic patients were diagnosed as having infantile spasms.

Infantile spasms, which often indicate poor prognosis in the general population, do not seem to be associated with difficulties in long-term seizure control in children with DS. However, in children with DS who have infantile spasms, there seems to be a substantial association between treatment and developmental quotient as well as progression to autistic features. There are no long-term studies of the intellectual outcome of children with DS in whom infantile spasms were successfully controlled. In our experience, patients with Down syndrome with infantile spasms and abnormal but non-hypsarrhythmic EEG may have poor disease progression (figure 1 a and b & Figure 2 a and b), with persistence of seizures and severely impaired psychomotor development (Nascimento & Ortez 2009).

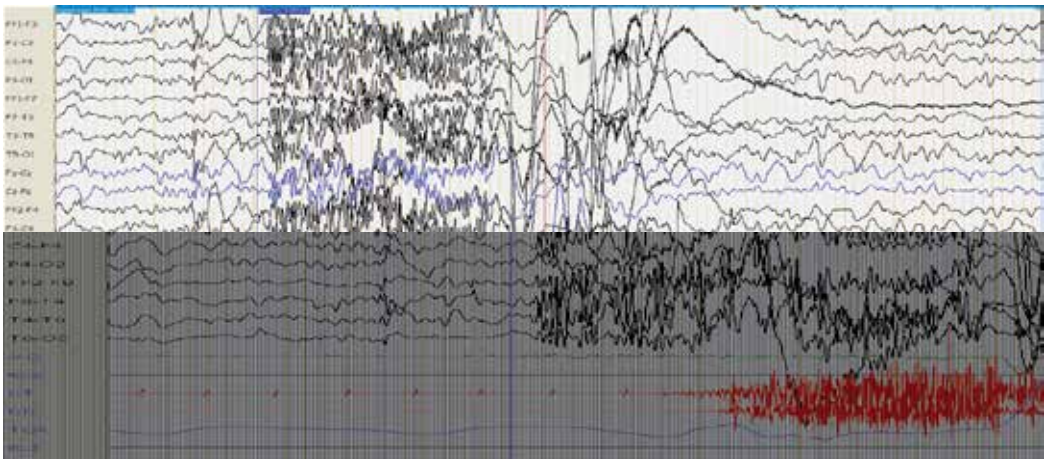


a)

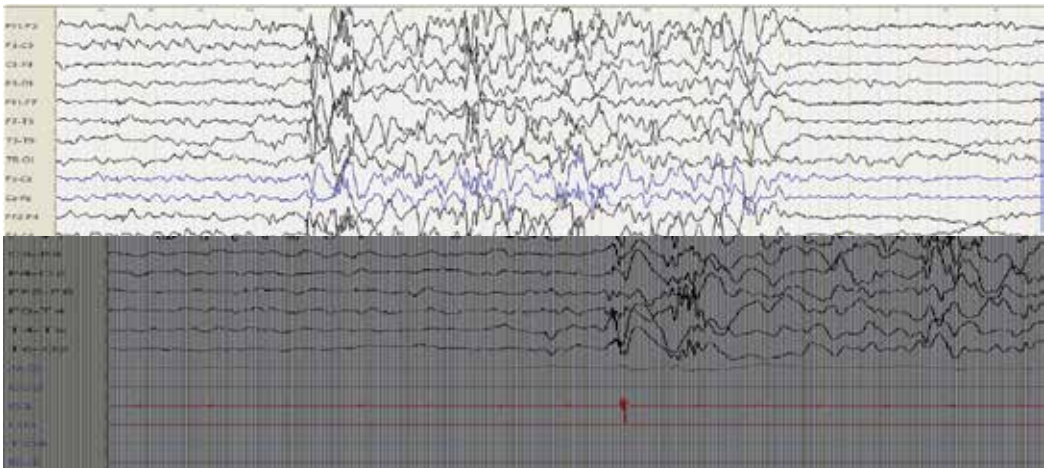


b)

Fig. 1. EEG of an 8m21d boy with DS. a) In a sleep stage, no physiological graphoelements were observed and there were very frequent multifocal paroxysms in the form of high-voltage spike-wave complexes; paroxistic alterations persisted during the waking stage. b) There was an electroclinical episode consisting of the emergence of a high-voltage spike-wave complex clinically accompanied by extension and abduction of the upper limbs and a slight extension of the lower limbs. Note the contraction recorded over both deltoid muscles.



a)



b)

Fig. 2. EEG of a 3-year-old girl with DS initially affected by West's syndrome who then evolved to Lennox-Gastaut syndrome. a) Tonic seizure: Ictal electroencephalography (EEG) shows a diphasic, mid-voltage slow wave, followed by recruiting low-voltage fast activity, replaced by spike and polyspike and wave discharges; tonic contraction was recorded over both deltoid muscles. b) Waking stage: brain activity constituted by an association of delta and theta waves and low-voltage beta rhythms, with a diffuse distribution. Frequent focal paroxysms were observed in the form of spikes and complex spike-waves of irregular medium and high amplitude, located independently in the frontal and parieto-occipital regions of both hemispheres.

There is a chance that DS in association with West's syndrome may evolve to Lennox-Gastaut syndrome (LGS). LGS has classically been defined by the triad of drug-resistant epilepsy with multiple seizure types, typically diurnal atonic as well as atypical absences, and mainly nocturnal tonic seizures; electroencephalography abnormalities, principally diffuse slow spike-wave or polyspike-wave discharges during wakefulness and bursts of diffuse fast rhythms at 10–20 Hz during sleep; mental deterioration of variable severity as well as behavioural disturbances may also occur (Gastaut et al., 1966; Dulac & N'Guyen, 1993). A series of DS patients with late onset LGS has been described, having a higher frequency of reflex seizures and more cognitive impairment (Figure 3). (Ferlazzo et al., 2009)

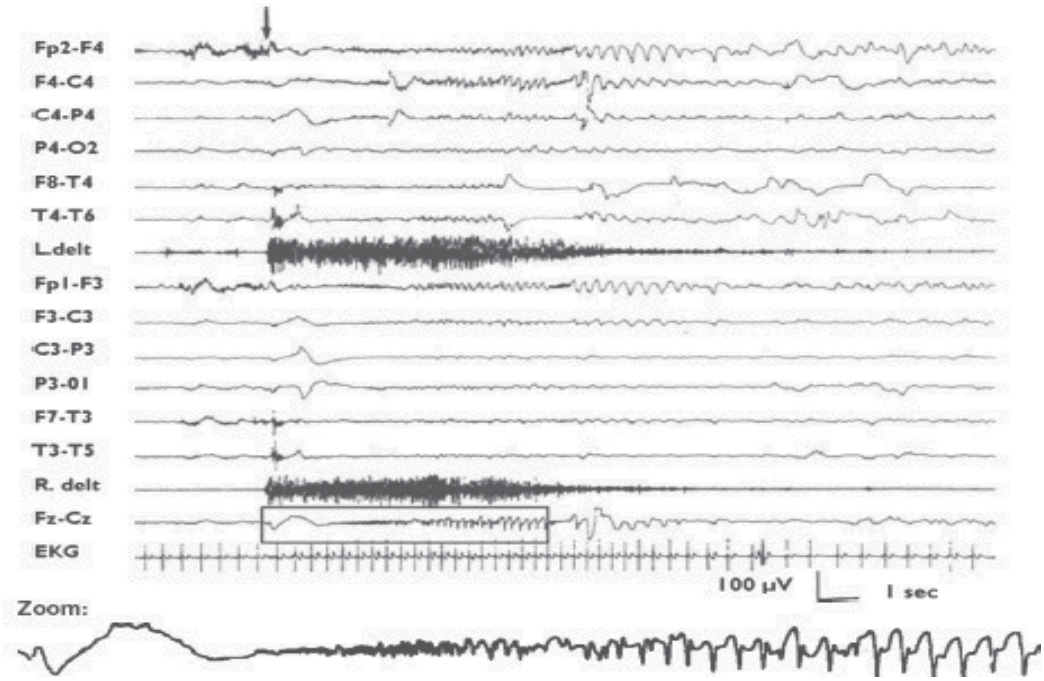


Fig. 3. 7-year-old boy. Tonic seizure triggered by a sudden noise (black arrow). Note the marked tonic contraction recorded over both deltoid muscles. Ictal electroencephalography (EEG) shows a diphasic, mid-voltage slow wave, followed by recruiting low-voltage fast activity, replaced by spike and polyspike and wave discharges, predominant over anterior leads, lasting for about 10 s. Electrocardiography shows an increased heart rate during seizure. Zoom: detail of the ictal discharge recorded over the vertex derivation, where muscular artifacts are less evident. (Ferlazzo et al. 2009)

Why do some infants with DS develop infantile spasms, whereas others with the same disorder do not? The cause has never been fully understood. Naming DS as the ''cause'' of the infantile spasms may, therefore, be at best inaccurate and at worst totally misleading, suggesting that we understand why the disorder has arisen in that particular child when DS has merely predisposed the child to infantile spasms. Only a few infants with Down syndrome develop infantile spasms, yet Down syndrome is accepted (Eisermann et al., 2003) as a ''cause,'' and frequently such infants are not given a brain scan. As a result, other authors have proposed adopting a terminology that distinguishes the underlying etiology

from the cause. According to this scheme, we use the term *proven etiology* to refer to any identified underlying neurological disorder, and employ *cause* as a more specific term that may be a complex and less well-understood sequence or combination of events. Perhaps because of the many different diagnoses that can be made in these infants and the developmental outcomes associated with them, classification into diagnostic groups has been commonly attempted. (Osborne et al., 2010)

The most frequent nomenclature has classified cases as either symptomatic, cryptogenic, or idiopathic, but unfortunately there is no clear definition of these terms (Lux & Osborne, 2005). "Symptomatic" is often used to indicate that a prior disorder exists. "Cryptogenic" is often used to mean that there must be an etiology, but that one has not been found. A recent report of the International League Against Epilepsy (ILAE) Commission on Classification and Terminology also suggests that the terms idiopathic, symptomatic, and cryptogenic should be replaced (Berg et al., 2010). It has suggested broad etiologic categories: genetic, structural–metabolic, and unknown.

4.2 Seizure in adulthood

The prevalence of epilepsy in DS has increased with longevity, reaching 46% in those older than 50. Descriptions of late-onset epilepsy in the absence of dementia in DS patients (LOMEDS) are rare, but since life expectancy of DS patients has markedly increased, LOMEDS may be more frequent than currently acknowledged and should be considered in the differential diagnosis of adult-onset myoclonic epilepsies. The electroclinical features are myoclonic jerks on awakening and generalised tonic-clonic seizures, with generalised spike and wave on EEG, and progressive dementia (Moller et al., 2002).

Familial Alzheimer's dementia (FAD) and progressive myoclonic epilepsy (Unverricht–Lundborg type) are both linked to chromosome 21. In an interesting study of 68 DS adults, it was found that among those with a history of seizures, individuals aged over 45 years were significantly more likely to develop AD than those under 45, and up to 84% of demented individuals with DS developed seizures. It suggests that late-onset epilepsy in DS is associated with AD, while early-onset epilepsy is associated with an absence of dementia (Menéndez, 2005).

Seizures in adults with DS differ from those in adults with Alzheimer's disease who do not have DS: myoclonic seizures usually occur late in the course of Alzheimer's disease whereas partial or tonic–clonic seizures occur in adults with Down's syndrome and are often precursors to cognitive decline. Adults with DS aged over 45 years who have seizures are substantially more likely to develop signs of Alzheimer's disease (Puri et al; 2001). Once seizures occur in the course of dementia in patients with DS, functional decline is often rapid, to the point where floor effects preclude further cognitive testing. Seizures are common in people with early-onset Alzheimer's disease associated with genetic defects, including mutations that result in overexpression of amyloid-β, such as those involving presenilin 1 genes.

Animals with overexpression of the *APP* gene have a lower threshold to induced seizures. High concentrations of amyloid-β caused by *APP* overexpression result in epileptiform activity in vivo, even in the absence of neurodegeneration, suggesting these high concentrations directly as a cause of aberrant neuronal network synchronisation. Slowing of the dominant occipital rhythm seems to be associated with dementia in individuals with DS, and the frequency of dominant occipital activity decreases as cognition deteriorates.

4.3 Seizure in Alzheimer´s and DS

Despite the apparent clinical heterogeneity in aged individuals with DS, age-associated AD-like neuropathology is a consistent feature. This is due to the fact that trisomy 21 leads to a dose-dependent increase in the production of the APP and subsequently the production of the amyloidogenic fragments leading to early and predominant senile plaque formation.
Ten percent of patients with AD have seizures, and another 10% have myoclonus. The incidence of seizures is about 10 times higher than expected in a reference population. Both seizures and myoclonus, individually or together, are manifestations of AD and may be seen at any time in the course of the illness, but myoclonus is often a late manifestation. (Volicer et al., 1995)

4.4 Electroencephalography (EEG) features

Individuals with DS have increased absolute power in all the EEG bands, independent of cognition functions. In the power spectrum of the resting EEG, there is a cognition-related increase in power at theta- and alpha-slowing. Furthermore, in the stimulated EEG, there are several cognition-related abnormalities, such as decreased responses to 12-Hz stimulation and decreased integral of beta- and gamma-band responses, indicative of decreased responsiveness to photic stimulation. Other reports note an increase in power at theta and delta in children with DS during sleep. (S'migielska-Kuzia et al., 2009). There is a significant increase in theta, delta, and beta power and a decrease in alpha compared with non-DS with epilepsy. (Figure 3)

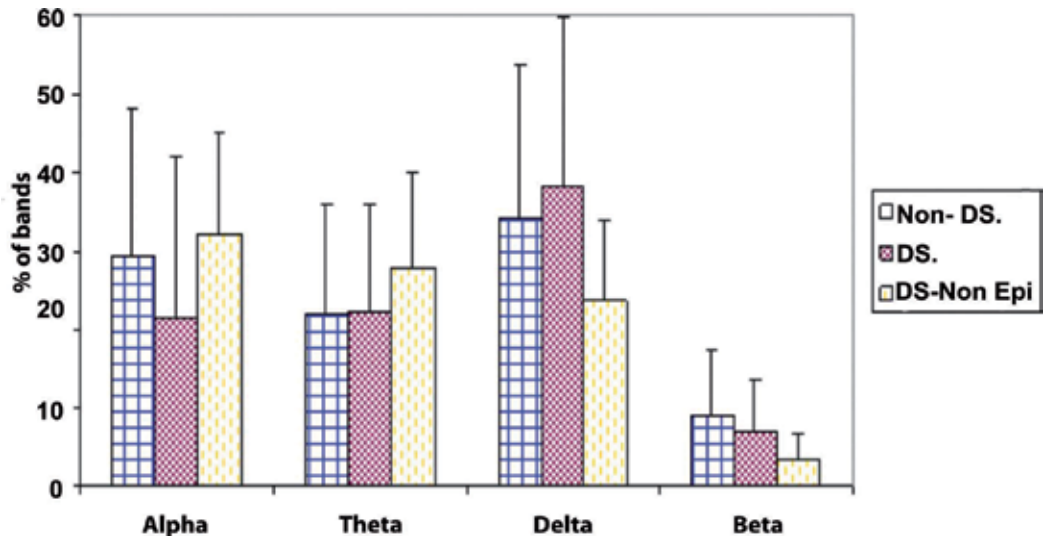


Fig. 3. Spectral power of alpha, theta, delta, and beta bands of DS, n = 12, DS Non-Epilepsy (Non-Epi) n=10 versus Non-DS (epileptic children) n = 28. Alpha Non-DS versus alpha DS P < .001; alpha Non-DS versus alpha DS Non-Epi P = .001; alpha DS versus alpha DS Non-Epi P < .001; theta Non-DS versus theta DS P = .767; theta Non-DS versus theta DS Non-Epi P < .001; theta DS versus theta DS Non-Epi P < .001; delta Non-DS versus delta DS P < .001; delta Non-DS versus delta DS Non-Epi P < .001; delta DS versus delta DS Non-Epi P < .001;beta Non-DS versus beta DS P < .001; beta Non-DS versus beta DS Non-Epi P < .001; beta DS versus beta DS Non-Epi P < .001. (S'migielska-Kuzia et al., 2009)

5. Treatment of epilepsy in DS

The pharmacological treatment of epilepsy in DS is no different from that of other patients diagnosed with epilepsy; the key is proper clinical and electrical classification to guide epilepsy treatment and thereby obtain good therapeutic results.

Although the cognitive profiles of newer antiepileptic drugs (AEDs) are in general better than those of older antiepileptic drugs, neurological adverse events do occur, including somnolence, distractibility, dizziness, and an altered pattern of sleep architecture. Individuals with DS have an unusually high number of side-effects from phenytoin (Tsiouris et al., 2002).

Over time, with the advent of advances in molecular biology, many AEDs have been uncovered, so they have come to be classified as first-, second- and third-generation drugs. (see table 2)

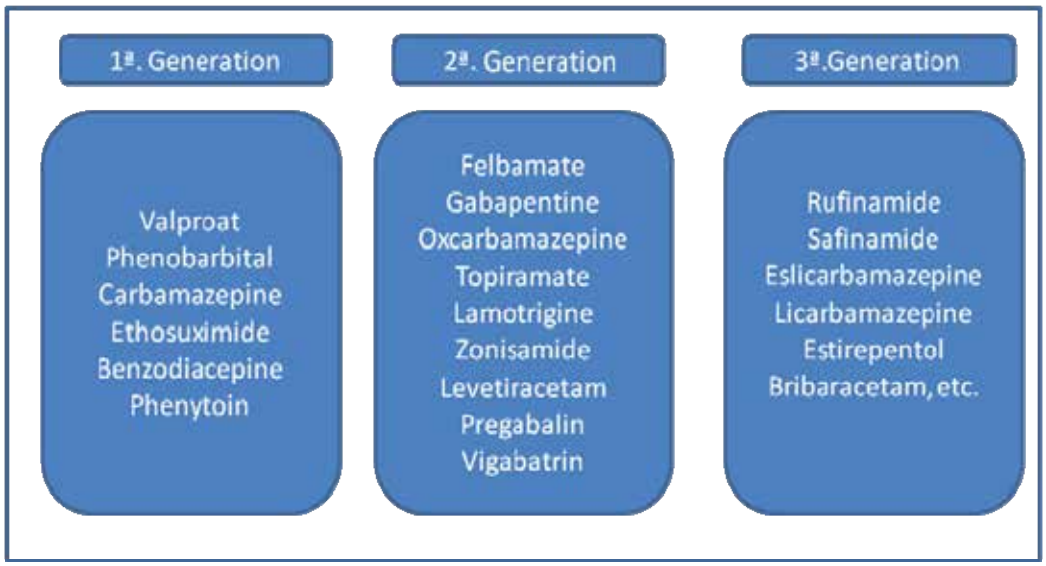


Table 2. Antipileptica drugs by generation

Figures 4 a & b and table 3 describe the different mechanisms of action demonstrated for antiepileptic drugs (AEDs).

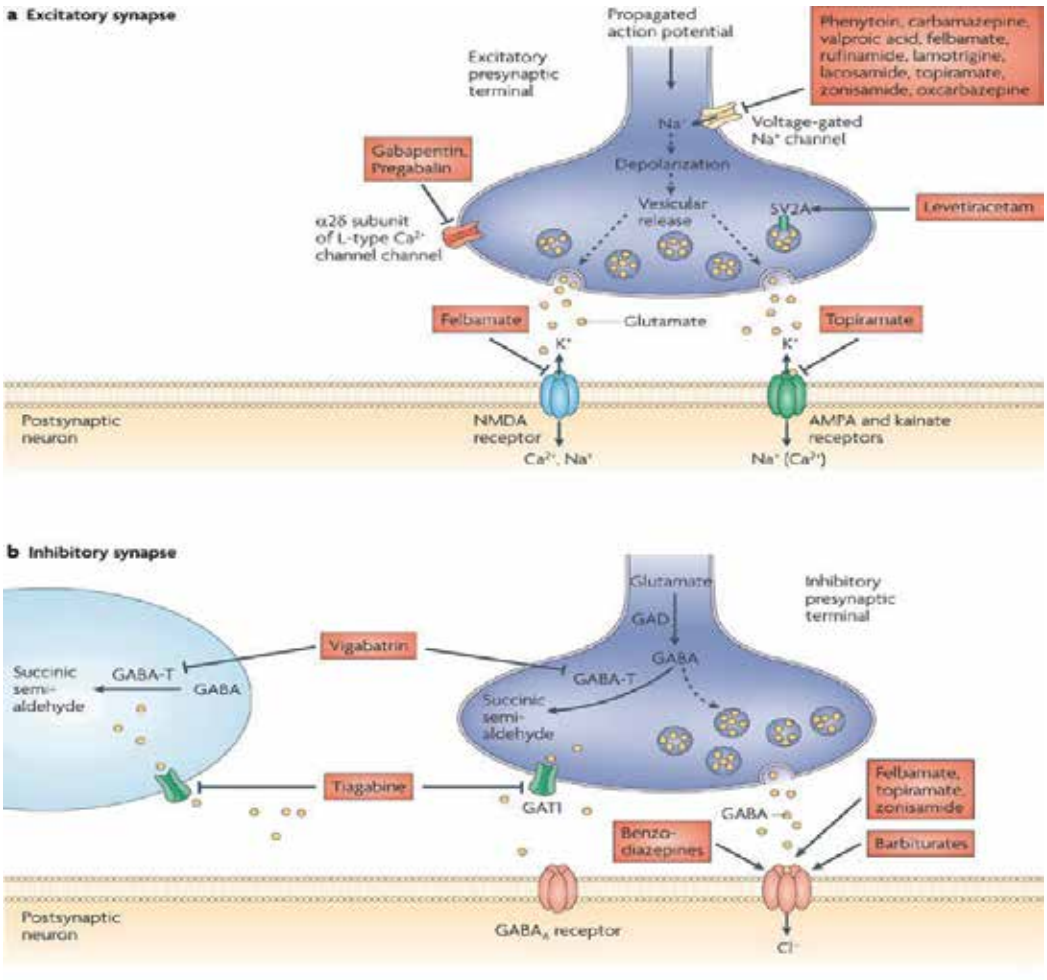


Fig. 4. a& b Proposed mechanisms of action of currently available AEDs at excitatory and inhibitory synapses.

a | Currently available antiepileptic drugs (AEDs) are thought to target several molecules at the excitatory synapse. These include voltage-gated Na^+ channels, synaptic vesicle glycoprotein 2A (SV2A), the α2δ subunit of the voltage-gated Ca^{2+} channel, AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptors, and NMDA (*N*-methyl-D-aspartate) receptors. Many of the AEDs can modulate voltage-gated Na^+ channels. This would be expected to decrease depolarization-induced Ca^+ influx and vesicular release of neurotransmitters. In addition, lacosamide is thought to enhance slow inactivation of voltage-gated Na^+ channels. This effect is different from that of other listed AEDs, which are thought to enhance fast inactivation. Levetiracetam is the only available drug that binds to SV2A, which might have a role in neurotransmitter release. Gabapentin and pregabalin bind to the a2δ subunit of voltage-gated Ca^{2+} channels, which is thought to be associated with a decrease in neurotransmitter release. Excitatory neurotransmission at the postsynaptic membrane can be limited by topiramate (acting on AMPA and kainate receptors) and felbamate (acting on NMDA receptors).

b | AED targets at inhibitory synapses have also been proposed. These include the g-aminobutyric acid (GABA) transporter GAT1 (also known as SLC6A1), which is inhibited by tiagabine, leading to a decrease in GABA uptake into presynaptic terminals and surrounding glia; and GABA transaminase (GABA-T), which is irreversibly inhibited by vigabatrin. This decreases the metabolism of GABA in presynaptic terminals and glial cells. The benzodiazepines, barbiturates, topiramate and felbamate have been found to enhance inhibitory neurotransmission by allosterically modulating $GABA_A$ receptor-mediated Cl^- currents. However, the action of each of these drugs is different and is dependent on the subunit conformation of the $GABA_A$ receptor complex. GAD, glutamic acid decarboxylase. (Bailer & White 2010).

	Blockade of voltage-dependent sodium channels	Increase in brain or synaptic GABA levels	Selective potentiation of $GABA_A$-mediated responses	Direct facilitation or chloride-ion influx	Blockade of calcium channels	Other actions
First generation AEDs.						
Benzodiazepines	-	-	++	-	-	-
Carbamazepine	++	?	-	-	+	+
Ethosuximide	-	-	-	-	++	-
Phenobarbital	-	+	+	++	?	+
Phenytoin	++	-	-	-	?	+
Valproic acid	?	+	?	-	+	++
Second generation AEDs.						
Felbamate	++	+	+	-	+	+
Gabapentin	?	?	-	-	++	?
Lamotrigine	++	+	-	-	++	+
Levetiracetam	-	?	+	-	+	++
Oxcarbamacepine	++	?	-	-	+	+
Pregabalin	-	-	-	-	++	-
Tiagabaline	-	++	-	-	-	-
Topiramate	++	+	+	-	+	+
Vigabatrin	-	++	-	-	-	-
Zonisamide	++	?	-	-	++	+

++ primary action; + secundari action; -, no action described; ?, controversial evidence.

Table 3. Main Mechanism of action of AEDs.

Table 4 describes the different AEDs and the main indication for the type of epilepsy diagnosed. Table 5 describes the AEDs, dose in children and adults and side effects.

FIRST GENERATION AEDs	MAIN INDICATION
Benzodiazepines	Status epilpeticus. Partial an generalizated seizures
Carbamazepine	Partial seizures (with and whithout secondary generalization) and primarily generalized tonic-clonic seizures.
Ethosuximide	Absence seizures, continuous spike – waves during slow sleep.
Phenytoin	Partial seizures (with and whithout secondary generalization) and primarily generalized tonic-clonic seizures. Status epilepticus.
Valproic acid	Generalized and partial seizures. Status epilepticus.
SECOND GENERATION AEDs	MAIN INDICATION
Felbamate	Severe epilepsies, particulary Lennox-Gastaut syndrome, refractory to all other AEDs.
Gabapentin	Partial seizures (with and whithout secondary generalization)
Lamotrigine	Partial and generalized seizures (may aggravate severe myoclonic epilepsy of infancy)
Levetiracetam	Partial and probably generalized seizures.
Oxcarbamacepine	Partial seizures (with and whithout secondary generalization) and primarily generalized tonic-clonic seizures.
Pregabalin	Partial seizures (with and whithout secondary generalization).
Tiagabaline	Partial seizures (with and whithout secondary generalization).
Topiramate	Partial and generalized seizures (efficacy against absence seizures not proven)
Vigabatrin	Infantile spasms and West Sindrome. Partial seizure (with and whithout secondary generalization) refractory to all other AEDs.
Zonisamide	Partial and, probably, generalized seizures.

Table 4. AEDs and the main indication for the type of epilepsy diagnosed

AEDs	Childrens Dose mg/kg/d	Adults Dose mg/day.	Side Effects.
Carbamacepine	15-30	600 – 2000 mg divided into up to 4 doses a day.	Skin rash, if allergic to carbamazepine. Diplopia (double vision), ataxia (unsteadiness) and nausea may occur initially or if the dose is too high.
Clobazam	0,1-0,2	20 - 50 mg divided into 1 or 2 doses a day.	Drowsiness may occur but this drug is less sedating than clonazepam or diazepam. Tolerance may develop.
Clonazepam	0,5-0,8	1 – 4 mg divided into 2 doses a day.	Drowsiness and sedation are quite common but these may wear off.
Ethosuximide	20-35	750 – 1500 mg divided into 2 or 3 doses a day.	Nausea and drowsiness may occur initially or if the dose is too high. Anorexia (weight loss).
Gabapentine	30	1800 – 3600 mg divided into 3 doses a day.	Drowsiness, dizziness, and headache
Levetiracetam	20-60	1000 – 3000 mg divided into 2 doses a day.	Dizziness, drowsiness, irritability, behavioural problems, insomnia, ataxia (unsteadiness), tremor, headache, nausea may occur in high dosages or when doses are increased, but will usually disappear after a few days.
Lamotrigine	5-15 1-5 + VPA	100 - 200mg if taken alone or if also taking sodium valproate. 200 – 400 mg if also taking phenytoin ,	Skin rash if allergic to lamotrigine. Drowsiness, diplopia (double vision), dizziness, headache,

AEDs	Childrens Dose mg/kg/d	Adults Dose mg/day.	Side Effects.
		phenobarbitone or carbamazepine.	insomnia, tremor and flu-like symptoms.
Oxcarbamazepine	15-30	1200 - 2400mg divided into 2 or 3 doses a day.	Skin rash, if allergic to oxcarbazepine. Diplopia (double vision), ataxia (unsteadiness), headache, nausea, confusion and vomiting.
Phenobarbital	3-5	30 – 180 mg divided into 2 doses a day.	Drowsiness may occur initially. Lethargy, sedation and slowing of mental performance may be long-lasting.
Phenytoin	5-10	150 – 600 mg divided into 1 or 2 doses a day.	Skin rash if allergic to phenytoin. Drowsiness, ataxia (unsteadiness) and slurred speech may occur if the dose is too high. Coarsening of facial features, overgrowth of gums, excess hair growth and acne may occur with prolonged therapy (over many years), as can some anaemias.
Topiramate	5-9	Up to 400 mg daily if taken alone. Usually 200 – 400 mg daily if taken with other anti-epileptic drugs, up to 800 mg.	Headache, drowsiness, dizziness, paraesthesia (pins and needles in hands and feet), loss of weight, and kidney stones. Speech disorder, impaired memory and concentration may occur when dose is increased

AEDs	Childrens Dose mg/kg/d	Adults Dose mg/day.	Side Effects.
			but will usually disappear after a few days.
Sodium valproate	20-50	400 – 2000 mg divided into 1 or 2 doses a day.	Drowsiness and tremor are infrequent side effects. Hair loss occurs in some people but is not usually severe and is usually reversible if the dose is reduced. Weight gain may occur. Liver. damage is rare. Sodium valproate has been associated with increased incidence of Polycystic Ovary Syndrome and menstrual irregularitiesthan other AEDs, if taken in pregnancy.
Vigabatrine	50-200	1000 – 4000 mg divided into 1 or 2 doses a day.	Drowsiness, behaviour and mood changes. Psychotic reactions have been reported. Visual field defects have been reported in one in three people taking vigabatrin in the long term.
Zonizamide	4-8	300 - 500mg divided into 1 or 2 doses a day.	Skin rash if allergic to zonisamide. Drowsiness, dizziness, weight loss, kidney stones, confusion, cognitive slowing, agitation, irritability, depression,

Table 5. AEDs, dose in children and adults. Side effects.

6. Conclusions

The findings of the last decade regarding the significant percentage of children with DS and epilepsy (approximately 1 in 10) highlight the importance of the awareness that physicians should start developing about this association so that they can intervene as early as possible when seizures are suspected, to maximize the patient's development and improve quality of life as much as possible.

The diagnosis, classification and treatment of epilepsy in DS must follow the guidelines applied to the general population.

Children with DS have a greater predisposition to epilepsy (specifically to West Syndrome and infantile spasms) attributed to structural and molecular abnormalities. In contrast, in adults with DS, epilepsy is associated with the accumulation of amyloid-β, due to the expression of APP that is observed in DS patients with early-onset Alzheimer-like dementia.

The prognosis of patients with DS and epilepsy will depend on several factors: type of epilepsy, age of initiation, etiology, early diagnosis and treatment's response. Epilepsy in patients with DS, as well as in the general population, in most cases represents an alteration of brain function that can be detrimental to neurological development; this produces a great amount of anxiety and concern in their parents and relatives. For this reason, all of the prognostic factors must be carefully taken into account when considering each individual case. The disease, its control and its management should be discussed in a clear and simple way with the family.

7. Acknowledgments

We would like to express our gratitude to all the patients with DS and their families; to the Library's department of the Fundació Catalana Sindrome de Down whom have made a good work in the translation of this chapter; to the members of the Epilepsy Unit from the Neurolgy Service of Sant Joan de Dèu's Hospital and to our families for their support and time.

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14

Endocrine and Autonomic Nervous Adaptations during Physical Exercise in Down Syndrome

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1. Introduction

One of the goals of Healthy People 2011 is to increase the quality of life and the years of healthy life of all citizens. As the life expectancy of people with Down Syndrome [DS] increases, changes in body function and structure due to ageing may lead to lead to activity limitations and participation restrictions for this population. The physical conditions seen in people with DS include endocrine dysfunction, cardiovascular disorders, obesity, and musculoskeletal disorders (Finesilver, 2002; Thompson, 1999). These physical problems can have a negative impact on the quality of life, regarding both professional and leisure activities.

Compared with their peers who are not mentally retarded, individuals with DS, regardless of their age, have lower cardiovascular fitness levels (Horvat & Croce, 1995; Tsimaras et al., 2003). This lower level of cardiovascular fitness may be the result of poor eating habits, a sedentary lifestyle, a lack of opportunity for recreational activities, a poor coordination, and a poor motivation for physical activity. In addition, the lack of cardiovascular fitness may be due to or caused by the high obesity rates observed among adults with mental retardation (Draheim et al., 2002). Poor cardiovascular fitness levels also may contribute to an increased risk of heart disease and stroke in adults with mental retardation (Sutherland et al., 2002).

Therefore, involvement in cardiovascular conditioning programs would seem to be essential, however, can the cardiovascular fitness levels of these individuals, be improved with exercise, without major health risks?

If exercise programs seem to be likely to improve the health of adults with DS, thereby increasing their quality of life and life expectancy, yet further research is needed to investigate the effects of exercise on adults, because some autonomic nervous regulations and endocrine adaptations are known to be inadequate in individuals with DS (Baynard et al., 2004; Figueroa et al., 2005; Iellamo et al., 2005; Gouloupoulou et al., 2006; Bricout et al. 2008).

2. Situation of the subject in international literature

One hypothesis explaining the improvements in life expectancy of DS individuals is based on the physical stimulation and psychological standing of these subjects (Eberhard et al., 1989; Draheim et al., 2002). Now, adults with trisomy 21 are living longer than previously

and individuals surviving into their sixth or seventh decade of life are encountered (Prasher & Krishnan, 1993). So, there is a need for an overall assessment of the health of adults with trisomy 21, and specifically as the sport activities helps improve the quality of life, and reduce risk factors, it seems important to know the possible effects of physical exercise on the health of these young people with Down syndrome.

In this order, few studies have investigated cardiac and hormonal responses in patients with Down syndrome subjected to acute physical exercise (Murdoch, 1977; Murdoch et al. 1978; Fernhall & Otterstetter, 2003; Baynard et al., 2004; Iellamo et al., 2005; Figueroa et al., 2005; Gouloupoulou et al., 2006). The conclusions of these few studies show that there are inappropriate responses that lead to the appearance of early fatigue, a premature stop of the effort, and/or a limitation to the activity. It was previously suggested that fatigue during exercise, or at least premature stop or the incapacity to do prolonged physical exercise in DS subjects, may partly be explained by a dysfunction of the autonomic nervous system [ANS] (Baynard et al., 2004; Iellamo et al., 2005; Figueroa et al., 2005; Gouloupoulou et al., 2006).

Secondly, others studies have investigated the hormonal adaptation's in specific physical tests and the results of all these works agree to show that heart rate [HR] is lower (-7 to 15 beats per minute) in DS individuals. Specifically, numerous studies hypothesized that the lower catecholamine responses (epinephrine and norepinephrine) in DS population to maximal exercise could explain the weaker heart rate (Fernhall et al., 2009).

These observations are reinforced by the results of a work achieved by Eberhard et al., (1989, 1991) and Bricout et al., (2008) evaluating catecholamine's kinetics during and after exercise. These authors showed that the plasma concentrations of epinephrine and norepinephrine after an exercise on bicycle or on treadmill were lower in subjects with DS than in control subjects, and could partly explain the relatively low elevation of HR during exercise, named "chronotropic incompetence".

However, some others factors may explain the reduced aerobic capacity and the possible incapacity to long physical exercise in DS. If endocrine dysfunction, cardiovascular pathologies, obesity, and musculoskeletal disorders are now well established as limiting to do prolonged physical exercise, DS individuals were also not highly motivating to repeat exercise, which contributes to more easily adopt a sedentary lifestyle, because the effort can be quickly perceived as painful, difficult and without sense for them.

The mechanisms of exercise intolerance in trisomy 21 require further consideration and in particular the relationship between the sympathetic nervous system, hormonal system which its importance in the adaptation effort is crucial.

Thus, many studies confirm that DS subjects present some endocrine diseases, such as diabetes, hypothyroidism, and insulin resistance ... (Murdoch, 1977; Murdoch et al. 1978; Sharav et al., 1991; Sasagawa et al., 1993; Konings et al., 2001). In a study conducted at the University Hospital of Grenoble between 2003 and 2005, on 14 young adults with trisomy 21, some of these endocrine disorders at rest (hypothyroidism and hypogonadism) were confirmed.

During muscular effort, it was shown that the responses of the adrenal axis (cortisol and catecholamine) and gonadal axis (testosterone) especially are the most affected when exercise is prolonged. Indeed, with exercise, the elevated catecholamine response, classically described in healthy subjects (Galbo, 1985) is lower in DS patients, and it can be suggested that the decreased response of catecholamine during effort in DS subjects explains the lesser capacity of these subjects to perform a muscular exercise which intensity and duration rise (Nagaoka et al., 1996; Fernhall & Otterstetter, 2003,).

In addition, some studies show that in patients with Down syndrome without adrenal insufficiency, basal cortisol is always lower (Murdoch, 1977; Murdoch et al. 1978). The pathogenesis of this observation remains difficult to explain, although it may have metabolic and physiological implications. During muscular exercise, the elevation of plasma cortisol, classically described in healthy subjects does not occur in DS subjects (Eberhard et al., 1993; 1996). Several hypotheses may be advanced to explain this fact:

- insufficient effort in intensity and / or duration, for subjects with trisomy are very reluctant to make long efforts,
- a possible adrenal dysfunction, which yet, remains unlikely (Konings et al., 2001). A more general dysfunction of the hypothalamic-pituitary-adrenal could exist, but the lack of studies on the regulation of secretion of CRF and ACTH in Down syndrome does not allow us to reverse or confirm this hypothesis. If dys-regulation of the HPA axis to stress in DS individuals partly explains the difficulty to use free fatty acids as energy substrates, there may be other endocrine factors directly related to adipose tissue, such as leptin, or in connection with the gonadotropic through testosterone, and its lipolytic action (Galbo, 1985).

Moreover, Bricout et al., (2008) show that DS subjects are characterised by an absence of elevated glycaemia during prolonged exercise, contrary to what is observed in healthy controls (Galbo, 1985). In addition, the concentration of lactatemia rises less than in controls suggesting that the peripheral utilisation of glucose is impaired. Therefore, it seems highly relevant to examine whether the gluco-regulation during exercise is impaired by a dysfunction of the autonomic nervous system response and / or impaired secretion of catecholamines and / or balance insulin / glucagon imbalance; and to know its impact on exercise capacity.

Furthermore, results of maximal exercise test show that 4 in 14 subjects presented chronotropic incompetence, and that these patients have an altered metabolic profile (increased fat mass, alterations in leptin, insulin, cortisol and plasma catecholamine at rest and during exercise) suggesting the close link between chronotropic incompetence, abnormal metabolic profile and endocrine regulation. These points deserve to be confirmed by future studies.

Recently, Fernhall et al., (2009) have conducted a study on catecholamine response to maximal exercise in persons with Down syndrome. This work reported an adrenergic drive largely absent in DS group, even though a sharp increase in catecholamine at high exercise intensities is largely responsible for increase in HR above the anaerobic threshold. In conclusion, the authors showed significant differences in baroreceptor sensitivity between individuals with and without DS, but not between DS compared with controls, suggesting that altered baroreceptor sensitivity does not fully explain the blunted HR response in DS population.

3. Exercise limitations and chronotropic incompetence

Meanwhile, investigation methods of the sympathetic nervous system are now well known and described in literature to be applied in this context (Nagaoka et al., 1996; Grote et al., 2004; Gouloupoulou et al., 2006). The measurement of heart rate variability is a non invasive method to assess both parasympathetic and sympathetic nervous activities (Task Force, 1996). The application of this method has revealed abnormalities associated with abnormal fatigue in athletes (Furlan et al., 1993). It has recently been used in trisomy 21 at rest,

exercise and orthostatic tests (Baynard et al., 2004; Fernhall et al., 2005; Iellamo et al., 2005; Figueroa et al., 2005).

The common hypothesis found in literature is that the individual with Down syndrome, has a reduced exercise capacity associated with chronotropic incompetence, expressed by maximum heart rate values (HRmax) decreased in response to a standardised exercise (Fernhall et al., 2001). Because peak exercise HR is correlated with age, the traditional approach has been to consider a patient as chronotropically incompetent when 85% of the age-predicted HR is achieved (Lauer, 2004). This measure is still confounded (Lauer et al., 1996) by resting HR and functional capacity, and Wilkoff & Miller, (1992) proposed the concept of HR reserve which is the difference between maximal predicted HR (or 220 beats /min minus the patient's age) and resting HR. Failure to use 80% of HR reserve constitutes chronotropic incompetence (Lauer et al., 1996).

In DS population, reduced HR response to prolonged exercise has been identified as the primary contributor to the low physical work capacity and cardiorespiratory fitness (Fernhall et al., 1996; 2001), but chronotropic incompetence is also an independent predictive factor of sudden death even in adult DS population without underlying coronary heart disease. Although it is debated (Guerra et al., 2003); understand the chronotropic incompetence in DS may be clinically relevant because it could partially explain the higher incidence of premature cardiac death in this population (Day et al., 2005).

Nevertheless, the mechanism of this alteration of cardiac response remains unknown, although dysregulation of the ANS appears to be the most probable explanation, by a decrease in sympathetic stimulation during exercise, and vagal tone incompletely removed (Fernhall et al., 2000; 2001). The rise in heart rate, normally induced by the removal of vagal inhibition and a gradual increase in sympathetic activation, is lower in DS population during a muscular effort (Fernhall & Otterstetter, 2003). This autonomic dysfunction has been studied by Figueroa et al. (2005) who used the HR variability [HRV] to explore the autonomic modulation of the sinoatrial node at rest and during exercise. Spectral components of HRV analysis in the low frequency [LF] and high frequency [HF] bands reflect sympathetic activity [LF/HF ratio] and parasympathetic tone [HF spectrum] (Task Force, 1996). These authors report an attenuated HR in individuals with DS due to blunted vagal withdrawal.

The results of these studies show significant abnormalities of ANS, with an observed increase in parasympathetic tone at rest (Baynard et al., 2004) a decrease of sympathetic stimulation during stress and during the tilt test, evoke a possible recall impaired baroreflex (Fernhall et al., 2005).

So, the mechanism responsible for the impairment of the autonomic cardiac regulation in DS population remains unclear. Even if the dysfunction of the ANS and /or the hormonal system is the main areas of research, we can also suggest that neurological damage are involved in the impairment of this cardiac regulation. The consistent findings of abnormalities in brain stem auditory evoked potentials, failure of growth and maturation in the brain from an early age, with loss of neurons and dentrites, and damage to the neurotransmitter system would provide the anatomic substrate for the autonomic dysfunction occurring at a central, brain stem site as a result of the genetic disorder (Yates et al., 1980; Mann et al., 1985; Wisniewski, 1990).

In the future, this dysfunction of the ANS must be supplemented by further investigations to better understand the possible mechanisms, especially through the involvement of hormonal factors.

4. Exercise limitations and obesity

One of the characteristics of the person with trisomy 21 is the propensity for becoming obese (Murray & Ryan-Krause, 2010). It affects between 15 and 20% of children and develops in most cases early in age; some studies have shown that nearly 10% of the children with Down syndrome were already located beyond the 95° percentile at 4 years old (Beange et al., 1995). The development of physical activity in the management of the obesity of young Down syndrome could play a major role. Indeed, the benefits of physical activity (combined with dietary measures) on cardiovascular risk have been clearly demonstrated in these populations, whether they live in special centers or in a family environment (Draheim et al., 2002; Montgomery et al., 1988).

Thus, in the general population, as in that of Down's syndrome, cardiovascular risk factors are associated with metabolic abnormalities, which are grouped under the generic common name 'metabolic syndrome' (Grundy et al., 2000). Among these metabolic abnormalities we can note a decrease in insulin resistance and excess body fat. Being overweight or obese can increase insulin resistance, which is characterised by a lack of insulin to stimulate glucose uptake by peripheral tissues and muscles, even though the plasma insulin concentration is abnormally high. The numerous endocrine studies showed that hyper-insulinemia stimulates the sympathetic nervous system, as is found in obese subjects (Monroe et al., 2000). This activation of the sympathetic nervous system is paradoxical, because it is part of a vicious circle in which there is a desensitisation of adrenergic signalling pathways (Lohse, 1996) hence a decrease in energy expenditure easing body fat storage. Simultaneously, this activation of the sympathetic nervous system adrenergic effects on the heart, blood vessels and kidneys promotes the development of hypertension. All these factors contribute highly to rise cardiovascular risk factors.

However, the factors restricting the effort in obesity are still insufficiently explored, especially for subjects with a mental disability. It seems that the place of regular physical activity in this population is highly relevant, especially since many studies have shown smaller performance in trisomy 21 (Pitetti & Tan, 1991a; Pitetti & Campbell, 1991b; Guerra et al., 2003; Fernhall & Otterstetter, 2003). The reasons for this disability to exercise are not clear and may reflect an insufficient stimulation (motivation problem) and / or the onset of fatigue induced by a limitation of use of energy substrates during prolonged exercise (Bricout et al., 2008) or to increased oxidative stress (Flore et al., 2008).

In adults, excess body fat may induce inflammation called 'low grade' and excessive oxidative stress (Wellen & Miller, 2005). Adipose tissue, then produce pro-inflammatory cytokines that alter insulin sensitivity. On the other hand, an inverse relationship between fitness level and markers of inflammation (high-sensitivity CRP) suggest a protective role of physical activity. Thus, the decrease in circulating CRP in regular physical activity suggests that it may limit this low-grade systemic inflammation (Oberbach et al., 2006). The effects of acute exercise have also been clearly demonstrated on the action of TNF-α (suppressive effect). Thus in young Down syndrome often over-weight and with a high percentage of body fat, the study of low-grade inflammation, its relationship with insulin sensitivity, biological markers of metabolic risk and cardiovascular inactivity and fitness need to be clarified. Several authors have demonstrated the positive impact of endurance exercise training on these markers. This reduced level of sensitivity CRP could reach 40% and was distinct from body composition and the level of initial CRP (Colbert et al., 2004). The checking of such a systemic effect in favour of a specific population "at risk" is therefore

highly pertinent. This reduction in plasma levels of inflammatory markers may represent a relevant therapeutic target.

5. Hormonal results during physical exercise in people with Down syndrome and possible link with an exercise limitation?

Historically, people with intellectual disabilities have long been away from sports associations. Then gradually, with the development of adapted physical activities, awareness of the benefits of such control on health has raised, and attitudes to integrate these people have evolved (Eberhard, 2006). The first work that reports on the involvement of subjects with mental disabilities in sport is those of Bell and colleagues in 1977, demonstrating the feasibility of integrating these people into sport. In the following decade, many studies will have demonstrated that training and regular codified sports practices are not only possible but present real health benefits (Pitetti et al., 1989; 1991a; 1991b; 1993; Draheim et al., 2002). In this analysis, we must differentiate the effects of practice on health indicators (*e.g.* on body fat, the risk of obesity ...) from indicators of performance, not specifically looked for in this context.

Thus, for subjects with trisomy 21, regular physical activity can for example:

- promote fat loss, and positively influence lipid parameters (cholesterol, lipoproteins; (Eberhard et al., 1993))
- reduce the risk of developing diabetes (Marliss & Vranic, 2002; McGavock et al., 2004)
- reduce the risk of developing hypertension (Fernhall et al., 2001)
- help perform long-term efforts.

For twenty years, the department of Sports Medicine, at the hospital of Grenoble, has been focusing on hormonal adaptations during exercise in young trisomy 21. Early works attached in 1989 (Eberhard et al., 1989) tested the effects of a short physical exercise on a cycle ergometer to evaluate the physical abilities of young DS teenagers. These preliminary results showed that DS subjects had a less important physical ability to make efforts not only because of a lack of motivation or behavioural problems, but also a possible cardiovascular dysfunction. Two years later the major result led, to propose further investigations on the relationship between "the biochemical adaptations and catecholamine responses after a maximum incremental exercise in these young DS teenagers (Eberhard, et al., 1991). This study has highlighted that exercise performed by young DS individuals is reflected by elevated plasma catecholamine lower than that traditionally seen in control subjects, also correlated with a maximum heart rate always lower than control subjects at the end of exercise. The hypothesis of a blunted adrenergic response in this population therefore emerged. In 1993, a new study was proposed in the department with the aim to assess metabolic parameters at rest then an effort, but also and after 12 weeks of training, the idea was to make the possible link with adaptive responses to exercise (Eberhard, et al., 1993, 1996, 1997). The results obtained in these studies confirmed the previous ones results, but also demonstrated that endurance training over a period of three months led to a better balance of lipid profile frequently observed to be abnormal in a sedentary population with Down syndrome.

This promotion of physical activity has becomes a real concern for this specific population. Relayed by local associations, it has given rise to a major support of young Down syndrome, involving them more in physical activities. Over the last years, adaptive sports clubs which include a large population of mentally handicapped people, have regularly organized

weekly training sessions that allow these young people to compete at a national level, and some of them achieve excellent results. These benefits had an immediate impact, improving measurable physiological parameters during exercise testing.

For example, in a last study proposed in 2002 in France (Grenoble), VO_{2max} values (gold index of aerobic fitness) obtained by a group of young DS adults, are excellent (44.2 ± 3.0 $ml.min^{-1}.kg^{-1}$ - Min = 39 Max = 60). For higher values, some young DS subjects get even better results than some control subjects of similar age. However, in this work, hormonal and metabolic investigations have shown that DS subjects present some alterations in adaptive response to maximal and sub-maximal exercise. Specific factors appear to be associated with result, such as obesity, a high percentage of body fat, hyper-insulinemia, and hyper-leptin levels (Bjorntrop & Rosmond, 2000). During the sub-maximal exercise, hormonal responses of young DS adults (cortisol, catecholamine) do not follow the observed rise in controls, whereas these adaptations allow glycolysis, gluconeogenesis and lipolysis when muscular exercise continues.

This result is expressed in a moderate elevation of blood glucose and HR, which could explain why people with Down syndrome can not continue muscular effort even at levels below maximum (Bjorntrop & Rosmond, 2000; McMurray & Hackney, 2005). This is also confirmed by low values of lipid oxidation in DS group and may be related to lower VO_{2max} for these subjects, and by free fatty acids values that remain high, suggesting that peripheral utilisation of lipids is insufficient when intensity and duration of the effort increases.

Compared with controls, hormonal responses to exercise in young adults with trisomy 21 were thus attenuated, which could explain cardiovascular and metabolic limitation of these subjects to continue muscular exercise (Bricout et al., 2008; Flore et al., 2008). The aim of these two studies was to analyse if hormonal responses could explain an exercise limitation in DS, and if it exist an oxidative stress in this population. DS is a risk factor for metabolic syndrome and cardiovascular disease. The greater oxidative stress described in DS can increase this risk owing to its potential deleterious effects on insulin sensitivity. We hypothesised that metabolic syndrome or its markers, at rest and during exercise, are more pronounced in young adults with DS.

Fourteen young men with DS (mean age 22.5 ± 0.7 years) took part to the study. During a treadmill sub-maximal incremental test, blood samples were collected for determination of hormonal and metabolic variables.

Compared to controls, DS individuals showed lower VO_{2max} (60.8 ± 2.4 *versus* 44.4 ± 3.3 $ml.min^{-1}.kg^{-1}$respectively; $P < 0.05$) but it was close to the predicted value (95 ± 6%). They presented a lower duration of sub-maximal incremental exercise ($P<0.001$).

At rest, DS individuals showed greater catecholamine, insulin and leptin values ($P<0.05$), but lower testosteronemia and cortisolemia ($P<0.05$) compared to controls. During sub-maximal incremental tests, catecholamine and cortisol did not increased, whereas the insulin concentration of DS individuals was significantly higher ($P<0.01$) compared to controls. Glycaemia increased significantly at the end of sub-maximal incremental test for controls but not for DS individuals ($P<0.01$). In DS participants, as expected, oxidative stress was greater than in controls (+ 15%; $P<0.001$) at rest and throughout the exercise protocol. Although a greater fat mass (DS: 19.9 ± 1.3%; controls: 13.5 ± 0.9%; $P<0.001$), and a lower insulin sensitivity (HOMA-IR DS: 1.09 ± 0.16; HOMA controls: 0.64 ± 0.13; $P<0.05$) was observed for DS participants, a metabolic syndrome could not be shown. Maximal fat-oxidation rate was lower in DS participants (DS = 394.2 ± 69.9 *versus* controls = 486.1± 134.8 $mg.min^{-1}$; $P <0.01$) but it was in the normal range.

These endocrine and metabolic profiles at rest and during exercise may limit endurance performance in DS individuals.
So now, the aim of our research team is to assess chronotropic adaptation to exercise in non obese young physically active adult males with DS and its relationships with exercise tolerance and catecholamine response. In a preliminary study, fourteen DS and healthy age-matched controls initially performed a graded maximal exercise test. Chronotropic incompetence (CI) was characterised if less than 80% of the chronotropic reserve was achieved.
Only four DS subjects (28%) had CI whereas none in controls. DS with CI showed exercise intolerance with HR peak and peak oxygen uptake lower than those without CI. Conversely, DS with CI had impaired epinephrine and norepinephrine responses to exercise. The association between CI, exercise intolerance and impaired catecholamine response in a subgroup of DS, indicate that biological effects of catecholamine are necessary for optimising acute exercise adaptations. Nevertheless, the results suggest that reduced chronotropic response and aerobic power in DS without CI are likely to be due to the metabolic consequences of fat mass excess and inherited oxidative stress rather than to impaired catecholamine drive.
However, the results of that work are still being acquired and it will be necessary to obtain results on a more important number of subjects to confirm our current results.

6. Interest of the promotion and guidance of an active lifestyle in populations with specific needs such as Down Syndrome

Direct assessment of health

There is no pharmacological treatment for trisomy 21. Nevertheless, the knowledge of physiological and biological parameters of trisomy 21 by means of studies based on protocols of effort is an original path that could bring: ***(1)*** load response adapted to the daily lives of subjects with trisomy 21, ***(2)*** results directly applicable to the health of these subjects (treatment of endocrine diseases, nutritional advice, and rehabilitation training ...).
By promoting a physically active life, as it has been shown in various reports across the Atlantic (A Report of the Surgeon General. U.S. Department of Health & Human Services, 1996) a reduction in health costs is logically expected for all categories of "persons with special needs", usually victims of the adverse effects of inactivity. The qualitative accompanying aging people with disabilities, is an important goal for our wealthy societies. The basic criteria for this support which actually extends from childhood to old age, is provided by professionals who educate, guide and look after these populations. These professionals should benefit from the results of this completed investigation on the effects of physical activity. Indeed, while renewing their knowledge and professional practices, they should be able to provide better care, better quality of life and even better end of life for all the elderly, disabled or not.
In addition, regular physical activity is widely recognised as a means of improving the health and the quality of life of people with disabilities. These benefits have already been demonstrated, at least temporarily. Over the last decade, the advanced educational media have helped give these people a more active lifestyle that has immediately impacted on their recognition and social integration. But as physical exercise is considered as a stress, questions remain as to the metabolic and hormonal adaptations of these DS subjects.

Similarly, long-term benefits of an active lifestyle, with a perspective of delaying the ageing of the person to maintain a certain level of mental and physical well-being, must be better identified.

Propositions could be considered, and should aim at finding the best conditions (nature, intensity, frequency) of an intervention based on physical activity among this specific population. It seems likely that the physical training of people with trisomy 21 should be scheduled, monitored and controlled in order to derive benefits for their health and quality of life.

In this context, Rimmer et al., (2004) have shown that a combined resistance and aerobic training program can improve physical fitness in this population; however, it remains unclear whether this transfers to improved performance on functional tasks of daily living.

Persons with DS have also reduced muscle strength and muscular endurance compared with their peers without disability and also compared with their peers with an intellectual disability but without DS (Croce & Horvat, 1992). Muscle weakness can also impact the ability of DS subjects to perform everyday activities, such as walking, maintaining balance while standing, and achieving all the movements of daily life. Further, because their workplace activities typically emphasize physical rather than cognitive skills, decreased strength can negatively impact the vocational and social development of adults with DS in the workforce. It has been shown that resistance training can improve work productivity in individuals with intellectual disability (Croce & Horvat, 1992). It might be expected that adults with DS have difficulty taking part in or being motivated to continue with a progressive resistance exercise program. Compliance with a training program can be very variable depending on the intellectual disabilities. To obtain a positive impact, it is necessary that the training program appeared to be a safe intervention for people with DS. No major adverse events should be reported by the participants, their families, or the fitness trainers involved in the program, during or after the training sessions.

To support the opinion that the activity is required in DS population, Heyne et al. (1997) have shown that the absence of a meaningful leisure program will foster maladaptive or aggressive behaviours. These authors point out the significance of leisure programs but also the importance of physical activities which will expand the social network, promote friendships and, introduce a sense of autonomy. Songster et al. (1997) reinforce this argument and emphasise that consistent training and sports competition in Special Olympics are essential to the physical, mental and social developments of young person with Down syndrome.

7. Conclusion

Children and young adults with Down syndrome have the opportunities to learn the different aspects of physical education and thus the means to become a physically educated person. They are capable of learning sports techniques, can progress, and participate in sports competition.

Nevertheless, physical activities need to be carefully taught and chosen for their fitness benefit. It is necessary to encourage enough recreational activities to maintain a sufficient motivation, so physical educational programs for this specific population should include all activities to learn, to understand both kinaesthetically and cognitively movements and abilities. Finally, the physical activity becomes enjoyable, social, repeatable, valued for its own sake, and we contribute to the formation of autonomous adults, physically active and educated.

CARDIOVASCULARA DATA		age	FC rest	FC peak	VO2 peak	SBP
Authors	*Review*	years	bpm	bpm	ml/min/kg	mmHg
Baynard et al., (2004)	Arch. Phys. Med Rehabil.	20,8 +/- 0,9	71 +/- 3,4	161 +/- 5,3	27,4 +/- 1,8	nd
Baynard et al., (2008)	M.S.S.E	25 +/- 2	nd	167 +/- nd	24 +/- nd	nd
Bricout et al., (2008)	J. NeuroEndocrinol.	22,5 +/- 0,7	57,7 +/- 3,7	180 +/- 2	44,2 +/- 3,04	116,0 +/- 2,8
Draheim et al., (2002)	Mental Retard.	34,5 +/- 10,0	nd	nd	nd	111,4 +/- 13,6
Eberhard et al., (1989)	J. Ment. Defic. Res.	14,8 +/- 2,2	67,1 +/- 12,6	123,6 +/- 19,7	47,6 +/- 8,1	107 +/- 14,6
Eberhard et al., (1997)	Adapt. Phys. Activ. Quater.	17,5 +/- 1,7	nd	162,2 +/- 18,9	48 +/- 1	nd
Fernhall et al., (1996)	M.S.S.E	26,7 +/- 5,9	nd	164 +/- 14,4	27,6 +/- 6,4	nd
Fernhall et al., (2003)	J.A.P	23,8 +/- 1,8	66,4 +/- 4,6	154, +/- 4,2	18,1 +/- 1,6	112,2 +/- 6,6
Fernhall et al., (2005)	Arch. Phys. Med Rehabil.	25,1 +/- 7,3	72,5	159 +/- 16,8	19,7 +/- 6,4	117,5 +/- nd
Fernhall et al., (2009)	Am. J Cardiol.	24 +/- 7	nd	170 +/- 10	27,3 +/- 5,6	nd
Figueroa et al., (2005)	Clin. Auton. Res.	27,8 +/- 8,1	76 +/- 3	nd	nd	115 +/- 4
Guerra et al., (2003)	Arch. Phys. Med Rehabil.	24,3 +/- 3,5	69,0 +/- 11,2	165,0 +/- 14,7	31,9 +/- 6,6	nd
Gouloupoulou et al. (2006)	Am. J. Ment. Retard.	24 +/- 0,9	70 +/- 1,5	162 +/- 2,4	24,5 ++/- 1,0	nd
Hefferman et al., (2005)	M.S.S.E	25 +/- 2,4	72 +/- nd	nd	nd	106,1 +/- 2,9
Iellamo et al., (2005)	Am. J. Physiol.	26,3 +/- 2,3	nd	nd	nd	116,1 +/- 2,7
Mendonca et al., (2011)	Arch. Phys. Med Rehabil.	36,5 +/- 5,5	69,6 +/- 12,2	167,6 +/- 19,1	31,7 +/- 7,9	nd
Mendonca et al., (2011)	Res. Dev. Disabilities	34,9 +/- 1,1	71 +/- nd	171,5 +/- 4,9	30,4 +/- 2,1	nd
Millar et al., (1993)	M.S.S.E	18,4 +/- 2,9	nd	172,8 +/- 15,1	26,95 +/- 7,92	nd
Rimmer et al., (2004)	Am. J. Ment. Retard.	40,6 +/- 6,5	nd	133 +/- 21	14,7 +/- 4,5	nd
Tsimaras et al., (2003)	Percept. Motor Skills	24,7 +/- 2,7	nd	181,0 +/- 3,8	30,5 +/- 4,3	nd

nd : not determined, SBP : systolic blood pressure.

Table 1. Cardiovascular data.

A.N.S DATA		age (years)	LF	HF	LF/HF
Authors	*Review*				
Baynard et al., (2004)	Arch. Phys. Med Rehabil.	20,8 +/- 0,9	1264,1 +/- 226,2 ms^2	1418,1 +/- 268,6 ms^2	1,81 +/- 0,42
Figueroa et al., (2005)	Clin. Auton. Res.	27,8 +/- 8,1	5,4 +/- 0,2 $ln.ms^2$	6,3 +/- 0,2 $ln.ms^2$	1,18 +/- 0,06
Iellamo et al., (2005)	Am. J. Physiol.	26,3 +/- 2,3	51,3 (35,5 to 59,4) n.u	34,6 (27,4 to 49,9) n.u	nd
Gouloupoulou et al. (2006)	Am. J. Ment. Retard.	24 +/- 0,9	6,93 +/- 0,12 $ln.ms^2$	6,57 +/- 0,16 $ln.ms^2$	0,35 +/- 0,11 $ln.ms^2$
Mendonca et al., (2011)	Res. Dev. Disabilities	34,9 +/- 1,1	6,8 ln ms^2	5,8 ln ms^2	1,1

LF: low frequency power expressed in ms^2 or ln ms^2 or n.u (normalised units)
HF: high frequency power; LF/HF ratio reflected the Autonomic Nervous System balance (sympathetic/parasympathetic).

Table 2. Autonomic Nervous System Data.

ENDOCRINE DATA		age (years)	Testosterone	TSH	T3	T4
Authors	*Review*					
Sakadamis et al., (2002)	Eur. J. Obstetr. Gynecol	26.4 +/- 3.9	31.26 +/- 14.1nmol/L	nd	nd	nd
Suzuki et al., (2010)	Int. J. Urol.	28 (15 - 54)	3.68 +/- 1.34 ng/mL	nd	nd	nd
Bricout et al., (2008)	J. NeuroEndocrinol.	22.5 +/- 0.7	15.45 +/- 1.63 nmol/L	6.33 +/- 0.93 mUI/L	4.35 +/- 0.11 pmol/L	16.22 +/- 0.60 pmol/L
Oliveira et al., (2002)	J. Pediatr (Rio J)	3.4 +/- 1.8	nd	7.2 ± 4.2 µU/mL	9.8 ± 2.1 ng/dL	1.7 ± 0.4 ng/dL
Murdoch et al., (1977)	J. Clin. Endocrinol. Metab	adults	nd	7.6 +/- 10.7 mU/L	1.61 +/- 0.47 nmol/L	69.1 +/- 22.2 nmol/L
Konings et al., (2001)	Eur. J. Endocrinol.	1.5 to 13	nd	6.5 +/- 1.3 mU/L		14.3 to 18.5 pmol/L

TSH: thyroid stimulating hormone; T3: tri-iodothyronine; T4: thyroxine; nd : not determined,

Table 3. Endocrine data.

ENDOCRINE DATA		age	cortisol (rest)	Epinephrine	NorEpinephrine	Epinephrine	NorEpinephrine
Authors	*Review*	years	nmol/L	rest (pmol/mL)	rest (pmol/mL)	post ex (pmol/mL)	post ex (pmol/mL)
Murdoch et al., (1979)	J. Ment. Defic. Res.	39 (22 - 57)	476.6 +/- nd				
Bricout et al., (2008)	J. NeuroEndocrinol.	22.5 +/- 0.7	365.1 +/- 14.7	0.67 +/- 0.14	3.62 +/- 0.53	1.19 +/- 0.22	10.21 +/- 2.64
Eberhard et al., (1991)	J. Ment. Defic. Res.	17.5 +/- 1.7	nd	0.38 +/- 0.23	1.08 +/- 0.78	0.92 +/- 0.41	6.32 +/- 3.41
Eberhard et al., (1997)	Adapt. Phys. Activ. Quater.	Boys: 18 +/- 1.7	nd	0.45 +/- 0.3	1.3 +/- 0.9	1 +/- 0.45	6.4 +/- 4.4
Eberhard et al., (1997)	Adapt. Phys. Activ. Quater.	Girls: 16.7 +/- 1.5	nd	0.4 +/- 0.3	0.63 +/- 0.25	0.78 +/- 0.3	6.9 +/- 1.6

TSH: thyroid stimulating hormone; T3: tri-iodothyronine; T4: thyroxine; nd : not determined,

Table 4. Endocrine data.

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15

Language and Visuospatial Abilities in Down Syndrome Phenotype: A Cognitive Neuroscience Perspective

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1. Introduction

Down syndrome (DS) is the most common genetic disorder with a prevalence that ranges from 1:700 to 1:100 live births and accounts for 25–30% of people with intellectual disability (Nadel, 1999; Rogers, Roizen, & Capone, 1996; Rondal, 1988, 1998; Rondal, Perera, & Nadel, 1999). Trisomy 21, one of the three forms of DS, is caused by the presence of a third chromosome at band 21q22 of the long arm. The other two forms, namely translocation 21 and mosaicism, account for only 6% of children with DS (Rogers et al., 1996). Recent medical advances suggest that over 300 genes are affected, making the genetic etiology of DS a complex mechanism that implicates gene interactions that are not clearly understood (Pennington, Moon, Edgin, Stedron, & Nadel, 2003; Antonarakis et al., 2004). The syndrome is characterized by specific phenotypic characteristics, health related problems, cognitive and language impairments, neuromotor dysfunction and early aging, often associated with increased prevalence of Alzheimer's disease.

Interest in the cognitive profile of DS has been robust during the past decades, with a large number of published studies discussing the atypical and unique profile of cognitive abilities in this population. Yet, there is no clear understanding of the cognitive profile of individuals with DS and how this differentiates from other forms of intellectual disability. The uniqueness of the cognitive make-up of individuals with DS will contribute to a better understanding of the specific strengths and impairments of this population, as well as to the development of more effective educational programs suitable for them. This chapter will focus on language and visuo-spatial abilities of individuals with DS from a cognitive neuroscience perspective. The chapter is organized in four parts. The first part discusses language abilities in individuals with DS, with particular emphasis on expressive and receptive vocabulary and grammar. In the second part, research in visuospatial abilities in individuals with DS is reviewed. The third part deals with the concept of atypical cerebral laterality (ACL) in DS individuals and, in particular, how ACL may affect their language and visuospatial abilities. Finally, the last part discusses the contribution of laterality research in understanding the unique pattern of cognitive abilities in DS.

2. Neurobiology of DS

At birth, the total brain volume of individuals with DS is close to normal, although microscopically some changes have been observed as early as 22 weeks of gestation that become more obvious by 6 months of age (Nadel, 2003). This condition has been linked to neural density reduction in the cerebral cortex (Florez, 1992). Indeed, the most affected area of the brain of individuals with DS is the cortex, both in neural density and in weaker neural synapses, caused by fewer dendritic spines. (Florez, 1992; also see Lubec & Engidawork, 2002 for a review). Although, the reduced brain volume cannot be directly associated with the observed intellectual disability of this group, it is suspected that these abnormalities in the neural density of the cortex may contribute to the weak cognitive deficits associated with the syndrome (e.g., memory and attention). Shapiro and colleagues (1992) reported abnormal neuronal interactions between the frontal and parietal lobes of individuals with DS, suggesting the involvement of Broca's area. More specific differences in the brain's neuroanatomy of individuals with DS include reduced growth of the frontal lobes (Kesslak et al. 1994), narrowing of the superior temporal gyrus, smaller brain stem and cerebellum (Cole et al., 1993).

Research using magnetic resonance imaging (MRI), comparing demented and non-demented individuals with DS, indicates the presence of reduced volume of left hippocampus and amygdala in the former group (Pearlson et al., 1998). In a different MRI study, measuring hippocampal and amygdala volumes, non-demented individuals with DS exhibited smaller hippocampal size compared to age-matched typically developing controls, but there were no differences in amygdala volumes between the two groups (Pinter, Brown, Eliez, Schmitt, Capone, & Reiss, 2001). In their study, Pinter and colleagues (2001) confirmed that individuals with DS have smaller brain volumes, both in gray and white matter, smaller cerebellar volumes, and relatively larger subcortical gray matter volumes, both in parietal and temporal structures. Finally, Teipel and colleagues (2003) found decreased size of the corpus callosum in a group of non-demented individuals with DS, even when total brain volume was controlled for (Teipel et al., 2003). Thus, although the neuropathology of individuals with DS is widespread, particularly in older adults, it is also selectively affecting mainly cortical areas that are involved in higher cognitive processes.

3. Cognitive profile of individuals with DS

Individuals with DS demonstrate a unique cognitive profile of strengths and weaknesses that is characteristic to the syndrome. The general intellectual ability of individuals with DS ranges from mild to severe mental retardation, with a decline as they grow older (e.g., Hoddap & Zigler, 1990; Pennington, Moon, Edgin, Stedron, & Nadel, 2003). This decline, which begins early in adulthood, has been related to the gradual deterioration of several brain areas, such as the hippocampus and the cerebellum (Pennington et al., 2003). Alternatively, this decline has been linked to the increased prevalence of dementia in adults with DS (Takashima, Ieshima, Nakamura, & Becker, 1989).

Hippocampal function that supports tasks that tap short-term and long-term memory are severely affected in individuals with DS, as opposed to those supported by prefrontal areas (e.g., executive functions) (Pennington et al., 2003). Explicit memory (verbal and non-verbal) is particularly weak when compared both to typically developing (TD) individuals matched for mental age, and non-Down syndrome individuals with intellectual disability (ID)

(Carlesimo, Marlota, & Vicari, 1997). These findings suggest that memory is disproportionally affected in DS due to the specific neurological abnormalities mentioned earlier (i.e., hippocampal dysfunction, reduced brain volume) (Lubec & Engidawork, 2002).
Sustained attention has been also examined in infants with DS (Brown, Johnson, Paterson, Gilmore, Longhi, & Karmiloff-Smith, 2003). Results showed that infants with DS exhibit poor engagement and less duration in sustained attention than TD mental-age controls (Brown et al. 2003). These results are unlikely to reflect lack of motivation or task difficulty. In older individuals with DS, this condition has been associated with other dementia symptoms, such as lack of orientation (e.g., Granholm, Sanders, & Crnic, 2000).
Language is the most extensively studied domain within the cognitive system in individuals with DS. Most authors agree that language is severely affected in these individuals, with expressive vocabulary, grammar and syntax being the weakest areas of function. Indeed, individuals with DS document an unusual disparity in performance in expressive and receptive vocabulary tasks, with the latter being more affected than the former. Particular emphasis has been placed in identifying the specific nature of the expressive language difficulties, their neural substrates, as well as the developmental changes throughout the life span.
Grammar and syntax are disproportionally affected in individuals with DS as well. Spoken language is characterized by a systematic omission of tense-related grammatical morphemes that persists throughout development. Surprisingly, in most studies examining English-speaking children with DS, regular past tense formation is more affected than irregular past tense formation, suggesting a difficulty with understanding and applying grammatical and syntactic rules, as opposed vocabulary entry and retrieval.
Visuo-spatial abilities have been less extensively investigated in DS. Some authors propose that visuo-spatial working memory is relatively preserved compared to verbal working memory in DS individuals (e.g**.,** Lanfranchi, Carretti, Spanò & Cornoldi, 2009). In fact, they have often been characterized with relative strengths in visuo-spatial processing and poor verbal processing skills (Jarrold, Baddeley & Hewes, 1999; Klein & Mervis, 1999; Wang & Bellugi, 1994). The relative strength of visuo-spatial skills is supported by strong fine motor skills documented through the use of gestures in early development (Bilovsky & Share, 1965). More specifically, it has been reported that children with DS are better at the construction subtests of the Stanford-Binet (e.g., block building, drawing line, copying, folding), compared to mental age controls, due to their strong visuo-motor abilities (Silverstein, Legutski, Friedman, and Takayama, 1982). Pueschel and collegues (1987) documented that young children with DS were better at figure closure and hand movement tasks, compared to verbal tasks (using the Kaufman Assessment Battery for Children), while TD children exhibited the reverse pattern of performance and children with IDs without DS exhibited equally weak performance in both tasks.
Bellugi and colleagues (1994) proposed that individuals with DS are better at sustaining the global configuration of objects, as opposed to local forms, a pattern that is reversed in individuals with intellectual disability of different genetic origin. Similar findings have been reported in drawing, where individuals with DS tend to reproduce the global features of a stimulus and omit the local details of the object. This pattern of performance is often encountered in patients with left hemisphere damage, suggesting lateralized brain lesions in individuals with DS. However, MRI data do not exhibit asymmetrical damage in

individuals with DS. This evidence supports the existence of an atypical functional lateralization in this clinical group (Jernigan & Bellugi, 1990).

4. Language of individuals with DS

One of the earlier studies on language of individuals with DS explored speech reproduction and observed weak expressive skills in this population (Dodd, 1975). During the same decade Gibson (1978) discussed the language production of individuals with DS during the first two years of life. He suggested that some aspects of language, particularly expression, fall behind their chronological age. Almost a decade later, Cardosa- Martins and colleagues (1985) found weaknesses in speech production in individuals with DS. Since then, it has been repeatedly documented that children with DS exhibit an unusual disparity between expressive and receptive language, compared to what would be expected, based on their mental age, with the latter being less affected than the former (e.g., Jenkins, 1993; Chapman, 1997). This delay in expressive performance is evident from infancy, even prior to development of formal vocal speech.

Likewise, it has been found that infant gesture development in DS population is delayed, compared to TD infants (e.g., Miller, 1992). Longitudinal studies examining one-to-two word formations, found that individuals with DS exhibit a delay in speech production up to 18 months, with large individual variation that reached 19 months (e.g., Oliver and Buckley, 1994). The "vocabulary spurt", typically observed in TD 24-month old infants, was commonly absent in individuals with DS. As the syntax becomes more complex and the vocabulary for speech production more demanding, the discrepancies between DS and TD individuals become more pronounced (Miller, 1992).

Receptive language and comprehension appear to be more advanced than expressive speech in DS adolescents (Chapman, Schwartz, & Kay-Raining Bird, 1991). However, some evidence suggests that comprehension declines with age in non-demented individuals with DS (Chapman, Hesketh, & Kistler, 2002). In our study, receptive and expressive vocabulary was investigated in DS and TD individuals matched for mental age (Ypsilanti, Grouios, Alevriadou & Tsapkini, 2005). Four subtests of the Test of Word Knowledge (TOWK) (Wiig & Secord, 1992) were used to assess naming pictures (expressive vocabulary), matching a spoken word to a picture among four semantically related detractors (receptive vocabulary), relational word knowledge and ability to provide definitions to orally presented words. We found no differences between the two groups in three of the four tasks of expressive and receptive vocabulary. The only reliable weakness of individuals with DS was evidenced in the word definitions subtest that demands complex syntactic constructions, requirement which is challenging for these individuals.

Surprisingly, narrative production, which is closely related to expressive language and syntax, seems to be less affected in individuals with DS. Chapman, and colleagues (1998) compared the performance of 33 individuals with DS with two TD control groups matched for syntactic comprehension and for mean length of utterance (MLU), respectively, and failed to find reliable differences in number of episodes (stories) described by the three groups. Individuals with DS documented syntactic errors that included verb omissions, suggesting that narrative production may be weak due to syntactic deficits and not vocabulary restrictions.

Grammatical word formation, particularly the omission of tense-related morphemes, is another area of difficulty for individuals with DS (e.g., Laws & Bishop, 2003). Regular past

tense formation is more affected than irregular in individuals with DS (Laws & Bishop; Eadie, Fey, Douglas, & Parsons, 2002). This peculiar finding suggests that irregular past tense formation resembles accessing vocabulary entries, which does not rely on understanding and applying grammatical rules (Pinter, 1991). This theorization has been subsequently supported by evidence indicating significant correlation between irregular past formation and vocabulary level in individuals with DS (Laws and Bishop, 2003).
In summary, most published studies agree that expression is more affected than reception in individuals with DS (for a review see Ypsilanti & Grouios, 2008) and that phonological short-term memory may be mediating the performance on such tasks. This hypothesis is supported by research findings indicating that auditory short-term memory predicts early vocabulary entry in individuals with DS (Chapman & Hesketh, 2001). Several investigators have proposed that children with DS may exhibit difficulties in naming and sentence repetition tasks due to strong anatomical and functional deficiencies in the corpus callosum (e.g., Wang, Hesselink, Jernigan, Doherty, & Bellugi, 1992). Bunn et al. (2002) argued that if interhemispheric communication is necessary for picture naming before speech production, then deficiencies in the corpus callosum, such as those observed in individuals with DS, would influence performance on naming, but not on reading tasks.

5. Visuospatial abilities in DS

Studies on visuospatial abilities of individuals with DS reveal the existence of strengths and weaknesses within this domain. In particular, individuals with DS are stronger in visuomotor integration and visual memory, compared to spatial memory and spatial construction (Fidler, 2005). Visuospatial short-term memory was found to be relatively unaffected in individuals with DS (e.g., Jarrold & Baddeley, 1997). Bellugi and colleagues (1999) found a unique dissociation in the visuospatial abilities of individuals with DS and Williams Syndrome (WS) (a genetically linked disorder associated with intellectual disability). Individuals with DS tended to maintain a holistic strategy in drawing tasks, while those with WS matched for mental age, reproduced local features of the drawing objects (Bellugi, Lichtenberger, Mills, Galaburda, & Korenberg, 1999). Moreover, individuals with DS exhibited increased difficulty in depicting internal details of objects in block construction and integrating simple shapes. In the same study, a clear dissociation in global/local features of individuals with DS was illustrated using the Navon task, in which participants were asked to maintain the global or local form of letter stimuli. Individuals with DS were unable to reproduce the local details of the stimuli, maintaining only the global aspect of the presented letter.
In another study, Bellugi and colleagues (2000) presented comparative data from drawings of individuals with DS and WS demonstrating that individuals with DS produce drawings conforming with gestalt rules (such as, closure and continuation) that lack detail, but are easily recognizable (Bellugi, Lichtenberger, Jones, Lai, & St. George 2000). Interestingly, the two groups were equally weak in the block design subtest of the WISC-R (Wechsler, 1974). However, further analysis of the process of block design indicated that although erroneously, individuals with DS maintained the global configuration of the block arrangements, while individuals with WS exhibited a more local perspective in their arrangements (Bellugi et al., 1999). The characteristic profile of the visuospatial abilities of individuals with DS implicates the neural substrates supporting these processes that are ultimately linked to the chromosomal abnormality of the disorder and its unique

phenotype. This profile may also indicate variations in neuronal plasticity in different disorders with intellectual disability (Bellugi et al., 2000).

6. Atypical cerebral laterality in individuals with DS

Atypical cerebral laterality (ACL) has been associated with the cognitive deficits of individuals with intellectual disability since the early 1920s (Gordon, 1921). ACL refers to the reverse or weak or bilateral representation of language in the two cerebral hemispheres (Geschwind & Galaburda, 1985). The majority of right-handed individuals (97%) exhibit left-hemisphere lateralization for language. Only about 60% of left-handed individuals exhibit left-hemisphere lateralization for language, 30% bilateral lateralization and 10% right-hemisphere lateralization for language (Bishop, 1990). Geschwind & Behan (1982) termed atypical laterality any laterality pattern that differed from the "standard dominance pattern" (pp. 70). According to Geschwind & Galaburda (1985a; 1985b), atypical dominance may involve the inverse or weak dominance of three features; hand dominance, language dominance and visuospatial dominance.

Several accounts have been put forward to explain the increased incidence of atypical laterality in individuals with neurodevelopmental disorders, which fall into four main categories: hormonal, genetic, pathological, and developmental. According to hormonal theories a number of exogenous and endogenous factors increase the secretion of prenatal testosterone in the fetus (Geschwind & Galaburda, 1987). This increment enhances the growth of the right cerebral hemisphere and inhibits that of the left cerebral hemisphere. In particular, their posterior regions, cortical areas tightly linked to intellectual disability and poor language development (Geschiwind & Galaburda, 1985a, 1985b, 1985c). This speculation has been repeatedly challenged during the past decades for its complexity (e.g., McManus & Bryden, 1991; McManus, Bryden & Bulman-Fleming, 1994; Annett, 1994; Previc, 1994), although the implication of testosterone levels in the establishment of cerebral lateralization remains well supported (e.g. Witleslon, 1985). For example, Witelson (1985, 1989) suggested that larger callosal isthmus is related to increased left-handedness in TD individuals, resulting from less axonal loss in the corpus callosum during foetal development, which is influenced by prenatal testosterone levels. Specifically, decreased levels of prenatal testosterone may cause decreased axonal loss in the corpus callosum, causing increased bilateral representation of cognitive functions and enhanced prevalence of non-right-handedness (Witelson, 1989, 1991).

Genetic theories argue that ACL results from genetic variation determined by a single gene (Annett, 1985; McManus & Bryden,1982). Individuals who are not carriers of this gene will exhibit random hand preference (right or left). Any pathology during development may inhibit the expression of the right-hand gene causing ACL (Annett & Alexander, 1996). Alternatively, Satz (1973) proposed that ACL may be the result of early brain damage in the left hemisphere, causing a mild dysfunction of the contralateral hand for motor activities, which in turn, forces a genetically right handed person to switch to non-right handedness. According to this account, individuals with ACL are "pathological" left handers, genetically programmed to become right handers, but brain pathology altered this biological expression. From a developmental perspective, ACL may be caused by an atypical maturational process in motor development that initiates from the trunk, followed by the shoulders and then the hands. If this maturational process is arrested, or lagged, it could cause increased randomness which would be documented by lack of hand preference (i.e.,

ambiguous handedness) (Palmer, 1964). Bishop (1983; 1990) speculated that non right handedness is an indicator of an immature development of the motor system, caused by diffuse brain abnormalities in individuals with intellectual disability.

The characteristic dissociation of language being disproportionately affected to visuospatial abilities in individuals with DS that cannot be accounted by the hearing impairments (Laws, 2004) provides suspicion for the link between ACL and language problems in DS population. Indeed, an extensive review of the literature indicates that individuals with DS document an ACL pattern in speech perception and oral motor movements (e.g., Elliott & Weeks, 1993; Elliott, Weeks, & Chua, 1994; Heath & Elliott, 1999).

In a typical dichotic listening paradigm, two different auditory verbal stimuli are simultaneously presented in each ear using headphones. The participant is asked to repeat the stimulus that was best heard. TD right-handed individuals document a right-ear advantage (REA) for speech stimuli, which is indicative of left hemisphere lateralization for speech perception. According to the original account, auditory information incoming from the right ear is predominately represented in the opposite hemisphere (i.e., the left hemisphere) due to anatomical contralateral auditory pathways and, thus, closer to the language centers of the same hemisphere (Kimura, 1961). In order for verbal stimuli incoming from the left ear to reach the left-hemisphere's language centers, information crosses from the right to the left cerebral hemisphere via the corpus callosum, causing significant delays. In individuals with DS, a left-ear/right-cerebral hemisphere preference for speech sounds (Hartley, 1981; Pipe, 1988), or no ear advantage (Sommer and Starkey, 1977; Tannock et al., 1984) has been observed, instead of the typical right-ear/left-cerebral hemisphere preference found in TD controls. Using dichotic listening tasks, Welsh, Elliot, and Simon (2003) verified this hypothesis with right-handed individuals with DS exhibiting a left-ear/right-cerebral hemisphere advantage for speech perception, unlike the right-ear/left-cerebral hemisphere laterality for speech perception typically observed in TD adults. Similar results were obtained in a Japanese study with individuals with DS (Shoji, Koizumi & Ozaki, 2008).

Recently, Groen, Alku and Bishop (2008) used T-complex auditory event-related potentials to investigate whether individuals with DS show ACL, as reflected by atypical patterns of brain activity elicited by verbal and tone sounds presented in each ear. The relationship between atypical lateralization, language deficits and handedness was further explored. The laterality patterns of individuals with DS were significantly different, compared to TD individuals, confirming the existence of ACL for auditory processing in this population. However, this atypicality was not specific to verbal stimuli, but also to non-verbal stimuli, suggesting a more "generalized abnormality in auditory processing in individuals with DS" (Groen et al., 2008, p. 155).

Overall, in line with behavioral studies of language lateralization in individual with DS, there is reasonable evidence for the existence of ACL in this group.

Laterality studies indicate that individuals with DS exhibit increased non-right handedness, and inconsistent hand preference compared to healthy adults (Groen, Yasin, Laws, Barry & Bishop, 2008). This finding is in accordance with laterality studies examining handedness in other populations with intellectual disability of different etiology (e.g., Grouios, Sakadami, Poderi & Alevriadou, 1999).

7. Discussion

An overview of studies examining language and visuospatial abilities in individuals with DS suggests that there is a unique profile of cognitive abilities characteristic to the syndrome

that cannot be explained by intellectual disability. Individuals with DS exhibit diverse levels of strengths and weaknesses in both cognitive domains. Namely, the deficits in language abilities usually exceed impairments in visualspatial abilities. Recent studies demonstrate a more complex neuropsychological profile in this population, suggesting that the dissociation between language and visuospatial abilities is too simplistic. Indeed, we presented research that indicates that there are strengths and weaknesses in the visuospatial domain and the level of performance rarely exceed that of TD individuals matched for mental age. Moreover, the strength in visuospatial ability may be an artifact of comparisons with another genetic syndrome with intellectual disability, namely WS.

The brain abnormalities of individuals with DS result in a complex pattern of a behavioral phenotype that involves a number of interacting cognitive systems. It also implicates atypical lateralization of function reflected in the abnormal auditory perception for speech stimuli and increased incidence of non-right handedness. According to the neuronal network hypothesis, the cognitive deficits of individuals with intellectual disability are the result of weak connectivity of different areas of the cerebral cortex (Ramakers, 2002). If this holds true, then processing difficulties are not limited to cognitive systems, but also perceptual systems such as auditory pathways. Indeed, difficulties in perceptual organization have been correlated with severity of intellectual disability, which is consistent with the hypothesis of a more general deficit in information processing (Ramakers, 2002). Future research should investigate the development of language and visuospatial abilities longitudinally to provide information on when the divergence of these cognitive domains becomes apparent.

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Part 4

Prenatal Diagnosis and Screening

16

Prenatal Diagnosis of Down Syndrome

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1. Introduction

Chromosomal aberration is a phenomenon occurring relatively commonly in the development process. Chromosomal aberration is known to have various causes, and its frequency has been reported to vary particularly according to maternal age. Though different among reports, the frequency was around 0.6-0.8% in analysis with all childbirths[1] and around 2-3% in case childbirths were reclassified based on maternal age of 35.[2] In addition, the frequency increased when the maternal age was relatively young, artificial insemination was used, etc.[3] Spontaneous abortion related to such chromosomal aberrations occurs usually in the first trimester, and consequently, the prevalence of trisomy 21, 18 and 13 decreases with the advance of gestational age.[4] Chromosomal aberrations that can be found in prenatal diagnosis are largely divided into aneuploidy, structural aberration, mosaicism, uniparental disomy, small defect, etc., and additionally, there can be marker chromosomes. Marker chromosomes are observed in around one out of 1000 cases of chromosomal analysis, and are known to be related to old maternal age.[5] It is reported that around 80% of marker chromosomes detected are new ones, and 13% of marker chromosomes are associated with physical abnormalities or cognitive disorders.[6]
The frequency and type of chromosomal aberration are known to be different depending on the time of evaluation, population group under the analysis of structural chromosomal aberration, and the banding level of the laboratory performing cytogenetic evaluation.[7] Thus, it is increasingly important to conduct adequate prenatal genetic screening and analyze its results properly. Among chromosomal aberrations detected in pregnant women who received amniocentesis because of their age, 64% were trisomy (21% trisomy 21), 11% translocation, 17% sex chromosomal aberration, and 8% other types of chromosomal aberration.[2] Thus, considering the increasing number of old age pregnancies in Korea, careful attention should to paid in analyzing the results of prenatal genetic tests. Thus, this study purposed to discuss how to analyze the results of maternal serum marker tests and sonography used in prenatal genetic evaluation.

2. Maternal serum screening

2.1 Alpha fetoprotein

Brock and Sutcliffe [2] found in 1972 that the level of alpha fetoprotein in amniotic fluid increased when the fetus had a neural tube defect, and from the 1980s, the maternal serum alpha fetoprotein test began to be used for screening fetal anomalies in pregnant women.

Alpha fetoprotein, which is glycoprotein, is produced in the yolk sac in the early stage, and later in the fetal gastrointestinal tract and liver. A small portion of alpha fetoprotein in fetal serum may be discharged to urine and amniotic fluid, and it may diffuse through the fetal membrane and the placenta and enter maternal serum. Whereas the alpha fetoprotein level in fetal serum and amniotic fluid increases until 13 week and then decreases thereafter, the level in maternal serum increases continuously until 32 week because the size of the fetus keeps growing.[8] Between 16-20 week of gestational age, the level of alpha fetoprotein in maternal serum is only around 1/100,000 of that in fetal serum.

2.1.1 Analysis of serum alpha fetoprotein data

As is known, the concentration of alpha fetoprotein in maternal serum or amniotic fluid is measured as a test for screening fetal neural tube defects. When the measurements of alpha fetoprotein are analyzed, we should be careful not to attach clinical importance to absolute values. It is because the test method of each laboratory or company may seem to have been stabilized by itself, but absolute values from the method are often different from those obtained by other laboratories or test methods. For this reason, the measurements of alpha fetoprotein are often reported as the multiples of the median (MoM). A median is the value in the middle when result values are put in order of size, so it can reduce errors and allow the comparative analysis of result values without being influenced by laboratory or test method.

On the other hand, the measurements of alpha fetoprotein in maternal serum can be affected not only by multiple pregnancy and gestational age but also by maternal weight, diabetes, race, etc.[9,10], so these factors should be confirmed before the analysis of such measurements. Because the absolute level of serum alpha fetoprotein rises along with gestational age[8], underestimated gestational age is the most common cause of increased alpha fetoprotein concentration. In such a case, we can avoid unnecessary additional tests by correcting gestational age and recalculating the median.

2.1.2 Causes of increased alpha fetoprotein in maternal serum

Fetal anomalies

The rise of the alpha fetoprotein level in maternal serum is often accompanied by fetal anomalies. This is not because of a special function of alpha fetoprotein but because fetal tissue leaks to amniotic fluid due to neural tube defect, abdominal wall defect, sacrococcygeal teratoma, cystic hygroma, skin defect, etc. and, as a result, an increasing amount of alpha fetoprotein flows into amniotic fluid and maternal serum[11] or because alpha fetoprotein is not reabsorbed but discharged to urine due to fetal kidney anomaly.[12]

In fetal neural tube defect, alpha fetoprotein exudes through exposed nervous tissue, so the level of alpha fetoprotein in maternal serum is relatively higher in spina bifida aperta or anencephaly than in spina bifida occulta. In most laboratories, those whose median alpha fetoprotein level in maternal serum is over 2.0-2.5 are classified into a high-risk group of fetal neural tube defect. When median 2.5 was used, spina bifida aperta was detected at a detection rate of 80% and the false positive rate was 3-4%.[13]

The anomaly found second most commonly to neural tube defect in alpha-fetoprotein screening is abdominal wall defect. Particularly in case of gastroschisis, organs removed out of the abdominal cavity contact amniotic fluid directly, and therefore, the alpha fetoprotein

level tends to be higher than that in omphalocele.[11] Maternal serum alpha fetoprotein screening detects around 85% of gastroschisis and around 50 % of omphalocele.[11]

Placental abnormalities

Because the concentration of alpha fetoprotein in fetal serum is 100,000 times higher than that in maternal serum[8], even a small inflow of fetal blood increases the fetoprotein level in maternal serum rapidly. In case of chorioangioma in the placenta and hemangioma in the umbilical cord, fetal serum exudes into amniotic fluid and the maternal body due to mass bleeding[14], and in case of placenta accreta, placental infarct, etc. as well, alpha fetoprotein increases in the same way.[16]

2.2 Maternal serum test for aneuploidy screening

2.2.1 Second trimester multiple marker screening test

In 1984, Merkatz et al.[16] found that the risk of Down syndrome was high when the level of serum alpha fetoprotein was low during the second trimester, but this finding alone was not sufficient for using alpha fetoprotein as an accurate Down syndrome marker.[16] Later, double marker test that added human chorionic gonadotropin (hCG) test, and triple test that added also estriol (E3) to the double test were introduced as Down syndrome screening tests in the second trimester.[18] Recently, quad test that added inhibin A was developed[18] for higher accuracy of screening. The mean median (MoM) of each marker in trisomy 21 and 18 is presented in Table 1. There were large-scale prospective studies that compared accuracy among a number of multiple markers in the U.K. (Serum, Urine and Ultrasound Screening Study; SURUSS)[19] and the U.S. (First and Second Trimester Evaluation of Risk for Fetal Anueploidy; FASTER),[20] and in both studies the detection rate of quad markers was reported to be 81 %. Table 2 summarized the Down syndrome detection rates of multiple markers from the two studies.

Aneuploidy	AFP	hCG	uE3	Inhibin A
Trisomy 21	0.74 * (↓)	2.05 * (↑)	0.70 * (↓)	2.548 * (↑)
Trisomy 18	0.65** (↓)	0.32** (↓)	0.42** (↓)	-

AFP, α-fetoprotein; hCG, human chorionic gonadotropin; uE3, unconjugated estriol
* Modified from the results of the FASTER trial
** Modified from the results of Benn PA et al. Obstet Gyenecol 1999;93:707-11.

Table 1. The mean MoM (multiples of the median) values for the second trimester maternal serum quad markers.

		Detection rate at a 5% false-positive rate	
		SURUSS[19]	FASTER[20]
Double markers	AFP + hCG	66%	-
Triple markers	AFP + hCG + uE3	74%	70%
Quad markers	AFP + hCG + uE3 + inhibin A	81%	81%

AFP, α-fetoprotein; hCG, human chorionic gonadotropin; uE3, unconjugated estriol

Table 2. The detection rate of second trimester serum markers for Down syndrome.

2.2.2 First trimester screening test

Maternal serum triple marker test

Among first trimester serum markers, pregnancy-associated plasma protein A (PAPP-A) and free β-hCG are known to have the highest discrimination for haploidy. In Down syndrome, the MoM of maternal serum PAPP-A is 0.38, decreasing by 60%, and that of free β-hCG is 1.83, increasing by around two times.[21] Accordingly, both of the two markers are used in first trimester screening, and with this test, the Down syndrome detection rate is 60-74% and the false positive rate is 5%.[19,20] The detection rate is lower than that of the quad test in the second trimester but similar to that of the triple test.

Nuchal translucency measuring

The thickness of nuchal translucency in the first trimester is related to fetal haploidy, in particular, to Down syndrome, apart from maternal serum markers.[22] Accordingly, the accuracy of screening can be enhanced through the first trimester combined test that measures the thickness of nuchal translucency in addition to serologic tests that measure PAPPA-A and free β-hCG.[22] In the results of a meta-analysis with 209,603 subjects, the combined screening test showed a detection rate of 86% (84-88 95%, CI) with a false positive rate of 5.1% and Down syndrome was detected in 785 subjects.[23] When only nuchal translucency was measured without serologic tests, the Down syndrome detection rate was 77% with a false positive rate of 6.0%, so it was less accurate than the combined test. The positive predictive value of the combined screening test was 16.8 (16.3-17.4, 95% CI), which means that one out of 17 pregnancies with a positive result of the combined screening test is found to have a Down syndrome fetus.[23] On the other hand, because PAPP-A decreases but free β-hCG increases for 11-14 weeks of pregnancy, the detection rate varies significantly according to gestational age.[23] In the results of FASTER, the Down syndrome detection rate was 73% and the false positive rate was 1% in 11 weeks of pregnancy, but 67% and 5%, respectively, in 13 weeks, and based on this result the research reported that the accuracy of the combined test was highest at the gestational age of 11 weeks.[20] However, it was reported that if second trimester screening would be performed additionally, it would be more efficient to have the combined test in 10 weeks of pregnancy.[23]

2.2.3 First and second trimester integrated test

Wald et al.[24] proposed integrated test, which uses information on first and second trimester markers in sequence. They expected that if nuchal translucency and serum PAPP-A are measured in 10-13 weeks, and alpha fetoprotein, total hCG, estriol and inhibin A in 15-18 weeks, Down syndrome can be detected at a rate of 94% with a false positive rate of 5%, or 85% with a false positive rate of 1%.[25] Accordingly, because Down syndrome can be screened with a false positive rate of 1%, it reduces the need of additional diagnoses such as amniocentesis, and prevents fetal death resulting from invasive examination. In the results of SURUSS[19] and FASTER,[20] the integrated test was most accurate as a Down syndrome screening test (Table 3).

However, the integrated test has a number of shortcomings to be an alternative general screening test in prenatal examination. First, most of pregnant women who receive a screening test in the first trimester want the termination of pregnancy immediately if abnormalities are found in the fetus, and it is safer to terminate pregnancy in the first trimester. Second, it is hard to distinguish pregnant women who cannot be followed up after

first trimester serologic tests. In SURUSS [19] as well, 20% of pregnant women who had a first trimester test did not appear in the second trimester test. In such a case, there could be the legal risk of not telling the results of the first trimester test, so it was not an adequate alternative at present. Third, if the risk is unusually high in the results of the first trimester test the results are not provided until the second trimester, and this may raise an ethical issue. Thus, the integrated test can be the method of highest sensitivity and specificity if a pregnant woman receives prenatal examination from the beginning of pregnancy and takes both the first and second trimester screening tests, but is not adequate for those who want to get invasive chorionic villus sampling immediately based on the results of the first trimester screening test.

	FASTER[20]					SURUSS[19]				
	FPR(%) for DR of			DR(%) for FPR of		FPR(%) for DR of			DR(%) for FPR of :	
	75%	85%	95%	1%	5%	75%	85%	95%	1%	5%
1st trimester										
NT only	8.1	20	55	54	68	12.9	25	55	33	60
PAPP-A + f-βhCG	7.1	16	42	46	67	5.5	12.1	33	52	74
Combined†	1.2	3.8	18	72	85	2.3	6.1	22	66	83
1st + 2nd trimester:						.				
Serum integrated‡§	1.2	3.6	15	70	86	0.8	2.7	12.5	77	90
Full integrated§	0.2	0.6	4.0	87	95	0.3	1.2	7.2	84	95
2nd trimester										
Triple (AFP+hCG+E3)	7.0	14	32	45	69	2.9	7.1	22	51	74
Quad (Triple+inhibin A)	3.1	7.3	22	60	81	2.6	6.1	18	63	83

DR, detection rate; FPR, false positive rate; NT, nuchal translucency; PAPP-A, pregnancy associated plasma protein-A; f-βhCG free beta subunit of human chorionic gonadotropin; Quad, second trimester quadruple screen; AFP, alphafetoprotein; uE3, unconjugated estriol

† Combined: NT, PAPP-A, and f-βhCG in the first trimester

‡ Serum integrated: PAPP-A in the first trimester and quad screen in the second trimester

§ Full integrated: NT and PAPP-A in the first trimester with quad screen in the second trimester

Data from Rosen T et al. Semin Perinatol 2005;29:367-75.

Table 3. Direct comparative data for the first and second trimester Down syndrome screens from the prospective FASTER and SURUSS trials.

2.2.4 Alternatives to the integrated test

Sequential screening

If the result of the first trimester screening test is positive, chromosomal analysis is performed immediately and if the result is negative, the second trimester screening test is performed and if the result is positive, chromosomal analysis is performed. A characteristic of this method is

that the result of the first trimester screening test is provided to the pregnant woman and then the second trimester test is performed additionally. It can enhance the Down syndrome detection rate up to 98% but its false positive rate is also high as 17%.[26]

Contingency screening

If the first trimester screening test indicates high risk, chromosomal analysis is performed, and if the result indicates low risk no additional test is performed, but if it indicates moderate risk integrating screening is performed.[27]

3. Genetic sonographic markers of aneuploidy

Most of fetuses with chromosomal aberration have organs whose appearance is anomalous or abnormal, and such defects can be detected through prenatal sonography. A fetal structural anomaly may be caused by multiple factors, but it can be the result of chromosomal aberration. Therefore, if a fetal anomaly has been found, we should determine whether to perform cytogenetic analysis through amniocentesis after checking if other abnormalities accompany. Using genetic sonographic findings, we can detect pregnancies with high risk of aneuploidy at a sensitivity of 50-93%[28], and the absence of genetic sonographic findings may be regarded as sure evidence for the low risk of haploidy. Genetic sonographic findings can be divided into major structural anomalies and minor anomalies. In particular, anomalies that are observed frequently also in normal fetuses and occasionally disappear with the advance of gestational age are called sonographic markers. It is generally accepted that a major anomaly is a sign of high risk of chromosomal aberration and thus chromosomal analysis is required, but it is still controversial whether additional tests are required when only a minor anomaly has been found. It is important to determine in consideration of the likelihood of chromosomal aberration on a case basis rather than applying a uniform rule to every case, and for this, we need to be familiar with the risk of chromosomal aberration and representative types of haploidy in connection to each genetic sonographic marker (Table 4).

3.1 Major anomalies related to haploidy

The characteristic and frequency of sonographic findings in fetuses with chromosomal aberration are various according to gestational age, and the detection of abnormal findings is affected by the reason of sonography, criteria for positive finding, the level of sonographic equipment, etc. Structural major anomalies are detected mostly by sonography in trisomy 13 and 18, but are missed relatively often in trisomy 21. Table 5 summarized structural major anomalies detected commonly in pregnancies with trisomy 13, 18 and 21 and Turner syndrome.

3.2 Sonographic markers related to haploidy

The most common sonographic markers in the second trimester include nuchal thickening, hyperechoic bowel, short limbs, pyelectasia, echogenic intracardiac focus, and choroid plexus cysts. In general, the risk of chromosomal aberration is higher when the number of markers is large. Table 6 summarized the likelihood ratio of haploidy when each sonographic marker has occurred singly. The likelihood ratio was calculated by sensitivity / false positive rate, and if two or more sonographic markers have occurred together, the combined likelihood ratio is the product of their respective likelihood ratios.

Structural defect	Population incidence	Aneuploidy risk	Most common aneploidy (Trisomy)
Cystic hygroma	1/120 EU–1/6,000 B	60-75%	45X (80%),21,18,13,XXY
Hydrops	1/1,500–4,000 B	30-80%	13,21,18,45X
Hydrocephalus	3–8/10,000 LB	3-8%	13,18,triploidy
Hydranencephaly	2/1,000 IA	Minimal	
Holoprosencephaly	1/16,000 LB	40-60%	13,18,18p-
Cardiac defect	7-9/100 LB	5-30%	21,18,13,22,8,9
Diaphragmatic hernia	1/3,500–4,000 LB	20-25%	13,18,21,45X
Omphalocele	1/5,800 LB	30-40%	13,18
Gastroschisis	1/10,000–15,000 LB	Minimal	
Duodenal atresia	1/10,000 LB	20-30%	21
Bladder outlet obstruction	1-2/1,000 LB	20-25%	13,18
Facial cleft	1/700 LB	1%	13,18, deletions
Limb reduction	4-6/1,000 LB	8%	18
Clubfoot	1.2/1,000 LB	20-30%	13,18, 4p-, 18q-
Single umbilical artery	1%	Minimal	

B, birth; EU, early ultrasonography; LB, livebirth; IA, infant autopsy.
Data from Shipp TD, et al. Am J Obstet Gynecol 1998; 178: 600–2. and Nyberg DA and Crane JP. Chromosome abnormalities. In: Nyberg DA, et al. Diagnostic ultrasound of fetal anomalies: text and atlas. Chicago (IL): Year Book Medical; 1990. p. 676–724.

Table 4. Aneuploidy risk of major structural fetal malformation.

Aneuploidy	Sonographic finding	Aneuploidy	Sonographic finding
Trisomy 13	heart (VSD, dilated right ventricle) CNS (ventriculomegaly, holoprosencephaly) Face (midline defects, ocular abnormalities) Kidney(enlarged cystic kidneys) IUGR with polyhydramnios	Trisomy 21	Heart (septal defects) Abnormal fluid accumulation (thickened nuchal fold, cystic hygroma, fetal hydrops) Abdominal malformations IUGR with polyhydramnios
Trisomy 18	Heart (VSD) CNS (posterior fossa cyst, abnormal head shape, ventriculomegaly) Facial anomalies Choroid plexus cyst Abnormal fluid accumulation (thickened nuchal fold, cystic hygroma, fetal hydrops) Abdominal malformations IUGR with polyhydramnios	Turner syndrome	Heart (Coarctation of aorta) Abnormal fluid accumulation (increased nuchal translucency/ thickened nuchal fold, cystic hygroma, feta hydrops) Renal anomalies Short femur or humerus

CNS, central nervous system; IUGR, intrauterine growth restriction; VSD, ventricular septal defect

Table 5. Sonographic finding with high incidence in different fetal chromosomal abnormalities

	Likelihood ratio (95% confidence interval)	
Sonographic marker	Nyberg et al.	Smith-Bindman et al.
Nuchal thickening	11.0 (5.5-22.0)	17 (8-38)
Hyperechoic bowel	6.7 (2.7-16.8)	6.1 (3.0-12.6)
Short humerus	5.1 (1.6-16.5)	7.5 (4.7-12.0)
Short femur	1.5 (0.8-2.8)	2.7 (1.2-6.0)
Echogenic intracardiac focus	1.8 (1.0-3.0)	2.8 (1.5-5.5)
Pyelectasis	1.5 (0.6-3.6)	1.9 (0.7-5.1)
Normal ultrasound	0.36	-

Data from Nyberg DA, et al Ultrasound Obstet Gynecol 1998; 12: 8-14. and
Smith-Bindman R et al. JAMA. 2001; 285: 1044-55.

Table 6. Likelihood ratio of sonographic soft marker for fetal aneuploidy

3.2.1 Choroid plexus cysts

Choroid plexus cyst is found relatively commonly during the second trimester, showing a prevalence of 0.3-3.6%.[29] This type of cyst is known to be benign, disappearing spontaneously at the later stage of pregnancy without affecting the development of the fetus. As a sonographic marker of haploidy, choroid plexus cyst is found in 50% of fetuses with trisomy 18[30] but most of the cases are accompanied by another anomaly, and it is reported that no particular prenatal care including chromosomal analysis is necessary if only isolated choroid plexus cysts are found.[29]

3.2.2 Mild cerebral ventricular dilatation

Mild cerebral ventricular dilatation, in which the diameter of the cerebral lateral ventricle is over 10-15 mm, is related to aneuploidy like trisomy 21.[32] Bromley et al.[32] reported that 12% of pregnancies with mild cerebral ventricular dilatation are related to abnormal karyotype (trisomy 18, 21). In addition, mild cerebral ventricular dilatation was observed in 4.3% of fetuses with trisomy 21[33] and all of them had accompanying anomalies. It is known that the presence of cerebral ventricular dilatation alone does not increase the risk of chromosomal aberration.

3.2.3 Nuchal thickening

Excessive skin on the back neck is a characteristic finding of trisomy 21. It is observed in 80% of neonates with trisomy 21 and can be found in other chromosomal aberrations (trisomy 13 and 18, 45X).[34] Although the sensitivity and the false positive rate vary according to gestational age and criteria for positive value are different among institutions, its sensitivity for detecting chromosomal aberrations is generally within the range of 20-40%.

3.2.4 Hyper-echoic bowel

Hyper-echoic bowel is found in 0.5% of normal fetuses, but in fetuses with aneuploidy like trisomy 21, it is known to be observed more frequently and increase the risk 6-7 times higher.[35] Assuming that the likelihood ratio of isolated hyper-echoic bowel is 6.7 and the total risk of Down syndrome in the entire population is one per 500, isolated echoic bowel is

expected to be related to the risk of Down syndrome in around 1-2% of the general population. Hyper-echoic bowel is also known to be related to ileus, congenital infection, and rarely to secondary meconium ileus in cystic fibrosis.[36]

3.2.5 Skeletal abnormalities such as shortened limbs

Shortened limbs are a characteristic observed in fetuses with trisomy 21[37] and the length of shortened humerus seems to be a slightly more specific marker than the length of shortened femur. These results may be different according to gestational age, racial group, expected sex of the fetus, and used criteria.

3.2.6 Echogenic intracardiac focus

Echogenic intracardiac focus is a common finding in the second trimester. It is observed in 3-4% of normal fetuses and disappears spontaneously in the third trimester.[39] If the size of echogenic intracardiac focus is large or there are multiple of them, the risk of aneuploidy increases. Bromley et al.[40] reported that the risk of aneuploidy is 2 times higher when echogenic intracardiac focuses are in the right ventricle or bilateral than when they are only in the left ventricle.

4. Conclusions

Chromosomal aberration is closely related to the in intrauterine fetal death and perinatal prevalence. However, amniocentesis and chorionic villus sampling used for prenatal diagnosis of haploidy are invasive methods, and the reported fetal loss rate resulting just from the examinations is around 1-2%. Accordingly, it is very important to screen those with high risk of chromosomal aberration before such invasive examinations.

Serologic test of pregnant women for the screening of trisomy 18 and 21 is an important diagnostic process in prenatal care, and in particular, second trimester serum alpha fetoprotein is used as an important screening marker to detect anomalies such as fetal neural tube defect. If the concentration of alpha fetoprotein is over 2.5 MoM, sonography should be performed in order to detect neural tube defect and other problems. Recently, because sonography shows a high detection rate for neural tube defect, amniocentesis is used less frequently in diagnosing neural tube defect.

The preferred type of serum screening test is various among countries and institutions. In general, the first trimester screening test is increasingly preferred, but second trimester screening is still used frequently at hospitals that cannot measure fetal nuchal translucency or perform chorionic villus sampling. The triple test has been used commonly as second trimester screening, but recently the use of the quad test, which adds inhibin A, is increasing. If both first and second trimester tests are performed, they can increase the detection rate of fetal anomalies, but because the two tests have to be applied at an interval of 3-4 weeks, it is quite important to provide an adequate explanation to the patient. The serum screening test can be performed in various ways depending on the combination of serum factors. Thus, rather than using a uniform test for every case, we need to understand the characteristic, detection rate and false positive rate of each test, and individualize the test for each case according to maternal age, sonographic findings, and compliance.

In conclusion, in order to determine the risk of chromosomal aberration, we should analyze each case based on maternal age, nuchal fold thickness in the first trimester, the results of

maternal serum screening in the first or second trimester, and sonographic findings in the second trimester. Through this approach, we may reduce the number of unnecessary chromosomal analyses, lower the fetal loss rate resulting from invasive examination, and enhance the accuracy of screening for fetal chromosomal aberrations.

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17

First Trimester Screening for Trisomy 21 by Maternal Age, Nuchal Translucency and Fetal Nasal Bone in Unselected Pregnancies

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1. Introduction

Screening programs play a significant role in the assessment of fetal chromosomal defects and provide the appropriate prenatal counseling and diagnostic tests.

Every pregnant woman has a risk that her fetus might be affected by trisomy 21. At the beginning of the 1980's the screening based on a woman's age was introduced. With the cut-off age of 35 years, 5% of the pregnant women population were classified as "high-risk". However, only 30% of fetuses with trisomy 21 were detected in this group while the majority of trisomy 21 babies were born to mothers from the "low-risk" group.

Later, biochemical screening tests in the second trimester became widely used. The test is based on the concentration of various fetoplacental markers in the maternal circulation: alpha-fetoprotein (AFP), unconjugated estriol (uE3), human chorionic gonadotropin (hCG) and inhibin-A. This method of screening is proved to be more effective than maternal age alone and at the same rate of invasive testing (about 5%) it can identify about 50 to 70% of the fetuses with trisomy 21 (Nicolaides KH 2004).

In the 1990's screening tests were moved to the first trimester. The woman's age was first combined with sonographic measurement of fetal nuchal translucency and fetal heart rate. Later sonographic screening was upgraded by the measurement of maternal serum free β-human chorionic gonadotropin (β-hCG) and pregnancy-associated plasma protein-A (PAPP-A) (Wright D et al, 2008; Kagan KO et al, 2008). Fetal nuchal translucency screening identifies 75 to 80% of fetuses with trisomy 21 at a false positive rate of 5%.

In the last 10 years, several additional first trimester sonographic markers have been described (nasal bone, tricuspid flow, ductus venosus flow) which improve the detection rate of chromosomal abnormalities and reduce the false positive rate. At a risk cut-off of 1 in 100, the detection rate of trisomy 21 is about 95% at a false positive rate of 2.5%.

The ability to achieve reliable measurements and evaluation of sonographic markers depends on an appropriate training of sonographers, adherence to a standard ultrasound technique in order to achieve uniformity of results among different operators (Nicolaides KH 2011). Health professionals undertaking the first trimester scan have to be adequately

trained and their results have to be subjected to an audit. The Fetal Medicine Foundation (FMF) has introduced a process of training and certification to help establish high standards of scanning on an international basis (www.fetalmedicine.com).

1.1 Nuchal translucency

Nuchal translucency (NT) is the assessment of the amount of fluid behind the neck of the fetus, also known as the nuchal fold. An anechoic space is visible and measurable sonographically in all fetuses between the 11th and 14th weeks of pregnancy (Figure 1). Underlying pathophysiological mechanisms for nuchal fluid collection include cardiac dysfunction, venous congestion in the head and neck, altered composition of the extracellular matrix, failure of lymphatic drainage, fetal anemia or hypoproteinemia and congenital infection (Nicolaides KH 2004). Accumulation of nuchal fluid decreases after the 14th week. Enlarged NT helps us to identify the high-risk fetuses for trisomy 21 and other chromosomal abnormalities (Nicolaides KH et al, 1992; Nicolaides KH et al, 1994).

NT is evaluated in fetuses with crown-rump length (CRL) range from 45 to 84 mm. The scan may be performed transabdominally but in some cases transvaginal approach may be beneficial. During the measurement, the fetus lies in a neutral position, with the head in line with the spine (www.fetalmedicine.com). The assessment of the NT is obtained in a mid-sagittal view of the fetal profile. The measurements are taken with the inner border of the horizontal line and callipers are placed on the line that defines the NT thickness. Normally, NT increases with the CRL.

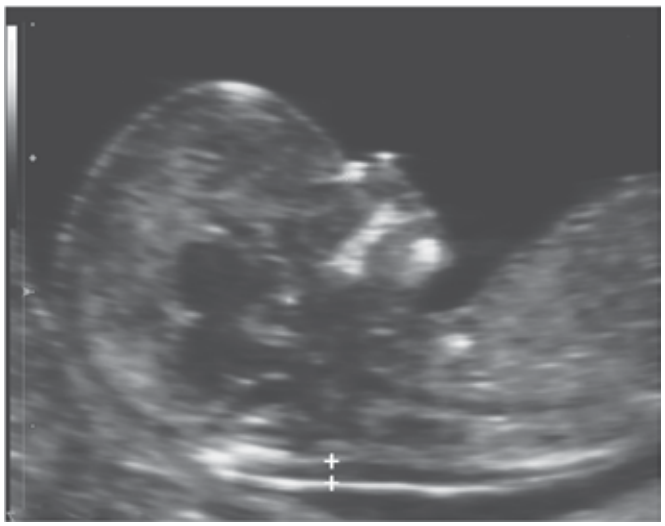

Fig. 1. Measurement of nuchal translucency behind the neck of the fetus.

1.2 Nasal bone

The nasal root depth is abnormally short in 50% of trisomy 21 cases (Cicero S et al, 2003). Sonographic studies at the 15th to 22nd weeks of gestation reported that about 65% of trisomy 21 fetuses had an absent or abnormally short nasal bone. The fetal nasal bone can be visualized between the 11th and 14th weeks of gestation, when CRL is between 45 and 84 mm. The assessment of the nasal bone should be obtained in a mid-sagittal view of the fetal

profile (Figure 2). The image of the nose includes three lines. The top line represents the nasal skin, in continuity with the skin is the tip of the nose, and the bottom line represents the nasal bone which is thicker and more echogenic.

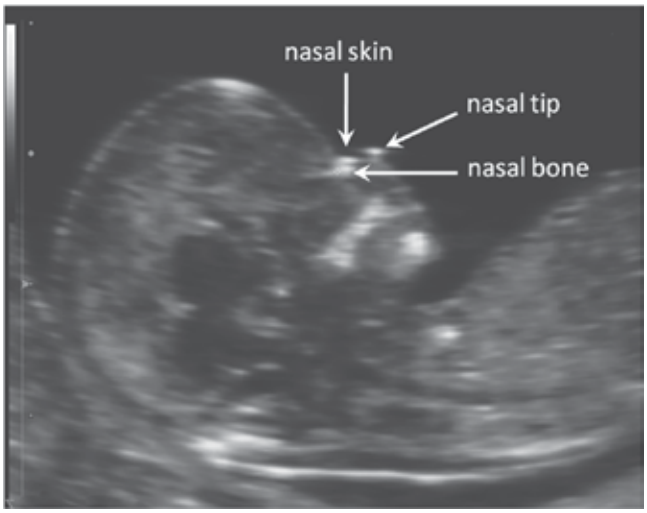

Fig. 2. Assessment of the nasal bone in a mid-sagittal view of the fetal profile.

2. Sonographic screening for trisomy 21 by maternal age, nuchal translucency and fetal nasal bone

The best performance of first trimester screening is achieved by a combination of maternal age, serum biochemical testing and multiple sonographic markers. But Doppler assessment of tricuspid and ductus venosus flow can be time consuming and it requires properly trained sonographers. As a first stage policy the assessment only by maternal age, nuchal translucency and fetal nasal bone, without biochemical testing, has some advantages (Nicolaides KH 2011). Beside the examination of fetal anatomy which leads to early diagnosis of many abnormalities in all pregnancies, the major advantage is the reduction in the cost of screening. The measurement of biochemical markers is undertaken only in the subgroup with positive first stage screening results.
Our study reports the results of the first trimester trisomy 21 risk assessment by a combination of maternal age, sonographic measurement of fetal NT thickness and assessment of fetal nasal bone in unselected pregnancies in Slovenia.

3. Subjects and methods

The study included all pregnant women appointed for the first trimester ultrasound screening examination at a single outpatient clinic between January 4, 2005 and April 30, 2010. Before the screening they all received counseling by their level one gynaecologists and an information leaflet about the ultrasound examination and the aim of screening.
At the time of the visit, they gave details about their demographic characteristics and medical history, which were entered into the computer database. The examinations were carried out by two experienced sonographers, certified by the Fetal Medicine Foundation Certificate of Competence in the 11th to 14th weeks scan.

In the majority of the cases the examination was performed transabdominally within 20 minutes. In less than 1% of the cases a transvaginal ultrasound examination had to be carried out.
In the fetal CRL less than 45 mm, the pregnant woman was given a new appointment, while in the fetal CRL more than 83 mm, only a detailed ultrasound scan was performed and the pregnant woman received further information about the possibility of the second trimester biochemical test.
For the examinations we used 2-5 MHz and 3.7-9.3 MHz transducers GE Healthcare Voluson 730 Pro, Milwaukee, USA, and 4–6 MHz, 4–7 MHz, 5–9 MHz and 7–9 MHz transducers Acuson S2000, Siemens Medical Solutions, Mountain View CA, USA.
Only singleton pregnancies with live fetus from the 11th to 14th weeks of gestation with the CRL of 45-83 mm were included in the further analysis. Pregnancy outcomes were obtained from the participating women, referred by level one gynecologists and paediatricians. Karyotype results were reported by the three cytogenetic laboratories.

3.2 Statistical methods

Risks were calculated according to the FMF program, following the FMF guidelines (Snijders RJM et al, 1999; www.fetalmedicine.com). The distribution of maternal age of the examined women was compared to the age distribution in the pregnant population in Slovenia for the time interval 2005-2010 (www.stat.si).
We calculated the sensitivity, false positive rate, positive predictive value and negative predictive value for a cut-off risk of 1 in 300. We accompanied the most important results with a 95% confidence interval. The balance between the false positive rate and the detection rate was studied and the trends were inspected graphically. The cut-off risk that would yield 5% false positives was calculated for trisomy 21.
Additionally, the expected numbers for the Slovene population were estimated by weighting the values of each woman according to the frequency of her age in the Slovene population.

4. Results

4.1 Study population

A total of 13,535 pregnant women were offered first trimester screening for detecting fetal chromosomal abnormalities at a single outpatient clinic between January 4, 2005 and April 30, 2010.
The sample represented unselected population of pregnant women of Caucasian ethnic origin. Twin pregnancies (3.6% of the cases, 486 of 13,535) were excluded. All further analyses included 13,049 women with singleton pregnancies.

4.2 Age distribution

The average maternal age (at the time of screening) was 29 years (range 11 to 47, SD=3.9) with a distribution as shown in the Figure 3 (black columns). When compared to the age distribution in the pregnant population in 2005-2010 in Slovenia, the average maternal age was the same - 28.9 years (Figure 3, red columns). The pregnant population however varied more, in particular there was a larger proportion of women aged 36 and more in the Slovene pregnant population (10.2%) as compared to only 3.6% in our sample. The sample on the other hand included a considerably larger percent of the women between 26 and 30 (47.9%

compared to 40.6%). Only 2.5% were 37 and older compared to the 7.2% in the population of Slovene pregnant women.

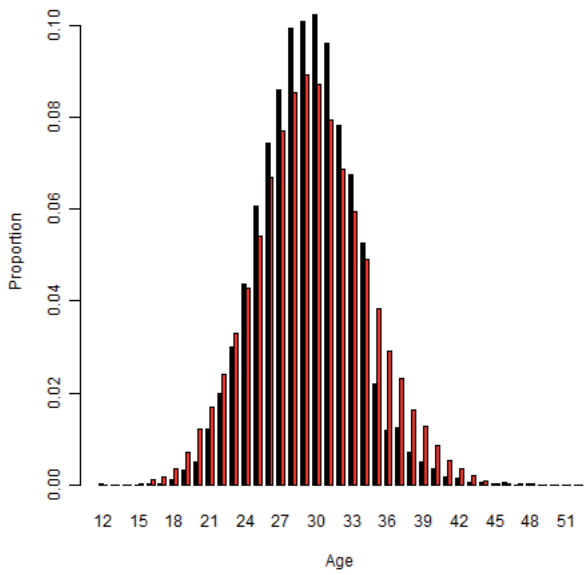

Fig. 3. Age distribution comparison at screening; Slovene population (red columns), our sample of unselected population of pregnant women (black columns).

4.3 The screening results

The average gestation was 12 weeks 4 days (range 11 weeks 1 day to 14 weeks 0 days). The average fetal CRL was 63.2 mm (range 45 to 83 mm). And the average NT thickness was 1.7 mm (range 0.9 to 13.4 mm).

The distribution of NT for fetal CRL in normal pregnancies and pregnancies with fetuses affected by chromosomal abnormalities is shown in Figure 4. The NT was above the 95th centile of the normal range for the CRL in 75% (15 of 20) of trisomy 21 pregnancies and in 64% (16 of 25) pregnancies with other chromosomal abnormalities.

At the time of testing in 3% of all pregnancies (394 of 13,049) the estimated risk for trisomy 21 was 1 in 300 or higher, considering the calculation based on FMF program. Three hundred and sixty cases (2.8%) turned out to be false positive. At the invasive testing, chromosomal abnormalities were identified in 8.6% of high risk cases (34 of 394), which represents one case of fetal chromosomal abnormality detected per 12 invasive diagnostic procedures.

4.4 Detection rate

We detected 34 out of 45 cases with chromosomal abnormalities (75.6%), diagnosed prenatally or postnatally.

Trisomy 21 was detected in 20 cases (Figure 4). Among women with an estimated risk of 1 in 300 or higher there were 17 cases of trisomy 21. The detection rate for trisomy 21 was 85% (17 out of 20), the 95% confidence interval for the detection rate was [69.4, 100]. The positive predictive value for the trisomy 21 was 4.3% (17 of 394); and the negative predictive value was 99.98% (12,652 of 12,655).

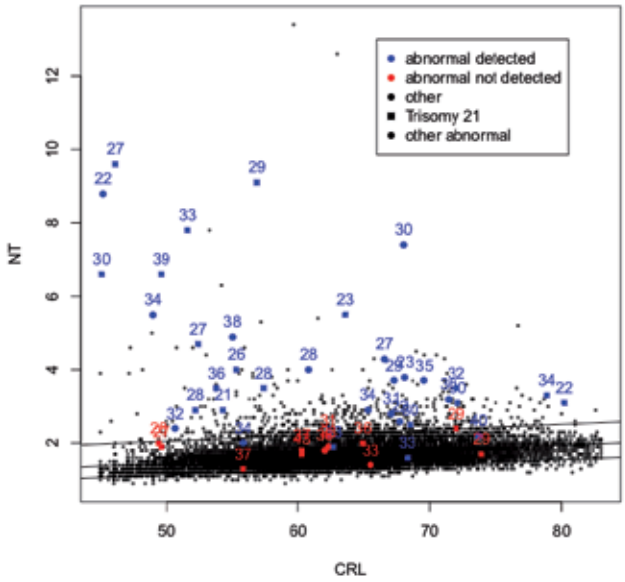

Fig. 4. Distribution of NT (in mm) with respect to CRL (in mm) in our sample. The curves present the 5th, 50th and 95th centiles.

Other chromosomal abnormalities were diagnosed in 25 cases (Figure 4). Among women with an estimated risk of 1 in 300 or higher there were 17 cases of other chromosomal abnormalities. The detection rate was 68% (17 out of 25). The 95% confidence interval for the detection rate was [49.7, 86.3]. The positive predictive value for the other chromosomal abnormalities was 4.3% (17 of 394); and the negative predictive value was 99.94% (12,647 of 12,655).

The diagnosed chromosomal abnormalities and their frequencies are presented in Table 1. The assessment of nasal bone was performed in 98.5%of the cases (12,847 out of 13,049). In 12 cases nasal bone was absent (0.1%), and in 3 out of those 12 cases (25%), the chromosomal abnormality was detected.

Karyotype	**No.**
Trisomy 21	20
Trisomy 18	10
Trisomy 13	2
45,X (Turner syndrome)	3
47,XXY	2
Mosaic structure	3
Unbalanced structural rearrangements	5
Total	**45**

Table 1. Chromosomal abnormalities in fetuses and newborns in our sample of 13,049 women with singleton pregnancies.

4.5 The risk threshold

The risk values of the undetected cases of trisomy 21 were as follows: 1 in 1979, 6365, 7435; for other chromosomal abnormalities they were 1 in 493, 872, 1102, 1279, 2016, 2180, 3816, 6731.

It was thus clear that a less conservative limit for probability threshold (for example 1 in 500) would not yield different detection rate in case of trisomy 21, but would rather improve the detection of other chromosomal abnormalities.

These changes are explored in Figure 5, which shows how detection rate would change by changing the risk threshold. The black curve shows how detection rate of trisomy 21 changes by changing the risk threshold (value 200 implies probability threshold set at 1/200). The red curve represents the same information for other chromosomal abnormalities. The numbers above the points indicate the percentage of false positives that would occur in our sample at that threshold.

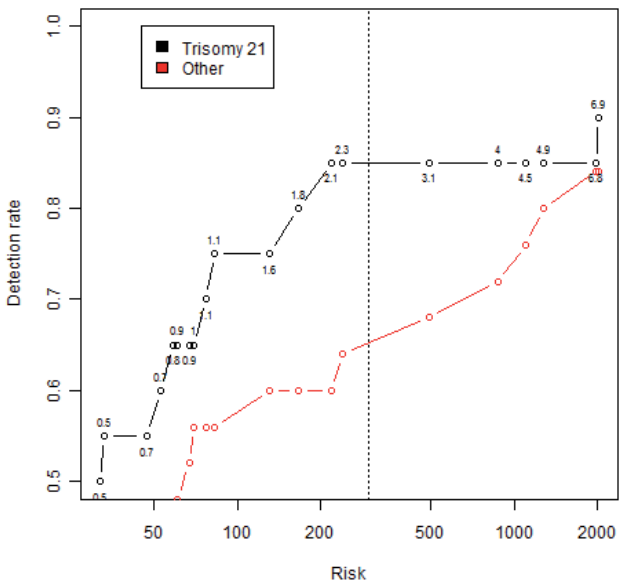

Fig. 5. Detection rate of trisomy 21 (black curve) and other chromosomal abnormalities (red curve) with respect to the risk threshold set (value 200 implies probability threshold set at 1/200). The numbers above the points give the false positive rate.

Inversely, Figure 6 shows the detection rate with respect to the percentage of false positives, the numbers above the points indicate the risk threshold (value 200 implies threshold 1/200).

4.6 Prediction for the Slovene population

Since the age distribution in our sample was different from the age distribution in the Slovene pregnant women population, we can expect all the above results to be slightly different. In particular, the rate of false positives can be expected to change. Figure 7 gives the false positive rate in our sample with respect to calendar year and compares it to the predicted false positive rate in the Slovene pregnant women population.

Inversely, Figure 8 gives the probability threshold that would result in the 5% false positives rate.

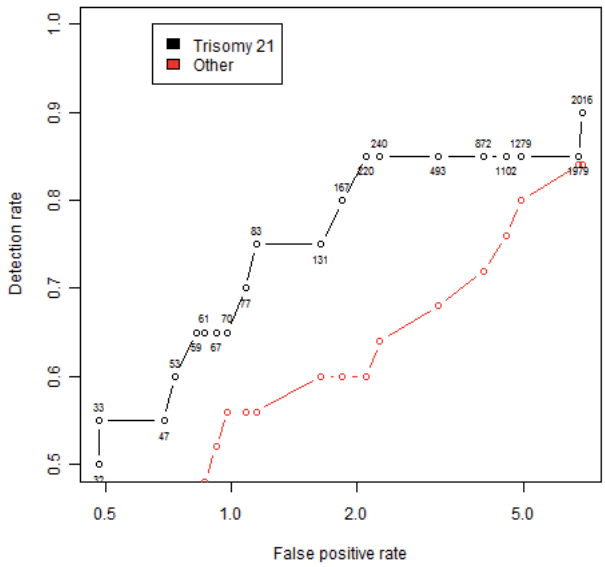

Fig. 6. Detection rate of trisomy 21 (black curve) and other chromosomal abnormalities (red curve) with respect to the false positive rate. The numbers above the points give the risk threshold (value 200 implies probability threshold set at 1/200).

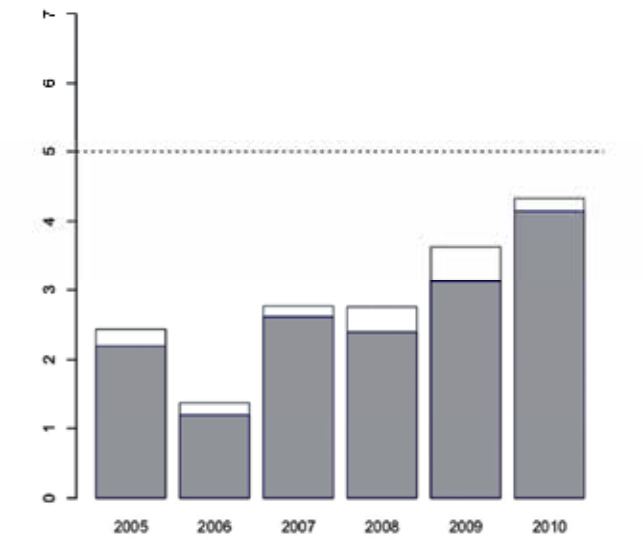

Fig. 7. The percentage of false positives in our sample (gray columns) and the predicted false positive rate in the Slovene pregnant women population (white columns) with respect to calendar year.

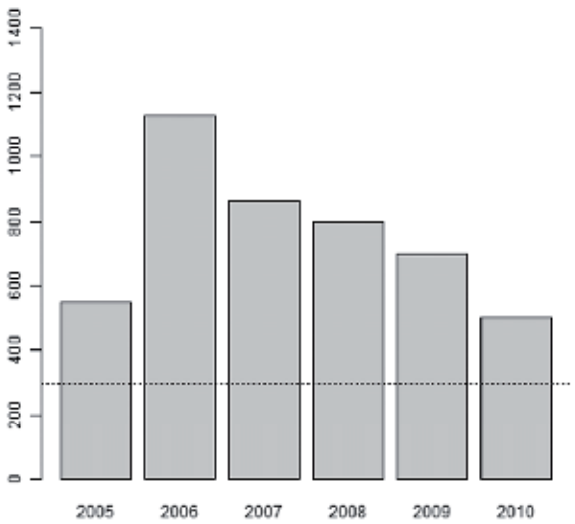

Fig. 8. The risk threshold that would result in a 5% false positive rate with respect to calendar year.

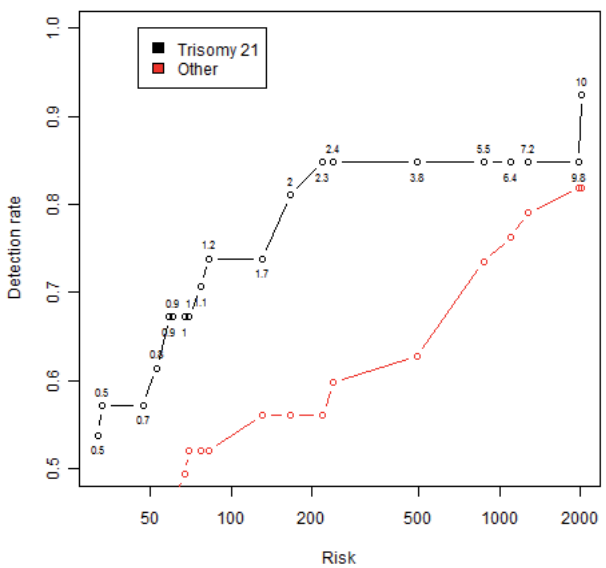

Fig. 9. Detection rate of trisomy 21 (black curve) and other chromosomal abnormalities (red curve) with respect to the risk threshold set (value 200 implies probability threshold set at 1/200) - prediction for the Slovene population. The numbers above the points give the false positive rate.

5. Discussion

The retrospective study of the first trimester screening for trisomy 21 in 5-year period from 2005 to 2010 by a combination of maternal age, sonographic measurement of fetal NT thickness and assessment of the fetal nasal bone represented a detection rate of 85% at a false positive rate of 2.8%.

5.1 Detection rate

The findings of many studies suggest that effective first trimester screening for trisomy 21 can be provided only by the combination of maternal age and measurement of fetal NT (Pajkrt E et al, 1998; Economides DL et al, 1998; Bindra R et al, 2002; Liu SS et al, 2004; Rozenberg P et al, 2006; Westin M et al, 2006; Czuba B et al., 2007; Kagan KO et al, 2010). At a risk cut-off of 1 in 100, the detection rate of trisomy 21 is about 75%, at a false positive rate of about 2%.

The detection can be improved to 85% by the additional assessment of the fetal nasal bone and even more by the Doppler assessment of blood flow across the tricuspid valve or blood flow in the ductus venosus, which increased the detection rate to about 95% at a false positive rate of 2.5% (Kagan KO et al, 2010). The disadvantage of the Doppler assessment of tricuspid and ductus venosus flow is that it is time consuming and it requires appropriately trained sonographers (Nicolaides KH 2011).

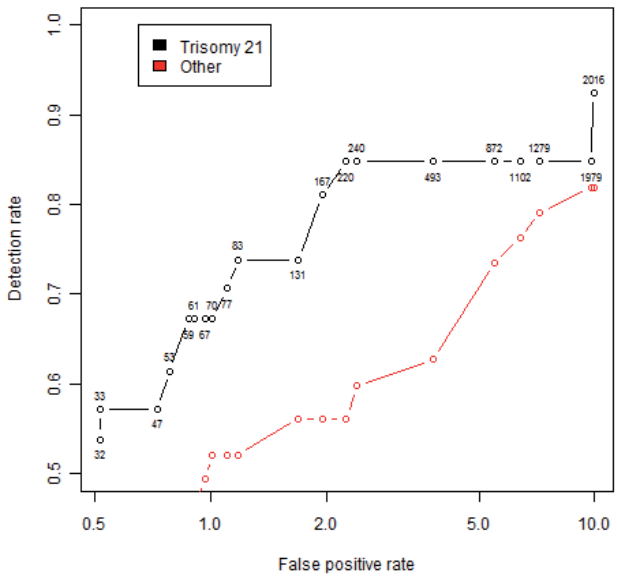

Fig. 10. Detection rate of trisomy 21 (black curve) and other abnormalities (red curve) with respect to the false positive rate - prediction for the Slovene population. The numbers above the points give the risk threshold (value 200 implies probability threshold set at 1/200).

By the measurement of fetal NT thickness and the assessment of the fetal nasal bone we were able to achieve the expected results at a risk cut-off of 1 in 300. In our sample the relationship between the false positive rate and the detection rate was proved to be in line

with the prediction from the Nicolaides study (Nicolaides KH 2011) and highly comparable to other reports (Prefumo F et al, 2006; Kagan KO et al, 2010).
Choosing sonographic assessment rather than biochemical testing as a first stage policy has a few advantages. In all sonographically screened pregnancies in addition to chromosomal markers, fetal anatomy is observed and both provide early diagnosis of many major abnormalities, not just in the subgroup with a positive first stage biochemical screening results (Nicolaides KH 2011). Further measurements of maternal serum free β-hCG and PAPP-A can be limited only to high risk subgroup.

5.2 Prediction for the Slovene population

But the trend of ageing the population of pregnant women is noticed and the false positive rate at the risk cut-off 1 in 300 is changing almost linearly with time (Figure 7).
Allowing ourselves 5% false positive rate in the population, we could lower the risk limit. But, as our detection rate is more than adequate according to FMF guidelines, at the moment we have no reason to actually change it, and we believe that any such change should also be weighted against the risk of pregnancy loss due to invasive testing.
Our calculations show that the FMF recommendations about the risk limit cannot be directly translated to our specific population distribution. We believe the limit that ensures a 5% false positive rate is constantly changing and could be adjusted within each country. To accurate the changing, the weighting could be performed using a large data set, preferably the data set on which the FMF algorithm was developed, using the respective population maternal age distribution. The only assumption needed for such estimation would be, that conditional on the maternal age, the NT and CRL distributions do not vary among the countries, which we believe is an assumption we can safely make.

6. Conclusion

The 11th to 14th weeks scan includes confirmation of the viability of the fetus, accurate dating of the pregnancy, and an early diagnosis of multiple pregnancies and identification of chorionicity.
Effective screening for trisomy 21 can be achieved in the first trimester of pregnancy by a combination of maternal age, sonographic measurement of fetal NT thickness and assessment of fetal nasal bone, with detection rate of 85% at a false positive rate of less than 3%.

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18

Noninvasive Prenatal Nucleic Acid Diagnostics of Down Syndrome

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1. Introduction

Molecular composition of pregnant women blood plasma/serum is an indispensable tool for biochemists in the field of routine prenatal diagnostics aimed at the detection of the most common aneuploidies.
Predictive power of some biochemical markers respectively their sum is an important guide for the assessment of the status of the fetus and pregnancy.
But the diagnostic potential of maternal plasma is much broader.
Fragmented and apoptically degraded fetal cells and nucleic acid molecules, which overcame the placental (feto-maternal) barrier and got under the influence of the immune system of the mother, offer other options for molecular biologists.
Some of them will focus on the clarification of rules between the quantity of fetal molecules and pathology of the placenta and the fetus others are engaged in direct diagnostic potential.
In the maternal blood circulation can be detected fetal genetic material of a different integrity ranging from the nuclear DNA of living cells through fetal apoptically partially degraded molecules more or less packed in nucleoides to completely fragmented DNA molecules mostly of size between 100 - 400 bp. Cell free fetal (cff) mRNA molecules from trophoblast are also presented in detectable concentration in maternal plasma.
While the presence of fetal cells in maternal circulation is quite rare (1 fetal cell in 1 million maternal cells), the quantity of cffDNA molecules in plasma is considerably higher (typically 3 % - 6 %).

2. Source and transport of cff nucleic acid molecules into maternal plasma

The ways in which cffDNA forms and how gets into the maternal circulation, are not yet fully clarified. There are several hypotheses on the origin of cff DNA.
The main source of free DNA appears to be a placenta (Wataganara & Bianchi, 2004). Most likely the cff DNA forms by disintegration of placental cells and the direct transition to the maternal circulation.
Another possibility is that the placenta functions as an mediator of transition for DNA circulating in the fetal blood circuit into maternal tissues.

In this case, there should be a two-way traffic, however, the amount of analyzed fetal DNA in maternal plasma is much greater than the amount of free maternal DNA in umbilical plasma in childbirth (Sekizawa et al., 2003). It is indicative of an unequal transfer of free DNA from fetus to the mother. Another source of fetal molecules may be fetal hematopoietic cells which get into the maternal circulation (Bianchi, 2004). The fetal DNA is apoptically released into maternal plasma due to the effect of maternal immune system (Pertl et al., 2000). Apoptotic fetal cells were found in detectable concentrations also directly in the plasma (Van Wijk et al., 2000). Apoptosis proven in maternal plasma (Sekizawa et al., 2003) or in cord blood (Hristoskova et al., 2001) suggests that this mechanism could be an important source of fetal material. Another hypothesis assumes a simple diffusion of shorter DNA molecules from the amniotic fluid through placenta or membranes into maternal circulation. This DNA then comes from various fetal tissues.

Cff DNA was also demonstrated in the maternal plasma even before feto-maternal circulation establishment. This means that the DNA could be of the trofoblastic origin (Bianchi et al., 2004). In general, it could be assumed that cff DNA in maternal circulation is of different origin but its large portion likely comes from the trophoblastic placental tissue.

The transfer of fetal molecules through the feto-maternal barrier is continuous during pregnancy and after the delivery they are quickly eliminated from plasma (16 min halftime of degradation (Lo et al., 1999).

Free fetal RNA allows non-invasive prenatal profiling of gene expression and offers a number of research and diagnostic applications on the basis of easily detectable mRNA transcripts from placentally expressed genes in maternal plasma (Ng et al., 2003).

Considering serious degradation as a result of the activities of the ribonucleases, the stability of mRNA molecules in fetal free plasma is surprising. Plasma RNA molecules are much more stable than isolated and purified RNA molecules. Mechanisms that protect the circulating free fetal RNA are not fully clarified at present time. However their conjunction with the sub cellular particles could prevent their degradation (Hasselmann et al., 2001).

They can form complexes with proteins, lipids, lipoproteins, phospholipids bound to the DNA in nucleosomes or within protected apoptotic corpuscles or other vesicular structures (Halicka et al., 2000; Hasselmann et al., 2001; Tsui et al., 2002; Sisko et al., 2001).

The stability of placental mRNA molecules in maternal plasma is a promising assumption that fetal markers at the level of mRNA will be clinically useful.

It could be useful for prenatal detection of some pregnancy pathologies, including pre-eclampsia and certain chromosomal aneuploidies (Ng et al., 2004).

3. Placental trophoblast and trisomy 21

Trophoblast with chromosome 21 trisomy shows disturbed both cell fusion and formation of syncytiotrofoblast (ST) (Frendo et al., 2000b; Massin et al., 2001). An activation of caspases is necessary for formation of ST in early stage of differentiation (Huppertz and Kingdom, 2004). If ST is inadequate, the individual trophoblastic cells with trisomy 21 could continue on in the cascade of apoptic events and release more fragmented cff DNA than disomic trophoblastic cells. Another possible cause for the release of larger amounts of fetal molecules in pregnancy with trisomy 21 is a continuation of trophoblastic cells in the improper proliferation, without initiating events leading to the formation of ST. In this case maternal immune system would regulate the division by increased degradation of fetal cells.

4. Fragmentation profile of cffDNA

Publications, relating to the characterization of the DNA fragments in the plasma, are aimed to the level of fragmentation of fetal molecules related to the maternal ones in the course of pregnancy. For pregnant women were described in plasma longer fragments in comparison with non-pregnant women (Chan et al., 2004).
Real-time PCR is the most commonly used procedure for the analysis of fetal DNA fragments in present time (Alberry et al., 2009; Ariga et al., 2001; Lo et al., 1998). Honda et al. (2002) described the sensitivity of the detection of fetal DNA for this method and determined it at 5,38 copies/ml of the peripheral blood.
Molecules of fetal origin are more degraded and shorter than the maternal and their size is approximately in the range from 100 bp - to 700 bp. (Zhong et al., 2000; Koide et al., 2005). Fragments above 1 kb belong mostly to the mother (Li et al., 2004).
We have evaluated fragment analysis using three methods (capillary electrophoresis of STR loci, capillary electrophoresis and Real-time PCR of gonozomal sequences) from two different perspectives. The first relied on direct analysis of the size of fractions and the second assessed PCR efficiency with respect to the size of amplified DNA molecules (Vodicka et al., 2010).
Direct detection of fetal molecules has confirmed a large heterogeneity in the individual fractions both by capillary electrophoresis and Real-time PCR.
A direct dependency of fetal molecules on the week of pregnancy has been suggested only in the size fraction of 500 - 760 bp. Regression modeling at STR and gonozomal analyses was the most accurate in the pD system, where the size of molecules ranged from 395 bp to 440 bp. Statistical significance of regression declined towards the smaller molecules.
The fragment size was 200 bp to 223 bp in the D21S1446 system and 157-188 bp in D21S1435. Regression analysis was inconclusive in the AMELY (molecule size 109 bp).
If we compare analysis of the size of fractions to the results of the effectiveness of the STR and gonozomal analyses (Vodicka et al., 2008a), where was demonstrated statistically significant increase of fetal molecules in all 3 STR systems, we found out that it was confirmed an interesting trend, which indicates an increase in the larger fetal molecules during pregnancy while the number of smaller molecules of fetal origin does not change.
In the case of quantification using SRY probe, whose length is 64 bp it was observed even reverse trend and the number of amplified molecules in 150-300 bp fraction was in indirect relation to the week of gestation.
The tendency has decreased for larger fractions and in total plasma the trend has stopped even slightly reversed.
On the basis of these observations, we can therefore assume the most sensitive detection of fetal material using short probes. But these probes are not much suitable for quantitative analysis. Fractions from 400 bp above are the most appropriate for the assessment of the amount of fetal material, although in these fractions the relative amount of fetal DNA is less.
The importance of this study resides in decision-making, whether, from a diagnostic point of view, it is more important a detection of fetal molecules or their quantification (Vodicka et al., 2010).

5. The extraction of cffDNA/RNA from maternal peripheral blood

5.1 CffDNA isolation procedures

An essential precondition for the success of all subsequent procedures is a sufficient yield, reproducibility and the purity of the isolated plasma cffDNA. Isolation procedures are most

often based on double centrifugation and subsequent binding (adsorption) of DNA molecules on a silica surface of membrane or magnetic particles.

5.1.1 Isolation by binding to a silica-gel membrane

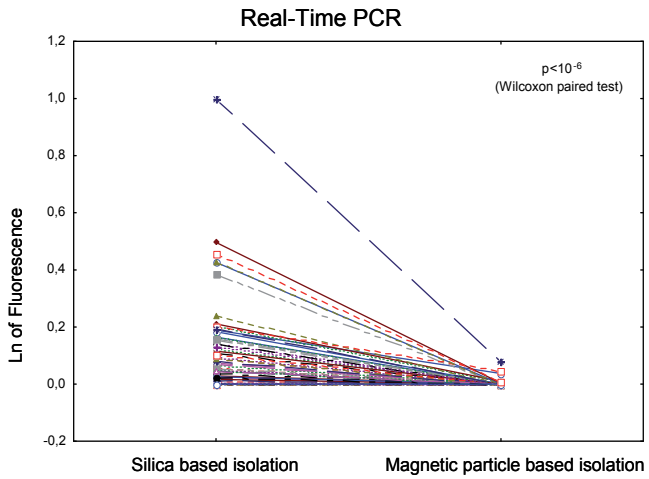
The adsorption on silica surface is one of the most common techniques which is able to capture free fragmented DNA.
Silicate polymer in the presence of chaotropic salts (e.g. sodium iodide or guanidine thiocyanate) specifically binds to the DNA molecule on the surface of silicon dioxide (usually specially prepared glass) and in the presence of water or other elution reagent, which has a very low content of salt, this molecule releases from its surface.
High specificity of the link between DNA molecule and silicate material is useful in eliminating other extracellular substances.

5.1.2 Isolation by binding to the magnetic particles

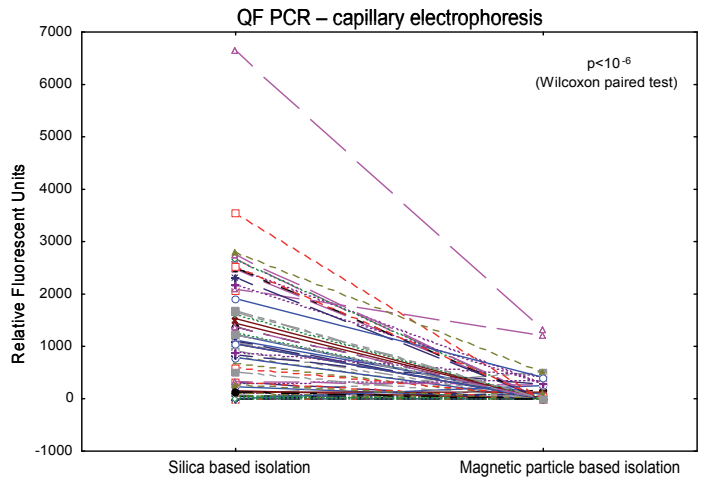
Methods based on the (para) magnetic particles (MPs) are other approach to the cffDNA isolation. MPs are particles of size from 5 nm to 100 μm, consisting of a metal core, it is usually γ-Fe2O3 (maghemit) or Fe3O4 (magnetite) but could be also for example Au.
The core is coated with a layer that has specifically prepared surface for binding the molecules that we want to isolate.
The size of the MPs can be adapted according to the molecules which we are isolating: 5-50 nm proteins; 20-450 nm nucleic acids; 10-100 μm viruses.
MPs respond to the external magnetic field and are capable to bind different bioreaktive molecules, because of their affinity to the modified surface, directly from the biological material. The isolation is then as follows.
MPs are added to the sample where bind with the targeted molecule. Modified MPs are then attracted by magnet to the wall of the tube and the remaining solution with non-bind substances is removed.
The MPs with joined molecules are subsequently washed and released in added solution.
Bound molecules are separated from the MPs by some physical-chemical step (denaturing or change in pH).
This way we obtain targeted molecules which could be afterward analyzed (Húska et al., 2008).

5.1.3 Comparison of silica-gel to magentic particles methods

Both above mentioned isolation procedures were tested in our workplace.
For a comparison, cff DNA (male fetus) was measured and quantified both by Real-time PCR in SRY locus and by quantitative fluorescent (QF) PCR and capillary electrophoresis separation in AMELY locus in 38 samples from the 1st and 38 samples from the 2nd trimester. While the concentrations of total DNA measured spectrophotometrically were similar there were substantial differences in yield of cffDNA between tested DNA extraction methods (graph 1,2).
Isolation based on the separation of magnetic particles had significantly lower recovery compared with the amount of cffDNA extracted by binding on the silicate membrane.
According to our current findings "silica membrane" based method seemed to be clearly more suitable for isolation of cffDNA from the plasma of pregnant women.
DNA extraction using magnetic particles should present a simple way how relatively easily and without contamination obtained the cffDNA.



Graph 1. Comparison of silica gel and magnetic particles isolation effectiveness by Real-time PCR quantification.

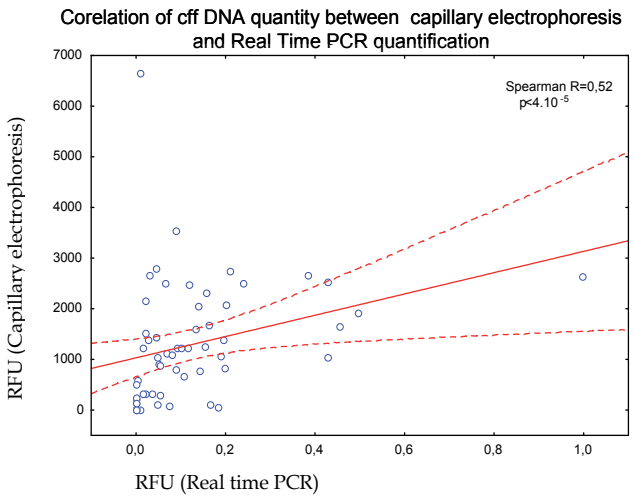


Graph 2. Comparison of silica gel and magnetic particles isolation effectiveness by capillary electrophoresis quantification

However, for the isolation of short fragments of cffDNA this method did not work, although the manufacturer declares, that it should be able to detect the DNA molecules of the size from 50 bp to 1,5 kb. Low efficiency of cffDNA capture can be caused by strong bond of free fragmented fetal DNA on magnetic particles, or by contraries, by weaker bond of these fragments on magnetic particles and their wash out during isolation.

To explain this, we re-isolated unbound DNA from supernatant. The subsequent quantification by capillary electrophoresis has demonstrated again only the maternal sequences, which suggests a stronger link of fragmented fetal DNA on magnetic particles.

If cffDNA was succsessfully detected by both methods, the amount of DNA has correlated to each other (Graph 3).



Graph 3. Correlation of the results of free fetal DNA quantification by capillary electrophoresis and real-time PCR (RT-PCR). The Y axis shows the Relative fluorescence units (RFU) obtained from capillary electrophoresis, X axis shows the fluorescence scanned in Real time system.

5.2 Cff mRNA isolation procedures

To maintain integrity of obtained free fetal RNA, it is important to use isolation techniques which have minimum isolation steps, are quick and contain RNA stabilizers.

Currently there are no commercially produced kits standardized for isolation of free fetal RNA directly from maternal plasma, but there are available kits specialized for isolation of viral RNA, which could be, with little modification, applied to the isolation of free fetal RNA.

Double centrifugation in cooled centrifuge or ultra centrifugation is necessary for separation of cellular component of blood prior to the isolation of free fetal RNA.

The combination of viral RNA Kit and vacuum pumps seems to be an optimal system for obtaining free fetal RNA in the highest possible quantity and the quality, with minimal risk of contamination during isolation, unlike centrifugation steps. Microfilters can be used to increase the concentration of free fetal RNA.

6. The use of cff DNA for diagnostic purposes

The resolution of the fetal genotype and precise quantification of cffDNA are crucial for diagnostic purposes.

6.1 Noninvasive fetal sex determination by cffDNA

So far, the most common and relatively simple for the analysis of fetal DNA was the use of Y specific sequences from male fetuses.

These sequences can be quantified by methods of Real-time PCR and QF PCR.

The methodology for fetal sex detection in maternal plasma is already relatively well developed and reliable (Lo et al., 1998; Honda et al., 2002; Wei et al., 2001; Zhong et al., 2000, Vodicka et al., 2008b).

Our quick and simplify fetal sex detection is based on refined QF PCR. We tested 475 DNA samples isolated from maternal plasma in different weeks of pregnancy ranging from 4th w.g. to 37th w.g. Y chromosomal sequences in AMELY were tested and quantify by comparison to AMELX using capillary electrophoresis.
The method is able to distinguish even less than 1 % of Y chromosomal sequences of artificial mixtures. Fetal sex was detected with 4.05 % of false positivity and 7.15 % of false negativity (Vodicka et al., 2008).
The methodology has very high detection sensitivity, which is comparable with a sensitivity of real-time PCR methodology (Sekizawa et al., 2001; Honda et al., 2002).
Possible contamination of the samples during collection and in the course of the isolation procedure is the main cause of false-positive detection of the male sex.
Minimization of any contamination is fundamental in sensitive DNA analyses in which it is necessary to capture the genotype in quantities of about 5%.
Mistakes in determination of male fetus (false-negative results) come mostly from early stages of pregnancy, when the concentration of cffDNA is still very low, so rather than non-compliance could be assessed these results as missing fetal DNA.
Capillary electrophoresis has proven to be uniquely able to capture even 1 % genotype artificial admixture.
The result of examinations of our group of pregnant women can be considered very satisfactory. The methodology is prepared for practical use, however, further improvement of isolation robustness and following laboratory procedures may increase specificity, sensitivity, and the yield of fetal DNA.

6.2 Noninvasive RHD genotyping by cffDNA

RHD genotype of the fetus can be identified from maternal plasma already at the beginning of pregnancy using molecular analysis of cffDNA.
Clinical significance of determination of fetal RhD status is dual:

1. Noninvasive fetal RHD determination from peripheral blood of Rh- alloimmunized pregnant women at the beginning of pregnancy allows to determine the fetuses which are really threatened by development of hemolytic disease of fetus and newborn.
2. If anti-D antibodies are not present at the beginning of pregnancy, RhD negative woman should be provided with anti-D immunoglobulin during pregnancy in sufficient dosage for each potentially sensitizing events including completion of antepartal prophylaxis at 28th week of gestation. Potentially sensitizing event means any situation in which may occur intersection of RhD positive fetal erythrocytes to the mother's blood, and subsequent development of the RhD alloimmunization (chorionic villus sampling, amniocentesis, cordocentesis, another intervention of the prenatal diagnosis and fetal therapy, obstetric hemorrhage in the 2nd and 3rd trimester, intrauterine death of fetus). Prevention of RhD alloimmunization should be provided to RhD negative pregnant woman by delivery of anti-D immunoglobulin in sufficient dosage in all above-mentioned cases. The administration of anti-d immunoglobulin is necessary if the fetus is RhD-positive, on the contrary, if the fetus is RhD negative, the pregnant woman is not threatened by the development of the RhD alloimmunization and administration of anti-D immunoglobulin is not necessary.

RHD genotyping of cffDNA is most often done using Taq man Real time PCR with probes from exone 7, 10, 4 or 5. System of negative and positive PCR control in addition to internal

control of PCR amplification is necessary for the reliability of detection considering great sensitivity of the method. At the same time, you must have created calibration quantitative profile for exact specification of detection limit of RhD positivity.

6.3 Noninvasive cffDNA analysis of monogenic inherited diseases

CffDNA can be also used for examination of monogenic inherited diseases.
Many genetic abnormalities, for example autosomal dominant myotonic dystrophy (Amicucci et al., 2000), Huntington's disease (Gonzalez-Gonzalez et al., 2003) or gene mutations such as achondroplasia (Saito et al., 2000) has already been examined from cffDNA. Furthermore, it is possible to detect some autosomal recessive diseases, such as cystic fibrosis (Gonzalez-Gonzalez et al., 2002) or β-talasemia (Chiu et al., 2002).

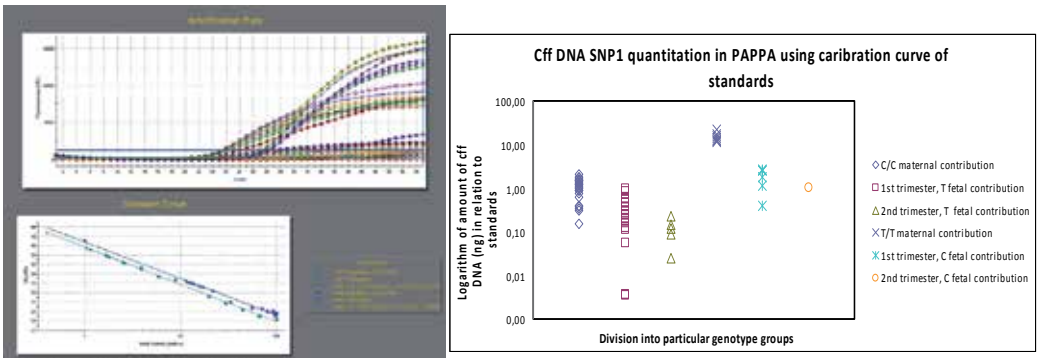
7. Cff nucleic acid quantitation for noninvasive Down syndrome diagnostics

Targeted molecular analysis of fetal aneuploidy with maternal plasma utilization is more complicated and applicable differentiation of maternal and fetal genotypes within the desired chromosome brings with it many challenges.
Considering differences between the quantity of total cffDNA and cfDNA, in the fetuses with Down syndrome (DS) pathology can be observed a greater quantity of cffDNA compared to the physiological pregnancies (Vodicka et al, 2008a).
The total amount of cffDNA cannot be considered as unambiguous marker due to large variation in the amount of cffDNA in physiological pregnancies and, rather, it is possible to include it to the system of ultrasound and biochemical markers.
Direct examination of the loci responsible for the DS brings a substantial improvement of the diagnosis. Currently, there are several approaches or their combinations respectively, aimed to unambiguous resolution of pathological fetus with the DS.

7.1 Noninvasive cffDNA diagnostics of Down syndrome using genotype differences between paternal and maternal genotypes

DS analyses and quantifications of cffDNA are based on the resolution of paternal from maternal genotypes. The fetus must be always heterozygous for the monitored loci. Previous genotyping of the parents is therefore important for the selection of appropriate markers.
Targeted resolution assumes a sufficient number of reliable analyzable markers of paternal (fetal) origin on chromosome 21 and at the same time a sufficient number of reliable markers outside the chromosome 21.
The genotype of the fetus can be distinguished by means of DNA sequence variation using male specific sequences for male fetus, short tandem repeats (STR) or single nucleotide polymorphisms (SNP).
For the estimation of the relative amounts of cffDNA originating in chromosome 21 it is necessary to choose a strategy that combines the creation of sensitive and accurate calibration standards for each marker and reciprocal measuring of cffDNA amount from the locus on chromosome 21 and cffDNA quantity from autosomal locus or locus on chromosome Y respectively.
If the measurement is accurate enough, then the amount of cffDNA of chromosome 21 related to the quantity of the cffDNA locus outside the chromosome 21 should differ in the

case of trisomy 21 from disomy 21. It is clearly distinguishable in the case of trisomy of paternal origin, where the extra chromosome comes from the father.
The methodology of quantitative calculations for STR loci on chromosome 21, where was mainly taken into account the relative length of alleles and preferential amplification of the shorter alleles has been developed at our department.
In pregnancies with trisomy 21, it was found statistically more cffDNA for systems in which the length of PCR products ranged mostly between 200 bp - 400 bp (Vodicka et al., 2008).
In total six genes that are specifically expressed in trofoblastic tissues (placenta) has been selected for quantification and genotyping on the DNA level in loci responsible for Down syndrome and for reference SNP genotyping (Böhmova et al. 2010).
These genes are DSCR4, KRTAP26-1, PLAC4, PLAC1, PAPPA and PSG11 of them DSCR4, KRTAP26-1 and PLAC4 are localized on chromosome 21 in the area responsible for Down syndrome. In total 12 SNPs were selected for genotyping in these genes.
Balanced allelic frequency for particular SNP alleles reported in the database of the NCBI website was the criterion for the SNP selection. For individual SNP variants have been defined TaqMan probes. Individual genotypes were identified initially by sequencing of the SNP regions for 30 randomly selected maternal DNA samples. Subsequently TaqMan Real Time PCR optimization and calibration was carried out.
Quantifiler kit (Applied Biosystems) has been used for verification of the quality of the dilutive series of calibration standards. The quantification itself was carried out on 240 DNA samples isolated from plasma of pregnant women in the 1st and 2nd trimester.
Heterozygozity in particular SNP ranged from 14 % to 55 % and the probability of appropriate genotypes capture for each SNP was stated in the range from 12 % to 28 %. Sensitivity of minor genotype capture depends on fluorescence background of non-specifically hybridized probe.
Taqman SNP probes were able to capture as low as 1 percent of artificial genotypic mixture and by the most sensitive systems we have detected 0,22 % artificial genotypic mixture.
R^2 values were in the range of 0,995 to 0,999 and the inclination (angular coefficient) assessing the effectiveness of the PCR (tendency around the values of -3.3 shows the optimum, 100% PCR amplification) from -3.2 to -3.6. All standard calibration curves were appropriate for the cffDNA quantification (Picture 1).



Picture 1. Quantity assessment of cffDNA using calibration curve in SNP1 of PAPPA

Cff DNA was analyzed only for the detectable genotypes. The detection ability ranged from 11.8 % to 100 %. The amount of cffDNA was very variable (from about 0.02 ng to 13 ng

cffDNA in 200 ul of the plasma). All 12 selected SNP are able to detect cffDNA with the desired probability. Parameters of the calibration curve are suitable for the precise quantification of cffDNA (Picture 1) (Böhmova et al., 2010).

7.2 Noninvasive cff mRNA analysis for Down syndrome diagnostics

Placental tissue is ontogenetically much unrelated to blood cells and may express other genes whose expression is missing in maternal blood cells. The first work, which confirmed the RNA of fetal origin, was published in 2000 (Poon et al., 2000).

Using microarray technology in the maternal plasma have been detected RNA markers whose expression is specific to the placenta (Tsui et al., 2004). The same approach was used in the study, which described the cff mRNA transcribed from gene PLAC4 (placenta-specific 4), which is located on chromosome 21.

On the basis of the assumption of uniform batch theory (3 alleles of PLAC4 gene, without allele-specific preferential transcription, express 1/3 more mRNA than 2 alleles) quantitative detection of disomic and trizomic dose was developed. With the use of the SNP in the heterozygous state in the gene coding for the PLAC4 there were detected disomic fetus ratio 1: 1 and trizomic ratio 2: 1 with 90% of the diagnostic sensitivity and 96.5% specificity of trisomy 21 capture by mass spectrometry technique (Lo et al., 2007a).

The disadvantage of this method is relatively labour-intensive preparation of high-quality and reproducible matrix for mass spectrophotometry RNA analysis. Another limiting factor is the real amount of cff mRNA molecules in the isolate from plasma, which could affect the evenness of distribution of mRNA molecules transcribed from individual alleles. Only sufficient number of SNPs can assure desired heterozygozity. Therefore it is necessary to test and introduce new mRNA SNP markers (Go et al., 2007; Yang et al., 2008).

Another approach of measuring allelic SNP ratios in cff mRNA has been tested using digital PCR. It is based on the assumption that, in the PCR reaction is either 1 or no molecule of cDNA. PCR is carried out in parallel in many hundreds to thousands of mixtures. According to the number of positive responses, you can estimate an initial number of cff RNA molecules with a particular SNP and by measuring of the ratios between the SNP from RNA derived from chromosome 21 and another autosome, you can detect chromosome 21 trisomy with high sensitivity (Lo et al., 2007b).

7.3 Noninvasive Down syndrome diagnostics by cffDNA epigenetic detection and quantification

Methodology relies on different methylation of maternal and fetal or placental sequence. Sequences, which are subject to methylation are on principle situated in CpG islands of promoter areas. These islets may regulate the transcription of the gene by variable degree of methylation. In different tissues there are promoters of genes whose expression is tissue specific, and which are methylated differently, and specifically for the type of tissue.

Different fetal (placental) and maternal methylation of regions on chromosome 21 were tracked for the purposes of Down Syndrome non-invasive prenatal diagnostics. Different methylation showed 22 out of 114 assessed genomic regions on this chromosome (Chim et al., 2008).

This relatively large number of methylation specific fetal DNA sequences offers a promising source of prenatal biomarkers independent on maternal and fetal genotype.

Large loss of genetic material (90 %) in the course of DNA modification, based on cytosine deamination using bisulphite, is a disadvantage of this epigenetic way of examination.

The modification procedure, somewhat laborious, in addition increases the risk of contamination. Additional distortion of results could be due to heterogeneous methylation of CpG islands.
Some laboratories are trying to test methods that would eliminate the a forementioned shortcomings (Chan et al., 2006; Tong et al., 2007).
Digital methyl sensitive PCR, methyl sensitive HRM and, probably, massive parallel DNA sequencing are the most promising prospective technologies for precise quantification of epialleles.

7.4 Combination of genetic/epigenetic/cff mRNA methods

To increase the diagnostic sensitivity it is possible to combine methodological approaches, based on cffDNA, cff mRNA and hyper/hypomethylated placental DNA.
Tong et al (2010) published the epigenetic quantification of hypermethylated placental DNA from chromosome 21 (locus HLCS) using digital PCR and compared it to the male specific genetic marker of the Y chromosome.
The relative quantities of the epigenetic marker were shown to be increased for fetus with trisomy 21 compared to disomic fetus. This approach is very sensitive even for the DS diagnosis in the first trimester.
It will be appropriate to use different paternal genetic polymorphic markers at the level of the SNP or STR instead of male specific genetic marker.
To increase the reliability of resolution of trisomy 21 from disomy 21 is also possible to perform relative quantification of cffDNA/cff mRNA using exonal SNP of chromosome 21 and exonal SNP from the reference autosome.

8. Tasks for future

For the successful development of high accurate detection of DS and other frequent trisomies it is necessary to attend to the following tasks:

- Choice of isolation procedures for plasmatic DNA fragments length of which corresponds to the fetal molecules.
- Protection of plasmatic molecules from the disintegration of maternal lymphocytes.
- Prevention of free plasmatic fetal RNA degradation.
- Loss minimization during modification of hyper/hypomethylated DNA.
- Provision of sufficient number of reliable genetic and epigenetic fetal markers.
- More sensitive optimization of calibration and quantification for HRM, Real time PCR and QF PCR.
- Introduction of automation of digital PCR and digital HRM for quantification of CffDNA/cff mRNA/modified cffDNA.

9. Conclusion

Almost 15 years have passed since the first work, which pointed to the presence of the cff DNA in maternal plasma of pregnant women (Lo et al., 1997).
Since then many authors gradually contributed more and more theoretical and methodological knowledge.

Nowadays, also thanks to introduction of new and more robust technologies we can boldly predict that direct clinical outcome of this multi-year effort of noninvasive prenatal diagnostics of Down Syndrome will be feasible in very near future.

10. Acknowledgment

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This book provides a concise yet comprehensive source of current information on Down syndrome. Research workers, scientists, medical graduates and paediatricians will find it an excellent source for reference and review. This book has been divided into four sections, beginning with the Genetics and Etiology and ending with Prenatal Diagnosis and Screening.

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