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NOVEL RISK MARKERS IN THE CHRONIC KIDNEY DISEASE PATIENT

NEW INSIGHTS INTO THE WASTING-INFLAMMATION AXIS

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ABSTRACT

Patients with chronic kidney disease (CKD) have a risk of cardiovascular morbidity and mortality that is 20-30 fold higher than that of a similar person without CKD. While traditional (i.e Framingham) risk factors certainly contribute, they cannot by themselves explain this exacerbated mortality burden. Instead, novel factors such as inflammation and protein-energy wasting (PEW; i.e. a newly proposed term for loss of body protein mass and fuel reserves), may play a far more important role for vascular disease than in the general population. This work tries to further characterize the inflammation-PEW interplay as well as other possible consequences of uremic inflammation.

Study I explores the major determinants and clinical consequences of anorexia in hemodialysis patients. We found that self-reported poor appetite was associated with inflammation and poor outcome. The severity of symptoms associated to poor appetite was increased in men as compared to women.

Study II explores the major determinants and clinical consequences of muscle atrophy in incident and prevalent dialysis patients. We found that visual signs of muscle atrophy are more common in female dialysis patients and progressively associated with inflammation, poor nutritional and anthropometric status and increased mortality.

Study III investigates the prognostic impact of the novel biomarker soluble tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) on mortality in patients undergoing hemodialysis. We found that sTWEAK may be an additive, but not a primary marker, of the high mortality rate seen in hemodialysis patients with systemic inflammation.

Study IV assesses the clinical and biochemical implications of low triiodothyronine levels in end-stage renal disease patients with normal thyroid function. We found that low triiodothyronine levels in biochemically euthyroid CKD patients are independent predictors of all-cause and cardiovascular-related mortality, perhaps due to an intimate association with inflammation.

Study V discerns the major determinants and plausible contribution of reduced telomere length on the mortality of dialysis patients. Age, male gender and systemic inflammation were important contributors to reduced telomere length in CKD patients, which may constitute a new mortality risk factor in this population.

Keywords: Chronic kidney disease, inflammation, protein-energy wasting, dialysis, sex, anorexia, muscle atrophy, telomere, thyroid hormones

LIST OF PUBLICATIONS

I. **Carrero JJ,** Qureshi AR, Axelsson J, Avesani CM, Suliman ME, Snaedal-Jonsdottir S, Bárány P, Alvestrand A, Lindholm B, Heimbürger O and Stenvinkel P. *Comparison of nutritional and inflammatory markers in dialysis patients with poor appetite.* Am J Clin Nutr. 2007 March; 85(3):695-701

II. **Carrero JJ,** Chmielewski M, Heimbürger H, Axelsson J, Snaedal S, Suliman ME, Bárány P, Lindholm B, Stenvinkel P and Qureshi AR. *Muscle atrophy, protein-energy wasting, inflammation and clinical outcome in pre- and hemodialysis patients.* Clin Nutr. 2008 Aug; 27(4): 557-564

III. **Carrero JJ,** Ortiz A, Qureshi AR, Martin-Ventura JL, Bárány P, Heimbürger O, Marron B, Metry G, Snaedal S, Lindholm B, Egido J, Stenvinkel P, Blanco-Colio JL. *Additive effects of soluble TWEAK and inflammation on mortality in hemodialyzed patients.* Clin J Am Soc Nephrol (*In Press*)

IV. **Carrero JJ,** Qureshi AR, Axelsson J, Yilmaz MI, Rhenmark S, Witt MR, Bárány P, Alvestrand A, Heimbürger O, Lindholm B and Stenvinkel P. *Clinical and biochemical implications of low thyroid hormones (total and free forms) in euthyroid patients with chronic kidney disease.* J Intern Med 2007 Dec;262(6):690-701

V. **Carrero JJ,** Stenvinkel P, Fellström B, Qureshi AR, Lamb K, Heimbürger O, Bárány P, Radhakrishnan K, Lindholm B, Soveri I, Nordfors L, Shiels PG. *Telomere attrition is associated to inflammation, low fetuin-A levels and high mortality in prevalent hemodialysis patients.* J Intern Med. 2008 Mar;263(3):302-12

CONTENTS

LIST OF ABBREVIATIONS

CKD	Chronic kidney disease
CRP	C-reactive protein
CVD	Cardiovascular disease
ESRD	End-stage renal disease
fT3	Free triiodothyronine
fT4	Free thyroxine
GFR	Glomerular filtration rate
HD	Hemodialysis
IGF-1	Insulin-like growth factor-1
IL	Interleukin
PBMCs	Peripheral blood mononuclear cells
PD	Peritoneal dialysis
PEW	Protein-energy wasting
SGA	Subjective global assessment
sTWEAK	Soluble Tumor necrosis factor-like weak inducer of apoptosis
T3	Total triiodothyronine
T4	Total thyroxine
TNF-α	Tumor necrosis factor-α
8-OHdG	8-hydroxy-2'-deoxyguanosine
SHBG	sex hormone-binding globulin

1 INTRODUCTION

1.1 CHRONIC KIDNEY DISEASE – STILL MANY UNKNOWNS

The prevalence of chronic kidney disease (CKD) has reached epidemic proportions. Nationally representative samples from USA and Taiwan report a CKD prevalence of 12-13% [1, 2], though most of these individuals may not be aware of their condition. CKD contributes to around a tenth of all deaths, which is a similar proportion to that from smoking or obesity [2]. The main cause for premature death in this population group is cardiovascular disease (CVD) [3]. At the moment there seems to be little hope in sight for improvements as several large randomized controlled trials have consistently been unable to show a survival benefit of new treatment strategies, such as increased dialysis dose[4, 5], intensified nutrition[6], homocysteine lowering therapy [7], normalization of hemoglobin with erythropoietin [8, 9], lipid lowering with statins [10] and treatment with ACE-inhibitors [11]. It is clear that there are still too many unknowns in the pathophysiology of CKD and its complications. Risk factors and therapeutic targets in the general population do not seem to explain the CKD patient's prognosis. We may have been looking at the wrong place [12].

There is an undeniable link between kidney dysfunction and cardiovascular risk [13]. The cardiovascular risk is increased early in the evolution of CKD (already at a glomerular filtration rate [GFR] of about 75 ml/min), and increases continuously with the deterioration of renal function [14-16]. In light of these results, even subtle kidney dysfunction it has been proposed as a condition needing intensive preventive CVD strategies [16]. Recent data shows that CVD is independently associated with kidney function decline [17, 18], so it could be hypothesized that the relationship between CKD and CVD is, indeed, reciprocal or bidirectional, probably leading to a vicious circle [19]. The biological changes that occur in CKD may promote CVD at an accelerated rate which cannot be fully explained by conventional risk factors [17]. Also, with a further reduction in renal function, retention of uremic toxins and metabolic alterations are likely to contribute further to the high risk of CVD.

1.2 CARDIOVASCULAR RISK FACTORS IN CKD – A COMPLEX PUZZLE

Many risk factors and metabolic alterations observed in the uremic *milieu* may contribute to the excessive risk of CVD in this population. ***Figure 1*** depicts risk markers that have been demonstrated (or speculated) to be associated with and/or to promote CVD. Traditional (i.e. Framingham) risk factors (age, hypertension, smoking, left ventricular hypertrophy, dyslipidemia and diabetes mellitus) predict cardiovascular mortality in patients with mild-moderate CKD [20].

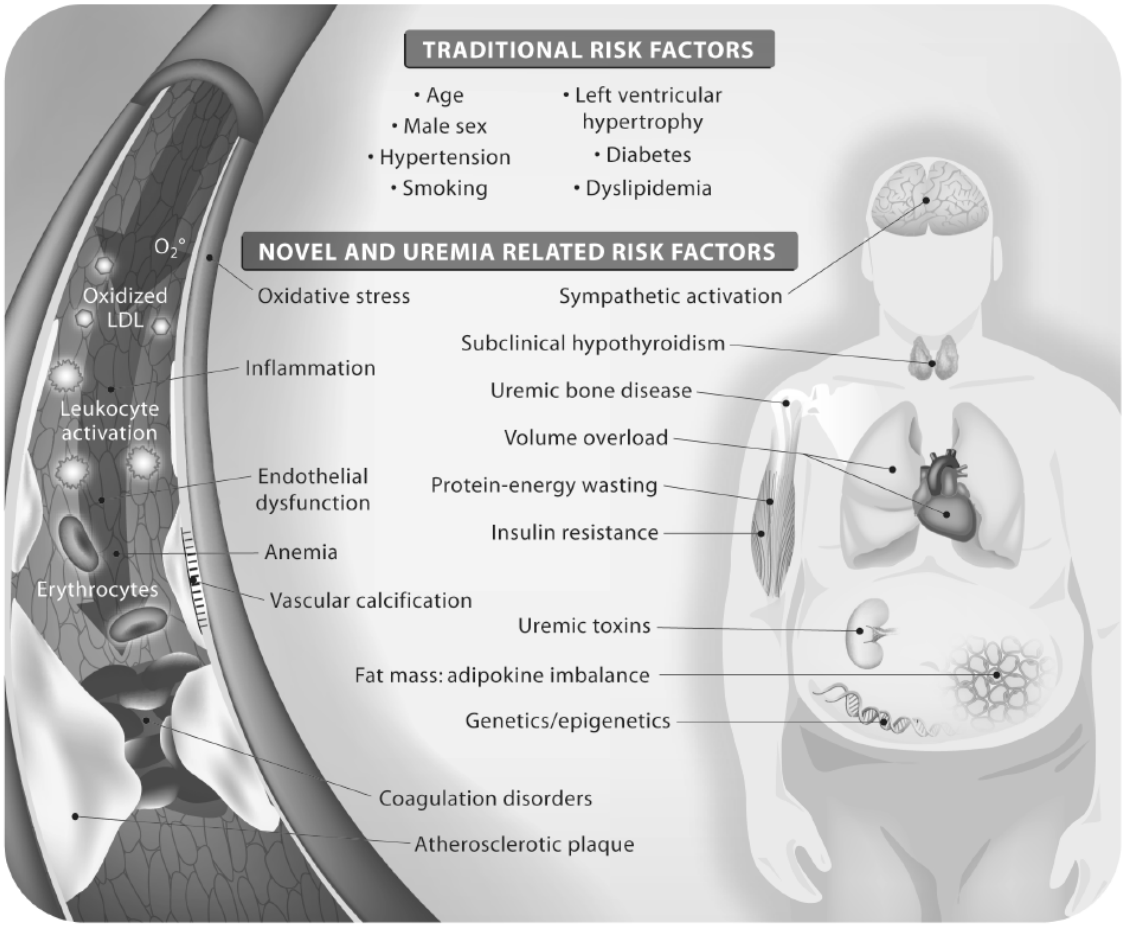


Figure 1. Schematic representation of traditional and novel (or uremia-specific) cardiovascular risk factors in chronic kidney disease. Printed with permission from [21].

However, "novel" risk factors for CVD, such as inflammation, endothelial dysfunction, sympathetic overactivation, protein-energy wasting (PEW; i.e. a newly proposed term for loss of body protein mass and fuel reserves[22]), oxidative stress, vascular calcification, volume overload and hyperhomocysteinemia, are highly prevalent in these patients and seems to play a far more important role for vascular disease than

in the general population [12, 23, 24]. Some studies have reported that in advanced CKD, traditional risk factors for CVD seem to explain only partially the increased cardiovascular risk [24-26]. However, when discussing the complicated interplay between traditional and novel risk factors one should keep in mind that risk factors seldom operate in separate rigid compartments; i.e. strong associations are usually found between traditional and novel risk factors.

The association between traditional risk factors and cardiovascular outcome becomes even more complicated with the so-called "reverse epidemiology" phenomenon in CKD: i.e. the well-known association between established risk factors in the general population (such as hypertension, hypercholesterolemia, obesity and hyper-homocysteinemia) does not exist, or even appears to be reversed, in patients with advanced CKD[27] and also in those CKD patients not yet on dialysis [28]. One reason for this could be that different time profiles exist for different risk factors in the different populations as premature deaths in CKD patients preclude the impact of complications, which are more important for long-term mortality. Furthermore, the common occurrence of persistent inflammation and/or PEW in advanced CKD seems to a large extent account for this seemingly paradoxical association between traditional risk factors and cardiovascular outcome in this patient group [29-31].

1.3 PROTEIN-ENERGY WASTING AND INFLAMMATION – KEY PLAYERS

The mechanisms leading to PEW of advanced kidney disease are not fully elucidated. Clearly, the nutritional and metabolic derangements in advanced CKD cannot be solely attributed to "inadequate" nutrient intake but also, for instance, inflammation, pre-existing comorbidities, insulin resistance, metabolic and hormonal derangements, inadequate dialysis dose, nutrient losses in the dialysate or increased energy expenditure [22, 32]. Irrespective of the specific etiologic mechanisms, it appears that the common pathway for all the metabolic derangements is related to exaggerated protein degradation relative to protein synthesis [32]. Furthermore, the metabolic and nutritional effects of chronic inflammation are many and closely mimic the PEW observed in patients with advanced CKD especially with regard to exaggerated protein catabolism, suggesting a cause and effect relationship [22, 32-34].

The mean concentrations of most cytokines and pro-inflammatory molecules are several-fold higher in end-stage renal disease (ESRD) patients than in normal healthy controls [35-44]. It is becoming more apparent that the etiology of inflammation is also multifactorial in patients with advanced CKD much like uremic wasting [22, 32-34]. These causes may include, among others, intercurrent clinical events, renal retention, oxidative stress, sympathetic overactivity, overhydration, dialysis procedure and/or bacterial infections [45, 46].

Owing to its high prevalence in patients with ESRD, chronic inflammation is proposed as a potential catabolic factor that worsens the nutritional status of these patients. When one considers the metabolic effects of chronic inflammation, the nutritional consequences are evident (***Figure 2***). Pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α, are thought to play integral roles in muscle catabolism in models of inflammatory diseases [34, 47, 48]. Elevated levels of IL-6 are associated with increased muscle proteolysis [49-51] and the administration of IL-6 receptor antibody in mice can block this effect [52]. Further, animal studies have also shown an increased skeletal muscle protein breakdown with TNF-α administration [53]. This issue is further developed in *Study II and III*.

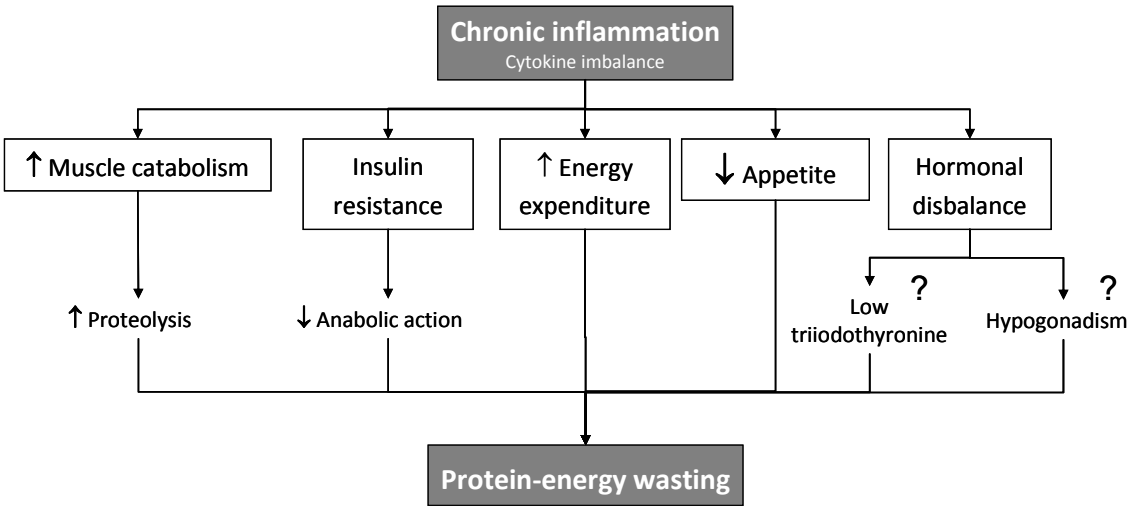


Figure 2. Potential mechanisms linking chronic inflammation with the development of protein-energy wasting in CKD. Extended from [32].

Anorexia or suppression of nutrient intake is another well established metabolic effect of inflammation. Clearly, pro-inflammatory cytokines such as IL-6, IL-1 and TNF-α are capable of inhibit the desire to eat [54, 55]. Animal studies suggest that the direct effects of these cytokines on the satiety center probably explain this finding. A cross-sectional

study by Kalantar-Zadeh *et al.* [56] showed that the extent of anorexia is closely and directly related to the level of plasma pro-inflammatory cytokine concentrations in patients with coronary heart disease. In addition, inflammation may contribute to the abnormal plasma amino acids profile present in many uremic patients [57]. This finding is not only limited to circulating cytokines, but also to the novel adipokines leptin [58], visfatin [59] and adiponectin [60]. This issue is further developed in *Study I*.

The altered metabolic *milieu* in CKD affects the secretion of hormones and the response of target tissues, causing endocrine dysfunctions [61]. These may include the growth hormone and insulin-like growth factor (IGF)-1 axis, the presence of subclinical hypothyroidism and/or hypogonadism. Very recently, the commonly observed low thyroid hormone levels in CKD patients have been suggested as an intermediate link between the inflammatory stress, subsequent protein-energy wasting and impaired cardiovascular response [62]. CKD *per se* causes alterations in thyroid hormones [63, 64] mainly characterized by a decrease in total triiodothyronine (T3) and free triiodothyronine (fT3) concentrations. This condition is present in about one fourth of CKD patients [65]. Traditionally, decreases in plasma T3 concentration have been interpreted as an attempt to conserve body energy stores by reducing the metabolic rate [66]. However, it has been suggested that decreased active thyroid hormone levels could be part of the deranged neuroendocrine/pro-inflammatory system that is associated with CKD [62]. Thus, low T3 levels may not just be an innocent bystander and could be involved in the increased mortality risk in this patient group. This could be due to plausible links between a state of subclinical hypothyroidism and low-grade persistent inflammation [62]. This issue is further developed in *Study IV*.

Other suggested inflammatory pathways leading to PEW not explored in this thesis are the increase of resting energy expenditure [67-69] (which would in turn lead to sarcopenia), and the impairment of insulin action by pro-inflammatory cytokines [70, 71].

1.4 INFLAMMATION AND PREMATURE CELL SENESCENCE

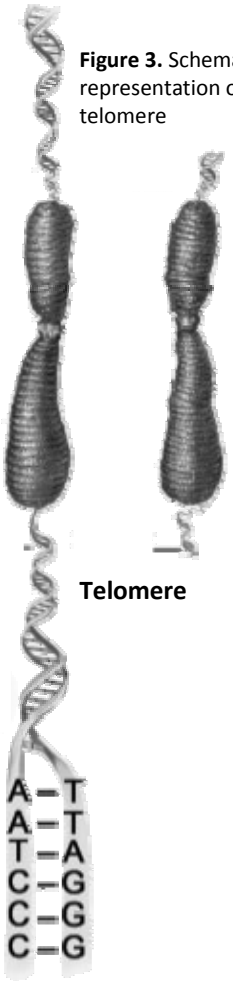


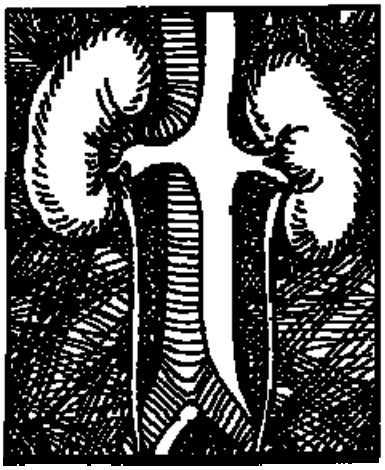
Figure 3. Schematic representation of a telomere

The length of telomeres, the specialized terminal regions of chromosomes, from circulating peripheral blood leukocytes has been proposed as a systemic index of biological ageing. Human telomeres consist of tandem repeats of a noncoding TTAGGG hexamer (***Figure 3***). Telomere length is to a large extent genetically determined, yet in proliferating cells telomeric sequences are lost with each cell division due to the inability of DNA polymerase to complete the chromosome termini, which is reflected in age-dependent telomere attrition. In addition, telomere length can be further modulated by environmental factors, such as oxidative stress [72, 73] or, as recently suggested, inflammation [74, 75].

Interestingly, leukocyte telomere length has been identified as a novel risk factor for CVD in the general population [75-78], supporting the hypothesis that premature biological ageing might contribute to the risk of coronary events. It has been recently suggested that leukocyte telomere length chronicles the cumulative burden of oxidative stress and inflammation over a life course [79]: First, inflammation, which enhances the turnover rate of leukocytes, increases the pace of leukocyte telomere length shortening. Second, as oxidative stress increases the rate of telomere attrition per cell division, it also accelerates leukocyte telomere length shortening. Both inflammation and oxidative stress are major determinants in atherosclerosis, a process that is contingent on the continued recruitment of leukocytes [80] and increased oxidative stress at the interface of the endothelium with blood [81]. Such a process usually takes place over the course of many years, and it would be expressed by a higher pace of telomere length attrition and shorter telomere length.

CKD patients are subject to a process of accelerated atherogenesis due to uremia-associated factors, mainly inflammation and oxidative stress [12]. These factors likely affect the concordance between the occurrence of cardiovascular events and age of manifestation of CKD [3], as both are known features of senescent tissues. Thus, inter-

individual variation in risk for CVD might result, to some extent from variation in the rate of biological ageing [77]. Recently, a small number of publications focused on the investigation of telomere and telomerase biology in peripheral blood mononuclear cells (PBMCs) of patients with CKD and premature cellular senescence has recently been proposed as an emerging CVD risk factor for patients with CKD [82]. To date, this hypothesis had not been proven. This issue is further developed in *Study V*.



2 AIMS

The overall aim of the investigations summarized in this thesis was to increase our understanding of some of the risk factors involved in the high mortality rates seen in patients with chronic kidney disease, with special emphasis on the characterization of the PEW-inflammation interplay.

The specific aims of the studies were:

- To investigate the prognostic impact and main determinants of impaired feeding behavior and muscle atrophy in ESRD patients **(*Studies I and II*)**.
- To investigate the prognostic impact of the novel biomarker soluble Tumor necrosis factor-like weak inducer of apoptosis (sTWEAK) on mortality in patients undergoing hemodialysis **(*Study III*).**
- To assess the clinical and biochemical implications of low triiodothyronine levels in end-stage renal disease patients with normal thyroid function **(*Study IV*).**
- To discern the major determinants and plausible contribution of reduced telomere length on the mortality of dialysis patients **(*Study V*).**

3 PATIENTS AND METHODS

The thesis work has been developed with data obtained from two patient materials whose collection is/has been coordinated by the Division of Renal Medicine, Department of Clinical Sciences, Intervention and Technology (CLINTEC), Karolinska Institutet. Therefore, patient phenotype was analyzed *post hoc* using collected data and, in some cases, by making new analysis from frozen samples.

3.1 PARTICIPANTS

The **Mapping of Inflammatory Markers in Chronic Kidney Disease 1 *(MIMICK-1)* cohort** is a patient population material consisting of prevalent patients undergoing hemodialysis (HD) at the Karolinska University Hospital at Huddinge, Stockholm (including as well its satellite dialysis units at Kungsholmen Södersjukhuset), Sophiahemmet, Danderyds Hospital and Uppsala Academic Hospital. This study originally aimed at investigating the variability of inflammatory parameters in prevalent HD patients over time. Recruitment of the patients occurred from October 2003 through March 2004. All patients who were currently receiving regular therapy at any of the units were invited to participate (n=254); 6 patients declined, and one patient with HIV infection was excluded. The 247 eligible patients were then followed up for 12 weeks, during which time the concentration of high-sensitivity C-reactive protein (hs-CRP) was measured weekly. Patients were further excluded from the study if presenting less than six of these weekly hs-CRP determinations. Eleven patients were excluded because of insufficient baseline clinical information; seven were excluded because of insufficient hs-CRP measurements; and one patient died. The remaining 228 patients were further followed up for assessment of overall and cardiovascular mortality in relation to biochemical markers, and constitutes the basis of *Studies I, II, III and V*, which only contain baseline measurements. Because of some missing values (due to impossibility to make an assessment or lack of plasma available), the number of patients considered in each of the studies varies as summarized in **Table 1.** The Ethics Committee of the Karolinska Institutet, Sweden, approved the study (no. 03/415).

The **Malnutrition, Inflammation and Atherosclerosis *(MIA) cohort*** is a cohort consisting of incident patients with CKD stage 5 (GFR<15 mL/min) sampled close to the start of renal replacement therapy (either HD or peritoneal dialysis) from the renal program of the Karolinska University Hospital at Huddinge, Sweden. Patients are further followed up till death or transplantation. Also, patients are invited to attend additional visits approximately after 1 year and 2 years of dialysis. This ongoing prospective cohort study started in 1994, and a descriptive protocol has been described in more detail [19]. The study exclusion criteria were age below 18 years or above 70 years, clinical signs of acute infection, active vasculitis or liver disease at the time of evaluation, or unwillingness to participate. This cohort constitutes the patient material included in *Studies II, III and IV*. The number of patients considered in each of the studies considered in this thesis is summarized in ***Table 1*** and corresponds to availability of the assessment and/or plasma for future determinations. The Ethics Committee of the Karolinska Institute, Sweden, approved the study (no 273/94 and 008/98).

Table 1. Basic description of the individual studies

Study	**Cohort**	**Subjects**	**Main variables studied**
I	MIMICK-1	223	*Primary variable:* Self-reported appetite *Explanatory variables:* BMI, lean body mass, fat body mass, mid-arm muscle circumference, handgrip strength, IL-6, CRP
II	MIMICK-1 MIA (baseline)	221 265	*Primary variable:* Muscle atrophy assessment *Explanatory variables:* Handgrip strength, lean body mass, mid-arm muscle circumference, BMI, IL-6, CRP, IGF-1
III	MIMICK-1 MIA (1 year) CONTROLS	208 79 40	*Primary variable:* sTWEAK *Explanatory variables:* IL-6, IGF-1, handgrip strength, CRP
IV	MIA (baseline)	210	*Primary variable:* T3, fT3 *Explanatory variables:* Thyroid stimulating hormone (TSH), total thyroxine (T4), free thyroxine (fT4), IL-6, CRP
V	MIMICK-1	175	*Primary variable:* Telomere length *Explanatory variables:* IL-6, CRP, Fetuin-A

3.2 CLINICAL AND PHYSICAL EXAMINATION

Each patient's medical chart was reviewed, extracting data pertaining to underlying kidney disease, history of CVD, diabetes, other comorbid conditions, common

medication and survival. Besides, in the MIMICK-1 cohort, the comorbidity score was determined according to the Davies Comorbidity Index [83], which takes into account 7 specified comorbidity domains for patients receiving dialysis; malignancy, ischemic heart disease, peripheral vascular disease (including cerebrovascular disease), left ventricular heart failure, diabetes mellitus, systemic collagen vascular disease, and other significant pathology (eg, cirrhosis).

CVD was defined by clinical history or signs of ischemic cardiac disease, and/or presence of peripheral vascular disease and/or cerebrovascular disease. Smoking habits were recorded as current smokers, former smokers and non smokers. GFR in the MIA cohort was estimated as the mean of urea and creatinine clearances. In the MIMICK-1 cohort, total time on hemodialysis was annotated as vintage time.

Survival was determined from the day of examination and sample collection, with no loss of follow-up of any patient. Cardiovascular mortality was defined as death as a result of coronary heart disease, sudden death, stroke, or complicated peripheral vascular disease. Causes of death were registered by a nephrologist blind to other clinical or biochemical data of the patients and to the objectives of the studies.

Nutritional status and protein-energy wasting

The presence of protein-energy wasting was evaluated by means of the subjective global assessment questionnaire (SGA) [84]. This assessment was completed either at the time of or within 1 week of blood sample collection and after the HD session in the MIMICK-1 cohort. SGA includes six different components: three subjective assessments that are answered by the patients and concern the patient's history of weight loss, incidence of anorexia, and incidence of vomiting, and three assessments that are performed by the evaluators and are based on the subjective grading of muscle wasting, the presence of edema, and the loss of subcutaneous fat. On the basis of these assessments, each patient received a nutritional status score: 1 = normal nutritional status, 2 = mild malnutrition, 3 = moderate malnutrition and 4 = severe malnutrition. For the purpose of the studies, protein-energy wasting was defined as SGA>1.

The specific assessments regarding self-reported appetite and the presence of muscle atrophy from the SGA were analyzed in studies I and II. Regarding the self-reported appetite assessment, all patients were asked to grade their appetite themselves according to the following scale: 1=good, 2=sometimes bad, 3=often bad, and 4=always bad.

The grading of muscle atrophy was assessed by a specially trained nurse examining the temporalis muscle, the prominence of the clavicles, the contour of the shoulders (rounded indicates well-nourished; squared indicates wasting), visibility of the scapula, the visibility of the ribs, and interosseous muscle mass between the thumb and forefinger, and the quadriceps muscle mass. The signs of muscle atrophy were scored as follows: 1) no signs, 2) mild signs, 3) moderate signs and 4) severe signs of muscle atrophy.

Anthropometric evaluation

Body composition was assessed in both cohorts and in the healthy controls by using skinfold thicknesses of biceps, triceps, subscapular, and suprailiac with a conventional skinfold caliper (Cambridge Scientific Instruments, Cambridge, MD). Lean body mass (LBM) and fat body mass (FBM) were estimated by means of dual-energy x-ray absorptiometry (in the MIA cohort) using the DPX-L device (Lunar Corp, Madison, WI) or according to a theoretical formula (in the MIMICK-1 cohort) based on skinfold thicknesses and body density [85]. In any case, lean body mass index (LBMI) and fat body mass index (FBMI) was defined as the total lean body mass in kilograms divided by the square of the height in meters [86]. Midarm circumference (MAC) was measured with a plastic tape ruler and was normalized to measurements from healthy subjects; midarm muscle circumference (MAMC) was calculated by using the following formula: MAMC = MAC - (3.1416 x triceps skinfold thickness/10) [85]. Handgrip strength was measured using a Harpenden Handgrip Dynamometer (Yamar, Jackson, MI, USA) in the dominant hand (in the incident dialysis patients) or in the hand without fistula (in the prevalent HD group) and normalized with measurements from healthy subjects [87]. These assessments were completed either at the time of or within one week of blood sample collection and after the HD session in the MIMICK-1 cohort.

3.3 BLOOD VARIABLES

Blood samples were collected after an overnight fast in the MIMICK-1 cohort and before the dialysis session in the HD cohort after the longest interdialytic period. Plasma samples were kept frozen at –70 °C if not analyzed immediately. In both cohorts, determinations of creatinine, serum albumin (bromcresol purple), haemoglobin, hypochromic red blood cells, total, LDL- and HDL-cholesterol, triglycerides, white blood cells count and high sensitivity C-reactive protein (CRP) (nephelometry) were performed by routine procedures at the Department of Clinical Chemistry, Karolinska University Hospital Huddinge. The rest of biochemical markers were mostly performed at the Research Laboratory of the Department of Renal Medicine, Huddinge. Plasma IL-6, IL-10, IGF-1, T3, fT3, T4, fT4, TSH and thyroxine-binding globulin (TGB) concentrations were measured using commercial kits available for an Immulite Automatic Analyzer (Siemens Medical Solutions, Los Angeles, CA, USA). Plasma concentration of soluble vascular adhesion molecule (sVCAM)-1 was measured using commercially available ELISA kits (R&D System Inc., Minneapolis, MN, USA). The serum concentration of 8-hydroxy-2'-deoxyguanosine (8-OHdG) was determined by a competitive ELISA kit (Institute for the Control of Ageing, Fukuroi, Shizuoka, Japan), while serum fetuin-A levels were measured by a sandwich ELISA (Epitope Diagnostics, Inc, San Diego, CA, USA). Finally, plasma concentrations of sTWEAK were determined with commercially available ELISA kits (BMS2006INST; Bender MedSystems).

3.4 TELOMERE LENGTH

Telomere length was analyzed blindly at the Division of Cancer Sciences and Molecular Pathology, Dept. Surgery, Glasgow Royal Infirmary, Glasgow, Scotland, UK. DNA was extracted from peripheral blood leukocytes following standard procedures and telomere lengths in the DNA samples determined following the method of Cawthon [88]. This method measures the average telomere length in genomic DNA by determining the ratio of telomere repeat copy number to single copy gene copy number (T/S ratio) in experimental samples relative to a control sample DNA which

had mean terminal restriction fragment lengths determined previously by standard southern blotting procedures [89, 90].

3.5 STATISTICAL ANALYSES

All statistical analyses were performed using statistical software SAS version 9.1.3 (SAS Campus Drive, Cary, NC, USA 27513). Normally distributed variables were expressed as mean ± SD and non-normally distributed variables were expressed as median and range (minimum and maximum) or interquartile range (25^{th}-75^{th} percentile, IQR). Also, categorical values were expressed as number and percentage, unless otherwise indicated. Statistical significance was set at the level of $p< 0.05$.

Comparisons between two groups were assessed with the Student's unpaired t-test and Mann-Whitney test or χ^2 test, as appropriate. Differences among more than two groups were analyzed by analysis of variance (ANOVA) using one-way ANOVA or Kruskal-Wallis test, as appropriate. As many values were not normally distributed, Spearman's rank correlation (ρ) was used to determine univariate correlations. Multivariate associations were performed by multiple regressions, stepwise multiple regressions and multinomial logistic regression analyses.

To evaluate the sensitivity and specificity of selected parameters as predictors of mortality, Receiving Operator Characteristics (ROC) analyses were performed [91]. The optimum cut-off value, with the combination of the highest sensitivity and specificity, was calculated.

Survival analyses were made with the Kaplan-Meier survival curve or the Cox proportional hazard model. The relative risks for mortality were determined by multivariate Cox regression analysis and presented as hazard ratio (HR; 95% confidence intervals (CI)).

Other specific statistical analyses are discussed in each of the studies presented in this thesis.

3.6 STUDY CONSIDERATIONS

Strengths of the studies

Our analyses have a number of strengths, starting with the detailed phenotype of our patient materials and a long follow-up time. In this sense, we should acknowledge that thanks to the computerized national hospital registries, no patient was lost to follow-up. Another advantage not always considered in other epidemiological studies is that we censored for transplantation in our survival analysis; restoration of renal function cancels the prospective risk of dying. The cross-sectional nature of the studies in the present thesis does not allow us to infer causality from the results. However, in studies on etiology, diagnosis, prognosis, or adverse effects, observational studies are much more valid than randomized controlled trials [92].

Limitations of the studies

a) Regarding study design

The incident and prevalent nature of our cohorts (in some cases with explicit exclusion criteria) make them not representative of the general CKD population. The studies presented in this thesis are of *post hoc* nature. Also, the MIA cohort (*Studies II, III and IV*) was initiated in 1994 with recruitment still ongoing. As therapy recommendations have changed with time, the time of inclusion in the study may constitute a confounder in some aspects of our analysis. For instance, statin prescription may be higher now than 10 years ago (***Figure 4***).

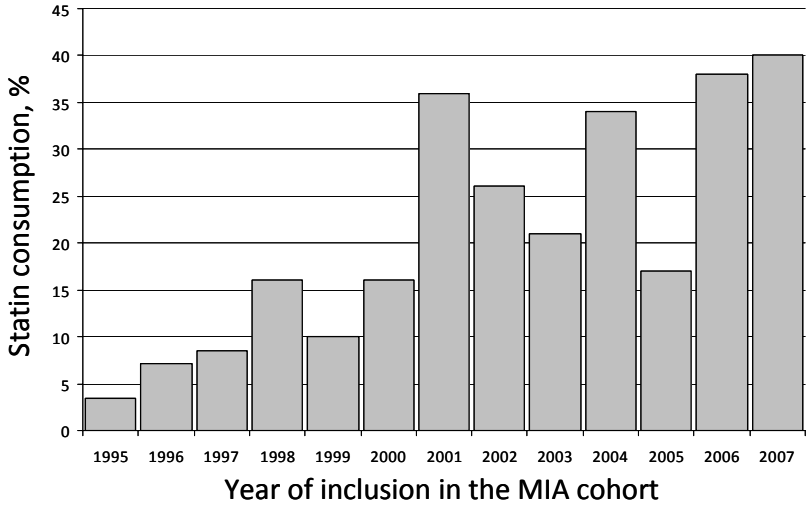


Figure 4. Proportion of patients consuming statins among newly yearly recruited patients in the MIA cohort.

b) Regarding clinical and biochemical characterization

Patient's diagnoses and causes of deaths rely on patient charts. For instance, clinical CVD or cardiovascular deaths have not always been confirmed by appropriate clinical investigations or autopsies, respectively. Therefore, we may have underestimated the true prevalence of CVD in our patient material [93].

We should acknowledge that body weight, skinfold thicknesses and DEXA may all be affected by fluid retention and hydration status in the ESRD setting [94]. BMI itself may not be a very precise parameter of nutritional status either, due to gross imbalance in fluid status in CKD. Also, PEW with loss of muscle mass (sarcopenia) is often associated with a relatively well-preserved fat mass in dialysis patients, resulting in small changes in BMI that may be obscured further by imbalances in fluid homeostasis [95]. Finally, PEW may also be present in overweight ESRD patients (BMI>25 Kg/m^2)[96].

SGA is commonly used to predict outcome in dialysis patients [97, 98]. SGA contains some patient reported questions that may have been under- or over-reported. Also, while we relied on trained nurses to conduct the SGA assessments, we cannot exclude the existence of intra- and inter-individual differences that may have influenced this assessment. In fact, the inter-interviewer agreement of SGA is only around 70% - mainly due to the differences of the patient's responses [99]. A recent validation report against the gold standard for nutrition, total-body nitrogen level, suggested that SGA differentiated severely malnourished patients from those with normal nutrition, but failed to reliably distinguish the degree of malnutrition [99]. However, we have not used the malnutrition gradation. Instead we dichotomized this variable into presence (SGA>1) or absence (SGA<1) of wasting signs. Furthermore, the reason for our analyses in *Studies I and II* was to show that even subjective and simple assessments, such as self-reported appetite and visual signs of muscle atrophy, may bring useful information into the clinic.

Measurements of thyroid hormones and sTWEAK have been done *post hoc* from frozen samples. Thus, we cannot exclude the possibility of sample degradation due to fridge storage or sample alteration due to repeated thawing and refreezing. However, when plotting these concentrations against sample age (time stored in the freezer) no differences were found, suggesting that serum concentrations were stable through storage time. It should be also noted that biochemical characterization is based on a

single time-point determination of molecules that are likely to vary over time and to be influenced by numerous factors and conditions. Despite this, there are to date dozens of studies showing that even a single cytokine measurement is a robust predictor of outcome [21, 33, 87]. Although CRP is a highly variable molecule, both single [21, 33, 87] and serial [100-102] measurements are able to predict the patient's outcome (***Figure 5***).

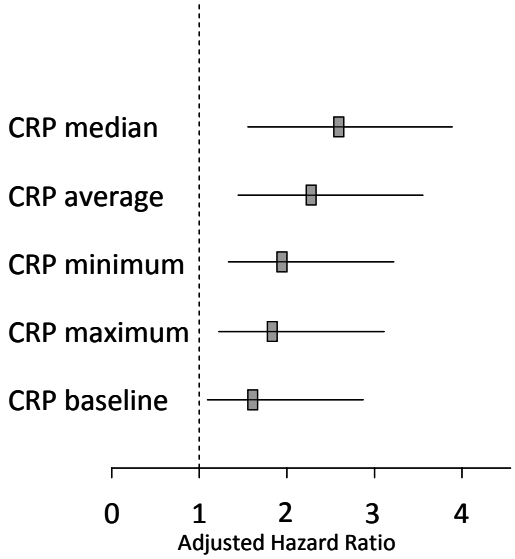


Figure 5. Adjusted Hazard ratios (adjusted for age, sex, vintage, comorbidity and access type) according to single or repeated weekly measures of CRP during a 12-week period in 224 prevalent HD patients. Single measurements consider baseline CRP, the maximum or the minimum value within the 12-month period. Repeated measurements consider the average or the median of 13 determinations. Adapted from [101].

c) Regarding statistical methods

Our sample sizes are relatively small and we cannot control for all possible confounders. In some cases, we may have introduced risk of overadjustment [103]. We have tried, to our best, to avoid the impact of collinearity by adjusting for factors pathophysiologically unrelated [104]. Although some researchers have argued against dichotomizing continuous variables in multiple regressions [105], we have sometimes done so in our analysis for simplification purposes, as we have a limited sample set not powered to discern risks per units of increase. We should also acknowledge the possibility of Type I errors (or a false positive) in decisions for which our patient materials were not adequately powered; and type II errors (or a false negative) in the case of random findings due to multiple testing.

4 MAIN RESULTS AND DISCUSSION

4.1 THE INFLAMMATION-WASTING INTERPLAY

The studies presented in this thesis further evidence for associations between inflammation, lack of appetite, muscle wasting and low thyroid hormone levels as well as the plausible interactions between low-grade persistent inflammation and PEW in patients with CKD, and how this dynamic interplay impacts on their survival rate.

Anorexia is a common finding in HD patients among whom its prevalence has been reported to range from 35 to 50% [56, 106-110], with a higher proportion of appetite loss on dialysis treatment days [111]. The immediate consequences of lack of appetite in HD patients are wasting and increased mortality [56, 106-108]. Also, appetite loss has been associated to higher non-access related hospitalization rates [56, 108]. As a good appetite is an important component of a satisfactory quality of life assessment, it would be expected that patients with poor appetite present a poorer quality of life [106] and increased symptoms of depression [108]. Multiple mechanisms may be involved in the pathophysiology of anorexia in CKD, and it is unclear how and to what extent a reduced appetite is a cause or a consequence of inflammation, PEW, or both. Confirming the observation of Kalantar-Zadeh et al [56], we could report in *Study I* that inflammation was strongly associated with self-reported appetite categories (***Figure 6***) in our prevalent HD patients [107].

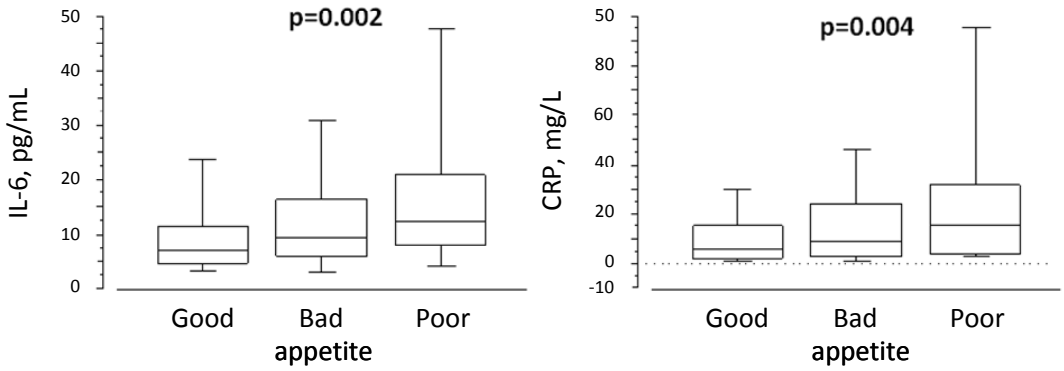


Figure 6: Levels of CRP and IL-6 according to self-reported appetite categories.

Indeed, levels of CRP, IL-6 or TNF-α have been linked to uremic anorexia [56, 107, 112]. Inflammatory cytokines may play an important role in the control of appetite, food intake, and energy homeostasis by interacting in several central nervous system pathways through both direct and indirect effects on specific brain areas [54, 113, 114]. On top of that, inflammation could also induce poor appetite through indirect mechanisms: this may include typical dental problems in CKD such as higher decayed, missing or filled teeth [115, 116] and the increased prevalence of periodontitis [117]. These issues will evolve into chewing and/or biting problems, making patients less likely to consume high-fiber foods such as bread, fruits or vegetables and therefore risking their essential nutrient intake. Because pro-inflammatory cytokines and oxidative stress may cause depressive behavior[118] and depression in CKD patients is closely linked to the presence of both inflammation and PEW [119], it has been hypothesized that depression could be another inflammatory mechanism leading to wasting through inhibition of the desire to eat. In fact, dialysis patients with poor appetite have been reported to be more often depressed [108]. Finally, recent evidence links the impairment of olfactory function with both the severity of malnutrition and the degree of inflammation in HD patients [120]. Normal olfactory function is required for full appreciation of the smell and taste of food, and this may be yet another mechanism leading to appetite loss in the uremic patient.

The development of muscle wasting may also be a result of chronic low-grade inflammation [121, 122]. Elevated levels of IL-6 in HD patients, for instance, are associated with increased muscle proteolysis [49-51] and the administration of IL-6 receptor antibody can block this effect [52]. We could further elaborate on this in *Study II*, as a subjective gradation of muscle atrophy related to increased concentration of inflammatory markers in both incident and prevalent dialysis patients [123]. TWEAK is a member of the TNF superfamily of structurally-related cytokines [124]. Although the mechanisms of action are not truly elucidated, it was recently demonstrated that sTWEAK acts as a strong muscle-wasting inducing agent through activation of the ubiquitin-proteasome and NF-kB pathways [125]. We could further elaborate on this hypothesis as higher levels of sTWEAK were linked to increased prevalence of PEW, decreased handgrip strength, and decreased IGF-1 levels in *Study III*. As several pro-inflammatory cytokines, including IL-6 also play a key role in the loss of muscle mass

and function [126], it is confirmatory that a condition with both increased IL-6 and sTWEAK was associated with significant reductions in two surrogate markers of PEW and muscle mass (i.e. IGF-1 and handgrip strength) in *Study III* (***Figure 7***). This phenotypic combination was associated to a worse outcome in an additive manner.

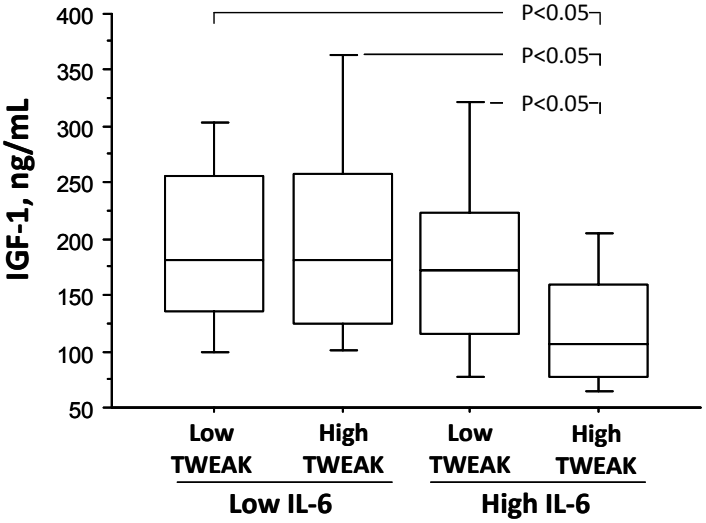


Figure 7. Levels of IGF-1 in prevalent HD patients with different levels of IL-6 and sTWEAK.

Finally, in *Study III* we could also show that low levels of T3, in the absence of thyroid alteration, were associated to inflammatory markers (***Figure 8***) and were a strong and independent predictor of the patient´s survival. Indeed, decreased active thyroid hormone levels are considered to be part of the deranged neuroendocrine/pro-inflammatory system that is associated with congestive heart failure [127]. This pro-inflammatory stimuli and in particular interleukin signaling, has been reported to downregulate the peripheral conversion of total thyroxine (T4) into T3 in both experimental [128, 129] and clinical studies [130, 131]. Additionally, several studies have shown that pro-inflammatory cytokines may inhibit T3 production or increase tissue turnover [128-132]. A proposed mechanistic rationale for this effect is that cytokine-induced competition for limited amounts of coactivators decreases hepatic type I iodothyronine 5´-deiodinase expression, resulting in decrease T3 production [133]. This observation may be of relevance for diseases like CKD where both persistent inflammation and PEW are common[19, 87].

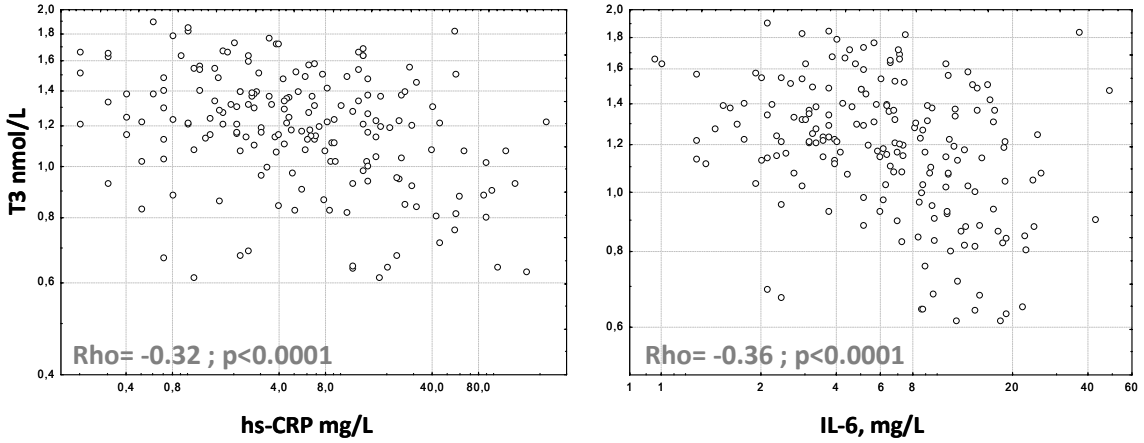


Figure 8. Spearman Rank univariate associations between T3 levels and markers of inflammation in clinically and biochemically euthyroid CKD patients.

4.2 INFLAMMATION AND PREMATURE CELL SENESCENCE

We could show in *Study V* significant associations between reduced telomere length and surrogate markers of inflammation (***Figure 9***).

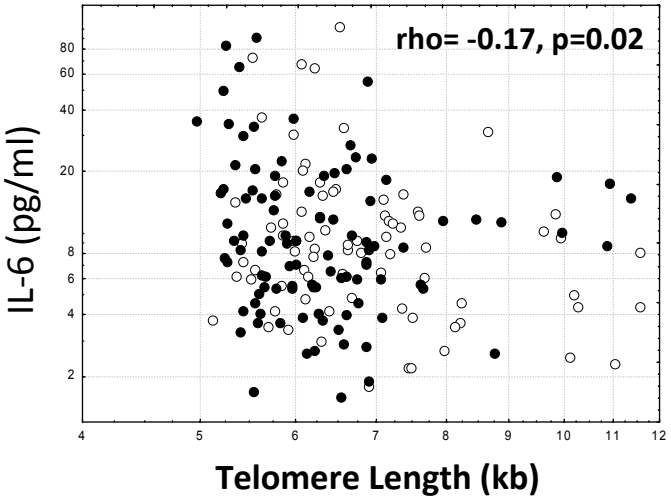


Figure 9. Spearman Rank univariate associations between telomere length and IL-6 levels in 175 prevalent HD patients

The consistent pattern of negative associations between inflammatory markers and telomere attrition as observed in our study further strengthens the hypothesis that the increased cell turnover due to inflammation is reflected in an increased telomere attrition and therefore shorter baseline telomere length. This was already been suggested by Aviv *et al*. [74] who described a negative correlation with CRP in women aged under 50 and by Fitzpatrick *et al*. [75] who found telomere length to be associated with IL-6 and CRP, albeit only for men or subjects aged 73 years or younger. Factors that affect telomere length are not well understood, but it has been demonstrated

that inflammation accelerates telomere attrition [134] and that prolonged oxidative damage inhibits telomerase activity and accelerates telomere shortening in vascular smooth muscle cells, an effect possibly mediated by the formation of 8-OH-dG [73], both features present in CKD. Approximately at the same time of publication as *Study V*, investigators from the Asklepios study [135] could study major determinants of telomere attrition in a population sample of 2500 Caucasian volunteers with a narrow age-range (35-55 years). While no significant associations were established with classical CVD risk factors such as cholesterol status and blood pressure, shorter telomere length was associated with increased levels of several inflammation and oxidative stress markers [135].

Confirming for the first time the hypothesis formulated by Tsirpanlis [82], we could demonstrate in *Study V* that reduced telomere length is associated with increased all-cause mortality in HD patients, independent of age, gender and IL-6. The loss of significance in the survival curves after adjustment for Fetuin-A levels may suggest a novel link between short telomeres, abnormal ossification of vessels and increased risk of CVD. In support of this, telomere length was associated with bone mineral density and inflammatory status, being shorter in women with osteoporosis [136]. On the other hand, it may represent the overall phenomenon of inflammation, as it is one of the major drivers of low fetuin-A levels in dialysis patients [137].

4.3 DOES SEX HAVE A ROLE IN UREMIC DISEASE RISK?

It is well known that the incidence of renal disease and the progression rate to ESRD is faster in men than in women [138-140]. However, the differences between the sexes in renal disease progression cannot be fully explained by differences in blood pressure or serum cholesterol levels. The underlying mechanisms for this sex disparity are potentially related to differences between the sexes in glomerular structure, glomerular hemodynamics, diet, variations in the production and activity of local cytokines and hormones, and/or the direct effect of sex hormones [141, 142]. Although female ESRD patients do not seem to have a survival advantage [143], the interaction with certain risk factors may be different than in males and that would entitle a risk factor protection. Thus, inflamed men on dialysis seem to have a worse survival as compared to women with the same condition [144]. Also, markers of

muscle mass are good predictors of survival in CKD men but not in women [145]. Some of the studies included in the thesis, pertained to variables or conditions potentially influenced by sex and the existence of sex differences has been hypothesized.

We could observe in *Study I* that, as confirmed by a later report [108], a lack of appetite is more commonly reported by women. In addition, a novel finding of this study is the observed sex differences in the relationship between inflammation and nutrition among patients with poor appetite. For men, but not for women, a poor appetite was accompanied by higher inflammation and worse nutritional status (evaluated by both biochemical and anthropometric measurements) (***Figure 10***). This may suggest that the severity of inflammation and the reduction in lean body mass is more severe among men with poor appetite as compared to women, and that the patient's sex is a contributing factor to the degree of appetite reported among these patients.

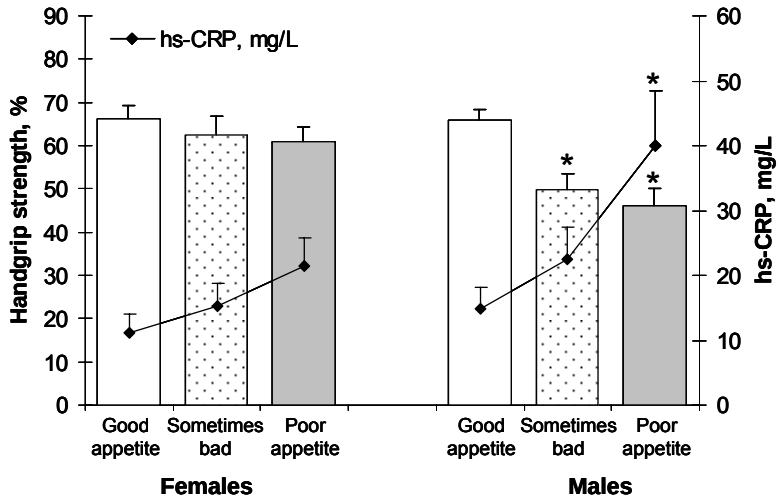


Figure 10. Levels of handgrip strength and CRP values among three self-reported appetite categories in male and female CKD patients. Only in men, a worsening in appetite categories was followed by increased inflammation and reduced handgrip strength values.

In *Study I*, we speculated, based on animal studies, on the existence of a plausible different regulation of leptin and ghrelin in men and women. We could further develop this hypothesis by measuring plasma ghrelin in the same cohort of prevalent HD patients [146]. When ghrelin levels were compared between male and female HD-patients with and without loss of appetite, they were significantly elevated in males only (**Figure 11**). Interestingly, recent data have shown that inflammation may influence ghrelin levels [147, 148]. Thus, the higher prevalence of inflammation present in anorectic males as compared to anorectic females in this cohort may, at least in part explain the differences observed in ghrelin concentration. In fact, whereas a significant correlation was found between ghrelin levels and both CRP (rho=0.16;

p<0.05) and IL-6 (rho=0.17; p<0.05) in men, no such correlations were demonstrated in women [146].

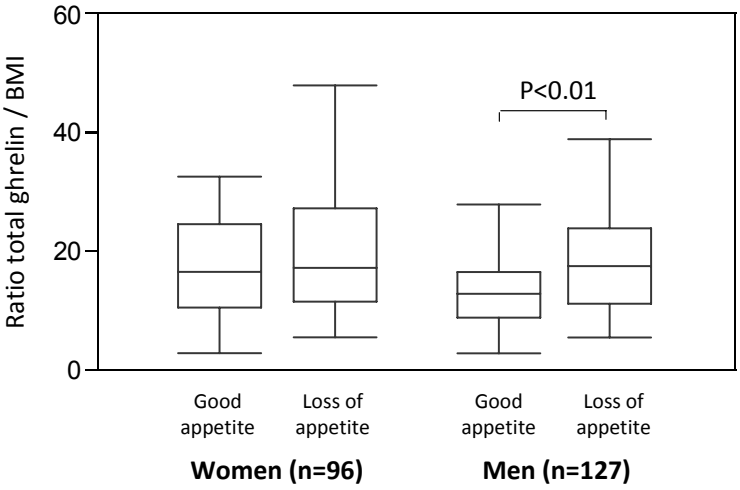


Figure 11. Ghrelin to BMI levels in men and women reporting good or reduced appetite. Modified from [146].

The prevalence of muscle atrophy was also higher among the female CKD patients in *Study II*, confirming previous reports [149, 150]. Although we do not have a clear explanation for this observation, it may be that different fat and muscle mass distribution in men and women may contribute to more clear visual signs of muscle atrophy in the female population.

In *Study V*, an increased telomere length in female HD-patients was observed as compared to their male counterparts (***Figure 12***), corresponding to the findings in both animal and human studies showing lower telomerase activity and less age-related telomere ablation in females [151, 152]. It has been hypothesized that estrogen may directly [153], or indirectly [154], exert protective effects on telomere length due to its anti-inflammatory and anti-oxidant properties.

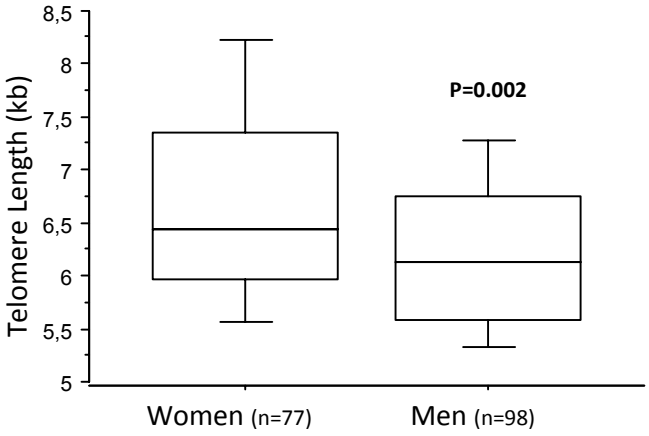


Figure 12. Telomere length in male and female prevalent CKD patients.

Interestingly, whereas a negative correlation between age and telomere length was found in female patients in our study, no such association was present in males,

whose reduced telomere lengths seem to be more closely associated with inflammation. Our results are in accordance with those by Fitzpatrick et al [75], showing that CRP was associated with reduced telomere length in aged males, but not matched females. In agreement with this, a recent study found age-dependent telomere attrition at a significantly faster rate in men as compared to women [135]. This may indicate that in both sexes, different mechanisms might have different implications for telomere erosion.

Altogether, these findings support the concept that the relation between inflammation, wasting, CVD and outcome may be different in male and female dialysis patients. In agreement with this hypothesis, a recent analysis from the PREVEND cohort [155] assessed which modifiable risk factors are associated with change in renal function during follow-up in a community-based study cohort. Different results were found for males vs. females. In males, urinary albumin excretion was the strongest independent predictor of greater renal function decline, together with plasma glucose and systolic blood pressure. In contrast, low waist circumference and cholesterol/HDL ratio were associated with a better renal function outcome in men. In females, on the other hand, systolic blood pressure and plasma glucose were independent risk predictors of renal function decline, whereas triglycerides were associated with better renal prognosis. Thus, there also seems to be gender differences in the standard predictors of the decline in renal function.

While some protective effects have been attributed to estrogen (as discussed above), testosterone levels have shown to actively induce muscle protein synthesis [156-158]. However, CKD causes endocrine dysfunctions at this level as well, over and above natural ageing [61]. As many as 50-70% of CKD stage-5 men have been reported to be hypogonadal based on low concentrations of total and free testosterone [159, 160]. Alterations on sex steroid production and metabolism (leading to primary hypogonadism and disturbances of the hypothalamic-pituitary axis) are already seen when moderate reductions in the glomerular filtration rate occur [161]. These disorders are not normalized by initiation of maintenance dialysis treatment; instead, they often progress [162]. Humoral factors, which accumulate in uremia, as well as other comorbid conditions and medications that frequently accompany CKD may contribute to suppressed sex hormone levels [160]. In light of the association between low

testosterone concentrations and the risk of developing future cardiovascular events recently reported in the general population,[163, 164] we have recently evaluated the possible contribution of low testosterone levels to the poor prognosis and the elevated CVD risk of male HD patients [165].

The main finding of this analysis (***Figure 13***) was the prognostic value of low testosterone levels for mortality by all-causes, but especially by CVD [165]. This impact persisted after adjustment for age, sex hormone-binding globulin (SHBG), baseline comorbidities, medication known to affect testosterone levels and IL-6, but it lost significance after adjustment for creatinine [165]. Thus, our study is in agreement with the majority,[163, 164, 166, 167] but not all[168] of the previous studies in non-renal patients supporting the hypothesis that testosterone deficiency is implicated in the progression of CVD. There is evidence suggesting mechanisms through which testosterone, *per se*, influences mortality and specifically cardiovascular end-points: At first, testosterone levels have been inversely related to the progression of atherosclerosis in the aorta [169] and carotid artery [170], perhaps due to an explicit vasodilatory effect [171, 172]. At second, testosterone has a well established effect on muscle protein synthesis [156-158]. Both muscle mass loss in wasted patients [173] or even rapid weight loss during weight reduction regimens [174] have been correlated with declining testosterone levels. Thus, our observation that adjustment for s-creatinine levels (used here as a crude surrogate marker of muscle mass) resulted in the loss of impact on mortality, may indirectly support this hypothesis.

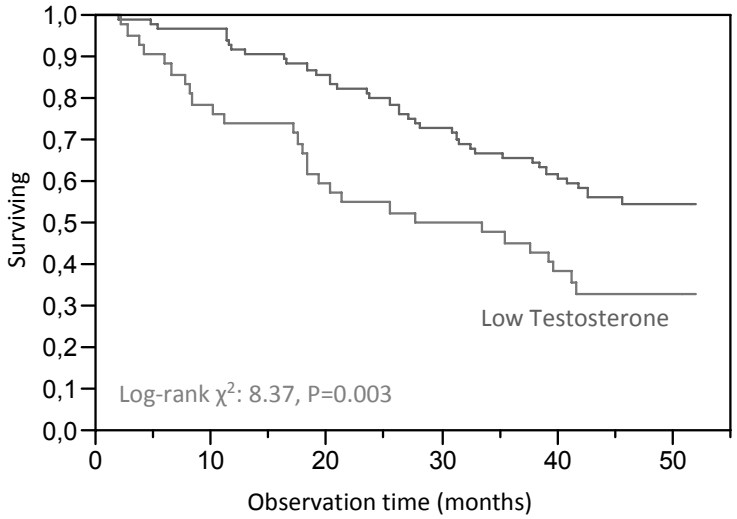


Figure 13. Kaplan Kaplan Meier Survival analysis for all-cause mortality in 126 prevalent men undergoing HD according to testosterone levels. Low testosterone was defined in our sample population as those levels below the 33th percentile of testosterone distribution. Modified from [165].

4.4 WHAT RISK MARKERS SHOULD WE USE?

Studies on the causes and consequences of inflammation in CKD are an ever-evolving field. Almost every month, new risk markers are introduced, and at a similar rate, new studies showing association between such new marker and clinical outcome is published. This thesis has contributed to that rate as well. An example of a recent (August 2008) risk biomarker is Fibroblast Growth Factor 23, a hormone that increases the rate of urinary excretion of phosphate and inhibits renal production of 1,25-dihydroxyvitamin D, thus helping to mitigate hyperphosphatemia in patients with kidney disease [175]. While it could potentially represent a novel future therapeutic target, its use in risk prognosis in a clinical setting may be doubtful, as it should represent the same phenomena as simple measurements of calcium, phosphorus, and parathyroid hormone (PTH) [176]. Nonetheless, cross-sectional and observational studies are needed to generate novel hypothesis and to stimulate further deepening into our understanding of molecular mechanisms and corresponding possible therapeutic strategies that should ultimately be tested in randomized clinical trials [92].

One aim of this kind of studies should be to offer an alternative source of useful information for clinician's prognostication. Prognostic estimates are a central element of both patient and physician decision making. Unfortunately, it was recently reported that physicians are inaccurate in their prognoses and systematically optimistic [177]. These phenomena may be adversely affecting the quality of care given to the patients. In this sense, S*tudies I and II* offer two simple assessments (a self-report of appetite and a visual gradation of the presence of muscle atrophy) that could constitute a valuable and inexpensive complement to other clinical measures in risk assessment and promotion of better health outcomes. In *Study IV* by comparison with ROC analysis, we propose that total (rather than free levels) T3 may be more useful in predicting future risk. Measurements of T3 and T4 are often run routinely in some clinics, in contrast to the free forms. It could be speculated that the superior predictive power of T3 in biochemically euthyroid patients is due to the fact that circulating hormone and the carrier protein might capture greater prognostic information, possibly beyond thyroid function *per se*.

The TWEAK and telomere biology research is emerging and although both markers were associated with increased mortality in S*tudies III and V*, we cannot yet propose

the generalized use of these biomarkers in predicting CKD risk. To start with, detection methods are not yet developed for routine or massive measurements. Also, if our hypotheses are true, they may represent, to some extent the same phenomenon of inflammation.

Finally, typically in epidemiology we tend to study factors associated with disease risk separately from their related factors or from their counterpart factors representing protection or resistance to specific insults. This phenomenon is suggested in *Study III* and may reflect our inability to see the complete picture. Unfortunately, such assessments may not always accurately represent the processes leading to disease development. Thus, it appears critical to simultaneously evaluate not only the risk factor exposure but also the body's reaction to the exposure. In *Study III* we aimed at introducing a new concept, namely the catalytic effect of persistent inflammation in accelerating mortality risk. The hypothesis would be that an individual's risk of disease may depend on the ability to counteract stress produced by increased inflammation. When that response is inadequate, then the risk is exacerbated. Thus, we can show that in the presence of persistent inflammation, high levels of sTWEAK represented a higher mortality risk than the expected by general additivity of effects. In other words, sTWEAK may be an additive, but not a primary marker, of the high mortality rate seen in HD patients with systemic inflammation (***Figure 14***). A similar behavior has been described for other risk markers, such as osteoprotegerin and fetuin-A, which only predicted mortality in the CKD population in the presence of inflammation [178, 179]. Another study supporting this concept recently demonstrated that uremia with inflammation, but not uremia *per se*, inhibits downstream growth hormone signaling contributing to muscle atrophy [180].

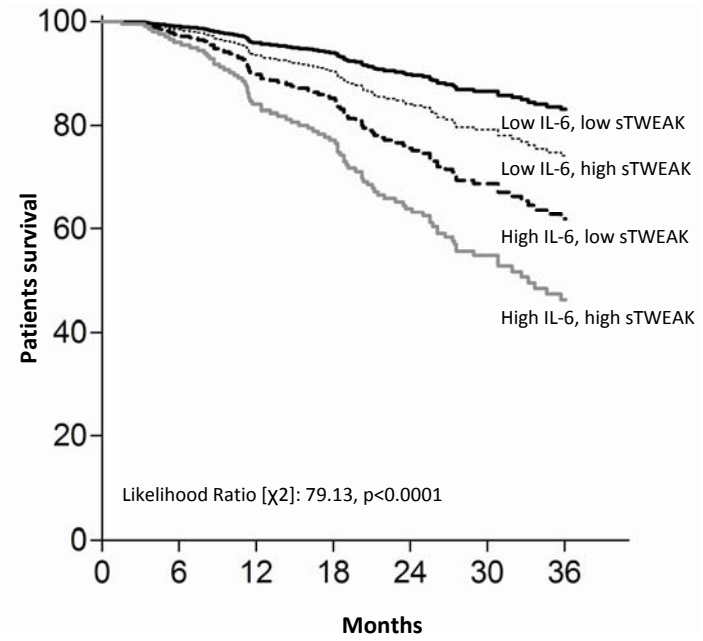


Figure 14. Adjusted mortality risk in 208 hemodialysis patients according to their levels of IL-6 and sTWEAK. Adjustment of the Cox was done for age, sex, Davies comorbidity score, intake of acetylsalicylic acid and dialysis vintage.

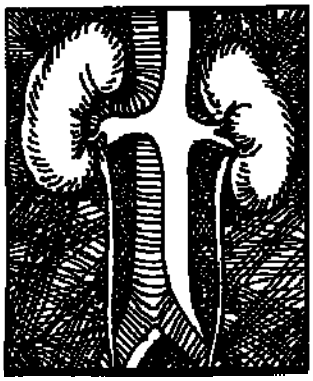
Given the large number of traditional, uremia-specific and novel biomarkers that have been shown to be associated with increased cardiovascular risk and mortality in CKD, it is natural that nephrologists feel confused and inundated with new information regarding which risk factors should be used for screening in clinical practice. The answer for that question is difficult, as in some cases we do not know whether we are studying a risk ***marker*** or a risk ***factor***. An example of this may be found in CRP itself. Although some *in vitro* data support a direct role for CRP in atherogenesis [181-183], there is as yet no definite evidence that CRP *per se* mediates atherogenesis *in vivo* [184, 185]. CRP may reflect, but not mediate, the risk factor of inflammation, as CRP production is stimulated by pro-inflammatory cytokines [186]. Decision making becomes even harder as it seems that genotypic[187, 188] and epigenotypic[189] risk factors may in the future further complicate this puzzle.

To this date, we would recommend risk markers that are easily measured in the everyday clinical practice and that represent a low economic burden. Although vascular calcification, oxidative stress and endothelial dysfunction seem to be significant determinants of poor outcome, we would primarily target markers from the following pathways:

1. ***Inflammation:*** Probably CRP is an easy and inexpensive inflammatory marker already routinely implemented in the clinic [190]. Both single [21, 33, 87] and serial measurements [100-102] provide a strong prediction of short-term and long-term survival. Because no additional power for mortality prediction was observed with high-sensitivity CRP as compared to non-sensitive CRP measurements [191], this could help to reduce costs and ease routine implementation. Although some studies have shown that IL-6 may be a more precise prognosticator [192, 193], IL-6 measurements are still impracticable and expensive in terms of routine measurements. Adding other inflammatory biomarkers or pro-inflammatory cytokines to that prognostication has shown to have little predictive gain to justify concomitant analysis [194].
2. ***Protein-energy wasting:*** The presence of PEW [22] is a strong predictor of short-time death [97, 98]. Frequent nutritional assessments reliably detect patients at risk of dying, and several therapeutic measures exist to treat this modifiable condition. SGA may be an easy and inexpensive validated tool to gain insight

into the nutritional status of the patients [97, 98] . However, it may be considered time consuming. Therefore, recent studies (some of them derived from this thesis) suggest that simple self-reports of appetite behavior [56, 107, 108], visual signs of muscle atrophy [123], as well as self-reports [195] or nurses assessments [196] regarding general well-being, may constitute an indirect way of assessing the patient's nutritional status and gather additional information for physician's decision making. Also, other biochemical markers of nutritional status, such as serum albumin, pre-albumin or IGF-1, may be valuable additional prognosticators.

3. ***Cardiac biomarkers of volume overload:*** Although outside the scope of this thesis, we should recognize accumulating evidence regarding cardiac troponin T (cTnT) as a useful serum cardiac biomarker for prognostication and cardiovascular risk stratification in CKD [197-201]. Despite being sensitive to GFR [202] or to dialysis [203, 204], B-natriuretic peptide (BNP)[205] and N-terminal-Pro-BNP (NT-Pro-BNP) [206-208] may be other promising candidates. At present, it is a critical task though to define the best cut-off levels at each stage of CKD including those on HD and PD[209]. Although they do not replace echocardiography, these markers may evolve to play an important and complimentary role in evaluating cardiovascular risk profile [205, 210].



5 CONCLUSIONS

I. Self-reported poor appetite in HD patients is associated with inflammation and poor outcome. The severity of symptoms may be increased in CKD men with poor appetite.

II. Visual signs of muscle atrophy are more common in female dialysis patients and progressively associated with inflammation, poor nutritional and anthropometric status and increased mortality.

III. sTWEAK may be an additive, but not a primary marker, of the high mortality rate seen in HD patients with systemic inflammation.

IV. Low triiodothyronine levels in biochemically euthyroid CKD patients are independent predictors of all-cause and cardiovascular-related mortality, perhaps due to an intimate association with inflammation.

V. Age, male gender and systemic inflammation were important contributors to reduced telomere length in CKD patients, which may constitute a new mortality risk factor in this population.

VI. Because the relation between inflammation, PEW, CVD and outcome appears different in male and female CKD patients, the sex perspective should be taken into account in future epidemiological studies on these risk factors in the setting of uremia.

6 DIRECTIONS OF FUTURE RESEARCH

The present thesis touches on a variety of intriguing hypotheses that could, ultimately, lead to new therapeutic CKD strategies. As our cross-sectional design precludes from causality, the next obvious step is to make longitudinal attempts, interventional and mechanistic studies.

It would be interesting to study appetite mediators through a gender perspective, given the fact that some of these molecules, such as leptin and adiponectin are sex dimorphic. One clear message from the studies of this thesis is the importance of improving nutritional status in this patient group. The use of sex hormones or derivatives in the treatment of eating disorders or nutritional deficiencies is not uncommon. Megestrol acetate has shown positive results in improving appetite, caloric intake, nutritional and inflammatory status in dialysis patients [211] via stimulation of neuropeptite-Y (NPY) in the hypothalamus or inhibition of pro-inflammatory cytokines [54]. Nandrolone decanoate (alone or in combination with resistance exercise training) has proved to have anabolic effects in CKD patients [212]. Low doses of testosterone in aged healthy men have reported favorable increases in lean body mass, overall nutritional status and other beneficial metabolic effects [158]. Therefore, it is exciting to hypothesize that sex-hormone replacement therapy could potentially improve the CKD patient well-being and outcome. As discussed in my previous thesis [213], a number of nutrients exert anti-inflammatory effects, such as fish oil [214-216] and soy [217, 218]. Therefore, the role of nutritional supplementation in targeting both the presence of PEW and the inflammatory state of CKD patients merits further consideration as well.

Although emerging, the evidence on thyroid abnormalities in increasing mortality risk is gaining strength [219, 220], longitudinal studies are needed to assess whether these cross-sectional observations reflect a causal relationship or not. Although the use of thyroid hormone therapy in non-thyroidal illnesses is controversial [221], interventional studies designed to reduce CVD and/or inflammation in renal disease is a tempting idea. New selective thyroid hormone receptor-β agonists may merit consideration in this regard [222]. Of note, a recent 8-week dietary intervention with low protein diet in CKD patients decreased levels of pro-inflammatory cytokines with

concomitant increases of both T3 and T4 [223]. It remains to be shown whether various anti-inflammatory strategies may correct low T3 levels and/or *vice versa*.

Because little is known about the mechanisms affecting TWEAK metabolism in CKD, it would be interesting to start by studying the conditions stimulating or repressing the expression of its receptor, Fn14, by isolating RNA from peripheral monocytes in inflamed dialysis patients. Also, some comparative data concerning sTWEAK removal, such as sTWEAK kinetics during the dialysis period would be of great interest. Because TWEAK may be another link between inflammation and muscle wasting, it would be important to confirm this causation and if so, to test whether anti-inflammatory treatments or anabolic regimes are able to restore these levels [224]. Shall this confirm true, it may represent an attractive therapeutic target in the pathogenesis of inflammatory diseases and CVD [225].

Regarding studies on telomere length, one important critical question still is whether CKD *per se* accelerates telomere attrition. Epidemiologically, this would need age- and sex- matched cohorts of patients and healthy controls and, alternatively, repeated telomere assessments within a significant time period. Experimentally, human cell culture studies with uremic serum would allow us to study not only the attrition rate but also telomerase efficiency. Another interesting aspect is the possibility of epigenetic modifications affecting telomere length [226]. Also, specific epigenetic alterations in the promoter of the telomerase gene may influence the attrition rate [227, 228]. As epigenetic DNA methylation may be influenced by inflammation, hyperhomocysteinemia, folate supplementation and redox control in uremia this may, in part, explain the observed telomere biology alterations [189, 229]. Also lifestyle factors, such as smoking, social status or a sedentary life style [230], could accelerate cell ageing through changes in the epigenetic status [231].

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