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Hemodialysis
Different Aspects

Edited by Maria Goretti Moreira Guimarães Penido

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Meet the editor

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Contents

Preface

This book is meant to provide an overview of the state of the art of hemodialysis. The pace of acquisition of new knowledge in hemodialysis is fast and the goal is to bring a thoughtful, well organized exposition of this burgeoning knowledge base to the readers. Each chapter has been thoroughly revised and updated and the readers will become acquainted with the latest thinking in some of the most important topics in hemodialysis.

The book is comprehensive and not limited to a partial discussion of hemodialysis. Chapters pertaining to nearly all aspects of the care of dialysis patients are included.

To accomplish this we are pleased to have been able to assemble a leading panel of expert contributors who have been challenged to summarize state of the art knowledge in each chapter of the book.

We wish to thank each author for taking considerable time and effort to ensure that each chapter provides state of the art information. We hope that readers achieve the same level of acquisition of new knowledge and enjoyment as we have attained by editing this book.

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1

Acute Renal Failure Induced by Adenovirus After Stem Cell Transplantation

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1. Introduction

Adenoviruses have been recognized as opportunistic and significant viral pathogens in immunocompromised patients such as recipients of hematopoietic stem cells or other solid organs treated with immunosuppressive agents, and among patients with acquired immunodeficiency syndrome. These patients are incapable of developing a normal immune response. Reactivation of adenoviruses in the impaired immunological response leads to acute or persistent infections with high morbidity or even mortality in these patients.

In the field of allogeneic stem cell transplantation in particular, adenovirus infection is one of the common complications. A representative consequence of adenovirus infection is hemorrhagic cystitis. The cumulative incidence of adenovirus-induced hemorrhagic cystitis was found to be 7%, which usually presents a favorable prognosis during the era of bone marrow and peripheral blood stem cell transplantation (El-Zimaity et al., 2004, Shields et al., 1985, Asano et al., 2003). Some cases, however, progress to a retrograde infection of the kidneys, and develop to acute renal failure and adenoviremia, resulting in a poor prognosis (Bruno et al., 2004). Lion et al. reported that mortality of adenoviremia after stem cell transplantation was 91% before the era of cidofovir (Lion et al., 2003).

In recent years, umbilical cord blood has been increasingly utilized as a source of hematopoietic stem cells for transplantation of patients without favorable donors despite its immature immune activity which often leads to a prolonged immunological deficiency and many kinds of early severe infectious complications such as bacteremia, cytomegalovirus disease, tuberculosis and fungal infection (Saavedra et al., 2002, Maeda et al., 2005). The

cumulative incidence of adenovirus-induced hemorrhagic cystitis after cord blood transplantation was, however, found to be relatively low (2.8%) among Japanese adults (Tomonari et al., 2006). It is important that cord blood transplantation is one of the risk factors of symptomatic adenoviremia (Robin et al., 2007), which develops to acute renal failure in the terminal stage (Abe T et al., 2009).

At present, there is no established consensus about the treatment for acute renal failure induced by adenovirus after stem cell transplantation. This chapter focuses on the recent advances in diagnosis, mostly due to the development of molecular methods, and therapeutic interventions. Furthermore, this chapter is intended to promote a consensus about the methods of early diagnosis and the treatment for this disease.

2. Serotypes of adenoviruses inducing cystitis and acute renal failure

The development of more sensitive diagnostic methods enabled us to increase awareness of this virus as a pathogen. At least seven human adenovirus species (A to F), including 52 serotypes, have been described.

Adenoviruses have mechanisms to escape from host immune responses, such as inhibition of interferon functions, inhibition of intrinsic cellular apoptosis in infected cells, and the prevention of major histocompatibility complex class I expression on the cell surface (Mahr et al., 1999) to reduce cytotoxic T-cell attack of the infected cells (Lichtenstein et al., 2004), especially in serotypes 1, 2, and 5 (species C). They persist focally in tonsils for years through low-grade replication. Surprisingly, T lymphocytes in tonsils and adenoids may harbor adenovirus-DNA (Garnett et al., 2007). This suggests that endogenous reactivation may occur during periods of immunosuppression after stem cell transplantation. Persistence of adenovirus species C is higher for younger age groups, in which primary infections predominantly with species C occur. The quantity of adenovirus-DNA decreases in an age-related manner, either from immune elimination or from depletion of latent stores. Persistent or latent infection of other species is still unknown.

Acute renal failure due to adenoviruses is caused by disseminated disease or retrograde infection from hemorrhagic cystitis. High persistence of serotype 2 at a young age is mentioned in lots of case reports regarding disseminated diseases of this serotype after stem cell transplantation. Other prevalent serotypes of disseminated diseases are 31 (species A), 11, 34, 35 (species B), 1 and 5. On the other hand, prevalent serotypes of hemorrhagic cystitis are 7, 11, 34 and 35 (species B) (Echavarría, 2008), especially serotype 11 (Asano et al., 2003). The organ tropisms of AdV serotypes remain unclear.

Multiple serotype infection was found to be more frequent in immunocompromised patients (30%) than in immunocompetent patients (5%) (Gray et al., 2007). Our group also experienced sequential adenovirus infection of serotype 14 hemorrhagic cystitis and serotype 35 disseminated infection after cord blood transplantation (Abe T et al., 2009). Serotype 14 is a group B adenovirus, which is associated with pharyngo-conjunctival fever (Tuck et al., 1957). This infection was infrequently reported as an acute respiratory disease in military recruits (Van der Veen & Kok, 1957, Hierholzer & Pumarola, 1976, Metzgar et al., 2007), and recently caused pediatric lower airway disease in Taiwan during 2001-2002 (Chen et al., 2002). A new variant of this serotype also caused life-threatening pneumonia in the United States (Louie et al., 2008). Serotype 35 has been detected in urine of patients with acquired immunodeficiency syndrome (AIDS) and other immunodeficiencies (de Jong et al., 1983, Shields et al., 1985, Stalder et al., 1977). Furthermore, an outbreak of serotype 35 pneumonia among residents and staff of a psychiatric facility was reported (Sanchez et al., 1997).

3. Pathological findings and methods of diagnosis

3.1 Pathological findings of acute renal failure induced by adenovirus

Hemorrhagic, necrotizing tubulitis with intranuclear inclusion bodies is observed in autopsy of patients with acute renal failure induced by adenoviruses after stem cell transplantation or intensive chemotherapy for acute leukemia.

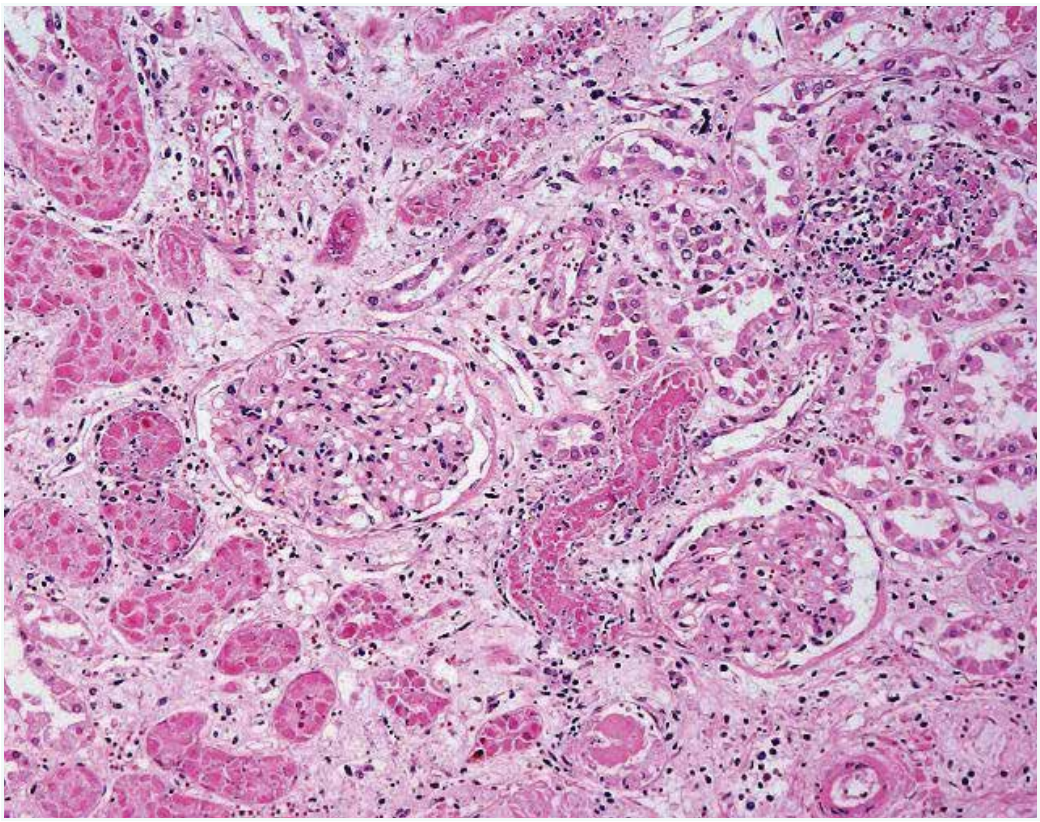

Fig. 1. Necrosis with interstitial hemorrhage of the kidneys of our autopsied patient. (hematoxylin–eosin stain, magnification x20)

Interstitial hemorrhage is specific to adenovirus (Figure 1) and is different from other viral nephritis after stem cell transplantation such as those with BK virus or cytomegalovirus (Colvin, & Nickeleit, 2007). Histopathologically, necrobiotic tubular cells are classified into inclusion-bearing cells of three types: 1) "smudge cells," 2) "Cowdry A" intranuclear inclusion cells including intranuclear eosinophilic amorphous or droplet-like bodies surrounded by a homogeneous clear halo, with marginations of chromatin on the nuclear membrane, and 3) "full-type" intranuclear-containing cells (Cowdry, 1934, Ito et al., 1991)(Figure 2).

Yuzawa et al. showed that these granular deposits contain adenovirus related antigens, immunoglobulins, and C3, which suggests that granular deposits may be formed "in situ" by viral antigens and circulating viral antibodies (Yuzawa et al., 1993).

It is definitive that immunofluorescent examination with anti-adenovirus antibody demonstrates specific fluorescence on the affected tubular cells (Figure 3).

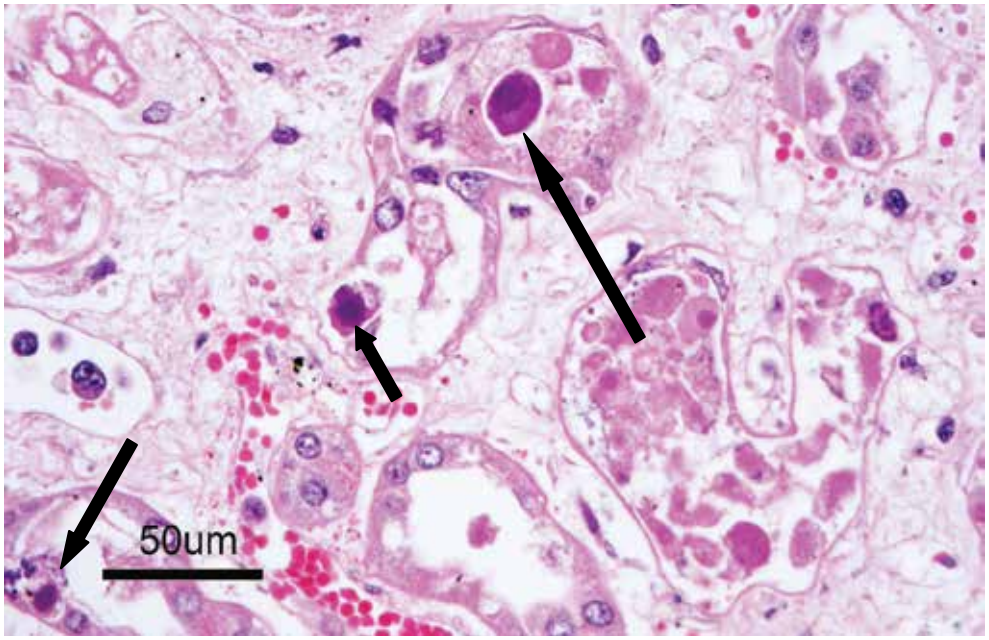

Fig. 2. The three types of inclusion bodies: Cowdry A(long arrow), full-type (intermediate-length arrow) and smudge-type (short arrow) are identified in the affected tubules (hematoxylin–eosin stain, magnification x40).

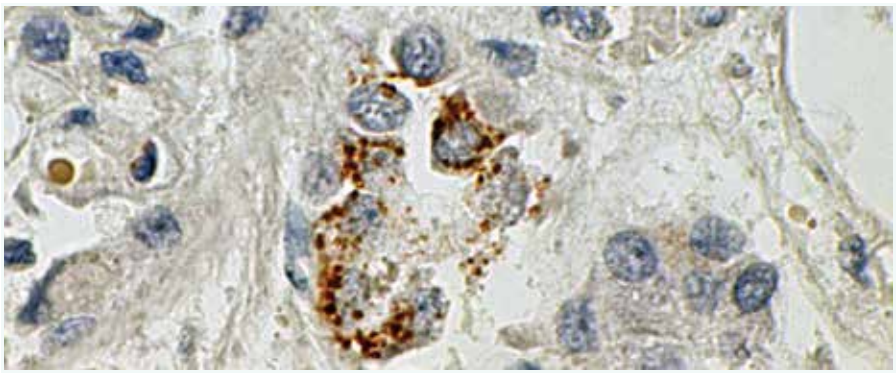

Fig. 3. Adenovirus antigens in affected tubular cells of our autopsied patient are revealed by immunofluorescent examination (magnification x100). Serotype-specific antigen can also be revealed by this method.

Electron microscopy can reveal intranuclear crystalline arrays of viral particles, 75 to 80 nm in diameter (Ito et al., 1991, Mori et al., 2003), but it is not routinely used in clinical laboratories.

3.2 Methods of diagnosis for acute renal failure induced by adenovirus

3.2.1 Clinical manifestations of acute renal failure induced by adenovirus

Adenovirus is usually detected in the recipients of stem cell transplantation within 100 posttransplant days. The mean time is 58 days, ranging from -44 to 333 days (Ljungman et

al., 2003). Adenovirus causes acute renal failure, which follows adenovirus-induced hemorrhagic cystitis or adenoviremia. At first, in hemorrhagic cystitis cases, the risk factors include acute graft versus host disease of more than grade II, male, old age, allogeneic stem cell transplantation and the use of busulfan in a conditioning regimen (Asano et al., 2003, Sencer et al., 1993, Seber et al., 1999, Leung et al., 2002). Common clinical symptoms are gross hematuria and lower abdominal pain. Some cases lead to hydronephrosis and acute post-renal failure due to obstruction of the terminal ureters by edematous change of the ureterovesical junction (Figure 4).

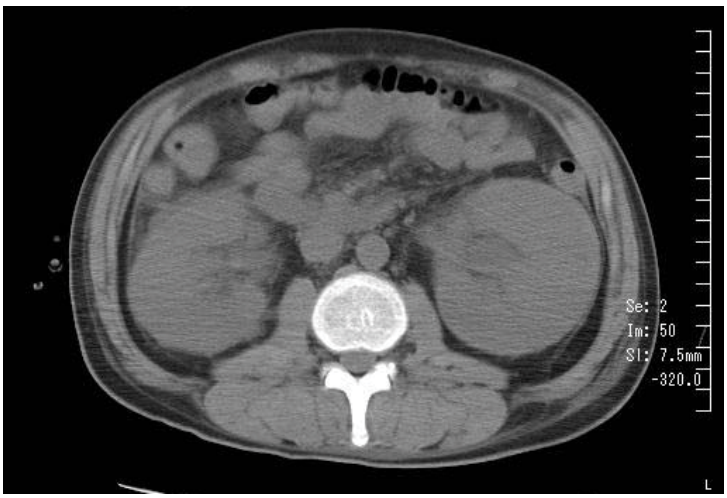

Fig. 4. Hydronephrosis and acute post-renal failure in our patient due to obstruction of the terminal ureters by edematous change of the ureterovesical junction after the onset of adenovirus-induced hemorrhagic cystitis.

Back or flank pain is a critical symptom that indicates a retrograde infection to kidneys, risk factor for which is graft versus host disease. Furthermore, adenovirus-induced nephritis is a high risk factor for disseminated adenovirus infection including symptomatic adenoviremia (Bruno et al., 2004). Clinical presentation of adenoviremia is low to high grade fever refractory to antibiotic or antifungal agents followed by multiple organ failure including acute renal failure which presents oliguria, elevation of serum creatinine and enlarged kidneys with hypo-density in CT scan. Risk factors of disseminated adenovirus infection including symptomatic adenoviremia are graft versus host disease, and, T-cell-depleted graft and cord blood transplantation (Robin et al., 2007, Kampemann et al., 2005). On the other hand, asymptomatic infections with detection of adenovirus from blood or urine have been observed (Lion et al., 2003).

3.2.2 Methods of molecular diagnosis for acute renal failure induced by adenovirus

Adenovirus-DNA has been detected in almost all clinical samples. In adenovirus-induced hemorrhagic cystitis cases before acute renal failure, the most rapid and handy qualitative diagnosis kit is immunochromatography, for example, adenocheck (Santen, Osaka, Japan, or SA Scientific, Texas, U.S.) (Nagafuji et al., 2004). This kit enables us to detect more than $1x10^4$ viral particles /ml in urine at the bedside, although it was developed for the diagnosis of adenovirus conjunctivitis.

Polymerase chain reaction (PCR) has been used as a rapid and very useful method, and is the most prevalent assay for the diagnosis of adenovirus infection. The clinical specificity of the PCR for urine was found to be 96% (Echavarría et al., 1998). To diagnose symptomatic adenovirus infection such as hemorrhagic cystitis, adenoviremia or acute renal failure, at first, DNA is extracted from the patient's urine, blood or renal biopsy sample. When acute renal failure and adenoviremia occur simultaneously, the patient may be diagnosed with adenovirus-induced acute renal failure without renal biopsy. Then nested PCR is performed to amplify the RNA gene, hexon gene or fiber gene of adenovirus with more than one pair of primers in a thermal cycler as shown in Figure 5. (Saito-Inagawa et al., 1996, Shimada et al., 2004, Lu & Erdomann, 2006, Leruez-Ville et al., 2004, Lion et al., 2003).

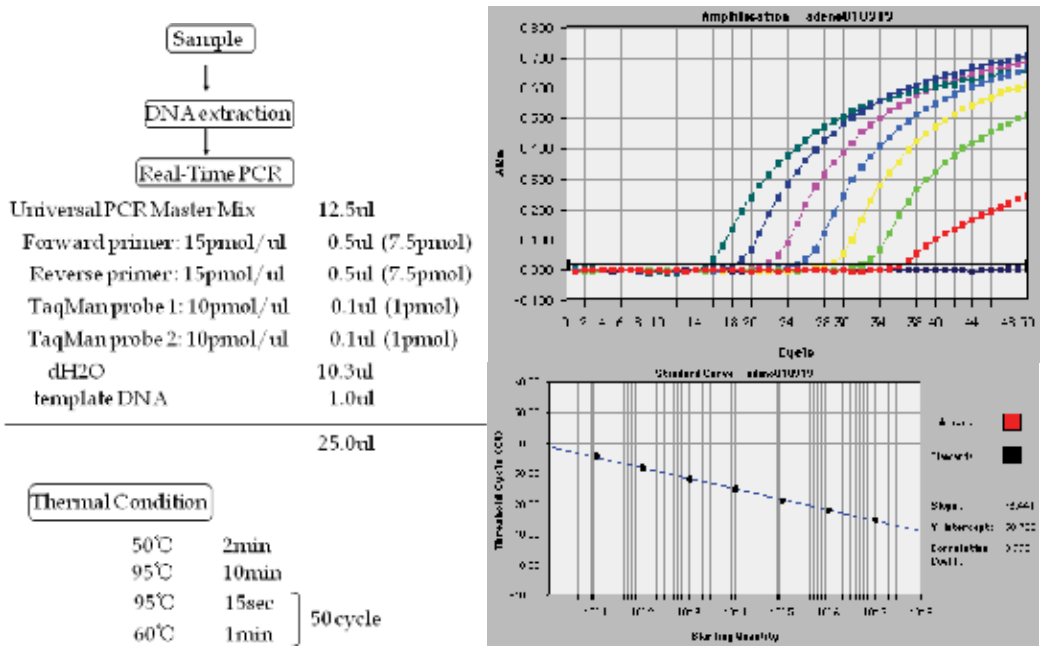

Fig. 5. Quantitative PCR using DNA extracts from the patient's samples is performed with a pair of primers, for example, as shown in the paper of Lion et al., and with a pair of TaqMan probes for quantification, for example, 5'-(FAM)-CCCATGGATGAGCCCACCCT-(TAMRA)-3' and 5'-(FAM)-CCCATGGACGAGCCCACCCT-(TAMRA)-3', in a thermal cycler. The thermal conditions were 50°C for 2 min, 95°C for 10 min, then 50 cycles at 95°C for 15 sec and 60°C for 1 min. Amplification standard was made for each number of control DNA copies from 10^1 to 10^7, which enabled us to quantify the number of viral DNA copies from the patient's samples.

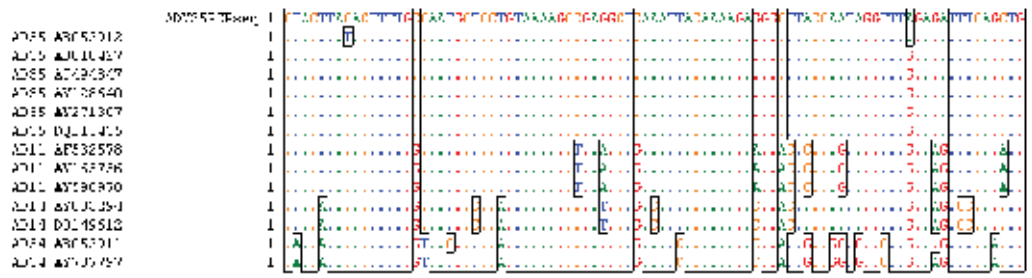

Fig. 6. The nucleotide sequences of amplified fragments were determined with a DNA auto sequencer with fluorescent dideoxy chain terminators. For example, the sequences of the amplified adenovirus-DNA from our autopsied patients (at the first line) were analyzed and compared with the 51 prototype strains in the GenBank database (below the next line), and finally the serotype was identified as 35.

This method is real-time PCR, which can be a qualitative or quantitative assay since amplification and detection of amplified products occur simultaneously. Then, typing is performed. Typing is primarily used for epidemiologic investigations, for studies on pathogenesis such as multiple serotype infections, for unusual or especially severe infections, or for treatment approaches such as high titer γ-globulin. The nucleotide sequences from these fragments were determined by a DNA auto sequencer with fluorescent dideoxy chain terminators. The sequences of the amplified adenovirus-DNA were analyzed and compared with the 51 prototype strains in the GenBank database, and finally the serotypes were identified (Figure 6)(Abe T et al., 2009). This method with sequencing is increasingly performed because it is rapid and now available in many laboratories with molecular equipment, and does not require expensive or difficult-to-obtain antisera. However, there is no clear viral load cut-off value that predicts disease outcome. Thus, it may be preferable to analyze the viral kinetics for each patient, considering the adenoviral load over time rather than the absolute value among symptomatic patients (Leruez-Ville et al., 2004).

PCR is highly sensitive and especially applicable for asymptomatic adenovirus infection surveillance of blood samples, perhaps weekly, and at present, it is commonly applied in stem cell recipients. The virus can be detected in blood 2 to 3 weeks before development of clinical symptoms, which offers the opportunity for intervention (Lion et al., 2003) to avoid acute renal failure and hemodialysis.

4. Treatment to avoid hemodialysis before and during hemorrhagic cystitis

Conventional treatments of hemorrhagic cystitis are hyper-hydration, diuresis and bladder irrigation to wash out viral particles and clots, and to prevent retrograde infection to kidneys and post-renal failure due to urethral obstruction caused by clots. High titer γ-globulin to specific adenovirus serotype is also administrated. In particular, serotype 35 specific antibody was present only at a very low titer in pooled gamma globulin (Flomenberg et al., 1987). Our group experienced sequential different serotype adenovirus infection of serotype 14 hemorrhagic cystitis and serotype 35 disseminated infection after cord blood transplantation, which is too confusing to treat because of the contrast between the improvement of adenovirus-induced hemorrhagic cystitis by serotype 14 high titer γ-globulin and the progression of acute renal failure (Abe T et al., 2009).

At present, Ribavirin and Cidofovir are antiviral agents used in the treatment of adenovirus. More evidence has been obtained for the efficacy of Cidofovir. Recent reports showed that 69-98% of patients with adenovirus disease were successfully treated with Cidofovir (Yusuf et al., 2006, Ljungman, 2003). Nagafuji et al. reported that 71% of patients with adenovirus-induced hemorrhagic cystitis were successfully treated with Cidofovir (Nagafuji et al., 2004). Therefore, Cidofovir is recommended as the first line treatment for adenovirus-induced hemorrhagic cystitis. Cidofovir is a monophosphate nucleotide analog of cytosine that can inhibit viral DNA polymerase and viral replication.

For a preemptive therapy before the onset of adenovirus-induced hemorrhagic cystitis, Ljungman et al. showed tthat, for 13 (81%) of 16 patients, asymptomatic infection was resolved when Cidofovir was given as a preemptive therapy (Ljungman et al., 2003). Lindemans et al. presented a practical guidelines for the treatment of adenovirus infections in recipients of stem cell transplantation by using Cidofovir including a preemptive therapy (Lindemans et al., 2010). The guidelines take three risk factors into consideration: cord blood transplantation, T-cell-depleted graft, and immune-suppression, and divide the patients into low-, intermediate-, and high-risk groups, each with a different treatment approach depending on the viral load measured by weekly or twice weekly peripheral blood quantitative PCR. The presence of these risk factors determines individual susceptibility to the development of adenovirus disease in the case of reactivation. At first, the low-risk group includes cases other than cord blood transplantation or T-cell-depleted graft, or at more than 4 months after stem cell transplantation for any donor source with immune-suppression by a lymphocyte proliferation inhibitor such as calcineurin inhibitor and/or less than0.5 mg/kg predonisolone. Furthermore, low risk group is divided into two groups by CD3 positive T cell count in peripheral blood. When adenovirus is detected in peripheral blood more than 100 copies/ml, absence of T cells (CD3+< 25/μL) or the immunoresponse failure to adenovirus shortly after adenovirus detection (CD3+ T cells < 300/μL within 2 weeks of adenovirus detection) leads to the same treatment of intermediate risk group because it has been associated with a poor outcome. The number of CD3-positive T cells has been shown to be critical in the development of infectious disease in the case of viral reactivation, and in the ultimate clearance of the virus (Chakrabarti et al., 2002, Heemskerk et al., 2005). The intermediate-risk group includes cases of cord blood transplantation or T-cell-depleted graft 1 to 4 months after stem cell transplantation with immune-suppression by more than two lymphocyte proliferation inhibitors (e.g., cyclosporine-A and mycophenolate mofetil) or a lymphocyte proliferation inhibitor and 0.5-1 mg/kg prednisolone. The high-risk group includes cases of cord blood transplantation or T-cell-depleted graft within a month after stem cell transplantation, and immune-suppression by more than one lymphocyte proliferation inhibitor and more than 1 mg/kg prednisolone. Critical viral load in peripheral blood for pre-emptive treatment is more than 10,000 copies in low-, more than 1,000 copies in intermediate-, and more than 100 copies in high-risk groups, respectively. Symptomatic adenovirus diseases are also critical for treatment. The treatment regimen for Cidofovir is 1 mg/kg per day three times weekly until viral load decreases to less than 400 copies/ml for more than two weeks and CD3+ T cells increase to more than 300/μl (Kampmann et al., 2005, Chakrabarti et al., 2002). If possible, immunosuppressive drugs should be tapered (Kampmann et al., 2005, Chakrabarti et al., 2002). Because of the marked nephrotoxicity of Cidofovir, hyperhydration together with oral Probenecid (2 g) given 3 h before Cidofovir administration is essential for nephroprotection (Nagafuji et al., 2004, Anderson et al., 2008, Hoffman et al., 2001). Viral load increases of more than 1 log /week or

patient presentation of adenovirus disease symptoms is indicative of therapy failure and immunotherapy (described later) should be considered.

There is weak evidence for the efficacy of Ribavirin against adenovirus, and its in vitro anti-adenovirus activity differs widely against different serotypes (most active against group C, subtype 1, 2, or 5) (Morfin et al., 2005). There are several case reports suggesting therapeutic benefit for some patients (Homma et al., 2008, Abe S et al., 2003, Lankester et al., 2004). Recently, a strategy combining prophylactic Ribavirin and preemptive Cidofovir treatment was described (Greil et al., 2006). Comparing the outcome with Historical controls in which no prophylaxis and Cidofovir alone was given as a preemptive treatment, the combined strategy with Ribavirin prophylaxis resulted in a significantly lower incidences of adenovirus infection (29% vs 66%) and adenovirus-associated mortality (0% vs 14%).

Although prophylaxis or preemptive therapy shows favorable effects on adenovirus infection, even Cidofovir has only limited efficacy when started as therapeutic treatment for disseminated adenovirus diseases in intermediate- and high-risk groups (Robin et al., 2007, Symeonidis et al., 2007). It is important that early detection of Adenoviremia enables us to initiate therapy before a significant increase in mortality (La Rosa et al., 2001).

Although less efficient than Cidofovir or Ribavirin, Ganciclovir can interfere with the function of adenovirus DNA polymerase, thus inhibiting viral replication in vitro (Lenaerts et al., 2008). In retrospective studies, lower incidences of adenovirus infections appeared to be reported in patients treated with Ganciclovir as cytomegalovirus prophylaxis (Bruno et al., 2003). Reports of a positive outcome in patients with adenovirus infection treated with Ganciclovir are uncommon (Chen et al., 1997). In some countries like Japan, Cidofovir is not available. Ganciclovir may be useful in these countries until Cidofovir becomes available.

Vidarabine is also active in vitro against adenovirus (Kurosaki et al., 2004). There have been a few case reports of successful vidarabine therapy of adenovirus hemorrhagic cystitis. Bordigoni et al. reported results obtained in seven recipients of stem cell transplantation treated with vidarabine, but none survived (Bordigoni et al., 2001).

5. Treatment for acute renal failure induced by adenovirus

Adenovirus causes acute renal failure through hemorrhagic cystitis or as a symptom of disseminated disease. In this phase, treatment options are severely limited. If possible, tapering or termination of immunosuppressive agents is indicated to prompt immune reaction against adenovirus, although the risk of graft versus host disease progression may increase. Of course, oliguria is an indication of hemodialysis. When post-renal failure with hydronephrosis is caused by clots or swelling of the ureterovesical junction in patients with adenovirus-induced hemorrhagic cystitis, percutaneous nephrostomy may be indicated (Mori et al., 2003). Regarding the administration of antiviral agents in severe renal dysfunction, serum elimination half-time of Cidofovir increases so significantly that its nephrotoxicity may force the patients into hemodialysis. Therefore, drug clearance should be carefully considered. Brody et al. showed that mean +/- SD of Cidofovir clearance in patients with renal dysfunction was 0.94 x cleatinine clearance (mL/min/kg) + 0.064 mL/min/kg while its clearance in normal control was 1.7 +/- 0.1 mL/min/kg (Brody et al., 1999). Ribavirin is contraindicated for patients with renal failure whose creatinine clearance is less than 50 ml/min. During the period of hemodialysis, high-flux hemodialysis resulted in the removal of 52% +/- 11% of Cidofovir administered (Brody et al., 1999). Ribavirin, however cannot be removed by hemodialysis (Kramer et al., 1990). In a case report,

reduction of immunosuppression and one dose of Cidofovir (2 mg/kg) were effective for adenovirus-induced acute renal failure on hemodialysis, and showed resolution of viremia and viruria and return of renal function to near baseline without coadministration of Probenecid to ensure adequate drug delivery to the proximal tubular cells (Sujeet et al., 2010). Nevertheless, treatment failure with Cidofovir indicates immunotherapy such as donor lymphocytes or adenovirus-specific cytotoxic T cells in the initial therapy of adenovirus-induced hemorrhagic cystitis or acute renal failure, and in the preemptive therapy according to the guidelines descrived above.

Donor lymphocyte infusion in allogeneic stem cell transplantation is an adoptive T cell transfer protocol based on the hypothesis that donor peripheral blood containing T cells can mediate antiviral activity. Hromas et al. reported a case of a 19-year-old man who underwent a T-cell-depleted allogeneic stem cell transplantation for T-cell lymphoblastic lymphoma. After treatment failure with antiviral drugs for adenovirus-induced hemorrhagic cystitis, he was given donor leukocytes (1×10^6 CD3 cells/kg) and subsequently cleared the virus (Hromas et al., 1994). A number of other case studies followed with similar positive outcomes (Chakrabarti et al., 2002, Howard et al., 1999, Bordigoni et al., 2001, Chakrabarti et al., 2000). Patients were infused with cell doses ranging from 1×10^5 to 3×10^7 CD3 cells/kg. Donor lymphocyte infusion is, however, impossible in patients after cord blood transplantation and often regarded as the last choice of the treatment option because the efficacy of this approach is limited by the low frequency of T cells specific to adenovirus and the relatively high frequency of graft versus host disease caused by alloreactive T cells. Graft versus host disease itself is a risk factor of disseminated adenovirus diseases because the therapy for graft versus host disease requires steroids, which suppress adenovirus-specific T cells. To reduce the risk of donor lymphocyte infusion, inactivation or selective removal ex vivo of alloreactive T cells, or suicide gene transfer has been investigated (Davies et al., 2008, Comoli et al., 2008, Andre-Schmutz et al., 2002, Solomon et al., 2002, Amrolia et al., 2003, Montagna et al., 1999, Ciceri et al., 2009, Tey et al., 2007).

To improve the safety and efficacy of the adoptive transfer approach to donor lymphocyte infusion, adenovirus-specific T cell selection from donor peripheral blood and expansion in vitro have been investigated. Feuchtinger et al. directly identified and isolated donor peripheral blood T cells that secreted IFN-γ in response to stimulation with adenovirus antigen, and expanded the cells with the stimulation of adenovirus lysate in vitro. Then the isolated T cells were transferred into 9 pediatric recipients of allogeneic stem cell transplantation with systemic adenovirus infection despite conventional therapy. The frequency of adenovirus-specific T cells in donor peripheral blood increased from 1.1% +/- 1% to 45.7% +/- 24% after selection. None of the infusions (range, 1,200-50,000 CD3+ cells/kg) was associated with toxicity in vivo, and 5 of 6 evaluable patients showed a significant decrease of adenoviral DNA in peripheral blood and stool with a corresponding increase in the frequency of adenovirus-specific T cells in vivo (Feuchtinger et al., 2006). Leen et al. has achieved similar success in adoptive immunotherapy by using in vitro–expanded adenovirus, EB virus, and cytomegalovirus-specific cytolytic T-cell (CTL) lines (Leen et al., 2006). For the production of the trivirus CTL lines, EB virus-transformed lymphoblastoid cell lines were used as antigen-presenting cells, which also presented EB virus antigens, and adenovirus vectors into which a cytomegalovirus antigen was introduced were transduced with these antigen-presenting cells. Cells were infused as a prophylaxis from day 30 after transplantation in recipients of HLA- haploidentical stem cell

transplantation as well as HLA-matched related, and matched unrelated cases within grade II acute graft versus host disease. The infused doses ranged from 1.7×10^5 to 4.5×10^6 cells / kg, and could control ongoing drug-resistant virus infections. No toxicity or acute graft versus host disease was observed. Adenovirus-specific T cells increased only in patients with recent or concurrent adenovirus infection, although the reactive T cells against the latent EB virus and cytomegalovirus routinely increased independently of detectable viral reactivation. None of the treated patients developed a de novo adenovirus infection for at least 8 weeks. All patients with detectable adenovirus in blood, stool, or tracheal aspirate (7 of 24) had a marked reduction in adenoviral load coincident with the rise in their adenovirus-specific T cells irrespective of infection serotype, including one patient who recovered from progressive adenoviral pneumonia requiring ventilatory support. Although adenovirus-specific CTL may be a safe and effective therapy, there are some serious problems such as high cost, preparation of large blood volume, and a prolonged period for the manufacturing process with special technical skill for 10-12 weeks. Therefore, the indication of this therapy should be limited in cases resistant to antiviral agents, but the preparation of this therapy should be started as early as possible.

6. Future perspectives (conclusion)

In the future, preemptive therapy for adenovirus will be established, and CTL therapies will be more standardized, rapid, cost-effective and available in all institutes. Other methods such as boosting immune recovery, dendritic therapy and new antiviral drugs are now also being developed. These new methods will enable resolution of the severe limitations in the treatment of adenovirus-induced acute renal failure.

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2

Plasma Total Nitric Oxide and Endothelial Constitutive Nitric Oxide Synthase (ecNOS) Inron 4 Gene Polymorphism: A Study in Children with Chronic Kidney Disease

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1. Introduction

Considerable progress has been made in the treatment of end-stage renal disease over the past 20 yr. Nonetheless, the dialysis population continues to carry an excess mortality from fatal cardiovascular events [Baylis et al., 2006]. One possible explanation derives from the association of uremia and accelerated atherosclerosis [Tripathi et al., 2008]. The latter may be related to pre-dialysis factors such as preceding arterial hypertension, glucose intolerance, secondary hyperparathyroidism, dyslipidemia, and others. However, dialysis itself may further contribute to atherosclerosis by oxidative stress, cytokine stimulation, and other events inherent to hemodialysis (HD). Endothelial dysfunction has been shown recently to be a major initiating factor in atherosclerosis [Kone et al., 1997]. Endothelial dysfunction is associated primarily with decreased nitric oxide (NO). However, endothelial dysfunction of uremia has received little attention.

Among the various factors involved in the deterioration of renal function, changes in the hemodynamic are thought to be important. Nitric oxide (NO), an important endothelium derived relaxing factor, is synthesized in the vascular endothelium by the NO synthase. NO is a potent regulator of intrarenal hemodynamics [Baylis et al., 2006 and Tolins et al., 1990]. At the release site it mediates local vasodilatation, antagonizes platelet aggregation, inhibits vascular smooth muscle cell proliferation and also, regulates some vessel-platelet interactions, limits the oxidation of atherogenic low density lipoproteins and has a vasoprotective effect by scavenging superoxide radicals and suppresses leukocyte adhesion to endothelial vessel wall. Multiple lines of evidence have suggested that NO plays a protective role in various important events during atherogenesis. Reduced NO levels are involved in the pathogenesis of the vascular endothelium due to the loss of its vasodilatory effect [Schmidt et al., 1994]. In the kidney, NO dilates renal blood vessels and modulates renin secretion [Kone et al., 1997 and Tripathi et al., 2008]. An impairment of NO production

causes abnormalities in vascular function in many diseases including human arterial hypertension and renal disease [Schmidt et al., 2000 and Tang et al., 2008].
The endothelial constitutive NO synthase (ecNOS), which produces NO from L-arginine, is encoded by a gene located on chromosome 7q35-36, expressed in endothelium [Baylis et al., 2006 and Nadaud et al., 1994]. Recently, several studies have shown that the polymorphisms of ecNOS are related to the haemodynamics. There are two alleles identified in intron 4 of the ecNOS gene. The larger allele, 4b, consists of five tandem 27-bp repeats and the smaller one, 4a, has four repeats [Wang el al., 1999 and Yilmaz et al., 2009].
Several allelic variants of eNOS gene have been identified and their association with human diseases states has been studied. The evidence suggests that NO may inhibit several key steps in the atherosclerosis process and that an alteration in NO production within the vascular endothelium could contribute to pathogenesis of atherosclerosis. Several eNOS gene polymorphisms have been reported as "susceptibility genes" in various cardiovascular (CVD) and pulmonary diseases. GT substitution in exon7 in codon 298, T786 mutation in the 5´ flanking region and high numbers of CVD , which have been repeated in intron 13 of eNOS gene are also known to be associated with an excess of risk of coronary artery disease (CAD) [Asakimori et al., 2001]. Among the reported polymorphisms of the eNOS gene, a significant association of the 4a/b polymorphism in intron 4 of the eNOS gene with CAD and arterial hypertension has also been reported (Yoon et al.2000).
Ichihara et al., in 1998 observed an association between the ecNOS4a allele and myocardial infarction in both a smoking and non-smoking Japanese population. As both coronary lesions and chronic renal failure are basically vascular disorders, we speculate that the gene polymorphism in ecNOS intron 4 might have some relevance to progression in chronic renal failure.
An association of the 4a allele of the ecNOS gene with renal disease was reported [Asakimori et al., 2001, Miyamotoet al., 1998 and Thaha et al., 2008]. Chronic renal failure is basically a vascular disorder and investigating ecNOS gene polymorphism might shed some light on the pathophysiology of renal disease and progression to end-stage renal disease (ESRD).

2. Aim of work

We investigated (a) the relevance of the ecNOS intron 4 polymorphism to the development and progression of chronic renal failure, (b) its relationship with arterial hypertension and cardiovascular complications in CKD pediatric patients undergoing maintenance hemodialysis (MHD) or conservative treatment (CT).

3. Subjects and methods

Seventy eight Egyptian pediatric patients with advanced CKD [stages 4 and 5 based on estimated glomerular filtration rate (eGFR) according to the National Kidney Foundation classification [National Kidney Foundation, 2002], were included in the study (Table 1). They were divided into two groups undergoing CT (n=32) or MHD (n=46). MHD children were selected from the hemodialysis Unit of the Center of Pediatric Nephrology and Transplantation (CPNT), while CT children were selected from the Nephrology Pediatric Clinic, Children's Hospital, Cairo University. The study was done from April 2009 to December 2009.

Stage	Description	GFR (mL/min. per 1.73 m2)	Related terms
1	Kidney damage with normal or ↑ GFR	≥ 90	Albuminuria Proteinuria Haematuria
2	Kidney damage with mild ↓ GFR	60–89	Albuminuria Proteinuria Haematuria
3	Moderate ↓ GFR	30–59	CKD Early renal insufficiency
4	Severe ↓ GFR	15–29	CKD Late renal insufficiency Pre-ESRD
5	Kidney failure	5 <15	Renal failure Uraemia End-stage renal disease

Table 1. Classification of chronic kidney disease (National Kidney Foundation, 2002)

In CT patients the causes of renal failure were renal hypoplasia or dysplasia (n=14), obstructive uropathies (n=8), neurogenic bladder (n=4), not known (n=4), or metabolic (n=2). In MHD, the causes of renal failure were hereditary nephropathies (n=17), obstructive uropathies (n=6), neurogenic bladder (n=2), glomerulopathy (n=2), renal hypoplasia or dysplasia (n=2), and unknown causes (n =17).The inclusion criteria for MHD patients included patients with onset of HD below 16 years with at least 6 months duration on MHD. They were treated with hemodialysis for 3–4 h three times weekly with a polysulfone membrane using bicarbonate-buffered dialysate. The duration of hemodialysis was 2.75 ±1.59 years. Measurement of HD adequacy was as follows: the delivered dose of HD was described as the fractional clearance of urea as a function of its distribution volume (Kt/V) and was determined by using the Kt/V natural logarithm formula according to the equation

$$Kt / V = Ln(R - 0.008\,xt) + (4 - 3.5\,R)\,UF / W \quad (1)$$

Where Ln= natural logarithm, R = post-dialysis BUN/ pre-dialysis BUN. t=dialysis session length in hours, UF= ultrafiltration volumes in liters. W=patients post-dialysis weight in kilogram.

Thirty two MHD patients and 16 CT patients were taking antihypertensive treatment. The following classes of drugs were employed: *a*-adrenoceptor antagonists in two MHD and two CT, *ß*-blockers in nine MHD, ACE inhibitors in seventeen MHD, and six CT patients and Ca channel blockers in twenty-nine MHD and ten CT patients. Subjects were taking their medication when ACE activity was measured and no influence of medication on the measurement.

All control subjects (n=30) were healthy with no clinical signs of vascular or renal disease and no family history of renal disease, as well as lack of medications taken at the time of the

study. Control subjects were selected to be matched for age and gender to the patient groups, as well as within the same BMI limits. They were collected from the pediatric clinic of National Research Centre (NRC), Cairo, Egypt. An informed consent for genetic studies was obtained from parents of all participants. The protocol of the study was read and approved by the Ethics Committee of NRC in Egypt.

3.1 Diagnostic criteria of vascular disease

We studied the prevalence of vascular disease in children with CKD according to the following criteria [Kimura et al. 1999].

Cardiac disease: the presence of primary dilated cardiomyopathy previously diagnosed clinically and by echocardiography.

Cerebral vascular disease: cerebral vascular disease was suspected on clinical grounds, i.e., rapidly developing signs of focal disturbance of cerebral function such as hemiparesis and hemisensory impairment. The diagnosis was confirmed by computed tomography or magnetic resonance imaging. Brain hemorrhage and subarachnoid hemorrhage were excluded.

A patient was considered to have a vascular disease when at least one of these two defined vascular disease was present.

3.2 Biochemical markers

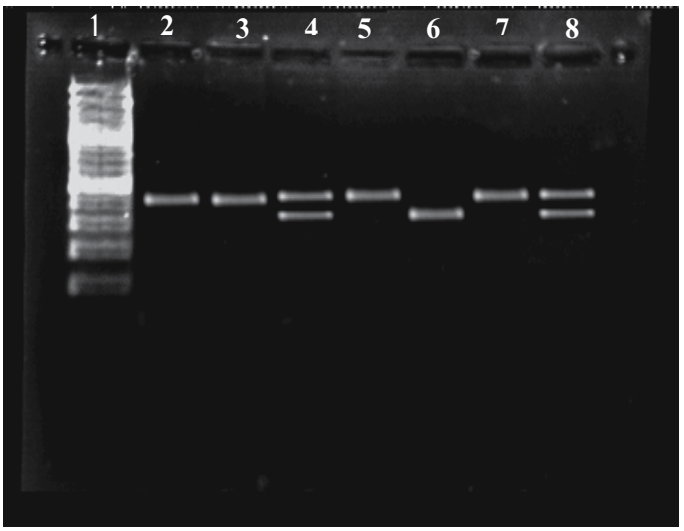
Venous blood samples were collected in the morning after an overnight fast on a midweek dialysis day, before the dialysis session. Three ml of venous blood sample was collected in EDTA vials for the extraction of genomic DNA. Complete blood count and pre- and post-dialysis kidney function test were determined by standard laboratory methods. Estimations of the plasma concentration of total cholesterol (TC), triglyceride (TG) and HDL cholesterol were made by using an Olympus AU400 (Olympus America, Inc., Center Valley, Pa., USA).

3.3 Quantitative determination of nitric oxide concentration in serum

It is done by using the Griss reaction after ultrafiltration via the immunosorbent assay (R&D system. Inc. Minneapolis, MN55413, USA) [Tsikas, 2005] .

3.4 Determination of ecNOS genotype

DNA was extracted from peripheral blood using a QIAamp Blood miniprep extraction Kit (QIAGEN, Germany) and was stored at 4^{o}C until analysis. eNOS genotypes were determined by the polymerase chain reaction (PCR). Briefly, the oligonucleotide primers (the forward primer sequence was 5′-AGGCCCTATGGTAGTGCCTTT -3′ which was located at position 5111-5130 base pairs of the genomic sequence of NOS, and the sequence of the reverse primer was 5′-TCTCTTAGTGCTGT GGTCAC-3′ which its position within the genomic sequence of NOS was 5530-5511 bp) that flank the region of the 27-bp direct repeat in eNOS intron 4 were used for DNA amplification [Salimi et al., 2006]. Each reaction mixture was heated to 94°C for 4 min for denaturation and underwent 35 cycles at 94°C for one min, annealing at 56°C for one min with an extension at 72°C for two min, and a final extension at 74°C for seven min. The PCR products were analysed by 2% agarose gel electrophoresis and fragments were visualized by ethidium bromide staining and ultraviolet transillumination [Figure 1].



Lanes (2,3,5&7) show a single band at 420bp representing the homozygous allele B pattern ($NOS^{B/B}$).
Lanes (4&8) represent the heterozygous pattern for allele A and allele B ($NOS^{A/B}$).
Lane (6) shows one band at 393bp representing the homozygous allele A pattern ($NOS^{A/A}$).
Lane 1 denotes molecular weight ladder marker 100-1000bp (Sigma, St. Louis, USA).

Fig. 1. Genotyping of NOS polymorphism photographed on a 2% agarose gel electrophoresis resolving the wild type allel B at 420bp and the polymorphic allele A at 393bp.

3.5 Statistical analysis

Statistical package for social science (SPSS) program version 11.0 was used for analysis of data. Data were summarized as mean ± SD, range or percentage. Histograms and normality plots were used for evaluating the normality of data. For those data with abnormal distribution, log transformation was performed before a t-test. The number of subjects required for each subgroup was determined by survey sampling basics: Analytical Plan, Population Variability & Confidence Intervals. Data were valuated between the experimental groups by One-Way Analysis of Variance (ANOVA) followed by Tukey's multiple comparison test. Allele and genotypic frequencies for ecNOS alleles were calculated with the gene counting method. Hardy–Weinberg equilibrium was tested with the X^2 test. Comparison of the categorical data i.e. different ecNOS genotypes among patients was done by independent-samples *t* test where appropriate after evaluating the normality of data. Fisher's exact test and X^2 test were done to confirm the results. . Multiple regression analysis was performed to assess the influence of ecNOS alleles on hypertension and vascular diseases. A p value of < 0.05 was considered significant.

4. Results

Clinical and biochemical characteristics of the studied groups were summarized in Table 2. There were no significant differences between groups with respect to age, gender ratio and total serum cholesterol level. Serum NO level was significantly higher in both MHD

and CT groups than the controls and the level was significantly higher in MHD group than CT group (143.93±35.99 µmol/L, 91.48±28.80µmol/L, 51.84±12.39 µmol/L, respectively).

The distribution of genotypes and allele frequencies were compared between patients and controls (Table 3). The genotype frequencies were in agreement with Hardy–Weinberg equilibrium. The Hardy-Weinberg equation is in the following form

$$p^2 + 2pq + q^2 = 1 \quad (2)$$

Where p is the frequency of the **A** allele in the population and q is the frequency of the **a** allele in the population.

The frequencies of genotypes in the population are given by

- p^2 for genotype **AA**,
- 2pq for genotype **Aa**,
- q^2 for genotype **aa**.

Dialyzed and CT patients had significantly higher frequency of the aa genotype and ecNOS4a allele ($P<0.05$) compared with control subjects. The multiple logistic regression analysis with correction for age and sex revealed that the frequency of the ecNOS4a allele carriers was significantly higher in dialysed and CT patients than in healthy subjects. There was no significant difference between MHD and CT groups as regard to aa genotype ($p>0.05$).

Comparing clinical and biochemical characteristics of carriers of the ecNOS 4a allele (aa+ab genotypes) and non carriers (bb genotype) were shown in Table 4. Hypertensive % was found to be higher in carriers of the ecNOS 4a allele than in non carriers - bb genotype - (85.95%*vs* 60.67%, p=0.04). Also, cardiovascular % was significantly higher in carriers of the ecNOS 4a allele than in non carriers - bb genotype-, (35.90%*vs* 5.13%, p=0.01). There were significant differences between the two subgroups as regard to triglycerides and HDL cholesterol (190.00±57.15 mg/dL vs 116.25±48.49 mg/dL, p=0.02 and 21.22±7.05 mg/dlL vs 32.00±15.96 mg/dL, p=0.03, respectively). Serum NO level was found to be lower in carriers of the ecNOS 4a allele than in non carriers (100.29±27.32 (µmol/L) vs 152.73±60.39 (µmol/L), p=0.04).

To examine the survival bias in evaluating the effect of the ecNOS4 polymorphism on renal disease, we analyzed allele and genotype frequencies in MHD subjects shorter than 3 years on dialysis and those dialyzed 3 years or longer (data not shown). There were no statistically significant differences between these two groups (p=NS), suggesting little or no influence of survival bias on the outcome of the study.

We performed a multiple linear regression analysis with backward stepwise selection using all the clinical risk factors for hypertension and the mutant allele of the eNOS 4 a gene. This analysis revealed that the most predictive independent risk factors for hypertension were the mutant allele (ß=0.50, p=0.03), NO level (ß =-0.47, p=0.03) and TG (ß=0.60, p=0.02) in Table 5.

A multiple linear regression analysis using a model identical to the one used to test hypertension was used to test vascular disease for the relationship with the mutant allele of the eNOS 4 a gene. This analysis suggested that the allele has a dominant effect on the risk of vascular complications in these children (ß =0.99, p=0.04) in Table 6.

	CT (n=32)	MHD (n=46)	Controls (n=30)
Age	9.14±7.59	10.62±3.49	8.7±4.51
Gender (M/F)	15 (46.88%)/17(53.12%)	25(54.35%)/21(45.65%)	20(66.67%)/10(33.33%)
SBP (mmHg)	98.66±6.66	127.13±18.37 b*	94.55±9.80
DBP (mmHg)	64.66±6.67	85.15±13.76 b*	60.59±10.11
Creatinine (mgdL)	3.93±3.75 a*	6.32±1.55 b*	0.77±0.34
Predialysis urea (mg/dL)	51.12±10.45 a*	71.56±20.61 b*	7.80±2.64
Dialysis, Yrs		2.75±1.59	
Kt/V		1.69±0.42	
Total cholesterol (mg/dL)	164.44±50.10	193.04±51.37 b*	160.31±18.74
Triglycerides (mg/dL)	160.78±57.33 a**	147.00±66.98 b**	65.31±18.35
HDL cholesterol (mg/dL)	21.35±1.17a*	27.34±9.88 b*	39.55±7.94
Serum NO level (μmol/L)	91.48±28.80a*	143.93±35.99b**c	51.84±12.39

Data was evaluated by ANOVA test. Values are presented as means ±SD or percentage as applicable. CT=conservative treatment, MHD=maintenance hemodialysis, SBP=systolic blood pressure, DBP=diastolic blood pressure, Kt/V=adequacy of hemodialysis, a*P<0.05 or a**P<0.01 vs controls and CT b*P<0.05 or b**P<0.01 vs control and MHD, cP<0.01 vs CT and MHD.

Table 2. Clinical and biochemical data of the studied groups

Gene		CT (n=32)	MHD (n=46)	Controls (n=30)
Alleles	b	43(67.19%)	61(66.30%)	47(78.33%)
	a	21(32.81%)	31(33.70%) b	13(22.67%)
Genotypes	bb	16(50%)	23(50.00 %)	17(56.67%)
	ab	11(34.38%)	15(32.61%)	13(43.33%)
	aa	5(15.62%) a	8(17.39%) b	0(0.00%)

Data was evaluated by the gene counting method. Values are presented as percentage. CT=conservative treatment, MHD=maintenance hemodialysis.
ap<0.05 vs control and CT
bp<0.05 vs control and MHD

Table 3. Frequencies of ecNOS intron 4 genotypes in patients and controls

	aa + ab (n=39)	bb (n=39)	P value
Age	11.66±4.77	9.33±3.25	NS
SBP(mmHg)	120.25±22.47	114.00±15.95	NS
DBP(mmHg)	79.37±13.40	77.47±13.80	NS
Hypertensive%	85.95%	60.67%	0.04*
Cardiovascular disease%	14(35.90%)	2(5.13%)	0.01*
Total cholesterol(mg/dl)	183.00±38.96	180.00±68.67	NS
Triglycerides(mg/dl)	190.00±57.15	116.25±48.49	0.02*
HDL-cholesterol(mg/dl)	21.22±7.05	32.00±15.96	0.03*
Creatinine(mg/dl)	5.99±2.06	5.44±2.15	NS
Predialysis urea(mg/dl)	69.88±27.84	67.00±23.01	NS
Serum NO level (µmol/l)	100.29±27.32	152.73±60.39	0.04*

Significance was estimated using independent t-test. Data are means ± SD. SBP=systolic blood pressure, DBP=diastolic blood pressure, NO=nitric oxide, p is significant if <0.05, NS=non significant.

Table 4. Clinical characteristics of CKD patients with different ecNOS intron 4 genotypes

	ß	P value
Age	0.06	NS
Triglycerides(mg/dl)	0.60	0.02*
Serum NO level (µmol/l)	0.47	0.03*
Mutant allele (a allele)	0.50	0.03*

NO= nitric oxide, P<0.05 was considered significant

Table 5. Risk factors affecting hypertension in CKD patients based on multiple linear regression analysis

	ß	P value
Age	0.07	NS
Hypertension%	0. 60	0.02*
SBP	2.19	0.03*
DBP	2.60	0.02*
Serum NO level (µmol/l)	0.41	0.03*
Mutant allele (a allele)	0.99	0.04*

SBP=systolic blood pressure, DBP=diastolic blood pressure, NO= nitric oxide, p<0.05 was considered significant.

Table 6. Risk factors affecting cardiovascular disease in CKD patients based on multiple linear regression analysis.

5. Discussion

Chronic renal failure is a multifactorial disease with different prevalence and clinical phenotype in different populations. This study is an attempt to describe the functional state of the NO system in patients on chronic HD, using carefully matched control subjects.

More than 99% of human DNA sequences are the same across the population, however, variations in DNA sequence can have a major impact on how humans respond to environmental insults such as toxins. The single nucleotide polymorphisms (SNPs) or snips are DNA sequence variations that occur when a single nucleotide in the genome sequence is altered. A point mutation is considered as SNP if it occurs in at least in 1% of the population. The SNPs are responsible for about 90% of all human genetic variation and occur every 100–300 bases along the 3-billionbases of human genome both in coding and non-coding regions. Two-third of known SNPs involves the replacement of cytosine with thymine. The SNPs that have no effect on cell function could predispose people to disease or influence their response to a toxicant [Agarwal et al., 2003].

Gene and environment play a critical role in many genetic disorders of multi-factorial origin. Epidemiological studies have shown statistically significant association of several factors among which genetic susceptibility is the common factor as indicated by familial aggregation and greater concordance in monozygotic twins with the onset of the diseases such as obesity, cancer, hypertension, and diabetes [Agarwal et al.,2003]. The PCR- and sequencing-based techniques have been used to assess the presence of SNPs in a particular gene. Genome–environment interaction among individuals for the susceptibility to a disease greatly varies and Fosmid library allele-specific haplotypes analysis (FLASH) and large insert genome analysis (LIGA) has improved the understanding of genetic variations in various populations.[Kaul, 2003] FLASH allows to isolate and sequence fosmid clones that tile a given loci in a haplospecific manner over along genomic region of interest and LIGAN allows the use of end sequence and fingerprint data to identify the variant genomic regions from multiple individuals.[Kaul, 2003] FLASH and LIGAN have overcome the limitations of PCR-based re-sequencing methods to screen thousands of individuals [Agarwal et al., 2003] .FLASH does not provide phase information for the underlying haplotypes structure of a diploid genome and LIGAN provides a limited resolution at the chromosome level.[Kaul, 2003]. Telomeric repeat amplification protocol (TRAP), a sensitive, high throughput PCR-based assay provides a reliable tool for the experiments that requires massive quantitation of telomerase activity, however, a pyraseme diated allele-specific extension reaction (AMASE) is a novel tool used for microarray-based mutation detection and allows the simultaneous, efficient, and accurate analysis of several samples from different stages of skin malignancies [Kaller et al., 2004].

In this study the frequency of a allele in the ecNOS intron 4 was significantly higher in both dialyzed and CT patients compared with in healthy controls indicating that the ecNOS 4a allele is a risk factor for ESRD in children with CKD. Such an association was observed earlier by others. Asakimori et al, in 2001, found a significantly higher frequency of the ecNOS 4a allele in haemodialysis patients, both non-diabetic and diabetic, and therefore suggested that the polymorphism in intron 4 of the ecNOS gene may have influence on the progression of renal disease. Wang et al, in 1999, in their study of 302 subjects with end-stage renal disease and 248 healthy controls found a significantly higher frequency of the ecNOS 4a allele in patients with ESRD caused by non-diabetic primary renal diseases. Freedman et al.2000 evaluated the role of four NOS gene polymorphisms in ESRD patients

and found that the a allele of the ecNOS 4 polymorphism in the NOS gene was associated with all-cause ESRD in probands and their siblings compared with healthy subjects.
One study showed that the basal concentration of NO metabolites (nitrate plus nitrite) in the plasma was reduced in individuals with essential hypertension [Asakimori et al., 2001 and Yilmaz et al., 2009]. Tsukada et al.,1998 reported a strong association between the a allele of the ecNOS gene and the plasma NOx (nitrate and nitrite) levels.
In our study, there was a significant elevation of serum NO levels in patients with CKD either on MHD or on CT than the controls and the level was significantly higher in MHD group than CT group. Nitric oxide, an extensively studied endothelium relaxing factor, is reported to be a very potent regulator of intrarenal hemodynamics [Kerkeni et al., 2009 and Möllsten et al., 2009] . It plays a major role in the regulation of cardiovascular homeostasis both in health and disease. Many studies have published the relation between NO and renal failure. An impaired response to NO may contribute to the initiation or maintenance of the increased intra-glomerular high pressure state. The impaired response to NO appeared more in MHD patients as they are to exposed to increased oxidative stress and this may explain the higher level in this group [Nguyen-Khoa et al., 2001].
In the present study, serum NO level was found to be lower in carriers of the ecNOS 4a allele than in non carriers. Gururajan et al., (2010) reported a strong association between the a allele of the ecNOS gene and the plasma NOx (nitrite and nitrate) levels. The mean plasma level of NOx of the subjects who were homozygous for a allele was nearly 20% lower than in the subjects with the b allele. Although it is disputable whether the NO metabolites in the blood are derived entirely from ecNOS in the endothelial cells of the blood vessels, it is in fact that plasma NO levels are different depending on ecNOS gene polymorphism. They concluded therefore that the ecNOS gene locus might be responsible for variations in the genetic control of plasma NOx. Failure of vascular endothelium to elicit NO-mediated vasodilation may be due to decreased formation, increased degradation, decreased sensitivity to nitric oxide formed, or a mixture of these factors. Decreased levels of nitric oxide may also be due to the increased activity of the enzyme myeloperoxidase, which consumes nitric oxide as a substrate and also promotes endothelial dysfunction.
The molecular mechanism by which ecNOS gene polymorphism acts to affect the occurrence of ESRD is not known, and it is also unclear whether this polymorphism is a causative variant or a marker of another functional variant. However, the fact that the distribution of a allele in the ecNOS intron 4 showing a significantly higher incidence in children with CKD and plasma NO metabolite levels are reported to be different in depending on ecNOS gene polymorphism, suggests that the ecNOS intron 4 is a useful marker for studying the relationship between NO and the progression of renal disease.
The subjects of the MHD group have been dialyzed for relatively long periods. Therefore we examined the survival bias. The frequencies of ecNOS intron 4gene polymorphism in the long dialysis period group did not differ from those in the short period group. It appears therefore, that neither death nor survival is factors in estimating the role of gene polymorphism in disease progression.
In this study, hypertensive % was found to be higher in carriers of the ecNOS 4a allele than in non carriers (bb genotype) and on correlating the diastolic blood pressure (DBP) to the a allele and other individual variables by multiple linear regression analysis, the a allele, NO level and TG concentration were variables that were independently associated with DBP ($p<0.05$). In a study using mice with disrupted eNOS gene revealed that eNOS function is

required for vascular and hemodynamic responses to acetylcholine and that the disruption of the eNOS gene leads to hypertension [Huang et al., 1995 and Tripathi et al. 2008]. Clinical and experimental studies suggest that an alteration in nitric oxide (NO) metabolism may be a contributing factor in the pathogenesis of hypertension. Thus, abnormalities in the activity of the enzyme endothelial NO synthase (eNOS) that synthesizes NO in endothelial cells may lead to NO abnormality with severe consequences.[Möllsten et al., 2009]. Inhibition of eNOS elevates blood pressure in healthy humans [Haynes et al., 1993]. Furthermore, NO production is diminished in patients with essential hypertension, under basal conditions [Forte et al., 1997 and Möllsten et al., 2009].

Our study revealed that the cardiovascular disease % was significantly higher in carriers of the ecNOS 4a allele than in non carriers (bb genotype) and that the allele has a dominant effect on the risk of vascular complications in children with CKD by multiple linear regression analysis. Apart from controlling the coronary blood flow, there is now an emerging consensus that NO generally acts to fine-tune and optimize cardiac pump function [Cotton et al., 2002]. Excessive NO depresses systolic function by decreasing myocardial contractility and shortening the ejection period [Cotton et al., 2002]. Elevated circulating levels of oxidative products of (NOx) and myocardial NO synthetase expression have been seen in patients with heart failure due to contractile dysfunction [Balat et al., 2003 and Kerkeni et al., 2009]. We had a previous study on the relation between plasma NO level and left ventricular diastolic function and its etiology in heart failure patients in the pediatric age group. We performed echocardiographic Doppler studies in 47 patients with congestive heart failure. Left ventricular diastolic dysfunction was classified as either a restrictive (RFP) or non restrictive filling pattern (non-RFP). Plasma NOx level was significantly higher in the studied patients than the control group. Plasma NOx levels are elevated in patients with isolated diastolic heart failure, in addition, in patients with LV systolic failure, the severity of LV diastolic dysfunction determines the amount of NO production [Elshamaa et al., 2006]. Many studies reported a correlation of this polymorphism with vascular diseases [Mitsuke et al., 2001, Thibaud et al., 2004 and Wasson et al., 2004].

The intron 4ab insertion/deletion genotype was associated with isolated lacunar infraction. Protective effect of the 4a variant could be mediated through changes in eNOS promoter activity and increased NO levels has been suggested through Haplotype and functional studies [Hoffmann et al., 2000]. Another study by Markus et al., 1998 of a Turkish population has shown that carriers of the minor 'a' allele of intron 4 VNTR had significantly elevated risk for stroke (a). An over representation of 4c allele of intron 4 VNTR in ischemic stroke patients was suggested in a study involving African American population. In pooled analysis of all patients, intron 4c, but not intron 4a, intron 4b, or 894G/T alleles are associated with stroke. In subgroup analysis by race, the intron 4c allele is most strongly associated with large artery ischemic stroke in African Americans [Yemişçi et al., 2009]. The association of SNP in exon 7 with stroke was seen only in the study of Hoffmann et al., 2000, while other studies indicated negative finding in white population. Further more Hingorani et al. (1999) also measured carotid stiffness in stroke patients and found it was unrelated to the exon 7 variant.

The limitation of this study includes the small sample size as we collected cases from one centre. Thus, a large number of patients and controls need to be examined to confirm the association of this polymorphism in the Egyptian pediatric population.

6. Conclusion

A allele of the ecNOS intron 4 gene polymorphism showed a significantly higher frequency in children with CKD, both on MHD and CT. These results suggested that the ecNOS gene polymorphism could serve as a useful genetic marker for evaluation of susceptibility to chronic renal failure. Plasma NO levels showed a significant decrease in aa alleles patients and also could independently predict the risk. However, the interactions between this genetic predisposition and environmental factors as well haplotype analysis require further studies. A large number of patients and controls need to be examined to confirm the association of this polymorphism in the Egyptian pediatric population.

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3

Renal Cell Carcinoma in Dialysis Patients with End Stage Renal Disease: Focus on Surgery and Pathology

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1. Introduction

In 1977, Dunnill et al. from Oxford at first reported that 14 of 30 dialysis patients with end stage renal disease (ESRD) examined at autopsy had acquired cystic disease of the kidney (ACDK) and that six of these 14 patients had renal cell carcinoma (RCC), including one with distant RCC metastatsis.[1] It is now well established that patients with ESRD are more prone to RCCs with an incidence of approximately 3 to 5 %.[2-5] These studies may misrepresent the true incidence RCC because they primarily relay upon screening radiology, particularly ultrasonography (US), for detection. Better estimate was provided by a single-center study in which most renal transplant patients undergo ipsilateral native nephrectomy at surgery. Based upon strict pathologic criteria reported by Denton et al., prevalence of ACDK, renal adenoma and RCC and oncocytoma were found in 33%, 14%, 4.2% and 0.6% of 260 patients[6], which may be lower than the true incidence given that only one kidney was removed.

Chen et al. found higher incidence of RCCs vs. the general population, with a standardized incidence ratio of RCC in dialysis patients of 24.1 ($p < .01$)[7]. Ishikawa et al. were able to demonstrate that time spent on haemodialysis was the most important risk factor for ACDK and also for the development of RCC. This important observation did highlight the key features of ACDK developing on haemodialysis and apparently increasing the likelihood of RCC[2,8,9]. Hughson's work suggested that during this time there was a progression of cystic lesions from simple to complex or hyperplastic cysts and on to renal tumor formation[10].

A recent nation-wide survey in Japan revealed a 15-fold increase in the number of dialysis patients with RCC in the last 2 decades[8]. Reasons for this rapid increase can be postulated as follows: the increasing number of dialysis patients: the increasing duration of dialysis in these patients: and the prevalence of tumor screening in dialysis patients by imaging studies. The prevalence of ACDK in the haemodialysis population in Japan appears to be higher than that in the USA or Europe and patient survival on dialysis in Japan is significantly longer. These are probably because of different patterns of primary renal disease and reduced cardiovascular comorbidity compared with Western populations[11]. The presence of RCCs also appears to vary within different populations. Kojima's study of 2624

dialysis patients found that 81.8% of their patients had ACDK on a median dialysis time of 11 years with 44 patients (1.68%) of RCCs. This compares with studies from the USA suggesting approximately one-fifth of the 30% or so patients with ACDK will have RCC, therefore close to 6% of the overall dialysis population[5]. This 3- to 4-fold difference in the risk of RCC in ACDK between Japan and the USA may be real or may be a consequence of differences in study populations or of methods used to screen patients.

Dialysis patients with RCC are one of the representative groups of patients with considerable surgical risks. According to the American Society of Anesthesiologists (ASA) classification (http://www.asahq.org/clinical/physicalstatus.htm)[12], surgical risk of patients with chronic renal failure (CRF) is assigned to at least physical status (PS) 3. Dialysis patients often have multiple complications such as respiratory or cardiac problems in addition to CRF. Since the number of RCCs in dialysis patients associated with high surgical risk is also expected to increase, a safe and less invasive radical nephrectomy is warranted.

Laparoscopic radical nephrectomy (LRN) has been used for the management of renal mass in ESRD patients and showed acceptable surgical outcomes[13-16]. Since 1998, we have developed gasless single-port retroperitoneal RN for RCC designated minimum incision endoscopic surgery, MIES[17-19]. This operation is completed via a single port that narrowly permits extraction of the kidney with perinephric fat, without CO_2 gas insufflation, and without injury to the peritoneum[17-19]. This operation was certified as advanced surgery by the Japanese government in 2006, and was covered by the Japanese universal insurance system from April, 2008[18,19]. MIES RN has been shown to be a suitable treatment modality for an expanding spectrum of high risk patients such as RCC in ACDK patients. We already reported the experience of this operation for initial 8 ESRD patients[20] and 7 bilateral cases in ESRD patients[21].

In this context, we will review the recent data about renal neoplasm in ESRD patients including our published papers and recent experiences.

2. Minimum incision endoscopic surgery (MIES) for dialysis patients

2.1 Patients and sources

We reviewed our single-center consecutive cases undergoing MIES RN for RCC in ESRD patients between 1998 and 2009. This database contains information regarding a large number of variables relating to patient characteristics and their surgical procedures. Evaluable parameters included age, gender, side of surgery, duration of dialysis, symptoms, radiological findings, body mass index (BMI) calculated by height and weight, physical status according to the American Society of Anesthesiologists (ASA) scoring system[12]. Time to feeding and walking after the surgery were also recorded.

2.2 Surgical treatment

The surgical technique of MIES RN, was previously demonstrated[17-19]. Briefly, the minimum incision, around 4 to 6 cm depending on the size of the specimen that narrowly permits extraction of the kidney covered with perinephric fat is made obliquely forward following the line of the 12th rib (fig.1). Muscles are separated without cutting and a small space is made under the laterocoronal fascia to allow positioning of a Wound Retractor. After a single port was made, the working space is created by dissecting along the anatomical planes extraperitoneally with long retractors and spatulas under the guidance of both

endoscope and direct vision. The peritoneum is kept intact during the operation. When isolated, the kidney with perinphric fat is put into the pouch (flexible catcher) in the surgical field to prevent rupture of the specimen and extracted through the incision. All procedures are carried out without trocar ports, without gas insufflation and without the insertion of the hands of operators into the operative field. For reducing the bacterial contamination, operative field and subcutaneous space were rinsed with approximately 2000ml and 100ml sterile saline, respectively, before skin closure. Skin is closed by subcuticular suture using polydioxanone, followed by Dermabond. There were no additional dressing or treatment applied postoperatively. Only two inexpensive devices, the wound retractor and the specimen catcher, are disposable in this operation which results in low equipment cost.
The most dialysis patients subject haemodialysis the day before surgery, as well as one day postoperatively, to maintain their routine dialysis schedule. Serum electrolytes were closely monitored, both preoperatively and postoperatively. Patients were discharged home when they met standard discharge criteria and were seen at 1 month, 6 months, 12 months, and at appropriate intervals (relating to their cancer diagnosis and other urological issues) thereafter, in the out-patients clinic for follow-up.

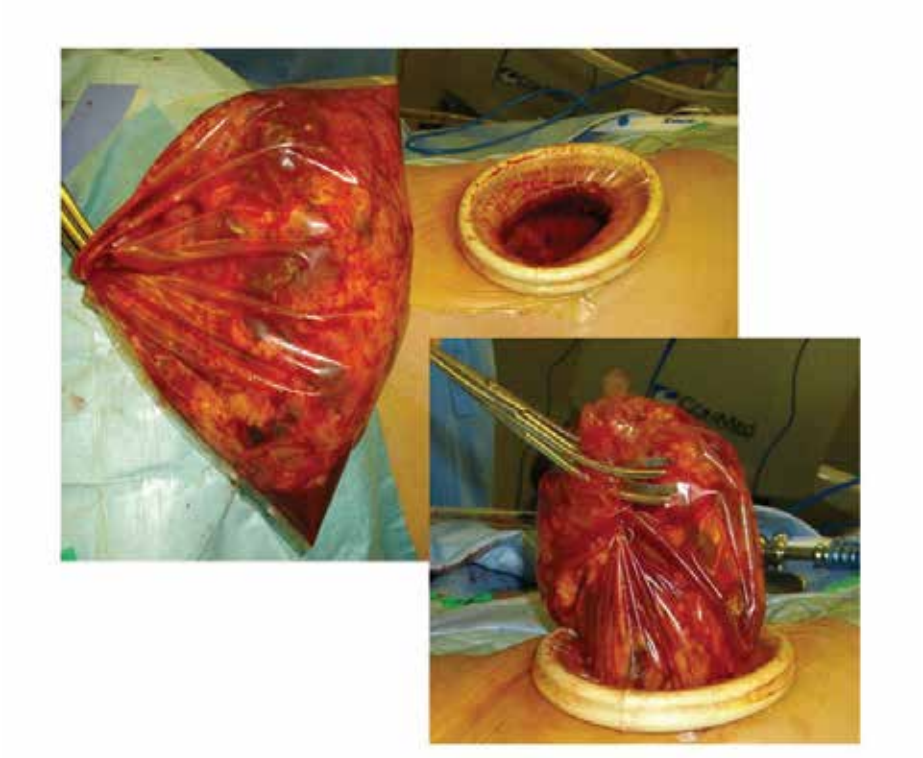

Fig. 1. Resected specimen of ACDK-RCC covered by flexible catcher and 5cm single-port.

2.3 Outcomes of MIES- RN for dialysis patients

We conducted 57 MIES RN for 50 ESRD patients including 7 bilateral RCC cases. For bilateral RCC cases, we have performed sequential operation. When bilateral RCCs are suspected concomitantly, we performed the first RN on the kidney that harbors the larger tumor. After confirming the diagnosis pathologically, the second RN is performed sequentially[21].

MIES RN was successfully completed in all 57 cases with a mean (SD; range) age at RN of 58.6 (10.2; 35-80) years. The mean (SD) operative time was 170 (47) minute with mean (SD) estimated blood loss of 218 (231) cc. The mean (SD; range) length of the extraction incision was 6.1(1.2; 4-9) cm and all kidney specimens with mean (SD) weight of 358 (303) gram were removed intact via single-port. 1 patient required blood transfusion (1.8%). Other complications were not found during operation. The average (SD; range) days to oral feeding and walking were postoperative 1.4 (0.5; 1-3) and 1.3 (0.6; 1-3) days, respectively. Circulatory and respiratory problems did not occur after operation.

Several studies have been performed involving patients with ESRD undergoing laparoscopic RN for RCCs (table.1).

Authors	yr	n	operation	Trans-peritoneal (Renal units)	Retro-peritoneal (Renal units)	Mean EBL (ml)	Mean operative time (min)	Others
Current Study		57	MIES (gasless single-port)[17-19,22]	0	57	218	170	
Bird et al[16]	2009	16	laparoscopic	16	0	153	–	
Reza Ghasemian et al[15]	2005	10	laparoscopic	20 (bilateral)	0	164	390 (bilateral)	
Gulati et al[14]	2003	5	laparoscopic	4	2	120	294	Open conversion in one case
Iwamura et al[13]	2001	6	laparoscopic	0	6	58	162	

Table 1. Summary of minimally invasive radical nephrectomy for RCC patients with ESRD

3. Pathological findings of RCC in dialysis patients

3.1 Characteristics of dialysis patients

We evaluated 57 cases consisting of 43 cases in 38 men and 14 cases in 12 women. Seven bilateral RCC (five men and two women) cases were found. The median (range) duration of dialysis was 12 years (0-27). ACDK was found in 35 patients (70%). On the basis of the duration of dialysis, all cases were classified into four groups: group A, 0-3 years (n=11); group B, 4-10 years (n=15); group C, 11-20 years (n=21) and group D, 21-30 years (n=10).The effects of duration of dialysis on the ACDK state in fig. 2. With increasing duration of dialysis, % proportion of ACDK kidney increased ($p< 0.0001$). Also, univariate and multivariate analysis incorporating into age, gender and duration of dialysis indicated that longer duration of dialysis was an independent predictor of development of ACDK ($p< 0.0001$).

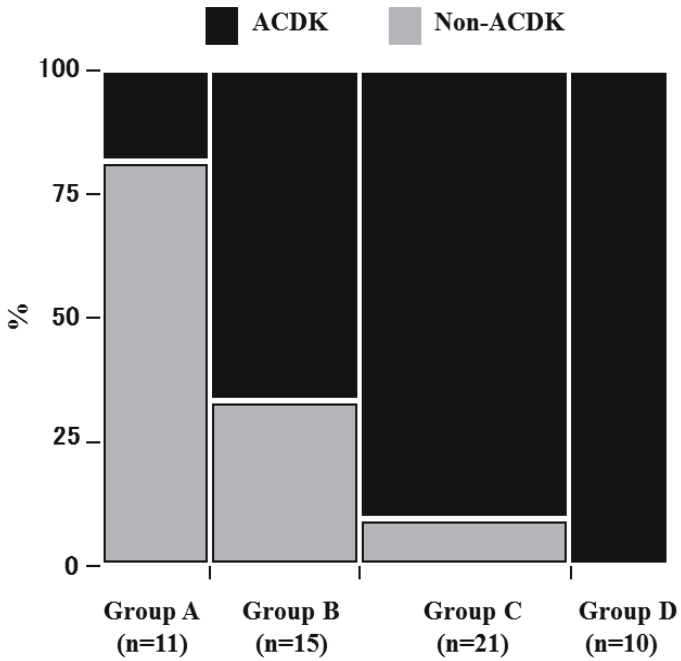

Fig. 2. Transition of proportion of acquired cystic disease of kidney (ACDK) in each group. As the duration of hemodialysis becomes longer, the proportion of ACDK increases ($p < 0.0001$).

3.2 Macroscopic and microscopic findings

There was no correlation between the average size of the tumors and the duration of hemodialysis in all cases. Only in ACDK-RCCs, maximal tumor diameter tend to increase with longer duration of haemodialysis but did not reach to a significant difference ($p=0.09$).

The main tumors and its associated foci (previously categorized according to the WHO 1998 classification system) were re-evaluated histopathologically according to the WHO 2004 classification of renal neoplasms and the 2009 TNM staging classification system (http://www.uicc.org/tnm). Although there is no consensus on the terminology of these special tumors, we used the term of Tickoo *et al*[23]. As a result, the current 57 cases were classified into 32 ACDK-RCCs corresponding to unclassified carcinoma in the current WHO classification, 22 clear cell RCCs and 3 papillary RCCs.

As regards the histological type of the tumors, ACDK-RCCs were more frequent (32/57 lesions, 56.1 %) than clear cell RCCs (22/57 lesions, 38.6%) or papillary RCCs (3/57 lesions, 5.3%). The ratio of ACDK-RCCs in the haemodialysis-related RCCs of each group increased concomitantly with the increase in the duration of haemodialysis; group A, 0/11 (0%); group B, 7/15 (46.7%); group C, 17/21 (81.0%); and group D, 8/10 (80%). Multivariate analysis incorporating into age, gender and duration of dialysis indicated that longer duration of dialysis was an independent predictor of development of ACDK-RCCs ($p < 0.0001$). Male gender also had a tendency to predict ACDK-RCCs but did not reach to a significant difference ($p=0.08$).

ACDK-RCCs had papillary, tubular or tubulocystic structures covered by neoplastic cells with large round or oval to irregularly shaped nuclei with or without prominent nucleoli and eosinophilic granular or focally relatively clear cytoplasms (Figure 3). All of them appeared to develop in close relation to the cystic regions and occasionally showed intratumoral calcium oxalate crystals deposition. Because the above-stated histopathological architecture of ACDK-RCCs was usually absent in conventional RCC, these ACDK-RCCs appeared to be difficult to classify into the known subtypes of RCC.

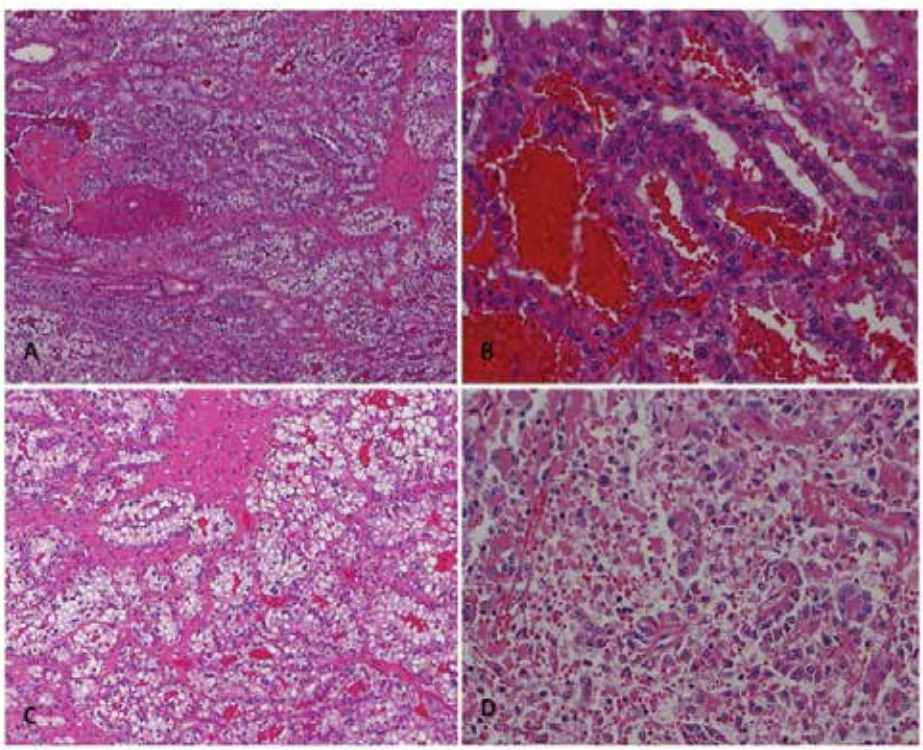

Fig. 3. Acquired cystic disease-associated renal cell carcinoma (ACDK-RCC), the most common tumor identified in ESRD, but only in cases with ACDK. A, This tumor shows a variegated architecture, including papillary, solid, and clear cell like areas. B, The tumor reveals papillary or tubular growth pattern of neoplastic cells with eosinophilic cytoplasms and round nuclei. C, Clear-cell RCC-like areas were focally present. D, ACDK-RCC with sarcomatoid changes were focally present.

3.3 Comparison of ACDK RCCs and non-ACDK RCCs

The mean duration of haemodyalysis was significantly longer in the ACDK-RCC cases (15.9 years) than in the non-ACDK-RCC (clear cell RCC and papillary RCC) cases (6.8 years) ($p < .0001$).There were no significant differences of age, laterality, tumor size, pathological T between two groups. % of male patients tend to be higher in the ACDK-RCC group (85%) than in in the non-ACDK-RCC (63%) ($p=0.07$). Two cases of ACDK-RCCs revealed

aggressive behavior, i.e. death from cancer. Those ACDK-RCCs with aggressive behavior tend to be detected in patients with longer duration of haemodialysis; both cases belonged to group D.

4. Discussion

4.1 Development of ACDK

During the past 20 years, ACDK has become more prevalent as patients with ESRD live longer, undergo more sensitive diagnostic imaging of the kidneys, and are less likely to undergo pretreatment bilateral nephrectomy. ACDK has been defined as macroscopic cystic structures compromising at least 25% of the renal parenchyma or greater than 3 cysts per kidney in a patient in renal failure who was not known to have cysts prior to the onset of renal failure and in whom there is no family history or other evidence of an inherited cystic disease[1]. Of patients on dialysis for less than 3 years 10% to have ACDK, 40% to 60% on dialysis for 3 years have ACDK and more than 90% have ACDK after 5 years on dialysis[24]. The current study also demonstrated that duration of dialysis was closely related with development of ACDK. After three years, proportion of ACDK dramatically increased. Cysts may be found in some patients with renal impairment prior to the initiation of dialysis treatment[25]. Ishikawa et al. reported that when male patients with ESRD were introduced to hemodialysis, the kidney volume was minimized because of the loss of hypertrophied nephrons 3.6 years after the start of haemodyialysis, and thereafter, the kidney enlarged due to the development of ACDK. The maximum kidney volume was obtained at 21.1 years after the start of hemodialysis[9,26]. ACDK may occur less frequently in those who are on peritoneal dialysis and may regress after transplantation[27].

4.2 Incidence of RCC in ESRD patients

the published incidence of RCCs in patients depends on the investigation method (radiologic, surgical, or autopsy). It is well established that patients with ESRD are more prone to kidney neoplasms with an incidence of 4.2% to 5.8%[28]. Those patients with ESRD who also have ACDK are even more prone to the development of carcinoma, with an incidence of 7%[23]. In United States, approximately 20% of those with ACDK will have RCC[5]. In Japan, Terasawa et al.[4], Satoh et al.[29] and Kojima et al.[11] reported 2.6% (41/ 1603), 0.61% (38/6201) and 1.68% (44/2624) of RCC in their patients on hemodialysis,respectively. Such an incidence of RCC appears significantly high, compared with that reported in the general Japanese population where RCC develops in 7.1 of 100000 men and 3.1 of 100000 women, and age-standardized incidence rates per 100 000 population for men and women were 4.9 and 1.8, respectively[30]. In Japan, longer dialysis patients have been dramatically increasing probably because of low cardiovascular comorbidity and low incidence of renal transplantation, leading to development of ACDK and RCC.

4.3 Diagnosis of RCC in ESRD patients

Diagnosis of RCC in dialysis patients may sometimes be difficult because the majority of the RCCs arise from multiple cysts of ACDK and theses RCCs are sometimes not enhanced well in computed tomography. Schwarz et al.[5] recommended a screening and management protocol in transplant recipients, incorporating the the Bosniak Renal Cyst Classification System[31].Complex cystic lesions were defined as those with irregularly thickened cyst walls, hyperdense or nonhomogenic cyst content and/or pronounced intrarenal calcifications,

and/or positive enhancement after intravenous application of contrast media (Bosniak category IIF to III). Ultrasound was followed by computed tomography (CT) scan or magnetic resonance imaging (MRI) when a moderately complex cystic lesion of the kidney was found (Bosniak category IIF) or in case of suspicion of renal cell carcinoma (category III or IV). Importantly, they recommended the nephrectomy in the case of progressive lesions, even if not reaching category III or IV. This is true especially for cystic lesions of category IIF. Fundamentally, we also underwent follow up with ACDK for screening of RCC according to the Bosniak Renal Cyst Classification System. Recently, we introduced diffusion-weighted MRI for detection of ACDK-RCC, which is now under investigation.

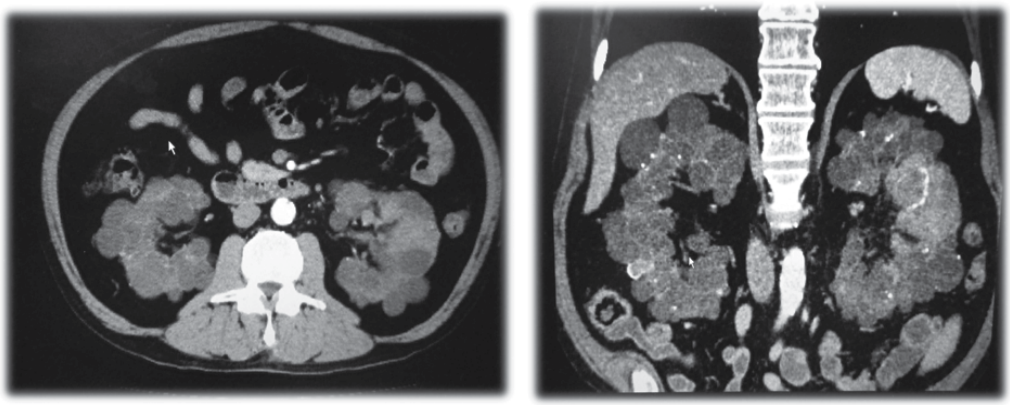

Fig. 4. Multidetector computed tomography for ACDK-RCC. Enhanced mass was found in left kidney (Bosniak category III).

Important problem is, how frequent is multifocality and bilaterality of tumors in ESRD. In the current study, bilateral RCCs were pathologically confirmed in 14% (7/50) of the dialyzed patients. This figure, consistent with previous reports[11,23], is considerably higher than in sporadic RCC in non-dialyzed patients which is reported as being approximately 4%[32]. Ghasemian et al. reported that because of the increased risk of developing RCC in the contralateral kidney, they performed bilateral laparoscopic RN in 10 patients. Of them, 2 patients had bilateral RCC[15]. Kojima et al. reported that satellite RCC nodules were detected in 29.5% of their patients with ACDK, whereas bilateral tumors were present 11.4%. When bilateral RCCs are suspected concomitantly, we performed the first RN on the kidney that harbors the larger tumor. After confirming the diagnosis pathologically, the second RN is performed sequentially[21]. We think that prophylactic nephrectomy should be avoided and followed up by imaging even after unilateral nephrectomy.

4.4 Minimum incision endoscopic surgery, MIES for RCC ; gasless single-port retroperitoneal RN

The perioperative management of ESRD patients is often complex. These patients are at increased risk of postoperative complications secondary to bleeding diathesis, hemodynamic instability, and immunosuppression. They also have higher risk during

anesthesia due to the multiple comorbidities, which include concomitant cardiovascular and respiratory issues. Preparation of the dialysis patient before the operation should include early withdrawal of drugs that affect platelet function, such as aspirin and non-steroidal anti-inflammatory drugs[33]. Preoperative optimization of platelet function and hematologic status may reduce intraoperative bleeding and the need for blood transfusion. Fornara et al. noted an increased transfusion rate in 19-dialysis-dependent patients undergoing laparoscopic nephrectomy (32%) compared with a similar group without renal failure (0%). They attributed this difference, not to increased blood loss or bleeding diatheses, but to a lower initial serum hemoglobin[34]. In our series, only one (1.8%) patients received transfusion. Recently, there were few patients with severe anemia even in ESRD patients by utilizing erythropoietin. Serum electrolytes should be closely monitored, both preoperatively and postoperatively. In addition, patients should not receive excessive amounts of intravenous fluids. Early dialysis may be necessary if serum electrolyte abnormalities or volume overload is present postoperatively.

Laparoscopic radical nephrectomy (LRN) for ESRD patients may offer an acceptable treatment modality with less invasiveness when compared with open RN[35,36]. Despite the several benefits of laparoscopic surgery such as reduced post-operative pain, shorter hospital stay, more rapid return to daily activities and so on, intra-abdominal CO_2 insufflation has various potential risks that may affect the cardiovascular and respiratory system. The pressure effects of pneumoperitoneum decrease cardiac output and stroke volume. The pressure effects also decrease respiratory compliance and increase airway pressure, with possible barotraumas, pneumothorax, and increased intracranial pressure. Gulati et al. reported a case of unexplained hypercarbia and hypotension developed during attempted retroperitoneal LRN requiring termination of the operation[14]. If CO_2 retention is problematic, the intra-abdominal pressure should be reduced and minute ventilation increased. Some have proposed performing laparoscopic procedures using abdominal wall retraction, rather than insufflation, in high-risk patients[37,38]. Bird et al. suggested that insufflation pressure for ESRD patients should be lower as compared with that for that for non-ESRD patients[16].

In patients with prior peritoneal dialysis, significant intra-abdominal adhesions can be encountered. Moreover, transperitoneal surgery itself could result in intraperitoneal adhesion which is not desirable for future peritoneal dialysis for ESRD patients or other abdominal operation. Retroperitoneal LRN approach has been shown to be a safe treatment modality for renal masses in ESRD patients[13].

Venous CO_2 embolism is a recognized risk during laparoscopic procedures. Its clinical presentation ranges from asymptomatic to neurogenic injury, cardiovascular collapse or even death depending on the rate and volume of gas entrapment and patient condition. Venous CO_2 embolism of laparoscopy occurs in 15 per 100,000 cases per year[39,40]. Incidences of subclinical embolism during various laparoscopic procedures have been reported to occur in as much as 6% of nephrectomy cases[41] and 17.1% total prostatectomy cases[42] when transesophageal echocardiography (TEE) was used for monitoring. Serious clinical events related with venous CO_2 embolism have been reported during laparoscopic nephrectomy[43,44] but not during laparoscopic radical prostatectomy. Gas embolism occurred during 2 distinct periods; first, during peritoneal insufflation, and second, during venous complex dissection[45]. Early signs of gas embolism include a rapid drop in end-tidal CO_2 and PaO_2 and an increase in $PaCO_2$ and can be followed by hypotension, hypoxia, cyanosis,

arrhythmia, or cardiac arrest. Elderly or high-risk patients with limited cardiopulmonary reserve might not be able to tolerate these situations.
Based on these above findings, non-use of CO_2 gas and retroperitoneal approach are considered to be key points for lesser invasive surgery for ESRD patients with renal tumors.
In this study, we demonstrated that MIES RN is a feasible treatment for RCC in ESRD patients requiring dialysis. Bleeding and operation time were comparable to LRN, as shown in Table.2. We already reported that this operation has minimal invasiveness similar to that of LRN[17] and an oncological outcome similar to that of open surgery[22]. Operative time and blood loss are similar to those in open surgery and complications including blood transfusion are very rare[17]. Postoperative data, days to oral feeding and days to walking are reported to be equal or sooner compared with LRN[17] and surgical site infection is extreamly rare despite the lack of use of prophylactic antibacterial agents[46]. These findings hold true even in ESRD patients. We stress that this operation has the following advantages over LRN especially for high risk group including ESRD: 1) this operation does not impose circulatory and respiratory stress on ESRD patients and avoids risks of venous embolism, air embolism, and venous thrombosis, which are actually rare, but can be lethal when they occur because of non-use of CO_2 during operation, 2) this operation leaves peritoneal cavity intact, leading no concern about intra-peritoneal adhesion after nephrectomy which is not desirable for possible future peritoneal dialysis and other operations, and 3) this operation can be performed even in patients with a history of intra-peritoneal surgery. In Japan, patients with ACDK-RCC have been increasing now. The cost of disposable instruments in this operation is much lower than that in LRN[47]. Based on these advantages, gasless single-port retroperitoneal RN seems to be ideal minimally invasive surgery for ESRD patients.

4.5 Pathology

In the present study, ACDK development in patients with ESRD/dialysis is associated with a higher risk of RCC and that the duration of dialysis is the main determinant of this risk. Papillary RCC has been previously reported to be the most common histological subtype found in the background of ACDK in dialysis patients, according for 42-71% of cases[28,48]. Our reevaluation showed that ACDK-RCC, but not papillary RCC, was the major histological subtype, accounting for 56.7% tumors in kidneys harbouring ACDK (50% patients), while papillary RCC was found only in 5.3%. Nouh et al. also reported a lower frequency of papillary RCC in dialysis patients (11%)[49].
The present study clearly showed that the histological spectrum of RCC differed according to the duration of dialysis, i.e. conventional clear cell RCC was the predominant subtype in patients with shorter duration of dialysis. Especially, within three 3 years, 91% cases were clear cell RCC, which is similar to histological spectrum of sporadic RCC. On the other hand, ACDK-RCC was the major histological subtype in those on dialysis for ≥ 10 years. These findings were identical to the findings reported by Nouh et al.[49] in Japanese population.
Generally, the biologic behavior of RCCs in ESRD is reported to be less aggressive than the RCCs in sporadic or non-ESRD setting[6,50]. However, some cases have been reported to behave aggressively and metastasize[23,49]. In the present study, two death from cancer were detected. All these two tumors were ACDK-RCC with long term dialysis more than 20 years, which is in line with other reports[23,49].

5. Conclusion

In conclusion, ACDK in patients with ESRD is a potential risk factor for the development of RCC. The risk is further increased by a longer duration of dialysis, which might increase the possibility of developing more aggressive histological subtypes of RCC with an unfavorable prognosis. The spectrum of RCC histological subtypes arising in ESRD is distinct from that of sporadic tumors. We believe that MIES RN, which is completed via a single port that narrowly permits extraction of the kidney with perinephric fat without CO_2 gas insufflation and without injury to the peritoneum, is a feasible treatment option for ESRD patients.

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4

Methylene Blue and Dialysis-Related Hypotension

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1. Introduction

End stage renal disease (ESRD) affects over 400,000 Americans, and over 8 million have chronic renal insufficiency [1]. Chronic renal insufficiency (CRI) describes a continuum of impaired renal function based on several parameters including glomerular filtration rate and serum creatinine. As renal function deteriorates, the risk of all-cause mortality increases [1]. The most prevalent form of renal replacement therapy is hemodialysis (HD), with over 75% of ESRD patients being treated with this modality [2, 3]. Patients requiring chronic HD have been noted to experience an increase in cardiovascular morbidity and mortality [4]. In fact, between 10-20% of patients who require HD die each year and approximately 45% of these deaths are attributable to cardiovascular causes [2, 3]. Hemodialysis itself is associated with substantial morbidity, including complications related to vascular access and those inherent to the HD procedure itself.

One of the most common complications of hemodialysis is intradialytic hypotension (IDH), which occurs in approximately 25% of HD sessions [5]. There are several therapeutic options available for the treatment of IDH. However, "resistant" forms of IDH do occur and a multimodality approach is usually necessary in such cases. The aim of this chapter is to discuss IDH and outline existing clinical approaches to IDH. Specifically, we will focus on the use of methylene blue (MB) in the treatment of IDH. Methylene blue, a nitric oxide pathway mediator, has shown promise in prevention or treatment of IDH in difficult-to-treat cases [6, 7].

2. Overview of intradialytic hypotension

IDH is seen in approximately 20-30% of hemodialysis sessions and is an independent risk factor for mortality [8]. During a typical HD session, an ultrafiltrate volume of 5 liters or greater may be removed with a concomitant 10-20% reduction in plasma volume [9, 10]. Intradialytic hypotension is likely multifactorial, and the inability of the cardiovascular system to adequately respond to the reduction in circulating plasma volume is among the leading factors.

Most patients are able to compensate for the ultrafiltration fluid losses by "mobilizing" fluid from extravascular/interstitial space, and tissue hydration state has a strong influence on changes in plasma volume during fluid removal and subsequent repletion [11]. In cases

where IDH occurs, available strategies include a combination of vasopressor administration, intravascular volume expansion with intravenous colloid or crystalloid solutions, positional patient changes, and discontinuation of ultrafiltration [12]. Detailed discussion of the underlying physiology and therapies available to treat IDH will now follow.

2.1 Physiology of intradialytic hypotension

Intradialytic hypotension can have very serious sequelae, up to and including the development of life-threatening end-organ (i.e., cardiac, cerebral) hypoperfusion [12]. Factors contributory to IDH can be broadly divided into those related to the HD procedure and those related to underlying patient condition(s) [12]. Patient related factors, the focus of this chapter, can be further divided into cardiac (i.e., left ventricular hypertrophy, chronic volume overload, anemia (acute or chronic), diastolic and systolic dysfunction, cardiac arrhythmias, ischemic myocardial syndromes) and vascular (i.e., vasoplegia or impaired maintenance of appropriate vascular tone/systemic vascular resistance) [7, 12]. Hemodialysis related factors include the ultrafiltration rate, possible intra-procedural blood loss, and dialysate temperature profile [12, 13].

We will focus on the vascular-related aspects if IDH, concentrating on the vasoplegia associated with the HD procedure. The amount of fluid available in the interstitial space for "vascular refilling" is the one of the primary determinants of IDH. In the presence of excess fluid in the interstitial space, the patient is theoretically more likely to tolerate higher volumes of ultrafiltrate removal [11]. Conversely, patients with relatively smaller amounts of volume in the interstitial space are more susceptible to even small amounts of volume removal. Therefore the determination of the patient's true dry weight is important to planning the amount of ultrafilatrate removal [14, 15]. It is important to remember that fluid shifts from the interstitial space to the intravascular space constitute a dynamic process and exhibit anatomic variations. For example, intravascular refilling is more vigorous in lower extremities because of the relative excess of extracellular fluid [16].

During ultrafiltration, the body's initial response to volume reduction involves sympathetic mediated vasoconstriction [17]. This vasoconstriction shifts blood flow away from dermal circulation and reduces heat loss. However, this response may not always be sufficient, either due to impaired sympathetic response itself or excessive production of endogenous vasodilators [18]. Chromogranin A levels, which are co-released with catecholamines, are decreased significantly in the post-HD plasma of patients with IDH compared to patients with stable blood pressures [19]. In addition, it has been suggested that patients with significant uremia have autonomic dysfunction that may be due to chronic hyperkalemic depolarization [20].

Endothelial cells and vascular myocytes release adenosine, which also has been implicated in the development of IDH [21]. It is hypothesized that during dialysis there are local areas of tissue ischemia that release adenosine in response. Antagonism of the A1 and A2 adenosine receptors in patients with frequent IDH has been shown to reduce the incidence modestly [21]. Also, peripheral blood mononuclear cells show greater expression of A2A receptors in IDH-prone patients [22].

Patients undergoing HD tend to experience a net increase in body temperature [23, 24]. Core temperature continues to increase throughout the dialysis procedure and may be associated with impaired sympathetic response before the development of IDH [25]. Isothermic HD may improve patient hemodynamics and may help prevent IDH [24]. It is important to note

that the protective hemodynamic effects of cool dialysate may be most pronounced among patients with lower baseline (i.e., pre-HD) body temperatures [26].
Additional aspects of vasoplegia associated with HD in the context of nitric oxide (NO) pathways will be discussed in subsequent sections, along with the role of methylene blue (MB) as a potential therapeutic agent in this setting.

2.2 Determination of dry weight and intravascular volume

The dry weight of a given patient can be difficult to determine and can fluctuate depending on any concurrent acute and chronic illnesses [14]. Several methods of estimating dry weight exist and will be discussed in this section. Commonly used techniques are based on intravascular pressure measurements, bioimpedance determination, arterial waveform analysis, and various sonographic techniques [27-29]. Specific methods include blood volume monitoring with ultrafiltration pulses, central venous and other invasive vascular pressure measurements, ultrasound measurements of the inferior vena cava (diameter and/or collapsibility) or direct bioimpedance measurements (whole-body, segmental, calf) [27-29]. Of note, the use of invasive blood volume monitoring devices has been associated with greater nonvascular and vascular access related complications and mortality [30]. Recent developments in the area of intravascular monitoring and estimation of dry weight and intravascular volume favor the use of minimally invasive or non-invasive modalities such as ultrasonography [31]. Techniques such as the pulmonary artery catheter are becoming less popular.

2.3 The role of nitric oxide in intradialytic hypotension

Analyses of plasma from patients identified to be at high risk of IDH suggest that this group may have chronic elevations in plasma nitrites as well as significantly elevated nitrite production [32]. Hemodialysis itself is associated with increased nitric oxide (NO) production [33]. When blood is exposed to hemodialysis membranes, endothelial cells show enhanced expression of inducible nitric oxide synthase (iNOS) mRNA in murine models [34]. In addition, uremic platelets produce increased amounts of NO [35]. It is speculated that cytokines, including IL-1β and TNFα, released during HD by activated mononuclear cells cause activation of NOS. Interestingly, significant increases in NO production are noted in patients who experienced a hypotensive episode during HD when compared to those that did not experience hypotension, suggesting a causal relationship [6]. However, the exact nature of this relationship remains to be elucidated.
NO is produced by two types of NO synthase (NOS) [7]. One exists in the endothelium and is constitutively active (eNOS). The other type is the inducible (iNOS) and exists in various tissues and cell types. Upregulation of iNOS results in increased NO production and generation of cGMP [7]. This can have profound effects on both the vasculature and myocardium.
In end stage renal disease, not only is there in increase in baseline NO production, there is also an increase in inhibitors of NO, namely asymmetric dimethylarginine (ADMA) [36]. It turns out these inhibitors are dialyzable, which suggests that disturbances within the baseline interplay between specific activators and inhibitors during HD may contribute to hemodynamic instability [36, 37]. Direct serum measurements during HD confirm higher nitrate generation, which combined with a decrease in inhibitory factors may lead to IDH.

In addition to the vasodilatory effects of NO, hypotension may also result from NO's negative inotropic properties [38]. Cholinergic agonists are known to elevate cGMP levels in cardiac myocytes and cGMP analogs may also produce profound negative chronotropic effects [39]. Exposure of cardiac myocytes to carbachol, a cholinergic agonist, results in suppressed contractility and chronotropy as well as profoundly increased levels of cGMP. This effect can be ameliorated by co-administration with methylene blue (MB) which suggests that NO dependent pathways may be involved [39]. Schematic representation of MB mechanism of action as well as pertinent metabolic pathways can be seen in Figure 1.

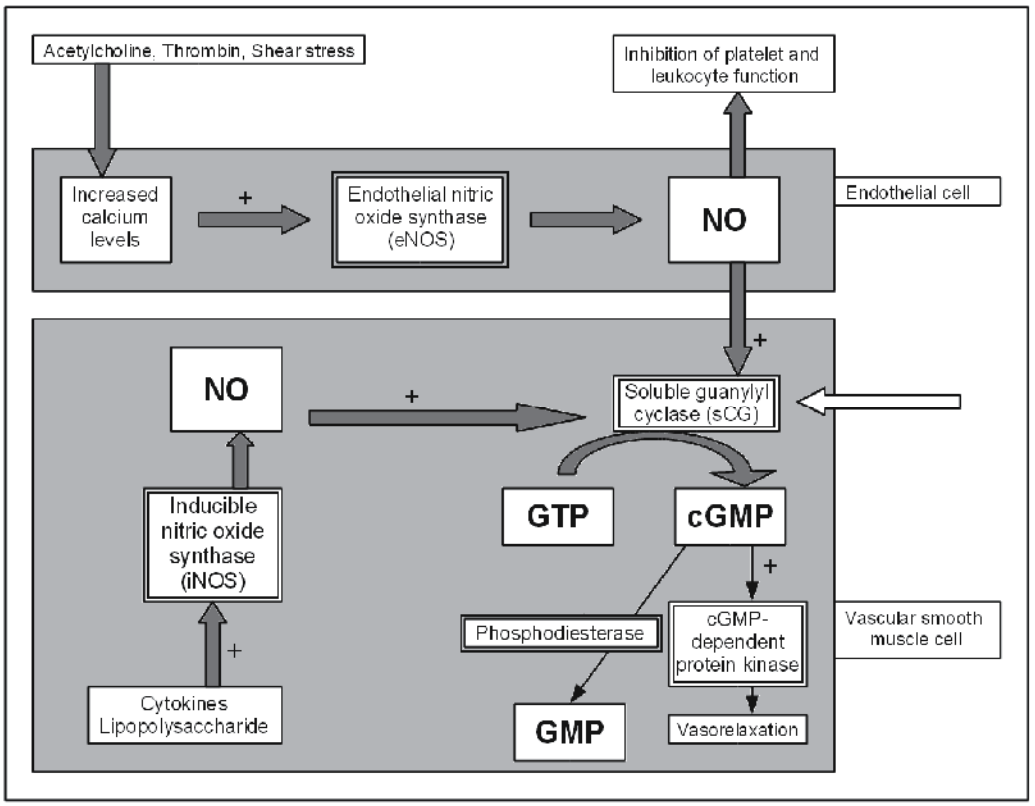

Fig. 1. Schematic representation of NO/cGMP-dependent pathways. Note the endothelial (eNOS) and inducible (iNOS) isoforms of the nitric oxide synthase (NOS) and their associated functional steps. Methylene blue inhibits (white arrow on right) the action of soluble guanylyl cyclase (sGC) and thus prevents vasodilation. Adapted from Bosoy et al [65].

2.4 Treatment for intradialytic hypotension

Evidence shows that episodes of intra- and post-dialytic hypotension are associated with increased morbidity and mortality [8]. This makes treatment and prevention of IDH an important part of both short- and long-term HD strategy. Several approaches to this problem exist and will be discussed in this section.

At the core of IDH is uncompensated response to reduction in circulating blood volume [40]. The patient may exhibit a blunted response to this reduction or the reduction may be too large to mount an effective response. Active monitoring of the dialysis patient's blood volume can provide real-time data to the clinician concerning the patient's volume status. Monitoring usually includes hematocrit, total protein and hemoglobin measurements, which cumulatively provide an estimate of total blood volume [41]. While the physiologic response to blood volume reduction varies considerably between patients, blood volume monitoring allows the clinician to tailor the treatment so the maximal tolerated blood volume changes are not exceeded. Clinical evidence shows that based on blood volume monitoring physicians are able to identify this maximum tolerated blood volume change in approximately 70% of patients, and the majority of hypotensive episodes occurred when this value is exceeded [42]. However, in almost 30% of patients such value could not be determined, suggesting that blood volume monitoring alone may not be sufficient for predicting risk of IDH in all patients. Ultrafiltration rate is also a very important determinant in the incidence of IDH. Altering the ultrafiltration rate to achieve a predetermined blood volume profile can reduce the incidence of IDH [43].

It has also been shown that limiting the reduction in plasma osmolarity during hemodialysis by altering the dialysate sodium concentration can enhance hemodynamic stability. In this paradigm, maintaining higher dialysate sodium concentration facilitates the maintenance of adequate intravascular blood volume [44]. The disadvantage to this method is that the higher sodium concentrations increase the amount of sodium available for exchange and may actually increase weight gain and precipitate hypertensive episodes.

As discussed earlier, HD is associated with an increase in core body temperature and concomitant propensity for IDH. Preventing increases in core body temperature allows for a more stable blood pressure throughout the dialysis process [23]. Low temperature diasylate settings (approximately 37-38°C) significantly decrease the severity and frequency of symptomatic IDH [13]. In addition, the low temperature improves capacitance and resistance of peripheral blood vessels and may result in improved cardiac contractility [45]. The use of thermal neutral dialysis reduces the frequency of IDH events by approximately one-fourth.

Several pharmacologic interventions also exist for the treatment of IDH. Midodrine is a prodrug that when metabolized acts as an alpha-1-receptor agonist. It can provide modest increases in both peripheral vascular resistance and cardiac output [46]. It is a well-tolerated medication with the most common side effects being piloerection, scalp itching/burning and nausea. Caution should be taken when administering midrodrine concurrently with negative chronotropic agents including β-blockers, digoxin and calcium channel blockers. Treatment regimens incorporating midodrine on the days of HD result in significant reductions in both intra- and post-dialytic hypotension [47].

3. Overview of methylene blue characteristics

Despite different therapeutic approaches to IDH, a significant proportion of patients undergoing dialysis continue to experience HD-related hypotension. In a quest for effective therapy for IDH, methylene blue (MB) has been evaluated as a potential adjunct in the setting of refractory hypotension. Methylene blue, a "natural pressor", is an

aromatic chemical compound used in analytical chemistry, biology and medicine. It is a soluble compound that can be administered intravenously or orally/enterally. It functions by inhibiting guanylyl cyclase in vascular smooth muscle, and decreases the levels of cGMP [7, 48-51]. Methylene blue also scavenges nitric oxide and inhibits nitric oxide synthesis [7, 50].

3.1 Pharmacology of methylene blue

Methylene blue is available in both an intravenous and oral form. Both forms undergo primarily urinary excretion with a half-life of approximately 4-5 hours [52]. Small amounts of MB are excreted in bile and feces as well [53]. Distribution appears to differ substantially between the two enteral and intravenous forms. Intravenous administration results in significantly higher concentrations within the blood and brain 1 hour after infusion when compared to the oral route. Bioavailability from oral administration ranges from 53-97% [54]. Oral administration results in much higher concentrations within the bowel wall and liver, with <3% of the administered MB remaining within the intestinal lumen after ingestion. Once in the blood, MB readily enters erythrocytes where it is reduced to leucomethylene blue at low concentrations. In high concentrations it can act as an oxidizing agent, potentially leading to hemolysis, methemoglobinemia and hyperbilirubinemia [53].

Most side effects of MB appear to be dose-dependent and do not occur with doses below 2 mg/kg [55]. Methylene blue can turn urine greenish-blue, and while this may be alarming to patients, it usually resolves within a few days of discontinuing MB. In addition, mild skin discoloration is common, but is self-limiting and treatable with administration of dilute hypochlorite solution [56]. Other side effects may include abdominal pain, nausea, vomiting, headaches, fever, confusion and diaphoresis [54]. Subcutaneous and intradermal injections should be avoided because they have been associated with necrosis and abscess formation [57]. Encephalopathy has been noted in one series that included five patients that received preoperative intravenous MB (3-5 mg/kg in 500 mL saline) for parathyroid adenoma localization. All five cases of encephalopathy occurred in female patients taking serotonin-metabolism modifying agents [58].

In the neonatal and pediatric populations, enteral MB administration of >2 mg/kg has been associated with severe methemoglobinemia, hemolytic anemia, Heinz body anemia, and hyperbilirubinemia [53]. Anemia following MB administration typically manifests within 24 hours, peaks at 4-5 days and can persist for up to 12 days [59]. Futhermore, photosensitive epithelial desquamation after MB administration has been reported among infants undergoing phototherapy. This may be due to lysosomal membrane breakdown after interacting with light in the presence of photosensitizing MB [60]. Practitioners caring for patients receiving MB infusions have to be aware of falsely depressed oxygen saturation readings due to methylene blue interfering with the pulse oximeter's light emission [61].

3.2 The use of methylene blue in hypotensive patients

Because of its ability to lower plasma levels of the endogenous vasodilator NO, methylene blue has been investigated in the clinical setting of difficult-to-treat hypotension. Investigational studies of MB in various hypotensive settings have been carried out, with some of the most compelling evidence coming from the areas of cardiac surgery, trauma, renal failure, and other forms of distributive shock.

Animal models of acute shock have been developed that facilitate objective testing of the role of MB in various forms of shock. Refractory hemorrhagic shock is seen most commonly in trauma patients and carries a high morbidity and mortality. In canine models, untreated animals usually die within 30 minutes of onset of refractory hemorrhagic shock. When treated with an initial bolus of MB and volume resuscitation the mortality was 0% at 120 minutes, compared to 75% for animals treated with volume resuscitation alone [62]. Animals treated with MB and volume resuscitation maintained significantly higher mean arterial pressures, increased cardiac output, better tissue perfusion, and increased oxygen delivery. Furthermore, MB administration has been associated with significant neurologic and myocardial protective effects during cardiopulmonary resuscitation. This is thought to be due to the effect of MB contributing to improved coronary perfusion pressure and cardiac index, as well as reduced cerebral perioxidation and inflammation [63].

Hepatic failure may also be complicated by vasoplegia and hypotension. Hepatic failure is commonly associated with increased plasma concentrations of endotoxin in addition to other endogenous vasodilators. Moreover, up to 40% of patients with cirrhosis develop hepatorenal syndrome within 5 years of initial diagnosis [64]. Published case reports describe the use of MB in refractory hypotension associated with hepatorenal syndrome, where vasoplegic patients with refractory hypotension were able to become vasopressor free within 5 days of intiation of MB therapy, suggesting a role of increased NO activity in the pathogensis of hepatorenal syndrome-associated vasoplegia [65]. See Figure 2 for a clinical vignette demonstrating MB use in the setting of difficult-to-treat vasoplegia. For additional information regarding clinical uses of MB for hypotension in various clinical settings the reader is referred to Table 1.

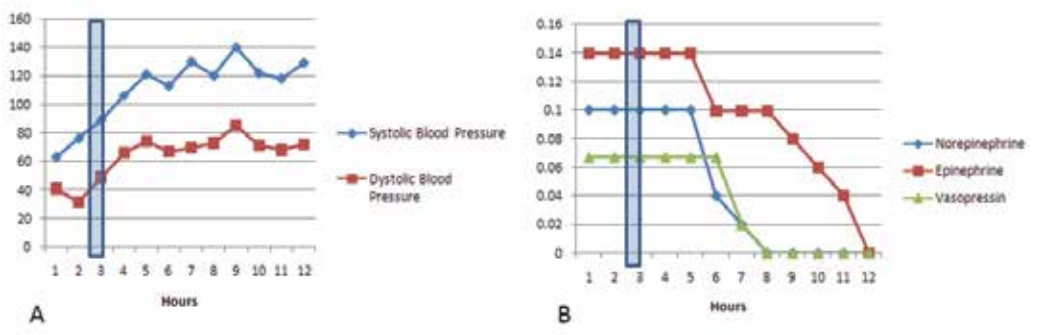

Fig. 2. Hemodynamic profile and vasopressor requirements of a middle-aged vasoplegic male patient after methylene blue (MB) administration. The patient experienced profound hypotension refractory to conventional management on day #2 following repair of type A aortic dissection. After the patient became essentially unresponsive to escalating vasopressor support, he received an intravenous injection of 2 mg/kg MB over 30 minutes (A and B, MB administration timing shaded in blue). A) Blood pressure response to MB injection showing increase in both systolic and diastolic blood pressures. B) Vasopressor doses immediately prior, during, and after MB infusion. All vasopressors were weaned completely within 9 hours of MB administration. Legend: MB – methylene blue; Infusion rates – Vasopressin units/min; Epinephrine – mcg/kg/minute; Norepinephrine – mcg/kg/minute; Blood pressure listed in mmHg.

Author (ref, year)	Clinical Setting	Major results/findings	Comments
Peer (6, 2001)	Investigational study of MB administration in HD patients. (n=41, 18 HD patients with hypotension, 18 HD patients without hypotension, and 5 healthy controls). MB was given as a bolus (1mg/kg) followed by an infusion (0.1 mg/kg) for 210 minutes until the end of HD session. On non-dialysis days, only the bolus dose was given.	In hypotension-prone patients, MB prevented the hypotension during dialysis and increased both systolic and diastolic blood pressure on non-dialysis days. In normotensive patients, MB increased blood pressure during the first hour of dialysis and during the first 90 minutes on non-dialysis days. The blood pressure in healthy controls remained unchanged.	Study based on the finding that elevated plasma NO levels were found in HD patients, likely contributing to HD-related hypotension. Nitrate generation was significantly higher in the hypotensive group than in the normotensive group. No side effects were noted.
Preiser (67, 1995)	Prospective medical-surgical ICU study involving patients with hypotension refractory to vasopressor therapy (n = 14). MB was given as a bolus (2 mg/kg) over 15 minutes. Additional dose was needed in 6 patients due to transient response to the initial bolus.	MB administration was associated with increased mean arterial pressure and increased systemic vascular resistance. A decrease in serum lactate following MB administration was noted.	A decrease in serum lactate following MB administration may be due to a reducing effect rather than improved tissue oxygenation.
Daemen-Gubbels (68, 1995)	Non-randomized clinical trial involving MB administration in the setting of sepsis. The trial involved consecutive patients with a pulmonary catheter in place (n = 9). MB was administered as an intravenous bolus (2 mg/kg).	MB administration resulted in increased mean arterial pressure and oxygen uptake as well as a decrease in arterial complaiance. MB use was also associated with increased myocardial function and oxygen delivery.	

Table 1. Clinical studies and reports describing the use of methylene blue for hypotension in various clinical settings. Legend: HD – hemodialysis; MB – methylene blue; NO – nitric oxide.

Author (ref, year)	Clinical Setting	Major results/findings	Comments
Bosoy (65, 2008)	Report describing MB use in vasoplegic patients with simulataneous hepatic and renal failure. One patient received MB bolus (1 mg/kg) followed by another dose of 0.5 mg/kg at 12 hours. Another patient received MB infusion of 1 mg/kg, followed by an enteral-based MB regimen (1 mg/kg every 12 hours).	Both patients experienced significant hemodynamic improvement following MB administration. The first patient was successfully weaned off dual vasopressin-norepinephrine regimen. The second patient was able to leave the hospital and receive palliative care at home due to his ability to be vasopressor-free utilizing enteral (oral) methylene blue regimen.	Central venous pressure was noted to increase following MB administration. Successful palliative use of enteral MB in a patient with end-stage hepatorenal dysfunction and refractory vasoplegia is described. No adverse effects noted.
Jaskille (69, 2008)	Case series of patients with severe burns (80-95% TBSA, n=2) and persistent hypotension despite simultaneous vasopressin and norepinephrine. Patient received single intravenous infusion (2mg/kg) of MB.	Response to MB noted within 30 minutes. Significant reductions in pressor requirements, including complete resolution of vasoplegia within 2 hours in 1 patient.	No adverse effects were noted.
Del Duca (70, 2009)	Case series of patients with intraoperative anaphylaxis during cardiac surgery. One case was a reaction to protamine, the other was a reaction to aprotinin. The patients were treated with intravenous MB (2mg/kg).	Both patients had prompt resolution of hypotension and did not require additional MB doses. There were no adverse effects.	

Table 1. Clinical studies and reports describing the use of methylene blue for hypotension in various clinical settings. Legend: HD – hemodialysis; MB – methylene blue; NO – nitric oxide. (Continuation)

3.3 The use of methylene blue in hemodialysis and intradialyitc hypotension

The use of MB may reduce the incidence of IDH in patients who are prone to this hemodynamic disorder [6]. Initial studies utilized a pre-HD bolus of MB and noticed lower incidence of intra- and post-dialytic hypotensive episodes. When given continuously to hypotension prone patients during HD, episodes of IDH were significantly reduced. Of interest, MB administration to normal controls did not cause appreciable changes in blood pressure, which corroborates the role of nitrate generation during HD as contributory to IDH.

As discussed earlier, there is usually a reduction in circulating plasma nitrates during HD procedure [66]. However, following the procedure certain patients appear more prone to the development of circulating nitrate increases. Among patients who are more prone to develop IDH, a significant increase in nitrate production was noted on post-dialysis day 1 compared to dialysis patients who are not prone to hypotension (1.21 +/- 0.13 μmol/min versus 0.61 +/- 0.11 μmol/min) [6]. In addition, when patients with propensity for IDH were treated with MB during the procedure, their 24-hour plasma nitrate production decreased significantly compared to untreated individuals, further supportiny the role of NO production and cGMP mediated pathways for the development of IDH.

4. Conclusions

Intradialytic hypotension continues to be a significant challenge. Despite multiple therapies, no single agent or modality has been proven universally effective in the management of IDH. For patients who are refractory to traditional therapies (fluid infusions, vasopressor administration, modification of ultrafiltration rates) adjunctive treatments may hold promise. One of those approaches is the use of methylene blue, an inhibitor of guanylyl cyclase in vascular smooth muscle, as well as nitric oxide synthesis inhibitor and scavenger. Although early research in this promising area is encouraging, further investigation is needed before more widespread implementation of this therapy is undertaken.

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5

Identification of Hemodialysis Patients' Physical and Psychosocial Problems Using the International Classification of Functioning, Disability and Health (ICF)

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1. Introduction

We aimed to identify hemodialysis (HD) patients' physical and psychosocial problems using the International Classification of Functioning, Disability and Health (ICF)-based checklist we developed. The ICF belongs to the WHO's family of international classifications, and it is the instrument for comprehensive understanding of patients. HD patients have diverse physical and psychosocial problems, and ICF-based approach may be useful to improve management and quality of life (QOL) of patients on HD. In this article, we introduced the new data associated with physical and psychosocial problems of 222 patients in HD, which extends our previous report (Tsutsui et al. 2009).

In Japan, the number of patients on HD was 36,397 in 1980 and increased to 290,675 in 2009 (Patient Registration Committee, Japanese Society for Dialysis Therapy, 2010). In addition to the physical limitations in functioning caused by renal failure and its comorbidities, HD patients have various restrictions resulting from HD therapy requiring radical lifestyle changes. Therefore, HD patients tend to have both physical and psychosocial problems. Thus, evaluation of QOL is especially important, and the Kidney Disease Quality of Life (KDQOL™) (Hays et al, 1994) and the Kidney Disease Quality of Life-Short Form (KDQOL-SF™) (Hays et al, 1994) have been widely used. The KDQOL-SF™ includes multi-item scales targeted at the particular health-related concerns of individuals who have kidney disease and are on dialysis. In the present study, we aimed to investigate the use of the International Classification of Functioning, Disability and Health (ICF) (WHO 2001), which is the instrument for comprehensive understanding of patients. In addition to evaluate physical and psychosocial problems of patients, ICF can be used as a tool for team medical treatment to make plans for treatment and care of patients. We have reported the checklist for HD patients based on ICF (Tsutsui et al. 2009). The data in this article include those of patients on HD with diabetic nephropathy, which was excluded in the previous report.

2. International classification of functioning, disability and health

The ICF was published by the World Health Organization (WHO) in 2001 to standardize descriptions of health and disability. The ICF and International Classification of Disease-10th

Revision (ICD-10) constitute the core classification in the WHO's family of international classifications, which provides a valuable tool to describe and compare the health of populations in an international context. The information on mortality (provided by ICD-10) and on health outcomes (provided by ICF) may be combined in summary measures of population health.

The overall aim of the ICF classification is to provide a unified and standard language and framework for the description of health and health-related states. It defines components of health and some health-related components of well-being (such as education and labor). An ICF-based approach can also be useful to collect information on a broad set of impairments, activity limitations and environmental factors that contribute to improve or worsen patients' functioning and disability status. Such information could provide a common framework for research, clinical work and social policy and help in improving the identification of needs related to health and social services, and related interventions.

The ICF provides a description of situations about human functioning and its restrictions and serves as a framework to organize this information. The ICF is based on the biopsychosocial model, an integration of medical and social models. The patient's functioning is conceived as a dynamic interaction between the underlying health condition and specific personal and environmental factors. The following diagram is one representation of model of disability that is the basis for ICF (Figure 1).

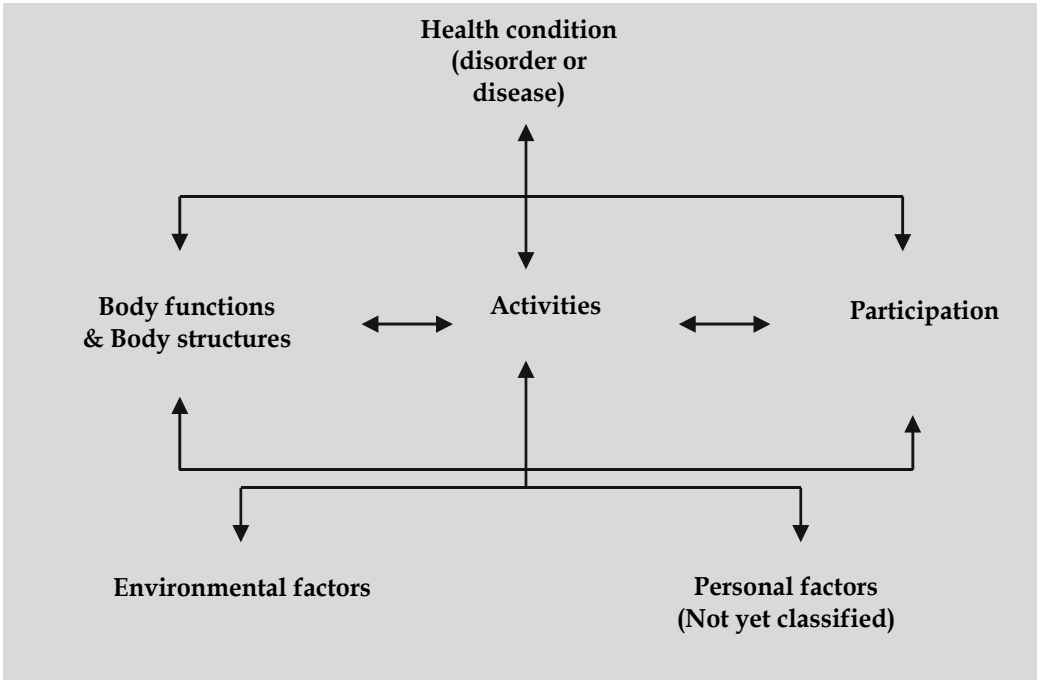

Fig. 1. Interactions between the components of ICF (WHO, 2001)

The ICF organizes information in the two main subdivisions: Part 1 covers functioning and disability and Part 2 covers contextual factors. Each of these two parts is divided into components. Components of functioning and disability consist of "Body functions and structures" and "Activities and participation". Components of contextual factors consists of

"Environmental factors" and "Personal factors". "Body functions" relate to the physiological and psychological functions of the body. "Body structures" are anatomic parts of the body such as organs, limbs and their components classified according to body systems. "Activities" is the execution of a task or action by an individual. It represents the individual perspective of functioning. "Participation" is a person's involvement in a life situation. It represents the societal perspective of functioning. "Environmental factors" refer to all aspects of the external or extrinsic world that from the context of an individual's life such as social attitudes and values, social systems and services, policies, rules and laws. "Personal factors" are those related to the individual such as age, gender, social status, and life experiences, which are not currently classified in ICF, although users may incorporate in their applications of the classification (WHO 2001).

Every component of the ICF has a hierarchical structure. The categories of ICF are classified by the code in which the letters (b, s, d, and e) is combined with the number. The letters b, s, d and e refer to the components "Body functions" (b), "Body structures" (s), "Activities and participation" (d), and "Environmental factors" (e). The letters are followed by a numeric code that defines the chapter number (first digit) and the category levels up to the fourth level (suffix of two, three, or four digits).

2.1 ICF checklist

The ICF in its current version consists of 1424 codes. Therefore, it is necessary to select a subset of the codes as needed for any given purpose. One of such activities is the development of the ICF checklist (WHO, 2003). The ICF checklist consists of a selection of 128 first- and second-level categories from the whole ICF classification system. It provides a relatively simple-to-use questionnaire, and is a generic template for a structured interview. The ICF checklist makes it possible to generate a profile of the individual patient on the functioning and disability in clinical practice. Of the 128 categories, 32 belong to "Body functions", 16 to "Body structures", 48 belong to "Activities and participation", and 32 to "Environmental factors". The ICF checklist utilizes a "qualifier" to evaluate each component, which is considered to be positive when patients have any level of impairments (i.e. mild, moderate, severe, or complete) in "Body functions" and "Body structures"; any level of activity limitations or participation restrictions in "Activities and participation"; and any level of barrier in "Environmental factors" (WHO 2003, Ewert et al. 2004).

2.2 ICF core sets

The ICF core sets are developed for medical conditions that have high impact on a patient's functioning and disability (Stucki et al. 2002). They have been developed in a formal decision making and consensus-based process integrating evidence gathered from studies for chronic conditions (Weigl et al. 2004, Brockow et al. 2004, Ewert et al. 2004). The ICF core sets for patients with a determined health condition represent a selection of ICF categories out of the whole classification that can serve as minimal standards for reporting of functioning and health for clinical studied and clinical encounter or as standards for multiprofessional. The ICF core sets contain categories not only on anatomic and pathophysiologic changes but also on functioning in every-day activities and relevant environmental factors.

These ICF core sets are to be developed in two levels: A brief and comprehensive ICF core sets. The brief ICF core sets includes only the most important ICF-categories and is intended to be rated in all patients of a clinical study. However, the comprehensive ICF core sets

include all categories that are typically limited in the selected health condition, and are created to guide multidisciplinary assessment (Stucki et al. 2002).
The ICF core sets have been developed for many health conditions including diabetes mellitus (Ruof et al. 2004), obesity (Stucki et al. 2004), and stroke (Geyh et al. 2004).

3. Identification of HD patients' physical and psychosocial problems

The process of developing the checklist for the HD treatment is briefly described.
Initially, we interviewed 32 HD patients using ICF checklist. They were interviewed for each category of the ICF checklist whether they had problems since starting HD treatment. For example, in the category of *b134 Sleep*, patients were interviewed "Have you ever experienced insomnia, nocturnal awakening, or hypersomnia since starting HD treatment?" The interviewer questioned about the details of their problems when patients answered "yes". The interview was done by the first author who was the medical social worker. All categories that at least 1 patient reported a problem were selected as problem categories. As a result, 57 categories of the ICF checklist were selected for the checklist for the HD treatment. Thirty-five categories in the ICF that were not included in the ICF checklist were chosen based on the consensus of the conference that included physician, nurses, and medical social worker. These 92 categories consisted of 39 categories from the "Body functions" component, 13 from the "Body structures" component, 20 categories from the "Activities and participation" component, and 20 categories from the "Environmental factors" component. Finally, we added 8 categories that are not included in the ICF categories considering the specificities of HD. These categories are following; *Functions of vascular access* in "Body functions" component, *Vascular access* in "Body structures" component, *Going to hospital, Managing weight, Angiostasis by oneself after drawing out needle,* and *Preparing a dialysis diet* in "Activities and participation" component, *Dialysis professionals* in "Environmental factors" component. Taken together, the checklist for the HD comprises 100 categories.

	Body Functions	Body Structures	Activities and Participation	Environmental Factors	Total
ICF checklist Version2.1α	19	8	15	15	57
Additional ICF categories for HD	20	5	5	5	35
Categories specific for HD	1	1	5	1	8
Final Checklist for HD	40	14	25	21	100

Table 1. Component of checklist.

3.1 Physical and psychosocial problems of maintenance HD patients

We interviewed 222 maintenance HD patients using the checklist for HD patients. The characteristics of them are shown in Table 2.

3.1.1 Body functions

The percentage of patients on maintenance HD who reported problems in each category of "Body functions" component is described in Table 3.

	Total n=222
Sex (men/women)	152/70
Age (years)	61±11
Age of HD introduction (years)	52±13
Duration of HD (years)	9.1±8.0
Underlying disease	
Diabetic nephropathy	86
Chronic glomerulonephritis	46
Nephrosclerosis	44
Polycystic kidney	9
Gouty kidney	6
Interstitinal nephritis	2
Obstructive urinary disorder	2
Reflux nephropathy	1
Cystinuria	1
Pregnancy-induced kidney disease	1
Pyelonephritis	1
Systemic lupus erythematosus	1
Unidentified	22

Table 2. Maintenance HD patients' characteristics.

Body Functions	%
b110 Consciousness functions	25.7
b1300 Energy level	39.2
b1302 Appetite	26.6
b134 Sleep functions	46.4
b140 Attention functions	32.9
b152 Emotional functions	26.6
b210 Seeing functions	50.9
b240 Sensations associated with hearing and vestibular function	37.8
b250 Taste function	20.7
b260 Proprioceptive function	36.9
b265 Touch function	16.7
b270 Sensory functions related to temperature and other stimuli	12.2
b280 Sensation of pain	45.9
b410 Heart functions	45.9
b415 Blood vessel functions	22.5
§ Functions of vascular access	27.2
b420 Blood pressure functions	75.7
b430 Haematological system functions	14.0
b440 Respiration functions	26.6
b4550 General physical endurance	50.5

Body Functions	%
b4551 Aerobic capacity	45.9
b4552 Fatigability	57.2
b515 Digestive functions	24.8
b525 Defecation functions	51.4
b530 Weight maintenance functions	22.5
b535 Sensations associated with the digestive system	33.8
b545 Water, mineral and electrolyte balance functions	26.6
b555 Endocrine gland functions	14.0
b610 Urinary excretory functions	61.3
b620 Urination functions	47.7
b64 Sexual functions	18.0
b670 Sensations associated with genital and reproductive functions	12.6
b710 Mobility of joint functions	43.2
b730 Muscle power functions	12.6
b735 Muscle tone functions	40.5
b780 Sensations related to muscles and movement functions	64.4
b810 Protective functions of the skin	59.9
b820 Repair function of the skin	36.9
b840 Sensation related to the skin	74.8
b850 Functions of hair	29.7

§; Categories specific for HD (Not ICF categories)

Table 3. Percentage of maintenance HD patients who reported impairment in each category of "Body functions" component.

In the "Body functions" component, problems of patients on maintenance HD are associated with sleep, fatigue, defecation, blood pressure, urination, muscle, skin, and those related to the symptoms or complication of kidney disease. Itching of the skin (Danquah et al. 2010, Caplin et al. 2011), sleep (Čengić et al. 2010, Danquah et al. 2010), blood pressure (Van Buren et al. 2011, Caplin et al. 2011), muscle cramps (Danquah et al. 2010, Weisbord et al. 2008), and constipation (Wu et al. 2004, Yasuda et al. 2002) have been reported as significant problems in patients with maintenance HD. According to the interview, these patients tend to have problems such as itching, muscle cramp, or low blood pressure not only in everyday life but also during HD treatment, which seem worry them substantially.

3.1.2 Body structures

The percentage of maintenance HD patients who reported problems in each category of component of "Body structures" component is described in Table 4.

In the "Body structures" component, a high percentage of patients on maintenance HD reported problems related to nail, disorder of urinary system, and eye disease. Disorder of nail structure such as half-and-half nail and tinea unguium (Saray et al. 2004, Dyachenko et al. 2007), disorder of urinary system such as pyuria and loss of urination (Vij et al. 2009, Fasolo et al. 2006), and eye disease such as diabetic retinopathy and glaucoma (Chiu et al. 2008, Varbec et al. 2005) have been reported.

Body structures	%
s220 Structure of eyeball	41.0
s410 Structure of cardiovascular system	30.2
s4100 Heart	9.5
§ Vascular access	33.8
s550 Structure of pancreas	0.9
s5801 Thyroid gland	12.1
s5802 Parathyroid gland	14.7
s610 Structure of urinary system	61.3
s6100 Kidneys	100.0
s630 Structure of reproductive system	6.8
s730 Structure of upper extremity	28.4
s750 Structure of lower extremity	13.5
s770 Additional musculoskeletal structures related to movement	5.0
s830 Structure of nails	56.3

§; Categories specific for HD (Not ICF categories)

Table 4. Percentage of maintenance HD patients who reported impairment in each category of "Body structures" component.

3.1.3 Activities and participation

The percentage of maintenance HD patients who reported restrictions in each category of "Activities and participation" component is described in Table 5.

In the "Activities and participation" component, a high percentage of patients reported restrictions related to actions that use upper limbs, job, and hobby. Consistently, actions that use upper limbs (Tander et al. 2007, Namazi et al. 2007), job (Panagopoulou et al. 2009, Kutner et al. 2010), and hobby (Al Eissa et al. 2010) have been reported to be highly restricted. The time restriction due to the regular dialysis and the need to protect vascular access seem to be major factors to affect patients' restriction in activities and participation.

3.1.4 Environmental factors

The percentage of maintenance HD patients who reported barriers in each category of "Activities and participation" component is described in Table 6.

In the "Environmental factors" component, a high percentage of paients reported as barriers in categories related to transportation service, social security, and labor. Transportation (Diamant et al. 2010, Gorden et al. 2003), social security such as medical fee (Holley et al. 2006, Gracia-Gracia et al. 2005) and labor (Neri et al. 2009, Muehrer et al. 2011) have been reported as barriers. Maintaining employment is one of the most serious problems. We reported that 41% of the patients in the previous study (Tsutsui et al, 2009) were terminated, transferred to a different position, demoted, or changed their employment agreement (from

Activities and Participation	%
d220 Undertaking multiple tasks	14.0
b240 Handling stress and other psychological demands	17.1
d430 Lifting and carrying objects	35.1
d440 Fine hand use	23.0
d450 Walking	13.1
d465 Moving around using equipment	9.9
d470 Using transportation	15.8
d475 Driving	19.4
§ Going to hospital	5.4
d510 Washing oneself	9.9
d520 Caring for body parts	9.5
d550 Eating	7.2
d570 Looking after one's health	15.8
§ Managing weight	14.0
§ Confirmation of vascular access	0.5
§ Angiostasis by oneself after drawing out needle	9.9
d630 Preparing meals	7.2
§ Preparing a dialysis diet	17.1
d640 Doing housework	9.5
d660 Assisting others	2.7
d845 Acquiring, keeping and terminating a job	24.8
d850 Remunerative employment	21.6
d9201 Sports	18.5
d9204 Hobbies	32.0
d9205 Socializing	24.8

§; Categories specific for HD (Not ICF categories)

Table 5. Percentage of maintenance HD patients who reported restrictions in each category of "Activities and participation" component.

full-time to part-time employment). According to Japanese statistics, 37.7% of men HD patients and 43% of women HD patients were terminated or retired in the past 5 years (Japan Association of Kidney Disease Patients, Japanese Association of Dialysis Physician. 2007). The problem related to the payment of medical fees is another serious concern for patients. Patients on HD had received a total exemption of medical fees until the coming into force of the "Law for Independence of Persons With Disabilities" in 2006. According to a report (Japan Association of Kidney Disease Patients, Japanese Association of Dialysis Physician. 2007), 75.2% of Japanese HD patients greatly hope for "continuation of medical security of HD treatment".

Environmental Factors	%
e110 Products or substances for personal consumption	27.5
e310 Immediate family	8.1
e320 Friends	7.7
e325 Acquaintances, peers, colleagues, neighbors and community members	5.0
e330 People in positions of authority	5.9
e350 Domesticated animals	14.4
e355 Health professionals	16.7
§ Dialysis professionals	5.4
e410 Individual attitudes of immediate family members	4.1
e420 Individual attitudes of friends	4.1
e425 Individual attitudes of acquaintances, peers, colleagues, neighbors and community members	6.3
e430 Individual attitudes of people in positions of authority	3.6
e440 Individual attitudes of personal care providers and personal assistants	2.3
e450 Individual attitudes of health professionals	18.5
e465 Social norms, practices and ideologies	17.1
e540 Transportation services, systems and policies	10.4
e555 Associations and organizational services, systems and policies	14.9
e560 Media services, systems and policies	23.4
e570 Social security services, systems and policies	23.8
e580 Health services, systems and policies	32.4
e590 Labour and employment services, systems and policies	35.1

§; Categories specific for HD (Not ICF categories)

Table 6. Percentage of the maintenance HD patients who reported barriers in each category of "Environmental factors" component.

4. Conclusion

We developed the ICF-based checklist for the HD treatment, and identified the physical and psychosocial problems that the HD patients had. We showed the features of HD patients with problems associated with disease or impairments as well as daily life activities. The checklist based on ICF, which is an integrated model of the medical and the social models, enables us to understand HD patients comprehensively. We will continue efforts to identify more relevant ICF categories to complete the final version of the checklist.

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6

Selenium (Se), Blood Glutathione Peroxidases and DNA Damage in Chronic Kidney Disease Patients on Hemodialysis and After Kidney Transplantation - The Effect of Se Supplementation

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1. Introduction

1.1 Generation, role and destruction of reactive oxygen species

Chronic kidney disease (CKD) is characterized by complex changes in cell metabolism leading to increased production of oxygen radicals which, in turn, may play a key role in numerous clinical complications of this pathological condition. Several reports have focused on the identification of biological elements involved in the development of systemic biochemical alterations in CKD (Granata et al., 2009). Under normal conditions in living organisms oxygen metabolism (above all reduction) leads to the formation of highly reactive intermediates called reactive oxygen species (ROS) that are dangerous for the cell (Yu, 1994). As long as the balance between ROS production and ROS scavenging is maintained, no disorders are observed. ROS include superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($OH^{\bullet}$), perhydroxyl radical ($HO_2^{\bullet}$) and singlet oxygen ($^1O_2$). According to some scientists, hydrogen peroxide (H_2O_2) also belongs to this group since it easily penetrates the membranes and in the presence of transition metals (copper or iron) it can be reduced to $OH^{\bullet}$ as in the example below (Yu, 1994):

$$H_2O_2 + Cu^{+} \rightarrow OH^{\bullet} + OH^{-} + Cu^{2+}$$

Hydroxyl radical is the strongest oxidant generated in biological systems because of its extremely short half-life (Yu, 1994; Fantel, 1996). Highly reactive hydroxyl radicals readily react with a variety of molecules. In general, the harmful effects of excess ROS on the cell most often include damage of DNA, oxidation of polyunsaturated fatty acids (PUFA) in lipids (lipid peroxidation), oxidation of amino acids in proteins and oxidatively inactivate specific enzymes by oxidation of co-factors (Brenneisen et al., 2005; Valko et al., 2007). ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling. However, during times of environmental stress (e.g., UV or heat exposure), ROS levels can increase dramatically, which may result in significant damage to cell structures. This

accumulates into a situation known as oxidative stress. ROS are also generated by exogenous sources such as ionizing radiation. In recent years, a substantial body of evidence has accumulated supporting a key role for ROS in many fundamental cellular reactions and suggesting that oxidative stress may be important in the pathophysiology of common diseases including atherosclerosis, malignant diseases, chronic kidney disease and diabetes mellitus as well as in the aging process (Young & Woodside, 2001; Sies, 1991).

It has been shown that CKD patients are at substantially higher risk of cardiovascular diseases, atherosclerosis and cancer than age-matched subjects in the general population (Mandayam & Shahinian, 2008). In CKD patients, the efficiency of the antioxidative defense system declines with the progress of the disease and reaches its peak in the end-stage. The extent of oxidative stress can be slowed by increased efficiency of the natural antioxidant system (Meydani, 2002). Mammalian cells are protected against ROS by two lines: endogenous mechanisms (mainly enzymes) and exogenous low molecular weight compounds (free radical scavengers) (Joseph, 1995). The antioxidant enzymes are: superoxide dismutases (SOD), catalases (CAT) and glutathione peroxidases (GSH-Px) and probably selenoprotein P (Joseph, 1995; Zachara et al., 2006). The share of these three enzymes that work closely together in ROS neutralization is shown in Fig. 1.

SODs play a central role in catalyzing the spontaneous dismutation of superoxide producing O_2 and H_2O_2. Marklund (1984) studied SOD isoenzymes in human tissues and has shown that liver contains very little extracellular SOD and a lot of the other isoenzymes, whereas all isoenzymes are abundant in the kidney. Tissue homogenates from chronically rejected human renal allografts demonstrated decreased activity of MnSOD and increased expression of MnSOD protein (MacMillan-Crow et al., 1996). The molecular mechanisms involved in the induction of MnSOD during oxidative stress have yet to be elucidated.

Protection by SOD is incompletely achieved if H_2O_2 is not subsequently degraded. H_2O_2 accumulation, if not efficiently recycled, will lead to the appearance of the very aggressive $OH^{\bullet}$. Decomposition of H_2O_2 is the function of CAT, which generates oxygen and water (Joseph, 1995). However in many cells CAT activity is very low, and frequently unavailable for H_2O_2 dismutation. Thus, in most tissues, the degradation of hydroperoxides is effected by GSH-Px. This enzyme contains essential trace element Se at the active site and reduces H_2O_2 to water and organic hydroperoxides to alcohols. Two forms of GSH-Px have been identified in blood: cellular, called cytosolic or classical (GSH-Px 1), and extracellular (present in plasma; GSH-Px 3) (Arthur and Beckett, 1994). GSH-Px 3 is synthesized in the kidney and shows a significant diagnostic value in kidney diseases (Zachara et al., 2006).

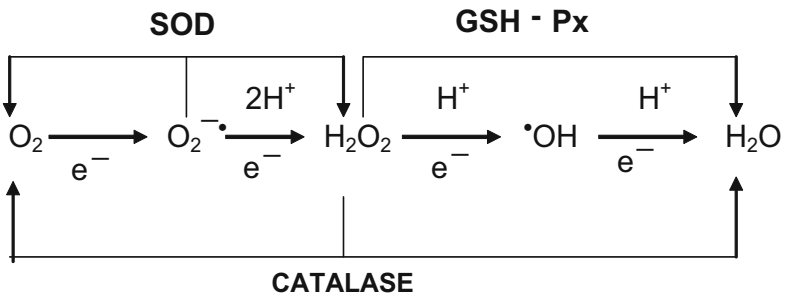

Fig. 1. Antioxidant enzymes. All three enzymes are not localized in the same compartments of the cells: SODs are either in the cytosol (Cu-Zn SOD) or in mitochondria (Mn SOD); GSH-Px is cytosolic; catalase is mainly found in peroxisomes (Adapted from Joseph, 1995).

Exogenous low molecular weight antioxidants include vitamins (A, C and E), carotenes, glutathione (GSH), uric acid, bilirubin and several trace elements (selenium, copper and zinc) (Young & Woodside, 2001, Joseph, 1995). The tripeptide GSH is generally considered an intracellular antioxidant and it plays a crucial role in the cells (Fantel, 1994). Being the most abundant antioxidant inside the cells, GSH can directly scavenge free radicals ($OH^{\bullet}$, $O_2^{\bullet-}$ and organic free radicals) (Yu, 1994) or act as a cosubstrate in the GSH-Px-catalyzed reduction of H_2O_2 and lipid hydroperoxides, a constituent of cell membranes (Loughrey et al., 1994; Zachara et al. 2005). Vitamin E is the most widely distributed antioxidant in nature. Among at least eight structural isomers of tocopherols, α-tocopherol is the best known and possesses the most potent antioxidant activity. It is highly effective in providing protection against membrane lipid peroxidation by reacting with lipid peroxyl radicals (Yu, 1994). In patients with CKD vitamin E level is reduced and supplementation suppresses oxidative stress. Similarly, vitamin C may be effective in reducing complication events in HD patients (Del Vecchio et al., 2011). In maintenance HD patients, plasma malondialdehyde (MDA), which is the terminal compound of oxidation of PUFA, is elevated (Boaz et al., 1999; Paul et al., 1993; De Vecchi et al., 2009). Loguhrey et al. (1994) have shown that in the serum of CKD patients MDA level is 42% higher, and in patients on HD – 76% higher, as compared with the control group. Hemodialysis did not change the elevated MDA concentration (Turi et al., 1991), however it is notable that HD combined with Vitamin E supplementation to patients prevented the breakdown of PUFA and reduced MDA level (Roozbeh et al., 2011). Vitamin E supplementation to patients on HD resulted in progressive increase in red blood cell vitamin E and a concomitant progressive decrease in MDA concentration, suggesting associated diminution in oxidative stress. However, vitamin E supplementation did not affect some other protective mechanisms, namely GSH and SOD (Cristol et al., 1997).

2. Selenium content and glutathione peroxidases activities in different stages of CKD

Numerous studies suggest that the formation of trace elements-containing compounds (mostly enzymes) and not the elements *per se* are critical to biological activities. Trace elements, Se, Cu and Zn play an essential role in the antioxidant defense system. They perform antioxidative functions through proteins in which they are incorporated (El-Bayoumy 2001). Se is a component of about 25 enzymes including the GSH-Px family, thioredoxin reductases and selenoprotein P which provide antioxidant protection against ROS-driven cancer initiation and promotion, as well as some others (Zachara et al., 2006; Brozmanova et al., 2010). The other two elements, Zn and Cu are incorporated *inter alia* into SOD. Cu is also a component of several other enzymes, for example, ceruloplasmine and cytochrome oxidase (L'Abbe and Friel, 1992). Cu deficiency leads to increased generation of free radicals and can promote lipid peroxidation (Lai et al., 1996). Zn is incorporated into many proteins, of which several hundred are enzymes (Valee, 2001). All mentioned enzymes participate in the metabolism of nucleic acids, proteins, carbohydrates and others.

In a healthy person the kidneys play an important role in the homeostasis of several trace elements, including Se (Wasowicz and Zachara, 1987). When Se is consumed at rates close to the human nutritional requirement, its highest levels are found in the kidneys and followed by the liver (Lockitch, 1989; Zachara et al., 2001c; Zachara et al., 2006). The Se level in tissues depends on its daily intake and the ingested chemical form. In people living in seleniferous regions the level is much higher than in those living in low Se areas e.g. New Zealand and

the Keshan region in China (Casey et al., 1982; Yang et al., 1989). Se is excreted from the organism mainly in the urine and feces, but urine contains 50-70% of the ingested element (Sanz Alaejos and Diaz Romero, 1993). Some authors (Yang et al.,1989) studied the relation between Se intake and urinary Se excretion in Chinese people living in different regions (low and high Se levels in the soil) and found a linear, significant correlation ($r = 0.886$; $P < 0.001$) between these two parameters. The amount of urine Se excretion increased progressively with increasing Se intake, but the form in which selenium was excreted in the urine remained unknown for a long time.

It was thought that the main form of Se excreted with urine is the metabolite called trimethylselenonium ion (TMSe) (Byard, 1969; Suzuki, 2005). In fact, this compound was isolated from rat urine and it represented 20-50% of Se excreted (Palmer et al.1970). Later on TMSe was identified in human urine (Francesconi and Pannier, 2004). Recent analytical advances based on HPLC separations coupled with atomic and molecular mass spectrometric detection have provided new insights into this area. New Se-containing carbohydrates (selenoshugars) are now known to be the major urinary metabolites in humans and in rats. The major metabolite is beta-methylseleno-N-acetyl-D-galactosamine (Suzuki et al, 2005). This selenosugar plateaus with a dose higher than 2.0 μg Se/mL water or g diet, and TMSe starts to increase in higher Se intakes, indicating that TMSe can be a biomarker of excessive and toxic doses of Se. TMSe is now considered to be a less significant metabolite (Francesconi, 2006). More recently it has been shown that after Se supplementation to mice the element is metabolized mainly in the liver and kidney (Suzuki et al, 2010). Excess Se was associated with selenosugar in the liver, transported to the kidney and the excess amount was excreted mainly as a selenosugar in urine. Quite recently French authors have identified, for the first time, a novel Se metabolite in human urine – Se-methylselenoneine (Klein et al., 2011). It cannot be ruled out that the use of other methods of analysis will discover more selenium compounds in urine.

2.1 Selenium concentration in the blood components of chronic kidney disease patients

Free radicals damage different tissues or organs, hence the trace and toxic elements behave differently in various organs. Available data suggested that in patients with CKD the levels of cadmium, chromium, copper, lead and vanadium are higher and the levels of selenium, zinc and manganese are lower than in healthy subjects (Tonelli et al., 2009). Among various important elements in CKD patients, Se is the focus of this study, therefore attention will be concentrated on this element and on GSH-Pxs present in blood.

Although some authors did not find any differences between Se concentration in the blood components between CKD patients and controls (Milly et al., 1992) many others found significantly lower values (for a review see Zachara et al., 2006). Several authors have shown a gradual decrease of Se level along with the progress of the diseases (Ceballos-Picot et al., 1996; Zachara et al., 2004). An example is shown in Fig. 2 (taken from Zachara et al., 2004a).

Several authors have shown that in non-dialyzed CKD patients the overall plasma Se concentration from incipient to the end stage of the disease is 12.5 to 44% lower than in healthy subjects (Ceballos-Picot et al., 1996; Richard et al., 1991; Zachara et at., 2001a). It has been shown that in the early stage of CKD, Se concentration in whole blood and plasma does not differ significantly from the values found in the control group (Ceballos-Picot et al., 1996; Zachara et al., 2001b). Along with the progression of the kidney impairment, Se concentration decreases in whole blood and plasma. This was particularly evident in the end-stage of the

disease, where Se concentration in whole blood and plasma was lower by 47 and 50%, respectively ($P < 0.0001$) as compared with the control group (Zachara *et al.*, 2000b).

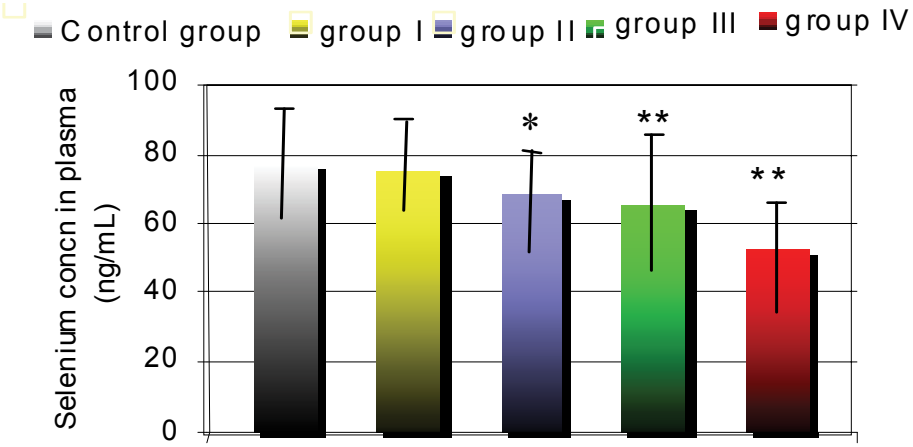

Fig. 2. Selenium concentration in plasma in controls and in different stages of CKD patients; (*p < 0.01; **p < 0.001 vs ctr.).

Methods of assessing the degree of renal damage in these patients include serum creatinine level, creatinine clearance and glomerular filtration rate (Krol and Rutkowski, 2008). The data presented in this paper are based mainly on the concentration of creatinine in plasma. Creatinine levels in CKD patients increase gradually with the development of the disease. Generally in CKD patients the concentration is several times higher than in healthy subjects (Zachara et al., 2004b; Rohun et al., 2011) and is highest in ESKD patients. In HD and in peritoneal dialysis patients the concentration is even higher (Zagrodzki et al. 2007; Mekki et al., 2010). Hemodialysis usually does not change the elevated concentration of creatinine (Mekki et al., 2010). Zachara et al. (2004a) have shown, in the entire group of CKD patients, a significant negative correlations between plasma selenium and plasma creatinine levels ($r = -0.380$; $P < 0.0001$; Fig. 3).

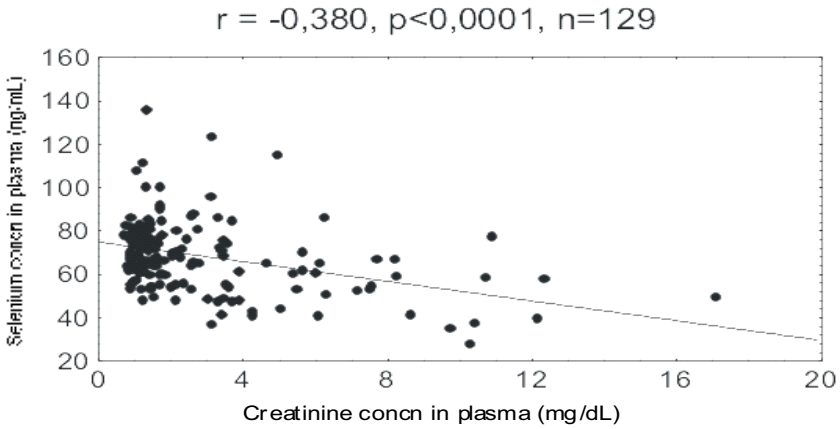

Fig. 3. Correlation between plasma selenium and ceatinine concentrations in control group and in different stages of chronic kidney disease patients (taken from Zachara et al., 2004a).

Schiavon et al. (1994a) studied 34 patients with different degrees of renal impairment, and found higher negative correlation between plasma selenium concentration and serum creatinine values ($r = -0.55$; $P < 0.001$). A negative correlation between Se concentration in plasma and creatinine clearance ($r = -0.47$, $P < 0.001$) was found by Ceballos-Picot et al. (1996). Ceballos-Picot et al. (1996) have shown that plasma Se concentration in patients on HD was more than 50% lower as compared with patients in advanced stage of the disease. However, many other authors found that the degree of Se reduction in dialyzed patients was similar to that observed in non-dialyzed patients who are in more advanced stages of the disease (for details see Zachara et al., 2006).

The biological significance of low Se concentrations in the blood is not fully understood, but severe deficiency leads to cardiomyopathy, as for example in the Keshan region in China (Ge et al., 1983). Lower concentrations of serum Se without severe deficiency have been associated with hypertension, heart disease and coronary disease in the general population, and with cardiomyopathy among dialysis patients. Mild Se deficiency appears to increase the susceptibility to oxidant stress, which may be relevant to hemodialysis patients in whom oxidative stress is markedly increased (Tonelli et al., 2009).

The question arises as to the cause of the low concentration of selenium in patients with CKD. Se levels in blood components are influenced by diet and food intakes, which are the principal source of this element. The lower Se levels in whole blood and plasma in uremic patients can be associated with the decreased intake of protein and increased protein loss with urine. Usually the patients are advised to consume limited amounts of protein to relieve uremic symptoms and to decrease the progression of kidney function (De Luis and Bustamante, 2008). Protein is the main source of Se, therefore the quantity of the element supplied is reduced (De Luis and Bustamante, 2008). Marchante-Gayon et al. (1996) indicated that the majority of Se (about 95%) seems to be bound to serum proteins. The most abundant selenoproteins in the blood components are selenoprotein P and GSH-Px (Ashton et al., 2009). Deagen et al. (1993) have shown that the Se is incorporated, in the form of selenocysteine (Sec), to selenoprotein P (≈ 50% of plasma selenium) and GSH-Px (which accounts for 10-30% of plasma selenium) or bound in the form of selenomethionine to albumin (≈ 23%). Very similar results have been obtained for blood plasma in healthy subjects by Koyama et al. (1999). They have shown a preference for supplemented Se to be taken up as selenoprotein P, the protein that incorporates up to 10 selenocysteine residues into the polypeptide chain (Persson-Moschos et al., 1995).

In CKD patients protein, and especially albumin concentration in plasma is lower than in healthy subjects (Dworkin et al., 1987; Zachara et al., 2004b; Zima et al., 1998). The precise cause remains unknown, but it is believed that among others the restricted diet, urinary or dialytic losses, impaired intestinal absorption, abnormal binding to Se transport proteins or drug therapy may all be responsible (Olson and Palmer, 1999; Vendrely et al., 2003; Bonomini and Albertazzi, 1995; Lockitch, 1989; Pasaoglu et al., 1996). Since protein foodstuffs contain the largest amount of Se, reduced protein intake seems to be a substantial cause of low Se concentration (Bonomini and Albertazzi, 1995). A strong positive correlation was noted between plasma total protein and albumin and Se concentration in healthy subjects and CKD patients (Dworkin et al., 1987; Zachara et al. 2004b). **The results of my group are presented in Fig. 4.**

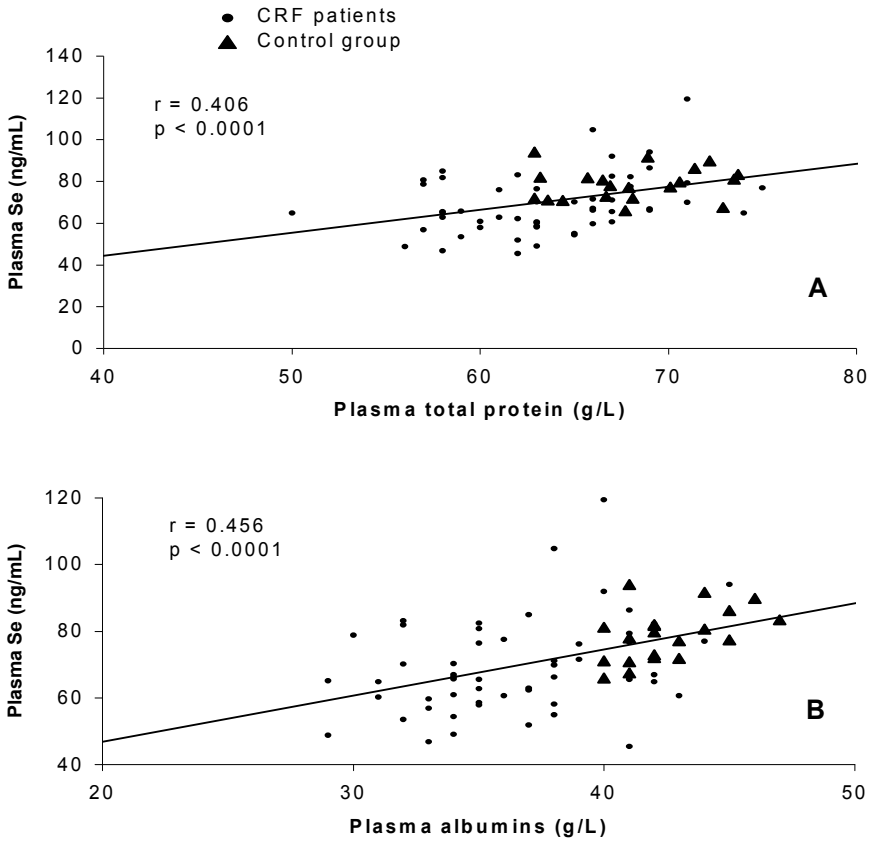

Fig. 4. The relationship between plasma total protein (A) and albumin (B) levels and plasma selenium concentrations in the control group (▲) and in patients at different stages of CKD patients (•) (taken from Zachara et al., 2004b).

3. Glutathione peroxidase activities in the blood of patients in different stages of chronic kidney disease and on hemodialysis

In human tissues, among about 25 selenoproteins (Papp et al., 2007), five are glutathione peroxidases: cytosolic (cellular, classical), (GSH-Px 1), gastrointestinal (GSH-Px 2), plasma (extracellular) (GSH-Px 3), phospholipid (GSH-Px 4) and sperm nuclei (GSH-Px 5) (Behne and Kyriakopoulos, 2001; Brigelius-Flohe, 2006). All of them can metabolize hydrogen peroxide and organic hydroxides (Arthur, 2000). Two of GSH-Pxs are present in the blood: GSH-Px 1 present in red blood cells and GSH-Px 3 present in plasma. Both have a tetrameric form and contain one selenium per subunit (or four gram atoms of Se per mole of enzyme) in the form of Sec (Maddipati and Marnett, 1987, Zachara, 1992). Se supply has been shown to induce the synthesis of these enzymes (Beilstein and Whanger, 1986). The relative position of the selenoproteins within the hierarchy is believed to reflect the relative biological importance. In the GSH-Pxs family the two extremes are represented by GSH-Px 1 and GSH-Px 3 , which disappear rapidly in even moderate Se deficiency (Brigelius-Flohe, 2006).

Cellular GSH-Px is present in all tissues, and was detected by Mills (1957) as an enzyme involved in the protection of red blood cells against oxidative hemolysis. It was the first protein with selenium in the polypeptide chain to be identified independently by Flohe et al., (1973) and Rotruck et al. (1973). Plasma GSH-Px was recognized as a distinct from the red cell enzyme. Later on it was purified and characterized from human plasma (Broderick et al., 1987). This enzyme is structurally, enzymatically and antigenically different from that in erythrocytes and other cells (Zachara, 1992). GSH-Px 3 is widely used as the marker of Se status (Papp et al., 2007).

Studies on red blood cell GSH-Px activity in CKD patients produced inconsistent results. Some authors found significantly lower (Zachara et al., 2001a; 2004a; Bulucu et al., 2000; Martin-Mateo et al., 1999), some - significantly higher (Ceballos-Picot et al., 1996; Mimic-Oka et al., 1992) and still others did not observe any differences (Zachara et al., 2004b; Temple et al., 2000). It can be concluded that bone marrow in CKD patients is not damaged and the synthesis of this enzyme does not deviate from the norm. In patients on dialysis the activity of this enzyme is similar as in ESKD (Ceballos-Picot et al., 1996).

In CKD patients plasma GSH-Px seem to be much more important than red cell enzyme. Therefore it is worthwhile to concentrate primarily on plasma GSH-Px in CKD patients. Studies on this enzyme have shown that although GSH-Px 3 is synthesized in a range of tissues, the renal proximal tubular epithelial cells are the main source from which it is secreted into plasma (Avissar et al., 1994; Whitin et al., 1998). Many reports indicate that patients with CKD have very low plasma GSH-Px activity (Yoshimura et al., 1996, Zachara et al., 2004a, Ceballos-Picot et al., 1996) including those undergoing HD (Yoshimura et al., 1996, Roxborough et al., 1999, Ceballos-Picot et al., 1996, Zachara et al., 2001a). Reports by several authors have shown that plasma GSH-Px activity in uremic patients is reduced by 34-52% as compared with healthy controls (Richard et al., 1991; Schiavon et al., 1994a; Ceballos-Picot et al., 1996; Yoshimura et al., 1996; Zachara et al. 2004a). Some of the authors indicated a gradual decrease in the activity with advancing stage of the disease. An example is shown in Fig. 5 B (Zachara et al., 2004a).

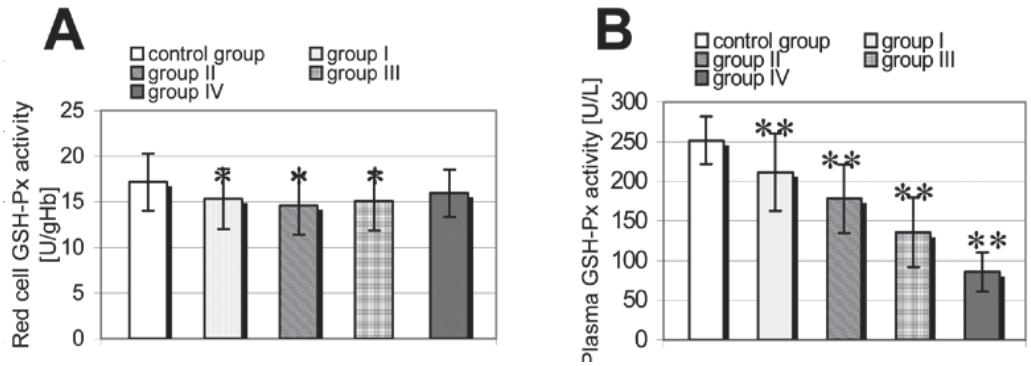

Fig. 5. Glutathione peroxidase activity in red blood cells (A) and plasma (B) in the control group and in patients with chronic kidney disease (CKD) in various stages of the disease. Patients were divided according to plasma creatinine (cr.) level: group I, incipient (n = 55) with cr. up to 1.36 mg/dL; group II, moderate (n = 40) with mean cr. level 1.72 mg/dL; group III, advanced (n = 40) with mean cr. level 3.96 mg/dL; group IV, end stage (n = 15) with mean cr. level 4.87 mg/dL. Statistics: *, $P < 0.01$ vs. control; **, $P < 0.0001$ vs. control (Adapted from Zachara et al., 2004b).

In contrast to the concentration of selenium, which does not differ in the initial stage of the disease from healthy subjects, the activity of GSH-Px already in the incipient stage was significantly lower ($P < 0.0001$) than in the control group. In the end stage of CKD, the activity of this enzyme decreased to one third of the value observed in the healthy subjects. A negative, statistically significant, correlation between plasma GSH-Px activity and creatinine concentration ($r = -0.657$) as well as between plasma GSH-Px activity and urea nitrogen ($r = -0.653$) in plasma was revealed (in both parameters $P < 0.0001$) (Fig. 6). A progressive decline in the activity of this enzyme is linked with the fact that GSH-Px 3 is primarily synthesized in the kidney and the progressing damage of this organ is reflected in diminished enzyme activity. Low GSH-Px 3 activity found in ESKD may depend on its synthesis in the residual, non damaged cells of the kidney and in other tissues/organs that synthesize small amounts of this enzyme. It has been shown that in humans this peroxidase is synthesized also in the liver, heart, lung and breast (Chu et al., 1992).

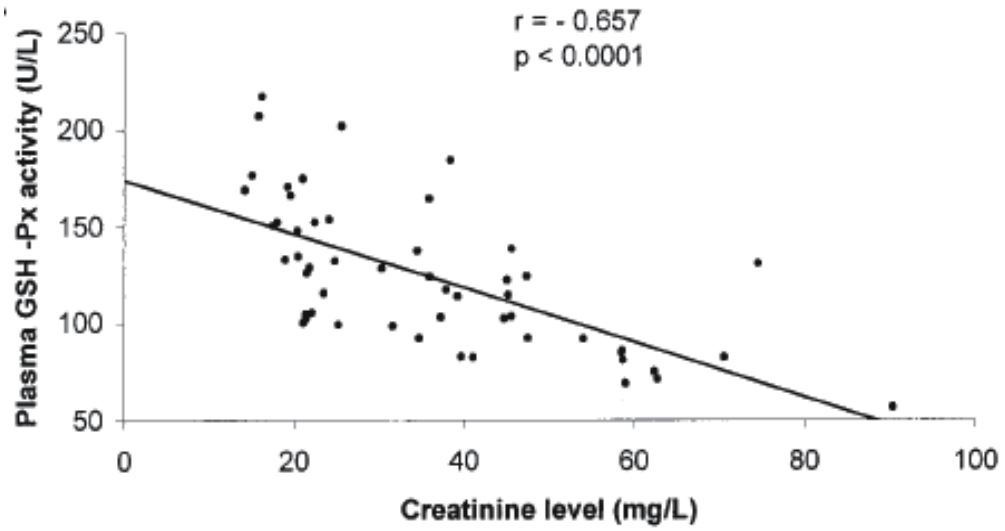

Fig. 6. Relation between plasma GSH-Px activity and creatinine level in CKD patients (taken from Zachara et al., 2004b).

4. The effect of selenium supplementation to CKD and HD patients on plasma GSH-Px activity and protein level

In healthy persons, Se supplementation leads to increased GSH-Px activity in red blood cells, plasma, other body fluids and tissues (Trafikowska et al., 1998; Iwanier and Zachara, 1995; Zachara et al., 1993). In plasma it reaches plateau value after 3-4 weeks of supplementation (Trafikowska et al., 1998; Iwanier and Zachara, 1995; Bonomini et al., 1995). In red blood cells, after Se supplementation, the GSH-Px activity increases much more slowly than in plasma (Trafikowska et al., 1998; Iwanier and Zachara, 1995) because of long life span of these cells. Thus plasma GSH-Px activity is a more sensitive index of short-term Se status, whereas red cell GSH-Px activity gives a long-term index status (Thomson and Robinson, 1980).

There is a dearth of publications on the effect of Se supplementation to CKD patients. Saint-Georges et al. (1989) were probably the first to supplement uremic, hemodialyzed patients

with sodium selenite administered orally. After supplementation (3 months with 500 μg/day and then for 3 months with 200 μg/day) plasma GSH-Px activity increased to a plateau after 16 weeks but remained about 30% lower than the control values. Studies of the effects of Se supply on plasma GSH-Px activity in patients on HD, carried out by various authors, have produced ambiguous results. Some authors show statistically significant increase in this enzyme activity after Se supplementation to dialyzed patients (Richard et al., 1991; Richard et al., 1993; Bellisola et al., 1994), others have shown slightly raised activity at the end of the HD session (Schiavon et. al., 1994a) or did not report any substantial differences (Zachara et al., 1994, 2001). Zachara et al. (2004b) supplemented CKD patients with Se in the incipient stage of CKD and in the end stage and have shown that in patients in the initial stage of uremia Se supplementation enhances plasma GSH-Px activity, whereas in the more advanced stages, the increase is much lower or does not occur at all. Schiavon et al. (1994a, 1994b) suggest that the measurement of plasma GSH-Px activity in CKD patients should be complemented by the determination of serum creatinine level and creatinine clearance, and may have a considerable diagnostic value, which means that this enzyme may be regarded as an additional marker useful in assessing the extent of CRF progress.

Yet there is no conclusive evidence whether CKD patients show a lowered plasma GSH-Px activity, or decreased synthesis of this enzyme. It is thought that endogenous, toxic compounds in the blood, which exert an inhibitory effect on this enzyme, are responsible for the decrease in GSH-Px activity. During dialysis, these compounds are removed so that the enzyme activity is enhanced. To explain whether this group of patients demonstrates a decrease in peroxidase activity or its synthesis, Yoshimura et al. (1996) determined simultaneously plasma activity and protein GSH-Px concentration in several non-dialyzed patients. In the study group, the enzyme activity was over 50% lower than in controls, and plasma concentration of GSH-Px protein was reduced or undetectable. The authors conclude that the decreased plasma activity of this enzyme in CKD patients was associated with its low concentration. The data reported by them (Yoshimura et al., 1996) show that the lowered plasma concentration of GSH-Px protein may be attributed to impaired biosynthesis of this enzyme in the kidney. The authors made other interesting observations. Since it had previously been thought that the decreased plasma GSH-Px activity in CKD patients may result from Se deficiency (Richard et al., 1991), they determined Se concentration in plasma and GSH-Px activity in red blood cells, and observed normal Se concentration and higher GSH-Px activity in red blood cells in patients than in the control group. The conclusion was that the lowered plasma GSH-Px activity cannot be attributed to blood Se deficiency. With regard to the concentration of GSH-Px protein, Roxborough et al. (1999) presented results completely opposite to those reported by Yoshimura et al. (1996). They obtained specific antibodies against GSH-Px of human plasma and determined plasma concentration of GSH-Px protein in the control group and in patients on dialysis. The significant findings were as follows: enzyme activity in patients before dialysis was over 50% lower than in healthy persons and it increased significantly after dialysis, but did not reach the value observed in the control group; secondly, before and after dialysis, plasma concentration of GSH-Px protein was at the same level, and, even more importantly, it did not differ from that observed in healthy persons.

Zachara et al. (2009) supplemented 30 patients on HD for 3 months with 200 μg Se/day (yeast Se) and have shown that plasma element concentration increased from 42 ng/mL (0 day) to 102 and 132 ng/mL after 1 and 3 months, respectively ($P < 0.0001$), but plasma GSH-Px protein level did not change significantly and was 11.4 μg/mL at the beginning (4.2 times

lower as compared with the control group – 48.4 μg/mL) and 11.8 μg/mL after 3 months. This level was similar to the group on placebo. These data show that Se supply to CKD patients on HD has no effect on the level of plasma GSH-Px protein (Fig. 7).

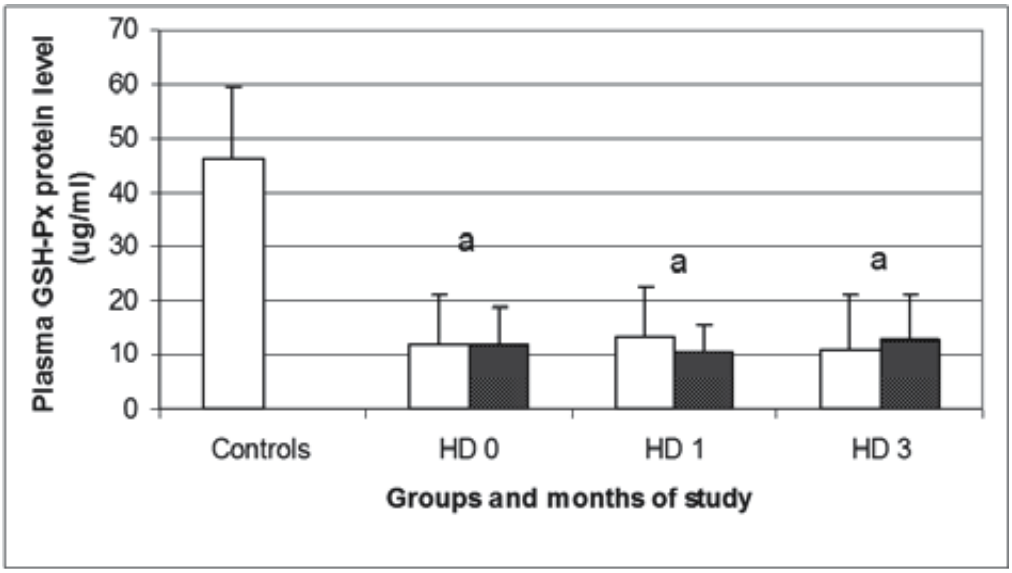

Fig. 7. Plasma GSH-Px protein level in healthy subjects and in CKD patients on HD supplemented with selenium and placebo. CKD patients on hemodialysis at the beginning of the study (HD 0) and after 1 and 3 months (HD 1 and HD 3, respectively) of Se or placebo supplementation. HD patients: white columns = placebo, black columns = + Se. Statistics: a, $P < 0.0001$ vs. controls (taken from Zachara et al., 2009).

Our results on the effect of Se supplementation to HD patients on GSH-Px protein level are the first published to date. Thus the question of the effect of Se on GSH-Px level in HD patients remains open and the matter requires further study.

5. Antioxidants in patients after kidney transplantation and the effect of Se supplementation

Kidney transplantation is one of the ways of kidney replacement therapy. Currently, kidney transplantation is the treatment of choice in patients with ESKD, showing higher survival rates than dialysis (Luque Galves et al., 2005). Kidney transplantation is now the only treatment that may restore plasma GSH-Px in patients suffering from ESKD. Data on Se concentration and GSH-Px activities, and some other parameters, in blood of patients after kidney transplantation is still rather scarce. To the best of my knowledge there were, except ours, two research centers reporting the results on the antioxidant status in patients in early stages after kidney transplantation: one in the United States (Avissar et al., 1994; Whitin et al., 1998) and one in Spain (De Vega et al., 2002; 2003). In this article I will focus on the GSH-Px.

In 1990 Avissar et al. (1994), searching for the source of plasma GSH-Px, studied the activity of this enzyme in the serum and plasma of anephric patients (cf. Zachara et al., 2006). They found that the activity was 22.6% of that noted in matched controls and was also significantly lower than plasma GSH-Px activity in HD patients. The authors also examined

GSH-Px activity and Se concentration in plasma of HD patients and controls. The patients' plasma Se levels were within the normal range, while GSH-Px activity of HD patients was 42% of the activity of the control group ($P < 0.001$). In this way the authors proved that the reduction in plasma GSH-Px activity could not be attributed to the lowered Se concentrations because: 1) the Se level in the patients' plasma was not significantly different from that in the controls; 2) when the patients were divided into two subgroups by Se levels (with the level below 96 ng/mL, and higher than 138 ng/mL), there was no significant difference in their plasma GSH-Px activity, and 3) the patients showed high (142% of the control value) red blood cell GSH-Px activity. In the sera of patients who had undergone transplantation, Se levels and GSH-Px activities were measured 22 and 30 days after surgery. Se level was the same as before transplantation, while plasma GSH-Px activity was eight times higher than before surgery and two times higher than in the controls. Several months after transplantation, plasma GSH-Px activity returned to normal values of healthy persons. Whitin et al. (1998), from the same center, transplanted kidneys in three groups of patients: 1) sixteen adults with renal disease who received a kidney transplant from related donors; 2) six adult patients who received cadaveric kidney transplants; and 3) three pediatric patients undergoing bilateral nephrectomy with subsequent kidney transplantation from related donors (cf., Zachara et al., 2006). Before transplantation, the HD patients had plasma GSH-Px activity of 34% (group 1) and 50% (group 2) of the control plasma, while the anephric individuals (group 3) had plasma GSH-Px activity ranging from 2 to 24% of that recorded in controls. After transplantation, plasma GSH-Px activity increased very rapidly: in group 1, the enzyme activity was two times higher three days after transplantation than before the operation, and 21 days post-transplant the averaged activities were within the normal values. In group 2, plasma GSH-Px activity increased rapidly over the first two weeks post kidney transplant. In six patients of this group, plasma GSH-Px activity reached normal levels after 9.8 days, and 27 days post-transplant the plasma enzyme activity reached a maximum level – 144% of the control value. The maximum level was temporary and after several weeks it decreased to a range similar to that of healthy individuals.

Our group studied the status of some antioxidants in the blood of patients before and in the early stages after kidney transplantation. I will focus my attention on some of the antioxidant parameters studied by us, namely on Se concentrations, red cell and plasma GSH-Px activities and some other parameters (Wlodarczyk et al., 2003). We found that whole blood and plasma Se concentration in patients before transplantation (79.2 and 64.3 ng/mL, respectively) was significantly lower as compared with healthy subjects (93.5 and 78.8 ng/mL, respectively; $P < 0.001$). The element concentration decreased significantly both in whole blood and in plasma ($P < 0.05$) within a week period after transplantation but returned to the values of the control group after 30 days. In the next two months there was a further, slight increase in Se concentration. Morris-Stiff et al. (2005) have shown that in 30, out of 40 patients before transplantation Se level in plasma was lower than in healthy subjects, but this was normalized in the majority of patients within 3 months. A decrease of Se in the first few days after surgery in our study is very likely associated with the restricted protein intake (the main source of Se) just after surgery. During the first 36-48 h after surgery, the patients received only fluids containing no protein or selenium. From the second/third day, a diet containing protein was introduced, so that the Se level increased slowly. GSH-Px activity in the red cells of patients before transplantation (15.3±3.6 U/g Hb) was the same as in controls (16.3±2.7 U/g Hb) and did not change

during the entire period of the study (it ranged from 15.3 to 16.3 U/g Hb). However, plasma GSH-Px activity of patients before surgery (76.7±20.6 U/L) was lower by 61% as compared with healthy controls (196±23.4 U/L $p<0.0001$). After transplantation, plasma GSH-Px activity increased very rapidly: by 53% after 3 days ($p<0.0001$), 90% after 7 days, and 2 weeks following surgery it almost reached the value of the control group (177 U/L). Two weeks later (at day 30 after transplantation) the activity exceeded the value of the control group. This higher activity was temporary and, similar to Whitin's study (1998), after several months it decreased to a range similar to that of healthy controls (Fig. 8).

Our results for the rapid increase in GSH-Px activity in plasma after kidney transplantation are in accord with the data of Whitin et al. (1998) who found that at 21 days post-transplant, the averaged plasma GSH-Px activity reached the normal value. De Vega et al. (2002, 2003) have shown that before transplantation plasma GSH-Px activity was – similar to our study – significantly lower than in the controls. The surprising results of these authors were that 48 hours following transplantation plasma GSH-Px activity decreased by 11% as compared to the pre-transplant value, whereas 7 and 14 days after surgery the enzyme activity increased by only 11 and 17% as compared to the initial value ($P > 0.05$). The cause of the decreased GSH-Px activity two days after transplantation and the very slow increase at a later stage remains unclear and open to speculation.

The plasma creatinine level, which in patients before surgery (6.93±2.29 mg/dL) was significantly higher ($p<0.0001$) than in the control group (0.8±0.1 mg/dL), decreased during the study and after 3 months it was 1.57 mg/dL. These data (plasma GSH-Px activity and creatinine concentration) indicate that the transplanted kidney takes up its function very rapidly. A highly significant negative correlation was found between creatinine level and plasma GSH-Px activity ($r = -0.588$, $p<0.001$).

According to my knowledge there are only few reports dealing with antioxidants in the blood of patients several months – several years following kidney transplantation. Simic-Ogrizovic et al. (1998) have shown that in recipients with stable kidney function 5 years post-transplant, plasma GSH-Px activity was the same as in the controls but in patients with chronic rejection, the enzyme activity was still significantly lower. Juskowa et al. (2001), studying the red cell GSH-Px activity several years after transplantation, found normal or decreased activity of this enzyme, depending of the medical treatment. Turan et al. (1992) studied, among other antioxidants, plasma GSH-Px activity in 30 kidney transplants 5 – 53 months (mean 19.9) after transplantation and found that the activity was significantly ($P < 0.001$) lower as compared with healthy individuals (3.40 vs. 10.43 U/L). Cristol et al. (1998) studied two similar groups of patients more than one year after transplantation and showed that in both groups plasma GSH-Px protein level was higher as compared with controls, but in the group with no clinical signs of chronic rejection the enzyme protein level was higher by 60% as compared with the group of histologically proven chronic rejection. Our results on rapid increase in plasma GSH-Px activity in post-transplant patients and the data on increased synthesis of plasma GSH-Px protein (Zachara et al., 2005, 2009) clearly show that the decreased plasma GSH-Px activity is most probably a consequence of impaired synthesis of this enzyme in the kidney. A significant negative correlation between plasma GSH-Px activity and creatinine concentration found in our study also support this supposition.

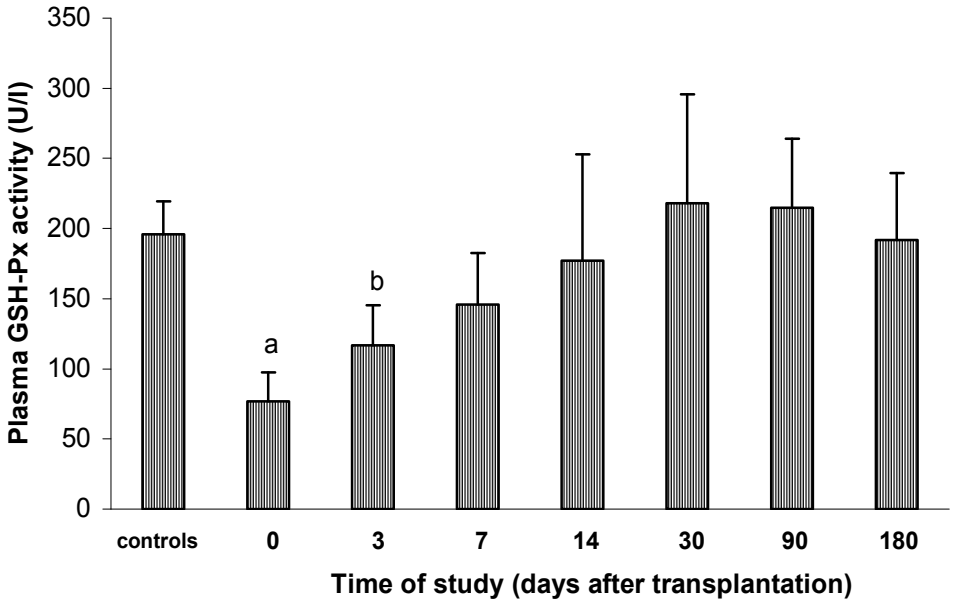

Fig. 8. Plasma GSH-Px activity in patients with ESKD before and after kidney transplantation and in healthy subjects. Please note a very rapid increase in the activity of this enzyme (taken from Wlodarczyk et al., 2003).

In a number of our patients hemodialysis was introduced almost immediately after surgery (Zachara et al., 2004c) because of the absence of kidney function. There were 15 men and 15 women aged 26-67 yrs (mean = 47.6 ± 11.3 yrs). The patients received grafts from 1 related living donor, and 29 kidneys from cadavers. All of them received the allografts for the first time. The mean serum creatinine concentration of patients was 7.72±2.35 mg/dl. The control group comprised 20 healthy volunteers (mean age, 43.0 ± 9.92 yr). It is interesting that in the group of patients who underwent two or more dialyses during the study, in the third day after transplantation (group 1; n = 19) plasma GSH-Px activity was significantly lower ($P < 0.02$) than in the group which only had one or did not require dialysis at all (group 2; n = 11). We examined those patients up to three months following surgery and the activity of this enzyme in the dialyzed group, during the entire period, was significantly lower ($0.05 < P < 0.01$) as compared with group 2 (Fig. 9, left part). On the contrary, creatinine concentration in group 1, from 3 to 30 days after transplantation, was significantly lower ($0.0001 < P < 0.01$) than in group 2. (Fig. 9, right part).

When calculating the relationship between plasma GSH-Px activity and creatinine level in the entire period of the study, we found a highly significance negative correlation ($r = -650$; $P < 0.0001$). This correlation was lower in subgroup 1 ($r = -472$; $P < 0.001$) and higher in subgroup 2 ($r = -0.676$; $P < 0.0001$).

While at present we have no convincing explanation for these disparate results, one can suspect that the main factor could be the immediate or delayed function of the transplanted kidney. In patients with immediate graft function and subsequent plasma creatinine level decline, plasma GSH-Px activity increases relatively fast and reaches the levels for healthy

subjects very soon after operation. On the other hand, when delayed graft function is noted, the increase in GSH-Px activity is much slower, as shown by different correlation factors between these two groups. A satisfactory explanation of this situation will require further studies.

The amount of Se in the diet in Poland and in some European countries is low and, consequently, the dietary element intake is below the recommended value. Diet is the main source of selenium and approximately 80% of dietary Se is absorbed depending on the type of food consumed (Navarro-Alarcon & Cabrera-Vique, 2008). Patients with CKD are advised to consume limited amounts of protein (the main source of Se). Thus, patients with ESKD have low Se levels in the blood. That is why our group wanted to check the effect of Se administration on blood GSH-Px activity in patients after kidney transplantation (Wlodarczyk et al., 2005).

To my knowledge there is only one publication on the effect of Se supplementation to patients after organ transplantation (Bost & Blouin, 2009). The authors have shown that in the plasma of patients before kidney and heart transplantation (month 0) Se levels were similar (94 and 96 μg/mL). The authors studied the effect of Se (as yeast-Se) supplementation on plasma element concentration in patients of kidney and heart recipients over a period of 3 years. They found that recipients who were supplied with 200 μg Se/day, after 12 and 24 months after surgery had the same level of Se in plasma (176 μg/L), and after 36 months the level was almost the same (182 μg/L plasma). This means that in those patients Se level reached a plateau after 12 months and was 1.9 times higher than at month 0 ($P < 0.0001$).

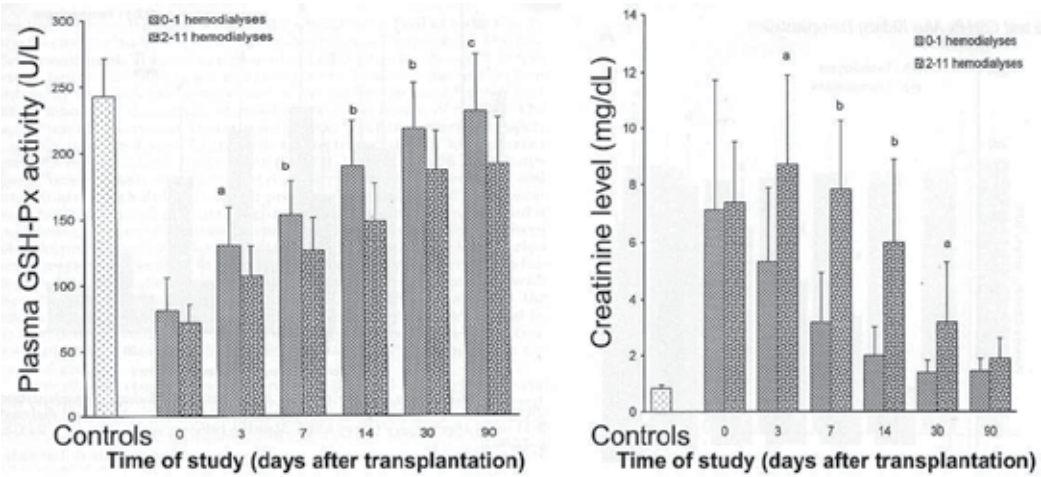

Fig. 9. (left part): Plasma GSH-Px activities before and after kidney transplantation in patients, who after surgery were not dialyzed (columns on the right) and those who had 2 – 11 HD sessions. Statistics between groups: a, $P < 0.02$; b, $P < 0.01$; c, $P < 0.05$. (right part): Creatinine levels in the same patients. Statistics between groups: a, $P < 0.01$; b, $P < 0.0001$.

Our study comprised thirty two patients: 17 were supplied with 200 μg Se/day (yeast-rich Se) and 15 patients were administered placebo. The study lasted 3 months. The data on plasma Se concentration and plasma GSH-Px activity are shown in Fig. 10. In the placebo group plasma Se concentration increased gradually from the 7th day and at day 90 it almost

reached the value of the control group, while in Se supplemented group at day 14 the level was the same as in controls and at day 90 it was higher than in controls by 39.0% ($P < 0.0001$). Kidney transplantation or Se supplementation to patients following surgery had no influence on red cell GSH-Px activity. During the entire period, in both groups (yeast-Se and placebo), this activity ranged from 14.5 to 15.7 U/g Hb and did not differ from the control group (16.7 U/g Hb; data not shown). In plasma, however, GSH-Px activity increased gradually in both groups, but after 30 and 90 days after transplantation in the Se supplied group the activity was significantly higher as compared to the placebo group. Se supplementation induces the synthesis of plasma GSH-Px in the transplanted kidney and, very likely, also in other tissues. It can thus be concluded that Se supplementation to kidney recipients has a positive effect on plasma GSH-Px activity and may be advisable in individuals affected with moderate impairment of kidney function.

6. The effect of selenium supplementation in hemodialyzed patients on the prevention of DNA damage in white blood cells

As stated in the introduction, oxidative stress, described as a disturbance in the prooxidant-antioxidant balance in favor of the former, leads to oxidative damage. It induces DNA damage, lipid peroxidation of PUFA in cell membranes, protein modification and others. Such effects are observed in CKD patients, especially in ESKD and in patients on HD. In those patients numerous diseases, such as cancer, cardiovascular complications, diabetes, atherosclerosis and neurological disorders are more frequent than in the age-matched subjects in the general population (Himmelfarb & Hakim, 2003). Many publications have shown that in patients undergoing HD some plasma antioxidant enzymes (mainly GSH-Px) activities are significantly lower (Zachara et al., 2000; Zachara et al., 2006; Yoshimura et al., 1996; Guo et al., 2009; Zwolinska et al., 2006). These enzymes, along with other antioxidants, protect the organism against ROS. ROS attack on DNA generates several modified DNA bases. The presence of these bases in cells may lead to mutagenesis. Such effects are observed in CKD patients, especially in ESKD and in patients on HD. Iseki et al. (1993) and Vamvakas et al. (1998) have shown that at the end-stage of CKD, the incidence of malignancies is higher than in the general population.. Kuroda et al. (1988) showed that in patients with diagnosed cancer of different sites (larynx, lung, stomach, liver, genitourinary tract) plasma Se concentration accounted for 99 ng/mL, whereas in the control group it was 145 ng/mL ($P < 0.001$). The evaluation of 15 extensive surveys indicated that in 10 surveys (72 484 patients at the ESKD) the cancer risk (the observed number of cancers compared with the expected number) was 7.6 (Vamvakas et al., 1998). In the majority of cases, tumors of kidney, prostate, liver and uterus were recorded. Although the pathogenic mechanisms of the enhanced incidence of cancer in CKD patients have not as yet been elucidated, Vamvakas et al. (1998) suggest that the impaired function of the immune system, reduced antioxidant capacity together with the increased ROS generation involved in DNA molecule damage and depression of DNA are the most essential factors. The diminished DNA antioxidative defense of the body leads to an intensified attack of free radicals on DNA molecules and finally to the development of malignant diseases (Ames, 1989). ROS induce various kinds of damage in DNA, e.g. oxidative damage and DNA strand cleavage (Wiseman & Halliwell, 1996). Hydroxyl radical reacts with DNA both with deoxyribose molecule, purine and pyrimidine bases (Fantel, 1996). The reaction of hydroxyl radicals with DNA results most frequently in damage to nitrogen bases, leading to the production of a

number of modified DNA bases, and finally to mutations. For example, 8-OH-guanine is a modified base. It may generate mutations contributing to a false reading of the modified base and its neighbouring bases. Transversion GC → AT is the most common change. Such a specificity of errors in 8-OH-guanine transcription plays a particular role in the mutation of gene that inhibits neoplastic changes (Fantel, 1996). Reactive oxygen species react both with nucleic and mitochondrial DNA (mtDNA). Human mtDNA is not shielded by histones, and thus it is easily damaged and the repair process is very slow (Shigenaga et al., 1994).

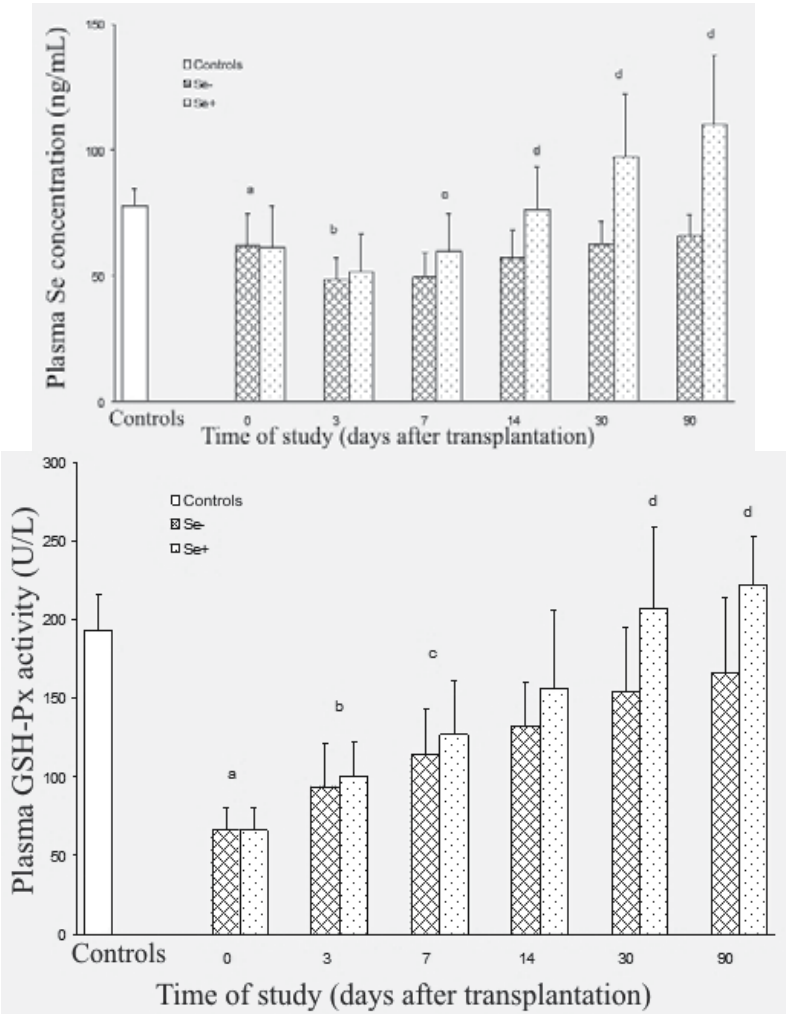

Fig. 10. Selenium concentration (left) and GSH-Px activity (right) in patients after kidney transplantation supplemented with 200 μg Se/day. Statistics: a, $P < 0.0001$ vs. controls; b, $P < 0.01$ vs. initial values; c (Se), $P < 0.02$ vs. placebo, and c (GSH-Px), $P < 0.0001$ vs. initial value; d, $P < 0.01$ vs. nonsupplemented group.

Except for the findings on frequent development of cancer in CKD patients listed above, reports on DNA damage in this group of patients are scanty in the literature. Lim et al.

(2000) investigated the frequency of mtDNA deletion in muscle sections taken from 22 CKD patients at the end-stage of the disease and in 22 healthy persons, free from any systemic diseases, matched by age and sex. Seventeen deletions (77%) were found in CKD patients and only five in the control group. These data show that mtDNA of skeletal muscles in CKD patients is extensively damaged and that these changes increase with age. Although the cause of DNA damage in CKD patients is still unknown, the authors (Lim et al., 2000) believe that accumulated uremic toxins, mostly of organic origin, are responsible for all the damage. These toxins stimulate ROS generation and damage cellular structures. ROS attack all structures, but mitochondrial genomes are thought to be most sensitive to the effect of free radicals.

The commonly used biomarkers of oxidative stress include measurement of oxidative damage to DNA (cf. Zachara et al., 2011). They can be assessed by determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) level in cellular DNA and the determination of urinary excretion of oxidatively modified bases/nucleosides (Dziaman et al., 2009). Worthy of attention is the fact that over the past decade another method – the comet assay – has become one of the standard methods for assessing DNA damage and repair (Collins, 2009). Being free of artifacts, it is one of the most sensitive and accurate methods, and it is also a valuable tool in assessing the role of oxidative stress in human diseases, and in monitoring the effects of dietary antioxidants (Collins, 2009). For that reason, we used the comet assay method which reflects the DNA content in the comet tail as well as the length of comet tail (Hartmann et al., 2003).

Nutrients constitute an important aspect of the antioxidant defense system with which humans have evolved (Ames, 2001). Selenium, as a nutrient, is the focus of this study and has, among others, two fundamental roles in cancer prevention: as a component of antioxidant defenses either as an agent able to scavenge free radicals or as an essential constituent of antioxidant enzymes such as GSH-Pxs (Combs, 1998; Schrauzer, 2003). Patients undergoing dialysis are at increased risk of many cancers, especially those of the kidney and urinary tract. Long periods of HD treatment are linked to DNA damage due to oxidative stress. In recent years, much attention has been paid to the role of selenium in the prevention of cancer of various organs (Valavanidis et. al., 2009; Reid et al., 2008; Gladyshev et al., 1998). Different forms of selenium compounds are administered to humans.

We did not find research in which selenium would be administered to persons with CKD on hemodialysis. Therefore we checked the effect of selenium supplementation in the prevention/repair of damaged DNA in patients with CKD on maintenance hemodialysis (Zachara et al., 2011). Forty-two CKD patients treated with regular HD were studied in a randomized, double-blind, placebo-control trials. Selenium (yeast-Se) was supplemented to 22 patients for 3 months with 200 μg Se/day, and placebo (beaker's yeast) was administered to 20 patients. The patients were dialyzed three times a week for 4 hours. The results were compared between the groups and with 30 healthy volunteers. Blood was taken from patients before the study and after 1 and 3 months, while from healthy subjects blood was taken only once. Several different parameters were analyzed, but here I will focus only on the results of DNA damage. DNA damage, including single-strand breaks (SSB) and alkali labile sites, were detected using alkaline single gel electrophoresis (SCGE; comet assay) according to the method of Singh et al. (1988) modified in our laboratory (Palus et al., 1999). The oxidative bases lesions in DNA were identified using formamidopyrimidine glycosylase

(FPG) enzyme which converts oxidized bases (Collins et al., 1997). The tail moment (tail length x tail % DNA/100) is the best indicator of DNA damage and will be presented here as the results achieved in the study.

Plasma Se concentration in patients at the beginning of the study (both groups taken together) was 23% lower compared with the controls (40.6 ng/mL vs. 52.7 ng/mL, respectively; $P < 0.0001$). In Se supplemented group it increased after 1 and 3 months to 94.6 and 115 ng/mL, respectively). In the placebo group there was no change in Se concentration throughout the study. Before the study the levels of single-strand breaks (expressed as the tail moment) were (in both groups taken together) before the study 3 times higher as compared with the control group (Fig. 11).

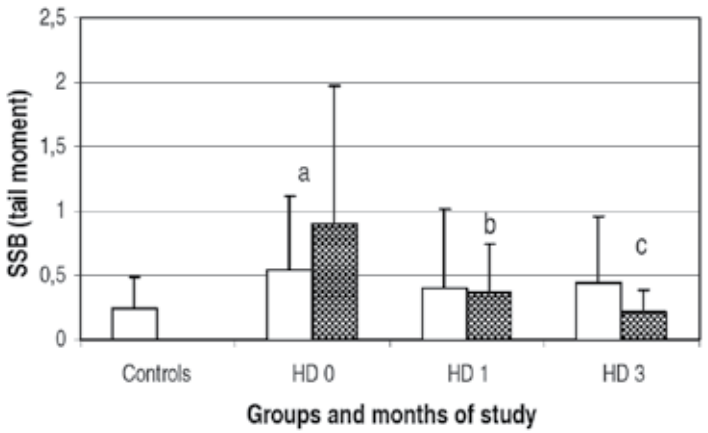

Fig. 11. Single-strand breaks (SSB) of DNA in white blood cells of healthy subjects and CKD patients on HD supplemented with placebo (white columns) and Se-yeast (black columns): HD 0 = start of the study, month 0; HD 1 and HD 3 = after 1 and after 3 months of tablets supply. Statistics: a, HD 0 both subgroups vs. controls, $P < 0.01$; b, HD 1 +Se vs. HD 0 +Se, $P < 0.02$; c, HD 3 +Se vs. HD 0 +Se, $P < 0.01$.

Almost the same values were obtained in relation to FPG: in patients they were 2.33 times higher than in the control group (Fig. 12). The higher, statistically significant levels of SSB and FPG in white blood cells of ESKD patients compared with healthy subjects at the beginning of the study shows that in those patients DNA is damaged, and that this may contribute to the development of cancer. The lower Se level in ESKD patients found in this study is probably responsible for DNA damage and may promote the development of cancer in such patients (Letavayova et al., 2006). Selenium supply in a dose of 200 μg/day led to the repair of damaged DNA. After 1 month the number of SSB was 2.4 times lower ($P < 0.02$) and after 3 months it was 4.3 times lower ($P < 0.01$). The FPG, after 3 months was 2.6 times lower compared with HD 0.

Epidemiological studies suggest that higher Se concentration reduces the risk of cancer (Letavayova et al., 2006) and increases DNA repair capacity in human fibroblasts damaged by H_2O_2 (Seo et al., 2002). Particular interest came from the clinical studies showing that dietary supply of organic Se, especially in the form of yeast enriched with Se, decreased the overall incidence of cancer twofold, above all of prostate, colorectal and lung cancers (Fisinin et al., 2009).

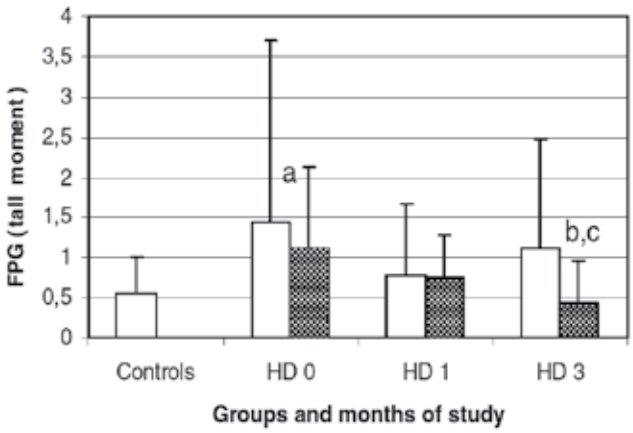

Fig. 12. Formamidopyrimidine glycosylase (FPG) in white blood cells of healthy subjects and CKD patients on HD supplied with placebo (white columns) and Se-yeast (black columns) at the beginning of the study (HD 0) and after 1 (HD 1) and 3 (HD 3)months. Statistics: a, HD 0, (both subgroups) vs. controls, $P < 0.01$; b, HD 3 +Se vs. HD 0 +Se, $P < 0.01$; c, HD 3 +Se vs. –Se, $P < 0.05$.

The data on the level of DNA damage in white blood cells of HD patients are in accord with the findings of Ribeiro et al. (2009) who have assessed the level of DNA damage by measuring the tail moment in various organs of Wistar rats in which the CKD was obtained by submitting the animals to 5/6 kidney mass ablation by ligation of kidney artery branches. The authors have also shown that CKD contributed to the damage of DNA not only in white blood cells but also in other tissues (liver, heart and kidney).

Interesting results concerning the protective effect of Se against oxidative damage to DNA in leukocytes of BRCA1 mutation carriers have recently been presented by Dziaman et al.(2009a). The authors demonstrated that BRCA1 deficiency contributes to 8-oxodG accumulation in cellular DNA which, in turn, may be a factor responsible for cancer development in women with mutations, and that the risk to developed breast cancer in BRCA1 mutation carriers may be significantly reduced in Se supplemented patients (300 μg/day) (Dziaman et al., 2009b).

The results of this study (Zachara et al., 2011) show that Se supplementation to HD patients reduced the level of DNA damage, as demonstrated by the progressive reduction of SSB and FPG in white blood cells. Longer term Se supplementation to patients on HD enhanced the protection of DNA against ROS, resulting in the reduction of the SSB and FPG levels. This dose (200 μg/day) and form (yeast enriched with Se) of the element was chosen because it had been shown that the inorganic form of Se is more toxic and less available than the organic form (Schrauzer, 2000). It is believed that the best form of Se for humans is the organic form – the selenomethionine (SeMet) (Schrauzer, 2001, 2003), and especially SeMet incorporated in yeast. It has been shown that SeMet enhances DNA repair and protects the cells against DNA damage (Laffon et al., 2010). Schrauzer (2009) has recently suggested that Se-yeast, in which the SeMet is protein-bound, shows a better effect than synthetic SeMet, since Se incorporated into protein is better protected from oxidation when exposed to air in the pure state. SeMet present in yeast is a protein-bound form, similar to the form normally present in food.

7. Conclusions

Patients with CKD and especially those treated by dialysis are at increased risk of many diseases and among others cancers, especially those of the kidney and urinary tract. Long periods of HD treatment are linked to DNA damage due to oxidative stress. It has been shown that in white blood cells of HD patients some biomarkers of oxidative damage to DNA [8-hydroxy-2′-deoxyguanosine content of leukocytes (8-OH-dG), and DNA strand breakage (alkaline comet assay)] are higher than in healthy controls. 8-OH-dG has been shown to have mutagenic properties. Reactions of ROS with DNA, proteins, and membrane lipids have been demonstrated and they can lead to mutations, inactivation of proteins and disruptions of cell integrity. Supplementation of Se to these patients resulted, as shown in this study (Zachara et al., 2011), in a significant decrease in DNA damage. The benefits of Se supply might be either through the prevention or repair of DNA damage, and they implicate at least one selenoprotein – GSH-Px 1 – in the process.

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7

A Systematic Review of the Effect of Vitamin C Infusion and Vitamin E-Coated Membrane on Hemodialysis-Induced Oxidative Stress

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1. Introduction

It is reported that oxidative stress plays an important part in the pathogenesis of cardiovascular diseases in chronic kidney disease (CKD) patients. This contributes to the increased cardiovascular morbidity and mortality in that group of patients. Clinical studies point to links between oxidative stress, inflammation, anemia and malnutrition (Panichi et al., 2011). The presence of inflammation and the length of duration of dialysis are the most important determinants of oxidative stress, which make them additional risk factors of cardiovascular disease. There are several deficiencies of antioxidant defense mechanisms which have been found in CKD patients. These include reduced levels of vitamin C, increased levels of oxidized vitamin C, reduced intracellular levels of vitamin E, reduced selenium concentrations, and deficiency in the glutathione scavenging system (Locatelli et al., 2003).

The main interest of this chapter is particularly focused on the effect of vitamin C infusion and vitamin E-coated membrane on hemodialysis-induced oxidative stress and, also setting this issue in the broader context of oxidative stress mitigation and the search for appropriate markers of oxidative stress.

The level of oxidative stress can be determined by several markers, because oxidants themselves have very short half-lives. Only lipids, proteins, carbohydrates, and nucleic acids modified by oxy-radicals are suitable as markers because of their long lifetime. According to the NKF KDOQI Clinical Practice Guidelines for Cardiovascular Disease in Dialysis Patients these markers include lipid peroxidation products (such as malonyldialdehyde (MDA), exhaled alkanes, oxidized low-density lipoproteins, advanced lipid oxidation products), oxidatively modified arachidonic acid derivatives (such as F2-isoprostanes and isolevuglandins); oxidatively modified carbohydrates (such as reactive aldehydes and reducing sugars); oxidatively modified aminoacids (such as cysteine/cystine, homocysteine/homocystine, 3-chlorotyrosine, 3-nitrotyrosine); oxidatively modified proteins (such as thiol oxidation, carbonyl formation, advanced oxidation protein products, 3-nitrotyrosine, advanced glycation end-products and oxidatively modified DNA (such as 8 hydroxy 2'deoxyguanine) (K/DOQI Workgroup, 2005). In the literature other oxidative stress markers are also mentioned, such as the lipid peroxidation products acrolein, 4-hydroxynonenal or thiobarbituric acid-reactive substances (TBARS); evaluation of

enzymatic anti-oxidant systems (erythrocyte content of SOD (superoxide dismutase) and GSH (glutathione), plasma levels of GSHPx (glutathione peroxidase)), non-enzymatic anti-oxidants (plasma levels of vitamin C, erythrocyte content of GSH and vitamin E) and inflammatory proteins (C-reactive protein (CRP), albumin) (Locatelli et al., 2003). With such a broad range of suitable markers of oxidative stress available, prioritizing them becomes difficult.

Several steps have been taken to minimize oxidative stress in CKD patients. They include:

1. supplementation of vitamin E

A daily supplement of 400–800 IU of vitamin E is recommended for secondary prevention of cardiovascular events and for prevention of recurrent muscle cramps (Fouque et al., 2007). In a double blind randomized trial, 60 hemodialysis patients divided into four groups received either vitamin E (400 mg), vitamin C (250 mg), both vitamins or a placebo daily for 8 weeks (Khajehdehi et al., 2001). Muscle cramps significantly declined in patients receiving both vitamins E and C (97%), vitamin E alone (54%), or vitamin C (61%) as compared with only 7% of placebo-treated patients (Khajehdehi et al., 2001). The SPACE trial tested the effect of vitamin E (800 IU/day) on a combined cardiovascular endpoint in 196 hemodialized patients with pre-existing cardiovascular disease, and showed a significant benefit from vitamin E supplementation (Boaz et al., 2003). However, the HOPE study showed no survival benefit of vitamin E in patients with mild to moderate CKD (Mann et al., 2004).

2. supplementation of vitamin C

Current recommendations for maintenance of hemodialysis patients advise supplementation with ascorbic acid 75-90 mg daily (Fouque et al., 2007) during dialysis. In addition to the potential benefits of vitamin C for anemia management, the importance of adequate vitamin C with regard to improving cardiovascular outcomes in hemodialysis patients is also the subject of research. A study by Deicher and colleagues of 138 incident hemodialysis patients examined baseline levels of plasma vitamin C and followed the cohort for occurrence of cardiovascular events (Deicher et al., 2005). Results showed that low total vitamin C plasma concentrations (less than 32 µmol/L) were associated with an almost fourfold increased risk for fatal and major nonfatal cardiovascular events compared with hemodialysis patients who had higher plasma vitamin C levels (greater than 60 µmol/L). On the other hand Nankivell and Murali reported oxalosis resulting in graft failure in a kidney transplant recipient who had been taking self-prescribed doses of vitamin C 2,000 mg daily as a dialysis patient for the three years prior to the transplant (Nankivell & Murali, 2008). Similarly, a case report by McHugh and colleagues (McHugh et al., 2008) describes mortality from vitamin C-induced acute renal failure. Vitamin C supplements appear to improve functional iron deficiency and hence the response to erythropoietin (EPO) (Keven at el., 2003). Vitamin C supplementation may help to relieve muscle cramps. In a double blind randomized trial the supplementation of vitamin C improved muscle cramps in 61% of patients as compared with only 7% of placebo-treated patients (Khajehdehi et al., 2001). In the study performed by Tarng DC. et al. vitamin C supplementation in chronic hemodialized patients resulted in the reduction of lymphocyte 8-OHdG levels and the production of intracellular reactive oxygen species (Tarng et al., 2004).

3. combination of vitamin C and E supplementation

In a double blind randomized trial the combination of vitamin C and E supplementation improved muscle cramps significantly in 97% of patients as compared with only 7% of

placebo-treated patients (Khajehdehi et al., 2001). In the Kuopio Atherosclerosis Prevention Study (KAPS) the supplementation of these two vitamins slowed the progression of carotid artery lesions (Salonen et al., 1995).

4. supplementation of acetylcysteine

In the randomized, controlled trial (Tepel et al., 2003) the antioxidant supplementation was associated with a reduced number of cardiovascular events in patients undergoing hemodialysis.

5. proper management of anemia

The literature is ambiguous with regard to anemia management. Red blood cells contain a high level of antioxidants. In their research, Siems W. et al. indicated that a rise in the red blood cell count may increase total antioxidative capacity (Siems et al., 2002). However, Drueke T. et al. in their study concluded that the intravenous injection of iron may induce an increase in protein oxidation and carotid atherosclerosis (Drueke et al., 2002).

6. modified type of dialysis membrane
7. high-flux hemodialysis

Ward RA. et al. showed the association between the use of high-flux hemodialysis and an improvement in some measures of protein oxidation (Ward et al., 2003). There is also evidence of reduced levels of cytokines such as IL-6 and C-reactive protein and a positive effect on oxidative stress of high-flux hemodialysis in comparison with conventional hemodialysis (Panichi, 2006).

2. Dialysis membranes

2.1 Modified type of dialysis membrane – the vitamin E-coated dialyser

The very first human hemodialysis therapy made use of a cellulose-based material with collodion tube membranes (Haas, 1888). Cellophane and Cuprophan membranes replaced collodion tube membranes later, due to their better performance In the 1960s regenerated cellulose was established as the principal membrane material. Dialysis with unmodified cellulose membranes is associated with such bioincompatibility phenomena as leukopenia, increased expression of adhesion molecules on leukocytes, and release of reactive oxygen species. Modifications in the structure of the hydroxyl groups in cellulose membranes led to the development of another type of membrane material, modified cellulose, with improved bioincompatibility. These modifications include replacement of the hydroxyl groups with other chemical structures, such as acetyl derivatives, or their bonding with different compounds, such as vitamin E or heparin.

Vitamin E, which is well-known for being a lipophilic antioxidant of cell membranes and lipoproteins (Traber & Atkinson, 2007), was introduced to the field of hemodialysis for the first time in the late 1990s to produce a cellulose-based vitamin E-modified dialyser (on the market as Excebrane dialyser, Asahi Kasei Medical Co., Ltd, Tokyo, Japan). It was used as a modifier (or coating agent) for the blood surface of cellulosic hollow-fiber dialyser membranes. In the last few years, studies have reported the positive effect of vitamin E-coated membranes on surrogate markers of oxidative stress and inflammation in hemodialysis patients (Cruz et al., 2008). Satoh M. et al. proved that vitamin E-modified cellulose dialysis membranes have a beneficial effect on reduction of oxidative stress (Satoh et al., 2001). Miyazaki H. et al. also found good results for endothelial dysfunction (Miyazaki et al., 2000).

Study	Study design	Sample size	Study duration [months]	Age (mean, range)	Males (%)	Mean duration of dialysis (mean) [years]	Membrane type (dialyser name)
Buoncristiani 1997	Single arm	10	1,0	67	40	4,9	• Excebrane (CL E 15) Low flux
Sommerburg 1999	Single arm	10	1,5	51	70	NR	• Excebrane (CL E) NR
Mune 1999	RCT	25	24,0	57	44	7,0	• Excebrane (CL E) Low flux
Bonnefont-Rousselot 2000	Single arm (cross over with conventional hemodialysis)	12	3,0	50	67	1,5	• PS, AN69, PMMA, CTA – High flux • Excebrane (CL E) High flux
Galli 2001	RCT	15	One single HD session or after 3 months	64	50	2,6	• CU (CL 15NL) Low flux • Excebrane (CL E 15NL) Low flux
Eiselt 2001	RCT (cross over with conventional hemodialysis)	10	1 month or 3 month	63	NR	3,6	• CU (CL S Series) Low flux • Excebrane (CL E) Low flux
Mydlik 2001	Single arm	8	0,8	54	38	NR	• Excebrane (CL E) Low flux
Shimazu 2001	RCT	6	9,0	59	50	11,2	• Excebrane (CL E) High flux
Satoh 2001	Single arm	18	6,0	68	55	>3,0	• Excebrane (CL E) High flux
Tsuruoka 2002	Randomized crossover	10	10,0	55	NR	9,5	• Excebrane (CL E) High flux
Usberti 2002	Single arm	10	7,0	22–75[a]	55	1,5–17,0[a]	• Excebrane (CL E) Low flux
Triolo 2003	Single arm	10	12,0	65	60	7,2	• Excebrane (CL E) Low flux
Hara 2004	Single arm	13	12,0	62	31	13,1	• Excebrane (CL E) Low flux
Mydlik 2004	Single arm	14	3,0	43	50	NR	• Excebrane (CL E) Low flux

AN69, acrylonitrile 69; PMMA, polymethylmethacrylate; PA, polyamide; CU, cuprophane; CTA, cellulose triacetate; CL, Clirans series; NR, not reported; a, range

Table 1. All study characteristics, outcome measures and dialyser characteristics of all studies included in the Sosa 2006 (Bonnefont-Rousselot 2000, Eiselt 2001, Galli 2001 provided data about TBARS level).

Only one systematic review conducted by Sosa MA. et al. provided comprehensive data that the transfer of dialysis patients to a vitamin E-coated dialyser is associated with an improvement in circulating biomarkers of lipid peroxidation, which is of potential clinical benefit (Sosa et al., 2006). Fourteen articles were included: 11 single arm, one randomized crossover and two randomized controlled trials, with a total of 37 to 158 evaluable patients. Metanalyses were conducted for malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS), combination of MDA and TBARS, low-density lipoprotein (LDL) and oxidized-LDL. There were three studies from Europe with analysis of data for TBARS[1] – Eiselt 2001, Galli 2001 and Bonnefont-Rousselot 2000. All three studies had an arm where E-coated membranes were used (single arm cross over design - Galli 2001 and Bonnefont-Rousselot 2000, randomized controlled trial – Eiselt 2001) with a total of 37 patients. Mean patient ages ranged from 44 to 65 years with mean durations of dialysis ranging from 3 months to 2,6 years. The studies lasted from 1 to 3 months.
In this systematic review, all results were metanalysed, which seems not to be methodologically correct. The summary estimate of all of the three studies revealed a non-significant decrease in pre-dialysis TBARS level of -0,4 mmol/L (CI95% -1,2; 0,4). In a sensitivity analysis excluding the study by Galli 2001, the overall mean decrease in pre-dialysis TBARS level was similar (-0.6 mmol/L, CI95% -1,4; 0,2), and expressed extremely low baseline values. Bonnefont-Rousselot 2000 had a younger group of patients, a shorter duration of dialysis, and in addition, high flux dialysis was used. Additionally, the results of all three trials were presented for 3 different periods – after one session – Eiselt 2001, after 1 month – Galli 2001 and after 3 months - Bonnefont-Rousselot 2000.

Study	Unit and parameter	Excebrane	Control	P value	Assessment
Bonnefont-Rousselot 2000	µmol/L Median (range)	1,72 (1,02-3,0)	1,65 (0,75 - 2,25)	ns	Pre-dialysis level and after 3 months of HD
Eiselt 2001	µmol/L Mean ± SEM	3,9 ± 0,15	4,09 ± 0,14	ns	Pre-dialysis level and after single session of HD
Galli 2001	nmol/l - measured by HPLC and espressed as malonaldehyde equivalents Mean ± SD	4,8 ± 2,4	12,9 ± 5,2	<0,001	Excebrane hemodialysis in comparison with CLS hemodialysis after 1 month

HD, hemodilaysis; HPLC, high performance liquid chromatography; SD, standard deviation; ns, not statistically significant.

Table 2. Thiobarbituric acid reacting substances (TBARS) – mode of measure, units and p value results in Sosa 2006.

The overall conclusion from the systematic review (Sosa 2006) based on the MDA and TBARS results was presented as the standardized effect size. The mean decrease in these biomarkers of lipid peroxidation (from 13 studies) was statistically significant at -1.7 units (CI95%, -2.7, -0.7).

[1] This is only one possibile biomarker to compare Excebrane with the results of data from trials regarding the efficacy of vitamin C infusion and vitamin E-coated membrane hemodialysis.

2.2 Vitamin C infusion and E-coated membrane hemodialysis

In order to assess the influence of vitamin C infusion and E-coated membrane hemodialysis a systematic review was conducted. The primary sources used to identify clinical studies included a literature search in PubMed (last search on 11th March in 2011) limited to clinical trials on humans using the searched terms 'dialysis', 'hemodialysis', 'renal replacement therapy', 'membrane', 'vitamin E', 'vitamin C' and 'infusion', and a literature search in the Cochrane Central Register of Controlled Trials, using the same searched terms without period limitations. Additionally, study references from relevant articles were reviewed and the relevant articles themselves. Non-English language studies were excluded. All primary studies meeting the following criteria concerning study population, interventions and reference interventions and study types were included: study population - patients with chronic renal failure (or renal transplantation), hemodialysis. Both patients with and without history of cardiovascular disease were included. Interventions and comparators: vitamin C alone or combined with defined dosage and vitamin E-coated dialysers for hemodialysis patients. Acceptable reference technologies include either placebos or a dialyser without vitamin E coating, with biocompatibility similar to the vitamin E-coated dialyser. We searched the data published as randomized controlled clinical trials. No restrictions were placed on minimal study duration or sample size. The inclusion and exclusion criteria defined above were used to pre-select articles by screening titles and abstracts primarily thematically, and a full text version of articles that might meet the inclusion criteria was ordered. Two independent reviewers (T.M. and M.W.)[2] evaluated the abstracts for relevance to the study topic. The data were extracted if they examined the impact on changes in circulating pre-dialysis biomarkers of lipid peroxidation, including malondialdehyde (MDA), thiobarbituric acid reactive substances (TBARS) and other parameters. The laboratory methods used to determine the levels of these biomarkers were also evaluated. The initial literature search returned 118 citations from PubMed, 368 citations from the Cochrane database. A total of 44 citations met the initial screening criteria and were retrieved and evaluated. Out of those, 40 studies were excluded for the following reasons: 5 studies examined the effect of oral vitamin E and vitamin C supplementation, 13 studies examined only the effect of vitamin C supplementation with or without additional substances (oral or infusions in comparison with placebo), 14 studies focused on the biocompatibility of the Excebrane dialyser or other E-coated membrane dialyser, 3 citations were review articles, editorials or case reports, 1 study documented changes exclusively in pre-dialysis hemoglobin level and/or recombinant human erythropoietin (rHuEpo) dosing requirement, 1 study measured biomarkers of oxidative stress in vitro, 3 studies compared a different type of membrane without vitamin C infusion. Finally, 3 studies (2 fully published trials (Yang 2006, Eiselt 2001) and 1 conference abstract (Racek 1999) met the review criteria and examined the effect of the vitamin C infusion and E-coated membrane hemodialysis on biomarkers of oxidative stress. In these two trials patients were randomized to groups with or without vitamin E-coated membrane dialyser and with or without vitamin C infusion. The overall duration of individual studies ranged from one dialysis session to 12 weeks. Duration of dialysis ranged from 3 to 153 months (no data for Yang 2006). Male gender distribution ranged from 50 to 70%. Mean age is about 65 (no data for Yang 2006). Causes of end-stage renal disease were documented in 2 trials.

[2] M.W. – Magdalena Wladysiuk, T.M. – Teresa Malecka-Massalska

Author and year	Country	Study design	Study duration	Sample size	Age (mean, range)	Males (%)	Mean duration of dialysis (mean, range) [months]	Outcome measure
Eiselt 2001A	Czech Republic	Randomized controlled trial	Single dialysis session (before/ after)	24 (4 groups with 6 persons)	66 (41-85)	58	41 (8-153)	●TBARS ●AOC in plasma ●GSH in erythrocytes ●SOD in erythrocytes ●GSHPx in blood
Eiselt 2001B	Czech Republic	Randomized controlled trial	12 weeks	20 (2 groups – 10 patients)	64 (37-79)	50	32 (3-84)	
Yang 2006	Taiwan	Randomized controlled trial	8 weeks	80 (4 groups for 20 patients)	NR	69	NR	●Plasma H2O2 counts ●Blood ROS reactive oxygen species counts ●RH2O2 ●Vitamin C, Vitamin E level ●Total antioxidant status TAS, ●Plasma oxalate level ●metHb level ●PCOOH, ●C-reactive protein (CRP)
Racek 1999	Czech Republic	NR	NR	24	NR	LD	NR	●MDA level ●VC level ●GSH in erythrocytes ●SOD in erythrocytes ●GSHPx in blood

NR, not reported; AOS, antioxidant capacity; GSH, glutathione; GSHOx, glutathione peroxidase; SOD, superoxide dismutase; MDA, malondialdehyde; VC, vitamin C; RH2O2, reference H2O2; ROS, reactive oxygen species; TAS, total antioxidant status; TBARS, thiobarbituric acid reacting substances; metHb, methemoglobin; PCOOH, phosphatidylcholine hydroperoxide.

Table 3. The characteristics of the individual studies and outcome measures in the present systematic review.

Author and year	Type of intervention in group - membrane type (dialyser name)	Vitamin C infusion dosage
Eiselt 2001A	Protocol O – CL C15NL cellulose dialyser (Terumo Corp., Tokyo, Japan) Protocol OC - CL C15NL and vitamin C infusion Protocol E - CL E15NL dialyser (vitamin E-modified cellulose membrane Excebrane, Terumo Corp.) Protocol EC - CL E15NL dialyser (Excebrane) with vitamin C infusion	Vitamin C (acidum ascorbicum, Biotika, Czech Republic) in 20 ml of saline before the blood entered the dialyser. The infusion rate was 2.1 mg/min - 504 mg per dialysis.
Eiselt 2001B	1 group - HD with vitamin C during dialysis 2 group – HD without any treatment The CL C15NL membrane was used for the first 4 weeks, to be followed by the E membrane (4 weeks), and again CL C15NL for the last 4 weeks of the study.	
Yang 2006	1 group - HD with vitamin C infusion 2 group - HD with VE-coated dialyser 3 group - HD with VE-coated dialyser and vitamin C infusion 4 group - HD with neither 1 and 4 group - PSN (Polysynthane membrane, Baxter Healthcare Co., Deerfield, IL, USA) 2 and 3 group – VE-coated - EE18 Excebrane (Terumo Co., Shibuyaku, Japan)	1 g of ascorbic acid in 250 ml of saline infused over 4 h of HD
Racek 1999	No data about the division patients to groups - CL C15NL cellulose dialyser (Terumo) conventional or E-modified membrane – with or without vitamin C infusion	500 mg of ascorbic acid infused over 4 h of HD

HD, hemodialysis; PA, polyamide; CU, cuprophane; CTA, cellulose triacetate; CL, Clirans series; NR, not reported.

Table 4. Type of interventions in group, membrane type (dialyser name) and vitamin C dosage in the present systematic review.

Eiselt 2001 presented the data for TBARS level in the short- and long-term observational period. After single session no change in the plasma levels of TBARS was observed after the dialysis with vitamin E-coated membrane with or without vitamin C infusion. In single session dialysis a rise from the baseline was only significant at the end of dialysis in the group with a nonmodified membrane (Protocol O - without vitamin C infusion).

TBARS level in plasma	before HD		after HD		
μmmol/L	Mean	SEM	Mean	SEM	P value
O	3,95	0,11	4,26	0,11	p<0,002
OC	4,28	0,15	4,18	0,15	Ns
E	3,90	0,15	4,09	0,14	Ns
EC	4,05	0,16	4,06	0,15	Ns

HD, hemodialysis; O, cellulose membrane; OC, cellulose membrane and vitamin C infusion; E, vitamin E-modified membrane; EC, vitamin E-modified membrane and vitamin C infusion, ns – non statistical significance

Table 5. Thiobarbituric acid reacting substances (TBARS) in plasma before and after hemodialysis (HD) in the short-term period in Eiselt 2001.

For long-term data on predialysis TBARS levels were comparable in two groups with and without vitamin C infusion. A significant decrease in TBARS, regardless of whether or not vitamin C was infused, was demonstrated in the results with the vitamin E-coated membrane after 4 weeks of nonmodified dialysis. Additionally, there was a significant difference in the group with vitamin E-coated membrane and vitamin C infusion also with baseline (p<0,02). TBARS returned to baseline in both groups after switching patients to a nonmodified membrane. In Eiselt 2001 no changes over time or differences between the study groups were noted with the intracellular antioxidants glutathione, superoxide dismutase, and glutathione peroxidase.

Parameter and units		Eiselt 2001			
		E-coated membrane		E-coated membrane and vitamin C infusion	
Mean and range		Before	After	Before	After
GSH (mmol/L erythrocyte)	Single session	1,40 (1,11–1,9)	1,42 (1,01–1,96)	1,39 (1,07–2,13)	1,46 (1,01–1,77)
	1 month	1,82 (1,13–2,05)	1,66 (1,31–1,90)	1,71 (1,41–2,02)	1,62 (1,36–2,41)
SOD (IU/g Hb)	Single session	908 (603–1 217)	920 (595–1 394)	958 (677–1 235)	930 (623–1 239)
	1 month	1 014 (893–1 284)	946 (873–1,469)	1 040 (854–1 269)	972 (771–1 383)
GSHPx (IU/g Hb)	Single session	40,2 (29,0–54,9)	38,8 (29,5–63,1)	36,9 (26,2–61,3)	37,0 (28,2–61,1)
	1 month	33,1 (21,8–73,7)	33,65 (21,7–72,1)	40,6 (19,8–51,9)	44,6 (25,2–51,3)

Table 6. Glutathione (GSH) and superoxide dismutase (SOD) in erythrocytes and glutathione peroxidase (GSHPx) in blood in the short-term and long-term study in Eiselt 2001.

In Racek 1999 conventional dialysis without vitamin C infusion was only significantly connected with increasing MDA level (from 3,8 0,5 to 4,2 0,6 μmol/l). Switching to vitamin

E-coated membrane did not increase the level of MDA neither with or without vitamine C infusion. The intracellular antioxidants glutathione, superoxide dismutase, and glutathione peroxidase during the study did not change in any of the group.

2.3 Vitamin E-coated polysulfone membrane

Lately, in its search for biocompatible biomaterials, hemodialysis technology has introduced a vitamin E-coated dialyser using polysulfonemembranes to the market (VitabranE ViE, Asahi Kasei Kuraray Medical Co., Tokyo, Japan). A significant reduction in plasma C-reactive protein in hemodialysis has been observed with the vitamin E-coated dialyser using polysulfonemembranes (Cruz et al., 2008). A reduced need for erythropoietin-stimulating agents (ESAs) has also been reported with a vitamin E-coated dialyser using polysulfonemembranes. This may be due to the longer red blood cell survival resulting from reduced oxidative stress and inflammation (Cruz et al., 2008). This new vitamin E-coated polysulfone membrane has been demonstrated to have a redox capacity and to actively react with redox-active substances in the blood (Floridi et al., 2009). It has also been reported that the vitamin E-coated polysulfone membrane combines the antioxidant and antithrombotic activities of α-tocopherol with the biocompatibility of polysulfone membranes, which have excellent clearance and permeability properties (Sasaki, 2006). Evidence show that vitamin E-coated polysulfone membrane plays a great role in the management of uremic anemia. However, there is a limited number of preliminary studies on that area. For example, one preliminary study published as a pilot study states a positive effect of vitamin E-bonded membranes on anemia in hypo-responder dialysis patients (Andrulli et al., 2010). Another study shows new positive effects of vitamin E-coated membranes on blood pressure level in dialysed patients (Matsamura et al., 2010). Recently, there are findings that these membranes may play an effective role in the management of uremic anemia and lead to a reduction in the chronic low-grade inflammatory response of patients with uremic syndrome (Panichi et al., 2011).

3. Discussion

In patients with CKD, markers of oxidative modification of lipids (Handelman et al., 2001; Oberg et al. 2004) and proteins (Nguyen-Khoa et al., 2001) are present at high levels. Moreover, various markers of oxidative stress seem to be associated with higher mortality and cardiovascular disease in dialysis patients (Bayes et al., 2005; Descamps-Latscha et al., 2005). When talking about the oxidative stress among hemodialyzed patients, there is a question that should be taken into great consideration; that is, what are the most appropriate biomarkers in relation to the disease state. The relevant biomarkers can be divided into three groups: enzymatic (superoxide dismutase, catalase, glutathione peroxidase), non-enzymatic (gluthatione, vitamin E, vitamin C, ferritin, transferrin, albumin) and the third group of lipids, proteins, carbohydrates and nucleic acids modified by oxy-radicals. None of these groups seem to be perfect for assessment of oxidative stress levels. The markers from the enzymatic group have a half-life of seconds, which makes their determination in vivo infeasible (Locatelli et al., 2003). The level of markers from the second group may vary a lot due to malnutrition, anemia and inflammation among hemodialyzed patients, common problems in that group of patients. The markers from the third group

have lifetimes from hours to weeks. From the diagnostic point of view this makes them ideal, but their large number makes it difficult to prioritize any one as the most ideal for the hemodialyzed patient group. Giustarini et al. describes the characteristic of the "ideal marker" (see Table 7; Giustarini, et al., 2009). Upon reviewing these characteristics, it becomes clear that finding "the ideal and the most reliable one" for that group of patients would be almost impossible.

No	Characteristic of biomarker
1	Chemically stable molecule
2	Directly implicated in the onset and/or progression of disease
3	Specific for the reactive oxygen/nitrogen species in question
4	Non-invasive assessment
5	Low intra- and inter-variability in humans
6	accurate, precise, specific, sensitive, interference-free, and validated assays for quantitative analysis
7	Consensus and establishment of reference intervals and values
8	Consensus and establishment of animal models

Table 7. Characteristics of and requirements for suitable biomarkers of oxidative stress (Giustarini, et al., 2009).

A review of the literature offers examples of how different markers were used to examine the oxidative stress among patients with end-stage renal disease. This makes it difficult to compare research on the same subject, because studies that measure the same process with different, nonstandardized markers. Here are examples: 8-hydroxy 2'-deoxyguanosine (8-OHdG) is known as a product of DNA damage (Tarng et al., 2000) and a novel marker for the assessment of oxidative DNA damage in reactive oxygen species-mediated diseases (Kasai, 1997). Zocalli et al. found asymmetric dimethyloarginine (ADMA) to be a strong and independent predictor of overall mortality and cardiovascular outcome in hemodialysis patients (Zocalli et al., 2001). In the study of Kamgar et al., the panel of following markers of oxidative stress were used: plasma protein carbonyl, F-2 isoprostane, C-reactive protein and plasma IL-6 (Kamgar et. al., 2009). Interestingly, the authors of this paper came to the conclusion that the addition of a potent antioxidant cocktail to conventional vitamin supplements had no effect on the severity of end-stage renal disease-induced oxidative stress, inflammation, hypertension, anemia or nutritional disorders in hemodialysis patients (Kamgar et al., 2009). There are some studies where TBARS were used as markers of oxidative stress (Ramos et al., 2008; Galli et al., 2001) whereas others made use of advanced glycation end products (AGEs), free pentosidine (FP), protein-bound pentosidine (BP), autoantibodies against oxidized LDL (ox-LDL-autoantibodies (Baragetti et al., 2006), and MDA (Giardini et al., 1984). The table below summarizes different biomarkers of oxidative stress used among hemodialyzed patients (Giustarini, et al., 2009). The table was created on the basis of studies of human subjects published on PubMed between January and June 2006 (Giustarini, et al., 2009). This indicates how many biomarkers have been used to assess oxidative stress; these studies used different methods and took samples from different tissues.

	Precursor	Bio marker	Method	Tissue	Healthy	Disease	Reference
1	**CRF**	MDA	TBA spectrophotometry	Plasma	6,20 nmol/L	11,9	Ece et al., 2006
2	**HD**	MDA	TBA spectrophotometry	Plasma	2,29 µmol/L	4,36	Zwolinska et al., 2006
3	**CRF**	15-F2t-IsoP	ELISA commercial kit, Assay Design	Plasma	84,3 pg/mL	328	Cottone et al., 2006
4	**HD**	PCO	Fluoresceinamine spectrophotometry	Plasma	2,06 nmol/mg protein	2,51	Mera et al., 2005
5	**HD**	SOD	ELISA commercial kit, BenderMedSystm	Plasma	58,6 ng/mL	305	Pawlak et al., 2006
6	**HD**	GPx	EIA commer cial kit, Byoxy tech	Plasma	116 U/L	N.A.	Huerta et al., 2006
7	**HD**	Ab anti oxi-LDL	ELISA commer cial kit, Bio medica	Plasma	210 mU/ mL	380	Pawlak et al., 2006
8	**HD**	Oxi-LDL	ELISA anti-oxidized phosphate dylcholine	Plasma	0,2 ng/µg LDL protein	1,8	Sasaki, 2006
9	**HD**	4-HNE	HPLC UV/vis detection	Plasma	3,0 µmol/mg protein	7,6	Odetti et al., 2006
10	**HD**	AGE	fluorescence	Plasma	232 fluorescece intensity	746	Mera et al., 2005
11	**HD**	HMA	HPLC fluorescence detection	Plasma	55,7%	45,3	Mera et al., 2005
12	**HD**	HNA1	HPLC fluorescence detection	Plasma	36,5%	44,8	Mera et al., 2005
13	**HD**	HNA2	HPLC fluorescence detection	Plasma	7,8%	9,3	Mera et al., 2005
14	**HD**	Organic hydro peroxides	Ferrous oxidation xylenol orange assay spectrophotometry	Plasma	0,65 µmol/L	1,99	Zwolinska et al., 2006
15	**CRF**	GSH	Acid deprote inization/DTNB spectrophotometry	Whole blood	1684 µmol/L	938	Ece et al., 2006
16	**CRF**	SOD	NTB/riboflavin spectrophotometry	Red blood cells	3457 U/gHb	2072	Ece et al., 2006
17	**HD**	SOD	Epinephrine spectrophotometry	Red blood cells	1750 U/g Hb	1240	Zwolinska et al., 2006
18	**HD**	Gpx	NADPH/ GSH spectrophotometry	Red blood cells	83,2 U/gHb	38,4	Zwolinska et al., 2006
19	**CRF**	Catalase	H2O2 spectrophotometry	Red blood cells	1705 k/gHb	1212	Ece et al., 2006
20	**HD**	Catalase	H2O2 spectrophotometry	Red blood cells	0,49 U/gHb	0,36	Zwolinska et al., 2006
21	**CRF**	GSH	Acid deproteinization/ DTNB spectrophotometry	Red blood cells	14,2 µmol/gHb	20,5	Stepniewska et al., 2006
22	**HD**	MDA	TBA spectrophotometry	Red blood cells	5,86 µmol/L	11,34	Zwolinska et al., 2006
23	**CRF**	GR	GSSG/ NADPH spectrophotometry	Red blood cells	1,95 U/gHb	2,57	Stepniewska et al., 2006
24	**CRF**	G6PDH	NADP spectrophotometry	Red blood cells	2,54 U/gHb	4,91	Stepniewska et al., 2006

Table 8. Summary of different biomarkers of oxidative stress used among hemodialyzed patients on the basis of studies of human subjects published between January and June 2006 (Giustarini, et al., 2009).

AGEs, advanced glycation end-products; CRF, chronic renal failure; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); EIA, enzyme immunoassay; G6PDH, glucose-6 phosphate dehydrogenase; GPx, glutathione peroxidase; GSH, glutathione; GSSG, glutathione disulfide; GR, glutathione reductase; HD, hemodialysis; HMA, albumin reduced; HNA1, mixed disulfide between albumin an low molecular mass thiols; HNA2, SH groups in albumin oxidized to sulfenic, sulfinic, sulfonic acid; HPLC, high-performance liquid chromatography; 4-HNE, 4-hydroxy-2-Nonenal; MDA, malondialdehyde; N.A., not applicable; oxi-LDL- oxidized low molecular lipoproteins; NBT, nitroblue tetrazolium; PCO, protein carbonyls; SOD, superoxide dismutase; TBA, thiobarbituric acid; TBARS, thiobarbituric acid-reactive substances. (Giustarini, et al., 2009)

In the systematic review conducted in 2006 (Schnell-Inderst 2006) only two studies analyzed the clinical endpoints of cardiovascular disease, results that are meaningful to patients. Due to the absence of clinically-meaningful endpoints, the relevance of studies analyzing the effect of anti-oxidative vitamins on intermediate endpoints like oxidative stress markers is fundamentally limited. In 17 studies with intermediate endpoints, supplementation with vitamins was associated with a change of one or several of the examined endpoints in the expected direction, such as a decrease in concentrations of biomarkers for oxidative stress, an improvement of the lipid profile, a reduced progression of aortic calcification or decrease of intimate media thickness. Due to the methodological quality of studies (heterogeneity of population, lack of standardization for interventions, and others) and contradictory results, the statement that anti-oxidative vitamins can play any role in secondary prevention for cardiovascular disease among patients with end-stage renal disease is neither supported nor rejected. Additionally, there is no relevant data for primary prevention of cardiovascular disease.

In the present systematic review the main objectives was to find any evidence that vitamin C infusion added to the vitamin E-coated membrane dialyser could change the level of biomarkers. The Sosa 2006 systematic review and the results for Excebrane should be a reference's level to assess the effect of additional C infusion. Varying conclusions for the TBARS outcome drawn by individual studies in Sosa maybe due to methodological differences. The cross over design of the same group of patients with small and insufficient sample size, different baseline risk of patients (comorbidity, time of dialysis) and, especially, the lack of parallel randomized control did not allow for the control of confounders (Sosa 2006). Additionally, a lack of standardized measurement of biomarkers and the time of measurement (single session, 1 month or longer) of biomarker assays probably does not allow any metanalyses to be performed. In this case only a qualitative systematic review could be made with separate conclusions for each of the subpopulations or patient groups. Single-arm groups (from observational or randomized trials) were metanalyzed as a measure of baseline level and then change over the time proposed by the authors of included studies. Natural fluctuations of biomarkers and their changes over time due to other interventions (comorbidities, diet, stress, age) could bias the results. There are existing head–to-head trials of Excebrane directly with other dialysers which could provide more reliable data. The same problems with population, interventions and measured outcomes were raised by the present systematic review. Additionally, the number of biomarkers and the lack of an answer to the question of which of them is the most ideal indicator of oxidative stress prevents us from coming to the final conclusion regarding the effect of

vitamin C infusion and vitamin E-coated membrane on hemodialysis-induced oxidative stress.

For our systemic review, TBARS was used as oxidative stress marker in order to compare the effect of vitamin C infusion and vitamin E-coated membrane on hemodialysis-induced oxidative stress. TBARS and MDA are recommended by the National Kidney Foundation (NKF) guidelines to assess oxidative stress; however, they are not perfect measures of oxidative stress. MDA is a product of lipid peroxidation. During that process there are other end-products such as pentane, isoprostanes, and conjugated dienes (Esterbauer, 1993). The TBARS determination is the most-used assay to assess lipid peroxidation. In this assay, thiobarbituric acid (TBA) reacts with aldehydes to produce a relatively stable chromophore that can be quantified using either spectrophotometry or high-performance liquid chromatography (HPLC) (Pompella et al., 1997). What is worth noting is that there are several problems with the use of TBARS in human fluids. Griffiths et al. underlines that aldehydes other than MDA may react with TBA to produce derivatives that absorb light in the same wavelength range; degradation of fatty acids can occur during the analysis, leading to false information about the actual MDA content in the fluid before testing (Griffiths et al., 2002). Halliwell and Chirico add that the presence of metal ions can increase the rate of this decomposition, whereas metal-chelating molecules can decrease this rate, making reliability a problem. What is more, the routine use of different anticoagulants to prevent blood clotting, *i.e.,*EDTA or heparin, may yield different values even on the same blood sample, and most TBA-reactive materialin human body fluids, including MDA, is not a specific product of lipid peroxidation and may produce false-positive results (Halliwell & Chirico, 1993). These problems led some researchers to use an HPLC modification of the TBARS method. This approach uses HPLC to separate the MDA-TBA adduct from interfering chromophores, thereby resulting in improved specificity (Halliwell & Chirico, 1993). Lipid hydroperoxides and aldehydes can also be absorbed from the diet and excreted in urine. It follows that measurements of MDA in plasma or urine can be confounded by diet and should not be used as an index of whole-body lipid peroxidation unless diet is strictly controlled (Wilson et al., 2002). This illustrates the difficulty in assessing which method is the best for assessment of oxidative stress. Moreover, it also underlines the need for analytical methodologies which validate the stability of the biomarker and extraction yield, accuracy and precision, selectivity and specificity, robustness and limits of detection and quantitation (Shah et al., 2000).

Currently, there is no consensus regarding an ideal marker of oxidative stress. So far, a panel of markers might be a better solution than just one (Locatelli et al., 2003). In order to analyze the process of oxidative stress there is no doubt that biomarkers should firstly be adequately validated, as too many have been proposed and used. Only under these circumstances can adequate conclusions be drawn and compared.

4. Conclusion

The available evidence is not sufficient to support an additional effect of the anti-oxidative role (measured by TBARS level) of a vitamin C infusion added to E-coated membrane dialyser in comparison to the vitamin E-coated dialyser alone. Methodological differences among three studies which used TBARS as a biomarker of oxidative stress prevent any

significant conclusion regarding this supplementation from being made. These results together with the systematic review of Sosa 2006 have limited importance in light of the lack of evidence that measurement of some biomarkers can give conclusive results regarding oxidative stress in cardiovascular morbidity of dialysis patients.
Finally, finding the most appropriate single biomarker or the most appropriate panel of biomarkers of oxidative stress in dialysis patients should be the first and most crucial step taken in order to decrease methodological uncertainty in future research on the issue.

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8

The Role of Ultrasonographic Monitoring for Hip Joint Changes in Patients with Chronic Renal Failure

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1. Introduction

Chronic renal failure (CRF) is associated with life-threatening accumulation of metabolic products due to the inability of their elimination. On hemodialysis, toxic substances are eliminated from the site of their high concentration (blood) via semipermeable membrane to a lower concentration medium (dialysis fluid) by the principle of diffusion. Hemodialysis has been widely used since the end of the 1960s. Although hemodialysis significantly prolongs life expectancy in CRF patients, hemodialyzers cannot compensate for all kidney functions. Hemodialyzer function has been greatly upgraded by technical innovations, however, many kidney functions in the regulation of homeostasis such as hormone production, e.g., vitamin D active metabolite (calcitriol) and erythropoietin, and many other functions remain unfeasible. Kidneys are involved in mineral metabolism and are target organs for the action of parathormone. In CRF patients, bone metabolism impairment occurs at creatinine clearance of 50-60 mL/min. Therefore, prolonged duration of hemodialysis is associated with development of numerous complications, in particular those involving the osteoarticular system. These complications are due to osteodystrophy and dialysis related amyloidosis (DRA). Tenosinovitis, in particular involving finger and hand flexors, snapping fingers and joint contractures are frequently present, however, tendon rupture may also occur. Muscle atrophy leads to the loss of strength, inability to perform fine finger movements, with a reduced function and range of movements and joint contractures. The hands assume a typical shape of so-called amyloid hand, while fingers look like "guitar players" (Farrell & Bastani, 1997; Fitzpatrick et al., 1996). Pain, swelling, degenerative joint changes and effusions are bilateral and symmetric, and may vary from very mild through severe where movements are painful and limited (Kelly et al., 2007; Zhang et al., 2002). Shoulders, hips and knees are mostly involved, however, all other joints

may also be affected (Yamamoto et al., 2008). Soft tissue and joint lesions lead to dysfunction of the musculoskeletal system. Subchondral cysts are seen on the bones and erosions on the joints; pathologic bone fractures may occur in advanced stages of amyloidosis. The number and size of the cysts increase with the length of dialysis. Amyloid bone cysts may frequently be misinterpreted as tumors. On x-ray study, amyloid cysts can be differentiated from "brown" tumors. Whereas the former are found in distal part of the bone, frequently bilateral, multiple, well delineated and with sclerotic edges, located subchondrally or in the area of ligament junction, "brown" tumors are found in the are of long bone diaphysis. β2-microglobulin is a polypeptide, molecular weight of 11800 daltons, found on the surface of most nucleated cells. Lymphocytes and T cells play a crucial role in the formation of β2-microglobulin. In normal individuals, β2-microglobulin is found in tissue fluids, synovia, serum and urine, filtered via glomerular filtration, resorbed and degraded in proximal tubules. Reduced function of proximal tubules results in elevated β2-microglobulin level in urine. Elevated serum β2-microglobulin levels may be due to its enhanced synthesis, as in some inflammatory diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus, Sjögren's syndrome, Crohn's disease, cytomegalovirus infection, infectious mononucleosis, hepatitis C, HIV infection, chronic osteomyelitis), or malignant and lymphoproliferative diseases (multiple myeloma, β-cell lymphoma, chronic lymphocytic leukemia), or because of its decreased secretion due to reduced glomerular filtration in CRF patients. A number of factors are responsible for elevated β2-microglobulin level in the blood of hemodialysis patients. About 100-200 g β2-microglobulin or 2-4 mg/kg body weight are synthesized *per* day, its reference plasma values ranging from 0.73 to 3.81 mg/L (Al-Taee et al., 2003; Farrell & Bastani, 1997;). In CRF patients, there is no β2-microglobulin elimination, thus its concentration being 15 to 60 fold normal values (Motomiya et al., 2001; Ohashi, 2001).

In human body, β2-microglobulin is found in extracellular space, circulates as a free monomer and does not bind to plasma proteins (Connors et al., 1985). By polymerization, β2-microglobulin is transformed from soluble to insoluble form and accumulated in the form of amyloid deposits in various tissues causing structural and functional organ lesions. The complications that develop due to amyloid accumulation involve visceral organs and osteoarticular system in particular. The visceral form of amyloidosis involves numerous organs, e.g., heart, lungs, gastrointestinal system and urogenital system; subcutaneous amyloid deposits are quite common, in particular those involving gluteal region, and may also be found intradermally around hair follicles, sebaceous glands and blood vessels (Jimenez et al., 1998). In amyloidosis involving osteoarticular system, amyloid is accumulated between cells (in the interstitium) but rarely in blood vessels, whereas in case of visceral complications in CRF patients, subendothelial amyloid accumulation is seen with deposits in vascular walls to produce intraluminal protrusions in blood vessels. In the second half of the 1970s, carpal tunnel syndrome was observed to occur in hemodialysis patients due to accumulation of a new amyloid type (Kenzora, 1978; Warren & Otieno, 1975). In 1985, Geyo et al. demonstrated β2-microglobulin to be responsible for the development of a new type of amyloidosis in CRF patients (Denesh & Ho, 1998; Geyo et al., 1985; Tsvetkova et al., 2007). According to the World Health Organization 1993 nomenclature, amyloidosis in CRF patients is termed Aβ2m-amyloidosis. It is the most common complication in CRF patients. In the literature, a number of synonyms have been used for this type of amyloidosis, i.e. AB amyloidosis, dialysis amyloidosis, and dialysis related amyloidosis (DRA). As clinical manifestations of this complication occur in the advanced stage of the disease, it is necessary to detect it in

its preclinical stage. Therefore, the lesions should be detected and followed-up before the clinical signs of the disease occur, and properly controlled during treatment. There are several diagnostic procedures used to follow-up the lesions involving osteoarticular system, e.g., x-ray, computed tomography (CT), magnetic resonance imaging (MRI), scintigraphy, biopsy, and ultrasonography (US). Changes of the osseous and articular structures are analyzed by use of x-ray studies. Radiological signs of arthropathy are present in patients undergoing hemodialysis for more than 5 years (Tsvetkova et al., 2007). X-ray is a presumptive method, i.e. bone lesions can be identified even before pain occurs. Changes are seen as typically distributed subchondral cystic bone lesions, usually of thin and sclerotic edges, joint erosions, and possibly pathologic fractures. After 10-year hemodialysis, 50%-60% of patients show bone cysts (Fitzpatrick et al., 1996). X-ray changes are not seen in early stages of the disease. Distribution and extension of amyloid pseudotumors and pseudocysts of bones and joints, around joints, small areas of osteolysis or cortical bone erosion are visualized by CT, however, this study is associated with exposure to higher irradiation doses (Kiss et al., 2005). MRI offers useful data on bone, joint and soft tissue changes and particular organ involvement (Fukuda & Yamamoto, 2001; Kiss et al., 2005), however, MRI is a more expensive, less available and more time consuming method, and therefore less commonly employed in clinical routine. The usual noninvasive diagnostic studies include US, classic x-ray and MRI. However, these are nonspecific and low sensitivity studies. Scintigraphy is a specific noninvasive diagnostic method used to identify amyloidosis distribution and extension all over the body (Floege et al., 2001; Hawkins et al., 1990; Ketteler et al., 2001; Linke et al., 2000). Three scintigraphy methods are most widely employed: standard bone scintigraphy by use of Tc-diphosphonates; scintigraphy with iodine 123I labeled serum amyloid P component (SAP); and scintigraphy by use of specific protein precursors in Aβ2M amyloid (^{131}I-β2m). Biopsy is an invasive diagnostic method providing an insight into the local area lesions; however, it may be associated with periprocedural complications. Joint effusion puncture, rectal biopsy, abdominal adipose tissue biopsy (aspiration) and salivary gland biopsy are most commonly performed. Histologic changes of soft tissue develop far before clinical signs of the disease. Clinical signs of hemodialysis related amyloidosis occur in advanced stages of the disease. As clinical symptoms are nonspecific, they may easily be misinterpreted as other joint diseases. Patients undergoing hemodialysis for less than 5 years rarely show clinical signs of amyloidosis (Al-Taee et al., 2003). Carpal tunnel syndrome frequently occurs as the first clinical sign of amyloidosis (Ikegava et al., 1995; Shin et al., 2008). The number and severity of clinical symptoms are known to increase with the length of hemodialysis. After 15-year hemodialysis, clinical symptoms of hemodialysis related amyloidosis are present in 100% of patients (Al-Taee et al., 2003). Therefore, it is of utmost importance to follow-up the course of disease in its preclinical stage. There are direct and indirect diagnostic procedures used to demonstrate amyloidosis. An ideal diagnostic study should be noninvasive, repeatable, highly specific, inexpensive and widely available. Tissue biopsy is considered as the 'gold standard' to demonstrate amyloidosis, however, other diagnostic methods are also employed to reach the diagnosis. US has been widely accepted in the diagnosis of pathology, of the osteoarticular system soft tissues in particular. New US high-resolution devices with many technological innovations (e.g., offering the possibility of changing the frequency, focus, 3D image, using probes of various shape and size, movement analysis, i.e. real-time study, etc.) can measure morphological changes with precision of tenths of millimeter.

Hemodialysis patients mostly show lesions of soft tissues, in particular tendons and synovial sheaths, with frequent tenosinovitis and joint effusion. On US, muscle lesions can be visualized, thickness of tendons, ligaments and joint sheaths measured, lesions of para-articular structures observed, and extent of joint effusion followed-up; tissue vascularization can be followed-up by Doppler US. Although not highly specific, US is a presumptive, widely available, noninvasive, repeatable and inexpensive study free from ionizing radiation that can be used to follow-up the course of the disease, therapeutic effects and possible complications involving the osteoarticular system in CRF patients. US can be used as a control tool on therapy administration or puncture of joints, bursae, cysts, ganglia, or on tissue biopsy. The aim of the present study was to assess the impact of hemodialysis duration, patient age and β2-microglobulin concentration at the onset of hemodialysis on morphological changes in the hip region.

2. Patients and methods

Bilateral hip US using a 7-cm linear probe of 7.5 MHz was performed in 106 hemodialysis patients aged >18 to measure articular sheath thickness and articular effusion in both hips. During the procedure, the patient was in supine position, with the leg in neutral position. We used anterior longitudinal approach where the probe is parallel to femoral neck axis. Thickness of the hip joint sheath measured in the concave segment of the femur was determined as distance from the joint sheath inner to outer edge. Synovial effusion in the hip region was expressed as distance between anterior cortex of the femoral neck concave segment and inner edge of the hip joint sheath. A total of 424 US measurements were performed in the study group and 204 bilateral measurements in the control group. Control group consisted of 51 healthy subjects that underwent the same measurements of hip joint sheath thickness and articular effusion. Study patients were divided into three groups according to the length of hemodialysis (<36, 36-72 and >72 months) and age (18-50, 51-65 and >65 years). In study patients, serum β2-microglobulin concentration was determined at the onset of hemodialysis, and in control subjects before US examination. All laboratory tests were performed at Laboratory of Biochemistry, Požega General Hospital, in Požega, Croatia. Beta2-microglobulin concentration was determined by use of the AxSYM β2-microglobulin microparticle enzyme immunoassay (MEIA; Abbott GmbH, Wiesbaden, Germany). Blood samples were obtained from cubital vein by use of Vacutainer system. Blood sample was centrifuged for 10 min at 3500 rpm within 5 hours of venepuncture. Subjects with a history of previous injury or operative procedure on the study joints, inflammatory (chronic osteomyelitis, tuberculosis, HIV infection, hepatitis C), malignant or rheumatic diseases, systemic lupus erythematosus, sarcoidosis, Sjögren's syndrome, Crohn's disease, or lymphoproliferative diseases (multiple myeloma, β-cell lymphoma, chronic lymphocytic leukemia) were excluded. All study patients were undergoing hemodialysis with low-flux hemodialyzers. All examinations were performed by the author himself in order to prevent inter-examiner finding variability. All study subjects were informed on the study protocol, objectives and methods used, expected benefits, possible risks, etc. Inclusion in the study was voluntary and all subjects signed the informed consent form. Study protocol was approved by the Ethics Committees of the Požega General County Hospital, Dr. Josip Benčević General Hospital from Slavonski Brod, and School of Medicine, University of Zagreb from Zagreb, Croatia.

3. Statistical methods

Kruskal-Wallis test was used to test significance of differences between different age groups. In addition, differences between pairs of groups were tested with Mann Whitney U-tests. Spearman's rank correlation coefficient was used to test correlation between age and concentration of β2M. Data were presented as mean, minimum and maximum in tables. The chosen level of statistical significance was $p<0.05$.

4. Results

Thickness of the hip joint sheath and articular effusion were measured bilaterally in 106 hemodialysis patients and the measured values were compared with those recorded in 51 control subjects. Measurement results were compared according to the length of hemodialysis, body side, age, sex, β2-microglobulin concentration, and measured thickness of the hip joint sheath and articular effusion in control subjects. The mean age of patients undergoing hemodialysis at the time of examination was 64.0 (range 28.2-87.4) years. There were 55 male patients, mean age 60.5 (28.2-87.4) years and 51 female patients, mean age 67.9 (30.7-82.0) years. The mean length of hemodialysis in 18-50, 51-65 and >65 age groups was 16, 53 and 123 months, respectively, mean 56.3 months. In control group consisting of 51 healthy volunteers, the mean age was 62.6 (40.0-86.4) years. There were 25 male subjects, mean age 61.6 (40.0-86.4) years and 26 female subjects, mean age 63.6 (40.5-81.0) years. There was no statistically significant difference in age between hemodialysis patients and controls (P□0.05). The US measured thickness of hip joint sheath and articular effusion in control group according to age groups is shown in Tables 1 and 2, respectively.

		Hip joint sheath thickness (mm)		
Age (yrs)	n	M	Min	Max
18-50	7	5.36	3.90	7.50
51-65	21	5.33	3.90	7.30
>65	23	4.69	3.10	7.50
Total	51	5.04	3.10	7.50

n = number of subjects; M = mean (mm)

Table 1. Number of subjects, age, and hip joint sheath thickness in control group.

		Articular effusion thickness (mm)		
Age (yrs)	n	M	Min	Max
18-50	7	4.11	1.10	6.40
51-65	21	4.85	0.80	7.10
>65	23	4.44	2.10	7.00
Total	51	4.56	0.80	7.10

n = number of subjects; M = mean (mm)

Table 2. Number of subjects, age, and articular effusion thickness in control group.

The US measured thickness of the hip joint sheath and articular effusion using anterior longitudinal approach with the probe parallel to the femoral neck axis is presented in Figure 1.

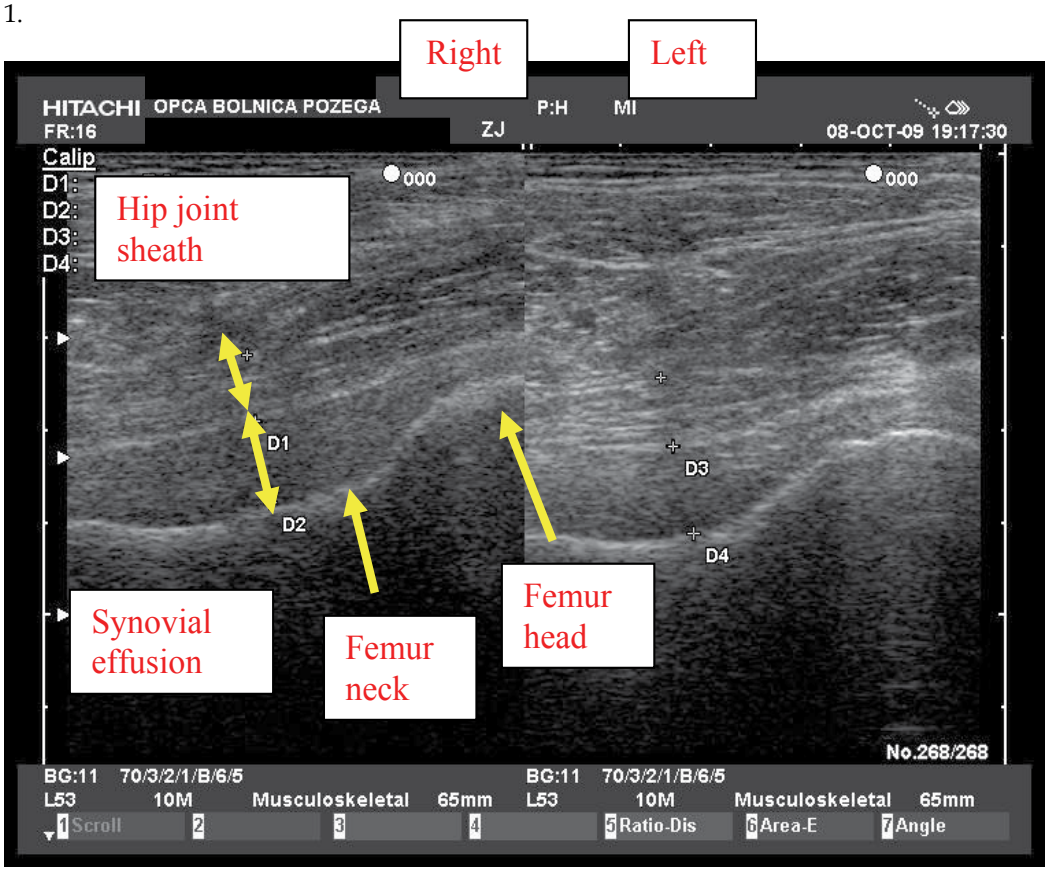

Fig. 1. US image of the hip joint sheath and intra-articular effusion.

The US measured thickness of hip joint sheath according to the length of hemodialysis and patient age is presented in Table 3 A and B.

Age (yrs)	Length of hemodialysis											
	<36 months				36-72 months				>72 months			
	n	M	Min	Max	n	M	Min	Max	n	M	Min	Max
18-50	8	5.30	3.90	6.50	5	6.32	5.10	7.40	3	7.93	6.70	9.00
51-65	10	5.45	4.00	6.90	9	6.44	5.00	8.70	7	6.86	4.20	8.50
>65	24	5.53	3.10	7.10	23	6.70	5.10	8.00	17	7.20	3.30	10.3
Total	42	5.47	3.10	7.10	37	6.59	5.00	8.70	27	7.19	3.30	10.3

n = number of patients; M = mean (mm)

Table 3A. Number of patients and hip joint sheath thickness according to patient age and length of hemodialysis.

Age (yrs)	Length of hemodialysis			
	Total			
	n	M	Min	Max
18-50	16	6.11	3.90	9.00
51-65	26	6.17	4.00	8.70
>65	64	6.39	3.10	10.3
Total	106	6.30	3.10	10.3

n = number of patietns; M = mean (mm)

Table 3B. Number of patients and hip joint sheath thickness according to patient age and length of hemodialysis (total).

Simple analysis of variance for independent samples with the length of hemodialysis (categorized into three groups) as independent variable, and hip joint sheath thickness and articular effusion as dependent variables was performed to assess the impact of hemodialysis duration on thickness of the hip joint sheath and articular effusion. A statistically significant difference was found in thickness of the hip joint sheath and articular effusion between groups of patients with different length of hemodialysis, as indicated by the results of simple analysis of variance: joint sheath thickness F=21.319; df=2/103; $P<0.01$ on the right; and F=21.011; df=2/103; $P<0.01$ on the left; and articular effusion: F=3.227; df=2/103; $P<0.05$ on the right; and F=4.479; df=2/103; $P<0.05$ on the left. Subsequent Tukey HSD test yielded a statistically significant difference in the hip joint sheath thickness among the three groups of hemodialysis patients. A statistically significant difference was found in the hip joint sheath thickness between the groups of patients with the length of hemodialysis <36 months and 36-72 months, <36 months and >72 months, 36-72 months and >72 months ($P<0.01$ all). Thickness of the hip joint sheath increased with the duration of hemodialysis. These differences were statistically significant on both the left and right side of the body. Subsequent Tukey HSD test yielded a statistically significant difference in hip joint intra-articular effusion between the groups of patients on hemodialysis for <36 months and >72 months ($P<0.05$). The magnitude of hip joint intra-articular effusion was significantly lower in the group of patients on hemodialysis for <36 months. Accordingly, the length of hemodialysis influenced the magnitude of articular effusion. Thickness of the hip joint sheath and articular effusion increased with the length of hemodialysis. Differences were statistically significant bilaterally. Comparison of the hip joint sheath thickness and articular effusion according to body sides yielded no statistically significant difference, as indicated by the nonsignificant t-ratio (joint sheath thickness: t=1.739; df=105, and articular effusion: t=0.633; df=105; $P>0.05$ both). Hip joint sheath thickness and articular effusion were compared according to patient sex by use of t-test for independent samples. A statistically significant sex difference was found in both hip joint sheath thickness and articular effusion bilaterally, as follows: hip joint sheath thickness t=2.479; df=104; $P<0.05$ (right) and t=3.138; df=104; $P<0.01$ (left), and articular effusion t=2.075; df=104 (right) and t=2.332; df=104 (left); $P<0.05$ both. The measured thickness of the hip joint sheath and articular effusion were greater bilaterally in male than in female patients. Simple analysis of variance for independent samples with age (categorized into three groups) as independent variable, and hip joint sheath thickness and articular effusion as dependent variables was

performed to assess the impact of age on hip joint sheath thickness and articular effusion. The analysis of variance yielded the following results: F=0.437; df=2/103 (right) and F=0.496; df=2/103 (left); *P*>0.05 both. There was no statistically significant difference in the hip joint sheath thickness among the three age groups on either side of the body. The same analysis of variance was also performed with various age groups and magnitude of articular effusion in the hip region, with the following results: F=3.252; df=2/103 (right) and F=3.023; df=2/103 (left); *P*>0.05 both. There was no statistically significant difference in the magnitude of articular effusion among the three age groups on either side of the body. Accordingly, patient age was not found to influence thickness of the hip joint sheath and intra-articular effusion.

Beta2-microglobulin concentration was determined before hemodialysis in the three patient age groups having undergone hemodialysis for a variable period of time. These results are shown in Table 4 A and B.

Age (yrs)	Length of hemodialysis							
	<36 months				36-72 months			
	n	M	Min	Max	n	M	Min	Max
18-50	8	31.020	19.874	42.662	5	34.331	25.580	43.688
51-65	10	28.586	10.115	50.864	9	29.241	11.032	41.376
>65	24	26.793	10.116	42.129	23	30.704	15.552	44.824
Total	42	28.025	10.115	50.864	37	30.838	11.032	44.824

n = number of patients; M = mean β2-microglobulin concentration (mg/L)

Table 4A. β2-microglobulin concentration in three patient age groups undergoing hemodialysis for <36 months and 36-72 months.

Age (yrs)	Length of hemodialysis							
	>72 months				Total			
	n	M	Min	Max	n	M	Min	Max
18-50	3	44.340	35.915	53.147	16	34.552	19.874	53.147
51-65	7	32.122	16.188	51.791	26	29.764	10.115	51.797
>65	17	32.069	10.328	47.997	64	29.600	10.116	47.997
Total	27	33.446	10.328	53.147	106	30.388	10.115	53.147

n = number of patients; M = mean β2-microglobulin concentration (mg/L)

Table 4B. β2-microglobulin concentration in three patient age groups undergoing hemodialysis for <72 months and total.

Simple analysis of variance for independent samples with the length of hemodialysis (categorized into three groups) as independent variable, and pre-hemodialysis β2-microglobulin concentration as dependent variable performed to assess the possible impact of the length of hemodialysis on pre-hemodialysis β2-microglobulin concentration produced the following results: F=3.262; df=2/103; *P*<0.05. Subsequent Tukey HSD test yielded a statistically significant difference in pre-hemodialysis β2-microglobulin concentration between patient groups with hemodialysis duration <36 and >72 months (*P*<0.05). Pre-

hemodialysis β2-microglobulin concentration was higher in the patient group with >72-month hemodialysis duration as compared with those with <36 month hemodialysis duration. Accordingly, longer hemodialysis duration was associated with higher β2-microglobulin concentration. In addition, β2-microglobulin concentration was correlated with patient age to assess the possible effect of age on pre-hemodialysis β2-microglobulin concentration. Simple analysis of variance for independent samples with age (categorized into three groups) as independent variable and pre-hemodialysis β2-microglobulin concentration as dependent variable was done to check for statistically significant differences in β2-microglobulin concentration among patients from different age groups. The analysis of variance yielded the following results: F=2.114; df=2/103; P□0.05. Accordingly, there was no statistically significant difference in pre-hemodialysis β2-microglobulin concentration among the three age groups; thus, patient age had no impact on pre-hemodialysis β2-microglobulin concentration in serum. The hip joint sheath thickness relative to β2-microglobulin concentration was compared between groups of patients with different length of hemodialysis. A statistically significant correlation between β2-microglobulin concentration and hip joint sheath thickness was recorded in the group of patients on hemodialysis for up to 36 months on the right side (r=0.310; P<0.05). In this group of patients, the higher the β2-microglobulin concentration, the higher was the hip joint sheath thickness. In the other two groups of patients on hemodialysis for 36-72 months and >72 months there was no statistically significant correlation between β2-microglobulin concentration and hip joint sheath thickness on either side of the body. The magnitude of hip joint intra-articular effusion and β2-microglobulin concentration were correlated in the groups of patients with different length of hemodialysis, yielding no statistically significant correlation between the two parameters in any of the patient groups. Pre-hemodialysis β2-microglobulin concentration had no effect on the magnitude of hip joint intra-articular effusion in any of the patient groups with different length of hemodialysis. The mean β2-microglobulin concentration was 1.662 mg/L in control group and 30.388 mg/L in overall patient group (28.025, 30.838 and 33.446 mg/L in patient groups on hemodialysis for <36, 36-72 and >72 months, respectively), i.e. 18.3-fold concentration in control group (P<0.05). The mean β2-microglobulin concentrations recorded in three control age groups are shown in Table 5.

Age (yrs)	Total			
	n	M	Min	Max
18-50	7	1.133	0.942	1.537
51-65	21	1.541	0.963	2.617
>65	23	1.934	1.130	2.836
Total	51	1.662	0.942	2.836

n = number of subjects; M = mean β2-microglobulin concentration (mg/L)

Table 5. β2-microglobulin concentration in three control age groups.

Hip joint sheath thickness was compared between patient and control group and tested for statistically significant between-group difference by use of t-test for independent samples, which yielded the following result: t=1.982; df=91; *P*>0.05. There was no statistically significant difference in hip joint sheath thickness between control group and patient group on hemodialysis for <36 months. However, comparison of hip joint sheath thickness between control group and patient groups on hemodialysis for 36-72 and >72 months yielded a statistically significant difference bilaterally, i.e. thickness of the hip joint sheath was statistically significantly greater in both patient groups. Thickness of articular effusion in the hip region was also compared between control group and patient groups with different length of hemodialysis. Intra-articular effusion was statistically significantly greater bilaterally in all three patient groups having undergone hemodialysis for <36, 36-72 and >72 months as compared to control subjects. The length of hemodialysis influenced the rate of hip joint intra-articular effusion. Thickness of the hip joint intra-articular effusion according to patient age and length of hemodialysis is shown in Table 6 A and B.

Age (yrs)	Length of hemodialysis											
	<36 months				36-72 months				>72 months			
	n	M	Min	Max	n	M	Min	Max	n	M	Min	Max
18-50	8	5.26	3.50	6.90	5	6.02	2.90	9.70	3	6.30	5.10	8.60
51-65	10	5.41	3.20	7.70	9	5.87	3.30	8.90	7	6.40	4.20	8.30
>65	24	6.14	3.90	11.5	23	6.86	4.40	9.40	17	7.06	2.70	11.0
Total	42	5.80	3.20	11.5	37	6.50	2.90	9.70	27	6.80	2.70	11.0

n = number of subjects; M = mean (mm)

Table 6A. Number of subjects and hip joint intra-articular effusion according to age and length of hemodialysis.

Age (yrs)	Length of hemodialysis			
	Total			
	n	M	Min	Max
18-50	16	5.69	2.90	9.70
51-65	26	5.84	3.20	8.90
>65	64	6.64	2.70	11.5
Total	106	6.30	2.70	11.5

n = number of subjects; M = mean (mm)

Table 6B. Number of subjects and hip joint intra-articular thickness according to age and length of hemodialysis (total).

5. Discussion

Complications involving the osteoarticular system are common in CRF patients. These complications develop at a slow rate, their clinical signs make only the tip of the iceberg that mostly indicate an advanced stage of the disease and are generally due to renal osteodystrophy and hemodialysis-related amyloidosis. Increased intra-articular effusion and greater hip joint sheath thickness are not specific changes and may occur consequentially to many other pathologic conditions. There are numerous diagnostic procedures to detect and follow-up changes in the osteoarticular system. As most of these methods are associated with high requirements related to duration, price, equipment, room, exposure to ionizing radiation, invasiveness and potential complications, ultrasonography imposes itself as an inexpensive, noninvasive, presumptive, widely available and repeatable examination free from ionizing radiation. As dialysis-related amyloidosis is a multifactorial disease and its pathogenesis has not yet been fully clarified, we embarked upon this study to assess the effect of patient age, hemodialysis duration and β2-microglobulin concentration on morphological changes in the hip region. Thickness of the hip joint sheath and magnitude of intra-articular effusion were found to be proportional to the length of hemodialysis. Thus, early lesions of the osteoarticular system can be detected by measuring the magnitude of articular effusion in the hip area. The increase in intra-articular effusion could be explained by changes involving joint sheath and cartilage due to hemodialysis-related amyloidosis and initial signs of chronic arthropathy in patients with dialysis-related amyloidosis. Biochemical and histologic tests of joint structures can explain the presence of articular effusion and lesions involving the osteoarticular system. In CRF patients, the level of advanced glycation endproducts (AGE) is considerably elevated. These endproducts are formed in the state of uremia *via* nonenzymatic pathway, primarily by oxidative reactions as part of chronic inflammation in CRF patients (Miyata et al., 1999; Sugiyama et al., 1998; Wada et al., 1999;). Elevated AGE levels are found in all tissues, in particular collagenous amyloid structures, which is produced at an increased rate due to impaired metabolism and higher production of β2-microglobulin as a major amyloid component. AGEs are deposited in collagen to cause its structural changes and making it liable to mechanical changes (Miyata et al., 1994). The amount of articular effusion can be influenced by AGEs, which attract monocytes *via* chemotaxis, stimulating them for synthesis of the tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) cytokines. The synovial sheath intima contains macrophages, while fibroblasts account for 70% of intimal cells. Cytokines stimulate synovial fibroblasts for collagenase synthesis. Thus, synovial fibroblasts may be involved in the cellular inflammatory reaction associated with joint damage and destruction in hemodialysis-related amyloidosis (Koski et al., 1989). Protein matrix is degraded by collagenase. Degraded collagen attracts monocytes *via* chemotaxis, stimulating them for further TNF-α and IL-1β synthesis, thus creating a vicious circle (Miyata et al., 1994; Koski et al., 1989). Cytokines act upon the joint cartilage collagen and irritate synovial sheath of the joint, resulting in greater amount of intra-articular fluid. These biochemical reactions are supported by histologic studies demonstrating the initial occurrence of amyloid deposits on the joint cartilage. Burgeson and Nimmi (1992) found these amyloid deposits to be initially accumulated in the cartilage predominated by type I collagen. Jadoul et al. (1997) used histology to detect initial accumulation of paucicellular amyloid deposits surrounded by macrophages on joint cartilage, supporting the inflammatory nature of amyloid deposits. Zhang et al. (2002) report on β2-microglobulin inhibitory action on joint cartilage

chondrocytes. Subsequent changes involve synovium, joint sheaths and tendons. Menerey et al. (1988) demonstrated the presence of amyloid in synovial fluid. These alterations may mark the onset of degenerative changes, erosions and effusions in the joints. Jadoul et al. (1993) found thickness of the hip joint sheath and supraspinous muscle tendon to correlate with the length of hemodialysis in 49 patients undergoing hemodialysis for a mean of 97 months. After 21 months of hemodialysis, measurement was repeated in 16 patients to show a statistically significant increase in the hip joint sheath thickness in nine patients (from 7.0±0.8 mm to 8.2±2.3 mm). On repeat measurement, there was no statistically significant increase in thickness of the supraspinous muscle tendon (from 6.6±0.4 mm to 7.0±0.8 mm). Jeloka et al. (2001) compared hip joint sheath thickness between 30 patients undergoing hemodialysis for a mean of 7.1 years and 20 control subjects. The mean hip joint sheath thickness was 8.19 mm and 5.06 mm, and mean serum β2-microglobulin concentration 25.6±4.12 mg/L and 2.6±1.73 mg/L in patients and control subjects, respectively. In hemodialysis patients, serum β2-microglobulin concentration was statistically significantly lower while on hemodialysis with low-flux hemodialyzers as compared with the time on hemodialysis with high-flux hemodialyzers. The authors demonstrated correlation between the hip joint sheath thickness and the length of hemodialysis. The longer the time of hemodialysis, the greater was the hip joint sheath thickness. The authors conclude that osteoarticular system changes in hemodialysis patients can be followed-up by measurement of the hip joint sheath thickness. Most authors agree that changes in the osteoarticular system are related to the length of hemodialysis (Jadoul et al., 1993; Jeloka et al., 2001). In the present study, we found no statistically significant difference in β2-microglobulin concentration among the three patient age groups, nor recorded any effect of age on morphological changes in the hip area. There are literature reports on the carpal tunnel syndrome, amyloid arthropathy and amyloid bone cysts to be more common in elderly patients (Denesh & Ho, 2001; Shin et al., 2008).

The mechanism by which patient age influences the prevalence of complications in hemodialysis patients remains unknown. The concentration of AGEs is higher in elderly subjects (Sell & Monnier, 1990). As AGEs influence collagen metabolism, it may explain the impact of age on anatomic structure changes in this population. Schiffl et al. (2000) investigated the prevalence of complications involving osteoarticular system in hemodialysis patients aged <55 and >55. The latter had a higher rate of complications including carpal tunnel syndrome, amyloid arthropathy and bone cysts as compared to patients aged <55 at the time of starting hemodialysis. The older patient age, the higher was the rate of complications. The time elapsed from starting hemodialysis to the onset of complications was shorter in older than in younger patients. Kurer et al. (1991) found carpal tunnel syndrome to be more common in elderly patients and in those starting hemodialysis at an older age. In older patients, carpal tunnel syndrome developed after a shorter period of hemodialysis than in younger ones. However, opposite results have also been reported in the literature. Using the Glasgow Ultrasound Enthesitis Scoring System (GUESS) scale, Kerimoglu et al. (2007) found changes of the osteoarticular system anatomic structures in hemodialysis patients to be related to the length of hemodialysis, while demonstrating no effect of patient age and β2-microglobulin concentration. Weybright et al. (2003) point to the possible misinterpretations and false-positive results of US measurement of articular effusion in the hip area. Ultrasonography performance is limited in overweight patients. Synovial membrane of the hip joint covers fibrous membrane and cannot be visualized on US. Hypoechogenic synovia may push as the

articular sheath, thus yielding an image of articular effusion. This is especially pronounced in obese subjects, where hypoechogenic synovia may appear as articular effusion on US. In our study, β2-microglobulin concentration influenced the hip joint sheath thickness in the group of patients on hemodialysis for <36 months, whereas no statistically significant correlation was observed in the other two patient groups. Serum β2-microglobulin concentration had no effect on the amount of articular effusion in the hip area in any patient group with different length of hemodialysis. In addition, there was no statistically significant difference in β2-microglobulin concentration among the three patient groups, however, β2-microglobulin concentration was higher in patients with longer hemodialysis duration. A high serum β2-microglobulin concentration is one of the preconditions for the development of hemodialysis-related amyloidosis; therefore, higher β2-microglobulin concentration is expected to be associated with more pronounced morphological changes of anatomic structures. There are no reports on hemodialysis-related amyloidosis in patients with β2-microglobulin concentration <10 mg/L (Farrell & Bastani, 1997). Because of different dialyzer membrane properties, β2-microglobulin concentration is by 30% lower in patients on hemodialysis with low-flux hemodialyzers (Jadoul, 1998). In their study including 50 patients, McCarthy et al. (1997) found residual kidney function to be preserved for a longer time in patients on hemodialysis with high-flux hemodialyzers. During the first 12-24 months of hemodialysis, β2-microglobulin concentration also depends on residual kidney function, i.e. the longer it is preserved, the later the onset of amyloidosis complications (Schiffl et al., 2000). The membrane of high-flux hemodialyzers is characterized by higher biocompatibility and lower rate of stimulating β2-microglobulin concentration production through complement activation. The high-flux dialyzer membrane can remove β2-microglobulin molecules and AGE modified proteins, thus influencing the process of amyloid formation (Henle et al., 1999; Jadoul, 1998). Therefore, the use of high-flux dialyzers is expected to be associated with a lower rate of complications involving the osteoarticular system (Geyo, 2001). Beta2-microglobulin concentration does not correlate with the activity of dialysis-related amyloidosis, thus serum β2-microglobulin concentration is not a diagnostic test to determine the severity of dialysis-related amyloidosis (Farrell & Bastani, 1997; Koski et al., 1989). A number of studies have confirmed that clinical symptoms of complications involving the osteoarticular system are more common in patients undergoing hemodialysis for a prolonged period of time, as well as in those with higher β2-microglobulin concentration (Barišić et al., 2007).

Shin et al. (2008) compared two groups of patients with different β2-microglobulin concentration and found the prevalence of carpal tunnel syndrome to be lower in patients with lower β2-microglobulin concentration. Barišić et al. (2007) report on a higher β2-microglobulin concentration in the group of hemodialysis patients articular pain as compared with those free from articular pain. In contrast, Kerimoglu et al. (2007) found no statistically significant correlation between GUESS scale and serum β2-microglobulin concentration. In the present study, intra-articular effusion in the hip area was statistically significantly greater bilaterally in all three hemodialysis patient groups as compared with control group. Difference in the hip joint sheath thickness did not reach statistical significance between control group and the group of patients on hemodialysis for <36 months, but was statistically significant in the groups of patients on hemodialysis for 36-72 and >72 months.

6. Conclusion

The present study demonstrated that changes involving osteoarticular system soft tissues in CRF patients could be followed-up by use of US. Although the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NKF KDOQI, 2003) recommendations do not recommend routine follow up of CRF patients by β2-microglobulin amyloidosis because there is no therapeutic option other than kidney transplantation for hemodialysis-related amyloidosis, we consider US a useful and widely available diagnostic method to detect and follow-up soft tissue changes in hemodialysis patients. In our study, pre-hemodialysis serum β2-microglobulin concentration did not influence the magnitude of articular effusion, but did influence hip joint sheath thickness in patients undergoing hemodialysis for <36 months, with no statistically significant correlation recorded in the other two groups of patients. Morphological changes correlated with the length of hemodialysis, but not with patient age. As the osteoarticular system changes are multifactorial, additional studies are needed to determine the effect of particular factors on these changes in CRF patients. Research should be focused on complications in patients undergoing hemodialysis with high-flux dialyzers in order to identify the possible impact of the type of hemodialyzers on osteoarticular system changes. US study is the method of choice to follow-up the dynamics of changes involving soft tissue structures (Bother et al., 2006; Drüeke, 1999; Kay et al., 1962; Kerimoglu et al., 2007; Kiss et al., 2005; Negi et al., 1995). Early and asymptomatic lesions of the joints, tendons and ligaments can be detected by US (Barišić et al., 2002; Jeloka et al., 2001; Lanteri et al., 2000; Takahashi et al., 2002). The need for the best possible care of hemodialysis patients points to the use of US as a adjunctive method to clinical examination to assess soft tissue changes of the osteoarticular system by providing better insight into the pathology of articular tendons, ligaments, cartilage and effusion (Backhaus et al., 2001; Takahashi et al., 2002). US analysis of morphological changes of the osteoarticular system can be of prognostic value in patients undergoing hemodialysis. Maintaining good function of the osteoarticular system helps the CRF patients achieve appropriate social inclusion and better quality of life, while reducing the cost of treatment for severe complications.

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9

Modulation of Iron Metabolism and Hepcidin Release by *HFE* Mutations in Chronic Hemodialysis Patients: Pathophysiological and Therapeutic Implications

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1. Introduction

Routine monitoring of body iron stores is an essential component of the management of patients with end-stage renal disease (ESRD) receiving chronic hemodialysis treatment (CHD). Maintenance of adequate iron stores is important for the prevention of iron overload as well as for the treatment of iron deficiency and anemia, and this goal is generally achieved by intravenous iron administration. Despite regular iron supplementation, anemia due to renal failure, blood losses related to the procedure, and chronic inflammation is a typical finding in CHD patients, and is associated with increased mortality, reduced physical and mental function, and poor quality of life. Treatment often requires the administration of erythropoiesis stimulating agents (ESAs), but many patients do not respond adequately and/or require high doses of these medications, with potential adverse cardiovascular and infective events.

A correct iron balance is required for the functionality of catalytic enzymes and proteins crucial for DNA synthesis, transport and storage of oxygen via hemoglobin and myoglobin, transport of electrons and cell respiration, oxidative phosphorylation, tricarboxylic acid cycle, and many other biochemical pathways. On the other hand, excess free iron is toxic to the cells due to its ability to catalyze free radical generation. Therefore, specialized transport systems and membrane carriers have evolved to keep iron in a soluble state that is suitable for circulation into the blood and transfer across cell membranes. Additionally, the absence of a physiological excretion mechanism requires systemic iron homeostasis to be regulated by intestinal absorption and iron recycling from erythrophagocytosis of senescent red cells.

Iron metabolism is frequently altered in CHD due to decreased saturation of transferrin (TF), the plasma iron carrier, resulting in reduced iron availability for erythropoiesis, because of chronic inflammation and/or blood losses. Furthermore, inflammation and oxidative stress induce iron retention in macrophages and the transcription of ferritin, a protein with anti-oxidant activity, and hyperferritinemia indicating increased iron stores, and chronic inflammation is frequently observed in these patients. Hyperferritinemia, reflecting iron overload, oxidative stress, and genetic factors, is associated with imminent death risk, mainly related to cardiovascular events, the leading cause of death in these subjects.

A major breakthrough in our understanding of iron metabolism has been the discovery that hepcidin, a peptide secreted by the liver in response to excess iron, inhibits intestinal iron absorption and iron recycling from monocytes by binding and inactivating the iron exporter Ferroportin-1 (Fp-1). Furthermore, hepcidin is an acute phase reactant induced by inflammation, and is downregulated by anemia, hypoxia and erythropoietin. Hepcidin is increased in patients with ESRD, because of chronic inflammation and of reduced urinary excretion due to kidney injury. Hepcidin levels reduce intestinal iron absorption and iron recycling from monocytes, decreasing serum iron available for the erythropoiesis, thereby playing a causal role in the anemia of inflammation and ESRD.
Recent data are consistent with the hypothesis that iron mediated vascular damage involves hepcidin upregulation with consequent accumulation of iron in macrophages, being hepcidin associated with carotid vascular damage independently of other known risk factors in high-risk populations. Moreover, *in vitro* studies suggest that hepcidin synergizes with excess iron, by favoring intracellular accumulation of this metal in macrophages and oxidative stress, and the induction of the release of pro-atherogenic cytokines, in particular macrophage chemoattractant protein-1 (MCP-1).
Mutations of the *HFE* gene responsible for the iron overload disorder hereditary hemochromatosis are frequently observed in the general population and uncouple the regulation of intestinal iron absorption from iron stores by determining altered hepatic iron sensing and a relative deficit in hepcidin release. These physiological alterations result in an attenuation of the effects of inflammation on iron metabolism, including reduced iron absorption, and iron sequestration in macrophages. Here we review novel data suggesting that *HFE* mutations are associated with reduced hepcidin release in CHD patients, resulting in an increased sensitivity to ESAs and iron supplementation, and possibly in a reduced risk of death due to the decreased incidence of complications related to excessive iron and ESAs dosages, including cardiovascular events and infections.
These results may have important clinical implications, as targeting the HFE/hepcidin/Fp-1 axis by pharmacological treatment may further improve the long-term outcomes of CHD, by reducing the amount of iron and ESAs supplementation needed and by improving iron utilization for erythropoiesis.

2. Iron metabolism

2.1 Role of iron in the cells

Iron, like other metal ions, is an essential nutrient, playing a crucial role in vital biochemical activities, as components of enzymes and other molecular complexes. Excess iron promotes noxious free-radical reactions (Kell 2010), so that it has to be compartmentalized and maintained at a fixed level to avoid any toxic effects, largely based on its ability to catalyze the generation of radicals, which attack and damage cellular macromolecules and promote cell death and tissue injury. A delicately balanced iron homeostasis is achieved by coordinated interaction among highly evolved and regulated uptake, storage, and secretion processes (Papanikolaou and Pantopoulos 2005; Andrews 2008).

2.2 Chemical properties, biological functions and toxicity of iron

The biological functions of iron are based on its chemical properties, in particular on the ability to form a variety of coordination complexes with organic ligands in a dynamic and flexible mode, and by its favorable redox potential to switch between the ferrous, Fe(II), and

ferric, Fe(III), states. Iron is associated to heme and non-heme complexes. The hemoproteins are hemoglobin and myoglobin, cytochromes and some enzymes, such as oxygenases, peroxidases, nitric oxide (NO) synthases, or guanylate cyclase. All these proteins contain heme as prosthetic group, which is composed by protoporphyrin IX and a ferrous ion, and is involved in oxygen transport to muscles and tissues (hemoglobin and myoglobin), in the respiratory chain (e.g., cytochromes a, b and c), in the activation of substrates by oxygen (e.g. cytochrome oxidase, cytochrome P450, catalase) and as a NO sensor (guanylate cyclase). The most prevalent forms of non-heme iron are metallo-proteins with iron-sulfur clusters, which are involved in the respiratory chain (e.g., in complex III), DNA synthesis (e.g. ribonucleotide reductase), and in the inflammatory response (e.g., cyclo-oxygenases and lipoxygenase). It should also be noted that non-heme iron has a central function in a recently discovered mechanism for oxygen sensing, via the hypoxia-inducible factor (HIF), that controls the transcription of a wide array of genes involved in erythropoiesis, angiogenesis, cell proliferation/survival, glycolysis, and iron metabolism in response to oxygen availability.

The efficiency of the redox reaction between ferrous and ferric ions is a fundamental feature for many biochemical reactions. However, this very property turns iron into a potential biohazard in the cell, because under aerobic conditions, iron can readily catalyze the generation of noxious radicals. Iron toxicity is largely based on the Fenton reaction, where catalytic amounts of iron are sufficient to yield hydroxyl radicals, collectively known as reactive oxygen intermediates (ROIs), from superoxide and hydrogen peroxide. ROIs are inevitable products of aerobic respiration in mitochondria and can be also generated during enzymatic reactions in peroxisomes, in the endoplasmic reticulum or in the cytoplasm. ROIs are also produced by the membrane-bound NADPH oxidase complex, mainly expressed in phagocytic neutrophils and macrophages during inflammation, and involved in the antimicrobial defense of the organism. Free radicals are highly reactive species that promote the oxidation of proteins, peroxidation of membrane lipids, and modification of nucleic acids. An increase in the steady state levels of reactive oxygen species beyond the antioxidant capacity of the organism, so called oxidative stress, is observed in many pathological conditions, such as chronic inflammation, ischemia–reperfusion injury, or neurodegeneration. Excess of redox active iron aggravates oxidative stress and leads to an accelerated tissue degeneration. Thus, under physiological conditions extracellular iron is exclusively bound to TF, which maintains iron soluble and nontoxic.

2.3 Iron distribution and absorption

The human body contains approximately 3–5 grams of iron (45–55 mg/kg of body weight in adult women and men, respectively). Approximately 60–70% of body iron is employed within hemoglobin (Hb) in circulating red blood cells; other iron-rich organs are the liver and muscles. Approximately 20–30% of body iron is stored in hepatocytes and in macrophages, to a large extent within polymers of ferritin. The remaining is primarily localized in myoglobin, cytochromes, and iron-containing enzymes. A healthy individual absorbs daily 1–2 mg of iron from the diet, which is utilized to compensate non-specific iron losses by desquamation of enterocytes and epidermis and, in childbearing aged women, by period. Erythropoiesis requires approximately 30 mg of iron per day, mainly provided by the recycling of iron via macrophages, which ingest senescent red blood cells (RBCs) and release iron, which binds to circulating TF.

An average daily Western diet contains approximately 15 mg of iron, from which only 1–2 mg is absorbed in the jejunum: in spite of such an apparently low requirement, dietary deficit of iron is a worldwide problem. Two thirds from absorbed iron derives from heme, mainly from myoglobin and hemoglobin of animal origin. The inorganic iron is not efficiently absorbed, but each transmembrane transport step is mediated by specific set of transport proteins and accessory enzymes that change the oxidation state of iron to facilitate the transport process. The low gastric and duodenal pH dissolves ingested inorganic iron and facilitates its enzymatic reduction to the ferrous form by the ferrireductase on the enterocyte brush border (DCYTB, duodenal cytochrome b), whose expression increases during iron deficiency. Ferrous iron is transported across the apical membrane by DMT1 (divalent metal transporter-1). Inside absorptive enterocytes, heme iron is enzymatically released by heme oxygenase-1 (HO-1), after having been transported by heme-carrier protein-1 (HCP-1), and follows the fate of inorganic iron: it is either stored in ferritin or transported across the basolateral membrane to plasma TF. The transport across basolateral membrane is mediated by Fp-1, also known as IREG1 (iron regulated transporter 1), or SLC40A1. Fp-1 is expressed in Kupffer cells and on the basolateral membrane of enterocytes, macrophages, placental cells and hepatocytes, where it works as iron exporter in association with the plasma ferroxidase ceruloplasmin (Cp), even if enterocytes depend heavily on the expression of an analogous transmembrane protein called hephaestin. Ferrous iron crosses the plasmatic membrane through a facilitate diffusion mechanism and Cp / hephaestin converts it to ferric state while it is still associated to the transporter protein. Ferric iron bounds to plasmatic apotransferrin (the iron-free form of TF, a glycoprotein synthesized in liver, retina, testis and brain) to form ferric iron-TF complex, which is the major type of iron present in blood. A small amount of iron circulates bound to albumin or other small molecular weight ligands, such as citrate salts. The TF complex facilitates the transport of iron to cells that express transferrin receptors, including erythroid progenitors, and limits the ability of iron to generate toxic radicals. Transferrin saturation % (TSAT) varies according to diurnal cycle and local circumstances. For example, TSAT is high in portal circulation and low in the blood leaving bone marrow, and is strongly influenced by inflammation, which decreases plasma iron availability in order to prevent iron utilization by pathogens for their replication.

2.4 Delivery of iron to tissues and intracellular transport

Iron uptake occurs primarily by the endocytic pathway, which involves the interaction between TF and transferrin receptors (TFR). Two different TFR are known, namely TFR-1, which is found in all cells and shows an elevated affinity for circulating TF, and TFR-2, mainly expressed in the liver and in the hematopoietic cells, which bind the TF complex with a lower affinity. Once the TF complex binds to TFR-1 at the cell surface, it is internalized in clathrin-coated pits: in the endosome the luminal pH is maintained at about 5.5 by a vacuolar H^+-ATPase (V-ATPase). This acidification process induces conformational changes in the TF-TFR-1 complex, with consequent release of iron in the endosome, while different ferrireductases operate the transformation of ferric iron into the ferrous state. The endosomal transporter DMT1 allows then the passage of ferrous iron in the cytoplasm. The acidic endosomal pH maintains apotransferrin bound to the TFR-1 and this complex is recycled on the cellular surface. At the more neutral plasmatic pH, apotransferrin dissociates and it can bind to plasmatic iron again. Not much is known about the mechanisms of intracellular iron transport

to organelles, but there is strong evidence for the existence of a transit pool of iron in the cytosol (Petrat, de Groot et al. 2002), which presumably remains bound to low-molecular-weight chelates, such as citrate, ATP, AMP, or pyrophosphate. It is believed that the levels of this elusive chelatable or "labile iron pool" (LIP) reflect the overall iron status of the cell and its variations activate adequate homeostatic responses for iron availability. The LIP constitutes also a source of iron susceptible of redox-state variations, with consequent generation of free radicals toxic for cells.

2.5 Iron storage and recycling

Not all absorbed iron is utilized in metabolic processes, but it is partly stored as reserve, both for use when iron levels are low, and to prevent toxic effects of free iron in the cell. Stored iron accounts for 20-30% of body iron in physiological conditions, and the major part of it is bound to ferritin. This is a ubiquitous multimer of 24 subunits with a central core that contains up to 4,500 atoms of iron, and that possesses an important ferroxidase activity (the H form, whereas the L form is the storage subunit) that facilitates the oxidation of the cytosolic ferrous iron to the ferric state. Iron is also stored in an insoluble form into a protein, named haemosiderin, likely derived from the lysosomal degradation of ferritin. Under iron overload conditions, ferritin levels increase dramatically, particularly in liver, pancreas and heart. Intestinal absorption accounts for only a fraction of TF-bound iron in the circulation. Recovery of iron from senescent erythrocytes also plays an important role in iron maintenance. At the end of their lifespan, human erythrocytes undergo surface alterations that mark them to be phagocytosed and digested by reticulo-endothelial macrophages in the spleen and liver. In the macrophages iron is predominantly recovered from heme by the action of HO-1.

2.6 Regulation of intracellular iron homeostasis

Regulated expression of proteins essential for cellular iron homeostasis, such as TFR-1 and ferritin, is achieved by a post-transcriptional mechanism, which is dependent on biologically active iron levels. The messenger RNAs of these peptides present regulatory sequences named IRE (iron responsive elements) in the untraslated regions (UTRs), the target of iron regulatory proteins (IRP-1 and IRP-2), which are rapidly degraded in iron-rich condition. Under iron deficiency conditions, IRPs actively bind multiple IREs localized at the 3'UTR of some mRNAs, such as TFR-1 mRNA, determining mRNA stabilization and increased translation of the protein, and simultaneously decrease the translation of ferritin mRNA by binding to 5'UTR IRE sequences, thereby maximizing the uptake and availability of iron in the cell. Conversely, when the iron levels are high, decreased IRE binding facilitates efficient translation of ferritin mRNA and decreases the stability of TFR-1 mRNA, leading to iron sequestration over uptake.

2.7 Hepcidin and systemic iron homeostasis

Systemic iron homeostasis is achieved by modulation of the amount of iron absorbed, since physiological mechanisms for the regulation of iron excretion are presently unknown. Intestinal iron absorption is regulated in response to iron need and availability and erythropoiesis activity. Stewart et al first disclosed the so-called mucosal block, detecting that a large oral dose of iron reduced the absorption of smaller dose of iron administered several hours later (Stewart, Yuile et al. 1950). These observations suggested that enterocytes

receive signals from other tissues or cells that are involved in either utilization (erythroid precursors) or storage (hepatocytes, macrophages) of iron. Signals that originate from storage sites to balance intestinal absorption were termed "storage regulators", while "erythroid regulators" signal when the consumption demand for iron in bone marrow, erythroid precursors and circulating erythrocytes, exceeds the amount present in stores. On the other hand, "inflammatory regulators" communicate signals in response to infection or inflammation, resulting in accumulation of iron in macrophages. Iron homeostasis is also influenced by hypoxia regulatory signals. Adding to the complexity, these diverse regulatory signals may not be completely independent of each other and elicit quantitative differences in response to common molecules. The amount of body storage modulates iron uptake: it is well established that in iron-deficient conditions, iron absorption is significantly stimulated by two- to three-fold compared to basal conditions, which are restored when iron storage are reconstituted. The erythropoietic regulation participates when iron demand for Hb synthesis increases, independently of body iron stores. This mechanism can explain the pathological iron accumulation observed in disorders characterized by ineffective erythropoiesis (such as thalassemia syndromes, congenital dyserythropoietic anemias, sideroblastic anemias).

A small antimicrobial peptide synthesized by the liver (and to a lesser extent by macrophages and adipocytes), named hepcidin (Pigeon, Ilyin et al. 2001), is now retained to be the principal effector of the modulation of iron availability by iron stores and inflammation in humans. The human hepcidin gene (*HAMP*; OMIM 606464), located on chromosome 19q13.1, encodes a precursor protein of 84 amino acids (aa). During its export from the cytoplasm, full-length pre-prohepcidin undergoes enzymatic cleavage, resulting in the export of a 64 aa prohepcidin peptide into the endoplasmic reticulum lumen (Wallace, Jones et al. 2006). Next, the 39 aa pro-region peptide is post-translationally removed by a furin-like pro-protein convertase, resulting in mature bioactive hepcidin-25. In human urine, hepcidin-22, a N-terminally truncated isoform of hepcidin-25 considered the urinary degradation product of hepcidin-25, can be detected. Structural analysis of human synthetic hepcidin by nuclear magnetic resonance spectroscopy revealed that this 8 cysteine-containing peptide forms a hairpin-shaped molecule with a distorted beta-sheet, which is stabilized by four disulfide bridges between the two anti-parallel strands. One of the disulfide bridges is located in the vicinity of the hairpin loop which points to a possible crucial domain in the activity of the molecule (Hunter, Fulton et al. 2002). Structure-function *in vivo* and *in vitro* studies on synthetic hepcidin have shown that the iron regulating bioactivity is almost exclusively due to the 25 aa peptide, suggesting that the five N-terminal amino acids are essential for this activity. *In vitro* experiments have shown that especially human hepcidin-20 (the other N-terminally truncated isoform detectable in human serum) exerts anti-bacterial and anti-fungal activity in a concentration range 10- fold higher than that measured in healthy individuals. Therefore, it is not clear whether *in vivo* hepcidin levels can reach values in which it can be anti-microbial, or whether this function is of biologic importance or only rudimentary in its evolutionary origin from the antimicrobial peptides of the defensin family. Hepcidin is excreted in the urine; in a murine model, its effect starts within 4 hours and lasts for more than 48 hours (Rivera, Nemeth et al. 2005). Fractional excretion of hepcidin-25 in 3-5%, because is not freely filtered or it is reabsorbed by the tubules like other small peptides.

Hepcidin mediated regulation of iron metabolism has been demonstrated to depend upon its ability to bind Fp-1 on cellular surface blocking its iron transport activity, and to increase Fp-1

endocytosis, JAK2 mediated phosphorylation, and consequently its degradation by lysosomes (Nemeth, Tuttle et al. 2004). In enterocytes, Fp-1 internalization on the basolateral surface causes the retention of absorbed iron with subsequent loss by desquamation, while the same process in macrophages causes the failure to release iron (Pietrangelo, 2007). The final effect is the reduction of plasma iron availability. Besides liver hepcidin controlled reduction of iron uptake and release at systemic level, there is also evidence for local production of hepcidin by macrophages, fat cells and cardiomyocytes, suggesting a different regulatory mechanism related to hepcidin to control iron balance (Theurl, Theurl et al. 2008). Indeed, mutations of *HAMP*, the hepcidin gene, cause severe early-onset HH in humans, whereas deletion of *Hamp* in animal models brings to severe iron accumulation (Lesbordes-Brion, Viatte et al. 2006). Conversely, hepcidin producing hepatic adenomas in patients and overexpression of hepcidin in animal models lead to hyposideremia and iron deficiency anemia.

Importantly, hepcidin is upregulated by both increased iron stores and inflammation. Indirect hepcidin inductions by IL-6 or LPS in humans displayed the same fast response in urinary hepcidin excretion, thereby acting like an acute phase protein with a peak value after 6 hours, followed by a steady decrease. When hepcidin is administered orally, a peak in urinary excretion appear in less than one day, suggesting a fast clearance of this peptide from the circulation, with a paradoxical sustained inhibitory effect on iron uptake, shown by the iron parameters which remained unchanged over the following days (Nemeth, Rivera et al. 2004). Hepcidin secretion is reduced in response to signals that cause an increase in iron release from cells, such as iron deprivation, and stimulus to erythropoiesis, whereas it rises when iron secretion is inhibited, as for iron load or for a flogistic state. Thus, hepcidin can represent the common effector of the homeostatic regulation of intercellular iron fluxes in response to the iron stores, erythroid, and inflammatory regulators.

It is still not clear how systemic iron demand modulates hepcidin release by the liver. A number of molecules have been implicated in different ways of transduction of the signal: the alteration of each of these molecules causes an insufficient release of hepcidin resulting in a deregulated iron flux from macrophages and enterocytes, which finally brings to iron overload in blood circulation and finally in tissues. Hepcidin release may be impaired by genetic factors, i.e. mutations inactivating *hepcidin*, *HJV*, *HFE* or *TFR-2*, or by non-genetic factors: alcohol abuse (that inhibits hepcidin transcription), viral infections such as chronic HCV hepatitis, and acute liver insufficiency and cirrhosis (because of the reduced hepcidin synthesis by hepatocytes). In addition, mutations in the *Fp-1* gene, which bring to insensitivity to hepcidin action, are as well responsible for hereditary forms of overload.

The small size of hepcidin, the compact and complex structure of the molecule, and the highly conserved sequence among species, make problematical the quantitative assessment of serum levels of hepcidin in humans. Furthermore, about 90% of serum hepcin-25 appears to be bound to circulating proteins, mostly α2-macroglobulin. Recently, laboratory assays for hepcidin-25 (the biologically active form of the hormone) in serum and urine has been developed: they include competitive ELISAs using biotinylated or radio-iodinated hepcidin as tracers, and several mass-spectrometry-based assays using as internal standards isotopically labeled hepcidin or shorter hepcidin mutants. Interestingly, hepcidin levels were correlated with ferritin in patients with chronic kidney disease (Tomosugi, Kawabata et al. 2006; Kato, Tsuji et al. 2008; Kroot, Laarakkers et al. 2010). In contrast, measurement of serum prohepcidin, an immature form of hepcidin measured by ELISA, was found to be increased in patients with ESRD (Kulaksiz, Gehrke et al. 2004), but have not been found to

correlate with mature biologically active hepcidin and with iron stores (Kato, Tsuji et al. 2008; Swinkels, Girelli et al. 2008).

3. *HFE* mutations and iron overload

The transcription and secretion of hepcidin by the liver is regulated by a mechanism of body iron sensing and is finely regulated by a group of proteins, including the hereditary hemochromatosis protein called HFE, TFR-2, hemojuvelin (HJV), bone morphogenetic protein 6 (BMP6), matriptase-2 (TMPRSS6) and TF. Mutations in *HFE*, *TFR-2*, *HJV* and the hepcidin gene (*HAMP*) are responsible for hereditary hemochromatosis (HH), a common iron overload disorder characterized by a deficit of hepcidin release or activity (Camaschella 2005; Pietrangelo 2007).

HFE mutations represent the most frequent cause of HH in Caucasian adults (Feder, Gnirke et al. 1996). HFE structure resembles MHC class I molecules and forms a heterodimer with β2-microglobulin. TF and HFE seem to compete in vitro for binding to TFR-1, which is hypothesized to sequester HFE thereby altering iron sensing and hepcidin expression. In this way, increased iron-loaded TF levels would result in the release of HFE with possibility to interact with other proteins, in particular with TFR-2, which only binds saturated TF at physiological concentrations. However, the exact mechanisms underpinning HFE regulation of iron metabolism through SMAD (son of mother against decapentaplegic) and possibly ERK (extracellular signal-regulated kinases) signaling are still not clear. The most common *HFE* mutation responsible for HH is a single nucleotide substitution that causes the substitution of a cysteine with a tyrosine at position 282 (C282Y). The homozygous genotype is very frequent in Caucasians, particularly in people from Northern Europe (frequency 1/300-400), whereas the prevalence decreases towards Southern Europe. This substitution brings to HFE mysfolding, resulting in failed interaction with β2-microglobulin and cell surface expression. A second and most frequent mutation is a substitution at position 63 of a histidine with an aspartate (H63D), which likely interferes with the ability of HFE to interact with TFR-1. This is a very common polymorphism in the general population, as 25-30% of the population carries the H63D variant, but its contribution to the pathogenesis of HH and iron overload syndromes is negligible, with the exception of compound heterozygosity with the C282Y. The penetrance of HH depends on age, gender, environmental factors, and on the role of the so-called modifier genes (Wood, Powell et al. 2008): for example, iron overload and clinical phenotype are more serious in patients with beta-thalassemic trait (Piperno, Mariani et al. 2000; Valenti, Canavesi et al. 2010).

Mutations of *TFR-2* cause a rare recessive form of HH, clinically similar to HFE HH, which is consistent with the hypothesis that HFE and TFR-2 might be part of the same signaling pathway. Indeed, it has been hypothesized that TFR-2 binds iron-loaded TF, but not iron-depleted TF. Thus, TFR-1 and TFR-2, by binding iron bound and not bound to TF respectively, may contribute to modulate iron sensing and hepcidin release by the liver. However, deletion of *Tfr-2* and *Hfe* had additive effects on iron overload in mice, thus suggesting that the pathways through which these two genes regulate iron metabolism might not be entirely overlapping. In addition, TFR-2 was discovered to be associated to erythropoietin receptor (Epo-R) in the EpoR complex, and thus to exhibit an extra-hepatic function, being required for efficient erythroid differentiation and erythropoiesis (Forejtnikova, Vieillevoye et al. 2010). Furthermore, very recently it has been demonstrated that also HFE is expressed in erythroblast and plays a role in the regulation of erythroid

differentiation, and that HFE deficiency is associated with increased erythropoiesis partly due to enhanced iron absorption and delivery to the erythron due to decreased hepcidin levels and increased TSAT, and partly due to a direct effect of HFE on the modulation of iron uptake in erythroid cells (Ramos, Guy et al. 2011). These new exciting findings suggest that the TFR-2/HFE complex is directly involved in the regulation of erythropoiesis independently of hepcidin levels, and thus that genetic variations of HFE and TFR-2 may influence RBCs production and the development of anemia in conditions characterized by reduced iron availability, such as CHD.

Many other molecules have been implicated in the regulation of hepcidin secretion. Binding of the iron-regulated BMP6 ligand, a bone morphogenetic protein of the TGFβ superfamily, to its threonine/serine kinase receptors (BMPR-I and BMPR-II) activates a signaling cascade leading to hepcidin transcription via phosphorylation, nuclear translocation, and binding to the hepcidin promoter of SMAD 1/5/8 effectors (Babitt, Huang et al. 2006). HJV, a GPI-linked membrane protein synthesized by the hepatocytes, is a BMP6 coreceptor, which is required for its regulatory functions on iron metabolism. The critical role of the BMP6/HJV/SMAD pathway in iron homeostasis is supported by the loss of hepcidin expression and massive parenchymal iron overload observed in *BMP6*-/- and *HJV* -/- mice as well as in mice with targeted liver deletion of SMAD4 (Andriopoulos, Corradini et al. 2009), and by the fact that *HJV* mutations represent the major cause of juvenile HH. Furthermore, iron overload and increased TSAT have been associated with specific upregulation of BMP6 in hepatocytes and *in vivo*. However, *BMP6* mutations have not been associated with HH so far. Interestingly, it has been shown that BMP6 levels are increased in HFE-related HH both in mice and humans, suggesting that HFE might be involved in the transduction of BMP6 signaling, or that BMP6 levels are upregulated in the attempt to compensate for the lack of HFE function, but supraphysiological doses of BMP6 were sufficient to normalize iron metabolism in experimental models. Recently, the serine protease matriptase-2 has been connected to this iron regulatory pathway because of its ability to cleave HJV (Muckenthaler 2008). Matriptase-2 is a type 2 transmembrane serine protease that is predominately expressed in the liver and was characterized as a negative regulator of hepcidin gene expression (Ramsay, Hooper et al. 2009). Matriptase-2–deficient mice have very high levels of hepcidin, which lead to the inhibition of dietary iron absorption and cause a severe iron-deficiency anemia phenotype. This anemic phenotype is mirrored in patients with matriptase-2 mutations, who present with iron-refractory, iron-deficiency anemia (IRIDA). Indeed, patients with IRIDA show inappropriately high hepcidin levels, which explain the lack of dietary iron absorption and the only partial response to parenteral iron treatment (Finberg 2009). In addition, it has been recently recognized by means of genome-wide association studies that very common genetic polymorphisms in *TMPRSS6* represent, together with *HFE* mutations, a major source of variability in serum iron and hemoglobin levels in the general population (Benyamin, Ferreira et al. 2009; Chambers, Zhang et al. 2009; Ganesh, Zakai et al. 2009; Tanaka, Roy et al. 2009).

Severe iron overload in HH involves several organs, mainly the liver, endocrine glands, and heart. However, the involvement of a specific organ varies on the entity of iron accumulation, which depends in part also on the specific mutation at the base of the disease. Usually, the more severe phenotype is observed in the juvenile forms versus adult disease (Pietrangelo 2007), and secondary iron overload in the presence of physiological upregulation of hepcidin presents with a different organ involvement (mainly macrophages), and clinical phenotype (e.g. anemia, accelerated atherosclerosis, altered immune regulation) (Pietrangelo 2004).

4. Anemia in ESRD

Anemia is a common problem in patients with ESRD and increases mortality and morbidity in these patients, especially related to cardiovascular events. The cause of anemia in patients with chronic kidney disease is multifactorial (Lankhorst and Wish 2010), but it is believed that a deficit of erythropoietin (Epo) due to kidney injury plays a major role. Indeed, it is generally advised that erythropoiesis-stimulating agent (ESAs) should be given to all patients with ESRD with hemoglobin (Hb) levels consistently (i.e. measured twice at least 2 weeks apart) below 11 g/dl, or with haematocrit < 33%, where all other causes of anemia have been excluded. However, recent evidence such as those provided by the TREAT study (Pfeffer, Burdmann et al. 2009) warn against excessive ESAs doses, as an increased risk of stroke has been reported in those patients treated with ESA with a hemoglobin target of 13 g/dl. Thus, the recommendation of an Hb level of 10 to 12g/dl in chronic kidney disease (CKD) patients seems adequate.

Epo is a glycoprotein hormone that promotes the maintenance of committed erythroid progenitors cells, specifically the burst-forming units (BFU-E) and colony-forming units (CFU-E), by binding to surface receptors and preventing them from apoptosis, and stimulating these erythroid progenitors to differentiate into reticulocytes and RBCs. Epo is mainly released by the peritubular capillary endothelial cells in the kidney. Reduced availability of oxygen for tissue metabolic needs (during anemia, hypoxemia or impaired blood flow to the kidney) stimulates Epo production through HIF, whose spontaneous degradation is inhibited in presence of decreased oxygen delivery and iron deficiency. Furthermore, it has now been clearly demonstrated that Epo administration results in reduced hepcidin levels and increased intestinal iron absorption in CHD patients, although it is still debated whether Epo directly signals through its receptor in hepatocytes to downregulate hepcidin transcription, or its effect is indirectly mediated by hypoxia through stabilization of the HIF transcription factors, or by increased erythropoiesis through the induction of erythroid regulators of hepcidin release of the TGFβ superfamily, possibly including growth and differentiation factor 15 (GDF15) and twisted and gastrulation 1 (TWSG1) (Tanno, Bhanu et al. 2007; Kato, Tsuji et al. 2008; Pinto, Ribeiro et al. 2008; Costa, Swinkels et al. 2009; Morelle, Labriola et al. 2009; Srai, Chung et al. 2010). Anyway, this mechanism seems aimed at facilitating enhanced iron delivery to the bone marrow when is needed for accelerated RBCs production, and suggests that the increase in Hb levels in CHD patients induced by Epo may be partly mediated by normalization of hepcidin levels and of iron delivery. Therefore, the process of erythropoiesis needs an adequate renal secretion of Epo, an appropriate response of bone marrow, and sufficient supply of substrates for Hb synthesis, such as iron, folates, and cyanocobalamin. Epo levels are significantly higher in patients with advanced CKD compared to general population, but inappropriately low for the degree of anemia. In addition, the persistence of anemia despite Epo higher levels suggests a concomitant hyporesponsiveness of bone marrow in these patients.

Furthermore, in patients with CKD, RBCs life span is shortened to 60-90 days (versus 120 days), and there is an increase tendency of bleeding due to platelet dysfunction, both for the presence of uremic toxins. Other causes of anemia include chronic blood loss from gastroenteric system and blood trapping in dialyzers, dietary restrictions, loss of taste for iron-rich foods, secondary hyperparathyroidism (which is associated with bone marrow fibrosis), increased hemolysis and decreased erythropoiesis, nutritional deficiency, and accumulation of inhibitors of erythropoiesis related to uremia.

A major condition that contributes to the establishment of anemia in CKD is the inflammatory state, in which the pro-inflammatory cytokines decrease EPO production and induce in this way apoptosis in CFU-E. In the anemia of inflammation, high levels of acute-phase proteins, such as C-reactive protein (CRP), ferritin and in particular of hepcidin are detected. Recent studies unequivocally demonstrate a high prevalence of chronic systemic inflammation in dialysis patients. This condition has been associated with adverse clinical outcome (Stenvinkel, Ketteler et al. 2005), including accelerated atherosclerosis, malnutrition, and pronounced anemia. Most importantly, chronic inflammation and cytokines can worsen anemia by reducing iron availability for hematopoiesis, shorten erythrocyte life span, and directly inhibit erythrocyte progenitor proliferation. Inflammation and cytokines are independent predictors of Epo hyporesponsiveness (Gunnell, Yeun et al. 1999), whereas anti-inflammatory cytokines might be associated with less severe anemia (Stenvinkel and Barany 2002).

5. Iron metabolism in ESRD

ESRD, and in particular CHD, are typically associated with alteration in iron metabolism parameters, that is most frequently characterized by decreased TSAT, resulting in reduced iron availability for erythropoiesis, and is supposed to be related to chronic inflammation and/or blood losses (Kalantar-Zadeh, McAllister et al. 2004). Hyperferritinemia is also frequently observed, and although in the most severe cases excess iron administration is believed to play a role, in the majority of cases it is thought to reflect the malnutrition-inflammation-cachexia syndrome (Kalantar-Zadeh, Rodriguez et al. 2004), referring to the state of malnutrition, chronic inflammation and wasting frequently reported in CHD patients, which is associated with a greater risk of cardiovascular disease and finally with a worse outcome (Kalantar-Zadeh, Regidor et al. 2005). It has been shown that inflammation and oxidative stress induce iron retention in monocytes/macrophages, and the transcription of ferritin, a protein with anti-oxidant activity (Scaccabarozzi, Arosio et al. 2000; Torti and Torti 2002). However, recent data highlight that ferritin levels reflect also iron stores in CHD patients. Indeed, in these subjects ferritin correlates with TSAT, with the presence of common mutations of the *HFE* gene (Valenti, Valenti et al. 2007), and with bone marrow iron stores (Rocha, Barreto et al. 2008).

In CKD patients, three types of situation related to iron-metabolism are observed: 1) absolute iron deficiency occurs due to decreased total body iron stores. It is associated with serum ferritin levels <100 ng/ml and with TSAT <20%. Due to very low iron stores, serum hepcidin is relatively low. This situation is common in patients who undergo hemodialysis, due to low-grade but frequent blood losses (typically 1-3 g of iron/year). 2) Functional iron deficiency is associated with serum ferritin levels higher than 100 ng/ml but TSAT less than 20%. Iron stores are normal or even increased, but the Epo-stimulated bone marrow needs more iron from TF than the iron output from tissue stores, resulting in ESA resistance (observed in 10-20% of cases). Increased hepcidin can aggravate functional iron deficiency by decreasing the release of stored and macrophage iron and intestinal iron aborption, through Fp-1 downregulation. There is also a third type of iron deficiency-related anemia, the most severe and intractable form, historically termed 3) "reticuloendothelial blockage", which usually occurs in the setting of acute or chronic inflammation / infection. It can be considered as an extreme form of functional iron deficiency and is associated with increased CRP levels, TSAT <20%, and normal to very high levels of ferritin. Reticuloendothelial iron

stores are locked, likely by hepcidin, and there is no release of iron to transferrin. Resistance to Epo therapy easily develops, especially if iron administration is limited by adherence to the "official" opinion-based upper cutoff (see below). Thus, the pattern of anemia, hyposideremia, ESA resistance, and high serum ferritin is frequently observed in CHD patients.

As mentioned above, increased hepcidin levels are likely involved in mediating the effect of inflammation on iron metabolism in CHD patients. Over-expression of hepcidin, stimulated by inflammatory cytokines (Nemeth, Valore et al. 2003) and by the innate immune response through Toll-like receptor-4 (TLR4) (Peyssonnaux, Zinkernagel et al. 2006) would result in sequestration of iron into macrophages, decreased intestinal iron absorption and recycling, and finally in the decrease of iron availability for erythropoiesis. Studies in mice moderately overexpressing hepcidin indicate that hepcidin can also induce blunted erythropoietic response to Epo (Nemeth and Ganz 2009). The molecular pathway activated by the inflammatory response which brings to hepcidin overexpression involves the binding of interleukin-6 (IL-6), the major hepatic regulator of the acute phase response to inflammatory stimuli, to its receptor, that eventually leads to the translocation of phosphorylated signal transducer and activator of transcription 3 (STAT3) to the nucleus and binding to the hepcidin promoter, resulting in up-regulation of hepcidin transcription. It has been further proposed that STAT3 activation itself, without inflammation, can regulate hepcidin levels (Wrighting and Andrews 2006). Other pro-inflammatory cytokines may also be involved in modulating iron balance in chronic diseases, such as interferon-γ (IFN- γ), interleukin-1, and tumor necrosis factor-α (TNF-α), but the exact mechanisms are still not clear. Furthermore, recent evidences proved that also macrophages, in addition to hepatocytes, express hepcidin at low levels upon stimulation of the STAT3 pathway by IL-6, induced by lipopolysaccharide (LPS), or by TLR4 mediated pathways (Peyssonnaux, Zinkernagel et al. 2007; Theurl, Theurl et al. 2008). In addition, activation of endoplasmic reticulum stress response by oxidative stress due to inflammation and iron overload may also play a role (Vecchi, Montosi et al. 2009). However, whether hepcidin release by macrophages produces systemic or only localized effects at the site of inflammation or bacterial invasion remains to be determined.

6. Iron therapy in the anemia of ESRD

ESAs, the recommended therapy for ESRD related anemia, increase the need for iron, as they stimulate the synthesis of 2 million new red cells/second (Cavill 1999), so that bone marrow requests strips iron off the circulating TF faster than TF can replenish it, resulting in a relative deficit of iron that leads the reticulocytes to enter the systemic circulation with suboptimal quantities of Hb (Brugnara 2000). Evidence now prove that adequate iron availability increases erythropoiesis and reduces ESA requirements (Besarab, Amin et al. 2000).

According to K-DOQI guidelines (2006), iron status should be evaluated every month during initial ESAs treatment, at least every 3 months during stable ESAs treatment or in CHD patients not treated with ESAs. In clinical practice, no single test adequately monitors iron stores or availability. Serum ferritin is the only available blood marker of storage iron, but it is more reliable in non-dialytic patients than in those who underwent hemodialysis. Tests reflecting the adequacy of iron for erythropoiesis include TSAT, mean corpuscular volume (MCV), and mean corpuscular hemoglobin (MCH) and related indices, such as percentage of hypochromic red blood cells (PHRC) and content of Hb in reticulocytes (CHr).

MCV and MCH decrease to less than normal range only after long-standing iron deficiency and so they do not configure as good indicators of relative iron deficit. TSAT and serum ferritin are undoubtedly the most available serum test, but both show acute-phase reactivity and are poorly decodable for assessment of iron status in such state as chronic disease, malnutrition and inflammation; furthermore they exhibit also diuturnal fluctuations (Kalantar-Zadeh, Rodriguez et al. 2004). Therefore, there is an urgent requirement for novel markers (Wish 2006) more specific for iron deficit, especially in CHD patients, in which occult infections and malnutrition play a major role in determining response to therapy and influencing mortality. CHr is a measure of the amount of hemoglobin in red cells 1 or 2 days old and is a reasonably good reflection of how much iron was available to the bone marrow for incorporation into new red blood cells a few days before. The CHr compared favorably with serum ferritin and TSAT in predicting a response to intravenous iron, but the optimal cutoff value is still under investigation. Mean CHr in the general population is 32±3.3 pg, whereas levels of 26–29 pg indicate iron deficiency, which is diagnosed by the absence of stainable iron stores (Mast, Blinder et al. 2002). More recent evidence refers to a cutoff of 32 pg, and determined that a CHr cutoff of 29 pg tended to miss a number of patients who ultimately responded to intravenous iron. The PHRC is based on the Hb concentration in RBCs; it takes into account the absolute amount of Hb as well as the size of the cell, but is poorly reliable due to the alteration observed in case of long sample transport and storage times. Soluble transferrin receptor levels (sTfR) provide an esteem of the iron need of the proliferating erythron, reflecting both erythropoietic activity and iron deficit, based on the fact that erythroblasts in the bone marrow will increase the presentation of membrane TFR-1 in the setting of iron deficiency, as inadequate iron supplies in a patient stimulated with ESAs. Levels of sTfR correlate with TFR-1 membrane expression and also tend to be elevated in the presence of increased erythroid activity (Tarng and Huang 2002). However, there is not a wide consensus regarding cutoff and use of sTfR for the assessment of iron status in CHD patients.

In patients undergoing ESAs therapy, interpretation of iron status tests should incorporate consideration of the Hb level and ESAs dose, in order to provide information important to medical decision making, because they elucidate the status of both external iron balance (net loss or gain of iron) and internal iron balance (disposition of iron between stores and circulating red blood corpuscles). For example, decreasing ferritin levels in the presence of a stable or decreasing Hb level may signify external iron loss, and so iron therapy is indicated. Conversely, decreasing ferritin levels in the presence of increasing Hb denotes an internal shift in iron from stores to Hb, as would be expected in a patient responding to ESA therapy: if iron status remains within the target range, additional iron administration may not be required. Finally, an increase in ferritin levels accompanied by a decrease in TSAT suggests inflammation-mediated reticuloendothelial blockade and may be accompanied by a decrease in Hb and increase in ESA dose (2006).

KDOQI guidelines suggest that iron supplementation should be administered during ESA treatment to maintain serum ferritin >200 ng/ml and TSAT >20%, or CHr >29 pg/cell in CHD and serum ferritin >100 ng/ml and TSAT >20% in ESRD or in patients in peritoneal dialysis. The upper limit of serum ferritin besides which there is no recommendation to routinely administer iron was set as 500 ng/ml. When ferritin level is greater than 500 ng/ml, decisions regarding iron administration should weight ESA responsiveness, Hb and TSAT level, and the patient's clinical status. Indeed, iron determination by means of

magnetic susceptometry showed hepatic non–heme iron concentration greater than the upper limit of normal in patients with ferritin > 500 ng/ml (Canavese, Bergamo et al. 2004).
The preferred route of iron administration in CHD patients is by intravenous (IV) infusion, since iron absorption from the gastrointestinal tract may be impaired in uremic patients (Eschbach, Cook et al. 1970; Donnelly, Posen et al. 1991; Kooistra and Marx 1998), likely because of high hepcidin levels. In ESRD or peritoneal dialysis patients, iron can be administered either orally or IV. The lack of data from clinical trials does not permit to state if a continuous iron supplementation is better than a periodical one. Available intravenous iron formulations include iron dextran, sodium ferric gluconate, and iron sucrose: all these forms may be associated with acute adverse effects, occasionally severe, including hypotension with or without other symptoms and signs, possibly for immune-mediated mechanisms (mast cell–mediated processes leading to a clinical syndrome resembling anaphylaxis), or for the release of small amounts of bioactive, partially unbound iron into the circulation, resulting in oxidative stress and hypotension (labile or free iron reactions). The pathogenesis may differ depending on the type of IV iron: anaphylactoid reactions appear to occur more frequently with iron dextran and high molecular weight forms (Novey, Pahl et al. 1994), whereas labile or free iron reactions occur more frequently with nondextran forms of iron (Agarwal, Vasavada et al. 2004; Agarwal 2006). Thus, resuscitative medication and personnel trained to evaluate and resuscitate anaphylaxis should be available whenever a dose of iron dextran is administered (2006). As a result, FDA has issued a ''black box" warning, recommending that patients undergo a 25 mg test dose the first time the drug is given. If a patient does not have an adverse reaction to this dose, he/she is less likely to have an anaphylactic reaction to the therapeutic dose of iron dextran, but fatal anaphylactic reactions still occur with an uneventful test dose (Lankhorst and Wish 2010).

7. Iron toxicity in ESRD

Before the ESAs era, CHD patients often developed severe iron overload, frequently with ferritin levels >1000 ng/ml, due to polytransfusion. Nowadays, patients with anemia of CKD are supplemented with IV iron to correct the relative deficiency linked to the ESAs therapy. However, large doses of iron may exceed storage capacity leading to the accumulation of unbound iron in plasma, which as seen before, via the Fenton reaction, can produce reactive oxygen species (Michelis, Gery et al. 2003). In addition, different formulation of IV iron are associated with various degree of oxidative stress: iron sucrose, for example, determines a 60% greater elevation in tumor necrosis factor alpha (TNFα), a proinflammatory cytokine, compared with either iron dextran or ferric gluconate (Zager 2005).
Moreover, iron has been implicated in the pathogenesis of the accelerated atherosclerosis in CHD (Griendling and FitzGerald 2003), in addition to traditional and population specific risk factors such as anemia, hyperhomocysteinemia, hyperphosphatemia and inflammation. It has been suggested that iron overload may increase cardiovascular risk in the general population, by affecting LDL oxidation and endothelial dysfunction (Roest, van der Schouw et al. 1999; Zacharski, Chow et al. 2000; Wolff, Volzke et al. 2004) and administration of intravenous iron has been associated with increased oxidative stress (Michelis, Gery et al. 2003). In CHD patients, carotid intima media thickness (an early index of atherosclerosis and a strong predictor of cardiovascular events) and carotid plaques were positively correlated with serum ferritin and oxidative stress and reduced plasma anti-oxidant activity (Drueke,

Witko-Sarsat et al. 2002; Valenti, Valenti et al. 2007), and intima-media thickness was also associated with the dose of IV iron administered (Reis, Guz et al. 2005). Furthermore, hepcidin and TNFα levels have also been correlated with vascular stiffness, another reliable predictor of cardiovascular events in CHD (Kuragano, Itoh et al. 2011). The mechanism may involve iron trapping into endothelial cells, plaque macrophages, and vascular smooth muscle cells, with activation of the atherogenic process and progression of the plaque lesion. Indeed, a significantly higher iron content has been detected in atherosclerotic plaques than in healthy vascular tissue (Stadler, Lindner et al. 2004). Hyperferritinemia (beyond 800 ng/ml) was found to be associated with imminent death risk, and cardiovascular death in hemodialytic patients (Kalantar-Zadeh, Don et al. 2001; Kalantar-Zadeh, Regidor et al. 2005), even if this association might not reflect only iron toxicity, since hyperferritinemia associated morbidity is partially explained by non-iron-related factors, such as proinflammatory cytokine release and the malnutrition inflammation cachexia syndrome. However, even after adjustment for these confounding variables, high ferritin levels and the intravenous dose of iron were still associated with increased total and cardiovascular mortality (Feldman, Santanna et al. 2002; Kalantar-Zadeh, Regidor et al. 2005). In particular, recent data from our group confirm that in CHD patients, hyperferritinemia reflects a relative increase in iron availability and a decrease in iron-specific anti-oxidant activity, is favored by *HFE* mutations, and represents a risk factor for advanced cardiovascular damage, as evaluated by the presence of plaques, both at carotid and femoral arteries (Valenti, Valenti et al. 2007).

Recent evidence (Neven, De Schutter et al. 2011) confirm a strict relation between iron and vascular damage in CHD patients, suggesting that the mechanism underpinning this association may involve the predisposition to arterial wall calcification. Indeed, increased oxidative stress, associated with iron overload, promotes *in vitro* a shift in vascular smooth muscle cells from a contractile to an osteogenic phenotype (Byon, Javed et al. 2008), as indicated by an increased expression of Runx2, a key transcription factor for osteogenic differentiation. Meanwhile, ROIs seem to inhibit osteoblastic differentiation and mineralization in bones (the so called calcification paradox of bone-vascular axis) (Parhami, Morrow et al. 1997; Persy and D'Haese 2009); both these processes may depend on an iron-induced upregulation of ferritin and an increase in ferroxidase activity. Furthermore, iron overload itself has been associated with osteoporosis and increased bone reabsorption (Valenti, Varenna et al. 2009). Reactive species stimulates also apoptosis in vascular smooth cells, providing in this way a nidus for the deposition of calcium-phosphate crystals (Reynolds, Joannides et al. 2004; Shroff, McNair et al. 2008), which could be directly promoted by iron salts (Neven, De Schutter et al. 2011), and promoting thus vascular calcifications and an increased cardiovascular risk for ESRD patients.

Moreover, iron overload has been associated with an increased incidence and severity of infections, which, besides the known growth-promoting effect of iron on microbial pathogens, have been also related to inhibition of phagocytosis (Cieri 1999), and to inhibition of the anti-microbial molecule lactoferrin (Ellison and Giehl 1991). Both iron sucrose and ferric gluconate have been shown to result in impaired trans-endothelial polymorphonuclear leukocytes migration in vitro (Sengoelge, Kletzmayr et al. 2003). Iron is an essential nutrient for bacterial microorganisms, and several bacterial species (including *E. coli*, *Klebsiella* spp., *Pseudomonas* spp., and *Salmonella* spp) can compete with TF for unbound iron in the blood by means of bacterial iron chelators, named siderophores (Cieri 1999; Brewster and Perazella 2004). Other

bacterial species, such as *Staphylococcus aureus* and *Haemophilus influenzae* express transferrin receptors, which allow these bacteria to use iron for growth. Infection risk in CKD patients depends not only on iron, but also on the severity of anemia (Hershko, Peto et al. 1988), transfusions, secondary splenic dysfunction, and the presence of catheters for hemodialytic or endovenous therapies. Although human data are not conclusive, it is still recommended that, as a precaution, IV iron is stopped in patients with ongoing bacteraemia, as in vitro and in vivo studies demonstrated that administering IV iron during active infection may contribute to bacterial growth (Zager 2005; Zager, Johnson et al. 2005). Nevertheless, correction of anemia is effective in reducing oxidative stress and, consequently, cardiovascular risk, decreasing morbidity and mortality and also producing regression of left ventricular hypertrophy in patients with CKD (Ayus, Go et al. 2005), so that a carefully balanced management of iron supplementation is needed in ESRD patients.

8. Role of hepcidin in anemia of ESRD

Increased hepcidin levels have been hypothesized to contribute explaining many typical alterations of iron metabolism in CHD patients, and to the pathogenesis of anemia in ESRD (Kulaksiz, Gehrke et al. 2004; Tomosugi, Kawabata et al. 2006; Malyszko and Mysliwiec 2007; Kato, Tsuji et al. 2008; Kemna, Tjalsma et al. 2008), an almost universal finding associated with increased mortality (Paganini 1989; Locatelli, Pisoni et al. 2004). As mentioned above, although treatment with ESAs and IV iron formulations (Locatelli, Aljama et al. 2004) are generally prescribed, functional iron deficiency is a common finding, determining the need for high doses of ESAs and iron, both associated with adverse events. Indeed, high ESAs doses have been associated with mortality due to cardiovascular events related to hypertension and hypercoagulability (Miyashita, Tojo et al. 2004; Phrommintikul, Haas et al. 2007; Strippoli, Tognoni et al. 2007), whereas excess iron promotes vascular damage by inducing oxidative stress, and heightens the risk of infections (Seifert, von Herrath et al. 1987; Jean, Charra et al. 2002; Teehan, Bahdouch et al. 2004; Kalantar-Zadeh, Regidor et al. 2005; Valenti, Valenti et al. 2007).

The mechanism proposed to explain refractoriness to IV iron was previously related to the inhibition of erythropoiesis and iron recycling from macrophages by inflammation (Stenvinkel 2003). Increased hepcidin may be involved in mediating the effect of inflammation by reducing intestinal iron absorption and iron recycling from monocytes (Pietrangelo 2007), decreasing serum iron available for the erythropoiesis (Camaschella 2005) and the therapeutic effect of ESAs and intravenous iron (Malyszko and Mysliwiec 2007). Furthermore, in CKD the renal clearance of hepcidin diminishes gradually with the progression of kidney disease, with some studies reporting an inverse correlation between glomerular filtration rate and serum hepcidin. Thus, the severity of kidney injury directly influences hepcidin levels contributing to anemia via this mechanism. Importantly, preliminary data indicate that hemodialysis reduce serum hepcidin levels (Zaritsky, Young et al. 2010), contributing to anemia management, but whether specific dialytic techniques differentially affect hepcidin clearance is still unknown. Finally, chronic iron supplementation is a strong inducer of hepcidin also in CHD patients, thereby paradoxically hampering the optimal utilization of large doses of administered iron. Indeed, overall evidence indicate that serum ferritin and C reactive protein levels, reflecting iron stores and inflammation, are the major determinants of hepcidin levels in CHD and ESRD (Tomosugi, Kawabata et al. 2006; Kato, Tsuji et al. 2008; Valenti, Girelli et al. 2009). Supporting

a causal role of increased hepcidin in the pathogenesis of anemia, hepcidin-25 levels have been correlated with hyposideremia and the severity of anemia in patients with stable Hb values (Valenti, Valenti et al. 2008). Moreover, we recently reported that frequent mutations in the *HFE* gene, which decrease hepcidin response to iron stores (Piperno, Girelli et al. 2007; Vujic Spasic, Kiss et al. 2008), are associated with increased sensitivity to ESAs and iron in CHD patients, and may be associated with a better clinical outcome (Valenti, Valenti et al. 2008). However, in a preliminary report hepcidin-25 was not significantly associated with Epo responsiveness (Kato, Tsuji et al. 2008), and in another recent study an association between greater iron doses, increased darbepoietin resistance index, and low hepcidin levels, possibly downregulated by ESAs and anemia, was detected (Bratescu, Barsan et al. 2010), thus suggesting that other mechanisms besides increased hepcidin are involved in the pathogenesis of anemia in CHD. All in all, these recent studies suggest that hepcidin is involved in the pathogenesis of anemia in CKD and chronic diseases, but is not the only player involved.

9. Influence of *HFE* mutations on iron metabolism regulation, hepcidin levels, and erythropoiesis in CHD patients

9.1 *HFE* mutations, iron metabolism, and erythropoiesis

As in the general population, in CKD patients iron metabolism homeostasis and iron stores depend from the interaction between genetic and environmental factors, including iron administration. Among the candidate genetic factors that have been considered, the effect of *HFE* genotype on iron overload in CHD patients was analyzed in a few small studies with inconsistent results. In a recent study from our group, the presence of the C282Y or H63D *HFE* mutations was associated with increased iron stores in Italian patients (Valenti, Valenti et al. 2007), whereas previously other Authors found only a nonsignificant, modest effect of the more common H63D mutation on serum ferritin (Pericole, Alves et al. 2005), and lower iron requirement in the few subjects carrying the C282Y *HFE* mutation (Canavese, Bergamo et al. 2002). Since the C282Y and H63D *HFE* mutations uncouple the regulation of intestinal iron absorption from iron stores, by modifying hepatic iron sensing and defective release of hepcidin (Gehrke, Herrmann et al. 2005), we hypothesized that these genetic variants may also influence iron mobilization from macrophages after therapeutic iron infusion and ESAs treatment by influencing hepcidin release. Consistently with previous data (Canavese, Bergamo et al. 2002), we showed that the two common C282Y and H63D mutations, present in about one third of subjects, were associated with a lower requirement of ESAs and a trend to a lower requirement of iron (Valenti, Valenti et al. 2008). The lower baseline ESAs and iron requirement in mutation carriers indicate that the alterations in iron metabolism include not only increased iron stores, but possibly also iron handling by macrophages after infusion, and iron availability to the erythron. *HFE* mutations attenuate the effects of inflammation on iron metabolism, including reduced iron absorption, iron sequestration in macrophages, and erythroblast resistance to Epo, and protect against iron-related damage by favoring the delivery of intravenously administered iron to the erythron. Importantly, in this study iron stores were not lower, and the requirement of iron and ESAs were not higher in *HFE* H63D/wt compared to C282Y/wt and H63D/H63D patients. In subjects without ESRD, chronic inflammation, and liver disease, simple heterozygosity for the H63D or the C282Y *HFE* mutations was not found to be associated with either iron overload (Adams, Reboussin et al. 2005) or reduced hepcidin release (Bozzini, Campostrini et al. 2008), and previous data

obtained in a very limited number of CHD patients did not support an influence of increased hepcidin on Epo requirement (Kato, Tsuji et al. 2008). It could therefore be speculated that chronic inflammation and blood losses of CHD may provide enough environmental pressure to magnify subtle alterations in cellular iron handling in carriers of the milder and more common H63D mutation, which thus reach clinical significance, unlike to what we observed in general population (Adams, Reboussin et al. 2005), and that the effect of *HFE* mutations might not be limited to modulation of serum hepcidin levels. Thus, in the setting of HDT paradoxically *HFE* mutations protect against iron related damage by favoring the delivery of intravenously administered iron to the erythron.

9.2 *HFE* mutations and survival

Furthermore, although due to the limited number of patients considered and the inclusion of prevalent patients in the analysis results should interpreted with caution, our data strikingly highlighted that the presence of *HFE* mutations was associated with a reduced hazard ratio of death. An updated mortality curve for cardiovascular disease and infection in 127 Italian CHD patients subdivided according to the presence or not of the C282Y and H63D *HFE* mutations is shown in figure 1. It is worthy of note that, in patients negative for *HFE* mutations, we observed a higher mortality due to sepsis, previously associated with a higher iron dosage (Jean, Charra et al. 2002; Teehan, Bahdouch et al. 2004), and due to cardiovascular disease, possibly linked to hypertension and thromboembolic events related to ESAs (Miyashita, Tojo et al. 2004; Phrommintikul, Haas et al. 2007) and oxidative stress related to iron (Valenti, Valenti et al. 2007).

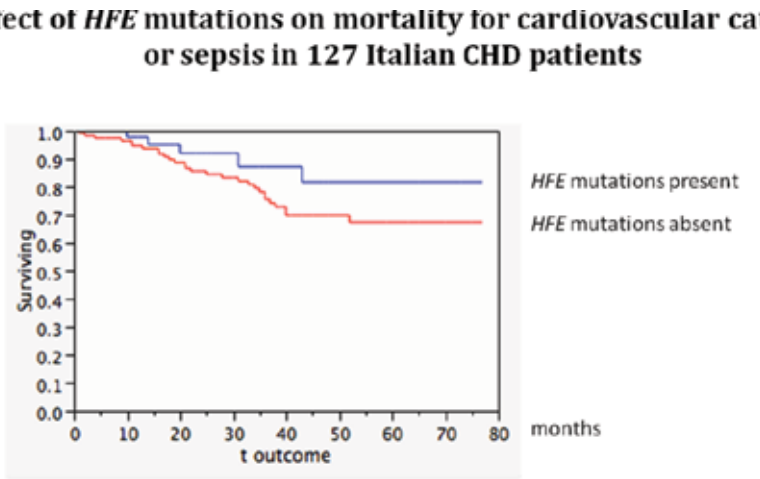


Fig. 1. Effect of *HFE* mutations on mortality for cardiovascular causes or infections in 127 Italian CHD patients.

9.3 *HFE* mutations and hepcidin release

In the hypothesis that altered regulation of hepcidin release by iron stores might explain the apparent protective role of the *HFE* mutations on cardiovascular complication and on the

response to ESAs therapy (Valenti, Girelli et al. 2009), we recently investigated in the largest series of CHD patients analyzed for serum hepcidin levels to date, and the first one in which a quantitative assay was used, whether the effect of common *HFE* gene mutations on hepcidin-25 could be involved in the pathophysiology of the alterations of iron metabolism and anemia. Our data clearly confirmed and extended previous reports based on a semiquantitative assay in smaller series, suggesting substantial hepcidin-25 upregulation in CHD patients compared to controls (Tomosugi, Kawabata et al. 2006; Kemna, Tjalsma et al. 2008), and preserved regulation of hepcidin-25 by iron stores and inflammation in this setting (Tomosugi, Kawabata et al. 2006; Kato, Tsuji et al. 2008). They also provided evidence of a modulating effect of *HFE* mutations, both the C282Y and the H63D mutations, on hepcidin-25 regulation by iron stores. First, we showed that hepcidin-25 levels are higher in patients receiving CHD than in healthy controls. Notably, while the groups studied were matched for gender, that is a major determinant of serum hepcidin levels (Ganz, Olbina et al. 2008; Swinkels, Girelli et al. 2008), CHD patients were significantly older than controls. However, this is unlikely to represent a substantial bias, since previous studies did not show a consistent increase in hepcidin levels with age in the general population (Ganz, Olbina et al. 2008). These data suggest that ESRD itself plays a role in hepcidin-25 accumulation in this setting. Also, we showed preserved regulation of hepcidin-25 levels by iron stores, as demonstrated by the very strong correlation with serum ferritin, and modulation by inflammation, as detected by CRP levels. Again, these data match closely those previously obtained showing a correlation of hepcidin-25 with ferritin and IL-6, but not CRP, in CHD, and, anecdotally, an increase in hepcidin-25 after IV iron administration (Tomosugi, Kawabata et al. 2006; Kato, Tsuji et al. 2008). Moreover, for the first time we were able to demonstrate a negative correlation between hepcidin-25 and serum iron, and we found that in a subgroup of patients with stable disease, selected to avoid the confounding effect of the frequent presence of acute inflammation, blood losses, cancer, and recent variation in the dosage of therapy, hepcidin-25 negatively correlated with Hb levels, and with a trend towards a negative correlation with lower serum iron. Since anemia and hyposideremia should rather decrease hepcidin levels, these findings suggest that hepcidin-25 plays a causal role in determining anemia by reducing iron availability to the erythron. To our knowledge, this is the first evidence supporting the theory that hepcidin is involved the pathogenesis of the anemia of CHD. These results imply that in CHD excessive iron administration may paradoxically hamper iron utilization for erythropoiesis by trapping iron in phagocytes, because of excessive hepcidin-25 induction favored by chronic inflammation, and that the effect of inflammation on altered iron metabolism and erythropoiesis may be mediated by increased hepcidin levels. They also suggest, together with the survival data in this same cohort shown above, that pharmacological downregulation of hepcidin (Babitt, Huang et al. 2007) may be beneficial in CHD. The subtle effect of *HFE* mutations on hepcidin release is likely magnified in CHD patients by the environmental pressure determined by chronic inflammation, and exposure to high amounts of iron and ESAs, thereby reaching clinical significance.

However, the protective effect of *HFE* mutations might not be limited to enhanced erythropoiesis related to relatively lower hepcidin levels. Indeed, as reported above, HFE protein seems to be directly implicated in the maturation of erythroblasts, and lack of functional HFE in erythroid precursors favors erythropoiesis and is associated with increased Hb levels independently of the effect on hepcidin levels (Ramos, Guy et al. 2011). In addition, another recently discovered role of HFE is the regulation of iron uptake and

inflammatory response in monocytes and macrophages. It has indeed been demonstrated that in the presence of mutated *HFE* human macrophages have a deficient response to inflammatory stimuli, which is likely related to an impaired ability to retain iron, NFκB activation, and cytokine release, and that hepcidin upregulation by inflammation is also impaired in the absence of functional HFE (Roy, Custodio et al. 2004; Wang, Johnson et al. 2008; Valenti, Dongiovanni et al. 2011; Wallace, McDonald et al. 2011).

9.4 Iron, *HFE* mutations and atherogenesis

Contrasting evidence suggest that iron deposition in the arterial wall may favor atherogenesis (Sullivan 2007). As a consequence of reduced hepcidin levels and lower inflammation, *HFE* mutations may play a protective role also in vascular damage related to atherosclerotic process, the major cause of mortality in CHD patients. In a large series of patients with metabolic syndrome alterations (Valenti, Swinkels et al. 2010), we observed that serum ferritin, reflecting iron stores, was an independent predictor of vascular damage, but only in patients negative for *HFE* genotypes predisposing to iron accumulation due to a relative decrease in the release of hepcidin. These data suggest that ferritin may represents a new marker of vascular damage, and support the controversial hypothesis that increased hepcidin favors atherosclerosis by inducing iron accumulation in arterial wall macrophages (Sullivan 2007). We found that ferritin levels were independently associated with common carotid arteries intima-media thickness, reflecting early vascular damage and a strong predictor of cardiovascular events, and very strongly associated with the presence of carotid plaques, possibly because of an additional effect of iron on the promotion of the complication of atherosclerosis by favoring endothelial damage and thrombosis (Day, Duquaine et al. 2003). The association between ferritin and vascular damage may thus be explained by the atherogenic effect of increased iron stores (Lee, Shiao et al. 1999; Duffy, Biegelsen et al. 2001; Wolff, Volzke et al. 2004; Lapenna, Pierdomenico et al. 2007), possibly mediated by increased hepcidin levels that determine iron trapping into monocytes, thus promoting transformation into foam cells in the presence of an atherogenic environment (Yuan, Li et al. 2004; Kraml, Klein et al. 2005; Sullivan 2007). Following this hypothesis, *HFE* mutations would be protective by decreasing hepcidin release and favoring iron egress from macrophages. Our data are consistent with the hypothesis that iron mediated vascular damage involves hepcidin upregulation, a mechanism that could be implied also in the enhanced atherosclerotic process in CHD patients, in which as explained before, hepcidin levels are more elevated than in general population.

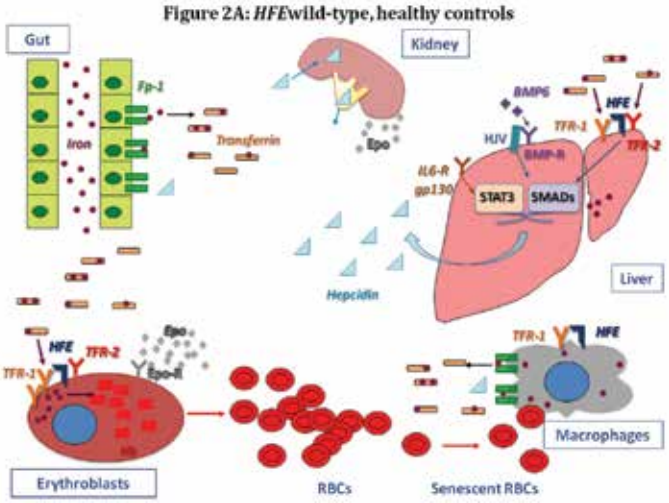
To further investigate the mechanisms involved in iron-mediated atherogenesis and explain the protective effect of *HFE* mutations, we next investigated the effect of iron treatment on the activation and secretion of atherogenic molecules in differentiating monocytes of subjects with different *HFE* genotypes. Treatment with iron salts, determining increased intracellular iron, enhanced the release of the macrophage chemoattractant protein-1 (MCP-1) and of IL-6 independently of oxidative stress in differentiating monocytes derived from patients with metabolic syndrome (Valenti, Dongiovanni et al. 2010). Furthermore, the iron-dependent induction of the MCP-1 and IL-6 was associated with the severity of vascular damage in these patients, suggesting that macrophage activation by iron may be involved in the pathogenesis of atherosclerosis progression in these patients also *in vivo*. IL-6 is a pro-inflammatory cytokine involved in the acute phase response, whose serum levels correlate with cardiovascular risk and have been linked to the inflammatory status within atherosclerotic plaques (Luc, Bard et al. 2003). MCP-1 (also known as CCL2), is a chemokine involved in macrophage recruitment at

inflammation sites released by macrophages, but also by smooth muscle cells and endothelial cells. MCP-1 plays a crucial role in both the initiation and progression of atherosclerosis, and its serum levels reflect the atherosclerotic plaque burden. Higher MCP-1 has also been reported to represent a negative prognostic factor in acute coronary syndromes (Nelken, Coughlin et al. 1991; Gu, Okada et al. 1998; Amasyali, Kose et al. 2009). A dose-dependent induction of MCP-1 transcription and release in the supernatant induced by iron treatment was confirmed in monocytes of healthy subjects with normal iron parameters, suggesting that this represents a physiological response to increase intracellular iron availability. These data are in line with previous experimental evidence, suggesting that iron may induce MCP-1 release by oxidative stress in macrophages. Indeed, intravenous iron treatment induces MCP-1 release by monocytes in mice and patients with ESRD (Zager 2005; Agarwal 2006), whereas the iron chelator deferoxamine have recently been shown to decrease NFκB and consequently MCP-1 release. Importantly, we also showed that treatment with hepcidin mimicked the effect of iron salts on MCP-1 release. This evidence led to another confirmation of the fundamental pathogenic role of hepcidin on atherogenic process. In addition, we obtained evidence that, as it occurs in the presence of homozygosity for the C282Y *HFE* mutation (Garuti, Tian et al. 2010), monocytes of patients with *HFE* genotypes predisposing to mild iron overload show reduced ability to accumulate intracellular iron. Iron induced upregulation of MCP-1 and IL-6 was prevented in monocytes of patients carrying these *HFE* genotypes "at risk", thus suggesting that the hampered upregulation of pro-inflammatory mediators may contribute to explain the lack of association between iron overload and accelerated atherosclerosis in HH patients, and the contradictory data on the effect of *HFE* mutations on vascular damage (Sullivan 2007; Engberink, Povel et al. 2010). Consistently, despite the fact that iron overload has been associated with increased serum MCP-1 levels, patients homozygous for the C282Y HFE mutation had lower levels than those whose iron overload was explained by other genetic/acquired factors and healthy controls (Lawless, White et al. 2007). We also measured serum MCP-1 levels in a series of patients at high risk for vascular disease because of metabolic syndrome, who typically display a high prevalence of altered iron metabolism (Valenti, Swinkels et al. 2010). In line with a possible induction of MCP-1 release by increased iron in monocytes/macrophages, serum MCP-1 levels, whose primary source is represented by activated macrophages, were significantly correlated with hepcidin-25, and MCP-1 levels were an independent predictor of the presence of carotid plaques, indicating an advanced atherosclerotic process. Thus, the emerging details of the physiology of hepcidin suggest a resolution of the apparent paradox of an important role of iron in atherogenesis in the absence of increased plaque burden in HH. Hepcidin, induced by iron and inflammation, acts to block iron recycling from macrophages by binding and causing internalization and degradation of ferroportin, the sole cellular iron exporter. Low hepcidin levels are observed in iron deficiency anemia and HH, both characterized by reduced macrophage iron stores. The failure of vascular wall macrophages to retain iron in HH may therefore prevent the progression of atherosclerotic plaques. *HFE* mutations could assume a protective role in the pathogenesis of atherosclerotic plaques and cardiovascular disease. These data support the hypothesis that MCP-1 release by intralesional monocytes/macrophages, which can be iron loaded because of intraplaque hemorrhage, systemic iron overload, and local inflammation is involved in the pathogenesis of iron-induced atherosclerosis.

9.5 Impact of *HFE* mutations in CKD: a model

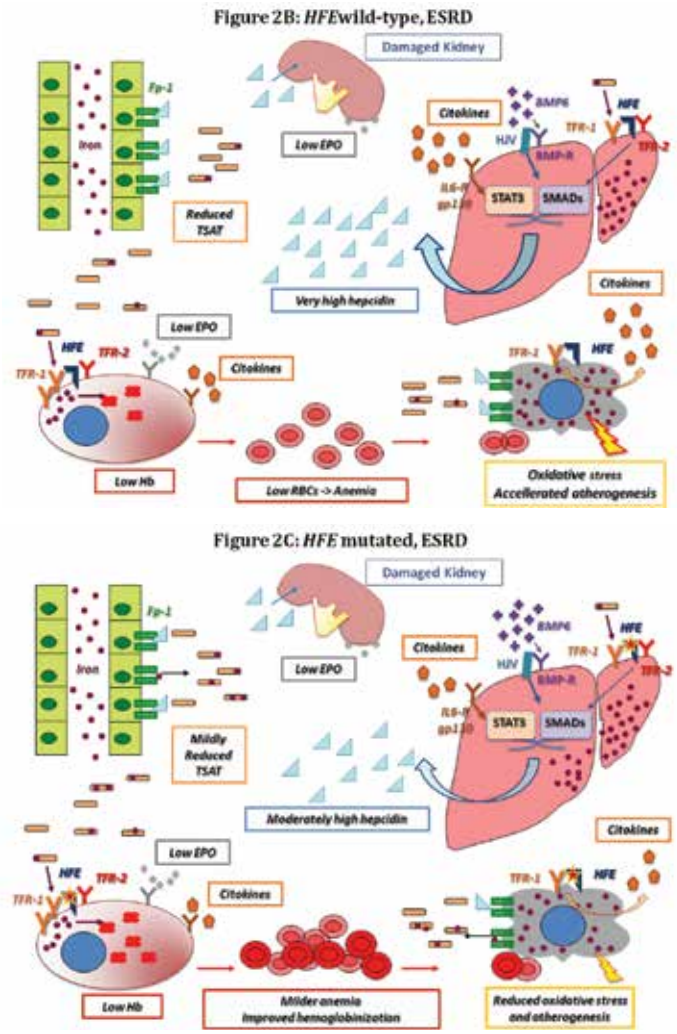
In healthy controls wild-type for *HFE* (i.e. not carrying mutations) (Figure 2A), iron absorption depends on the activity of Fp-1, regulated by hepcidin. Iron is necessary for the

production of Hb in erythroblasts, which are stimulated by Epo, secreted by the kidneys to stimulate the production of mature RBCs in response to anemia or a relative deficit of oxygen delivery to the cells. Uptake of iron-TF occurs by means of TFR-1. Iron recycled from senescent RBCs by macrophages exits these cells via Fp-1. Hepcidin, secreted by the liver and excreted by the kidneys, promotes the degradation of Fp-1, thus inhibiting enteric absorption and the release of iron from macrophages. HFE is bound to TFR- 1 and is involved in the transmission of signaling that finally brings to the finely tuned secretion of hepcidin, in agreement with iron status. This regulatory pattern involves also BMP6, HJV and many other molecules, and converges on the activation of SMADs. In patients with ESRD wild type for *HFE* mutations (Figure 2B), the presence of proinflammatory cytokines, through IL-6 release and the activation of STAT3, and the impairment of renal excretion enhance the concentration of hepcidin, and consequently cause the impairment of iron absorption from the enteric lumen and of the excretion from macrophages: iron stores are not depleted so ferritin levels are high, but few iron circulates into the blood, so that TSAT is low. The erythroblasts have not sufficient iron to support Hb production and, along with Epo deficiency due to renal dysfunction, this provokes a significant reduction in RBCs production, which consequently leads to anemia. Furthermore, iron overload in macrophages predisposes to enhanced oxidative stress and accelerated atherosclerosis, and increases the susceptibility to infections. Conversely, patients with ESRD and mutations of *HFE* gene (C282Y and/or H63D) (Figure 2C) display a milder degree of anemia, since these mutations uncouple the hepatic iron sensing and lead to a relative deficit in hepcidin secretion, to a reduced cytokines release and to an improvement of erythropoiesis. In this way, more iron is absorbed from the gut, TSAT is only moderately diminished, and less iron remains trapped into macrophages. Finally, less severe oxidative stress is observed, contributing to the possible reduction in mortality and better response to ESAs therapy.



Fp-1: ferroportin 1, EPO: erythropoietin, Epo-R: erythropoietin receptor, IL6: interleukine 6, HJV: hemojuvelin, BMP6: bone morphogenetic protein 6, BMP-R: bone morphogenetic protein receptor, TFR: transferrin receptor, HFE: hereditary hemochromatosis protein, STAT3: Signal transducer and activator of transcription 3, SMADs: small mother against decapentaplegic, Hb: hemoglobin, RBCs: red blood cells, TSAT: transferrin saturation.

Fig. 2. (A) A model of the impact of *HFE* mutations in chronic renal disease (see text).



Fp-1: ferroportin 1, EPO: erythropoietin, Epo-R: erythropoietin receptor, IL6: interleukine 6, HJV: hemojuvelin, BMP6: bone morphogenetic protein 6, BMP-R: bone morphogenetic protein receptor, TFR: transferrin receptor, HFE: hereditary hemochromatosis protein, STAT3: Signal transducer and activator of transcription 3, SMADs: small mother against decapentaplegic, Hb: hemoglobin, RBCs: red blood cells, TSAT: transferrin saturation.

Fig. 2. (B), (C) A model of the impact of *HFE* mutations in chronic renal disease (see text).

10. Hepcidin targeting therapies for the management of the anemia of ESRD and CHD

Overall evidence thus suggest that *HFE* mutations are associated with reduced serum hepcidin levels in CHD patients, and result in a reduced need of ESAs and iron supplementation, thus conferring a possible benefit on survival, in particular by reducing cardiovascular and infectious complications (Valenti, Valenti et al. 2008; Valenti, Girelli et al. 2009). These results may have important clinical implications, as they suggest that targeting

the HFE/hepcidin/ferroportin-1 axis by pharmacological treatment (Babitt, Huang et al. 2007), which is now under extensive investigation for the treatment of anemia of chronic disease (Nemeth 2010), may further improve the long-term outcomes of CHD, by reducing the amount of iron and ESAs supplementation needed and by improving iron utilization for erythropoiesis (Babitt and Lin 2010).

Several drugs are under development for the modulation of the release and activity of hepcidin. The approaches under study include neutralizing anti-hepcidin antibodies (Sasu, Cooke et al. 2010), inhibitors of BMP type I receptors such as dorsomorphin (Yu, Hong et al. 2008), soluble HJV, which acts as decoy receptor inhibiting BMP signaling (Babitt, Huang et al. 2007), and ESAs themselves, which can directly or indirectly inhibit hepcidin release (Pinto, Ribeiro et al. 2008).

In a mouse model of anemia of chronic disease (ACD) caused by injections of heat-killed *Brucella Abortus* (Sasu, Cooke et al. 2010), neutralizing antibodies (Abs) directed against hepcidin were able to restore the reticulocyte response and normal Hb levels in combination with ESAs, whereas ESAs alone, and importantly ESAs plus IV iron, were ineffective. Furthermore, over-expression of hepcidin in mice was sufficient to hamper the erythropoietic response to ESAs. It is therefore conceivable that administration of anti-hepcidin antibodies could restore ESAs susceptibility in the roughly 10% of CHD patients, who display ESAs resistance due to chronic inflammation and high hepcidin levels. These preliminary preclinical data suggest that it is unlikely that therapies that antagonize hepcidin would result in the control of anemia when administered alone, but they could restore susceptibility in patients with ESAs resistance or reduce the dose of ESAs and iron required to achieve anemia control, thereby minimizing side effects.

A different approach to control hepcidin activity is represented by the modulation of hepcidin expression by targeting bone morphogenetic proteins (BMPs) activity. Soluble forms of HJV (sHJV), deriving from cleavage by furin or TMPRSS6, have been detected both in cell cultures and in human sera. Administration of sHJV (HJV.Fc) has been demonstrated to inhibit BMP signaling and hepcidin expression, likely by binding to BMP6 and preventing the interaction with BMP receptors (Andriopoulos, Corradini et al. 2009), and could be possibly exploited as a treatment for anemia of inflammation (Babitt, Huang et al. 2007). It was also shown that anti-BMP6 Abs were similarly effective (Andriopoulos, Corradini et al. 2009). Notably, sHJV has been demonstrated to be downregulated by iron stores and induced by hypoxia, thus possibly being involved in hepcidin regulation by these stimuli. However, the physiological relevance of sHJV forms is still unknown; it has been hypothesized that it is released by the skeletal and cardiac muscle, where HJV is expressed, thus acting in response to iron deficiency and hypoxia to increase oxygen supply by hepcidin downregulation and increased iron availability for the synthesis of Hb, myoglobin, and mitochondrial cytochromes (Babitt and Lin 2010).

A recent and promising development was provided by the recognition of the hepcidin suppressive activity of heparin. Heparan sulfate proteoglycans (HSPGs) are expressed on the surface of various cell types and in the extracellular matrix, and modulate BMP osteogenic activity by binding BMPs, BMP antagonist, and BMP receptors. Heparin, a proteoglycan analog to HSPGs, has been reported to strongly down-modulate hepcidin release *in vitro* in hepatocytes and *in vivo* in mice, resulting in increased serum iron and decreased splenic iron levels (Poli, Girelli et al. 2011). The effect was long lasting and higher for unfractioned than for low-molecular-weight heparin and fondaparinux, and seemed to depend on the sequestration of BMP6, thereby preventing the interaction with HJV and the

induction of hepcidin transcription. Importantly, heparin not only hampered BMP6 dependent induction of SMAD phosphorylation and hepcidin transcription, but could also prevent the IL-6 and STAT3 dependent activation of hepcidin promoter, suggesting that it may overcome the effect of inflammation on hepcidin release also in patients with anemia of chronic diseases or ESRD. Due to the dose-dependent anticoagulant effects and the need for therapeutic monitoring, it is unlikely that unfractioned heparin could ever be implemented as a therapy for the anemia of chronic disease or CHD anemia in patients without thrombophilic conditions. However, it is conceivable that heparin could be modified experimentally to improve the anti-hepcidin while decreasing anticoagulant activity. This represents a very promising approach, because heparins with low anticoagulant, but preserved anti-inflammatory activity, have already been developed (Ceccarelli, Bani et al. 2009).

Another possibility consists in the downstream inhibition of BMP receptors. Dorsomorphin has been identified through a large scale *in vivo* screening approach as a selective inhibitor of BMP signaling through the antagonism of type I receptors activity (Yu, Hong et al. 2008). Interestingly, this small molecule compound was able to profoundly reduce basal hepcidin levels and increase serum iron in mice. However, due to the many roles of BMP signaling in the regulation of cell differentiation and homeostasis, it is likely that dorsomorphin, that for example effectively inhibits osteogenesis *in vitro*, would have unacceptable side effects in humans.

Anti-cytokines Abs, such as those neutralizing IL-6, a major inducer of hepcidin during inflammation via STAT3 activation, would likely reduce hepcidin transcription and inflammation-related ESAs resistance at the same time, but potential side-effects (altered immune function) will be again a limiting factor for their clinical utilization. Finally, it should be not be forgotten that large doses of ESAs can reduce hepcidin levels, even if the effect seem to require the effective induction of erythropoietic activity *in vivo*, and is therefore subjected to clinical resistance, and at the price of severe side effects.

In conclusion, the association of anti-hepcidin Abs, or possibly anti-BMP6 Abs or anti-inflammatory/anti-hepcidin heparins, to ESAs represents the most promising approach for the treatment of the anemia of CHD and chronic diseases. However, as *HFE* has been demonstrated to be directly involved in the maturation of erythroid progenitors (Ramos, Guy et al. 2011) inflammation and atherogenesis, it should be noted that the advantageous effect of the presence of *HFE* mutations on erythropoiesis in CHD patients may not be completely dependent on relatively decreased hepcidin levels (Valenti, Girelli et al. 2009). Therefore, even if the degree of hepcidin activity suppression achievable *in vivo* would likely play a major role in determining the clinical outcome, it is possible that anti-hepcidin therapies would result in a lesser improvement in anemia control than that conferred by the presence of protective *HFE* genotypes. A better comprehension of the mechanisms linking *HFE* mutations with altered protein function both related to iron sensing / hepcidin induction and to the direct control of cellular iron intake is needed to design new therapeutic approaches aimed specifically at inhibiting this molecule. Furthermore, the possible development of side effects, such as the promotion of carcinogenesis related to chronic exposure to high TSAT levels, which would confer easy access to this growth promoting and mutagen metal to susceptible cells in patients already exposed to high levels of oxidative stress, should be weighted against the potential benefits (Zacharski, Chow et al. 2008; Dongiovanni, Fracanzani et al. 2010; Fargion, Valenti et al. 2010).

11. Conclusion

Common polymorphisms in the *HFE* gene of HH represent a major determinant of iron metabolism balance in Caucasian subjects, and the subtle effect of the C282Y and H63D mutations is likely magnified in CHD patients by the environmental pressure determined by chronic inflammation, and exposure to high amounts of iron and ESAs, thereby reaching clinical significance. These genetic factors act by hampering hepcidin induction in hepatocytes in response to increased iron stores, thereby resulting in reduced serum hepcidin and inadequate inhibition of the activity of the iron exporter Fp-1, and consequently in inappropriately high duodenal iron absorption and iron recycling from erythrophagocytosis in macrophages and increased serum iron. As a consequence, homozygosity for these mutations may result in organ damage related to iron overload in parenchymal tissues.

ACD and the anemia of ESRD and CHD are conversely characterized by chronic inflammation with increased cytokines levels, resulting in increased hepcidin levels with consequent reduction in iron absorption, recycling, and availability to the erythron. This response proves advantageous in the short-term to restrain iron availability to pathogens during infections, but in the case of chronicization it leads to severe anemia, and may impair the response to ESAs and oral and even IV iron therapy. Furthermore, as hepcidin is filtered by the glomerulus, ESRD itself is a contributing factor to increased hepcidin levels in CHD. Evidence is also accumulating that *HFE* mutations directly favor erythroblast maturation and hemoglobinization independently of serum hepcidin and reduce macrophages activation in response to inflammation.

We showed that in CHD patients *HFE* mutations may confer an adaptive benefit by decreasing hepcidin release in response to iron and inflammation, thereby improving iron availability to erythropoiesis, anemia control, and the response to ESAs and IV iron therapies. This would translate in a decreased burden of side effects, mainly related to an increased susceptibility to cardiovascular events and infections, the latter limited to IV iron. Although data must be confirmed in larger prospective studies, this favorable shift in iron metabolism balance possibly results in reduced mortality, in particular because of cardiovascular and infective diseases.

These data suggest that anti-hepcidin therapies such as anti-hepcidin or anti-BMP6 Abs, which are currently under development for ACD, may improve anemia management in CHD, concomitantly sparing the doses and side-effects of ESAs and iron and resulting in better quality-of-life, and most importantly a survival advantage for these patients. However, as the beneficial effect of *HFE* mutations on iron metabolism in CHD does not seem to be fully explained by lower hepcidin levels, direct inhibition of HFE-mediated regulation of iron metabolism may represent another valuable new therapeutic target.

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10

Micronutrient Metabolism in Hemodialysis Patients

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1. Introduction

Patients with end stage renal diseases (ESRD) that require long-term dialysis treatment still have high morbidity and mortality rates. Risk factors that are associated with outcomes and survival rates include long dialysis duration, medical complications, oxidative stress, infection, inflammation, and impaired immune responsiveness. Some studies have been investigated associations of disturbances of certain micronutrients in patients undergoing dialysis. However, the homeostasis of these micronutrients plays a critical role in the maintenance of antioxidant status and immune function, and the amelioration of infection. This chapter will focus on the associations between micronutrients (trace elements and vitamins) status and these risk factors and their potential therapeutic uses in hemodialysis patients. In addition, we review other special nutrients (substrates) that are essential for patient management.

2. Main risk factors: Inflammation & oxidative stress

ESRD patients who undergo long-term hemodialysis can develop cardiovascular diseases, anemia, protein-calorie malnutrition, infections and altered immune function, renal osteodystrophy, and skin disorders, which are common complications (Himmelfarb, 2005). These patients already have a high incidence of cardiovascular disease, and half of the deaths among these patients can be attributed to induced cardiovascular disease (Bevc et al., 2006). In view of the accelerated atherosclerosis, some traditional risk factors as well as non-traditional factors that occur in dialysis patients have been reported.

In contrast to cardiovascular disease, other complications can be partly explained by these known risk factors. Increasing evidence shows that patients on dialysis have oxidative stress and inflammation. Relative to healthy controls, these patients have significantly higher plasma concentrations of lipid peroxidation products expressed as thiobarbituric acid-reactive substances (TBARS), malondialdehyde (MDA)(Guo et al., 2010; Kirmizis et al., 2010), advanced oxidation protein products (AOPP)(Taki et al., 2006; Chen et al., 2011), and oxidative DNA products (8-hydroxydeoxyguanosine, 8-OHdG)(Morishita et al., 2011). Chronic inflammation in hemodialysis patients is characterized by elevated concentrations of inflammatory markers and cytokines, such as C-reactive protein (CRP), interleukin-1β

(IL-1β), IL-2, IL-6, and tumor necrosis factor α (TNF-α)(Guo et al., 2010; Kirmizis et al., 2010). Cytokines are also important mediators involved in systemic inflammatory diseases.
Oxidative stress is closely associated with inflammatory status, and the maintenance of the redox balance is known to modulate immune system homeostasis in hemodialysis patients. Chronic inflammation can induce an increased production of free radicals that cannot be counterbalanced due to defective antioxidant capability; an altered redox state is responsible for the accelerated dialysis syndromes. Oxidative stress occurs at sites of active inflammation and as a part of the reaction to invasive microorganisms (Nihi et al., 2010). Increased oxidative stress is also markedly related to the retention of oxidized solutes, the dialyzer membrane, bacterial contaminants in the dialysate, mitochondrial dysfunction, and decreased levels of antioxidant enzymes, including superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx)(Dursun et al., 2002; Ward and McLeish, 2003; Himmelfarb, 2008).
Oxidative stress has been implicated in other long-term complications, including anemia, amyloidosis, malnutrition, and infection (Morena et al., 2005). Infection may aggravate micronutrient deficiencies by reducing nutrient intake, increasing losses, and interfering with utilization by altering metabolic pathways (Maggini et al., 2007). Poor nutritional status arises due to *i*ncreased oxidative stress, and the latter may be related to alterations in micronutrients' homeostasis in hemodialysis patients. Overall, lowered micronutrients' status can promote oxidative stress and inflammatory responses may lead to suppressed immunity, which predisposes to infections and aggravates malnutrition.

3. Trace elements

3.1 Zinc

Zinc (Zn), an essential trace element, is an integral part of many enzyme functions and is a cofactor in those signaling pathways involving Zn-requiring proteins (Ngom et al., 2011). Zn is also an antioxidant and has anti-inflammatory properties. For example, Zn plays a critical structural role for antioxidant enzyme SOD and can stabilize biological membranes to decrease their susceptibility to oxidative damage that can impair cell functions (O'Dell, 2000). Zn is also a part of Zn-fingers and increases metallothionein biosynthesis, which can act as an antioxidant.
Several studies have demonstrated that Zn homeostasis has a critical impact on the health of hemodialysis patients. These patients have significantly lower concentrations of Zn in the blood and leukocytes and have increased oxidative stress (Roozbeh et al., 2009; Dashti-Khavidaki et al., 2010). The possible causes of decreased Zn concentrations need to be clarified, although Zn removal during hemodialysis, decreased gastrointestinal absorption of Zn, inadequate Zn intake, and reduced Zn-binding proteins in these patients have been reported. Increased expressions of intracellular metallothioneins following oxidative damage can sequester plasma Zn, and up-regulation of Zn-importing proteins by pro-inflammatory cytokines can reduce the plasma concentrations of Zn (Liuzzi et al., 2005; Haase and Rink, 2009; Guo et al., 2011). CRP is considered to be a sensitive marker of the induction of inflammatory activity; an association between decreased plasma Zn concentrations with higher CRP levels in hemodialysis patients has been noted (Guo et al., 2010). Medications used by hemodialysis patients, such as calcium carbonate, calcitriol (Dashti-Khavidaki, 2010), and aluminum phosphate-binders (our unpublished results), may interfere with blood Zn concentrations.

Efficient immune function depends on the status of some micronutrients and an inadequate status may lead to depressed immune responses. Zn is an immune regulator with multiple functional activities for immune effector cells, such as natural killer cell activity, and chemotaxis of neutrophils and monocytes (Kreft et al., 2000). Disturbances in Zn homeostasis can lead to a shift in the Th1/Th2 balance towards a Th2 response. In hemodialysis patients with Zn deficiency, the percentages of circulating CD3 and CD4 T lymphocytes are significantly lower than in controls; plasma Zn concentrations are correlated with %CD3 and %CD4 T cells, and CD4/CD8 ratios (Guo et al., 2010).

Zn deficiency has also been found to be associated with some uremic symptoms, such as anorexia, impaired taste sensation, and sexual dysfunction. On the other hand, depression is a common psychological problem encountered among ESRD patients. Chronic inflammation is known to play an important role in the pathophysiology of depression, and the concentrations of pro-inflammatory cytokines are increased in these patients (Bilgic et al., 2007; Maes, 2008). An association between Zn deficiency with depression in hemodialysis patients has been noted (Roozbeh et al., 2011). Recent investigations suggest a positive association between blood Zn concentrations and albumin, hematocrit and prealbumin, which suggests that better nutritional status is a reason for higher Zn status in dialysis patients (Grzegorzewska and Mariak, 2001; Navarro-Alarcon et al., 2006). In addition, Zn promotes insulin-like growth factor-1 (IGF-1), which plays a role in the regulation of hematopoiesis and osteoclastogenesis (Nishiyama et al., 1999; Yamaguchi et al., 2010). Thus, Zn status also seems to be related to hemodialysis bone disease and anemia.

Zn supplementation can increase blood Zn concentrations, leading to an increased protein catabolic rate (Jern et al., 2000), decreased osmotic fragility and lipid peroxidation (Candan et al., 2002), and reduced CRP levels (Rashidi et al., 2009) in hemodialysis patients. Zn supplementation (50 mg of elemental Zn from Zn sulfate for 90 days) improved food intake and serum cholesterol status in hemodialysis patients (Chevalier et al., 2002). Furthermore, our unpublished results showed that hemodialysis patients who received 10 mg of elemental Zn (78 mg of Zn gluconate) for 2 months had increased plasma Zn concentrations, reduced oxidative stress, and improved immune function. Sexual dysfunction in chronic renal failure patients undergoing hemodialysis is also common. Zn administration (100 mg/day for 6 weeks) can significantly increase serum concentrations of Zn and testosterone (Jalali et al., 2010). Most maintenance hemodialysis patients suffer from anemia, and Zn-based polaprezinc (34 mg Zn/day for 21 days) has been confirmed to provide an improved response to erythropoietin and increase hemoglobin levels (Fukushima et al., 2009).

In summary, low Zn status is likely to be more common and Zn supplementation would appear to be beneficial for Zn-deficient patients undergoing chronic hemodialysis. Zn status may alter the risk of complications of hemodialysis, which results from improved nutrition, antioxidant, and anti- inflammatory properties.

3.2 Copper

Copper (Cu) is an essential trace element that is required for a number of enzymes. Cu has vital roles in hemoglobin synthesis and immune function and is also a cofactor for SOD, cytochrome c oxidase, and ceruloplasmin (Maggini et al., 2007). Regarding the antioxidant enzyme SOD, Cu provides its catalytic activity. However, excess blood Cu, particularly the free fraction, may lead to tissue injury apparently due to its pro-oxidant effects and the depletion of anti-oxidant reserves.

Although the actual cause for changes in Cu concentrations and distribution remains to be elucidated, long-term dialysis patients have elevated concentrations of plasma Cu and increased oxidative stress compared to healthy controls (Navarro-Alarcon et al., 2006; Tonelli et al., 2009; Guo et al., 2010). The prescription of different phosphate binders did not have any observable effects on serum Cu status (Veighey et al., 2011). However, Zn deficiency may increase the absorption of intestinal Cu and Cu can significantly inhibit the influx of Zn across the intestinal brush border membrane. The release of Cu during inflammatory tissue damage also can explain the enhancement of blood Cu concentrations (Guo et al., 2009). Moreover, mitochondrial dysregulation affects Cu chaperone expressions (Granata et al., 2009), which may result in impaired Cu homeostasis and increased oxidative stress in hemodialysis patients.

An increased Cu concentration in erythrocytes or blood is accompanied by an increase in oxidative stress and an increased TBARS concentration in hemodialysis patients (Bober et al., 2007; Guo et al., 2010). In addition, chronic inflammation, as indicated by an increased concentration of serum ceruloplasmin, is related to elevated concentrations of CRP and Cu (Panichi et al., 2004). Previous studies have shown that higher concentrations of CRP, Cu, Cu/Zn ratio, and ceruloplasmin are related to inflammatory status (Bo et al., 2008; Ghayour-Mobarhan et al., 2008). Elevations in plasma Cu can increase protein kinase C activation due to oxidative stress and these are associated with inflammation and the progression of renal diseases (Davis et al., 2001).

Recently, β-2-microglobulin deposits in the form of amyloid fibrils were found in the musculoskeletal systems of dialysis patients as a result of kidney failure (Srikanth et al., 2009). β-2-microglobulin accumulation with resultant tumor formation is also a known, albeit rare complication of long-term hemodialysis (Mendoza et al., 2010). β-2-microglobulin has been reported to be markedly correlated with oxidative stress and inflammatory biomarkers in hemodialysis patients (Filiopoulos et al., 2009). However, Cu binding to β-2-microglobulin may precede amyloid formation (Srikanth et al., 2009).

Elevated serum and leukocyte Cu concentrations were associated with cardiovascular disease; Cu may directly affect atherogenesis and is a marker of inflammation associated with atherosclerosis (Kinsman et al., 1990; Ford, 2000). Higher blood Cu status may subsequently promote the development of breast cancer, and the adjusted odds ratios for breast cancer were 1.8, 1.0, 1.6, and 3.2, respectively, in the 4 quartiles of Cu distribution (Overvad et al., 1993). Whether or not Cu homeostasis has an impact on the development of atherosclerosis and some types of cancer in long-term dialysis patients remains to be established.

Variations in the distributions of both Cu and Zn can actually indicate oxidative stress, inflammation status, and immune dysfunction in dialysis patients. There is no question that disturbance in blood Cu remains a major problem and that Zn supplementation may benefit hemodialysis patients.

3.3 Selenium

Trace element selenium (Se) is required for the functions of a number of Se-dependent enzymes. Se bound to the active sites of antioxidant enzyme GPx plays an important role in protecting cell membranes and sub-cellular components from oxidative damage. Thus, Se is a potent antioxidant that acts as an anti-inflammatory agent and is required for immune system function (Rayman, 2000). Se can simulate Th1 immune responses against viral

infections and is involved in thyroid hormone synthesis (Kocabaş et al., 2006). Adequate Se status is necessary for maintenance of cell-mediated immunity and humoral immunity. In addition, the association of plasma Se concentrations with subsequent risk of cancer has been described. We are currently conducting clinical trials in our laboratory; the anti-angiogenesis effects of supranutritional levels of Se are consistently observed in three types of cancer.

The kidney accumulates the highest amounts of Se and is the major source of plasma GPx (Adamowicz et al., 2002). The plasma concentrations of Se and GPx activities are significantly lower in dialysis patients than in healthy controls (Zagrodzki et al., 2007; Tonelli et al., 2009; Pakfetrat et al., 2010; Fujishima et al., 2011a). Reduced intake of Se lowers the blood Se status of dialysis patients (Andrew et al., 2008). Increased inflammation may decrease the absorption of Se and result in low Se status (Walston et al., 2006). Blood Se status is also significantly associated with albumin concentration and the dialysis process (Fujishima et al., 2011a); lower Se status may be related to malnutrition in these patients.

Further, statins (3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors) also have antioxidant and anti-inflammatory properties, which can further reduce the numbers of inflammatory cells and CRP concentrations (Ferms, 2003). In hemodialysis patients who used statins, blood Se concentrations were significantly higher compared with untreated patients (Taccone-Gallucci et al., 2010). Thus, this raises the possibility that low Se concentrations may be attributed to diminished Se retention due to increased oxidative stress.

Patients undergoing hemodialysis have low Se status that may increase their risk of anemia and certain cancers (Viron, 2002). On the other hand, a population-based prospective cohort study has conducted among hemodialysis patients showed that low blood Se status may contribute to immune system dysfunction and an increased risk of death, especially death related to infectious disease (Fujishima et al., 2011b). Se status was significantly associated with decreased IL-6 concentrations and altered erythrocyte membrane fluidity in these patients (McGrath et al., 1995; Walston et al., 2006), and Se supplementation stimulated erythrocyte GPx enzyme activity and reduced NF-kB activation in HIV-infected patients (Kupka et al., 2004). A previous investigation also showed an association between CRP and low Se concentrations in dialysis patients (Guo et al., 2010). This suggests a relationship between the reduced concentrations of plasma Se and the increased inflammatory responses of hemodialysis patients.

Se supplements such as sodium selenate, sodium selenite, L-selenomethionine, and Se-enriched yeast, are available to counter potential deficiencies. However, organic forms of Se are more bioavailable and are found to be less toxic than inorganic Se. Se-containing yeast (150 μg Se/day) supplementation, was effective for increasing blood Se concentrations in healthy adults, whereas the same amount of inorganic forms failed to raise blood Se concentrations (Schrauzer, 1979). For hemodialysis patients who received oral supplementations of sodium selenite (230 μg Se/day) for 3 months, followed by 200 μg/day for the next 3 months, their plasma Se concentrations increased until reaching a plateau similar to control levels (Saint-Georges et al., 1989). Hemodialysis patients who received supplements of 200 μg of Se (as Se-rich yeast) per day for 3 months had significantly higher plasma Se concentrations and decreased leukocyte DNA damage (Zachara et al., 2010). Se-rich yeast supplementation (200 μg Se/day for 3 months) for hemodialysis patients

significantly increased blood Se concentrations, but had no effect on plasma levels of GPx protein (Zachara et al., 2009). Rapidly increased plasma GPx activity was found following kidney transplantation (Whitin et al., 1998). Thus, the damaged kidney is unable to synthesize GPx, even after induction with Se. In contrast, erythrocyte GPx in the kidney is primarily synthesized in the proximal tubules (Whitin et al., 1998). Treatment with yeast-Se (900 μg of Se/week) caused an increase in Se concentrations and erythrocyte GPx activity (Zachara et al., 2001).

On the other hand, increased plasma Se is positively associated with indices of erythropoiesis and nutritional status (Zagrodzki et al., 2007). Significantly increased Se concentrations were seen after 4-months of recombinant human erythropoietin (rHuEpo) treatment (Celiker et al., 2001). In contrast, Se deficiency, particularly when combined with viral infection, can cause cardiovascular disease (DiSilvestro, 2005). Patients on hemodialysis have a higher prevalence of hepatitis C virus infection, may be attributed to the decreased plasma Se concentrations and GPx activities (our unpublished observations). Reduced serum Se and platelet GPx activity in hemodialysis patients are related to cardiovascular complications (Girelli et al., 1993). Se supplementation may help to prevent cardiovascular disease due to its antioxidant capacity, improvement in blood lipids, and the inhibition of platelet aggregation (Nève, 1996; Panicker et al., 2010; Rayman et al., 2011).

Se deficiency may alter immune function, increase oxidative stress, and increase the generation of inflammatory cytokines, which is complicating the clinical features in hemodialysis patients. The clinical manifestations of Se deficiency have a late onset; a deficiency may be present for a long time before these manifestations appear. Thus, there is a need for routine assessments of Se status and the efficacy of Se supplementation for hemodialysis patients.

4. Vitamins

4.1 Vitamin C

Vitamin C, also known as ascorbic acid, is a potent water-soluble antioxidant and co-enzyme in hydroxylation reactions and has anti-mutagenic properties. Vitamin C plays an important role in the synthesis of neurotransmitters that are critical for brain function and that are known to affect mood (see the web from the Linus Pauling Institute). Vitamin C is also required for the synthesis of collagen and carnitine.

Hemodialysis patients are particularly prone to vitamin C deficiency because of dietary restrictions, limited absorption, and poor nutritional status (Böhm et al., 1997; Richter et al., 2008). The dialysis process may decrease the blood concentrations of vitamin C (Deicher et al., 2005). Significantly decreased plasma vitamin C and increased oxidative stress were seen during hemodialysis, which could be exacerbated by intravenous iron treatment (Shi et al., 2005). Also, increased weekly hours of dialysis increase the loss of vitamin C (Coveney et al., 2010). Vitamin C homeostasis may have a significant impact on these patient outcomes.

Daily intake of 60-100 mg of vitamin C is sufficient to maintain health in the general population, but may not be adequate for dialysis patients (Richter et al., 2008). Although, short-term oral vitamin C supplementation (250 mg at 3 times/week for 2 months) did not alter oxidative stress and inflammatory markers in hemodialysis patients (Fumeron et al.,

2005). Every other day supplementation with 250 mg of vitamin C for 3 months increased blood vitamin C concentrations, decreased MDA, and improved LDL and total cholesterol concentrations (Abdollahzad et al., 2009). Several polypeptides associated with oxidative stress and inflammatory markers were also normalized after oral vitamin C supplementation (250 mg at 3 times/week)(Weissinger et al., 2006). For hemodialysis patients who were given 1,000 mg of vitamin C for 3 months (Washio et al., 2008) or for 1 year (Ramos et al., 2005), decreased LDL oxidation, increased plasma vitamin C and plasma SOD activity, but not SOD mRNA expression in leukocytes were noted. After intravenous administration of vitamin C, ESRD patients also showed increased paraoxonase activity and vitamin E, decreased AGE, isoprostances, 8-OHdG and lipid peroxidation concentrations, and reductions in pro-inflammatory mediators (Chien et al., 2004; Tarng et al., 2004; Shi et al., 2005; Chan et al., 2006; Ferretti et al., 2008).

A high dietary intake and high circulating concentrations of vitamin C may protect against ischemic heart disease, possibly due to its antioxidant properties and anti-inflammatory effects (Wannamethee et al., 2006). Inverse relationships between vitamin C status with CRP, IL-6, fibrinogen, coagulation factors VII, VIII, and IX, and thrombin-antithrombin complexes have also been noted (Deicher et al., 2005). Therefore, low total vitamin C plasma concentrations may be an independent predictor of adverse cardiovascular outcomes for hemodialysis patients; vitamin C supplementation has been suggested to reduce cardiovascular disease risk.

Hemodialysis patients with functional iron deficiencies often develop resistance to rHuEpo. For these patients, anemia prevention relies primarily on supplemental EPO and intravenous iron. Improved vitamin C status and decreased inflammation could lead to better utilization of iron and EPO (Handelman, 2007). Plasma concentrations of vitamin C are positively associated with hemoglobin concentrations, and vitamin C status may help patients to utilize iron for erythropoiesis during anemia management (Attallah et al., 2006; Deved et al., 2009; Finkelstein et al., 2011). Anemic hemodialysis patients who received oral daily vitamin C at a dose of 500 mg/day had decreased ferritin concentrations and EPO dose requirements, while hemoglobin and hematocrit concentrations were increased (Sirover et al., 2008).

On the other hand, long-term dialysis patients have a decreased quality of life compared to healthy controls (Mydlik and Derzsiová, 2008). Increased malnutrition-inflammation scores may be significantly associated with depression, sleep disorders, and poor quality of life (Bilgic et al., 2007). Lower quality of life scores were positively related to decreased blood status of the antioxidants Zn, Se, and vitamin C (Raimundo et al., 2006). When health-related quality of life was assessed using Short Form 36 (SF-36) scale scores, associations between plasma vitamin C status, MDA and life quality were also found in EPO-treated hemodialysis patients (Guo, C-H., unpublished results). Hemodialysis patients who were treated with EPO and were supplemented over 3 months with vitamin B6 (20 mg/day), folic acid (10 mg/week), and vitamin C (60 mg/day), showed increased quality of life scores (Mydlik and Derzsiová, 2008).

Oral or parenteral iron administration is a common practice for hemodialysis patients. However, a major concern is that an oversaturation of transferrin and subsequent propagation of redox-active iron can occur with the recommended dose of iron (Ardalan et al., 2007). Redox-active iron is a potent pro-oxidant that triggers free-radical chain reactions. Vitamin C may also act as a pro-oxidant via the reduction of ferric ion. Hemodialysis

patients who received 100 mg of iron sucrose and vitamin C intravenously had an increase in oxidative stress comparable to patients given iron alone (Eiselt et al., 2006); however, this finding was not confirmed by Shi et al (2005). This suggests that combination treatment of high doses of iron and vitamin C by intravenously route, should be avoided in anemic hemodialysis patients.

In addition, hyperoxaluria due to vitamin C supplementation (500 mg/day for 3 months) for chronic dialysis patients was found (Sirover et al., 2008). A concern has been raised that high plasma oxalate concentrations may lead to deposition in soft tissues. Nevertheless, one study found that there was no evidence that vitamin C supplementation increased the risk of kidney stone formation (Curhan, 1999). An earlier study involving 45,251 peoples with no history of kidney stones and who were followed for 6 years found that those who had consumed 1500 mg or more of daily vitamin C had a reduced risk of kidney stones compared to those who consumed less than 250 mg daily (Curhan, 1996). Vitamin C supplementation may address the potential protective effects on the clinical outcomes; however, hemodialysis patients should also be advised not to ingest excess amounts of vitamin C supplements.

4.2 Vitamin E

Vitamin E is a family of tocopherols (alpha-, beta-, gamma-, and delta-) and tocotrienols (alpha-, beta-, gamma-, and delta-). Vitamin E is a fat-soluble vitamin with a variety of cellular membrane stabilizing- antioxidant and non-antioxidant activities. Some studies have been suggested that vitamin E can be used as an erythropoietic agent for decreasing premature erythrocyte hemolysis by reducing membrane fragility and the oxidation of cell membrane polyunsaturated fatty acids (Jilani and Iqbal, 2011). Vitamin E also has anti-tumor effects and is anti-atherogeneic due to its antioxidant effects. Vitamin E supplementation has shown anti-proliferative, pro-apoptotic, and cyclooxygenase-2- inhibiting effects (Wada, 2011). Vitamin E is a nutrient known for promoting optimal immune function. When given orally, vitamin E has been shown to significantly enhance both cell mediated and humoral immune functions in humans and animals (Pekmezci, 2011). Vitamin E supplementation increases IL-2 production by T cells, enhances Th1 responses, and decreases the production of IL-4, a stimulator of Th2 responses (Maggine et al., 2007).

The plasma concentrations of vitamin E in chronic renal insufficiency patients may be decreased or normal (Yukawa et al., 1999; Hodkova et al., 2006; Zwolinska et al., 2009; Guo et al., 2011). In addition, abnormal distributions of vitamin E in different lipoproteins have been reported (Yukawa et al., 1999). After hemodialysis, the concentrations of high density lipoprotein (HDL)-cholesterol, vitamin E, and the total antioxidant capacity are significantly decreased (Montazerifar et al., 2010). In comparison to control subjects, LDL from dialysis patients contained less amounts of vitamin E and the MDA of LDL was significantly increased (Yukawa et al., 1999). Two studies evaluated vitamin E (600 IU) or multiple vitamins (800 IU of vitamin E, 250 mg of vitamin C, 100 mg of vitamin B6, 250 μg of vitamin B12 and 10 mg of folate) for possible effects on oxidative stress and inflammation in chronic hemodialysis patients; however, neither of these studies found any of the expected effects (Hodkova et al., 2006; Kamgar et al., 2009). This may have been due to not investigating patients with increased oxidative stress. However, oral administration of vitamin E (400 IU) and sodium selenide (600 μg), taken 6 h before a dialysis session, markedly reduced

oxidative stress in hemodialysis patients who were receiving iron infusions (Ardalan et al., 2007).

Alpha-tocopherol (800 IU) taken daily for 12 weeks decreased the susceptibility of LDL to oxidation in dialysis patients (Islam et al., 2000). The SPACE study, which examined hemodialysis patients, reported that 800 IU/day of alpha-tocopherol significantly reduced cardiovascular disease endpoints and myocardial infarction (Boaz et al., 2000). Moreover, oral vitamin E supplementation (> 800 IU) for 20 weeks significantly decreased the concentrations of plasma F2-isoprostanes in hemodialysis patients with increased oxidative stress (Reed et al., 2009). This suggests that adequate supplementation of vitamin E is needed in hemodialysis patients.

By comparison, a dialyzer membrane modified with vitamin E provided more effective antioxidant defense than oral administration of 600 IU of vitamin E for hemodialysis patients (Mydlík et al., 2001). Vitamin E bound to a dialyzer membrane resulted in lowered plasma MDA and oxidized LDL and MDA-LDL, and significantly increased the plasma vitamin E concentrations (Mune et al., 1999; Morimoto et al., 2005; Mydlik and Derzsiová, 2008). Although treatment with a vitamin E-coated dialyzer significantly reduced the percentage increase in an aortic calcification index after 24 months (Mune et al., 1999), whether a vitamin E-coated dialyzer can prevent cardiovascular disease and other dialysis-related complications needs further exploration.

4.3 Folic acid

Folic acid (folate) is a water-soluble vitamin essential for a number of critical metabolic pathways. The pteridine moiety of folate is the prosthetic group of many enzymes that are involved in the transfer of one-carbon unit in amino acid and nucleotide metabolism, mitochondrial protein synthesis, conversion of homocysteine to methionine, methylation of transfer RNA, and de novo purine nucleotide synthesis.

Folate is involved in the remethylation of homocysteine to methionine; therefore, a low folate status results in an accumulation of homocysteine. Hyperhomocysteinemia is recognized as an independent risk factor for atherosclerotic cardiovascular diseases, neurodegenerative conditions, osteoporosis, and cancers. Homocysteine and its by-products (homocysteine thiolactone) have been shown to damage the endothelial cells and exacerbate the cardiovascular injury (Jakubowski, 2008). Further, homocysteine increased the affinity of N-methyl-D-asparate (NMDA) glutamate subreceptors, which indirectly caused the calcium influx (Obeid and Herrmann, 206). Increasing evidence shows that homocysteine thiolactone induce protein unfolding and aggregation, and can lead to the formation of amyloid fibrils (Paoli et al., 2010). DNA methylation plays an essential role in maintaining cellular function, and reduced DNA methyltransfrerase may contribute to the development of certain cancers (Davis and Uthus, 2004). In addition, homocysteine increased bone resorption and stimulated p38 mitogen-activated protein kinase (MAPK) activity, and inhibited collagen cross-linking in bone may contribute to osteoporosis (Jamal et al., 2005; Koh et al., 2006).

Some studies have noted that a significantly lower plasma concentration of folate and a higher homocysteine concentration in these patients (Tamura et al., 1996; Stanford et al., 2000). The increased incidence of atherosclerotic vascular disease is correlated with the status of blood homocysteine in ESRD patients undergoing dialysis (Bachman et al., 1995; Heinz et al., 2009). Moreover, blood homocysteine concentrations were found to be

significantly above the cutoff of 13.5 μmol/L in chronic dialysis patients, with or without vascular diseases (Leblanc et al., 2000; Tremblay et al., 2000). An increase in homocysteine concentration was also associated with increased mortality among dialysis patients who were not receiving vitamins (Heinz et al., 2009). In addition, mitochondria have higher levels of several folate forms and tetrahydrofolate-synthesizing enzymes; folate deficiency impaired nuclear DNA and mitochondrial DNA synthesis, mitochondrial folate uptake, and enhanced mitochondrial oxidative decay, which may occur in hemodialysis patients.

Several studies have shown that folate deficiency is not observed in all patients on chronic hemodialysis (Tremblay et al., 2000; Coveney et al., 2010); although, blood folate loss can occur during the hemodialysis process (Tamura et al., 1996; Leblanc et al., 2000; Heinz et al., 2008). An inverse correlation between blood folate and total homocysteine concentrations has been shown (Koulouridis et al., 2001). This relationship between homocysteine and folate suggests that the concentrations of folate within the reference interval are inadequate for dialysis patients.

With regards to decreasing plasma folate concentration, it is strongly associated with increased homocysteine, TBARS, pro-inflammatory cytokines, and CD4/CD8 lymphocyte ratios (our unpublished observations). Also, a reduced folate concentration is inversely associated with colon tumorigenesis in these patients (Kaji et al., 2011). Plasma homocysteine concentrations are higher in dialysis patients; however, no significant inverse correlation between homocysteine and bone mineral density is observed (Kayabasi et al., 2010). By contrast, increased concentrations of homocysteine are associated with an increased risk fracture in hemodialysis patients (Jamal et al., 2005).

Chronic renal disease is associated with a relative resistance to the lowering effects of low-dose folate supplementation on homocysteine (Bostom et al., 1997). Folic acid supplementation (1-5 mg/day) may normalize plasma homocysteine levels in moderately hyperhomocysteinemic individuals with normal renal function; however, a similar effect has not been observed in ESRD patients (Tremblay et al., 2000). Supplementation with high dose of folate (> 5 mg/day) significantly reduces plasma homocysteine in hemodialysis patients, with or without atherosclerosis (Stanford et al., 2000).

Hemodialysis patients who received daily supplements of 15 mg of folic acid for 2 months had markedly increased in plasma folate concentrations and decreased blood total homocysteine, irrespective of their 5,10-methylene-tetrahydrofolate reductase (MTHFR) C677T genotypes (Billion et al., 2002). In hemodialysis patients who received oral supplementations of 15 mg/day of folic acid, had significantly lower homocysteine concentrations and found no evidence of adverse effects (Bostom et al., 1996). Treatment with folic acid (10 mg/day) for 6 months have normalized plasma homocysteine concentrations, and significantly increased total plasma antioxidant capacity and decreased TBARS levels in these patients (Chiarello et al., 2003; Alvares Delfino et al., 2007). For hemodialysis patients who were given 15 mg/day of 5-methyltetrahydrofolate for 12 weeks, increased plasma folate concentration and improved endothelial dysfunction were observed (Baragetti et al., 2007). On the other hand, intravenous 5-methyltetrahydrofolate (50 mg/day for 2-5 years) reduced inflammation status and prolonged survival rate in ESRD patients undergoing hemodialysis (Cianciolo et al., 2008). Low-dose intravenous folinic acid (1 mg/day, 3 times/week) for 3 months significantly reduced plasma homocysteine and MDA concentrations (Apeland et al., 2002).

Routine supplementations of folic acid over long time periods should be considered in order to reduce homocysteine concentrations, may be more beneficial in minimizing uremic complications in hemodialysis patients.

4.4 Vitamin B6

Vitamin B6, a water-soluble vitamin, is an essential coenzyme for numerous biochemical pathways and is a potent antioxidant. Vitamin B6 is involved in lipid metabolism, nucleic acid and protein biosynthesis, and helps to maintain normal nerve function and the formation of red blood cells (Hisano et al., 2010). Vitamin B6 is also critically required for absorption of vitamin B12 and synthesis of niacin. Moreover, it may inhibit platelet aggregation, and ameliorate the development of diabetic neuropathy (Metz et al., 2003; Kobzar et al., 2009). By contrast, vitamin B6 deficiency impairs lymphocyte maturation, growth and proliferation, and antibody production; it suppresses the production of Th1 cytokines and, thus, promotes Th2 responses (Maggini et al., 2007).

Pyridoxal-5'-phosphate, the active moiety of vitamin B6, is significantly depleted in most chronic hemodialysis patients without supplementation and high-efficiency hemodialysis contributes to its depletion (Leblanc et al., 2000; Tremblay et al., 2000; Busch et al., 2010). In addition, these patients need to consume more vitamin B6 for hemoglobin synthesis during rHuEPO treatment, which result in vitamin B6 deficiency (Mydlik et al., 1997). Hyperhomocysteinemia may be also caused by reduced vitamin B6 concentrations. Blood cystathionine status is major indicator for the trans-sulfuration pathway of homocysteine, which has been shown to be dramatically increased in hemodialysis patients due to the inhibition of cystathionine catabolism by low blood vitamin B6 contents (Herrmann and Obeid, 2005). On the other hand, high concentrations of homocysteine in blood associated with an increased risk of cardiovascular disease, but no significant difference in the pre-dialysis serum pyridoxal-5'-phosphate concentrations of patients with or without evidence of vascular disease (Leblanc et al., 2000).

There is increasing evidence that deficiency of vitamin B6 may cause hyperoxalemia and hyperoxaluria in dialysis patients; vitamin B6 treatment lower urinary oxalate excretion and inhibit calcium oxalate crystal formation (Chetyrkin et al., 2005; Mydlík and Derzsiová, 2010). In contrast, lower blood concentration of vitamin B6 was correlated with accumulation of the advanced glycation end-products (AGE) in hemodialysis patients (Busch et al., 2010). Increased oxidative stress by-product can induce calcium oxalate crystal aggregation and attachment in the renal tubules (Thamilselvan and Menon, 2005).

A previous study showed oral supplementation of folic acid (5 mg/day for 14 days), but not vitamin B6 (40 mg) or B12 (1 mg), was very effective in lowering total homocysteine in healthy subjects. However, in dialysis patients require much more aggressive B-complex vitamins therapy to achieve the effect of lowering total plasma homocysteine (Bostom et al., 1996). These patients treatment with vitamin B supplements (15 mg of folic acid, 100 mg of vitamin B6, and 1 mg of vitamin B12/day for 4 weeks), but not vitamin B6 alone, have significantly decreasing total plasma homocysteine. Vitamin B supplementation (40 mg of folic acid, 100 mg of pyridoxine hydrochloride, and 2 mg of cyanocobalamin) decreased plasma homocysteine concentrations, but did not improve survival or reduce the incidence of vascular disease in those patients (Jamison et al., 2007). Increased intake of B-complex vitamins (5 mg of folic acid, 20 mg of vitamin B6, and 50 μg of vitamin B12) given 3 times per week for an average of 2 years did not reduce mortality and had no significant effects on

the risk of cardiovascular events in patients with end-stage renal disease (Heinz et al., 2010). In contrast, high-dose intravenous B-complex vitamins (250 mg of vitamin B1, 250 mg of B6, and 1500 μg of B12; 3 times/week) reduced blood total homocysteine levels only when combined with 5 mg of folate given orally (Sombolos et al., 2002). Other results indicate that the folate (5 mg) and vitamin B6 (250 mg) supplementation, but not vitamin B6 alone, resulted in a reduction of homocysteine concentrations and improvement of the lipidemic profiles (LDL and HDL) in patients maintained with hemodialysis (Ziakka et al., 2001).
However, vitamin B6 (pyridoxine) supplementation (50 mg/day for 3-5 weeks) alone has been shown to improve the immune function of hemodialysis patients (Casciato et al., 1984). Vitamin B6 supplementation (60 mg/day for 4 weeks) was also effective in improving peripheral polyneuropathy symptoms of various etiologies, possibly because of the resistance to peripheral polyneuropathy that vitamin B6 provides for hemodialysis patients (Okada et al., 2000).
In summary, results above indicate that administrations of vitamin B6 and folic acid in combination are clinically beneficial for improving blood homocysteine status, lipid profile and peripheral polyneuropathy symptoms, and reducing calcium oxalate formation. The dose in 50 to 100 mg/day of vitamin B6 can be of great therapeutic value in hemodialysis patients. However, daily consumption of large amounts of vitamin B6 supplements (> 100 mg) should be carefully considered.

4.5 Vitamin B12

Another essential micronutrient that is crucial for health is vitamin B12 (cobalamin), which is involved in one-carbon (methyl) metabolism. Two forms of vitamin B12, methylcobalamin and 5-deoxyadenosyl cobalamin, are commonly used by the human body. Cobalamin is required for the methionine synthase that catalyzes the conversion of homocysteine into methionine, and insufficient amounts will result in hyperhomocysteinemia (Green and Miller, 2007). In addition, cobalamin is required by L-methylmalonyl-CoA mutase that catalyzes the conversion of L-methylmalonyl-CoA to succinyl-CoA, thus maintaining methylmalonic acid within its normal range.
Vitamin B12 status is typically assessed by plasma or serum vitamin B12 concentrations. For adults, values below approximately 170-250 pg/mL indicate vitamin B12 deficiency (Institute of Medicine, 1998). An elevated homocysteine concentration (values >13 μmol/L) and increased methylmalonic acid also suggest vitamin B12 deficiency (De Vecchi et al., 2000; Andrès et al., 2007). In a vitamin B12-deficient state, the irreversible reaction that forms 5-methyl tetrahydrofolate results in a secondary folate deficiency with concomitant impairments in thymidine and purine synthesis, which can lead to alterations in immunoglobulin production (Maggini et al., 2007). Individuals with vitamin B12 deficiencies may also have anemia, gastrointestinal symptoms, or peripheral neuropathies (Marar et al., 2001). Evidence has accumulated in recent years that vitamin B12 supplementation has beneficial effects on cardiovascular disease, dementia and cognitive function, depression, and some cancers.
In chronic dialysis patients, markedly increased concentrations of methylmalonic acid and total homocysteine have been found (Herrmann and Obeid, 2005). Statistically significant correlations were observed between homocysteine and vitamin B12 concentrations (De Vecchi et al., 2000). The uptake of vitamin B12 by peripheral blood mononuclear cells from hemodialysis patients was lower than by cells from controls (Herrmann et al., 2001).

Treatment with vitamin B12 and folic acid, but not vitamin B12 alone, appears to be effective for lowering the total plasma homocysteine concentration in hemodialysis patients who have either normal vitamin B12 concentrations or a deficiency (Pastore et al., 2006). In addition, adding vitamin B12 to a folate supplement can further enhance the reduction of plasma homocysteine, as compared to treatment with folate alone (Stopper et al., 2008). Oral supplementation with 15 mg/day of folic acid together with 1 mg/day of vitamin B12 was more effective for reducing homocysteine concentrations (Azadibakhsh et al., 2009). Further, genomic damage in the peripheral blood lymphocytes of dialysis patients due to oxidative stress can be ameliorated by supplementation with folic acid and vitamin B12, which is thought to contribute to homocystine reduction (Stopper et al., 2008).

Both folic acid and vitamin B12 supplements have desirable effects on blood homocysteine levels. However, a poor response to treatment with erythropoietin for renal anemia is common. Infections, oxidative stress, and inflammation have been shown to reduce the responsiveness to erythropoiesis- stimulating agents by increasing the release of pro-inflammatory cytokines (Stenvinkel, 2003). Oxidative stress and inflammation can be attenuated by vitamin B12 and folate supplementation. Whether vitamin B12 alone or in combination with folate is beneficial for altering erythropoiesis in hemodialysis patients may need further investigation.

5. Other nutrients (substrates)

5.1 EPA and DHA

A growing body of evidence suggests that the omega-3 fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) have immune modulating, anti-inflammatory, lipid-lowering, anti-arrhythmic, and anti-hypertensive effects (Kris-Etherton et al., 2002). Both EPA and DHA are present in mitochondrial membranes and are essential for mitochondrial function. It has been reported that omega-3 fatty acids could decrease the production of homocysteine; thus, they had a cardioprotective effect in patients with normal renal function (Pooya et al., 2010). Fish oil sources of EPA and DHA have also shown renal protective effects that prevented against the progression to chronic kidney disease in older adults (Lauretani et al., 2009). These could also significantly reduce inflammation, fibrosis and oxidative stress following renal injury (Soumura et al., 2010; Peake et al., 2011). Moreover, fish oil supplements (900 mg of EPA and 600 mg of DHA/day for 1 month) may decrease urinary oxalate excretion and the risk of calcium oxalate crystallization attributable to an altered oxalate transporter activity (Siener et al., 2011).

Chronic hemodialysis patients reportedly have significantly low concentrations of EPA and DHA in the plasma and cell membranes (Saifullah et al., 2007; Nakamura et al., 2008; Madsen et al., 2011). This abnormal status may be attributed to the dialysis process, which can enhance oxidative stress and potentially increase omega-3 fatty acid peroxidation. In addition, these patients consumed lower amounts of dietary fish and, consequently, had suboptimal blood concentrations (Kutner et al., 2002; Friedman et al., 2006).

Short-term supplementation with fish oil (2.4 g of EPA and 1.2 g of DHA) could significantly increase the EPA and DHA contents in leukocytes phospholipids of hemodialysis patients within one week (Faber et al., 2011). After oral administration of EPA/DHA (2.7 g/day for 3 months), decreased lipid peroxidation and leukotriene B4 production in peripheral blood mononuclear cells were found for ESRD patients on hemodialysis (Taccone-Gallucci et al.,

2006). Patients who were administered supplements of fish oil (2.4 g/day) for 2 months had significantly decreased inflammation and increased insulin sensitivity, HDL-cholesterol, albumin, and hemoglobin (Perunicic-Pekovic et al., 2007; Rasic-Milutinovic et al., 2007). Significantly increased serum HDL-cholesterol and decreased triglyceride concentrations were also observed in hemodialysis patients who were administered fish oil (2.4 g) supplements for 3 months (Svensson et al., 2004).

Supplementation with fish oils (EPA+DHA, 2 g/day) also had a beneficial effect on plasma HDL and triglyceride concentrations in those patients with serum triglyceride levels > 200 mg/dl and total cholesterol > 220 mg/dl (Taziki et al., 2007). Further, EPA (1.8 g/day) or fish oil (1.3 g/day) treatments for 3 months remarkably reduced the increased plasma concentrations of remnant lipoproteins and triglycerides and prevented the peroxidation of LDL in dialysis patients (Ando et al., 1999; Saifullah et al., 2007). When hemodialysis patients were given low doses of fish oils (1.6-1.7 g/day), however, there were no significant improvements in their blood lipid profiles (Poulia et al., 2011), lipoprotein (a) (Beavers et al., 2009), or homocysteine concentrations (Rasmussen et al., 2010).

Uremic pruritus, also known the renal itch, is quite common in patients undergoing hemodialysis or peritoneal dialysis (50-90%). The pathological processes that lead to uremic pruritus remain poorly understood, and there is no definitive treatment. Administration of fish oil (6 g/day for 8 weeks) could significantly decrease erythropoietin requirements (Jones and Kaiser, 2002) and improved the severity and distribution of uremic pruritus among hemodialysis patients (Peck et al., 1996). These authors speculated that the anti-inflammatory and anti-proliferative effects of fish oils may have contributed to these symptoms' reversal.

Abundant evidence suggests that EPA/DHA exhibit powerful lipid-lowering and anti-inflammatory capabilities, and are consequently involved with reduced uremic complications and an enhanced nutrition status in hemodialysis patients.

5.2 Coenzyme Q10

Coenzyme Q10 is a member of the ubiquinone family of compounds, which is found in virtually all cell membranes, mitochondria, and lipoproteins. Coenzyme Q10 is a vitamin-like substance that plays a crucial role in energy metabolism and in free radical scavenging (Thomas and Stocker, 2001). It is also recognized an obligatory cofactor for the functions of uncoupling proteins (Echtay et al., 2000).

Coenzyme Q10 has a direct anti-atherogenic property; oral coenzyme Q10 supplementation was shown to ameliorate cardiac contractility and endothelial dysfunction in patients with chronic heart failure (Littarru and Tiano, 2007). In addition, a recent study identified coenzyme Q10-sensitive genes that were regulated by peroxisome proliferator-activated receptor-alpha (PPARα) and were involved in cholesterol synthesis, lipoprotein metabolism, and inflammation (Schmelzer et al., 2010).

There is evidence to support the safety and efficacy of coenzyme Q10 in congestive heart failure, diabetes, atherosclerosis, hypertension, cardiomyopathies, migraine, and Parkinson's disease (Rosenfeldt et al., 2007; Nahas, 2008). Coenzyme Q10 supplementation may result in increased concentrations of coenzyme Q10 within circulating lipoproteins and increase the resistance of human LDL to oxidation (Littarru and Tiano, 2007). Coenzyme Q10 supplement (200 mg/day) improved blood pressure, and the endothelial functions of the conducting arteries of the peripheral circulation in dyslipidemia patients with type 2

diabetes (Hodgson et al., 2002; Watts et al., 2002). A combination of orally given fenofibrate and coenzyme Q10 (200 mg/day) was effective for improving the endothelium-dependent and endothelium-independent vasodilator functions of the forearm microcirculation (Playford et al., 2003). Further, coenzyme Q10 attenuated elevated blood pressure, renal membrane phospholipid degradation, and enhanced renal phospholipase A2 due to its antioxidant and anti-inflammatory actions (Okamoto et al., 1991; Ishikawa et al., 2011).

Evidence has shown that high coenzyme Q10 contents in human renal tissues (Dallner and Sindelar, 2000). However, the plasma coenzyme Q10 and coenzyme Q10/LDL-cholesterol ratios in both conservative therapy and hemodialysis populations were markedly lower (Lippa et al., 2000). Plasma coenzyme Q10 concentrations were significantly decreased and MDA concentrations were increased (Gazdikova et al., 2000); reduced coenzyme Q10 status probably due to renal impairment and removal by dialyses. However, statins are widely used cholesterol-lowering medications that may decrease the endogenous synthesis of coenzyme Q10, and are consequently involved with reduced mitochondrial respiration and perhaps mitochondria and cell death (De Pinieux et al., 1996; Colquhoun et al., 2005).

Hemodialysis patients always have an impaired mitochondrial respiratory system, and this may contribute to enhance oxidative stress (Granata et al., 2009). It has been reported that coenzyme Q10 administration suppressed oxidative stress in hemodialysis patients (Sakata et al., 2008). Supplementation with coenzyme Q10 (100 mg/day) for 3 months reduced the serum lipoprotein (a) in hemodialysis patients who were treated with statins (Shojaei et al., 2011); decreased serum lipoprotein (a) attributed to inhibition of expression of lipoprotein(a) receptor by coenzyme Q10 (Singh and Niaz, 1999). Coenzyme Q10 supplementation (90-120 mg/day) significantly improved the peripheral circulation and decreased the plasma concentrations of TBARS and carbonyl protein in patients on hemodialysis or peritoneal dialysis (our unpublished observations), although we were unable to determine coenzyme Q10 status at baseline. There was significantly lower salivary secretion in hemodialysis patients (Gavaldá et al., 1999); administered supplemental coenzyme Q10 (100 mg/day for 1 month) to these patients can improve salivary secretion (Ryo et al., 2011). Nahas (2008) indicated that a typical dose of 60 to 120 mg 1-3 times daily was not associated with any serious risks. In fact, the toxicity of higher doses of coenzyme Q10 has not been encountered in clinical use.

Low coenzyme Q10 status and increased oxidative stress in chronic dialysis patients may be ameliorated by coenzyme Q10 administration, which, therefore, can be considered as a complementary treatment. Although promising, administrations of coenzyme Q10 may improve peripheral circulation status and lipid profile, prevent against cardiovascular disease and neurodegenerative disease in patients undergoing hemodialysis require further study.

5.3 Probiotics, prebiotics and synbiotics

Chronic gastrointestinal symptoms are the most common in ESRD patients who are treated by hmodialysis or peritoneal dialysis. Disturbances in gastric and small intestinal motileity, small intestine bacterial overgrowth, gastric hypochlorhydria, diarrhea, abdominal pain, and irritable bowel syndrome are symptoms typically seen in these patients (Cano et al., 2007). The pathogenesis of these symptoms is probably multifactorial, and has also been attributed to changes in the small intestine microflora (Strid et al., 2003). It has been suggested that uremic toxins, commonly used medications, complications, and changes in dietary patterns have major influences on the microflora of the small intestine (Evenepoel et

al., 2009; Shu et al., 2009). Further, an abnormal distribution of microflora in the gastrointestinal tract can result in gastrointestinal permeability and inflammation. Oxidative stress and malnutrition frequently occur in people who suffer from gastrointestinal disturbances.

In addition to these considerations, uremic toxins include low-molecular weight solutes, medium-sized molecules (peptides and proteins), and protein-bound, low-molecular weight solutes. With regard to the latter, such as indoxyl sulfate and p-cresyl sulfate (originating from intestinal bacterial fermentation end-products of tyrosine and tryptophan), these cannot be efficiently removed by hemodialysis, even with a high-flux membrane (Niwa, 2011). Accumulation of these uremic toxins induces free radical production, and increases the expressions of transforming growth factor-β1, tissue inhibitor of metalloproteinase-1 and proalpha1(I) collagen; therefore, they play an important role in the development of cardiovascular disease and can promote the progression of renal dysfunction (Niwa, 2010).

Some studies in humans have indicated the potential efficacy of probiotic, prebiotic, or synbiotic preparations for gastrointestinal diseases (Madsen et al., 2001). Probiotics are defined as viable micro- organisms for which sufficient quantities can reach the intestine in an active state and exert beneficial health effects. Prebiotics are non-digestible food ingredients that are metabolized by the probiotics. Synergistically acting combinations of probiotics and prebiotics are designated synbiotics.

The reported beneficial effects of these preparations include stimulation of intestinal motility and intestinal immunity, recovery of a disturbed gut mucosa barrier, elimination of toxins and potential pathogens, the release of nutrients, antioxidants and growth factors, stimulation of mineral absorption, and reduced colonic transit times via the normalization of altered intestinal microflora (Madsen et al., 2001; Singhi and Baranwal, 2008). In recent years, the prebiotic oligo-fructose-enriched inulin was shown to reduce urinary p-cresyl sulfate excretion in healthy volunteers (De Preter et al., 2007). The same authors also demonstrated that oligo-fructose-inulin significantly reduced p-cresyl sulfate generation rates and serum concentrations in hemodialysis patients (Meijers et al., 2010).

However, an oral preparation of lactic acid bacteria did not reduce serum p-cresol concentrations in hemodialysis patients (Hida et al., 1996). Interestingly, the results of our preliminary study showed that synbiotics may be beneficial for decreased bacterial overgrowth and normal motility patterns in patients undergoing peritoneal dialysis and hemodialysis.

Intestinal therapeutic interventions with probiotics and prebiotics have provided some clinical benefits, but these have not been exhaustively studied in ESRD patients on hemodialysis or peritoneal dialysis. Nonetheless, it will be important to determine which probiotic genera and species possess beneficial traits, in addition to finding the optimal doses and possible synergistic combinations.

6. Conclusion

Renal dialysis patients appear to be prone to certain degrees of deficiencies for a number of micronutrients (Zn, Se, vitamin C, E, folate, B6, and B12) and nutritional substrates (coenzyme Q10 and EPA/DHA). This could be due to the significant losses of these factors in these patients or to their high needs. Increased oxidative stress and pro-inflammatory cytokines are important targets for nutritional and pharmacologic therapy for ESRD patients

who are undergoing hemodialysis; prolonged oxidative stress and pro-inflammatory cytokine production can exacerbate the severity of uremic complications and inadequacy residual renal function. These clinical features may be ameliorated by the use of supplements of these micronutrients. Probiotics and prebiotics may also provide benefits to these patients. Their use is not always a standard of care for hemodialysis patients. Therefore, uncertainty has arisen as to whether or not nutraceutical interventions are needed by hemodialysis patients.

7. References

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11

Differences in Erythrocyte Index and Hyporesponsiveness to Erythropoiesis in Hemodialysis Patients Treated with Different Erythropoiesis-Stimulating Agents

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1. Introduction

The majority of patients undergoing hemodialysis have anemia due to decreased production of erythropoietin. This condition is referred to as renal anemia, and is a type of normocytic and normochromic anemia. Renal anemia contributes to a worsening of quality of life (QOL) and has a poor prognosis for survival.

Clinical use of erythropoiesis-stimulating agents (ESAs) has markedly improved the QOL and prognosis for survival of hemodialysis patients. ESA is commonly used for treatment of renal anemia, but some hemodialysis patients subsequently develop macrocytic and hypochromic anemia that is not responsive to vitamin B12 and folic acids (Ogura et al., 2007). Macrocytic and hypochromic anemia after ESA treatment is also common in elderly hemodialysis patients (Murata, 1998). However, the mechanism underlying ESA induction of these changes in erythropoiesis is unknown.

The correlation between renal anemia and ischemic heart disease, which is referred to as cardiorenal anemia syndrome (CARS) (Silverberg, 2003), suggests that treatment of anemia may also suppress cardiovascular events. However, the results of the Correction of Hemoglobin and Outcome in Renal Insufficiency (CHOIR) study showed that patients with a high hemoglobin level in stage III-IV chronic kidney disease (CKD) after use of large doses of ESA have a poor prognosis for cardiovascular complications (Singh et al., 2006). Also, in a meta-analysis, Phrommintikul et al. reported an increased incidence of cerebrovascular disease in patients treated with ESAs at a high dose, with the incidence not related to the hemoglobin value (Phrommintikul et al., 2007). Recently, the results of a randomized control study (Trial to Reduce cardiovascular Events with Aranesp Therapy: TREAT) in diabetic CKD patients showed that darbepoetin did not have an inhibitory effect on the progression of renal dysfunction and the new onset of cardiovascular events (Pfeffer et al., 2009). Based on the results of the TREAT trial, it was concluded that the cause of the poor prognosis in patients receiving large doses of ESA is an increased incidence of cerebrovascular disorders. However, analyses in the CHOIR and TREAT trials were based on ESA treatment with

different intervals of administration, and there has not been sufficient investigation of the effects of different types of ESA on prognosis. Therefore, it remains unclear whether treatment of anemia leads to suppression of cardiovascular events.

2. Effects of erythropoiesis stimulating agents on hematopoietic response and cerebrovascular disorders

The mechanism through which large doses of ESA promote the onset of cerebrovascular disorders has not been fully elucidated, and it is unclear whether there is a direct causal relationship. Therefore, in this study, we evaluated the effects of different types of ESA on erythropoiesis, investigated the factors responsible for determining the poor response to ESAs, and determined the incidence of cerebrovascular disorders with different types of ESA.

2.1 Effects of different types of ESA on hematopoietic response

The subjects were 78 maintenance hemodialysis patients with ESRD (40 men and 38 women; age 63.9 ± 9.8 (mean ± SD) years old, range: 32-82 years old) who were treated at our clinic (HD duration 13.4 ± 9.8 years, range: 2-35 years). Inclusion in the study required that patients were taking epoetin for treatment of renal anemia and had no active inflammatory disease. The causes of ESRD were diabetes mellitus (n=20), chronic glomerulonephritis (n=42), renal sclerosis (n=5), IgA nephropathy (n=2), and unknown/uncertain (n=9). Patients were being treated twice or thrice weekly with standard bicarbonate dialysis with semisynthetic membranes (dialysis filter surface area 1.3-2.4 m^2). The mean weekly HD duration was 10.3 ± 1.5 h. Dry weight was targeted in each case to achieve a normotensive edema-free state. The study protocol complied with the ethical guidelines of our institution and informed consent was obtained from each patient.

The 78 patients were randomly divided into two groups in December 2008: those (N=30) who continued to take epoetin (epoetin group), and those (N=48) who switched to darbepoetin instead of epoetin (darbepoetin group). The dose of darbepoetin was determined using a ratio of 1:200 relative to the epoetin dose. In addition to a general physical examination and routine blood tests, the erythrocyte index, iron metabolism markers, normalized protein catabolic rate (nPCR) as a nutrition factor, and Kt/V as a dialysis efficacy factor were measured before dialysis every month for 1 year. Comparisons between the two groups were made using average values over 1 year. The doses of ESA and venous iron were modulated with the goal of maintaining hemoglobin (Hb) at 10-11 g/dL and ferritin at 100–200 ng/mL.

The general characteristics of the study population are summarized in Table 1. There were no significant differences in baseline values between the epoetin and darbepoetin groups.

Changes of parameters in the epoetin group are shown in Table 2. Ferritin, transferrin saturation (TSAT) and iron dose all decreased after one year, compared to the respective baseline values. There were no significant differences in the mean corpuscular volume (MCV) and the mean corpuscular hemoglobin concentration (MCHC) (Fig. 1).

Changes of parameters in the darbepoetin group are shown in Table 3. The Ht value increased significantly and MCHC decreased significantly after one year, compared to the respective baseline values. The change from epoetin to darbepoetin resulted in a significant decrease in MCHC (Fig. 2).

Parameter	Epoetin (N=30)	Darbepoetin (N=48)	P-value
Age (year)	62.3±10.9 (32-82)	65.0±9.8 (36-80)	n. s.
HD duration (year)	14.0±10.6 (2-35)	12.9±9.0 (2-34)	n. s.
Male / Female	12/ 18	28 / 20	n. s.
Cause of CKD			
Chronic glomerulonephritis	17	25	
Diabetic nephropathy	7	13	
Renal sclerosis	2	3	
IgA nephropathy	1	1	
Unknown	3	6	
Pre Hb (g/dl)	10.25±0.41	10.28±0.53	n. s.
Pre Ht (%)	31.13±1.12	31.42±1.60	n. s.
Pre MCV (fl)	98.01±5.23	96.90±3.09	n. s.
Pre MCH (pg)	32.28±1.86	31.70±1.24	n. s.
Pre MCHC (%)	32.93±0.51	32.71±0.45	n. s.
Pre Ferritin (ng/ml)	151.4±91.3	152.6±35.3	n. s.
Pre TSAT (%)	30.2±8.4	30.2±7.2	n. s.
Pre Iron dose (mg/week)	10.8±10.0	15.5±6.2	n. s.
Pre rHuEPO dose (IU/week)	4318±2219	3591±1792	n. s.

Table 1. General characteristics of the 78 hemodialysis patients.

Parameter	Baseline	After one year	P-value
Hb (g/dl)	10.28±0.53	10.21±0.35	n. s.
Ht (%)	31.42±1.60	31.17±0.99	n. s.
MCV (fl)	96.90±3.09	97.39±3.82	n. s.
MCH (pg)	31.70±1.24	31.90±1.43	n. s.
MCHC (%)	32.71±0.45	32.74±0.40	n. s.
Ferritin (ng/ml)	152.6±35.3	128.3±40.6	p<0.05
TSAT (%)	30.2±7.2	26.0±7.4	p<0.05
Iron dose (mg/week)	15.5±6.2	8.1±6.1	p<0.01
rHuEPO dose (IU/week)	3591±1792	3766±1942	n. s.

Table 2. Changes of parameters in the epoetin group.

Parameter	Baseline	After one year	P-value
Hb (g/dl)	10.25±0.41	10.23±0.49	n. s.
Ht (%)	31.13±1.12	31.77±1.34	p<0.05
MCV (fl)	98.01±5.23	98.60±5.92	n. s.
MCH (pg)	32.28±1.86	31.75±2.13	n. s.
MCHC (%)	32.93±0.51	32.18±0.59	p<0.001
Ferritin (ng/ml)	151.4±91.3	136.0±63.4	n. s.
TSAT (%)	30.2±8.4	28.3±9.2	n. s.
Iron dose (mg/week)	10.8±10.0	12.8±7.9	n. s.
rHuEPO dose (IU/week)	4318±2219	4131±3145	n. s.

Table 3. Changes of parameters in the darbepoetin group (DA dose: 20.7±15.7 μg/week).

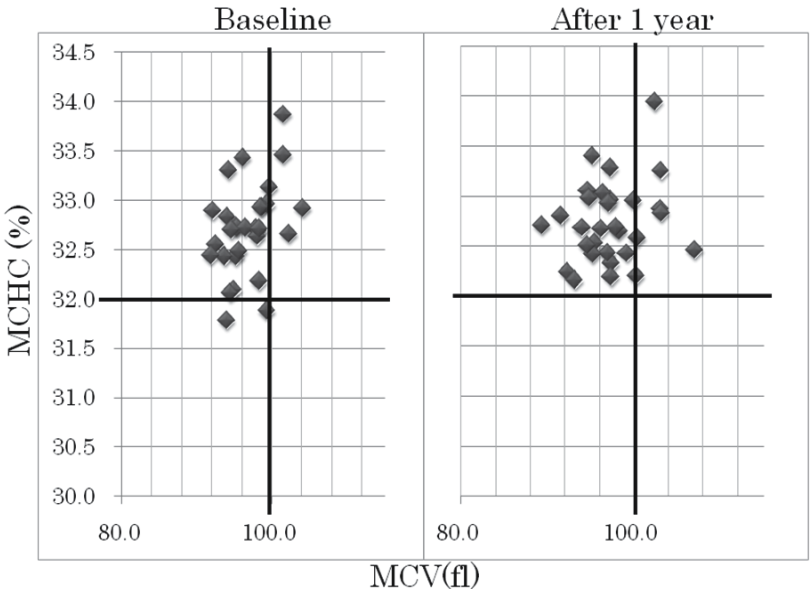

Fig. 1. Changes of annual mean values of MCV and MCHC in the epoetin group.

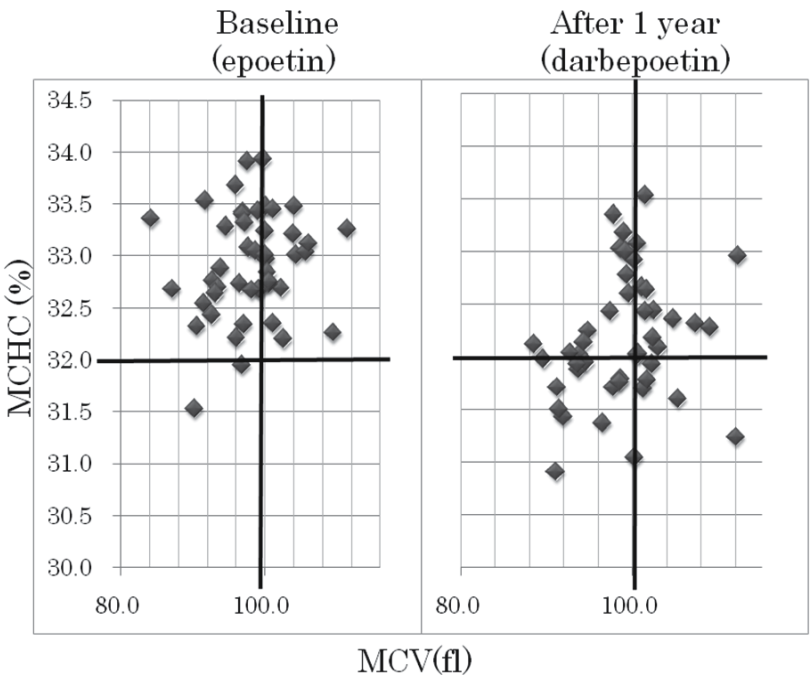

Fig. 2. Changes of annual mean values of MCV and MCHC in the darbepoetin group.

In this study, the change from epoetin to darbepoetin resulted in a significant increase in Ht and a significant decrease in MCHC, while continuation of epoetin did not change the erythrocyte index (EI). Since there were no significant differences in ferritin, TSAT and iron dose between the two groups, the changes of Ht and MCHC may have originated from the effect of darbepoetin. The increase of Ht and decrease of MCHC indicate macrocytic and hypochromic changes of erythrocytes. The mechanism of these changes induced by darbepoetin remains unknown.

2.2 Factors responsible for determining a poor response to an ESA

Next, the responsiveness to darbepoetin was evaluated by dividing the 48 patients in the darbepoetin group into two subgroups. The high dose (n=14) and low dose (n=34) subgroups comprised patients who required a DA dose of ≥60 μg/week and <60 μg/week, respectively, during the follow-up period. The annual mean values of EI, iron metabolism markers, nPCR and Kt/V and the incidences of complications were compared between the two subgroups (Table 4). A higher rate of complication of hepatic cirrhosis was found in the high dose subgroup ($p<0.02$). There were no significant changes in Kt/V, nPCR and other parameters between the two subgroups. There was also no significant difference in the given dose of iron; however, the ferritin level was lower in the high dose subgroup.

Parameter	Low dose subgroup (DA＜60ug/week)	High dose subgroup (DA≧60ug/week)	P-value
N	34	14	
Age (year)	64.88±10.57	65.31±6.00	n. s.
HD duration (year)	13.03±9.37	15.00±9.53	n. s.
Sex (Male / Female)	19 / 15	11 / 3	n. s.
Diabetes (%)	38.2 (13/34)	35.7 (5/14)	n. s.
Complication of liver cirrhosis (%)	2.9 (1/34)	28.5 (4/14)	P<0.02
Dry weight (kg)	50.48±8.33	53.35±7.84	n. s.
HD time (hour)	4.02±0.23	4.44±0.46	n. s.
Kt/V	1.53±0.17	1.58±0.22	n. s.
nPCR (g/kg/day)	0.89±0.15	0.85±0.19	n. s.
Alb (g/dL)	3.92±0.21	3.95±0.17	n. s.
Hb (g/dL)	10.31±0.47	10.11±0.25	n. s.
Ht (%)	32.05±1.19	31.48±0.58	n. s.
MCH (pg)	31.57±1.90	32.63±1.29	n. s.
MCV (fL)	98.16±5.20	101.60±4.02	n. s.
MCHC (%)	32.16±0.66	32.12±0.37	n. s.
TSAT (%)	29.19±9.27	30.31±7.12	n. s.
Ferritin (ng/mL)	153.55±66.45	110.25±21.88	p<0.01
CRP (mg/dL)	0.32±0.52	0.33±0.31	n. s.
Iron dose (mg/week)	10.73±6.57	14.62±4.75	n.s.
Average DA dose (μg/week)	13.53±5.19	33.77±16.95	p<0.02

Table 4. Comparison of the high dose and low dose darbepoetin subgroups.

The required dose of darbepoetin was significantly higher for patients complicated with liver cirrhosis. Such patients were not excluded from the CHOIR or TREAT study, which suggests that this complication affects the prognosis of patients receiving a high dose of darbepoetin. In Japan, ESA hyporesponsiveness is defined as a failure of improvement of anemia with a darbepoetin dose of 60 μg/week or an epoetin dose of 9000 IU/week without iron deficiency (Tsubakihara et al., 2010). Insufficient dialysis, malnutrition, unclean dialysate, and drug effects have been suggested as causes of ESA hyporesponsiveness (Ifudu et al., 1996). In this study, there were no significant differences in any parameters except ferritin between the low dose and high dose darbepoetin subgroups. Since there was no difference in the given iron dose, the patients in the high dose subgroup might be exhibiting hyporesponsiveness to iron.

2.3 Incidence of cerebrovascular events with different types of ESA

Finally, the incidence of cerebrovascular events was evaluated in follow-up of onset of cerebrovascular disorders for another year (a total observation period of 2 years) in patients in both groups. The Kaplan-Meier method was used to analyze the incidence of cerebrovascular disorders from the beginning of the observation period (Fig. 3). There was no significant difference in cerebrovascular event-free survival between the epoetin and darbepoetin groups. Although no difference in the incidence of cerebrovascular events was found following the change from epoetin to darbepoetin, the incidence of cerebrovascular events showed a tendency to increase in the high dose darbepoetin subgroup.

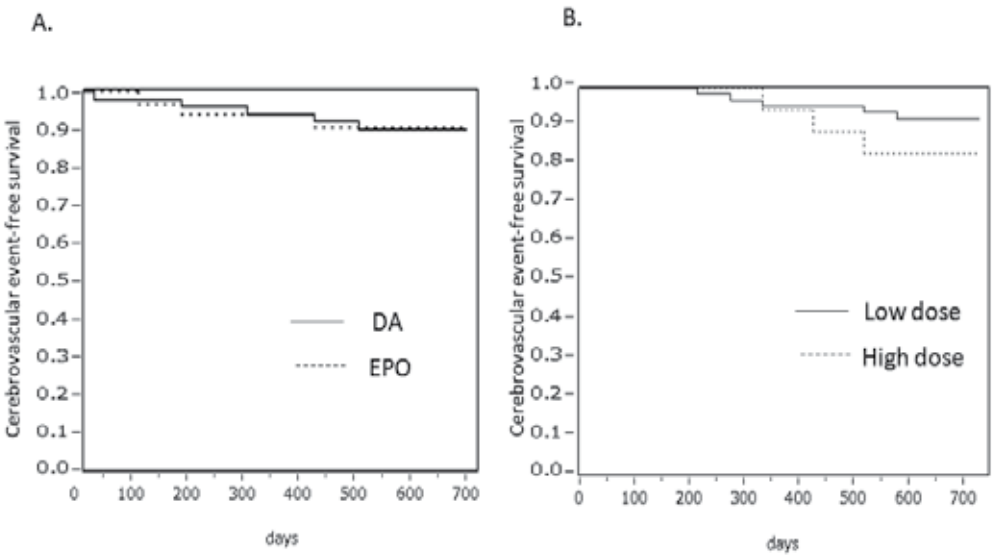

Fig. 3. A. Cerebrovascular event-free survival in the epoetin (EPO) and darbepoetin (DA) groups. B. Cerebrovascular event-free survival in the high dose and low dose darbepoetin subgroups.

A random prospective study showed poor mortality and a high incidence of cardiovascular events in hemodialysis patients with high Hb compared to those with normal hematocrit (Besarab et al., 1998). Moreover, the results of secondary analysis in the CHOIR study

showed that patients in whom anemia did not improve with administration of large doses of ESA had a worsened prognosis compared to patients in whom anemia did improve with a low dose of ESA (Szczech et al., 2008). Thus, it is currently thought that a lower response to ESA (ESA hyporesponsiveness) results in a poor prognosis. In the current study, we found no significant difference in the incidence of cerebrovascular events over a two-year observation period between patients treated with epoetin and darbepoetin. However, a higher, although not significantly higher, incidence of cerebrovascular events was found in patients who received a high dose of darbepoetin. Collectively, these data suggest that particular care is needed in treatment of patients with ESA hyporesponsiveness.

3. Conclusion

The ESA dose was significantly higher for patients complicated with liver cirrhosis, who were not excluded from either the CHOIR or TREAT study. This suggests that this complication affects the prognosis of patients receiving a high dose of an ESA. We found no significant difference in the incidence of cerebrovascular events with epoetin and darbepoetin during a two-year observation period; however, epoetin and darbepoetin might have different effects on the hematopoietic response.

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12

Focal Dental Diagnostic in Patients with Replaced Renal Function-One New Method in Dentistry

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1. Introduction

Patients with chronic renal disease CRD grow up in worldwide and Bulgaria makes no exception from this tendency. The people in ERSD increase too, and they require renal replacement therapy-hemodialysis or renal transplantation. With development of transplantation medicine these patients hope for better treatment and better quality of live.

In the end of the year 2009 FDM Sofia win a project which is financed by National Science Fund. This project is built on the PhD thesis of Dr. Maria Dencheva from FDM. So The Faculty of Dental Medicine (FDM), Sofia has been established as a leading scientific unit in the diagnostics and treatment of dental foci from the maxillofacial area of patients with progressive renal deficiency. The funding of various organizations proved fruitful in order for a team of experts to share their efforts and experience within the project, and also to move towards dental treatment of a much larger group of patients on hemodialysis and renal transplantation.

The objects of the project are to optimize the complex dental foci diagnostic and treatment of the patient on hemodialysis and renal transplanted and to prove the needs for prevention against fields of disturbance (dental foci) with the groups of patients formed under the project.

We are orientated to complex foci diagnostics of the field of disturbance in MFA by this population of patients, because that give to us possibility for registration not only carieses and periodontal diseases, but also energetic unintegrated areas in organism. They are not obligatory limited in tissue but they can stimulate negative the system common reactivity (SCR) and in this way to different nerve-vegetative reactions.

The complex focal dental diagnostic is as a result of a common scientific work of many years and medical specialists. The method is scientific defended and it practices from Prof. Kisselova in the Faculty of Dental Medicine Sofia. **We modify it for the needs of patients on hemodialysis and kidney transplanted through including infrared thermographic methods and laser equipment. So the effect is short with aseptic healing process -Fig1.**

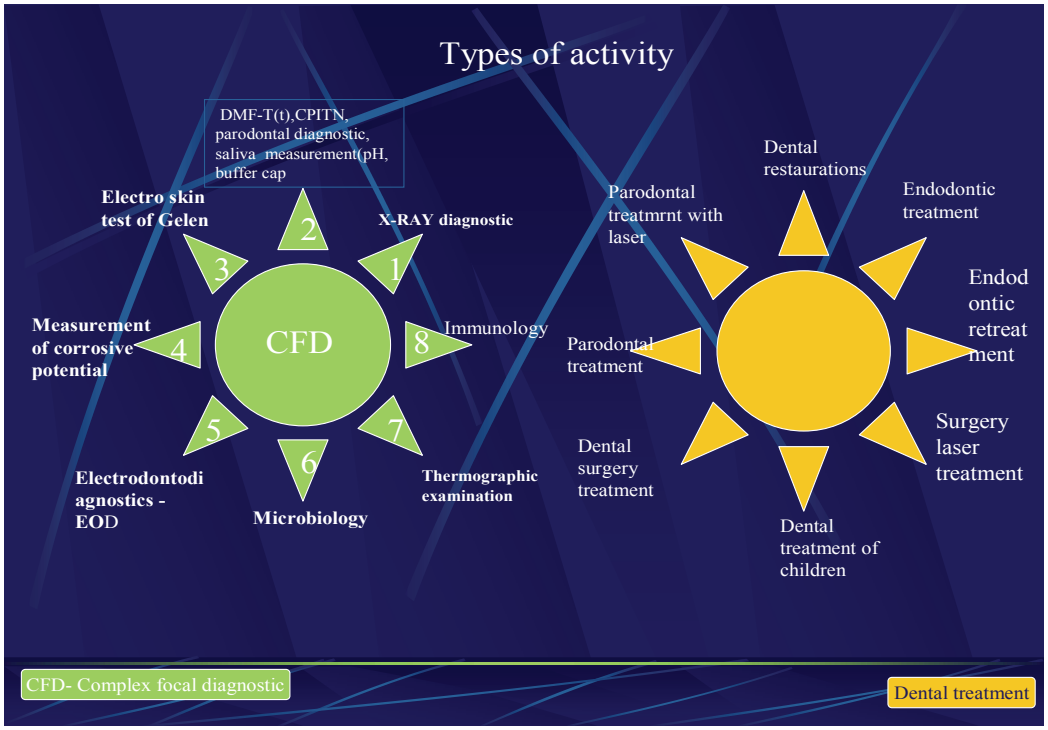

Fig. 1. Types of activity.

2. Method description

1. Focal directed medical history
2. Replenishment of individual diagnostic card
3. Examination of unstimulated whole saliva.
4. X-ray diagnostic.
5. Conductive methods–Electrodontodiagnostics - EOD, Measurement of corrosive potential
6. Electro skin test of Gelen
7. Thermovision research during Flir A310 in 6 six aspects.
8. Individual inquire
9. Register -The need of systematic registration of personnel diagnostics and dental treatment information, manipulation and profit of data, provides reasons to create an information system for exact analyzes and reports.

2.1 Focal directed medical history

The patient shall have to be asked about major diseases of the vital human systems, thus paying attention to certain reactions of a neurological- vegetative type in order to evaluate the general status of information. Medically compromised patients form a group one out of three patients that have to undergo a similar anamnesis directed straight against the source of information. Patients, who suffer from malfunctions of the immune system and changes of general reactivity, i.e. when their organism does not react adequately to minimum disturbances. In this sense any pathological process of the dental-jaw system – inflammatory

and degenerative disease of the dental pulp, periodontitis, oral mucousis – all these could be a cause for infirmities of certain organs by the connecting tissue that links them (Kisselova-Janeva, *2001)*.

System for general reactivity (SGR) is defined as a functional complex by Pischinger (Fig. 2)

1. Basic substance including extra- cellular fluids.
2. Cells of connective tissue.
3. Functional peripheral vascular system.
4. Neuro-vegetative bifurcations/endings.

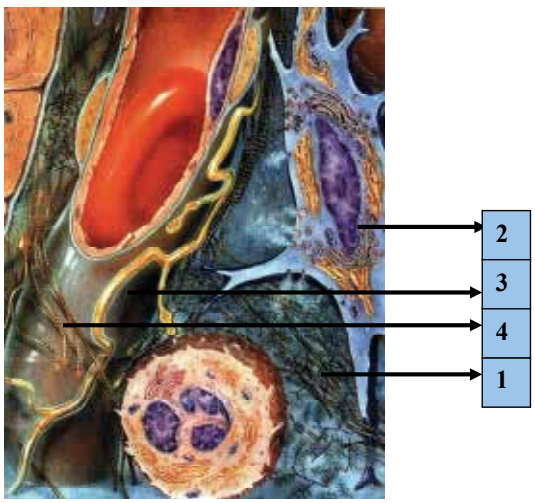

Fig. 2. System of general reactivity according to Pischinger.

The system of basic regulation nets the entire organism and represents its anatomic-physiological base. The inflammatory processes are, therefore, stirred. There appears an interjection, interacting and mutual simultaneous support of existing pathological processes, based upon exogen and endogen noxes. Via their unspecific parameters, SGR reacts according to any stimulate both locally and totally. The reactions differ according to the impulse, which is often inadequate according to the power of vexation. A most important peculiarity of the SGR is that it can react as per the vexation (left, right) whereof the injury is harder. Sources of inflammation arising from dental problems are just a part of the capabilities of the human body proves that 80% of them are located in the oral cavity (Bergsmann, 1973).

Based upon the most modern opinions regarding the inflammatory problem, the Bulgarian scholars Gerassi &Kiselova use the following definitions:

FIELD OF DISTURBANCE- energetically non- integrated part of the organism that is transformed into a source of functional disturbances that may, but not necessarily, have material fixations. The field of disturbance by means of its constant activity and influence over the system of basic organism regulation leads to certain structural changes within.

The term INFLAMATORY is specifically concerned with material findings and their impact and that is why it is considered incomplete.

The fields of disturbance can be **active** and **potential** (latent) (Kisselova-Yaneva, 2001).

Active are those that overwhelm the local reactive barrier and, as a result, there is a distant commencement influence over the general organism reactivity.

They may be within a compensation period without any clinical manifestation, so that only the data from different tests or the decompensate period of single symptoms can reveal the overall clinical picture.
The potential are chronically restricted information alterations whereas the local protective barrier is still intact, thus there is no distant disturbance. A number of patients are carriers of latent fields of disturbance, yet even a banal infection can lead to a trigger for inflammatory activity. The disturbances that infections and inflammation cause to the organism can be of paramount importance in life threatening situation.
The fields of disturbance may be present in any part of the organism.
The diagnostics of the fields of disturbance (FD) is of paramount importance in order to find out the domination lines. The domination field is the one that influences most the pathogenesis of the inflammatory disease (Kisselova-Yaneva, 2001). People, seemingly of good health, do possess latent fields of disturbance, yet even a banal infection can unlock inflammatory activity.
Thus, the theory of Pischinger on the system of general regulation and the paramount importance of inflammation of infection, being etiological factor for the immergence of the chronic overload syndrome (Kisselova-Yaneva, 2002, Part1 and 2, Kisselova-Yaneva, 2000), lead to an accent over their early detection concerning each one of the patients. Inflammation sources have multilateral impact over the organism, Fig. 3.

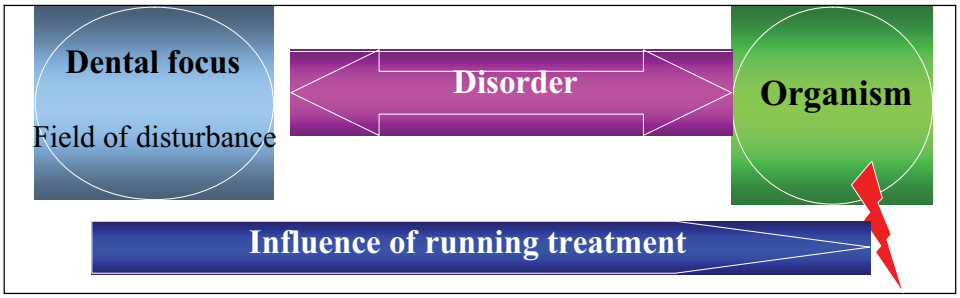

Fig. 3. Interconnection between "field of disturbance – organism".

It is a fact that the fields of disturbance can cause a disease process but, on the other hand, they can fail or seriously deteriorate the healing process by an infection process, blocking basic vital functions (Kisselova-Yaneva,2000).
Defining of obvious fields of disturbance in the maxillofacial area is the basis of a rational plan for dental treatment, combined with the schedule of prophylactic examination considering the general state of the organism, diseases and medication therapy of each patient.
Patients, suffering from a chronic renal insufficiency are just apt to micro- inflammations as a result of the uremia (Graig et al., 2007).
Inflammation, according to Scannapieco et al. (2008), Suzucki & Chialastri (2007), is considered a significant factor within the development of renal diseases and rejection of renal grafts. Gluhovschi et al. (2003), however, researches dental inflammation and the development of glomerolonephritis, taking into consideration the presence of other inflammatory processes. The facts present, however, are bifocal. Following antibiotic treatment and dental aid, there is a significant decrease of blood-urea and proteinuria, according to statistical data. There are some patients, who demonstrate indications

following treatment, where the above quoted indications have increased despite antibiotic treatment, which means that the interrelation between existing dental focuses and kidneys remains uninterrupted. On the other hand, Hujoel et al. (2001) suggest that the lack of higher risk of infections in patients with coronary- cardiac diseases and radically elimination inflammations of dental origin or untreated periodontitis are not to undergo more extensive treatment. Niderhagen et al., 2003 state that afore dental sanitation, patients with liver transplants need a series of tests in order to examine the oral cavity for fields of disturbance, backing up the opinion for a necessary dental extractions with periepical changes combined with the feeling of pain and other symptoms. Non-erupted teeth without inflammation, teeth with not exactly filled root channels and caries teeth should not be extracted. Osano et al. (2005), while studying asymptomatic post-endo-dental periepical alterations and the cases of infectious complications during the anti-leukemia chemotherapy with patients with malignant blood diseases, do not observe any interrelation, paying attention to the fact that there should be an optimum strategy developed: either endodontic treatment or antibiotics. The purpose is to minimize oral complications within the period of extensive immune suppression. Meyer et al. (1999) are absolutely positive in their research, stating that the patients in an end phase of cardiac insufficiency expecting transplantation shall not be subject of "vigorous" dental treatment, since they have found no connection between the mortality rate and the infection and the level of transplant rejection within the group of patients with dental inflammation and the group without focal dental infections.

According to Rustemeyer & Bremerich, 2007, (Rustemeyer et al. 2006), (Sarachev 2006) the removal of oral septic focus by means of extraction or another form of treatment is a necessary means in order to avoid transplant failure. They explore the necessity of dental and periodontal inflammatory sanitation with patients expecting renal and liver transplantation, as well as a cardiac implant. It becomes obvious that the need for surgical sanitation is grave within the group of renal transplanted patients. Since there are no set rules for inflammatory sanitation, the authors share the opinion that patients, who are about to undergo transplantation process, shall have to have their oral cavity sanitized in order to avoid systematic and local oral complications during the post- transplant period.

There are certain information releases regarding febrility states with unclear etiology following transplantation. The lack of precise diagnostics leads to improper treatment related to unnecessary antibiotics regime. Most often the medical personnel ignores the fact that the reason lies in the oral cavity – e.g. asymptomatic periodontal changes, unerupted and semierupted molars (Fig. 4) (Helderman, 1996), (Martinez- Tirado, 1982), (Samra et al., 1986), (Zeier, 2004, Zeier & Ritz, 2002). Barnett, 2006 expresses the opinion that it is necessary to further explore the connection between oral and systematic diseases and states.

Gugenheimer et al. (2005) have completed a research proving that there are not set protocols for dental treatment of patients, who are about to undergo transplantation. Since there is no standard for dental treatment for patients before and after transplantation. Segelnick & Weinberg(2009) describes a case of a patient, who has lost a number of options for transplantation because of untreated periodontal disease. Another important fact is that most transplantation centers offer a minimum of medical, social and psychological consultancies and even less- dental help. Only in case of infection dental help is required. Most often, it is too late to interfere, since the operation has already been planned and there is no time for optimal dental treatment. A dentist's visit and treatment has to take place log before the operation itself and not just a couple of days before, since the sanitation of the oral cavity require much more time. Segelnick& Weinberg (2009) states that the patients

under total dental and periodontal programs for prevention of infection in maxillo facial area shall have to undergo a quite problematic post- operational period. Djemileva, Bolyarova (2007), Bayraktar et al., (2007), Sulejmanagic et al. (2003) back up the same opinion. In Germany, the Deutche Gesellschaft für Zahn-Mund- und Kieferheilkunde (Otten, 1998) there is a protocol that separates the patients for dental sanitation before and after organ transplantation. There is an organization, established to control and motivate those patients and to report on the sanitation completed.

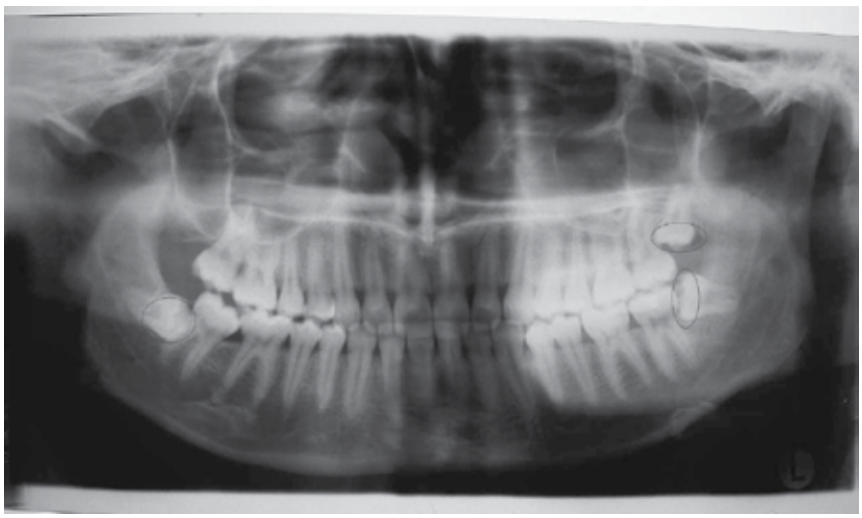

Fig. 4. Erupted and semierupted molars of a patient after a renal transplantation with a high temperature due to an uncertain reason.

The University of Old Dominion in the USA attaches a protocol of Coral Diaz(1996) for dental health during the post-transplantation period. It specifies the time for routine dental treatment, sustaining hygiene programs and first aid. The patients are distributed in three groups – one year after the transplantation, transplantation within period from one to three years and transplantations from 3 and more years. The protocol accentuates on the periodontal record of the patient.

2.2 Replenishment of individual diagnostic card

The individual diagnostic dental card includes the following dental and periodontal indices - DMF, PBI, PD, CPITN, OHI, and a detailed description of available dental restorative materials in the patient's mouth.

2.2.1 Dental status and dental materials available

DMFT- index – D – decayed, M – missing teeth, F – filling, crown.
Dental materials in patient's mouth are described by type, for example: amalgam, composite, metal alloy, ceramic, plastic, brackets, etc.

2.2.2 Periodontal diagnostics with the following indexes - CPITN, PBI, OHI, PD

Identification of periodontal status of participants and periodontal treatment needs by CPITN **(Community periodontal index of treatment needs**).

Public periodontal index of treatment needs was established as an epidemiological tool by the WHO (Ainamo et al.,1982)
During the clinical dental examination of patients we registered the oral status and the CPITN-index of every one of them, using a specially created index CITN probe*. Transplant patients are examined on the day of the initial visit as part of a complex focal diagnostics. Periodontal examination of patients on hemodialysis was done the day after the dialysis session in order to avoid protracted bleeding on probing. From the described methods for dental diagnostics we used the one which examined al the available teeth within the sextant. The wisdom-teeth are examined only in case of lack of molars. For the sextants in which there is a lack of at least two teeth with severe periodontitis and predict impending extraction , we used conditional code 9 .
Examined teeth in sextants:

1st (17, 16, 15, 14)
2nd (13,12, 11, 21, 22, 23)
3th (24, 25, 26, 27)
4th (34, 35, 36, 37)
5th (43, 42, 41, 31, 32, 33)
6th(47, 46, 45, 44)

The essence of the index is described in table 1 and 2 .The most serious finding in each sextant was code in table 1 according to requirements. The maximal code, in any sextant of each patient, defined the needs of periodontal treatment (table 2).
* A specially designed CPI probe with a 0.5-mm ball tip is used , with a black band between 3.5 and 5.5 mm and 8.5 and 11.5 mm from the ball tip.

Findings	*Code*
Healthy, no signs of periodontal disease	0
Bleeding observed, directly or by using mouth mirror after probing	1
Supragingival or subgingival calculus detected during probing, but all the black band on the probe visible	2
Pocket 4-5 mm(gingival margin within the black band on the probe)	3
Pocket 6 mm or more (black band on the probe not visible)	4

Table 1. CPI.

Maximum score CPITN	*Treatment recommendation*
CPITN 0	No need for additional treatment
CPITN 1	Need to improve personal oral hygiene
CPITN 2 CPITN 3	Need for professional cleaning of teeth, plus improvement in personal oral hygiene
CPITN 4	Need for more complex treatment to remove infected tissue

Table 2. Treatment needs.

Defining of Hygiene index:
Dental debris is visualized by means of coloring agent. The presence (+) or absence (-) of debris is detected on all four dental surfaces (vestibular, oral, mezial, distal). The index is calculated in a percentage of all dental surfaces debris free.

Defining of Papilla bleeding index (Index indicating papilla bleeding as an inflammation indicator):
By means of a periodontal probe bleeding is caused when using „sweeping" of the sulcus beside mezial and distal dental surfaces from the very base of the papilla to the top. 20 – 30 seconds later the intensity of bleeding is being detected. The indications are as follow: when there is a lack of bleeding the value goes to zero „0"; when there are indications for single bleeding spots – „1"; indications for a narrow bleeding line at the gingivitis fringe or several bleeding spots mean –„2"; upon filling the inter-dental space with blood the extent is – „3"; at profusion bleeding – „4".
Probing is completed by the quadrants: I quadrant – palatial, II – vestibular, III – lingual, IV – vestibular.
The index value of a patient represents an arithmetic mean of all values measured. The percentage of all bleeding papilla compared to the rest papilla defines the field of distribution of inflammation.
Defining of the periodontal depth. (PD)
The research is completed by means of a special periodontal probe (PCP-UNC 157, Hu-Friedy, Manufacturing Co.). The advantage of this probe is its graduation of up to 15 mm, in an interval of 1 mm, which grants us the opportunity to measure in high preciseness deep periodontal pockets. Measurement applies to any periodontal unit within the oral cavity at six points – central vestibular, central oral, a-medial and distal plus medial and distal – two points (vestibular and oral). Four values have been registered – vestibular, oral and a greater value for the measured medial and distal.
Defining of loss clinical attachment for each periodontal unit.
This measurement is completed once again by means of a special periodontal probe (PCP-UNC 157, Hu-Friedy Manufacturing Co.). The distance between the cementoenamel junction from the bottom of the pocket of all four surfaces of each periodontal unit.

2.3 Examination of unstimulated whole saliva

Examination of saliva secretion assists the analysis of dental and oral health with patients of transplanted renal functions. The results of the detailed analysis of the research on saliva indicators can be utilized in the form of predilection parameters for establishing of pathological states, and therefore, to define the present dental oral status.
Patients in an end status of chronic renal insufficiency suffer from characteristic xerostomy that is easily explained by the law levels of liquid absorption, side effects of basic antihypertensive and other medicaments, possible alterations in the saliva glands because of autoimmune diseases and changes due to aging processes (Djemileva,1998), (De la Rosa Garcia et al. 2006),(Gavalda et al. 1999), (Guggenheimer & Moore 2003), (Thelin et al. 2008). The research of Bots et al. 2007 proves that patients undergoing hemodialysis secrete less saliva (stimulated or not stimulated) temporarily, but after transplantation and reversing of the normal renal functions it is increased to its ordinary level. These same patients demonstrate a decrease in pH levels from 7.36 to 6.74 probably because of decrease of concentration of urea in the saliva and respectively a decrease of hydro isolation and the oral flora to ammonia (Burne & Marquis, 2000).
According to the research of (Panov, 2010) the application of dry urine tests, studying the saliva has its information value since the percentage of positive samples reaches a value of above 50 regarding nitrites, albumen, blood and relative weight. It is a very rare event to indicate positive samples of ketones and ureabilynogene. The average situation appears with the tests for bilirubine and glucoses. Over 40% of the cases are positive regarding the

leucocytes samples. According to research and the data accumulated by Panov we have to apply the same methods for our patients. Hereby, the research and the data of Panov make us apply the same methodology towards our patients. We use the dry tests with ten \10\ indicators - Deca PHAN leuco fig.5.
The conditions necessary for examination of patients incorporate the following factors: at least an hour of not consuming any food or beverages, no smoking, any form of dental and oral-hygienic procedures (brushing of teeth, mouth gargling, using of oral shower, etc.).
The standard band (sticks) is placed face down over the dorsal surface of the tongue for a period of up to 5 seconds or until it is entirely drenched. The results are recorded directly via a scale.

Fig. 5. Dry test sticks.

2.4 X-ray diagnostics

X-Ray diagnostics is absolutely imperative for each patient needing ortopantomography. It gives to us an initial X-ray structure of the maxilla-facial-area and represents an additional method indicating the presence of an active source of inflammation. Other additional methods in search of inflammation sources include:

2.5 Conductive methods

Electrodontodiagnostics - EOD, Measurement of corrosive potential (fig. 6).

Fig. 6. Equipment for EOD examination.

By means of "VITATEST" we examined dental vitality. It is a specialized portable apparatus for electrodontodiagnostics. Essentials of the examination: the passive electrode lies in the patient's right hand while the active one (the cathode) is placed at the characteristic spot of Rubin on the surface of a dried tooth that has to be examined. After pressing the start button the micro electrical mono-polar impulse begins to increase. When the patient reacts the active electrode is removed from the tooth. Then there is a check of the recordings displayed on the screen of the device. The following values are considered to be standard in Bulgaria:
2-6-10μA – reaction of a normal pulpa, up to 20 μA – acute caries, up to 30 μA – chronic caries, up to 35 μA – hyperemia of dental pulpa and partial pulpitis, from 40 – to 60 μA – necrosis of the coronary pulpa, between 100 and 200 μA – necrosis of dental root pulpa (Botushanov, 1994), (Kodukova et al. 1980).
Examination of corrosive potential by means of "CORPOTEST" apparatus (Fig. 7).

Fig. 7. Apparatus for measurement of corrosive potential.

Corpotest apparatus is essentially a direct current milivoltmeter with high input resistance of 25 MΩ ± 5%, allowing the measured electrical sources not to be loaded. It has a battery charger of 9 V. The range of measured potential differences varies from -1990 to +1990 mV. This test applies for all metals and alloys needed for obturations and denture constructions. Optimal values are considered to vary from 100 to 150mV/for each separate metal object and up to 800 mV – total for all metal objects in the oral cavity. The measurements of alloys of non- precious metals are marked with a minus (-), for those made of precious metal – with plus (+) (Petrunov et al.2009).
Measurement of corrosive potential - This test studies the pathogalvanic elements in the oral cavity in case there are metal objects – amalgam obturation metal bridge constructions, metal frame of removable denture.
Patients undergoing hemodialysis need an objective and almost obligatory finding in order for the uremic breath and the changed sense of taste in the oral cavity to be registered. These are a result of the increased concentration of urea in the saliva and its consecutive transformation into ammonia (Temnyalov,2004), (Cervero et al. 2008), (Kho et al. 1999). It is, however, possible that such complaints be observed in patients, whose laboratory blood and urine indications are still normal, e.g. after transplantation and this is due to the increased corrosive potential combined with poor personal and professional oral hygiene (Petrunov et al.2009).

2.6 Electroskin test of Gelen

EST of Gelen (electro-skin test of Gelen) (Fig. 8) for defining of local reactivity is completed by means of MICRODENT apparatus.

Fig. 8. Apparatus MICRODENT.

It is a specialized portable device for focal diagnostics. It is equipped with a system for graduate increase of electricity applied to the patient following interruption and post closure of the patient's circuit, thus avoiding pain during the test. The type of patient's electricity is galvanic. The input voltage varies from 3,0 V to 19,5 V (±0,5 V). The test's essentials consist of defining of local reactivity of the skin above the zone of the supposed or detected field of disturbance (source of infection) after a clinical examination and paraclinical tests (image diagnostics). This is done by means of subjective and objective findings. The subjective ones are registered by the patient as hyper-algesia, and the objective ones – as a hyperemic zone at or around the location of detected dental changes or adjacent dental tissues. The passive electrode of the apparatus (cathode) is held by the patient with their right hand, while the active is a small brush that is periodically moisturized with physiological solution/sodium chloride (NaCl), moving in circles around the facial skin. In the beginning of the examination the individual limen of sensitivity of each patient is identified and it is not changed until the very end of the test procedure.

2.7 Thermovision research in 6 six aspects

Since any object which is higher than the absolute temperature - 273 C emits infrared rays. Thermography is not invasive to the human body, and is a non-contact technique through which the temperature distribution of the human body surface can be monitored and recorded. In 1968 thermography was introduced as a method of diagnostic examination in the medical field (Miroshnikov 1980, Komoriyama et al. 2003).

The interest paid in the foccal infection as a "hidden" disease leading to the damage of different organs increases more and more because of the fact that patients are being directed to such an examination after all the diagnostic and therapeutic sequences have been run out - unnecessary loss of time and money. The contemporary diagnostics of foccal infection requires reducing the subjective factor in the measurement to zero. These conditions are fulfilled by thermography - contact or infrared - precise and reliable method objectively reading the changes in the temperature of definite points. In combination with the medical approach not to measure just the value of the t°, but the thermoregulations capacity of the organism in dynamics , the regulation thermography is unique method for prophylaxis of foccal infection by registrating "hidden" changes.

Medical analysis of the thermogram – it is based upon leading indications:

1. **Temperature gradient** – this is the quantity of temperature difference between two elements of the examined object.

Distribution of thermal emissions on the surface of human body is uneven, there exists the so called "physiological asymmetry", reasons for which is the heterogeneous structuring of the elements of the dermal system.

Clinical data indicate that the magnitude of physiological asymmetry is maximum 0,4 ° C . In this context the temperature gradient of up to this value is within the standard and should not be interpreted as a pathological thermal visional symptom.

2. **Qualitative characteristics of the temperature field of the object or a separate fragment**

The study of qualitative characteristics of termal distribution over the surface of the human body in the most widespread approach for evaluation of a thermogram. There are three different options for qualitative evaluation : Isothermic – when all or a predominant part of the elements of the tested object have identical or similar / within the limits of physiological asymmetry / temperature.

Hypothermia – detection of an area within the thermal field of the object with temperature less than that of the surrounding elements ; by degree of intensity the hypothermia can be : moderate / thermal gradient of up to 1,0 °C , expressed / gradient 1,0° –2,0° / and acutely expressed /with a gradient exceeding 2,0 °C/.

Hyperthermia – detection of an area within the thermal field of the object with temperature higher than that of the surrounding elements; By degree of intensity hyperthermia is graded likewise.

The laboratory for thermovision diagnostics has two separate rooms: medical office with a specific premise for the patent to stand during the examination and a room for achieving thermal adaptation / waiting room/.

Auer thermographic protocol is:

Basic requirements towards the environment in order to provide optimum conditions thermalvision diagnostics :

- Distance from the camera to the patient – from 0,3 m to 2 m ;
- Room temperature - 22° C ± 2° C ;
- No air draft with seep exceeding 1 m/sec ; that is why no ventilators and air conditioners are allowed;
- There should be no open sources of thermal radiation;
- The patient or the thermovision camera must no be exposed to direct sunlight or radiation from powerful artificial lights;
- Instruct the subject to refrain from smoking for at least 4 hr before thermographic examination.
- No anti-perspiration fluants are allowed

In order to guarantee maximum effect and reliability of the test and to reach a state of adaptation of thermoregulatory mechanisms the patient has to wait in a room of stable temperature comfort for approximately 10 minutes while taking off thick or tight clothes and trying to relax.

To measure temperature accurately, it is therefore necessary to compensate for the effects of different radiation sources - the emissivity of the object, the reflected apparent temperature, the distance between the object and the camera, the relative humidity, temperature of the atmosphere. This is done on-line automatically by the camera.

Thermal images were produced by an infrared – Flir A310, which detects temperature differences as low as 0.06 C, and analyzed using an imaging system- ThermaCAM Researcher Professional and Therma CAM Reporter 8.

Simultaneous photographs are taken by infrared camera a digital photo camera of the face, right and left profiles, neck, abdomen, back - static thermovision. The entire body is observed for about 5 minute. Should here be doubt for focal source additional photos are taken.
Then the data is processed by a computer and the function Fusion image applied. It is used for achieving greater specificity of the area with detected temperature discrepancy and a gradient exceeding 0,4 C ◦ is considered to be statistically significant and indicative for the presence of a pathological process.
In a table form the program Therma CAM Reporter 8 creates a protocol on the diagnostics.
Later on the X-ray findings are compared to the Gelen test. An opinion is issued on the present active field/s of disturbance due to MFA \maxillo facial area\. Thermal findings from other organs and systems are described and if necessary the patient is transferred to an expert – e.g. consultation with an otolaryngology specialist.
Regarding the active fields of disturbance in MFA there is a treatment plan prepared.

A Case

Hemodialysis patient. The patient goes to a prophylactic dental examination without any complaints. Following the above described tests it became evident that there was hyper thermal, asymmetric field in the area of upper left molars. Following clinical examinations findings probed that there were obturations at positions 26, 27, 28. Then sector X-ray graph and EOD were applied, as well as the electrical-skin test of Gelen. In this particular case there was no correlation between the test data and the results from the test of Gelen and the infrared camera. The EOD test of the three teeth proved as follows - 12µA, 70µA ,18µA. The values of tooth 27 indicate necrosis. Following endodentic treatment confirmed the diagnosis of asymptomatic pulp necrosis.
Infrared diagnostics can be applied with certain priority in the field of vascular diseases and control over the functioning of vascular fistulas in patients under dialysis Fig. 9.

Fig. 9. Thermovision of head, arms and body.X-ray of 26,27, 28.

2.8 Individual inquire

Sex/ m/ f age ……

date …….

Self assessment of dental health

- Very good
- Good
- Satisfactorily
- Bad
- Very bad

Education

- Basic
- Technical/Specialized
- Higher

Position towards dental health

- Dental visit only by pain and/or visible changes in maxillo-facial area
- 2 times a year
- 4 times a year
- Compliance with dental treatment plan
- Before transplantation

Did any medical person explain toYou the relationship between damaged tooth structures and common diseases?

- Yes
- No

It is better to visit:

- Private dental clinic
- A defined structur in hospitle
- Speciliced center for dental treatment of medical compromized patients

What kind of manipulations do you become in the dental office more often.

- Tartar cleaning
- Dental obturation
- Tooth extraction
- Treatment of pulpitis and periodontitis

Do you know the dental activities included from National health insurance fund (NHIF)

- Yes
- No

If you be clear about, do you mean that they are enough

- Yes
- No

What is your recommendation for enlarge of dental manipulations financed by NHIF

- Profesional dental cleaning of calculus and debrs
- More often dental examination
- Anything more (please describe)

What kind of resuorces for oral hygiene do you use

- Tooth- brush
- Tooth paste

- Oral douche
- Mouthwash
- Dental floss
- Intertooth brush
- Cheving gum
- Bonbons without sugar

How many times do you wash your teeth

- Once
- Twice
- 3 times daily
- Do not wash every day

The data from the presented individual inquiry are comparable to the objective findings of the clinical dental examination and besides give to us a broader view on the information of these patents regarding the importance of dental health. We also seek their opinion on a better organizing of dental aid.

Based upon the research of (Dencheva, 2010) 170 patients separated in three groups: hemodialysis (n=59), with renal transplants (n=51) and health controls (n=60) we defined the attitude of patients towards their dental health fig.10. It is an alarming fact the hemodialysis and renal transplant groups indicate the highest percentage of patients asking for dental help only after feeling pain or suffering from obvious changes in the facial-maxillary area

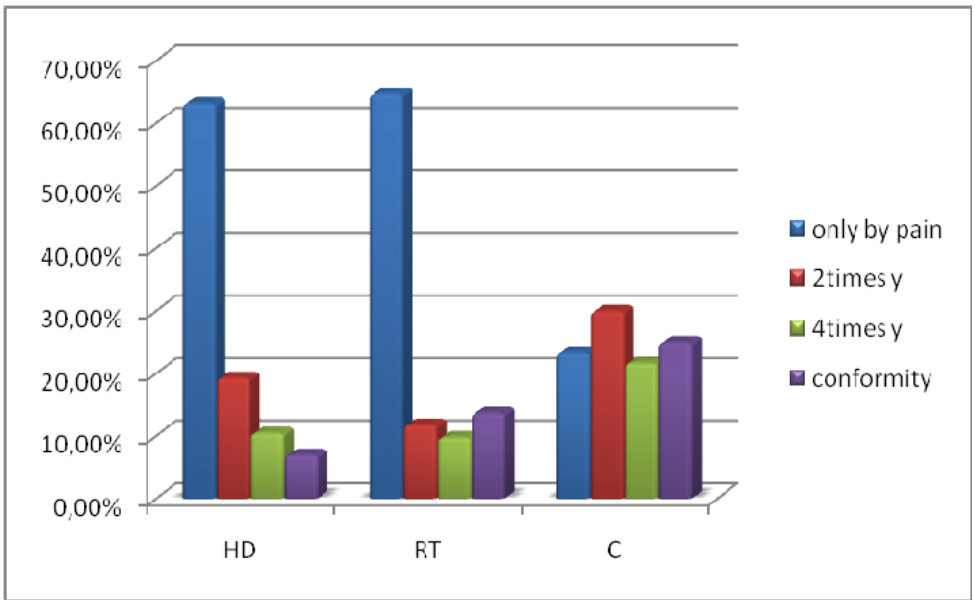

Fig. 10. Position towards dental health.

The active cooperation with all organizations of patients engaged with the problems of patients with renal transplants and those expecting other organ transplants makes us positive that the achievements of our team is of paramount social importance.

2.9 Register

For the purpose a data base was created using the Access 2007 software. The created data base contains information about the patient's medical history, dental status, periodontal status, x-ray images, photos, saliva tests, preclinical examinations, dental treatment. The created data base assures an easy registration of all personnel data; permits comparison of dental status before and after dental focal infection treatment; gives possibilities to provide statistical analyzes.

The necessity for systematic accumulation together with facilitated access and processing of information for the diagnostics and treatment measures motivates the development of an information system for the needs of this scientific project.

The structure has been designed and furnished in such a way that allows the easiest possible access to the date with regard to a single patient facilitating extrication of information. Initiation platform with command keys has been developed in order to provide direct access to information on the results of separate tested indications as well as for the completed dental focal sanitation (diagnostic and treatment) of all patients in total and separately according to groups suffering from a specific disorder.

The program is capable of filing records for activities completed that can be easily selected from the main menu. The files apply for all patients as well as for each separate group as per their specific disease. It is a flexible program allowing the formation of additional requests and reports according to the needed information in concern.

The information can be easily formatted into SPSS or MS Excel, which enables us to statistically process the data and graphically display them.

The establishment of a database for patients under hemodialysis and renal transplants makes the program applicable for all other patients suffering from system diseases subject to focal dental sanation – diagnostics and treatment.

Following the final completion of the project we expect that the system database shall also become a register of the patients undergoing dialysis and renal transplants, who have been treated by means of focal dental sanitation before a forthcoming transplantation or are included in a prophylactic dental program. The information will undoubtedly be useful not only for dental specialists but also experts in the field of dialysis and experts within the field of transplantation medical science.

3. Conclusion

The methodology offered can be successfully applied to patients suffering from another organic chronic disease and a forthcoming transplantation operation e.g. heart valve prosthesis, liver, marrow and cardiac transplantations. Its completion determines formulating of an optimum plan for dental treatment of patients with strict succession of eliminating of active fields of disturbance of MFA Fig. 11.

So far it is a part of the current project „Optimization of the complex focal dental sanitation in patients on hemodialysis and renal transplanted." This diagnostics methodology has been applied to 83 patients divided into two basic groups-group – hemodialysis and renal transplanted. For each of them there was a preliminary prepared focal dental schedule of treatment. 55 patients have successfully finished treatment procedures and are now a part of prophylactic dental programs for tracking a post-treatment phase\tracking. There is also a interim statistic phase of processing of the accumulated data.

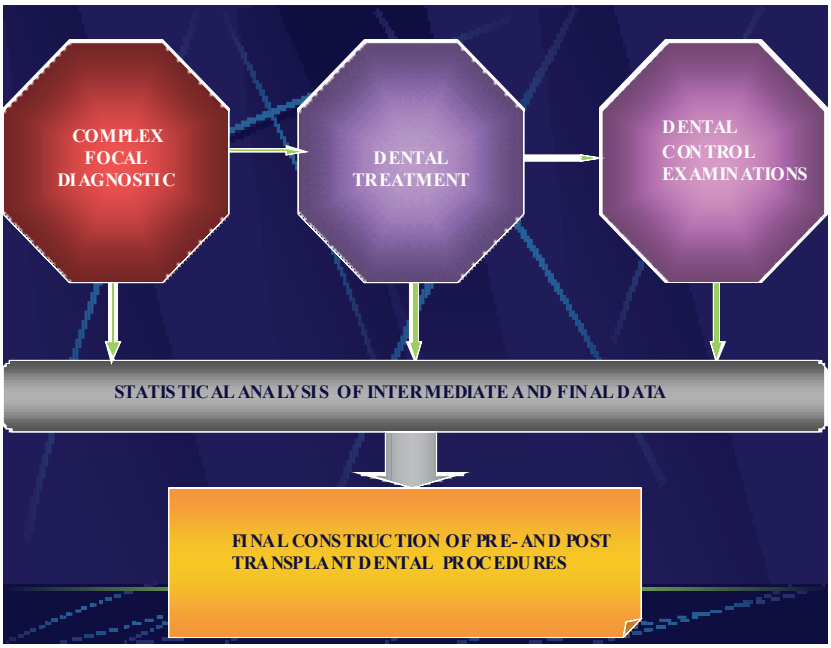

Fig. 11. The functional center of the specialized sector of dental focal diagnostics and treatment.

4. Acknowledgment

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13

Renal Aspects and Enzyme Replacement Therapy of Fabry Disease

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1. Introduction

Fabry disease (FD) is a lysosomal storage disorder caused by the deficient α-galactosidase A (α-gal A) activity. Fabry nephropathy typically progresses throughout the fifth decade to end-stage renal disease (ESRD) requiring hemodialysis and/or kidney transplantation. Except for ESRD development, a milder phenotype "renal variant" type is characterized with low plasma α-gal A activity. This survey improved the interest for FD screening among ESRD patients. After the description of this "renal variant" of FD, many studies were designed for ESRD patients.

The prevalence of 0.24 – 0.36 % found in Brazil express the importance of Fabry disease investigation among ESRD patients without known cause. Routine screening of male hemodialysis patients would enable earlier identification of other family members who might benefit from specific clinical treatment. The analysis of other epidemiological characteristics of regular FD could be used for the screening and detection of other kindred who might benefit from specific therapy as well as their offspring.

2. General aspects

Like most rare diseases, Fabry disease has long been a mystery disease, typically afflicting men of all ages and ethnic groups. With a poorly understood etiology and a disease affecting all major organ systems, little treatment other than symptomatic management has been available until recently. A patient may see many different specialists over a period of 5 to 10 years before an accurate diagnosis takes place.

Beginning in childhood, common symptoms include chronic or intermittent numbness; burning, tingling pain that can occur daily, usually in the fingers and feet; episodic pain that is incapacitating and may be brought on by stress, exercise, or temperature changes; recurring fever with elevated erythrocyte sedimentation rate; angiokeratomas that may appear in adolescence and increase as an adult; opacity of the corneal lens; inability to perspire; severe abdominal pain; and an intolerance to temperature (heat or cold) and exercise. The condition then progresses in adulthood to include renal, cardiovascular, cerebrovascular, and pulmonary complications that may lead to end-stage renal disease, stroke, myocardial infarction, breathing problems and obstructions, and more.
In this review, we intend to explain the major aspects of Fabry disease and comment some points relatives to the characteristic of progressive renal failure in these patients.

3. Epidemiological data

Fabry disease is a rare inborn error with a recessive X-linkage inherited pattern (Desnick *et al*., 2001). The estimated FD incidence is between 1:40,000 and 1:117,000 in general population (Desnick *et al.,* 2003; Rolfs *et al.,* 2005). Previous reports about the prevalence of end stage FD males on dialysis was estimated between 0.22% and 1.2% in several populations (Nakao *et al*., 2003; Linthorst *et al*., 2003; Kotamko *et al*., 2004; Thadhani *et al*., 2002; Grünfeld *et al*., 2003; Mehta *et al*., 2009).
Three similar studies explain the prevalence of FD among end-stage renal disease (ESRD) males in Brazilian population (Delgado *et al*., 2007; Biagini *et al*., 2007; Porsch *et al*., 2008). All studies screened about 30-40% of total patients who were submitted to dialysis treatment in three different Brazilian States: Rio de Janeiro (RJ), Paraná (PR) and Rio Grande do Sul (RS). Both first and second studies (RJ and PR) observed the same FD prevalence (0.24%) and the third study (RS) observed a comparable value (0.36%). FD prevalence in all studies was small compared with the results obtained by Nakao and colleagues (1.2%) (Nakao *et al*., 2003) and here it is important to note that many others studies never showed the same prevalence among hemodialysis patients. In fact, even other study in Japanese population found a small prevalence (0.22%) of FD among male dialysis patients (Ichinose *et al*., 2005). In addition, according to the nationwide screening for FD among dialysis patients performed in Austria (Kotamko *et al*., 2004), FD prevalence was really low (0.161%). In Latin American others studies also described lower FD prevalence, akin to Peru (0.3%) (Tumialán *et al*., 2005) and Colombia (0.4%) (Martínez *et al*., 2005). These results support the idea that true FD prevalence is in average less than 0.5% among ESRD male and the overestimation in some studies may be related to small populations groups and/or a selection bias of the patients screened. Further studies are required in different ethnical groups with greater samples, especially among ESRD males with unknown cause for chronic renal failure.

4. Genetic and biochemical outlines

The enzymatic defect in FD results from the deficient activity of the α-galactosidase A (α-gal A), a lysosomal hydrolase encoded by a gene (*GLA*) localized to Xq22. The *GLA* gene is 12 kb long and consists of 7 exons encoding 429 amino acids including a 31-amino acid signal peptide. The mature form of α-gal A is a homodimeric glycoprotein with molecular weight of ~46kDa synthesized from that point on cleavage of the signal peptides with ~50kDa (Bernstein *et al*., 1989).
The primary substrate of this enzyme is globotriaosylceramide (galactosyl$_{\alpha 1}$ → 4galactosyl$_{\beta 1}$ → 4glucosyl$_{\beta 1}$ → 1'ceramide), and the failure of α-gal A activity increase the deposition of

glycosphingolipids with terminal α-linked galatocsyl moieties (Desnick *et al.*, 2003). In FD this leads to progressive intracellular accumulation of glycosphingolipids, mainly in the form of globotriaosylceramide (Gb-3), in many cells, particularly in renal epithelial cells, endothelial cells, pericytes, vascular smooth muscle cells, cardiomyocytes, and neurons of the autonomic nervous system (Desnick *et al.*, 2001). The Figure resume the cleavage events in Gb-3 to Gb-2 transition.

The genetic defect occurs in all cell types, but involvement differs greatly among different organs and cell types. This heterogeneity likely reflects different rates of sphingolipid metabolism. Thus the minimum threshold requirement for α-gal A activity to prevent Gb-3 accumulation varies across cell types due to the type and amount of substrates that are recycled by the different cells (Alroy *et al.*, 2002).

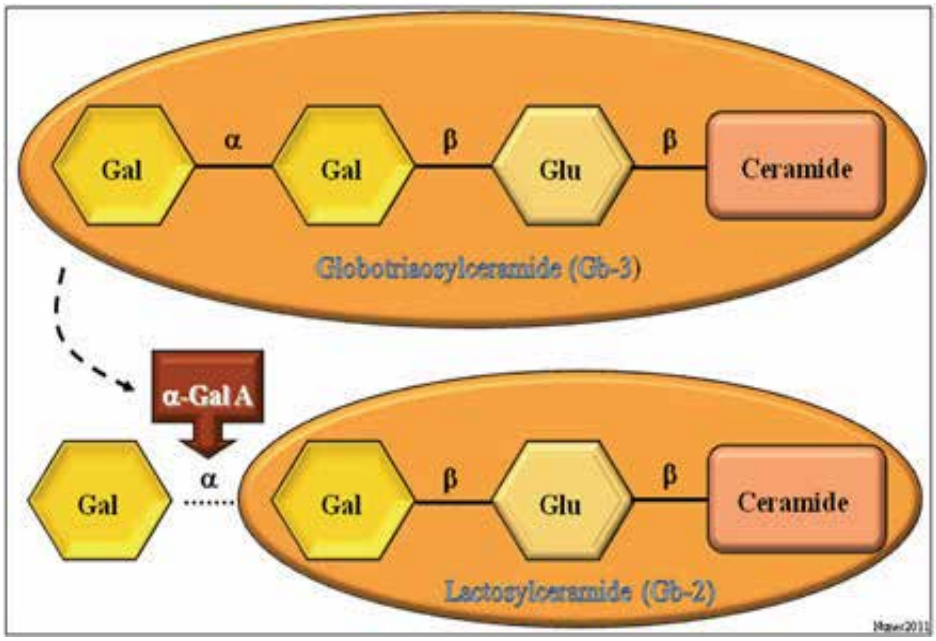

Fig. 1. Schematic representation of α-gal A action in Gb-3 to Gb-2 cleavage into lysosomal.

Renal lesions are found in both hemizygous (male) and heterozygous (female) patients. Renal symptoms in the latter are typically milder and delayed by 2 to 3 decades, but there is considerable variability (Gubler *et al.*, 1978). Although the disease primarily affects men, the genetic mechanism responsible allows the defect to be passed on by women. However, as women were presumed to be protected to the effects of the disease, this disorder has not been studied as comprehensively in them. The variability is likely the result of the random nature of X inactivation, resulting in considerable variability in α-gal A activity among carriers and within one carrier individual among various tissues or regions of a single tissue.

5. Clinical diagnosis markers

Clinical onset of the disease typically occurs during childhood or adolescence with recurrent episodes of severe pain in the extremities, characteristic cutaneous lesions know as angiokeratomas and a distinctive but asymptomatic corneal dystrophy (Clarke *et al.*, 1971).

Proteinuria and chronic renal disease occur with increasing age. Severe renal impairment leads to hypertension and uremia. Without dialysis, transplantation or enzyme replacement therapy (ERT), progressive renal failure is the main cause of death in the 4th decade of life in most hemizygous males with FD. However, a number of variants with residual α-gal A activity with late-onset manifestations primarily limited to the heart or kidney have been described (Desnick *et al*., 2001; Nakao *et al*., 1995; Meroni *et al*., 1997).

The 'classical phenotype' includes the pain and paresthesias in extremities, diffused angiokeratoma and hypohidrosis during childhood or adolescence, and also corneal opacities and renal failure (Clarke *et al*., 1971; Desnick *et al*., 2004). Fabry nephropathy typically progresses throughout the fifth decade of life to ESRD requiring hemodialysis and/or kidney transplantation. In view of this fact, hemodialysis patients represent an important target group for FD screening (Desnick *et al*., 2003; Nakao *et al*., 1995). Death usually occurs due to renal failure, cardiac or cerebrovascular disease. In addition, milder variants with residual α-gal A activity have been described (Desnick *et al*., 2004; Nakao *et al*., 1995). The cardiac and renal variants present with either late-onset manifestations primarily limited to the heart or kidney (Desnick *et al*., 2001; Meroni *et al*., 1997).

Since the description of the 'renal variant' (Nakao *et al*., 2003), a milder FD phenotype with either late-onset manifestations primarily limited to the kidney, important dialysis screening efforts of ESRD populations have been carried out (Nakao *et al*., 2003; Linthorst *et al*., 2003; Torra *et al*., 2003; Kotamko *et al*., 2004; Thadhani *et al*., 2002) and seemed to be worthwhile since kidney failure is an important outcome in FD.

While in an epidemiological point of view FD occurrence is low, on the other hand the FD diagnosis is very important for detection of family members. In view of this fact, dialysis patients represent an important target group for FD screening because they permit to identify FD patients and therefore others carriers among your family members. Each screened confirmed patient could allow early diagnosis of others related subjects, who can get treatment before or in the earlier symptoms manifestations. In these terms, FD screening among ESRD patients consists of an important tool for detection of FD patients and it could be followed by FD screening between family members of the index case. Both pedigree and population screening studies have been described and it can be carried out in subpopulations thought to be at higher risk of disease than the general population (Warnock *et al*., 2005).

6. Guess of renal damage

There are some references that the renal Gb-3 content, renal pathology and renal function correlate with residual α-gal A activity in leukocytes (Branton *et al*., 2002). If renal α-gal A activity correlates with leukocyte α-gal A activity (a reasonable but untested assumption), this suggests that residual enzyme activity in renal parenchymal cells retards progression of renal disease. One case report has suggested that renal α-gal A activity was reduced compared with liver α-gal A activity when each was expressed as a fraction of normal α-gal A activity in that organ; the mechanism for such a finding is unclear (Kano *et al*., 1974). Similarly, were found that Fabry patients with conservative missense mutations have delayed appearance of renal disease compared with patients with nonconservative missense mutations or others mutations those resulting in deletions, insertions, or premature stop codons (Branton *et al*., 2002).

According Alroy and colleagues (Alroy *et al.*, 2002) three mechanisms might explain the segmental and global glomerulosclerosis that characterizes Fabry disease: microvascular disease, podoctyte injury, and tubulointerstitial injury. Gubler and colleagues (Gubler *et al.*, 1978) observed that in older Fabry patients, those 25 to 50 year old, the progressive renal pathologic changes in the glomeruli and tubulointerstitium may be related to ischemic change. These changes include glomerulosclerosis, often with wrinkled and partially collapsed glomerular basement membrane, tubular atrophy, interstitial fibrosis, and vascular thickening. These changes were generally absent or mild in patients under 25 year of age. In particular, these investigators noted that the earliest and most consistent degenerative alteration was arterial "fibrinoid" deposits and suggested that these were due to necrosis of smooth muscle cells fatally overloaded with Gb-3 deposits.

Hypertension is not a common feature of Fabry disease, although it may occur with progressive renal dysfunction (Branton *et al.*, 2002). Therefore, according Gubler and colleagues (Gubler *et al.*, 1978), one mechanism of renal injury in Fabry disease is accumulation of Gb-3 within the arterial vessel wall and subsequent vascular compromise. In this regard, the renal vasculature is similar to the coronary and cerebral vessels, in which large vessel deposition of Gb-3 is associated with premature vascular disease that is responsible for premature death in many patients.

Toxic accumulation of Gb-3 within the podocyte may constitute a second important mechanism of glomerular injury. Podocytes are highly differentiated cells; their foot processes and slit-diaphragms constitute a critical portion of the glomerular filtration barrier that retards the entry large molecules into the urinary space. These cells are post mitotic and fail to undergo proliferation under most pathologic circumstances (an exception being the collapsing variant of focal segmental glomerulosclerosis), which means that they generally are not replaced when they are lost due to lethal injury. Kriz and Lemley (Kriz & Lemlev, 1999) have proposed that when podocytes are lost, the denuded glomerular basement contacts the parietal epithelial cells and forms a synechia. Within the synechia, there is activation and proliferation of cells, especially mesangial cells, the entry of immune cells, including macrophages, and the accumulation extracellular matrix protein. This repair response may be driven in part by the leakage from the circulation into the synechia of macromolecules, including cytokines, chemokines, and growth factors, via the impaired glomerular filtration barrier. Matrix expansion and subsequent collapse of the capillary loop appears as the focus of solidification. This constitutes the lesion of segmental glomerulosclerosis, which progresses to global glomerulosclerosis.

The Gb-3 also induces podocyte injury, resulting in focal and ultimately global glomerulosclerosis. Deposition of Gb-3 within tubular epithelial cells may lead to focal tubular atrophy and interstitial fibrosis. As this process progresses, the glomeruli upstream of more severely affected tubules may function poorly or not at all. Other glomeruli may undergo hypertrophy to compensate, and hyperfiltration in these glomeruli may trigger a secondary form of focal segmental glomerulosclerosis. Evidence in support of this mechanism would include demonstration of glomerular enlargement, particularly in the early stages of glomerular and tubular injury in Fabry disease.

Alternatively, Eng and colleagues (Eng *et al.*, 2001a) proposed the change in microvascular inclusions in interstitial endothelial cells of kidney, heart, and skin as a primary endpoint in their recent trial of α-gal A replacement. But the importance of this parameter as a potential substitute marker for the progression of renal dysfunction (impaired GFR) and other renal pathology (glomerulosclerosis, interstitial fibrosis) is uncertain and needs to be tested in

longitudinal studies. Some additional pathologic markers, as mesangial expansion, glomerulosclerosis, and interstitial fibrosis were included in another trial of α-gal A replacement (Schiffmann *et al.*, 2000), although these pathologic markers are also untested as appropriate markers for progressive renal functional decline in Fabry disease.

7. Urinary concentration and proteinuria changes

Urinary concentration defects may be the earliest functional manifestation of Fabry renal disease, leading to polyuria and nocturia. However, nephrology referral is more typically initiated by the development of proteinuria. Proteinuria may begin in the teenage years and becomes more frequent when patients reach their 20s and 30s.

In the NIH series (Branton *et al.*, 2002), 33 of 34 patients who had urine protein electrophoresis were found to have glomerular proteinuria, although the proteinuria did not usually reach nephrotic levels. Indeed, 23% of patients progressed to chronic renal insufficiency (CRI) without ever having nephrotic proteinúria and 50% of patients developed CRI by 43 year of age. The full presentation of nephrotic syndrome was not frequent even in those patients who developed nephrotic-range proteinuria. Only 26% of patients with nephrotic-range proteinuria developed hypoalbuminemia, and 21% developed hyperlipidemia. The onset of CRI may begin as early as the second decade of life. The mean age of onset of clinical nephropathy (CRI or proteinuria) has been reported as 27 year (Donati *et al.*, 1987).

End-stage renal disease was the most common cause of death in Fabry patients before the development of dialysis and renal transplantation and ESRD may rarely occur during the teenage years (Branton *et al.*, 2002).

Progression from onset of CRI to ESRD occur in mean of 4 ± 3 yr (range, 1 to 13 yr) and was not affected by patient age at onset of CRI or magnitude of proteinuria (Branton *et al.*, 2002). Besides that, patients with undetectable residual α-gal A activity had higher scores for glomerular pathology ($P = 0.027$) and tubulointerstitial pathology ($P = 0.007$), and they also had higher concentrations of Gb-3 in kidney tissue ($P = 0.039$) (Branton *et al.*, 2002).

8. Improve in treatment in recent years

Fabry patients with proteinuria or CRI should have aggressive treatment of hypertension is present and should probably be treated preferentially with angiotensin antagonist therapy; the latter recommendation is based on theoretical considerations, as definitely proof of efficacy has not been obtained yet.

Two different recombination α-gal A preparations are in use for treating Fabry disease (Schiffmann *et al.*, 2001a; Eng *et al.*, 2001a). One enzyme is produced by Chinese hamster ovary (CHO) cells with classic recombinant technology (agalsidase β, Fabrazyme – Genzyme Corporation), and the other enzyme is produced by cultured human skin fibroblast with an activated promoter of the α-gal A gene (Agalsidase α, Replagal – Shire Human Genetics Therapies). Both recombinant enzymes are quite comparable in properties and differ only alightly in glycan composition (Blom *et al.*, 2003). The two enzyme preparations have independently been examined in clinical investigations and are both registered in Europe for treating Fabry patients. Although both enzyme therapies were found to result in the desired Gb-3 from endothelium, the clinical effects are not robust as anticipated. In some

patients, stabilization of renal function and improvenment in cardiac hypertrophy occurs upon therapy, but a considerable number experiences progressive complications (Vedder *et al.*, 2007)

The NIH carried out a double-blind, randomized, placebo-controlled study of recombinant α-gal A produced in a human cell line (Replagal; Shire Human Therapies) and administered by biweekly infusion to 26 male Fabry patients for 6 months (Schiffmann *et al.*, 2001a). The primary endpoint of this study was a reduction in neuropathic pain. Significant reductions in the severity of "pain at its worst" and in the use of chronic pain medications were demonstrated. Enzyme replacement therapy was associated with relatively few side effects, chiefly mild transient infusion reactions, which became uncommon when the infusion duration was increased (from 20 to 40 min).

In the same study of Schifmann and colleagues, histologic assessment of renal biopsy samples showed that enzyme therapy was associated with more normal glomeruli ($P < 0.01$) and fewer glomeruli exhibiting mesangial matrix widening ($P < 0.01$), as well a slight increase in segmentally sclerotic glomeruli ($P < 0.05$) and no change in the number of globally sclerotic glomeruli. There were relatively few globally sclerotic glomeruli; therefore, the overall effect was an improvement in glomerular architecture. There was no change in the composite scores for tubulointerstitial damage or glycolipid deposits (the latter was assessed on toluidine blue– stained sections as the sum of scores in podocytes, glomerular endothelial/mesangial cells, proximal tubular epithelial cells, distal tubular epithelial cells, extraglomerular vascular endothelial cells, and vascular medial cells). Interestingly, there was however a significant decrease in glycolipid deposits in vascular endothelial cells ($P < 0.002$). Enzyme replacement therapy was associated with a significant fall in Gb-3 concentrations in plasma and urine sediment, but the findings in kidney tissue biopsies were not significant (NS) (Schiffmann *et al.*, 2001a). Six months of enzyme therapy had no consistent effect on proteinuria. With respect to the effect of enzyme replacement therapy on renal function, there was a trend toward a greater fall in GFR, measured by inulin clearance, with placebo compared with enzyme therapy ($P = 0.27$) (Schiffmann *et al.*, 2001a). Thus, this results was encouraging but insufficient to demonstrate a definitive benefit of ERT with α-gal A on kidney function in the group studied during a small period of treatment.

In another study, Thofhern and colleagues (Thofhern *et al.*, 2009), available 9 patients (7 male, 2 female) during the period between January 1, 2002, and August 1, 2005. They were treated according to protocol, receiving 0.2 mg/kg agalsidase alfa IV every two weeks. Over the course of 36 months of ERT, there was no change in kidney function and 24-hour proteinuria. This suggests that agalsidase alfa may slow or halt the progression of kidney disease when used before extensive kidney damage occurs. No significant side effects were observed with ERT during the course of this study.

Recent results from an open-label extension of enzyme replacement in patients enrolled in the original trial of NIH showed that patients in the initial treatment group continued to have stable or improved renal function measured by inulin and creatinine clearance after 8 months of enzyme therapy. In contrast, patients originally in the placebo group who had shown a decline in renal function demonstrated significantly improved inulin clearance and creatinine clearance after 12 months of enzyme replacement therapy (Schiffmann *et al.*, 2001b). Improved intracardiac conduction as evidenced by significant reduction of QRS complex duration in electrocardiogram (ECG) after 6 months of enzyme treatment was also noted. These new results with enzyme replacement are encouraging and results of continued therapy will be interesting to follow.

Eng and colleagues (Eng *et al.*, 2001b) evaluated the efficacy of recombinant human a-gal A produced in Chinese hamster ovary cell line (Fabrazyme; Genzyme, Boston, MA) in Fabry patients. In an open-label, doseranging study involving 15 patients, these authors found that biweekly infusions given for 10 weeks was associated with reduced kidney Gb-3 content (5 of the 15 patients underwent paired renal biopsies; no statistics given). On glutaraldehyde-fixed, methylene blue–stained kidney biopsy tissue, there was reduced storage material in interstitial capillary endothelial cells and mesangial cells. There was a lesser degree of improvement or no improvement in tubular epithelial cells and glomerular podocytes.

These results obtained by Eng and colleagues were used to design The International Fabry Disease Study, a double blind, randomized placebo controlled trial involving 58 male Fabry patients treated with biweekly intravenous infusions for 20 weeks, with the primary endpoint being clearance of interstitial capillary endothelial cell deposits (Eng *et al.*, 2001a). Complete clearance of interstitial capillary endothelial cell deposits occurred in 20 of 29 of α-gal A–treated patients and 0 of 29 placebo-treated patients ($P < 0.001$). Similar significant changes in endothelial cell deposits were seen in skin and heart capillaries. There was no beneficial effect on GFR assessed by inulin clearance after 20 weeks of ERT. There was a transient improvement in pain scores that did not persist to week 20, but pain results were no different than with placebo.

However, after 11 months of additional open label treatment with agalsidase beta, the renal effects of this ERT were more completely evaluated: clearance of Gb-3 from glomerular endothelial cells, clearance from smooth muscle cells, mesangial cells and cortical interstitial cells, and some clearance from podocytes (Thurberg *et al.*, 2002). These results showing a clear renal benefit with the ERT was followed by an extension for an additional 54 months of treatment with agalsidase beta: complete clearance from endotelial and other cells was maintained and renal function remained stable in all but six patients, in wich progressive disease was associated with significant baseline proteinuria and more glomerular sclerosis (Germain *et al.*, 2007). After that, agalsidase beta (Fabrazyme) was approved for marketing in Europe in 2001, and in the United States in 2003 with the main indication for the reduction in the accumulation of Gb-3 deposits in the capillary endothelium of the kidney. Agalsidase alfa (Replagal) is approved for marketing in Europe and many other countries. Both was recently approved for use in Brazil.

Data from the phase IV trial with agalsidase beta in 82 adults with Fabry disease and renal dysfunction at baseline (creatinine concentration between 1.2 and 3.0 or estimated creatinine clearance less than 80 mL/min), randomly assigned in a two to one treatment to placebo ratio during 35 months, showed a trend to better clinical effect with treatment, although no statistically significant (Banikazemi *et al.*, 2007). Importantly, a secondary analysis found benefits with therapy in those patients with renal function relatively well preserved (clearance ≥ 55mL/min/1.73m^2) for those with renal function less than 55mL/min/1.73m^2. In addition, an open label treating 58 patients with 1 mg/kg of agalsidase beta for 30 to 36 months demonstrated that baseline proteinuria less than 1 g and glomeruloesclerosis less than 50% showed better evolution and better renal prognostic (Wilcox *et al.*, 2004). All these data suggest that starting therapy before extensive renal damage has been identified might be beneficial for the prevention of progression to renal failure.

In summary, some placebo-controlled clinical trials have shown that 6 months of enzyme replacement therapy with α-gal A is associated with improved glomerular architecture and/or reduced glycolipid deposits in the kidney, and one study also suggested improvement in renal function. These trials have recently been reviewed (Pastores *et al.*,

2002). It makes sense that vascular endothelial cells are especially responsive to intravenously administered enzyme, in that these cells have direct access to the α-gal A present in the circulation. The majority of Gb-3 deposition in the kidney, however, does not appear to be within these cells. Indeed, Schiffmann and colleagues also demonstrated improved glomerular architecture, and results of follow-up studies of renal function are forthcoming (Schiffmann *et al.*, 2001a/b).

All the results of International Study of Fabry Disease with agalsidase beta, including double blind studies, open-label studies and extension studies has shown the best evidence of benefit on the renal function, with low rate of adverse events. Data of these studies demonstrated that 1mg/kg each other week of enzyme replacement are capable of stabilize the renal function mainly when the clearance is relatively well preserved (≥ 55 mL/min/1.73m^2), the glomeruli and possibly interstitial tissue is not extensive damaged and the proteinuria is less than 1 g per day.

Additional studies as to whether α-gal A therapy can prevent, slow, halt, or reverse declining renal function in patients with Fabry disease will likely be of 1 year or longer duration, possibly selecting only patients with renal insufficiency for inclusion to maximize the chance of showing a benefit in renal function. Nevertheless, at the present time enzyme replacement therapy holds considerable promise for patients with Fabry disease with and without kidney involvement.

9. Final considerations

As treatment for Fabry disease is now available the enzyme replacement therapy. This treatment, among other benefits, reduced Gb-3 depositions in renal tissue and could represent a better outcome for FD patients. However, this approach is important if applied before the onset of ESRD when the efficiency of treatment is more evidenced.

This fact also indicates the importance of FD diagnosis before the classical symptoms appear. A better awareness about FD among physicians and scientists should increase the number of patients that will be identified by different specialists and through population and family screening. Subsequently, FD diagnosis should be considered in every patient with unexplained renal disease, especially in patients with painful uncleared episodes, disseminate cutaneous lesions or when cardiac or cerebral complications suggest an underlying mutisystemic disorder.

The results presented here stresses the importance of Fabry disease reports since a single FD diagnosed patient by screening is a potential indicator for finding others Fabry carriers within his family. Because enzyme replacement therapy by recombinant α-gal A has emerged as a promising means to prevent and remove Gb-3 deposition, it is now necessary to make this diagnosis earlier.

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14

Chronic Inflammation and S100A12/ Receptor for Advanced Glycation Endproducts Axis: A Novel Risk Factor for Cardiovascular Disease in Patients with Chronic Kidney Disease?

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1. Introduction

Atherosclerotic cardiovascular disease (CVD) is a significant cause of morbidity and mortality in patients with chronic kidney disease (CKD), particularly patients undergoing hemodialysis (HD). The death rate from CVD in patients with advanced stage CKD is 10 to 40 times higher than that in the general population (Iliou et al., 2001; Sarnak et al., 2003). A majority of patients with CKD have traditional CVD risk factors such as old age, diabetes mellitus, systolic hypertension, smoking, and dyslipidemia. However, the interventions to address these traditional risk factors have failed to decrease the risk for CVD in patients with CKD (Stenvinkel et al., 2008).

Atherosclerotic lesions develop through chronic inflammatory processes, and chronic inflammation is a common feature of CKD. In addition, the metabolic milieu during the development of renal dysfunction appears to accelerate the atherosclerotic process by decades in patients with CKD. Given these findings, many researchers have emphasized that nontraditional risk factors such as oxidative stress and advanced glycation endproducts (AGEs), in combination with their receptor (RAGE), may play an important role in the development of CVD in patients with CKD.

RAGE is a member of the immunoglobulin superfamily of cell surface molecules. Enhanced RAGE expression has been observed in peripheral blood monocytes of patients with CKD, suggesting that RAGE may amplify ligand-induced monocyte perturbation and contribute to monocyte-mediated vascular inflammation in patients with CKD. The major alteration of glycated protein in patients with CKD is decreased clearance of glycation-free adducts and their markedly increased levels in plasma. However, the change of plasma AGEs into proteins in patients with CKD is relatively modest. Therefore, ligands other than AGEs for RAGE may be more important in RAGE-mediated atherosclerosis of patients with CKD.

Several RAGE ligands, including S100 proteins, the High Mobility Group Box proteins, and amyloid fibrils, have been identified. Among them, emerging evidence indicates that S100A12 is a proinflammatory cytokine (Pietzsch and Hoppmann, 2009). In this review, we

focus on chronic inflammation, which causes atherosclerosis in patients with CKD, and highlight the role of the S100A12 protein, which is upregulated in patients with CKD.

2. CKD: An inflammatory state

The inflammatory response is not only a local process but can also be reflected systemically as it is accompanied by increases in inflammatory markers including acute phase proteins, cytokines, and adhesion molecules. In recent years, some of these markers, such as C-reactive protein (CRP), interleukin (IL-6), serum amyloid type A, tumor necrosis factor (TNF)-α, adhesion molecule, and CD40 ligands, have been evaluated for their prognostic value in normal patients as well as in patients with CVD. Numerous studies have reported an association between renal impairment and different mediators and markers of inflammation including CRP, IL-6, and TNF-α, even among patients with moderate renal impairment, suggesting that CKD is a low-grade inflammatory process (Landray et al., 2004; Stenvinkel, 2006) and peripheral polymorphonuclear leukocytes and CD14+/Cd16+ monocytes are key mediators in the process (Merino et al., 2008; Sela et al., 2005). Approximately 30–50% of predialysis, HD, and peritoneal dialysis (PD) patients have serological evidence of an activated inflammatory response (Stenvinkel, 2001). Serum CRP levels are particularly high when renal function declines to the level of end stage of renal disease. Elevated serum CRP is a strong predictor of cardiovascular mortality in patients undergoing HD (Ma et al., 1992). Thus, it is possible that inflammation could promote both atherosclerosis and cardiovascular mortality. However, the precise mechanisms that contribute to the high prevalence of inflammation in patients with CKD are not well understood.

3. S100A12 protein

Human S100 proteins are the largest subgroup within the superfamily of EF-hand calcium-binding proteins. Currently, S100 proteins comprise a family of at least 25 low molecular weight proteins (9–14 kDa) (Santamaria-Kisiel et al., 2006). S100 proteins are characterized by the presence of two calcium-binding EF-hand motifs and display unique properties. It is thought that S100 proteins serve as a calcium trigger or sensor proteins that regulate the function and/or subcellular distribution of certain target proteins and peptides upon calcium-dependent activation. All S100 proteins, with the exception of S100G (calbindin D, 9 k), are organized as tight symmetric, antiparallel homodimers (some as heterodimers), and the noncovalent interface between the two monomers is formed mostly by hydrophobic amino acid residues. Each monomer is composed of a C-terminal, classic EF-hand, common to all EF-hand proteins, and an N-terminal, pseudo EF-hand, which has been found exclusively in the N-termini of S100 and S100-like proteins (Pietzsch and Hoppmann, 2009; Zhou et al., 2006).

In recent years, a subgroup of the S100 family (S100A12, S100A8, and S100A9) has been associated with acute/chronic inflammatory disorders (Foell et al., 2004a; Foell et al., 2004b). Human S100A12 was first described by Guignard and colleagues as a cytosolic protein, p6, in neutrophilic granulocytes and monocytes/macrophages that crossreacts with antibodies raised against S100A8 (Guignard et al., 1995). RAGE is of significant importance as a neutral target of S100A12 (Donato, 2007; Hofmann et al., 1999). Engagement of RAGE by S100A12 activates nuclear factor (NF)-κB, a central transcription factor involved in inflammatory

events that triggers the expression of multiple gene products contributing to the inflammatory response (Hofmann et al., 1999; Yang et al., 2001).

3.1 Gene and protein structure

The cytogenetic location of S100A12 is part of the tight S100 gene cluster on human chromosome 1q21. The S100A12 gene has been mapped to 1q21.2-1q22(1q21.3) and is located between the S100A8 and S100A9 genes (Ravasi et al., 2004). The S100A12 gene has a length of approximately 4.1 kbp and consists of three exons, which are divided by two introns of 900 and 400 bp. Exon 2 contains part of the 5′-untranslated region (UTR) and exon 3 contains the 3′-UTR. The mRNA size, exclusive of the polyadenylate stretch, is 466 Bp (Acc No. NM_005621).

The protein is encoded by sequences in exons 2 (138 nucleotides) and 3 (138 nucleotides), with the two EF hand motifs of the protein separately encoded by exons 2 and 3 (Acc Nos. X98289, X98290, D83657) (Wicki et al., 1996; Yamamura et al., 1996) and a 276-bp open reading frame encoding a 92-amino acid polypeptide with a predicted molecular mass of 10,575 Da. Homologous proteins have also been found in other species such as bovine, porcine, and rabbit. Human S100A12 has 70% sequence identity with both porcine and rabbit S100A12, and 66% sequence identity with the bovine protein (Dell'Angelica et al., 1994; Hitomi et al., 1996; Nonato et al., 1997; Yang et al., 1996). Human S100A12 shares 40% identity with human S100A8 (calgranulin A; myeloid-related protein 8), and 46% identity with human S100A9 (calgranulin B; myeloid-related protein 14), respectively. The S100A12 gene is not observed in rodents. The crystal structure of calcium-bound S100A12 closely resembles structures of other members of the calgranulin subfamily, presenting as an anti-parallel homodimer of four-helix subunits (Moroz et al., 2003). At low molecular calcium concentrations, human S100A12 also forms a hexamer consisting of three symmetrically positioned calcium-bound homodimers (Moroz et al., 2003). Most of the S100 proteins have been shown in various conformational and functional states depending on the intracellular or extracellular concentrations of calcium, zinc, and copper. In both EF-hands, the calcium ion is coordinated in a pentagonal bipyramidal configuration. Generally, the dimeric S100 proteins binds four calcium ions per dimer [~10^{-4} to 10^{-5} M (overall Kd)], with high affinity binding [~10^{-5} to 10^{-7} M (Kd)] at the C-terminal canonical EF-hand motifs and low affinity binding [~10^{-3} to 10^{-4} M (Kd)] at the N-terminal pseudo EF-hand motifs, respectively. An additional bound calcium ion per subunit occurs in the human S100A12 hexamer structure, in addition to the two calcium ions in the EF-hands. S100A12 also binds zinc ions at a binding site formed by both subunits that is closely located at the dimer interface. Of importance, divalent copper ions may bind at the same site. Coordination of both zinc and copper ions is supported by the N-terminal residues His15 and Asp25 from one subunit and two appropriately positioned imidazoles of a His-Tyr-His-Thr-His motif comprising residues 85–89 in the C-terminus from the other subunit (Moroz et al., 2003). These cations may regulate both intracellular and extracellular functions of S100A12. Studies on S100A12 monomers isolated from porcine granulocytes demonstrate substantial regulation of S100A12 calcium-binding affinity by zinc (Dell'Angelica et al., 1994). Upon copper binding, human S100A12 dimers form close contacts possibly enabling changes in their target binding sites or forming oligomers (Moroz et al., 2003). However, the particular role of S100A12 in zinc and copper binding in normal and disease states *in vivo* remains to be elucidated.

3.2 Expression, secretion and regulation

S100 lacks a signal peptide for secretion via the Golgi-mediated pathway and some debate exists regarding whether the high levels derived from inflammatory lesions are due to active secretion or passive release as a consequence of neutrophil necrosis. S100A8/9 release correlates with loss of neutrophil viability (Voganatsi et al., 2001), suggesting that necrosis probably represents a significant extracellular source of S100. In contrast, protein kinase C activation by pro-inflammatory stimuli, $[Ca2+]_I$ elevation by contact with activated endothelium (Frosch et al., 2000), and lipopolysaccharides from several bacterial species (Kido et al., 2005) cause a rapid release from neutrophils. S100A12 is constitutively expressed in neutrophils at low levels (~5% of cytosolic protein) (Guignard et al., 1995) and is expressed in myeloid cell lines (Vogl et al., 2004). We previously examined the amounts of S100A12 mRNA in cultured human THP-1 macrophages after treatment with various stimuli known to modulate atherosclerosis. First, IL-6, a proinflammatory cytokine, increased the level of S100A12 mRNA by ~2-fold in a time- and a dose-dependent fashion. The S100A12 protein was also detected in the culture medium and increased significantly after adding IL-6. Induction was abolished by pretreatment with a JAK kinase inhibitor and cycloheximide but not with an MEK kinase inhibitor. Second, pioglitazone, a thiazolidinedione, decreased the level of S100A12 mRNA by ~25% of basal in a time- and dose-dependent fashion. Pioglitazone also inhibited the induction of S100A12 mRNA by IL-6. These results indicate that S100A12 production is induced by IL-6 through de novo protein synthesis via the JAK-STAT kinase pathway and inhibited by activation of the peroxisome proliferator-activated receptor-γ (PPAR-γ) in human macrophages (Hasegawa et al., 2003). Ligand-bound PPAR-γ represses signal-dependent transcription of many inflammatory proteins (Pascual and Glass, 2006). The rapid upregulation by pro-inflammatory stimuli and repression by PPAR-γ agonists is consistent with the pro-inflammatory role of S100A12 (Goyette and Geczy, 2010). Nevertheless, further studies are needed to clarify the S100A12 regulatory mechanism.

3.3 Binding partners and function

Despite the large interest in the (patho-)physiological properties of human S100A12, knowledge of its intracellular targets is still limited. In bovine S100A12, annexin 5, aldolase, cytosolic NADP+-dependent isocitrate dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase show either a strict or weak calcium-dependent interaction with S100A12 (Hatakeyama et al., 2004).

The best known extracellular target protein is RAGE (Donato, 2007). RAGE is the first cell surface receptor that binds specifically to several members of the S100 protein family, including S100A12, S100A1, S100B, and S100P (Arumugam et al., 2004; Dattilo et al., 2007; Donato, 2007; Hofmann et al., 1999). The binding of S100 proteins, including calgranulins S100A6 and S100A18, is still debated (Goyette and Geczy, 2010). Engagement of the extracellular domain of the RAGE membrane by calcium-bound S100A12 activates intracellular signal cascades, including MAP kinase and NF-κB, which induce cytokine secretion (e.g., TNF-α and IL-1β), and expression of adhesion molecules (e.g., ICAM-1 and VCAM-1), and thereby mediate their pro-inflammatory effects on lymphocytes, endothelial cells, neutrophils, and mononuclear phagocytes (Yang et al., 2001), leading to the development of several chronic inflammation such as asthmatic lung (Yang et al., 2007), rheumatoid synovuim (Yang et al., 2001), inflamed mucosa in inflammatory bowel disease (Leach et al., 2007), diabetes mellitus (Kosaki et al., 2004), and atherosclerosis (Mori et al., 2009).

3.4 Association of plasma S100A12 level with atherosclerotic CVD in CKD

In 2009, we were the first to report that plasma S100A12 levels in 72 patients undergoing HD had mean levels 2.3-fold higher than those in control subjects. Furthermore, maximum carotid artery intima-media thickness correlated positively with plasma S100A12 levels in patients with HD, suggesting that plasma S100A12 levels are associated with atherosclerosis as a complication of CKD in patients undergoing HD (Mori et al., 2009). We also found that plasma S100A12 levels were elevated in patients undergoing PD (~2-fold), and in a high group of the peritoneal equilibrium test, suggesting the influence of chronic inflammation (Uchiyama-Tanaka et al., 2007). Consequently, a larger cross-sectional dataset of 550 patients undergoing HD at our affiliated hospital was analyzed to assess the relationship between plasma S100A12 levels and the presence of CVD (Shiotsu et al., 2011). We found that plasma S100A12 levels in patients undergoing HD with a history of CVD were significantly higher than those in patients with no history of CVD. Furthermore, plasma S100A12 level was identified as an independent factor associated with the prevalence of CVD, and higher plasma S100A12 levels were associated with an increased risk for CVD. These results suggest that plasma S100A12 protein may be a novel predictor of CVD events in patients undergoing HD. This finding was compatible with a report by another group (Nakashima et al., 2011) that conducted a prospective study including 184 patients undergoing prevalent HD. Plasma concentrations of S100A12 and sRAGE were studied in relation to risk profile and mortality after a median follow-up period of 41 months. The results showed that S100A12 and sRAGE levels were significantly elevated in patients undergoing HD compared with those in healthy controls. S100A12 had a strong positive correlation with CRP and IL-6, whereas sRAGE was negatively associated with CRP. S100A12, but not sRAGE, was independently and positively associated with clinical CVD. Further adjustment for inflammation made the predictive value of S100A12 disappear for all-cause mortality, but it still persisted for CVD-related mortality. In another population, the expression of RAGE and S100A12 in peripheral blood mononuclear cells (PBMCs) of subjects with pre-mature coronary artery disease (CAD) for the first time without CKD and diabetes were reported (Mahajan et al., 2009). Semi-quantitative RT-PCR was performed to determine RAGE and S100A12 transcriptional expression in PBMCs. Increased expression of RAGE and EN-RAGE in non-diabetic patients with pre-mature CAD was observed, suggesting a significant contribution of enhanced S100A12 expression in PBMCs to CAD pathophysiology.

4. Conclusion

Circulating S100A12 is elevated in patients with CKD and is associated with CVD events and CVD-related mortality, which is partly explained by its links to inflammation. Further studies are apparently needed from a therapeutic point of view. It is likely that a modification in uremic state affects S100A12 expression in neutrophils and/or monocytes. The identification of "activated" leucocytes and interventions by targeted therapy for such a leucocyte population may be beneficial to prevent CVD in patients with CKD.

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15

Choice of Renal Replacement Therapy and Role of Haemodialysis in the Intensive Care Unit

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1. Introduction

Renal replacement therapy (RRT) has become an established component of modern critical care. Approximately 60 - 70% of critically ill patients with severe acute kidney injury (AKI) are treated with RRT which represents ~5% of all intensive care unit (ICU) admissions (Ostermann & Chang, 2008; Uchino et al., 2005). Thirty years ago, intermittent haemodialysis for the critically ill patient with AKI was typically delivered three times weekly using dialysis machines without accurate volumetric control, acetate-based dialysate and unmodified cellulosic membrane dialysers. Peritoneal dialysis was performed with hard catheters and low dwell volumes. Since then, the types of RRT used in ICU and the available kits have advanced significantly.
Despite its worldwide use, the practice of RRT is variable. The main reasons are differences in expertise of nursing and medical staff, local availability of machines and lack of robust clinical data to support one technique over another (Pannu et al., 2008). Several important aspects related to the management of RRT, including mode, optimal indications and timing are the focus of ongoing discussion and opposing views.

2. Aims of RRT

AKI in critically ill patients manifests itself with varying degrees of uraemia, fluid accumulation, acid base disturbance and non-renal dysfunction. The clinical course can be very variable. RRT for AKI is predominantly supportive with the aim to maintain metabolic and volume haemostasis and to prevent uraemic complications and dysfunction of other organs during the critical illness until renal function recovers. It is important that these benefits of RRT are balanced by potential harm, including risks related to central venous access, infections and anticoagulation (Oudemans-van Straaten, 2007).

3. Practice of RRT

3.1 Indications for RRT

In the setting of chronic kidney disease, the European Best Practice Guidelines recommend starting chronic dialysis when a patient with an estimated glomerular filtration rate (GFR) of <15ml/min/1.73m^2 has symptoms or signs of uraemia, fluid overload or malnutrition in spite of medical therapy or before estimated GFR has fallen to

<6ml/min/1.73m² in an asymptomatic patient (European Best Practice Guidelines, 2005). The overall aim is to replace renal function. The situation is very different in the acute setting where RRT for AKI is predominantly viewed as organ support until kidney function recovers. Although acute RRT should be started before the onset of any potentially life threatening complications of uraemia, the optimal time and indications remain controversial. Potential benefits of early initiation are more rapid metabolic/uraemic control and more effective prevention and management of fluid overload (Gibney et al., 2008). Some data also suggest that RRT before the onset of severe AKI may attenuate kidney-specific and non-kidney organ injury from acidemia, uremia, fluid overload, and systemic inflammation, and potentially translate into improved survival and earlier recovery of kidney function (Clark et al., 2006; Matson et al., 2004). The obvious counterargument is that early RRT might subject patients who would recover renal function with conservative treatment alone, to the potential risks associated with RRT (Vinsonneau & Monchi, 2011). Delay of RRT until AKI is severe avoids treatment in patients with potentially recoverable AKI but increases the risk of uraemic complications and fluid overload.

3.1.1 Indicators for initiation of RRT

Studies exploring the optimal indication for starting RRT have used different parameters, including arbitrary cut-offs for serum creatinine, serum urea or urine output, fluid balance, time from ICU admission or time after onset of AKI. Several retrospective studies have shown an association between lower serum urea levels at time of RRT and better outcomes (Gettings et al., 1999; Liu et al., 2006; Shiao et al., 2009). Liu et al reported a lower mortality in 122 patients in a mixed ICU who had serum urea levels ≤27 mmol/L (≤76 mg/dL) on the day when RRT was started compared to 121 patients with higher urea values (Liu et al., 2006). When adjusted for age, hepatic failure, sepsis, thrombocytopenia and serum creatinine, the relative risk of death with a higher urea level at time of RRT was 1.85 (95% CI 1.16 – 2.96). Improved mortality was also reported in other retrospective studies when RRT was instituted with serum urea <21 mmol/L (Gettings et al., 1999) or <23 mmol/L (Shiao et al., 2009) compared to higher levels. The authors made the conclusion that "early RRT may be better than late RRT". However, 2 larger studies did not find any correlation between serum urea at time of RRT and outcome (Bagshaw et al., 2009; Ostermann & Chang, 2009). Instead, they showed that patients who had a serum creatinine < 309 µmol/L at time of RRT had a greater risk of dying compared to those with higher serum creatinine levels. Other studies found a better outcome in patients with a shorter time from ICU admission (Payen et al., 2008; Piccinni et al., 2006), a higher urine output at time of RRT (Sughara & Suzuki, 2004), and a shorter time between onset of AKI and RRT (Ostermann & Chang, 2009).

Despite the perception that early RRT may be better, the data are conflicting (Bagshaw et al., 2009; Bouman et al., 2002; Demirkilic et al., 2004; Elahi et al., 2004; Gettings et al., 1999; Liu et al, 2006; Ostermann & Chang, 2009; Payen et al., 2008; Piccinni et al., 2006; Shiao et al., 2009; Sughara et al., 2004). There are only 2 randomized controlled trials (RCTs) exploring the impact of timing of RRT (Bouman et al., 2002; Sughara & Suzuki, 2004). The majority of data stem from retrospective analyses with different biochemical cut-offs to distinguish between early and late treatment. Therefore, it cannot be excluded that patients in the "early" RRT group simply had less severe AKI and that patients in the "late" group received RRT too late, ie. after the onset of uraemic complications. Finally, the interpretation of non-

randomized studies is limited by the exclusion of patients who met criteria for early RRT but never received it.

In the largest prospective RCT investigating the role of timing, 106 predominantly cardiac surgical patients with AKI were randomized to three groups: early high-volume continuous venovenous haemofiltration (CVVHF) (n=35), early low-volume CVVHF (n=35) and late low-volume CVVHF (n=36) (Bouman et al., 2002). There were no significant differences in 28 day mortality or duration of AKI between the groups. All hospital survivors recovered renal function except for one patient in the early low volume CVVH group. Of interest, 4 patients in the late arm recovered renal function spontaneously and 2 patients died before the criteria for CVVH were met. The authors came to the conclusion that there was no significant benefit with either early CVVH or high filtration rates for patients with oliguric AKI. A large retrospective analysis of 1,847 ICU patients treated with RRT for AKI demonstrated that the most important independent risk factors for ICU mortality were mechanical ventilation, associated organ failure, pre-existing chronic health problems, acidosis, oliguria and age (Ostermann & Chang, 2009). There was no association between serum urea and creatinine at time of RRT and outcome. The paper concluded that the decision to start RRT should depend less on classic renal parameters but more on the clinical status of the patient, degree of acidosis, risk of fluid overload and associated organ failure.

The negative impact of fluid overload is increasingly being recognised (Bouchard et al., 2009; Payen et al., 2008). In a retrospective analysis of 398 ICU patients, Bouchard et al found a significantly higher ICU mortality in patients who had gained 1-20% or more in body weight between ICU admission and day of RRT compared to patients without any weight gain (Bouchard et al., 2009). Oliguria and fluid overload in the context of AKI should be viewed as triggers for RRT independent of actual serum urea and creatinine results.

In summary, based on the existing data in the literature, the decision when to start RRT should take into account the patient's severity of illness and its trends, degree of fluid overload and number and types of associated organ failure. Individual serum creatinine or urea parameters are not adequate in identifying the optimal time for RRT. Obviously, in patients with a futile prognosis RRT may not be appropriate. In this case, withholding RRT constitutes good end-of life care (Gabbay & Meyer, 2009).

3.2 Choice of RRT modality

Current RRT options for ICU patients with AKI are continuous modes [continuous haemofiltration (CVVHF), continuous haemodialysis (CVVHD) and continuous haemodiafiltration (CVVHDF)], intermittent haemodialysis (HD), hybrid techniques like slow extended haemodialysis (SLED) or prolonged intermittent renal replacement therapy (PIRRT) and acute peritoneal dialysis. Some characteristics of the different modalities are listed in Table 1.

Continuous RRT is often perceived to offer greater cardiovascular stability compared to traditional intermittent HD. However, intermittent techniques have evolved over the last 20 years. HD machines now have accurate fluid balance control modules with real-time indirect assessment of relative changes in blood volume which allow rapid fluid removal with less haemodynamic instability. A switch towards bicarbonate-based dialysate and less bioincompatible membranes, together with a recognition of the importance of higher dialysate sodium concentrations and cooled dialysate have also led to a reduced the risk of

intradialytic hypotension. Advantages of intermittent HD are the reduced need for anticoagulation, lower financial costs and greater flexibility which ultimately allows more time for investigations and therapies, including active rehabilitation. Small solutes such as potassium are removed more efficiently with HD which may be particularly beneficial in the setting of acute life threatening hyperkalaemia.

	CVVH	CVVHD	CVVHDF	Intermittent HD	SLED / PIRRT	PD
Solute transport	Convection	Diffusion	Convection + diffusion	Diffusion	Diffusion	Diffusion
Therapy time/day	24 hours	24 hours	variable	4 – 6 hours	6 – 12 hours	24 hours
Blood flow (ml/min)	100 - 250	100 - 250	100 - 250	200 - 350	100 - 300	none
Urea clearance (ml/min)	20- 40	25 – 45	25 – 45	150 – 180	90 – 140	15 - 35
Need for Anticoagulation	Yes	Yes	Yes	Not absolutely	Not absolutely	No

Table 1. Comparison between different modes of RRT (adapted from Mehta et al., 1993)

In addition to potentially better haemodynamic stability, continuous RRT offers the advantage of more sustained fluid removal and less fluctuations in fluid status and metabolic control. Obvious disadvantages are the need for continuous anticoagulation and less flexibility, including less time for the patient to participate in active rehabilitation. The rate of solute removal is lower than with intermittent HD. Over the last few years, there has been much debate whether haemofiltration offers additional benefits for patients with AKI and severe sepsis / septic shock beyond solute clearance (Peng Z et al., 2010). Proponents argue that RRT in the context of severe sepsis contributes to removal of soluble inflammatory mediators of sepsis and restores immune function through improved antigen-presenting capability and leukocyte responsiveness (Honoré et al., 2007). Although this concept of "blood purification" is conceptually appealing, the evidence is limited to data from animal models and small human studies (Bouman et al., 2007).
Although there are differences in capillary fibre structure and design, most ICUs use the same dialysers for CRRT, PIRRT and IHD. The resurgence of batch dialysis systems (for instance, Genius® , NxStage®) and the use of individual water purification systems (microfilters, water softeners, multiple pass reverse osmosis) coupled with endotoxin filters has made it possible to provide haemodialysis even without access to ultrapure water supply as in chronic haemodialysis units.

3.2.1 Continuous versus intermittent RRT

It is widely perceived that continuous modes may be better for critically ill patients with AKI. However, prospective randomised clinical trials have failed to confirm this. Based on 4 different systematic reviews, there is no convincing evidence that continuous RRT is superior to intermittent HD in terms of mortality or renal recovery (Bagshaw et al., 2008; Kellum et al., 2002; Rabindranath et al., 2007; Tonelli et al., 2002). Although these analyses included different studies of varying methodological quality and size, the authors came to

similar conclusions. The Hemodiafe Study, a multicenter RCT comparing intermittent HD versus CVVHDF in patients with AKI and multiple-organ dysfunction syndrome (MODS), showed that it is possible to treat almost all critically ill patients with AKI with intermittent HD, provided strict guidelines to improve tolerance and metabolic control are adhered to (Vinsonneau et al., 2006). In the intermittent HD group, haemodynamic stability was maintained by using a high dialysate sodium concentration and dialysate cooled to 35^0C. There was no difference in outcome between both groups.

Despite the lack of data that continuous RRT is superior to intermittent HD with regards to mortality or renal recovery, there is some evidence that fluid overload may be easier to control with continuous RRT compared to intermittent modalities. Retrospective analysis of data collected as part of the Program to improve Care in Acute Renal Disease (PICARD) study showed that patients treated with intermittent RRT continued to gain fluid in the subsequent days in contrast to patients on continuous RRT in whom the fluid balance was significantly better controlled (Bouchard et al., 2009). This observation makes intermittent HD less suitable for patients with major fluid overload and supports the recommendation to use continuous RRT for this patient group (Vanholder et al., 2011).

Comparative studies of continuous and hybrid techniques are limited (Abe et al., 2010; Kielstein et al., 2004; Kumar et al., 2000; Kumar et al., 2004; Marshall et al., 2004). In a small trial, 39 patients were randomised to either CVVHF or 12-hour SLED (Kielstein et al., 2004). Cardiovascular tolerability and urea clearances were equivalent. However, less heparin was needed and acidosis was corrected faster in patients treated with SLED. In another randomised trial in 60 patients, CVVHDF was compared to 6 – 8 hours of SLED (Abe et al., 2010). There was no difference in ICU or 30 day mortality but survivors in the SLED group had higher renal recovery rates and a shorter length of stay in ICU.

3.2.2 Peritoneal dialysis versus continuous RRT

Acute peritoneal dialysis in the ICU is possible but requires the insertion of a peritoneal dialysis catheter and relies on an intact peritoneum and adequate bowel movement. It is contraindicated in patients with abdominal pathology. Solute clearance and fluid removal are generally less predictable and controllable and may be reduced in patients with impaired mesenteric blood flow. Removal of large volumes of fluid may not be possible with small dwell volumes, especially in the first 2 weeks after insertion of the peritoneal catheter.

There are only limited data comparing peritoneal dialysis to continuous RRT in adults (Gangji et al., 2005; Swartz et al., 2005). In a Vietnamese study in patients with infection associated AKI, CVVHF was superior but the applied peritoneal dialysis strategies were different from current practice (Phu et al., 2002). Another study showed not difference but the clearance delivered by peritoneal dialysis was low (Gabriel et al., 2008).

In summary, analysis of the currently published studies shows no clear advantage of one modality over the other in terms of mortality or renal recovery. The decision regarding type of RRT should take into account the individual patient's clinical condition, medical and nursing expertise, and the availability of RRT machines. Regular education and training of those who provide RRT is essential with emphasis on the characteristics and values of each RRT technique. During the dynamic course of critical illness the type of RRT modality may have to change in response to the varying needs of the patient. Most clinicians choose continuous RRT for patients with cardiovascular compromise, significant fluid overload and multi-organ failure, and an intermittent mode for cardiovascularly stable patients

(Kanagasundaram, 2007; VA NIH Acute Renal Failure Trial Network et al., 2008) Although the risk of hypotension is increased during intermittent HD, the Hemodiafe study showed that the introduction of a relatively simple treatment algorithm including use of a high dialysate sodium concentration of 145 mmol/L in combination with cooled dialysate and stopping vasodilator infusions, markedly reduced the risk of intradialytic hypotension (Vinsonneau et al., 2006). Hybrid techniques like SLED / PIRRT offer the advantages of both CRRT and intermittent HD, including haemodynamic stability, increased patient mobility and reduced financial costs.

3.3 General care

Severe AKI results in hypercatabolism and altered drug metabolism. Provision of adequate nutrition and attention to drug dosing are essential when looking after patients on RRT. While no particular mode or filter is recommended, it is important to understand the characteristics of these different RRT techniques and potential differences in drug removal. Both, under-treatment and drug toxicity have been reported in patients on RRT.

4. Conclusions

RRT is a form of organ support for critically ill patients with AKI. The most commonly used modalities are continuous haemo(dia)filtration and intermittent modes. There is no evidence that one mode is superior to another in terms of mortality or renal recovery. Except for a few specific indications, each modality can be considered a valid RRT option for critically ill patients with AKI. The choice should depend on the patient's clinical condition, medical and nursing expertise and local availability of machines. Intermittent HD is an acceptable option for critically ill patients with AKI, including patients with MODS provided clear guidelines are adhered to in order to prevent haemodynamic instability. For patients with significant fluid overload, continuous RRT appears more appropriate.

It is widely acknowledged that the way RRT is delivered has an impact on quality and outcome of patients with severe AKI. The value of regular education and training of those who deliver RRT, and attention to concurrent interventions (drug dosing, nutrition, fluid management) cannot be emphasized enough. There is a clear role for each different modality in the management of AKI in ICU. RRT should be individualised depending on the patient's varying needs during the critical illness.

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16

Dry Weight and Measurements Methods

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1. Introduction

1.1 Why do we need dry weight for our patients?

The need for a concept of dry weight derives from an awareness of the dangers of being overhydrated or as better expressed, being fluid overloaded. These dangers in the hemodialysis patient are reflected by strain on the heart indicated by left and eventually right ventricular hypertrophy and dilatation, with gradual reduction in the efficiency of the heart. Eventually, heart failure occurs with increased hospitalization and mortality rates. More recently interest in attaining dry weight has been stimulated by awareness that an abnormally low fluid load is also harmful in that it might be associated with unacceptable degrees of low blood pressure and consequently of ischemia of vital organs such as the brain, gut and liver. A working definition of dry weight is required before further discussion. Charra modified earlier thoughts on this as follows: the post dialysis weight at which the patient is and remains normotensive until the next dialysis in spite of interdialytic fluid retention (without ant-hypertensive drugs) (Charra, 2007; Charra *et al*., 1996). This weight might be compared to the usual range of weights in a person with normal kidney function whose consumption of water in food or as liquids is balanced by loss of fluids through the skin lungs, gut and urine. However, there is always a range of fluid volumes within a liter or two around the true dry weight in patients with clinical dry weight assessment (Jaeger and Mehta, 1999).

Other definitions in dialysis patients have included the weight at which hypotension and symptoms such as muscle cramps, nausea and vomiting occurs (Agarwal and Weir, 2010; Leypoldt *et al*., 2002). Clinical judgment of dry weight is often based on an educated guess since the one to three liters fluid overload characteristic of many dialysis patients cannot be detected by current routine physical examination (Sinha *et al*., 2010; Zucchelli and Santoro, 2001). A more sensitive physical sign, which requires training and practice but is not widely taught, is the measurement of internal jugular vein pressure. This clinical sign faithfully represents right atrial pressure which is often increased with fluid overload, The equilibrium blood and interstitial fluid volumes is dependent on the differences between the interstitial and blood oncotic pressures, with accumulation of edema fluid, and increase of compliance (largely due to the normal gel structure being dissipated) As a result large volumes of fluid can accumulate with little increase in hydrostatic pressure. The effect of ultrafiltration (UF) is dependent on the degree of fluid overload in that the blood volume will decrease far more when the patient is close to dry weight (Merouani *et al. 2011*). Further, the blood volume change for the same volume of ultrafiltration is highly dependent on the ultrafiltration rate.

1.2 Blood pressure and hydration

The relationship of blood pressure level, which is a physical sign comparatively easy to measure with accuracy, to over hydration, is frequently discussed in the care of the dialysis patient. From work performed by many authors (Ok and Mees, 2010) (Charra and Chazot, 2003a), reduction in dietary sodium and in sodium loading during dialysis is associated with decrease of blood pressure to close to normotensive levels. While volume increase is associated with predominantly systolic pressure, diastolic pressure is also increased. It is not rare in older patients for the pulse pressure to be increased with low diastolic pressures associated with poorly compliant major blood vessels. This increased rigidity of the aorta and other large blood vessels occurs due to fibrosis with calcification possibly playing a major role. One confounding influence on the blood pressure and fluid overload relationship is a degree of cardiac damage over years so severe that blood pressure might be normal or even below normal (Charra and Chazot, 2003b). Reduction in fluid overload can actually increase blood pressure in such patients. Echocardiography is very useful in distinguishing patients with large failing hearts from those with normal blood pressure following adequate treatment with antihypertensive drugs or salt restriction (Santos and Peixoto, 2010).

1.3 Sodium and fluid overload

Sodium, in the form of salt, plays a major role in the fluid and electrolyte problems of the dialysis patient, to a large extent because the regulation of salt is almost completely absent once the kidneys are not functioning. The dialysis patient is at risk to the same extent as a healthy patient to increased dietary intake of sodium but in addition sodium is often added during the actual dialysis treatment (Matsuoka *et al.*, 1990; Schmieder, 1997). In health the balance of sodium is determined predominantly by renal regulation of tubular re-absorption as influenced directly or indirectly by plasma and extracellular volume. These functions are not present in the dialysis patients, and therefore expansion is obviously accompanied by relative inability to excrete water. Blood pressure increase occurs in most patients as the result of sodium and fluid retention and is considered to be a prime factor in the pathogenesis of the cardiac disease which is the major cause of hospitalization and mortality in dialysis patients, Left ventricular hypertrophy leads to ventricular dilatation and left-sided cardiac failure with pulmonary edema, and pulmonary hypertension. The right ventricle then also hypertrophies and fails with this being the basis for the congestive heart failure seen so often in dialysis patients (Fiaccadori *et al.2011*). Peripheral edema is obvious, especially in the legs but congestion of visceral organs may be of greater pathognomonic significance. An example is the gut where increased permeability to luminal endotoxin could promote the inflammation characteristic of most dialysis patients. While anti hypertensive drugs are successful in reducing blood pressure, control of salt and water may be more effective. Normal or low blood pressure in the absence of such treatment can be misleading since this can be due to the result of progressive cardiomyopathy and represents a poor prognosis. Recent research suggests that declines in blood pressure occurring in the absence of treatment are associated with poor outcomes (Silver *et al.*, 2008).

1.4 Sodium control

Control of salt intake becomes one of the most relevant aspects of the dialysis treatment; current recommendations are for less than six grams of salt to be ingested daily. Patient education concerning sodium content of individual items has variable effects but is often

swamped by the easy availability of fast foods. However, notable examples exist of the success of this approach particularly in Tassin in France (Charra *et al*., 2004) where the combination of compliance with a low salt diet, and relatively longer dialysis time has been associated over decades with well controlled blood pressure levels and low mortality (Chazot and Jean, 2008). Asking patients to reduce their fluid intake voluntarily is rarely successful because of the powerful effect of thirst and appeals to limit salt are far more likely to be successful.

Extra sodium may be administered in three ways during dialysis. The first is the use of dialysate sodium concentrations higher than the patient's level (Odudu *et al.2011*). As much as 3 to 5 g of sodium may be administered during a dialysis (Tetta *et al.2011*). This attribute of the dialysis treatment has been through a number of cycles. In the early days of dialysis sodium concentrations were low commensurate with long dialysis times and therefore low ultrafiltration rates (Silverstein *et al*., 1974). As dialysis times were reduced, dialysate sodium concentrations increased but currently are decreasing again with more awareness of the effects of excess sodium. One potentially more precise solution to obtain balance has been the alignment of the dialysate and serum sodium levels which is approximately possible because of the opposite effects of the Gibbs-Donnan equilibrium and the fact that the plasma water concentration is about 7% higher than that of plasma sodium. The second is during priming of the dialysis circuit when the volume of saline administered is variable, depending on the degree of care, but is often 100 to 500 ml per treatment. Adding this volume to the ultrafiltration requirements deals with the problem in part but cannot be relied on to occur due to homodynamic instability. In addition the sodium content of the ultrafiltrate is lower than that of isotonic saline. The third is the use of saline for the management of hypotensive episodes when reductions in ultrafiltration rate are unsuccessful (Santos and Peixoto, 2010). Since the quantities given can be substantial, replacing saline with hypertonic glucose is an acceptable replacement, perhaps with reservations in some diabetic patients.

2. Fluid volume measurements

2.1 Body fluid compartments

Total body water (TBW) can be divided into two compartments: extracellular fluid volume (ECV) and intracellular fluid volume (ICV). Further, the extracellular compartment is divided into interstitial fluid and blood plasma volume (Fig.1).

Accuracy of measurement of extracellular fluid volume (ECV) is an important issue in research and clinical practice. Currently, the standard reference for body fluid measurements are dilution method using deuterated water (D_2O) for total body water and NaBr for ECV measurements (Riek and Gerken, Pierson *et al*., 1978; Kim *et al*., 1999). However, with dilution method patients have to wait about 3 hours in order to obtain equilibrium of the diluting substances in the body. In addition, the accuracy of this method has been questioned for example, NaBr might overestimate the ECV(Thomas *et al*., 1991).

Bioimpedance analysis (BIA) techniques have been used as noninvasive and simple methods to measure body fluid volume. The principle of BIA methodology is based on the physical principle that fluid volume is negative related to electrical resistance, and therefore, ECV and ICV can be calculated as

$$ECV=\rho_{ECV}*L^2/R_E \quad (1)$$

$$ICV = \rho_{ICV} * L^2 / R_I \quad (2)$$

where R_E and R_I are the extracellular and intracellular resistances calculated with the Cole model using multifrequency spectroscopy (BIS), L is the length of the measuring segment and ρ_{ECV} and ρ_{ICV} are resistivity in extracellular and intracellular fluid considered as constant. TBW can be calculated by sum of ECV and ICV. Current accuracy of estimation of ECV is about 1.0-1.5 L by advanced bioimpedance method (Moissl *et al.*, 2006; Zhu *et al.*, 2006a; Ellis, 2000). Main factors affecting accuracy of BIA or BIS to estimate fluid volume includes 1) error from measurement and 2) error of calculation from inaccurate assumptions. For example, the current standard method is the wrist to ankle (so called "whole body") bioimpedance measurement (De Lorenzo *et al.*, 1997). This method assumes that arm, trunk and leg can be modeled as a cylinder with uniform conductivity when electrical current is injected from hand to foot and voltage is measured between wrist and ankle. Since the different cross sectional areas between trunk and limbs, limbs' resistance (the arm and leg) contribute about 90 % of whole body resistance but the limbs' volume is less than 30 % of total body volume (Zhu *et al.*, 1998a; Zhu *et al.*, 1998b; Bracco *et al.*, 1996). This inhomogeneous distribution between resistance and volume in segments leads to different degrees of error which are associated with variation in body composition and in degrees of body hydration (Thomas *et al.*, 1998). As a result, estimation of fluid volume with whole body bioimpedance methods does not provide accurate information (Cox-Reijven *et al.*, 2001; Earthman *et al.*, 2007).

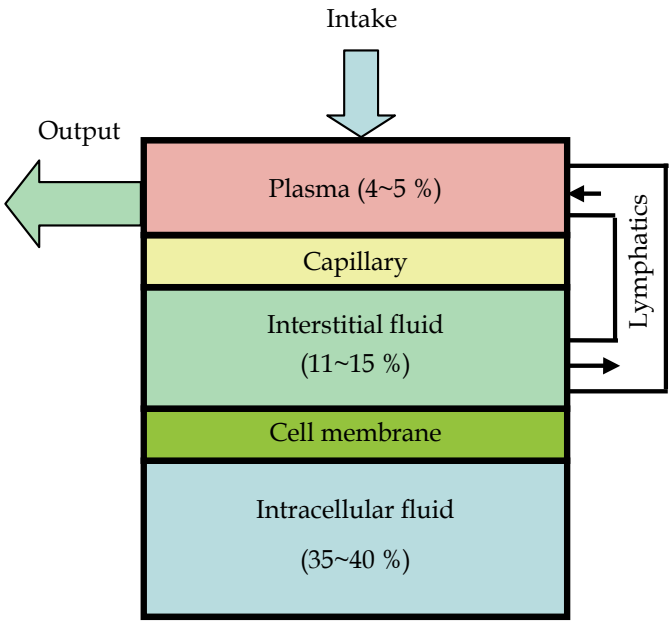

Fig. 1. Body fluid compartments, extracellular fluid volume (ECV) consist of plasma and interstitial fluid volume.

As an example, the problem of inhomogeneous distribution in fluid volume and cross-sectional area of body segments can be observed from an experiment of change in body position (Zhu *et al.*, 1998a).

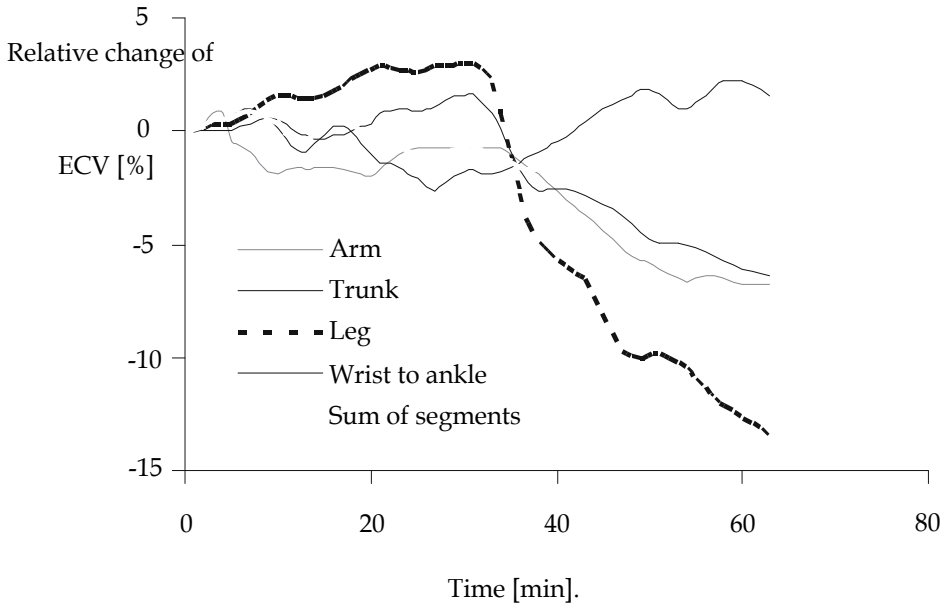

Fig. 2. Change in segmental ECV during change in body position (Modified from Zhu et, al., J Appl Physol 85:497-504, 1998)

Fig.2 shows segmental ECVs changes during 30 minutes standing and then immediately after 30 minutes supine. As shown in Fig.2, the ECV in the leg increased and the trunk decreased about 3 % during the standing position due to the fluid volume accumulating in the leg by gravitational force. After assuming the supine position, ECV shifted back from the leg to the trunk. Although total ECV in the body did not change during the position changes, difference in ECV measured by wrist to ankle increased about 2 % in standing and decreased more than 6 % after being supine. This example clearly demonstrates that change in distribution of fluid volume can make a disproportional change in resistance in the segments because of their different geometric sizes, and therefore, ECV calculated from the sum of the resistances cannot be correct. It has to be noticed that change in fluid redistribution can be made not only by change in body position but also by change in fluid overload (Zhu *et al.*, 2008b).

2.2 Measurement of fluid overload

Fluid overload can be generally defined as the ECV accumulated in the body of greater than a normal degree. Although the measurement of the normal range of ECV (normal hydration, NH) is difficult; degrees of fluid overload estimated by clinical practice have widely been used in clinical practice. Excess fluid volume can be roughly estimated through clinical sign such as hypertension and by a physician's assessment such as increased jugular vein pressure. Accurate assessment of fluid overload or quantitative calculation of excess fluid volume is a challenge due to: 1) lack of a reliable techniques to measure ECV and 2) the normal range of ECV is unknown because of the variability of excess fluid in individuals not only derived from differences in intake of salt but also by variability due to the age or gender in the healthy population (Silva *et al.*, 2008). Body fluid status is a dynamic equilibrium or steady state

condition controlled by physiologic functions. But in dialysis patients this function is lost. From engineering point of view, estimation of fluid status in normal level (dry weight) can be generally divided into two aspects of methods: static and dynamic approaches.

3. Static methods for estimating dry weight

The principle of the static method is based on the degree of fluid overload can be assessed by comparison of a parameter using healthy subjects as reference.

3.1 Bioimpedance vector analysis (RXc graph)

Resistance (R) and reactance (Xc) graph shows relationship of change in impedance vector at 50 kHz in R-Xc plane to estimate degree of hydration or nutrition with the range of tolerance ellipses from healthy subjects (Fig 3) (Piccoli, 2005). This method was suggested by Piccoli et al to identify dry weight in hemodialysis patients according to 50th, 75th and 95th % of vector in healthy population (Piccoli, 1998). The advantage of this method is that the state of tissue hydration and nutrition can be reflected by the length and angle of a vector which consist of resistance and reactance at 50 kHz in R-Xc plan. Since 50 kHz current is applied, the impedance can be affected by electrical properties in both the extracellular and parts of the intracellular fluid compartments.

The major disadvantage with this method is that the state of hydration cannot be separately obtained due to the vector being determined by resistance and reactance. Therefore, this method is useful to generally compare the status of hydration or nutrition in populations but it cannot provide quantitative information about degree of fluid overload.

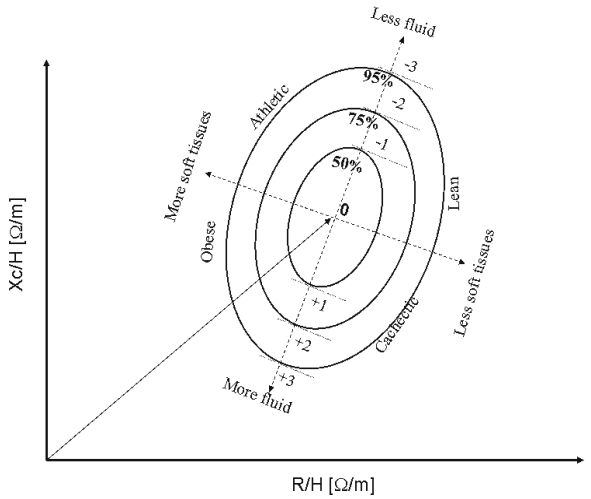

Fig. 3. Principle of phase ankle method (Modified from Piccoli, Contrib Nephrol. Basel, Karger, 2005, vol 149: pp 150–161)

3.2 The ratios of fluid volume

Ratios or percentage of ECV to body mass or one of components, for example ECV/TBW, ECV/ICV or ECV/body mass (BM) should be maintained in an approximately constant level controlled by normal physiologic function. Logically, these parameters should be able to

identify the body hydration state (Lopot *et al.*, 2002; Farnetti *et al.*, 2004; Park *et al.*, 2009; Koziolek *et al.*, 2006; Booth *et al 2011.*). However, in practice, there are two issues as mentioned above:1) accuracy of ECV measurement and 2) the normal range in healthy population limits its usefulness in clinical practice. The normal range has been reported as from 0.37 to 0.46 in ECV/TBW and from 0.22 to 0.24 in ECV/BM (Katzarski *et al.*, 1996; Woodrow *et al.*, 2005; Zhu *et al.*, 2011; Chen *et al.*, 2002). The problems of the methods using the ratio of resistance with different frequencies such as ratio of extracellular resistance to intracellular resistance (Re/Ri) or resistance at 5 kHz to resistance at 200 kHz (R5/R200) are similar problem to the volume ratios (Spiegel *et al.*, 2000; Zhou *et al.2010*). A comparison of the ECV/TBW or ECV/BM in HD patients with healthy subjects is showing in Fig 4 (a) and (b). In that study a bioimpedance spectrum analyzer system (Hydra 4200, Xitron Technologies, CA) was used to measure whole body BIS (wBIS) in NS and in (HD) patients to obtain ECV and ICV (Zhu *et al.*, 2011). There was no significant difference in wECV/TBW between BL and DW_{cBIS} in male patients although the patients had lost body fluid. The lack of sensitivity with this method limits the clinical application of wECV/TBW for individual patients (Fig. 4 (a)). The poor sensitivity of wECV/TBW can be explained that 1) since TBW is the sum of ECV and ICV, decrease in ECV produces the similar changes in the numerator and denominator of the ratio of ECV/TBW; 2) the accuracy of ICV measurements by wBIS is still not good enough for clinical use.

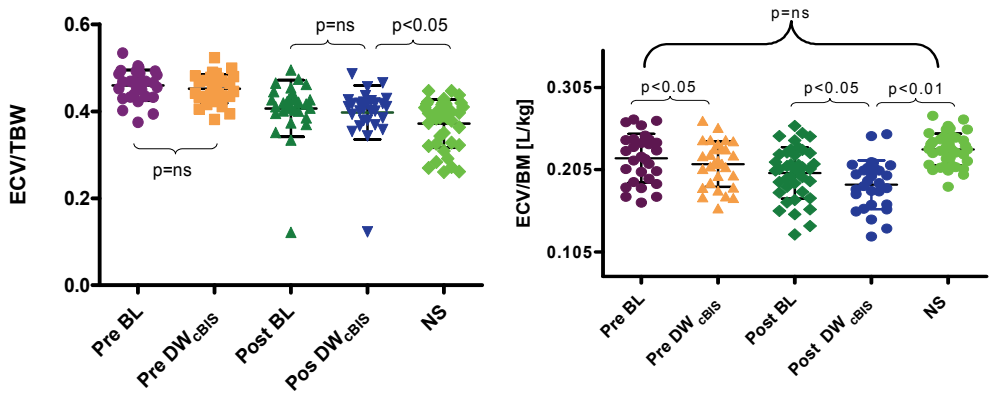

Fig. 4. Comparison of results in patient to NS with ECV/TBW (a) and with ECV/BM (b) (Modified from Zhu et al., Physiol Meas. 32:887-902, 2011)

In addition, accuracy of wrist to ankle (wBIS) measurements is affected by body composition and degree of hydration. (Sung *et al.*, 2001) (Bracco *et al.*, 1996) (Zhu *et al.*, 1998b). No difference in wECV/BM between NS and the patients at BL pre-HD might be explained that the ratios of ECV/BM depends on the body composition, such as more or less fat mass in the NS. (Fig.4 (b)).

3.3 Body composition model

Recently a whole body bioimpedance model (WBM) based on functions of body weight, ECV and ICV with wBIS has been developed to calculate degree of excess fluid volume (EFV) with the equation as following (Chamney *et al.*, 2007).

$$EFV = 1.136 \cdot ECV - 0.43 \cdot ICV - 0.114 \cdot BM \tag{3}$$

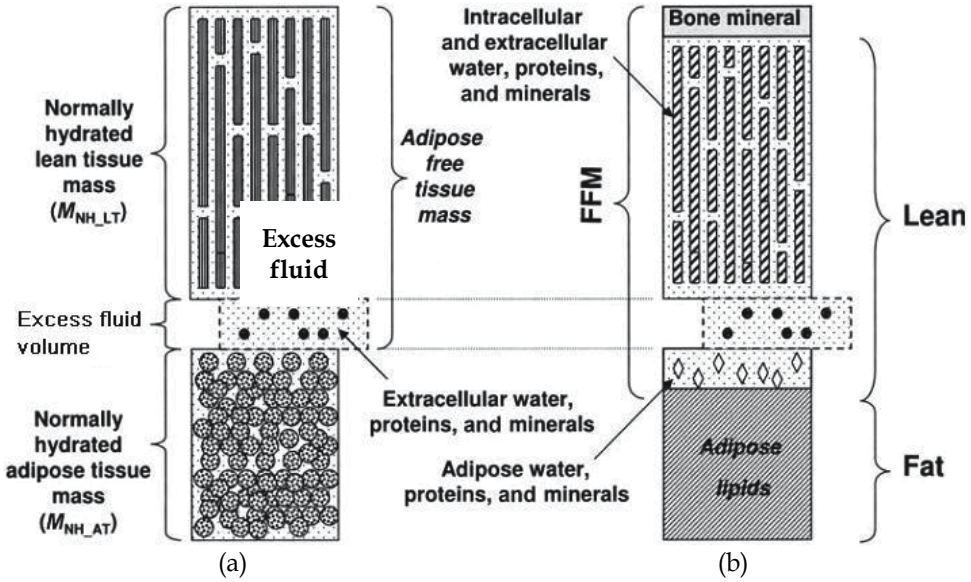

Fig. 5. Principle of whole body model (WBM) (Modified from Chamney et al *Am J Clin Nutr* 2007, 85:80 –9)

The weight at normal hydration (NHW_{WBM}) with whole body model can be calculated by the difference between pre HD BM and excess fluid volume (EFV).

$$NHW_{WBM} = \text{Pre-HD BM} - \text{EFV} \quad (4)$$

The principle of this method is based on the assumption that ECV is proportionally distributed in fat and other body components in healthy subjects (Fig.5). The main advantage with this method is that the model introduces relationships between fluid volume and fat mass which might reduce the error from variability of body composition. However, since this method is using whole body bioimpedance spectroscopy (wBIS) technique for collecting raw data, the problems with wBIS are inherent in the measurement so that accuracy of this method is reduced in subjects with great fluid overload.

3.4 Calf resistivity method

Calf bioimpedance measurement in dialysis patients was reported by Kouw et al to evaluate calf conductance between patients and NS (Kouw *et al.*, 1993). Recently, it was found that the value of calf conductance or resistivity (1/conductance) correlated with BMI (Zhu *et al, 2011*). To reduce the error, calf resistivity was normalized by BMI (resistivity/BMI) to reflect degree of body hydration. Generally, an assumption is correct that the calf is more hydrated than other segments of the body due to effect of gravity. Moreover, the calf has uniform structure of body composition so that it can reduce the error from bioimpedance measurement. Calf electrodes placement and measurements show in Fig.6.

Calf resistivity (ρ_5) was calculated from resistance (R_5) at 5 kHz and where *A* and *L* are the cross sectional areas of the calf and the distance (10 cm) between the sensing electrodes, respectively.

$$\rho_5 = R_5 \times A/L \ (\Omega \cdot cm) \quad (5)$$

The cross sectional area was calculated by Eq 6, where C_{ave} was the mean of the two measured calf circumferences ($C_{ave} = (C_{Max} + C_{Min})/2$).

$$A = C_{ave}^2/(4\pi) \quad (6)$$

To reduce the effect of differences in body composition, resistivity ρ_5 was normalized by the body mass index (BMI; calculated as BM [kg] divided by height^2 [m]^2), and reported as calf normalized resistivity ($\rho_{N,5}$) in units of $\Omega m^3/kg$ (Eq 7).

$$\rho_{N,5} = \rho_5/BMI. \quad (7)$$

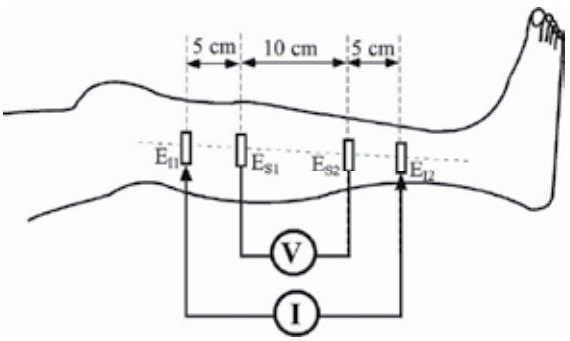

Fig. 6. Four electrodes are placed on the lateral side of calf to inject current (0.8 mA) and measure voltage. One sensing electrode (E_{S1}) is placed on the point of maximal calf circumference (C_{Max}); while the other sensing electrode (E_{S2}) was placed 10 cm below the E_{S1} which was defined at the point as minimal circumference (C_{Min})

Fig.7 shows that the standard deviations in normalized resistivity ($\rho_{N,5}$) was significantly less than the non-normalized values (ρ_5). The average of normalized resistivity in normal subjects (NS) was 20.5±1.99 *10^{-2} ($\Omega m^3/kg$) in males and 21.7±2.6 *10^{-2} ($\Omega m^3/kg$) in females respectively. It can be defined with the values of the mean minus the standard deviation as minimal levels of normal hydration males (18.5 *10^{-2} $\Omega m^3/kg$) and in females (19.1 10^{-2} $\Omega m^3/kg$) respectively (Fig.7).

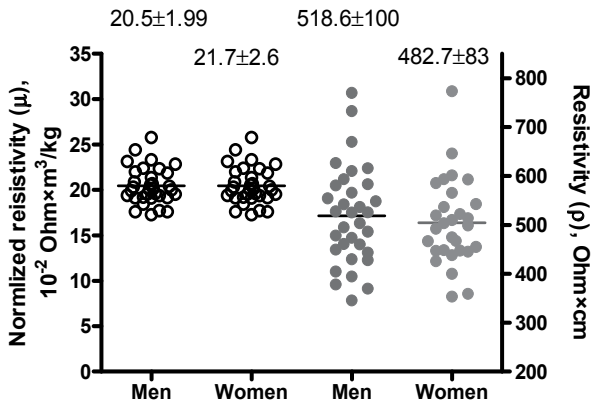

Fig. 7. Comparison of resistivity and normalized resistivity in NS

Normalized resistivity ($\rho_{N,5}$) in NS differed significantly between males and females, but there were no significant differences between the age groups (Fig. 8).

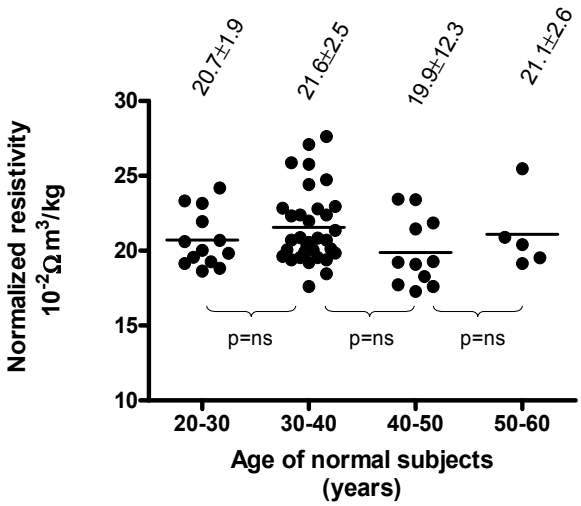

Fig. 8. Comparison of normalized resistivity in NS with different ages. (Modified from Zhu et al., Physiol Meas. 32:887-902, 2011)

Fig. 9 demonstrated the change in fluid volume in HD patients from BL with fluid overload to reaching the dry weight. There was no difference in normalized resistivity between NS and the patients who reached dry weight (DW).

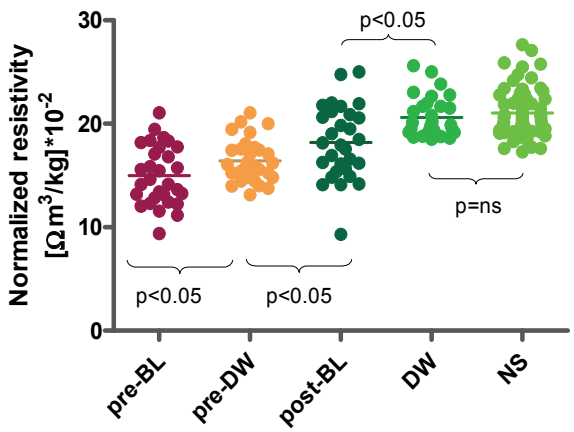

Fig. 9. Comparison of normalized resistivity between HD patients and NS. (Modified from Zhu et al., Physiol Meas. 32:887-902, 2011)

The unit of measurement of $\rho_{N,5}$ in $\Omega*[m^3/kg]$, can be interpreted also for its physical meaning. Since m^3/kg is equal to $1/kg/m^3$, which is the reciprocal of density, the parameter $\rho_{N,5}$ can be interpreted as ohm per density measured by bioimpedance, so that it does reflect the relationship of hydration to body density (Zhu *et al. 2011*).

3.5 Vena cava diameter index

Measurement of inferior vena cava diameter index (IVCD) has been used to indicate hydration by intravascular volume with ultrasound technique (Krause *et al.*, 2001). This is a noninvasive method for fluid volume in blood. However, it cannot indicate the state of interstitial fluid volume because the degree of hydration in intravascular space cannot proportionally reflect fluid overload in interstitial space (Katzarski *et al.*, 1997; Dietel *et al.*, 2000). In addition, variability of healthy subjects is so large that it cannot be applied in patients due to lack of precision of device (Bendjelid *et al.*, 2002; Agarwal *et al.2011*).

3.6 Biochemical parameters

Serum indicators Atrial Natriuretic Peptide (ANP), Brain Natriuretic Peptide (BNP), NT Pro BNP and Cyclic Guanidine Monophosphate (cGMP) have been suggested to indicate degrees of fluid volume expanded in dialysis patients (Ishibe and Peixoto, 2004; van de Pol *et al.*, 2007). The measurements might reflect the fluid status in the blood compartment but cannot be used to determine state of interstitial fluid because the relationship between plasma volume and interstitial fluid volume proportion is unknown for individual patient. As a result, the normal ranges of these parameters were reported with such large variability that they cannot be applied in clinical practice. Cardiac dysfunction and variable removal during dialysis are other problem for its application (Bargnoux *et al.*, 2008).

4. Dynamic methods

4.1 Two compartments model

Change in ECV during HD provides information about body hydration. Relative blood volume (RBV) has been used to monitor reduction of plasma in blood compartment during ultrafiltration (UF) (Leypoldt and Cheung, 1998; Chamney *et al.*, 1999). Since change in RBV reflects the state of vascular refilling, a number of authors have suggested that the curve of RBV either with the slope change or with maximum change in RBV value at the end of treatment might indicate degree of hydration based on change in the state of refilling rate (Sinha *et al.*, 2010; Agarwal *et al.*, 2008). Fig.10 shows the relationship between change in blood volume and interstitial volume (V_{IT}) by UF.

Relationship can be described with a simple two compartments model (Fig.10) by differential equations as following.

$$\frac{dBV}{dt} = k_{IB} \cdot V_{IT} - k_{BI} \cdot BV - UFR \tag{8}$$

$$\frac{dV_{IT}}{dt} = -k_{IB} \cdot V_{IT} + k_{BI} \cdot BV \tag{9}$$

where BV represents plasma volume in blood, k_{IB} and k_{BI} are transfer coefficients between two compartments; UFR is ultrafiltration rate which is the driving force in this dynamic system. From Eq.8 k_{IB} times V_{IT} represents refilling rate (RFR). Although we assume k_{IB} is constant which is determined by pressure gradient between two compartments in the same treatment, k_{IB} can be various in different hydration state. Since k_{BI} times BV is much less than k_{IB} times V_{IT}, the model can be considered as a four parameters system. Then, the equations can be rewritten as following

$$\frac{dBV}{dt} = k'_{IB} \cdot V_{IT} - UFR \tag{10}$$

$$\frac{dV_{IT}}{dt} = -k'_{IB} \cdot V_{IT} \tag{11}$$

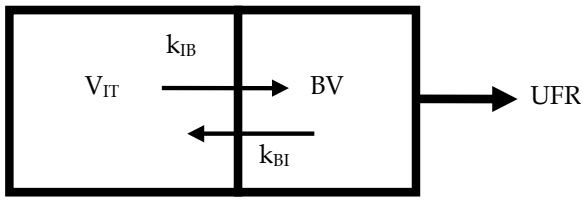

Fig. 10. Two compartment model: blood volume and interstitial fluid volume

4.2 Blood volume monitor

From Eq.10, the changes in RBV depend on the difference between UFR and refilling rate (RFR). RFR is determined by fluid status (V_{IT}) and pressure gradient (k_{IB}) which is associated with individual cardiac function and homodynamic parameters. In general, absolute or change in RBV have little relationship to body fluid status because RBV indicates the state of difference between UFR and refilling rate (Barth *et al.*, 2003). Even though constant UFR is used in the same patient, RFR is generally not associated with fluid status because the k_{IB} is affected by body hydration, fluid status and cardiac function in individual treatments. A sample shows the relationship between change in RBV and calf normalized resistivity during HD (Fig. 11).

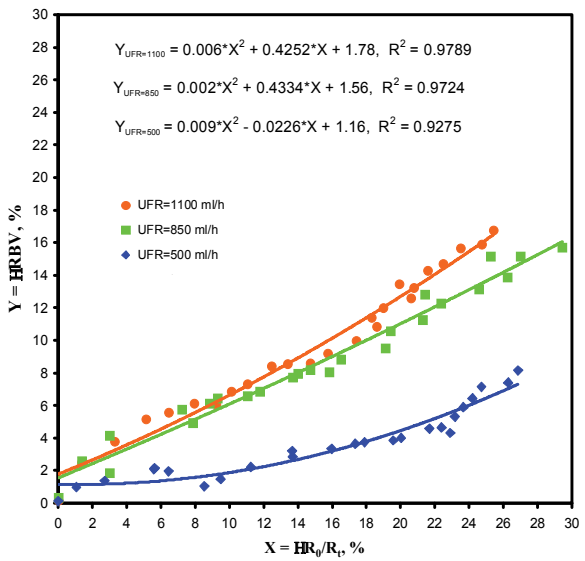

Fig. 11. Relationship between RBV and calf resistance ratio (R_{E0}/R_{ET}) with different UFR in a same patient (From *Sipahioglu etal.,* Blood Purif 2010;29:230–242.)

RBV method provides a simple way to monitor change in plasma volume which indicates change in RFR durring HD. Since the RBV shows a relative change, it cannot represent refilling rate from tissue so that it cannot reflect body hydration. Absolute refilling rate can only be calculated if we know the absolute blood volume or refilling volume. RBV can be useful to tell if UFR is correct in individual treatment. A recent study showed that neither in the slope nor maximal change of RBV was observed when patient reduction the post HD weight (Fig.12).

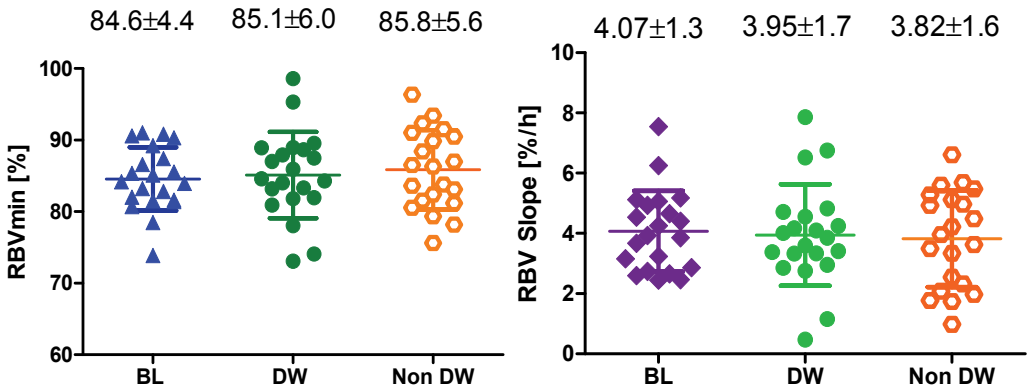

Fig. 12. RVB were compared between BL and dry weight in maximal change of RBV (RBVmin) and in RBV slope. No difference either in slope or in maximal change of RBV were obtained when body fluid reduced.

4.3 Continuous measurement of calf bioimpedance spectroscopy (cBIS)

Calf bioimpedance spectroscopy (cBIS) method provides a way to continuously measure calf ECV during HD. Relative change in ECV can be represented by R_{E0}/R_{Et} where R_{E0} and R_{Et} are extracellular resistance at beginning and at any time until the end of HD. Since the plasma volume only take about ten percent of ECV in the calf, change in R_{E0}/R_{ET} mainly indicate change in interstitial fluid volume. The ratio of R_{E0}/R_{Et} indicates the ratio of extracellular fluid volume in any time (ECVt) to the volume at the beginning (ECV0). The relationship can be described as following (Zhu *et al.*, 2004).

$$\frac{ECV_t}{ECV_0} = \frac{\rho_{ECV}\frac{L^2}{R_{Et}}}{\rho_{ECV}\frac{L^2}{R_{E0}}} = \frac{\frac{1}{R_{Et}}}{\frac{1}{R_{E0}}} = \frac{R_{E0}}{R_{Et}} \tag{12}$$

where ρ_{ECV} is the resistivity in extracellular fluid volume, L is the length of the measurement area of the calf and R_{E0} and R_{Et} are extracellular resistance at beginning and at any time during dialysis respectively. Since ρ_{ECV} and L are constant in this equation, they are canceled in the ratio of volume so that R_{E0}/R_{Et} is equal to ECVt/ECV0. This is the principle why decrease in calf R_{E0}/R_{Et} represents the reduction of extracellular fluid volume. In our previously study, when the curve of R_{E0}/R_{Et} is flattening, it indicates a limitation of excess

fluid in interstitial space so that the state of this hydration can be defined as normal hydration state. Fig.13 show the principle of the continuous monitoring R_{E0}/R_{Et} until reach the flattening in last 20 minutes.

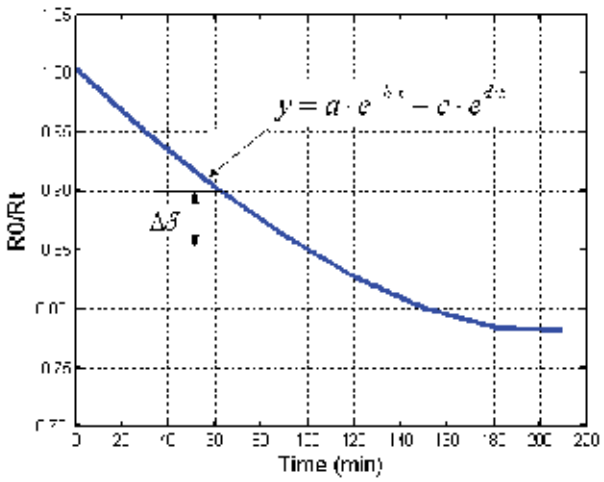

Fig. 13. Shows the principle of curve of R_{E0}/R_{Et} during UF. The curve can be represented as exponential function of two variables and two constants (a and c)

The main advantage with curve of R_{E0}/R_{Et} is the method does not require a normal range from a healthy population but utilizes its slope change as an indicator of tissue hydration. Flattening of the curve during 20 minutes has been defined as excess fluid volume being completely removed in the body when the patients reach dry weight.(Fig.14).

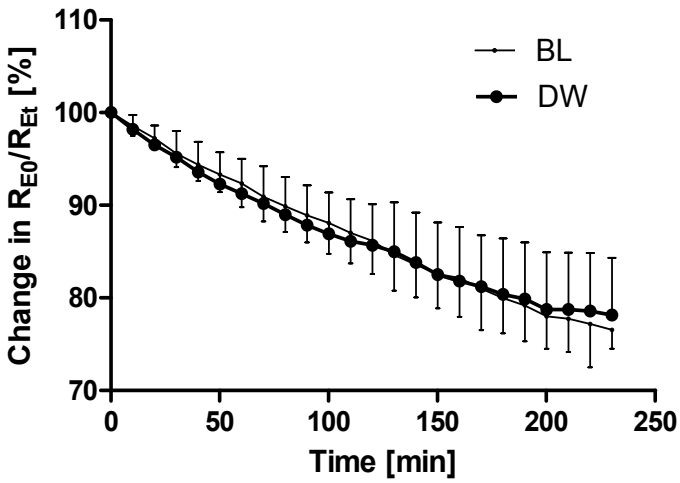

Fig. 14. Comparison of the curve of R_{E0}/R_{Et} in group patients between BL and DW (Modified from Int J Artif Organs 27:104-9, 2004).

Fig 14 shows change in curve of R_{E0}/R_{Et} in a group of patients with different hydration states. However, this method has a problem if the patients with deep vein thrombosis of the calf can stop or reduce the fluid volume from leg back to main circulation and this problem will cause less change in the curve R_{E0}/R_{Et} during HD treatment. In this case, R_{E0}/R_{Et} curve cannot indicate hydration change in the body.

4.4 Combination of static and dynamic method

Principle of the method using R_{E0}/R_{Et} curve and normalized resistivity is shows in Fig.15. The slope of change in resistance represents the removal of excess fluid volume in the calf. The flattening of the resistance curve means that the fluid exchange between intravascular and interstitial compartments has reached an equilibrium state. We hypothesize that DW has been reached if the curve is flattening and the normalized resistivity is in the normal range (Fig. 15). Approaching normalized resistivity value in the calf provides a secondary indication of DW. The system used is a refinement of the Xitron Hydra multifrequency device which measures both extracellular resistance and resistivity (Zhu *et al*., 2008a). The algorithm used to determine normal hydration state employs two criteria together: a) flattening of the change in resistance (R_{E0}/R_{Et}) curve; and b) normalized resistivity in the range derived from healthy subjects.

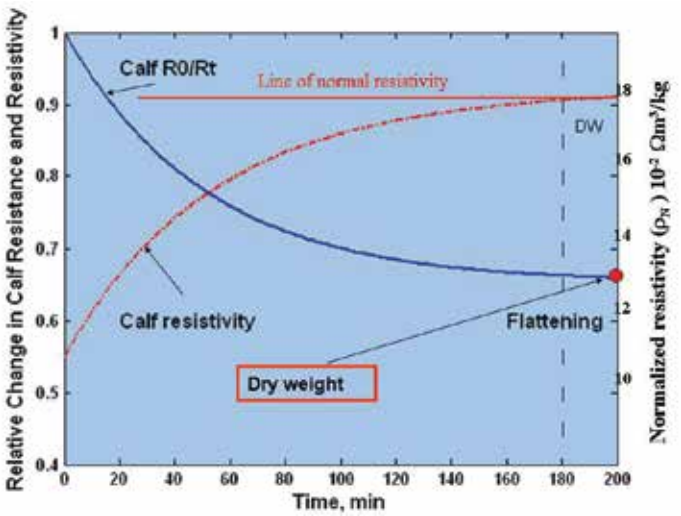

Fig. 15. Determination of dry weight with calf bioimpedance spectroscopy (cBIS)

4.4.1 Definition of flattening of the curve

R_{E0} and R_{Et} represent ECV at different times with change in ECV during whole HD. Since the curve may be affected by a movement of the patient's body during HD, the noise might influence the measurement of R_E. To reduce these interferences, a dynamic filter has been developed to reduce the error.

4.4.2 Continuous curve of the slope of R_{E0}/R_{Et} within specific time

The ratio of $R_{E,i}/R_{E,i+1}$ is collected from any two successive series of data, where subscript *i* (*i*=1, 2, 3, …N) represent *i*th value of measurement from a series of N data. The R_E at the start of HD is considered to be the reference of the initial hydration status (R_{E0}).

We have defined that the curve of continuous measurement of the λ is flattening as

$$\delta(\Delta t) < C_1 \tag{13}$$

where $\Delta t = t_m$ represents the time interval between t- t_m minutes which can be present in more general form as follows:

$$\Delta t = t_i - t_{i+m}, \tag{14}$$

where i represents the number of i-th measurement and i = 0, 1, 2, … N-m, N is the total number of measurements; m represents the number of measurements and t_m represents the time at m measurements. The flattening of the curve can be defined by Eq.13. At any specific time (t_{i+m}), when the $\delta(\Delta t) < 0.01$ the curve during this 20 minutes is considered as flatting.

4.4.3 Continuous measurement of resistivity

To continuously measure calf resistivity, calf circumference must be measured because at the same time the cross-sectional area is reducing during the treatment. The major issue of continuously calculate resistivity is how to measure the calf circumference during HD. The calf cross sectional area during HD can be calculated based on an assumption that change in circumference during dialysis is due to decrease in the fluid volume of the calf. Therefore, the value of circumference can be calculated by Eq.15

$$\chi_t = \sqrt{\chi_0^2 - \frac{4\pi\rho_0 L}{R_{E,0}}(1 - \frac{R_{E,0}}{R_{E,t}})} \tag{15}$$

where χ_0 and $R_{E,0}$ are measured at the start of dialysis. The ρ_0 is a resistivity with constant value which is experimentally calibrated by actual measurements of circumference , L is 10 cm, R_{E0} and R_{Et} are resistance measured by the bioimpedance device at the initial time and by continuous measurement until the end of the treatment. With Eq.15, circumference can be accurately calculated (Zhu *et al.*, 2006b).

4.4.4 Continuous calculation of resistivity change

With Eq.5 to Eq.15, calf resistivity during HD treatment can be calculated in Eq.17.

$$\rho_t = \frac{\chi_t^2 \cdot R_{5,t}}{4\pi L} \ \Omega\cdot\text{cm} \tag{16}$$

To compare the resistivity and reduce the variation, normalized resistivity is defined as

$$\rho_N = \frac{\rho_t}{BMI} \times 10^{-2} \ \Omega\cdot\text{m}^3/\text{kg} \tag{17}$$

where BMI is body mass index defined as body weight (Wt) divided by body height (H) squared. Since we know the range of normalized resistivity in NS, the patient's normal hydration level can be obtained by comparing the minimal level of normalized resistivity, which is given by

$$\rho_{N,P} \geq \rho_{N,H} \quad \Omega\cdot\text{m}^3/\text{kg} \tag{18}$$

where $\rho_{N,P}$ represent the patient's value for normalized resistivity and $\rho_{N,H}$ represents a minimal level of normalized resistivity in healthy subjects (18.3 $*10^{-2}$ $\Omega m^3/kg$ for male and 20 $*10^{-2}$ $\Omega m^3/kg$ for female respectively) from previous study.

4.4.5 Continuous calculation of body weight

Body weight (W_t) during HD treatment can also be continuously calculated by

$$W_t = W_{\mathrm{Pr}e} - \sum_i UFR_i \times \Delta t_i - W_V \tag{19}$$

where W_{Pre} is pre HD weight, UFR is ultrafiltration rate, Δt is the treatment time at *i*-th period of time with constant UFR (UFR>0), W_V is any possible change in weight including use of intravenous or oral fluids. Final, dry weight (DW_t) can be calculated in a general and continuous form as follows.

$$DW_t = W_{\mathrm{Pr}e} - \int_{t_0}^{t_{DW}} UFR(t) \cdot dt - W_V \tag{20}$$

where UFR(t) is a function of UFR with the time in case UFR is not constant. To obtain a numeric result it is assumed that the initial constant is zero. when the patient reaches dry weight, dry weight can be calculated by pre HD body weight minus ultrafiltration volumes during subsequent treatments (Zhu *et al.*, 2008a).

5. Summary

This chapter presents clinical issues and current new techniques to estimate fluid overload in dialysis patients. During the last decades, many technologies have substantially improved; these advanced tools provide the possibility to understand physiological mechanisms to control and adjust body fluid status in dialysis patients. Body fluid status can be explained with respect to terms such as dry weight, normal hydration and fluid overload.

Fluid overload can be specifically defined as extracellular fluid volume in dialysis patients greater than level of the normal ECV range. However, normal range of ECV must be clearly expressed and it must be a measurable value. Hydration is not the same as fluid overload. In chemistry, hydration is defined as a ratio of water to total mixed components of the system. In the body, hydration can be defined as the ratio of water to the mass of other body components. Since the water or ECV in healthy population is a range, normal hydration should be defined as a range and it is determined by 1) absolute ECV range; 2) total body mass and its components. The main challenge is to find the relationship between ECV and body mass components in a healthy population. The concept of dry weight in dialysis patient is defined as the patient having no excess fluid volume at post dialysis weight. However, body weight consists of different components, such as fat and muscle with variability of fluid content so that if body composition changes, the body hydration does not proportionally follow this change. The main question in the determination of dry weight is how to quantitate the degree of fluid overload.

Methodologies of measuring fluid overload can be generally divided into three techniques: static, dynamic and combination of both methods. With static methods, fluid status is measured in the intravascular compartment such as IVCD, peptides such as ANP, BNP and in both the intravascular and extravascular fluid compartment with methods such as such as

the BIA vector, WBM and calf resistivity. With dynamic method, currently, two methods are reported: RBV monitor measures changes in plasma volume only while continuous measurement of calf R_{E0}/R_{Et} provides information about change in plasma and interstitial fluid volume.

BIA phase angle bioimpedance at 50 kHz technique provides multi information about state of hydration and nutrition but it cannot produce quantitative value of fluid overload for individual. Whole body (Wrist to ankle) bioimpedance spectroscopy techniques have been largely improved to measure fluid volume, however, this method has the inherent problem of body composition influencing measurement of resistance so that its accuracy for measuring fluid overload cannot be further improved. Calf normalized resistivity provides a simple way to measure degree of fluid overload. Other measurements, such as biochemical or IVCD methods, cannot indicate the hydration of extravascular space directly so that they are not reliable in detecting degree of fluid overload. RBV measurement displays change in plasma volume but it cannot provide direct information about fluid overload in interstitial compartment. A general relationship exists between change in blood volume and refilling volume but this complicated by the effect of other factors on refilling such as autonomic tone, vascular tone, splanchnic vasoconstriction and temperature. Calf continuous measurement of R_{E0}/R_{Et} monitors the ECV changes including plasma and interstitial fluid volume. The main advantage of this method is that it does not require a control parameter to estimate fluid overload. However, calf R_{E0}/R_{Et} curve could be affected by lower limb venous thromboses. The combination of calf R_{E0}/R_{Et} curve and normalized resistivity can provide the best information concerning dry weight estimation the individual patient but this method may take a long time because post dialysis weight has to be reduced gradually over a long period of time if the patient is greatly fluid overloaded.

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17

Current Status of Synthetic and Biological Grafts for Hemodialysis

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1. Introduction

Patients with chronic kidney disease (CKD) stage 4 or 5 have the option of kidney transplantation, hemodialysis (HD), peritoneal dialysis (PD), or conservative management.[1] National Kidney and Urologic Disease Information Clearinghouse reported that in 2007, there were 368, 544 U.S. residents with ESRD who were receiving dialysis, of whom 341, 264 were undergoing HD.[2] Quality of life and long-term survival of patients with CKD who are on HD depends on the successful placement of vascular access, as autogenous arteriovenous access, prosthetic arteriovenous access, or tunneled central venous catheter. DOQI guidelines are emphatic that autogenous arteriovenous access placement should be considered first, as it provides the optimal vascular access, followed by prosthetic grafts if autogenous arteriovenous access placement is not possible. There is a great deal of controversy regarding the choice of synthetic or biological material, as the guidelines suggest that it should be based on surgeon's experience and preference. The evidence to support the superiority of tapered versus uniform tubes, thick- versus thin- walled characteristics, elastic versus non-elastic arterial, stretch vs. standard PTFE, externally supported vs. unsupported grafts, is still evolving.

An ideal vascular graft would have the following characteristics: 1) appropriate size to match host vessels, 2) mechanical strength, 3) low thrombogenicity/complete endothelialization, 4) rapid/complete healing, 5) ease of handling, 6) resistance to infection, 7) structural durability in face of repeated needle puncture, 8) low incidence of hyperplastic intimal changes and 9) low cost.[3]

In this chapter, we will review the development of vascular grafts over the years and discuss the advantages of one over the other.

2. Prosthetic grafts for hemodialysis

Although the first synthetic graft used for HD access in the United States was made of Dacron dating back to the early 1970s, unfavorable results and the availability of better prosthetic materials like expanded PTFE (ePTFE) forced its abandonment. In 1976, Dr. Baker presented the first results of ePTFE grafts in 72 HD patients. Since then ePTFE remains the graft of choice for vascular access.[4] ePTFE is considered the material of choice due to the fact that it is readily accessible, ease of implantation, good medium term patency, and relatively low complication rates compared to other synthetic and biological materials.[5] The medical community has made strenuous efforts to increase the use of autogenous arteriovenous access, prevalence has increased from 22% in 1995 to 57.7% in 2011. However, the use of synthetic grafts still remains significant (18.4% prevalence in 2011).[6,7]

2.1 Indications for use of prosthetic grafts

Autogenous arteriovenous access are clearly superior in terms of long term patency to grafts, but not feasible in many patients undergoing HD[8]. The indications for prosthetic grafts include: lack of suitable vessels particularly in elderly and diabetic patients, need for immediate cannulation and in children who cannot tolerate multiple painful venipunctures.[5]

2.2 Complications associated with prosthetic grafts

Graft failures are typically caused by stenosis (leading cause of graft failure) due to thrombosis and neointimal hyperplasia at site of anastamosis, as well as graft infection (contributes to 10-15% of graft failure).[9,10] Other less common complications of prosthetic accesses include; steal syndrome, seromas, aneurysm formation, central vein stenosis and bleeding.[11] Thrombosis seems to occur soon after implantation due to technical problem, with a clot typically forming at the surface of the graft when it is first exposed to blood. The clot is formed initially of platelet aggregates, and then fibrin and thrombin is laid down via activation of the coagulation cascade. Platelet activity is generally most intense during the first 24 hours and subsides to a very low level after 1 week.[9] Neointimal hyperplasia in prosthetic conduits can be attributed to upstream and downstream events. The upstream factors include; hemodynamic stress at the graft-vein anastamosis, compliance mismatch between the graft material and vein (more studies required to establish this factor), arterial injury at the time of graft placement, intrinsic properties of the synthetic graft itself (shown to attract macrophages which then secrete specific cytokines bFGF, PDGF, and VEGF), graft injury from dialysis needles, as well as the presence of uremia (causing endothelial dysfunction even prior to synthetic graft implant).[12-14] The downhill events are essentially a consequence of the upstream events. Pro-inflammatory cells release cytokines promoting the migration of smooth muscle cells and myofibroblasts from the adventitia media into the intimal layer, where they proliferate and cause lesions of neointimal hyperplasia.[13] These stenotic lesions are usually treated by percutaneous angioplasty (PTA) or open patch angioplasty, which unfortunately, predisposes the patient to restenotic lesions due to endothelial and smooth muscle cell injury.[15]

Infection is the second most common complication of synthetic grafts and can lead to further complications such subacute bacterial endocarditis, epidural or brain abscess.[11] These complications can lead to graft failure in up to 35% of patients.[16] Graft infections have an incidence rate as high as 2%, and are 4 times as prevalent in synthetic grafts when compared to autogenous veins.[9] Common causative organisms are Staphylococcus aureus (26.32% of

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Johnson et al.[47]	USA	1983	548 Impra® grafts	N/A	N/A	N/A	Early thrombosis (<6 weeks) 9.58%; Late thrombosis (>6 weeks) 17.52%; Infection 7.12%; Perigraft hematoma 2.01%; Steal syndrome 15	7- yr cumulative patency 55%
Munda et al.[48]	USA	1983	67 (55 Gore-Tex®, 12 Impra® grafts)	N/A	N/A	N/A	Graft thrombosis (21%), infection (25%), or stenosis by neointimal hyperplasia (34%), pseudoaneurysm formation (8%), steal syndrome (3%)	Cumulative patency of 67% ± 6% (12 mo), 50% ± 7% (24 mo), 43% ± 9% (48 mo).
Schanzer et al.[49,50]	USA	1989	65 (30 Vasutek vs. 35 control PTFE grafts)	63% vs. 66% at 1 yr	N/A	75% vs. 67% at 1 yr	Thrombosis 36.6% vs. 28.5%; Infection 13.3% vs. 11.4% Seroma 3% vs. 0% Steal 3% vs. 0% Pseudoaneurysm 0% vs. 17.1	In PTFE-silicone group, mean time to first use was 1.3 days vs. 2 to 4 weeks for conventional PTFE grafts. Bleeding after needle removal was significantly decreased after early and late punctures of PTFE-silicone grafts vs. conventional PTFE grafts ($p < 0.001$).
Tsuchida et al.[29]	Japan	1992	Experiment 1: 20 carbon lined (CL) vs. 20 control PTFE grafts Experiment 2: 12 high porous grafts vs. 12 control PTFE grafts	N/A	N/A	N/A	N/A	Graft platelet accumulation index (GPAI) of CL graft was significantly reduced.

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Tordoir et al.[20]	Netherlands	1995	37 (17 stretch ePTFE vs. 20 standard ePTFE grafts)	59% vs. 29% (1 yr)	N/A	N/A	Thrombotic events: 40% (standard) vs. 12% (stretch)	Stretch ePTFE has better primary patency rates and less stenosis due to intimal hyperplasia vs. standard ePTFE grafts.
Dawidson et al[22].	USA	1996	270 Gore-Tex® stretch graft (96 cannulated within 14 days, 174 cannulated after 14 days)	N/A	N/A	N/A	Thrombosis: 14% of early cannulation group vs. 7% in late cannulation group. Infection: 7% early cannulation group vs. 1% late cannulation group.	Graft survival in late cannulation group (beyond 14 days) was 93, 92, and 91 % vs. 80, 78 and 73% for the early cannulation group (earlier than 14 days) at 1, 2, and 3 yr. respectively (p=0.0008).
Taylor et al.[51]	USA	1996	45 lower extremity access grafts (39 ePTFE, 6 modified bovine heterographs)	59% (12 mo) 47% (24 mo)	N/A	N/A	Thrombosis (33%) graft infection (18%), distal limb ischemia (16%), aneurysmal dilatation of the graft requiring revision (7%), and fistula-induced congestive heart failure symptoms (4%)	Lower extremity AV dialysis accesses are associated with multiple complications and should be placed only if significant patient morbidity can be accepted and justified.
Hakaim et al.[21]	USA	1997	79 stretch ePTFE grafts (48 underwent early cannulation and 31 late cannulation)	N/A	N/A	N/A	Thrombosis occurred before cannulation in 2.0% of early cannulation and 3.2% of late cannulation group.	Cumulative primary patency estimates for early cannulation were 89%, 82%, and 70% vs. 86%, 78% and 74% for the late cannulation group at 3, 6, and 12 mo. respectively. Early cannulation of prosthetic dialysis grafts did not increase perioperative morbidity rates or decrease 12-month cumulative primary patency rates.

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Ritter et al.[52]	USA	1997	6 male rats per test group (control, Heparin irrigated and TDMAC heparin irrigated)	N/A	N/A	N/A	Mean number of emboli during a 20-minute period was 91 for the control group, 84 for the heparin-irrigated group, and 22 for the TDMAC-heparin group.	TDMAC-heparin coating of ePTFE microvascular prostheses reduces downstream microemboli.
Kaufman et al.[18]	USA	1997	129 (65 Impra® and 64 Gore-Tex® grafts	1 yr (Impra® 43%, Gore-Tex® 47%) and at 2 yr (Impra® 30%, Gore-Tex® 26%) (p = 0.78)	N/A	1 yr (Impra® 49%, Gore-Tex® 69%) and at 2 yr (Impra® 33%, Gore-Tex® 41%) (p = 0.15)	Thrombosis Infection: 11% Impra® and 14% Gore-Tex® Steal Syndrome: 6% in each group	No difference in the performance of 6-mm standard ePTFE grafts on the basis of manufacturer, whether Gore-Tex® or Impra®.
Khadra et al.[53]	Australia	1997	74 thigh PTFE grafts	N/A	N/A	N/A	Infection: 16%	Mean survival time for thigh PTFE grafts was 84.6 weeks (SD 76.1). Infection rate (16%) vs. forearm infection rates (20%).
Schuman et al[23]	USA	1997	632 grafts (438 reinforced Gore-Tex® vs. 194 nonreinforced Impra® grafts	Greater primary patency for nonreinforced Impra® grafts at 1 yr. (p=0.02)	N/A	80% nonreinforced vs. 77% reinforced at 1 year	Reinforced Gore-Tex® had a mean of 2.75 thrombosis/ graft and required 1.20 revisions/ graft, while non-reinforced Impra® only had a mean of 2.45 thrombosis/ graft with 1.09 revisions/ graft	Non-reinforced PTFE performed better than reinforced PTFE
Hurlbert et al.[19]	USA	1998	190 (100 Gore-Tex® vs. 90 Impra® grafts)	No difference between Gore-Tex® and Impra® grafts at 2 yr (P > 0.53)	N/A	No difference between Gore-Tex® and Impra® grafts at 2 yr (P > 0.13)	Ccomplications was similar between the two grafts.	No difference between Gore-Tex® and Impra® in the number of days before the first thrombectomy or in the number of thrombectomies or revisions per graft (P > 0.50).

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Jeschke et al[54]	USA	1999	69 [37 polycarbonate polyurethane (PU) vs. 32 ePTFE] grafts into abdominal aortas of male Sprague-Dawley rats	N/A	N/A	N/A	Neointimal cell proliferation lower in PU grafts compared with ePTFE at 56 days (1.4 +/- 0.1µ versus 8.6 +/- 1.5µ, P <.001) and at 6 mo (0.15 +/- 0.002µ versus 3.4 +/- 0.5µ, p <.001). Neointimal thickness at 6 mo after implantation was 3.2 +/- 0.8µ for PU compared with 10.3 +/- 3.1µ for ePTFE (P <.05).	Luminal surface of PU grafts took 4 weeks to completely endothelialize, whereas ePTFE grafts took 24 weeks (P <.05).
Longest et al.[25]	USA	2000	Computer Hemodynamic Analysis of Venaflo® II	N/A	N/A	N/A	N/A	Geometric design of the new graft-end was based on the reduction of three time- and area-averaged hemodynamic parameters (including the wall shear stress gradient, wall shear stress angle gradient, and radial pressure gradient).
Lemson et al.[24]	Netherlands	2000	120 (59 cuffed PTFE vs. 61 standard PTFE grafts)	Control: 69%, 56% , 42%, 34% vs. cuff group: 62%, 43%, 30%, and 19% at 6 mo, 18 mo, 2 yr respectively (P = .097)	Control: 76% , 62% , 52%, 43% vs. cuff group: 71%, 60%, 53%, and 41% at 6 mo, 18 mo, 2 yr respectively (p=0.53)	Control: 86%, 84%, 79% vs. cuff group: 89%, 81%, 76%, and 65% at 6 mo, 18 mo, 2 yr respectively (p=0.42)	Thrombotic occlusion lower in cuff group (0.68 ppy) than in control (0.88 ppy) (p=0.0007) Stenosis- CG: 0.22 ppy vs. control: 0.14 ppy; Infection- CG: 0.06 ppy vs. control: 0.01 ppy; Hemorrhage- CG: 0.10 ppy vs. control: 0.01 ppy; Pseudoaneurysm- CG: 0.04 ppy vs. control: 0.02 ppy; Ischemia- CG: 0.04 ppy vs. control: 0.00 ppy; Venous hypertension- CG: 0.00 ppy vs. control: 0.01 ppy; Seroma- CG: 0.04 ppy vs. control: 0.04 ppy	Cuffed PTFE did not result in a better patency rate. Initial vein diameter and local problems (edema, obesity, or skin atrophy) appear to be the most important risk factors for graft failure.

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Glickman et al. [35]	USA	2001	142 (71 Vectra® vs. 71 Gore-Tex® or Impra® grafts)	55% vs. 47% (6 mo) and 44% vs. 36% (12 mo) (P >.05).	N/A	87% vs. 90% (6 mo) and 78% vs. 80% (12 mo). (P >.05).	Higher incidence of graft kinking in PUU group (p<0.001) Infection, anastomotic obstruction or thrombosis, pseudoaneurysm, kinking, stenosis, wound healing	At ≥5 minutes, more PUU cannulation sites achieved hemostasis compared with ePTFE sites (P <.0001). 53.9% of all PUU grafts were cannulated before 9 days versus none with the ePTFE grafts (P <.001).
Nyberg et al.[27]	USA	2001	12 Venaflo® II grafts	N/A	N/A	Graft patency rates were 90.9% at 1 yr and 68.2% at 2 yr	No early postoperative complications. One graft was lost to thrombosis in the first yr; two grafts were lost to thrombosis in the second yr.	Cuffed ePTFE graft provided stable blood flow and satisfactory graft patency during 2 yr of follow-up, even in high risk patients with a prior history of vascular access thrombosis.
Sorom et al.[26]	USA	2002	48 (24 Venaflo® II vs. 24 Gore-Tex® grafts)	A significant advantage in primary graft patency was observed with the Venaflo® II at 12 months after placement (*P* <.01).	N/A	Significant improvement observed in the Venaflo® II group compared with the Gore-Tex® group at both 12 months (+32%, *P* = .037) and 24 months +37%, *P* = .021).	Graft-vein anastamosis stenosis: 15% Venaflo® II vs. 41% Gore-Tex®	Graft patency at 12 mo. was 64% vs. 32% (P =.037) and 58% vs. 21% at 24 mo. (P =.0213) for Venaflo® II and Gore-Tex® respectively. Incidence of graft failure was lower in the cuffed ePTFE graft group (P =.039).

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Huber et al.[55]	USA	2003	34 study meta-analysis (autogenous vs. various PTFE access)	72% vs. 58% (6 mo) 51% vs. 33% (18 mo)	N/A	86% vs. 76% (6 mo) 77% vs. 55% (18 mo)	N/A	Patency rate for upper extremity AVF in adults is superior to that for PTFE counterparts, although the overall quality of the studies in the meta-analysis was less than ideal.
Kiyama et al.[56]	Japan	2003	58 (30 Thoratec® PVG vs. 28 Gore-Tex® stretch grafts)	60.7% vs. 56.5% (1 yr); 54.7% vs. 51.8% (2 yr) PVG and Gore-Tex® stretch graft respectively	N/A	78.7% vs. 79.9% (1 yr); 78.7% vs. 69.3% (2 yr) PVG and Gore-Tex® stretch graft respectively	Thrombosis: 26.7% vs. 35.7%; Stenosis: 23.3% vs. 28.6%; Infection: 6.7% vs. 14.3%; Seroma: 0% vs. 3.6%; False aneurysm: 0% vs. 3.6%; Kinking: 3.3% vs. 3.6%; Arterial Steal: 0% vs. 3.6% PVG and Gore-Tex® stretch graft respectively	PVG is an acceptable alternative to the stretch ePTFE.
Peng et al.[57]	Singapore	2003	163 PVG	73% at 1 yr	N/A	86% at 1 yr; 64% at 3 yr	32.7% graft infection rate; 30% graft thrombosis rate. Infection caused 61.5% of graft loss	PVG were first cannulated at a median time of 19 days after implantation, with 12% used within 3 days.
Laredo et al.[28]	USA	2003	10 bilateral aortoiliac grafting in dogs (5 heparin adsorbed Carboflo® vs. 5 control Carboflo® grafts)	87.5% heparin coated vs. 50% control (7 days) P=0.28	N/A	N/A	N/A	8% of preimplantation heparin activity remained on heparin grafts after 2 hours and only 2% after 7 days.
Hazinedaroglu et al.[58]	Turkey	2004	5 PU grafts	N/A	N/A	N/A	N/A	Patients implanted with a PU graft were dialyzed within hours after surgery.

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Jefic et al.[34]	USA	2005	133 PUU grafts	51% and 33% at 6 mo and 1 yr respectively.	N/A	78% and 61% at 6 mo and 1 yr respectively	24% patients died (unrelated to graft placement), 49% (n=50) had graft thrombosis (94% or 47/50 underwent successful percutaneous thrombectomy)	Within the first 4 days after graft placement, 108 of 133 grafts (81%) were cannulated.
Liu et al. [59]	Taiwan	2006	39 (17 Venoflo II vs. 19 Gore-Tex® grafts)	N/A	N/A	N/A	Mean degree of stenosis in non-cuffed graft group (44.95%) was greater than that of the cuffed graft group (22.76%)	Degree of venous stenosis was significantly reduced.
Schild et al.[36]	USA	2007	30 Vectra® grafts in HIV + patients	42% at 12 mo	N/A	N/A	10% (n=3) developed infection	Infection was less (10% vs. 45%)
Tsoulfas et al.[60]	USA	2008	67 (41 Venaflo® II vs. 26 control ePTFE grafts)	37.7% vs. 25.7% at 1 yr, 35% vs. 10.3% at 2 yr, 28% vs. 5.1% at 3 yr (p = 0.086)	N/A	81.8% vs. 56.1% at 1 yr, 61.8% vs. 46.3% at 2 yr, 51.5% vs. 33.1% at 3 yr (p=0.047)	Thrombosis as a cause of complete graft failure was higher (34%) in the standard group than in the cuffed group (9%) (p = 0.0125). Infection of the graft was observed in 12% of the cuffed group and in 6% of the standard group (p = 0.55)	Cuffed ePTFE graft provided better long-term outcome.
Kakkos et al.[33]	USA	2008	117 (76 Vectra® grafts vs. 41 TBB)	92% vs. 100% (1 mo); 50% vs. 46% (12 mo); 26% vs. 31% (18 mo) P=0.62	93% vs. 100% (1 mo); 70% vs. 82% (12 mo); 58% vs. 78% (18 mo) P=0.033	97% vs. 100% (1 mo); 81% vs. 88% (12 mo); 87% vs. 84% (18 mo) P=0.91	6.6% graft infection rate Postoperative complications more frequent in TBB fistulas and late complications in Vectra® grafts.	Use of Vectra® grafts and TBB fistulas started after a median of 14 (7-30) and 70 (52-102) days, respectively (P < .001)

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Davidson et al.[30]	USA	2009	150 (83 Propaten® vs. 67 control ePTFE grafts)	CFS for all grafts was 69% at 12 mo. CFS for heparin bonded group was 88% and 78%, vs. 69% and 58% for the control group at 6 mo and 1 yr, respectively (p=0.007).	N/A	N/A	N/A	Heparin binding technology resulted in a 20% improved primary graft patency of about 80% at one yr.
Schild et al. [61]	USA	2010	31 Flixene grafts	78% at 6 mo	86% at 6 mo	N/A	N/A	94% of the patients were cannulated within 24 hours. 3% were cannulated within 24-48 hours 3% were cannulated within 48-72 hours.
Lioupis et al. [62]	United Kingdom	2010	108 (48 BBAVF, 15 ABBA, and 48 Flixene graft)	27%, 51%, and 55% for ABBAs, BBAVFs, and grafts respectively at 18 mo	N/A	N/A	Complications were not more frequent in AVGs than ABBAs and BBAVFs (p=0.127)	Median time to first use for AVGs was shorter (p<0.0001). Complications were not more frequent in AVGs than ABBAs and BBAVFs (p=0.127).

Abbreviations: polytetrafluoroethylene (PTFE), expanded polytetrafluoroethylene (ePTFE), carbon lined (CL), graft platelet accumulation index (GPAI), high porous (HP), tridodecylmethylammonium chloride (TDMAC), polycarbonate polyurethane (PU), per patient year (PPY), polyurethaneurea (PUU), polyurethane vascular graft (PVG), transposed brachio-basilic fistula (TBB), clot free survival (CFS), autogenous brachial vein-brachial artery access (ABBA), brachio-basilic arteriovenous fistula (BBAVF), Not available (N/A)

Table 1. Clinical trials of prosthetic grafts for hemodialysis.

infections cultured), methacillin resistant Staphylococcus aureus (21.05%), followed by Pseudomonas aeruginosa (5.26%).[11] The largest number of infections occur when patients are going under routine dialysis (more than 50% of patients) and as a complication of chronic cannulation, rather than postoperative complications.[11] Reducing *Staphylococcus aureus* carrier state in patients undergoing HD and improving antiseptic technique may reduce the rate of infections in grafts.

2.3 Characteristics of PTFE grafts

While PTFE is available in various configurations and is produced by various manufacturers, very few have proven to be more beneficial in improving patency in randomized clinical trials for long-term.[1]

2.3.1 Effect of wall thickness

In order to examine the effect of wall thickness on patency, Lenz et al.[17] investigated both standard wall and thin wall configuration of PTFE. Although the incidence of complications and mortality did not statistically differ amongst the 2 groups, standard ePTFE had better patency rates. Studies comparing 2 manufacturers of ePTFE grafts: Gore-Tex® (W.L. Gore and Associates, Flagstaff, AZ) and Impra® (C. R. Bard Inc., Murray Hill, NJ) did not find any difference in the performance of 6-mm standard ePTFE grafts.[18 19]

2.3.2 Effect of stretch characteristics

In an attempt to reduce kinking of the graft in areas of angulation and to improve intraoperative handling, the graft was modified to stretch (Gore- Tex® Stretch). Tordoir et al. reported a cumulative primary patency rate of 59% in the stretch ePTFE group compared to 29% in standard ePTFE group at 1 year ($p < 0.01$). In addition, there were significantly fewer thrombotic events for the stretch ePTFE grafts as opposed to the standard ePTFE grafts (40%vs. 12%, $p<0.001$).[20] Early cannulation of stretch ePTFE grafts was not found to increase peri-operative morbidity rates or decrease 12-month cumulative primary patency rates.[21] In contrast, another study comparing the patency of early cannulation with late cannulation in Gore-Tex® stretch grafts showed that graft patency after thrombosis formation was significantly higher in the late cannulation group ($p=0.0002$).[22]

2.3.3 Effect of ringed reinforcement

Ring reinforced grafts were created to reduce kinking at the apex of loop grafts and decrease incidence of thrombosis associated with external compression. In a retrospective study in which 632 reinforced and non-reinforced PTFE grafts were compared for patency and complications, it was found that non-reinforced grafts had higher primary and secondary patency rates.[23]

2.3.4 Effect of cuff or hood on venous ourflow

One of the few modifications that improved patency rates in PTFE vascular grafts was placing a cuff or hood on the venous outflow. The main objective of placing a cuffed PTFE graft is to enlarge the outflow, and reduce mechanical sheer stress in order to reduce thrombotic occlusion caused by neointimal hyperplasia.[1] In a computer simulated model, Venaflo® II (C. R. Bard Inc., Murray Hill, NJ), a flared-end ePTFE graft to simulate a vein

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Wellington [63]	Canada	1981	23 Dardik Biograft™	N/A	N/A	N/A	Thrombosis: 26.1%; Infection: 13.0%	Overall patency rate at 1 yr was 60% (no obvious superiority of umbilical vein over expanded Teflon grafts)
Hurt et al.[64]	USA	1983	140 (62 Artegraft® vs. 78 PTFE [Gore-Tex® or Impra®] grafts)	N/A	N/A	N/A	No difference between BCAH and PTFE	Survival rate: 3 mo. (93% vs. 84%); 1 yr. (84% vs. 65%); 2 yr (72% vs. 63%); 3 yr (54% vs. 56%) for BCAH and PTFE respectively.
Enzler et al.[65]	Switzerland	1996	720 (429 AVF and 291 grafts [150 bovine xenografts, 69 PTFE grafts, 59 sheep collagen grafts, 10 autologous and 1 homologous vein grafts])	AVF: 70%, 64%, 59%, 51%, 32% at 1, 2, 3, 5, and 10 yr. respectively. Grafts: Bovine Xenografts: 51%, 21%, 13%; PTFE: 41%, 24%, 24%; Sheep Collagen: 54%, 48%, 39% at 1, 2, and 3 yr. respectively.	N/A	AVF: 74%. 67%. 64%, 56%, 36% at 1, 2, 3, 5, and 10 yr. respective-ly. Grafts: Bovine Xenografts: 56%, 29%, 24%; PTFE: 58%, 47%, 40%; Sheep Collagen: 71%, 58%, 45% at 1, 2, 3 yr. respectively	Overall Complication rate: 20.71% AVF, 66.6% PTFE, 55.93% sheep collagen, 37.33 bovine xenograft Infection: 0% AVF, 10% PTFE, 2% sheep collagen, 1% bovine xenograft Thrombosis: 12% AVF, 45% PTFE, 44% Sheep collagen, 27% bovine xenograft..	AVF remains the procedure of choice. PTFE appears to be a reasonable second choice.
Bacchini et al. [42]	Italy	2001	53 PTFE, 10 reinforced PTFE, and 22 ProCol® grafts 404 native AVF	17.4 % (PTFE) vs. 23.9% (BMVG) at 12 mo AVF: 43% at 12 mo	N/A	50% at 12 mo and 6.6% at 50 mo for PTFE; 81.9% at 19 mo for BMVG; AVF: 52.4% at 50 mo and 46.5% at 90 mo	N/A	22 patients with a 20 mo follow-up confirms better survival of BVMG than PTFE ($p < 0.04$).

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Glickman et al. [43]	USA	2003	74 (59 ProCol® vs. 15 ePTFE grafts [Impra®, Gore-Tex® or Boston Scientific])	33% in BMVG vs. 18% in ePTFE group at 1 yr (p=0.120)	45% BMVG vs. 18% ePTFE at 1 yr (p=0.011)	67%/59% for BMVG group vs. 45%/15% for ePTFE group at 1 yr and 2 yr respectively (p=0.006)	Infection and thrombosis in the BMVG group were lower than the ePTFE group.	80% of the ePTFE grafts were abandoned vs. 34% of the BMVG group.
Matsuura et al.[39]	USA	2004	19 SynerGraft® in 12 canines (11 between carotid artery and jugular vein and 8 between femoral artery and vein)	72.6% and 58.6% for SG vs. 57.4% and 54.7% for the ePTFE grafts at 6 mo and 12 mo respectively.	N/A	N/A	None of the SG grafts became infected, but ePTFE graft group became infected within 54 days of implantation.	At 10 weeks, SG showed fibroblast cell migration and proliferation with incorporation into the surrounding subcutaneous tissue, and elongated cells expressing the contractile protein smooth muscle actin were also observed. After 24 weeks, procollagen synthesis was demonstrated in the fully colonized graft matrix.
Katzman et al.[44]	USA	2005	276 (183 ProCol® and 93 synthetic grafts [90 ePTFE, 1 silicone, 2 PUU])	35.6% ProCol® vs. 28.4% synthetic grafts at 12 mo (p=0.524)	N/A	65.6% ProCol® vs. 55.5% synthetic at 12 mo. 60.3% ProCol® vs. 42.9% synthetic at 24 mo (p =0.036).	Complication rates, including dilation, seroma, infection, and thrombosis, were lower for the ProCol® vs. synthetic grafts (p < 0.001).	Intervention rate was lower in the ProCol® group (0.97 versus 1.37) vs. synthetic grafts (p = 0.003).

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Madden et al.[66]	USA	2005	54 (27 SynerGraft® vs. 27 PTFE grafts)	No significant differences	No significant differences	No significant differences	No infections were seen in either group, but 2 aneurysms occurred in the SG group. No significant difference in the number of thrombectomies between the SG and PTFE groups (1.2 vs. 2.0, p = 0.3) Significantly more fistulagrams were performed in the SG group (p <0.05). Mild Steal syndrome: 1 SG vs. 4 PTFE recipient	Study interrupted by FDA. Considering the total number of interventions required by each group to maintain patency, patients with SG had a higher total cost than those with PTFE grafts. In patients without sufficient vasculature for native AVF, results do not support the routine use of SG in the general dialysis population.
Emrecan et al.[67]	Turkey	2006	4 total SynerGraft®	Patient 1 and 2: Radial artery-brachial vein implantation, and had a patency of 5 mo and 6 mo respectively. Patient 3 and 4: Brachial arteryaxillary vein implantation and had patency of 8 mo and 7 mo respectively.	N/A	N/A	Patient 4: True and pseudoaneurysm	N/A

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Darby et al.[40]	United Kingdom	2006	25 SynerGraft®	29% at 1 yr	45% at 1 yr	81% at 1 yr	5% infection rate at 1 yr	SG is an alternative when autologous vein is not available.
Tahami et al.[68]	Switzerland	2007	46 (23 ProCol® vs. 23 ePTFE grafts)	Patency rates did not differ	N/A	Patency rates did not differ	Less severe complications in the BMVG group	The BMVG is a viable alternative for HD access in patients where autologous construction is not possible, and should be given priority in patients with a failed ePTFE graft or high risk for infection.
Chemla et al.[41]	United Kingdom	2009	56 (29 SynerGraft® vs. 27 ePTFE grafts)	28% for SG vs. 48 % for ePTFE at 1 yr P = 0.290	52% vs. 64% at 1 yr P = 0.430	57% vs. 68% at 1 yr P = 0.370	4% vs. 9% infection rate for SG and ePTFE at 1 yr respectively (p=0.410)	Both grafts were adequate conduits for HD and amenable to repair. Anticipated advantages for SG were not seen in either patency or stability.

Abbreviations: bovine carotid artery heterograph (BCAH), polytetrafluoroethylene (PTFE), arteriovenous fistula (AVG), bovine mesenteric vein graft (BMVG), preserved saphenous vein (PSV), Endoscopic Vessel Harvesting System (EVHS), hemodialysis (HD), SynerGraft® (SG), polyurethaneurea (PUU), Not available (N/A).

Table 2. Clinical trials of biological conduits for hemodialysis

cuff, showed measurable improvements in reducing wall shear stress gradient, wall shear stress angle gradient, and radial pressure gradient.[25] Sorom et al.[26]found that Venaflo® II was associated with increased blood flow rates during HD and improved graft patency compared with ePTFE graft. Similarly, in a smaller study it was found that a flared-end ePTFE graft provided stable blood flow and satisfactory graft patency during 2 years of follow-up, even in high risk patients with a prior history of vascular access thrombosis.[27] However, a European study did not show improvement in patency rate despite a reduction in thrombotic occlusion and stenosis.[24]

2.3.5 Effect of coating PTFE

Another technique meant to improve PTFE graft has been coating the PTFE vascular grafts with carbon or heparin to prevent early graft failure and improve overall patency rates.[28] In a canine model, Tsuchida et al. showed that the graft platelet accumulation index (GPAI) was significantly ($p<0.05$) lower in the carbon lined PTFE group when compared to the control PTFE group.[29] They concluded that carbon lining decreases platelet accumulation on PTFE grafts. Propaten®, ePTFE with bioactive heparin covalently bound to it (W.L. Gore and Associates) has also been shown to be effective in improving graft patency. It is the only vascular graft of its kind approved for HD access on the market. Davidson et al. [30] found 20% improved primary graft patency of about 80% at one year when comparing Propaten® to standard ePTFE. In order to diminish the risk of neointimal hyperplasia. Cagiannos et al.[31] studied the effects of coating an ePTFE graft with rapamycin in a porcine model. They showed that the rapamycin coated grafts significantly ($P <0.0001$) lowered cross sectional narrowing in the outflow graft when compared to non-coated grafts; as well as no evidence of medial necrosis or aneurysmal degeneration. After a four week observation period, coated grafts showed features of diminished neointimal hyperplasia compared to untreated ePTFE grafts. Researchers have also analyzed the effect of a bioabsorbable vascular wrap mesh containing paclitaxel on neointimal hyperplasia in a sheep model. Paclitaxel coated mesh significantly reduced neointimal hyperplasia and neointimal capillary density without toxicity to adjacent vessels.[32]

2.3.6 Self-sealing grafts

K/DOQI recommends that PTFE grafts should not be routinely used until at least 2 weeks after placement and not until swelling has subsided so that palpation of the course of the graft can be performed. This time is needed for tissue-to-graft incorporation, reducing peri graft hematoma.[1] Due to this complication, newer "self-sealing" grafts have been designed that can be cannulated sooner.[11] Vectra® vascular grafts (C. R. Bard Inc., Murray Hill, NJ) made of a proprietary blend of segmented polyetherurethaneurea and a siloxane containing a surface-modifying additive (SMA), were designed to provide early cannulation, reducing the need for temporary central venous catheters to provide access until the graft matures. In a study of 76 patients, in which Vectra® grafts were compared to transposed brachio-basilic vein (TBB) autogenous access, Kakkos et al.[33] found that aggressive graft surveillance and endovascular treatment methods resulted in equivalent long-term secondary patency rates. The advantage of earlier use of Vectra® graft must be balanced against the need for more frequent secondary interventions and the risk of graft infection. In a single center study, Jefic

et al. obtained 81% (108 of 133 grafts) cannulation rate within 4 days of Polyurethane (PU) [Thoratec® Vascular Access Graft] graft placement in which none of the recipients required a temporary catheter. A shorter mean bleeding time after withdrawal of dialysis needle was also acquired in the PU graft group (4.0 minutes vs. 9.2 minutes in ePTFE group).[34] Similarly, Glickman et al. showed that PU grafts had achieved better hemostasis at cannulation sites compared to ePTFE sites when the two were compared at 5 minutes or less after dialysis ($P<0.0001$). Also, they showed that 53.9% of all PU grafts were cannulated before 9 days vs. none of the ePTFE grafts ($P<0.001$).[35] In the HIV- positive ESRD patient population, reduction of temporary catheter use and prevention of infection is critical. A study of 30 consecutive HIV positive patients receiving Vectra® graft implantation showed a lower infection rate (10% vs. 45%) than published reports of infection in PTFE grafts. It was concluded that the unique self-sealing property of the Vectra® grafts reduced the development of perigraft hematoma and may have accounted for decreased infection.[36]

3. Biological conduits for hemodialysis

Biological graft materials tend to have less intimal hyperplasia at the venous anastamosis, reduced tendency to thrombose, and a lower risk of infection when compared to PTFE.[11] Butler et al. compared bovine heterographs to PTFE and found that the synthetic graft had significantly fewer late thromboses, increased resistance to infection, easier to repair and had comparable longevity.[37] Anderson et al. found that bovine heterographs required twice as many revisions per dialysis month to maintain patency.[38]

SynerGraft® 100 (SG100 [CryoLife Inc., Atlanta, GA]) is a modified bovine ureter graft with some similarities to synthetic graft (similar internal diameter and strong tissue matrix). This graft has been processed to remove xenograft cells while maintaining a collagen matrix that is not chemically cross-linked by aldehydes allowing re-population by autologous cells. Matsuura et al. reported a primary patency rate of 72.6% and 58.6% for SG 100 vs. 57.4% and 54.7% for the ePTFE grafts at 6 months and 12 months, respectively. SG 100 graft showed fibroblast cell migration and proliferation with incorporation into the surrounding subcutaneous tissue after 10 weeks, and procollagen synthesis demonstrated at 24 weeks; while the ePTFE graft had no evidence of cellular ingrowth.[39] In a study of 23 patients receiving SG 100 grafts, Darby et al. found that the bovine ureter graft was a stable vascular access conduit, providing a suitable graft alternative when autologous vein was not available. Their study showed 29% primary, 45% primary assisted, and 81% secondary patency rates at 1 year, with only a 5% infection rate.[40] On the other hand, Chemla et al. found that both grafts were adequate conduits for HD, the anticipated advantages for SG 100 were not seen in either patency or stability.[41]

4. Future developments in prosthetic and biological conduits for hemodialysis

The use of pharmacological agents may hold the promise of long-term graft patency. Treatment with 200 mg of dipyridamole and 25 mg of aspirin twice daily resulted in significant improvement of patency rates while adverse effects in both the treatment and placebo groups remained the same.[45] Other agents, such as fish oil and anticoagulants have also been tried with limited success (table 4).

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Cagiannos et al[31]	USA	2005	22 Mongrel pigs into 3 groups: untreated ePTFE (n=6), adhesive-only coated ePTFE (n = 6), or adhesive- and Rapamycin-coated ePTFE (n = 10)	N/A	N/A	N/A	N/A	Rapamycin-eluting ePTFE grafts decrease neointimal hyperplasia in a porcine model.
Kohler et al[32]	USA	2007	40 male sheep into 5 groups: no mesh; or a 3-cm x 6-cm mesh with 0.0, 0.3, 0.7, or 1.2 μg/mm² of paclitaxel for a total dose of 0.0, 0.6, 1.3, or 2.2 mg, respectively.	N/A	N/A	N/A	N/A	Paclitaxel-eluting mesh significantly reduced neointimal hyperplasia and neointimal capillary density without toxicity to the adjacent vein.
Heise et al[46]	Germany	2010	In-vitro study of double outflow (bi-flow) grafts	N/A	N/A	N/A	N/A	New graft design addresses two major problems responsible for the development of venous stenosis of prosthetic grafts.

Abbreviations: expanded polytetrafluoroethylene (ePTFE), Not available (N/A)

Table 3. Clinical trials of experimental conduits for hemodialysis

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Sreedhara et al.[69]	USA	1994	Dipyridamole, Aspirin, Dipyridamole + Aspirin, or placebo in: 84 Type I (new ePTFE graft) patients 23 Type II (previously revised ePTFE graft) patients	N/A	N/A	N/A	Angina pectoris, GI bleeding, headache, nausea, and vomiting reported	Type I patients: At the end of the 18 mo follow-up, cumulative rates of thrombosis were: 21± 9% on dipyridamole, alone, 25±11% on dipyridamole and aspirin combination, 42±13% on placebo, and 80±12% on aspirin alone. RR of thrombosis with dipyridamole was 0.35 (P = 0.02) with 95% CI of 0.15 and 0.80. The RR with aspirin was 1.99 with 95% CI of 0.88 and 4.48 (not significant, P=0.18). Type II patients: high thrombosis rates regardless of treatment group. Overall, 78% thrombosis in Type II patients and no statistical differences between study groups.
Zibari et al.[70]	USA	1997	408 various vascular access procedures (206 pre-operation treatment with vancomycin vs. 202 non-medicated control group)	N/A	N/A	N/A	Access infection developed in 1% of vancomycin group and in 6% of control group (P = 0.006). All 14 infections occurred in upper extremity PTFE grafts.	Use of preoperative single-dose IV vancomycin prophylaxis for hemodialysis vascular graft procedures reduces the risk of postoperative access infection

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Schmitz et al.[71]	USA	2002	24 total new Gore-Tex® graft patients (12 fish oil vs. 12 control oil group)	75.6% fish oil-treated group vs 14.9% control group at 1 yr	N/A	N/A	Gas, bloating most common complaints	Survival analysis revealed a significant difference between fish oil-treated and untreated patients (P < 0.03), with a power of 90%. Fish oil treatment also decreased venous outflow resistance and systemic BP, compared with control values.
Lok et al.[72]	USA	2007	232 new grafts for access (116 fish oil treatment vs. 116 control group)	N/A	N/A	N/A	N/A	Results of study not yet published
Dixon et al.[45]	USA	2009	649 total patients: 321 in dipyrida-mole and aspirin group vs. 328 in placebo group.	23% placebo vs. 28% dipyridamole/ aspirin group at 1 yr	N/A	N/A	Graft failure, death, and serious adverse events (including bleeding) did not differ significantly between study groups	Treatment with dipyridamole plus aspirin had a significant but modest effect in reducing the risk of stenosis and improving the duration of primary unassisted patency of newly created grafts
Chan et al.[73]	USA	2009	Anticoagulant and antiplatelet medications on 41,425 dialysis patients (8.3% on warfarin 8.3%, 10.0% on clopidogrel, 30.4% on aspirin, 8.1% patients on at least two of these drugs, and 59.7% not medicated)	N/A	N/A	N/A	N/A	Warfarin associated with a 27% (P < 0.001) increase in mortality, clopidogrel with a 24%(P = 0.0002) increase in mortality, and aspirin with a 6% (P = 0.02) increase in mortality when compared with patients receiving none of these medications. Prescription of multiple drugs was associated with a 22% (P < 0.0001) increase in mortality. No statistically significant interaction was found among warfarin, clopidogrel, and aspirin on survival.

AUTHOR	COUNTRY	YEAR	NO. AND GRAFT TYPE	Primary Patency	ASSISTED PRIMARY PATENCY	Secondary patency	Complications	Comments
Dixon et al.[74]	USA	2011	649 patients with new AV grafts (272 on aspirin at baseline vs. 377 not on aspirin at baseline)	30% (aspirin group) vs. 23% (control) at 12 mo.	N/A	N/A	Serious adverse events in 56% of aspirin group vs. 53% of control group. Risk of bleeding, death, hospitalization, or vascular access events was not increased among participants on aspirin at baseline.	Use of aspirin at baseline associated with a dose-dependent prolongation of primary unassisted graft patency that approached statistical significance (p=0.06). Use of aspirin at baseline did not associate with prolongation of cumulative graft patency or participant survival.

Abbreviations: expanded polytetrafluoroethylene (ePTFE), polytetrafluoroethylene (PTFE), Not available (N/A).

Table 4. Effects of various medications on vascular access grafts

In an approach of reducing neointimal hyperplasia by decreasing mechanical sheer stress, a new double channel (Bi-Flow) graft was designed. These grafts showed laminar flow and lower levels of turbulence, leading to lower risk of stenosis.[46]

5. Conclusions

Prosthetic grafts should be reserved for situations where autogenous vein is not available to perform an access. The most commonly used prosthetic graft is e-PTFE based. Newer advances in medication bonding to decrease thrombosis and formation of intimal hyperplasia may be promising. In addition, various graft characteristics such as flared-end and stretch may provide better patency. Biologic grafts are being tested; however, at this point data are lacking to show superiority over prosthetic grafts. This area is a fertile ground for randomized clinical trials in the search for a man made or biologic graft that would equal autogenous vein in patency and complication rates.

6. Abbreviations

Chronic kidney disease (CKD), hemodialysis (HD), peritoneal dialysis (PD), arteriovenous fistula (AVF), polytetrafluoroethylene (PTFE), expanded PTFE (ePTFE), transposed brachialbasilic vein (TBB), percutaneous angioplasty (PTA)

7. Definitions

Primary Patency: Interval of time from access placement to any intervention necessary tomaintain patency of access. Assisted Primary Patency: Interval of time from access placement to time of intervention necessary to maintain the functionality of the access. Secondary Patency: Interval of time from access placement to access abandonment including intervening surgical or endovascular manipulations

8. Manufacturer and graft

Graft	Graft Material	Manufacturer
Artegraft®	Bovine Carotid Artery Heterograft	Artegraft Inc., New Brunswick, NJ
Atrium Adventa™ VXT	Reinforced ePTFE	Atrium Medical Corp., Hudson, NH
Boston Scientific	ePTFE	Boston Scientific Corp., Natick, MA
Carboflo®	Carbon impregnated ePTFE	C.R. Bard Inc., Murry Hill, NJ
CryoVein®	Cryopreserved femoral vein	CryoLife Inc., Atlanta, GA
Flixene™	Trilaminate membrane	Atrium Medical Corp., Hudson, NH

Graft	Graft Material	Manufacturer
Gore-Tex®	ePTFE	W.L. Gore and Associates, Flagstaff, AZ
Gore-Tex® Intering® graft	reinforced ePTFE with radial support	W.L. Gore and Associates, Flagstaff, AZ
Gore-Tex® stretch graft	Stretch ePTFE	W.L. Gore and Associates, Flagstaff, AZ
Gore-Tex® stretch graft with removable rings	Stretch ePTFE with removable rings	W.L. Gore and Associates, Flagstaff, AZ
Impra®	ePTFE	C.R. Bard Inc., Murry Hill, NJ
Dardik Biograft™	Modified human umbilical vein	Meadox Medicals Inc., Oakland, NJ.
ProCol®	Bovine mesenteric vein heterograph	Hancock Jaffe Laboratories Inc., Irvine, CA
Propaten®	Bioactive heparin convalently bound to ePTFE	W.L. Gore and Associates, Flagstaff, AZ.
SynerGraft® 100	Bovine Ureter Graft	CryoLife Inc., Atlanta, GA
Thoratec® Vascular Access Graft	Polyurethane	Thoratec Corp., Pleasanton, CA
VascuLink™	Self-sealing polycarbonate urethane graft	Lemaitre Vascular Inc., Burlington, MA
Vascutek®	Self-sealing ePTFE	Tarumo Interventional Systems, Somerset, NJ
Vectra®	Proprietary blend of segmented polyetherurethaneurea and a siloxane	C.R. Bard Inc., Murry Hill, NJ
Venaflo® II	Cuffed ePTFE	C.R. Bard Inc., Murry Hill, NJ

9. Disclaimer

The views expressed in this article are those of the authors and do not necessarily reflect the official policy of the Department of Army, the Department of Defense, or the US government.

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