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VASCULAR AND INFLAMMATORY MARKERS IN CHRONIC KIDNEY DISEASE

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Vascular and inflammatory markers in chronic kidney disease

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

In patients with chronic kidney disease (CKD), inflammation and malnutrition are harmful and highly prevalent conditions with influence on the atherosclerotic process and outcome. The risk of cardiovascular disease (CVD) is substantially increased in CKD patients compared with healthy individuals. In uremic patients, homocysteine (Hcy) is a marker of disturbed metabolism and is suggested as a potential cardiovascular risk factor. Several inflammatory markers are associated with cardiovascular morbidity and mortality. Together with CRP and serum amyloid P (SAP), pentraxin 3 (PTX3) is a protein belonging to the through evolution highly conserved pentraxin family and is involved in the regulation of the innate immune system. In contrast to CRP, which is synthesized in the liver, PTX3 is produced in the vasculature, and circulating levels mirror local inflammatory processes. The aim of this thesis was to study inflammatory and metabolic biomarkers associated with cardiovascular risk in *i)* elderly individuals in the general population, *ii)* patients with CKD and *iii)* dialysis patients. We also investigated homocysteine and PTX3 as predictors of mortality in dialysis patients. **In paper I** we measured the reduced, free and total forms of Hcy in plasma of patients with peritoneal and hemodialysis (HD) treatment and in patients with CKD stage 3 to 5. The more harmful form of Hcy, the reduced Hcy form (rHcy), was higher in all patients with impaired renal function than in healthy controls. In dialysis patients, the ratio of reduced and total Hcy (rHcy/tHcy) was higher than in CKD patients and the ratio further increased during HD treatment. **In paper II** we investigated PTX3 and estimated glomerular filtration rate (eGFR) in two large community cohorts of elderly women and men in Uppsala. In a cross-sectional analysis, higher PTX3 levels were associated with lower GFR estimated from plasma cystatin C levels. In a longitudinal analysis, PTX3 independently predicted incidence of CKD as estimated by a drop of eGFR below 60 mL/min*1.73 m^2 BSA. These findings suggest that inflammatory processes are activated and play a role in the early stages of CKD. **In paper III** plasma levels of PTX3, CRP, albumin and Hcy were measured twice over a three-month period in HD patients. PTX3 had the highest intra-individual variation followed by albumin, CRP and Hcy. Furthermore, persistently elevated PTX3, increasing levels of CRP, decreasing levels of albumin and persistently low Hcy levels over three months were associated with a high mortality risk after adjustment for other risk factors. **In paper IV** the release of PTX3 in response to inflammatory signals induced during a HD treatment was investigated. The plasma concentration of PTX3 was measured at the start and after 30, 60, 120, 180 and 240 minutes of the HD session. The increase of PTX3 was significant after 60 minutes, while CRP levels did not change during hemodialysis. We found that PTX3 is a sensitive marker of HD-induced inflammatory activity, probably because it is rapidly released from neutrophil granules on immune activation during HD. **In conclusion**, accumulation of reduced Hcy in CKD and dialysis patients has potentially toxic effects on the vasculature. However, Hcy is not a reliable risk marker in patients with chronic kidney disease. PTX3 has a high intra-individual variation over three months, but the levels in CKD patients are associated with CVD and mortality. In elderly individuals in the community, PTX3 was associated with CKD incidence over a 5-year follow-up period. PTX3 is a quick and sensitive biomarker that has the potential to be an important clinical tool in patients with early and late stages of CKD, as well as in dialysis patients.

LIST OF SCIENTIFIC PAPERS

I. Sjöberg B, Anderstam B, Suliman M and Alvestrand A. “Plasma reduced homocysteine and other aminothiol concentrations in patients with CKD”, American Journal of Kidney Disease, 2006, Vol 47; No 1, 60-71.

II. Sjöberg B, Qureshi AR, Heimbürger, Stenvinkel P, Lind L, Larsson A, Bárány P and Ärnlöv J. “Association between the inflammatory marker pentraxin 3, glomerular filtration rate and CKD incidence in two community-based cohorts”, manuscript.

III. Sjöberg B, Snaedal S, Stenvinkel P, Qureshi AR, Heimbürger O and Bárány P. “Three-month variation of plasma pentraxin 3 compared with C-reactive protein, albumin and homocysteine levels in haemodialysis patients”, Clinical Kidney Journal 2014; 7: 373-379.

IV. Sjöberg B, Qureshi AR, Anderstam B and Alvestrand A and Bárány P. “Pentraxin 3, a sensitive early marker of hemodialysis-induced inflammation”, Blood Purification 2012; 34: 290-297.

CONTENTS

LIST OF ABBREVIATIONS

ACEI	Angiotensin converting enzyme inhibitor
ACR	Albumin creatinine ratio
APD	Automated peritoneal dialysis
ARB	Angiotensin receptor blocker
AUC	Area under the curve
BMI	Body mass index
BW	Body weight
CAPD	Continuous ambulatory peritoneal dialysis
CKD	Chronic kidney disease
CRP	C-reactive protein
CVD	Cardiovascular disease
ELISA	Enzyme-linked immunosorbent assay
FBMI	Fat body mass index
fHcy	Free form of Hcy
GFR	Glomerular filtration rate
Hb	Hemoglobin
Hcy	Homocysteine
HDF	Hemodiafiltration
HD	Hemodialysis
HOPE	Heart Outcome Preventive Evaluation
hsCRP	High sensitive C-reactive protein
ICC	Intra-class correlation

IQR	Interquartile range
IL	Interleukin
kDa	Kilodalton
Kt/V	Dialysis treatment adequacy (K = Dialyzer clearance, t = dialysis time, V = volume of distribution of urea)
LBMI	Lean body mass index
MDRD	Modification of Diet in Renal Disease
MIMICK	Mapping of Inflammatory Markers in Chronic Kidney Disease
MBD-CKD	Mineral bone disorder in CKD
PD	Peritoneal dialysis
PEW	Protein-energy wasting syndrome
PIVUS	The Prospective Investigation of the Vasculature in Uppsala Seniors
PTH	Parathyroid hormone
PTX3	Pentraxin 3
Qb	Blood flow
rHcy	Reduced form of Hcy
RRT	Renal replacement therapy
SNR	Svenskt njurregister
TNF	Tumor necrosis factor
tHcy	Total plasma concentration of Hcy
UF	Ultrafiltration
ULSAM	The Uppsala Longitudinal Study of Adult Men

1 INTRODUCTION

Patients with chronic kidney disease (CKD) have a substantial increase in cardiovascular morbidity and mortality compared with the general population (1, 2). The cardiovascular risk is increased already in the early stages of CKD and increases several-fold during disease progression. Patients with hemodialysis or peritoneal dialysis have the highest mortality risk, especially in the first three months after dialysis initiation. Patients with a kidney transplant have a much higher survival rate than dialysis patients (3, 4). During the last decades, there has been some interest in non-cardiovascular risk factors, such as inflammation, and their association to the atherogenic process. Inflammatory markers can predict CVD and all-cause mortality in the general population as well as in dialysis patients (5, 6). The most frequently used inflammatory marker is C-reactive protein (CRP), but other markers such as pentraxin 3 (PTX3) may add information about the complex association between inflammation and cardiovascular disease (CVD) (7-9). Markers of metabolic disturbances in uremia, such as homocysteine (Hcy), have also been suggested as potential modifiable cardiovascular risk factors (10, 11).

1.1 KIDNEY, NORMAL STRUCTURE AND FUNCTION

In a young healthy person, each kidney is built up of one million nephrons, the functional units of the kidney, see schematic figures 1 and 2.

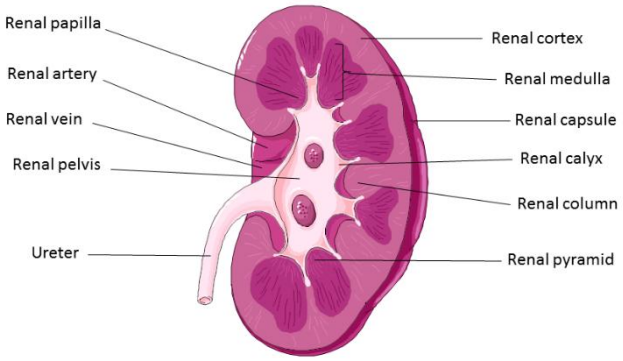


Figure 1. The structure of the kidney.

The kidneys have several important functions beyond the removal of toxic waste products (uremic toxins) and regulation of the fluid balance of the body. Blood pressure is regulated by

the renal vessels (oncotic and hydrostatic pressure gradient and auto regulation) and the synthesis of red blood cells is regulated by the hormone erythropoietin, produced in the juxtaglomerular cells in the kidney, which stimulates the synthesis of red blood cells in the bone marrow. Further, the kidneys handle the regulation of the acid-base balance and the conversion of inactive to active D-vitamin.

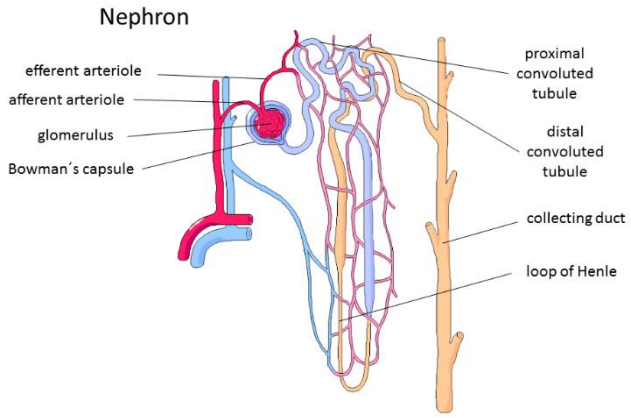


Figure 2. The structure of the nephron.

The glomeruli are built up of vessels and the renal blood flow is 1.2 L/min, 25 % of the normal cardiac output of 5 L/min in a young healthy adult. The renal plasma flow is 0.65 L/min, 20 % is filtered and the normal glomerular filtration rate (GFR) is 125 mL/min.

1.2 KIDNEY, PATHOLOGY, CHRONIC KIDNEY DISEASE (CKD) STAGE 1 – 5

1.2.1 Causes of CKD

Diabetes nephropathy is considered to be the most common cause of chronic kidney disease world-wide, followed by hypertension/renovascular disease (12). In Sweden, hypertension/renovascular disease is the most common cause of CKD, followed by diabetes nephropathy, but among prevalent patients with renal replacement therapy (RRT), chronic glomerulonephritis (25 %) is the most common cause of CKD, followed by diabetes nephropathy (18 %), even though diabetes nephropathy is the most common diagnosis for patients initiating dialysis treatment. The third most common diagnosis among patients with RRT is adult polycystic kidney disease (10 %) followed by hypertension (8 %), pyelonephritis (4 %), unknown cause (1 %) and others (33 %) (13).

1.2.2 CKD stages

Chronic kidney disease (CKD) is defined as a state of kidney damage and/or decreased glomerular filtration that lasts for at least 3 months (14). Patients with CKD often have a declining kidney function over many years. Current classification of CKD is based on cause (glomerular diseases, tubule-interstitial diseases, vascular diseases, cystic and congenital

diseases), GFR category and albuminuria category.

Table 1. GFR categories in CKD in current classification system.

GFR category	GFR (mL/min/1.73 m^2)	Terms
G1	> 90	Normal or high
G2	60 – 89	Mildly decreased
G3a	45 – 59	Mildly to moderately decreased*
G3b	30 – 44	Moderately to severely decreased
G4	15 – 29	Severely decreased
G5	< 15	Kidney failure

*Relative to young adult level. In the absence of evidence of kidney damage such as albuminuria, neither GFR category G1 nor G2 fulfill the criteria for CKD.

1.2.3 Prevalence, incidence and gender differences

In the general adult population, CKD is a global health burden and the world-wide prevalence of CKD is 10 – 15 % in both low-income and high-income countries (15, 16). The prevalence of RRT in Sweden was 938 individuals per million inhabitants in 2013. The majority of the 9,051 patients with RRT were renal transplanted (rtx, 57 %) and 3,857 patients had dialysis, hemodialysis (HD, 33 %) or peritoneal dialysis (PD, 9 %). The number of patients with RRT more than doubled in 20 years and the number of HD patients increased from 1,124 in 1990 to 2,881 in 2013 (13), even though the increase rate has diminished over the last decade. There is a consistent gender difference in patients with CKD in the late stages; in most populations, 35 – 40 % are women and 60 – 65 % are men.

1.2.4 Uremic syndrome, metabolic disturbances and corrective treatment

At the early stages of CKD, stage 1 – 3, there are often few symptoms and renal failure is often discovered during investigation of hypertension or CVD. In CKD stage 4 – 5 several symptoms appear.

A **Anemia** develops because of reduced renal synthesis of erythropoietin starts in CKD

stage 3 and develops slowly and the patient may not notice symptoms until Hb is below 90 g/L. Most CKD patients respond to anemia treatment in the form of erythropoietin and iron with increasing Hb levels to a target interval between 100 – 120 g/L (17, 18).

B **Increased catabolism and decreased synthesis of proteins** may contribute to muscular weakness and metabolic acidosis. Loss of appetite and prescribed low protein diet may induce insufficiency of essential amino acids, which aggravates the risk of malnutrition and weight loss (19). Medication with sodium bicarbonate can regulate the acidosis, supplementation with essential amino acids and help from a dietitian can reduce uremic symptoms.

C When GFR diminishes, **phosphate** accumulates and the plasma **calcium** level is low, because of reduced renal synthesis of the active vitamin D metabolite calcitriol $1.25(OH)_2$-vitD_3 (calcitriol). Disturbed action of the phosphaturic hormone fibroblast growth factor 23 (FGF23) and its co-factor klotho, as well as increased production of parathyroid hormone (PTH), all contribute to development of mineral bone disorder in CKD (MBD-CKD) (20, 21). MBD-CKD leads to skeletal changes, for example osteitis fibrosa, but also increased risk for calcifications in vessels and in the heart (coronary arteries), contributing to an increased risk for CVD (22). Calcifications in the muscles and joints may cause tenderness in muscles and arthralgia. Diet restrictions and dialysis regimens are important tools to correct calcium-phosphate imbalances. Furthermore, many patients need phosphate binders, vitamin D supplementation and/or calcimimetic medication to bridge the negative effects of an impaired calcium-phosphate balance (23).

D **Hypertension**, associated to salt and water retention in CKD patients, is an early consequence of impaired kidney function and adequate antihypertensive treatment is recommended. Overt fluid overload and disturbed electrolyte balance often appear late in CKD stage 5, with risk of life-threatening pulmonary edema and **hyperkalemia**.

E There are many **symptoms of uremia** and these change as GFR lowers and vary between individuals. Symptoms include fatigue, altered taste sensation, nausea, vomiting, weight loss, weakness, mental tiredness, decreased ability to concentrate, gastro-intestinal symptoms, sleeping problems, edema, bleeding tendency, restless legs and itchiness. Severe complications are lung edema with respiratory distress, uremic pericarditis with or without chest pain, seizures and unconsciousness.

1.2.5 Renal replacement therapy

When a patient with declining kidney function reaches GFR 5 – 10 mL/min/1.73 m², it is time to start RRT (24). The preferable choice is a kidney transplant, but limited availability of organs and high risks for elderly or patients with severe CVD are the reasons why most patients reaching CKD 5 start with dialysis. There are two different modalities of dialysis; the most common is hemodialysis, where blood passes through a dialyzer with a semi-permeable membrane and the uremic toxins and fluid volume are removed. Standard hemodialysis treatment is performed for four hours, three times a week. The other modality is peritoneal dialysis where exchange of dialysis fluid in the abdomen four times a day removes uremic toxins and fluid from plasma through the peritoneal membrane. After a successful replacement of a transplanted kidney, the recipient often reaches a GFR of 40 – 60 mL/min/1.73 m², while both dialysis types have less effective clearance, resulting in increased inflammation, malnutrition and a higher mortality risk (25, 26).

1.3 RISK FACTORS FOR MORTALITY

1.3.1 Cardiovascular disease and chronic kidney disease

Patients with chronic kidney disease have generally increased arteriosclerosis, left ventricular hypertrophy, congestive heart disease and coronary heart disease. Individuals with mild CKD (stage 1 – 2) have an increased cardiovascular risk (27, 28) and the incidence of cardiovascular death in dialysis patients is up to 20 times higher than in age- and sex-matched controls in the general population. For dialysis patients below 45 years of age, the cardiac mortality risk increases over 100-fold (29).The traditional risk factors for CVD and mortality in the general population, such as diabetes mellitus, hypertension, hypercholesterolaemia, smoking and physical inactivity, are highly prevalent in patients with mild CKD (stage 1 – 3). Patients with CKD also have a number of non-traditional risk factors such as fluid overload, anemia, inflammation, oxidative stress, and disturbances in calcium and phosphate levels, as well as hyperparathyroidism which may accelerate the arteriosclerosis process (30). CKD patients develop both atherosclerosis with calcium plaques in the intima layer and arteriosclerosis with hyperplasia of smooth muscle cells and subsequently often extensive calcifications in the media layer of the arteries. The vascular calcifications are widespread and affect the coronary arteries, aorta and heart valves (31).

However, compared with what is observed in the general population, several cardiovascular risk factors associated with nutritional status are reversed in dialysis patients, e.g. obese dialysis patients have lower mortality risk than non-obese (32) and low plasma cholesterol and low serum creatinine before hemodialysis are factors associated with increased mortality risk (19).

Patients with chronic heart failure without underlying kidney disease often have decreased GFR. This is, in part, a consequence of reduced cardiac output followed by reduced renal blood flow. There is a physiological compensatory increase of the renal vascular resistance and retention of fluid and sodium to maintain the intra-glomerular pressure. This often worsens the heart failure and when blood pressure cannot be sustained, the kidney function is affected. Treatment of patients with chronic heart failure followed by reduced GFR requires a balance of ACEI/ARB, beta blockers, furosemide and, in early stages, aldosterone antagonists. Patients also need frequent check-ups measuring creatinine, eGFR, electrolytes, blood pressure and edema level. The kidney function is dependent on the progress of the heart failure.

1.3.2 Malnutrition

Patients with CKD stage 4 – 5 and dialysis patients often suffer from protein-energy wasting which is linked to both inflammation and worse cardiovascular outcome. Protein-energy wasting also occurs among obese hemodialysis patients, together with significant skeletal muscle loss (obese sarcopenia) (33). Serum albumin is a poor marker of the nutritional state in dialysis patients since albumin is an acute-phase reactant and is associated with inflammation, rather than being a marker of protein-energy wasting (34). Fluid status also has some influence on albumin levels.

Obesity is associated with persistent low-grade inflammation in the general population and results in increased risk for inflammatory diseases including atherosclerosis and diabetes mellitus. Obese individuals have elevated plasma levels of CRP, IL6, IL10 and TNFα (35). In contrast, serum levels of PTX3 are inversely associated with BMI and waist circumference (36, 37). In community-based cohorts and longitudinal data over five years showed that weight loss was associated with an increase of PTX3 levels (37).

1.4 INFLAMMATION

1.4.1 Inflammation and CVD

Today, atherosclerosis is seen as an inflammatory disease involving the innate immune system. In CKD patients, persistent inflammation strongly contributes to the accelerated arterial disease, which results in an increased risk of cardiovascular morbidity and mortality, where dialysis patients suffer the highest risk (38, 39).

1.4.2 The role of inflammation in CKD patients

Independent of dialysis modality, exposure to dialysis membranes and/or contaminated dialysis fluid, dialysis patients have increased plasma levels of CRP, interleukin 1 (IL1),

interleukin 6 (IL6) and tumor necrosis factor alpha (TNFα) without clinical symptoms of on-going inflammation or infection. The long and persistent effect of inflammation in dialysis patients seems to be detrimental and the risk for mortality and incident cardiovascular complications is associated to plasma levels of inflammatory markers (40-46).

1.4.3 Inflammatory markers in CKD

1.4.3.1 CRP

The acute-phase protein CRP is the prototype marker for inflammation used in clinical settings and high CRP predicts cardiovascular mortality in the general population (47, 48), in CKD and in dialysis patients (49, 50). CRP is implicated in endothelial dysfunction and CRP synthesis in the liver is stimulated by IL6 in response to tissue damage or bacteria and can be detected systemically 6 – 8 hours after injury. In the clinic, increased circulating CRP levels are easy to detect, because CRP has a long half-life of 19 hours. CRP does not change during one HD treatment and for that reason CRP is not an ideal marker to assess inflammatory reactions during HD (51-53).

1.4.3.2 Albumin

Low-grade inflammation is an important cause of protein-energy wasting in CKD patients, a process that eventually leads to malnutrition and low serum albumin (54). However, albumin is mainly considered an inflammatory marker rather than a marker for nutritional status and because of this, hypoalbuminaemia is associated with poor outcome in dialysis patients (35, 55).

1.4.3.3 PTX3

The long pentraxin PTX3 is part of the innate immune system together with the short pentraxins CRP and serum amyloid P, see figure 3 below (56). Pentraxins are members of a family of ancient proteins with well-preserved structure throughout evolution (57-59). Pentraxin 3 is produced in the vasculature by different cell types, including endothelial cells, smooth muscle cells, fibroblasts, mononuclear phagocytes and epithelial cells, in response to IL1β and TNFα as well as lipopolysaccharides from bacteria (60). Neutrophil granules act as a reservoir of PTX3 and will rapidly release PTX3 in response to inflammatory signals or microbial recognition (61). PTX3 can, therefore, in thirty minutes systemically reflect the acute and local inflammation in the vasculature (62). PTX3 acts like a tuner for the immune system and is involved in pathogen recognition, complement activation and regulation, which improves the defense against various infections (63, 64). PTX3 is involved in the development of atherosclerotic plaque and in angiogenesis in some way, but its role is debated. It is unclear whether PTX3 is part of the atherogenic process, is a tuner of the immune system's protective qualities or a biomarker of processes in the vasculature (65). Studies show that PTX3 has an active part in foam formation in plaques and takes part in activation of the classical complement cascade (66-68), but on the other hand, a study from 2011 shows cardio-protective effects of PTX3 in healthy men (69). PTX3 also seem to be protective in acute myocardial infarction (70, 71).

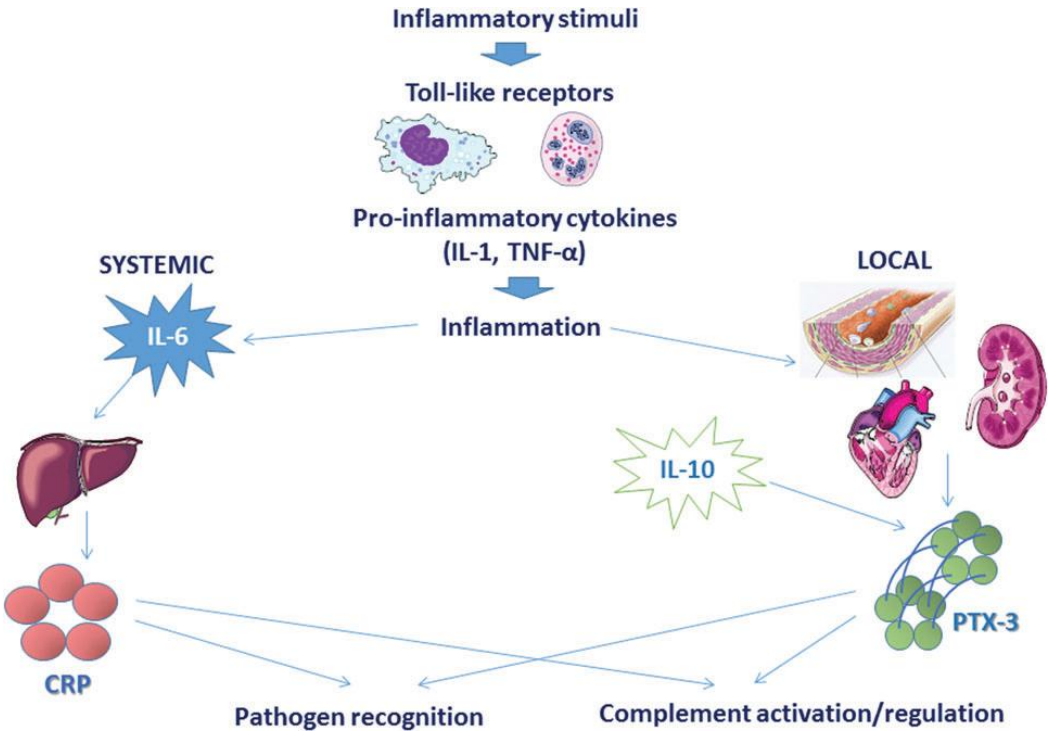


Figure 3. Role of PTX-3 in the innate immune system. Toll-like receptors are activated by inflammation. While CRP secretion from the liver is stimulated by systemic IL-6, PTX-3 is released locally in the vasculature. IL-10 amplifies PTX-3 secretion. PTX-3 and CRP are involved in pathogen recognition, complement activation and regulation. Published with permission (56).

1.4.3.4 TNFα

The pro-inflammatory cytokine TNFα is part of the regulation of pro- and anti-inflammatory mediators and provides rapid defense against infection, but is fatal in excess. In addition to being involved in plaque instability, TNFα is implicated in myocardial infarction and acute ischemic disorders (72). TNFα levels and activity are upregulated in patients with CKD stage 5 (45).

1.4.3.5 IL6

The pro-inflammatory cytokine IL6 has a direct inflammatory effect in the myocardial and peripheral vasculatures, due to the IL6-regulated activation of leucocytes and endothelial cells in the atherosclerotic process. An increased plasma level of IL6 predicts cardiovascular and total mortality in CKD stage 5 patients better than the most frequently used inflammatory marker CRP (73). The synthesis of CRP in the liver is induced by an increase of plasma IL6, see figure 3.

1.5 HOMOCYSTEINE

1.5.1 Mechanisms of Hcy

Homocysteine (Hcy) is a sulfur-containing amino acid not used in protein synthesis, but derived from the intracellular metabolism of methionine (fig. 4). Any excess of intracellular Hcy is transported into plasma, where approximately 70 % of Hcy is bound to albumin and 30 % is oxidized to disulfides (Hcy-Hcy), so-called free oxidized Hcy (74). A small portion of Hcy, 1 – 3 %, is in free reduced form (rHcy). The normal range of total Hcy (tHcy) in the healthy population is 3 to 15 µmol/L (75).

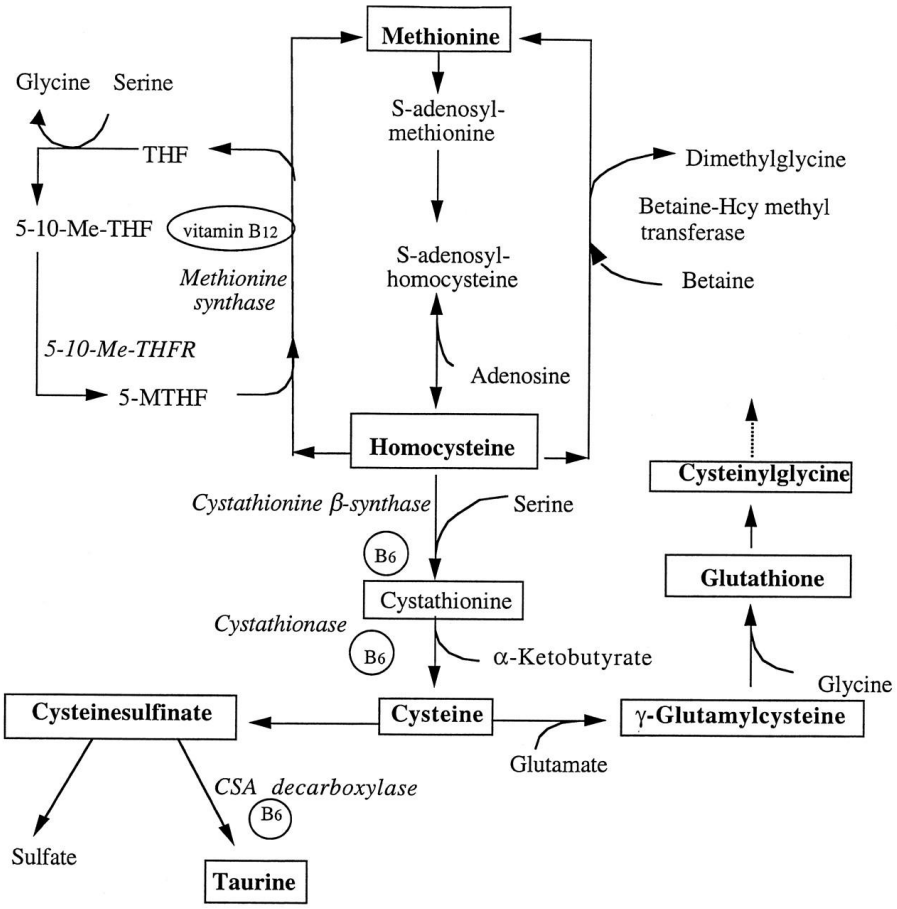


Figure 4. Homocysteine metabolism.

1.5.2 Hyperhomocysteinaemia

For decades it has been noted that high plasma concentration of Hcy is associated with CVD. However, although experimental studies have shown that Hcy causes endothelial dysfunction, reduced bioavailability of nitric oxide and increased smooth muscle cell proliferation, the causality remains unclear (76-79). Elevated plasma concentration of tHcy

has been suggested as an independent and graded risk factor for CVD in the general population as well as in CKD patients (80-83).
Circulating tHcy exists mainly bound to plasma albumin and as a consequence plasma levels of tHcy are lower in CKD patients with hypoalbuminemia than in those with normal plasma albumin. Low serum albumin is associated with protein-energy wasting, which is a confounding factor when analyzing tHcy in dialysis patients, as many patients have inflammation. Therefore, low concentration of tHcy, rather than high, indicates poor outcome in HD patients and Hcy is one of several reversed risk factors in CKD patients (84-86). In a clinical study, 459 HD patients were divided into two groups, one with protein-energy wasting and one without signs of inflammation. Here tHcy was inversely related to all-cause mortality in patients with protein-energy wasting, while a direct association was seen in patients without protein-energy wasting inflammation (87).

1.6 INTERVENTIONS

1.6.1 Vitamins

In the Heart Outcome Prevention Evaluation (HOPE) study, 5,522 patients with diabetes or vascular disease were randomized to placebo or a combination of B vitamins. The group treated with vitamins showed reduced tHcy levels, but there were no significant benefits in outcome over a 5-year follow-up period (88). A new Cochrane review (2015) confirms that supplementation with vitamin B6, B12 and folic acid does not prevent cardiovascular events in patients with or without pre-existing CVD and no more randomized trials are needed to assess this question (89, 90). In accordance with studies of patients with CVD, a study of 510 dialysis patients treated with high-dose folic acid for 24 months was performed. The patients treated with high-dose folic acid had the same rate of cardiovascular events as untreated patients (91, 92). Large intervention studies with supplementation of vitamin B12 and folic acid to dialysis patients show reduced plasma Hcy, but no improvement in the number of cardiovascular events or progression rate of atheroma plaques. There are no available data supporting the use of folic acid or B vitamins to improve survival in CKD patients (90, 93).

1.6.2 Dialysis

Despite improvements in hemodialysis and peritoneal dialysis technology, the prevalence of CVD is high and accounts for more than 50 % of premature death in this population (1). In addition to traditional risk factors like hypertension, dyslipidemia and diabetes mellitus, non-traditional risk factors like disturbed mineral metabolism, vascular calcification, hyperhomocysteinaemia, persistent inflammation and oxidative stress are highly prevalent in dialysis patients (94, 95). The low-grade inflammation is usually detected via CRP, but other markers like PTX3 and IL6 contribute to understanding the inflammatory process in the vasculature (96). In Stockholm, 80 % of prevalent hemodialysis patients in 2005 had CRP > 2 mg/L (Fig. 5).

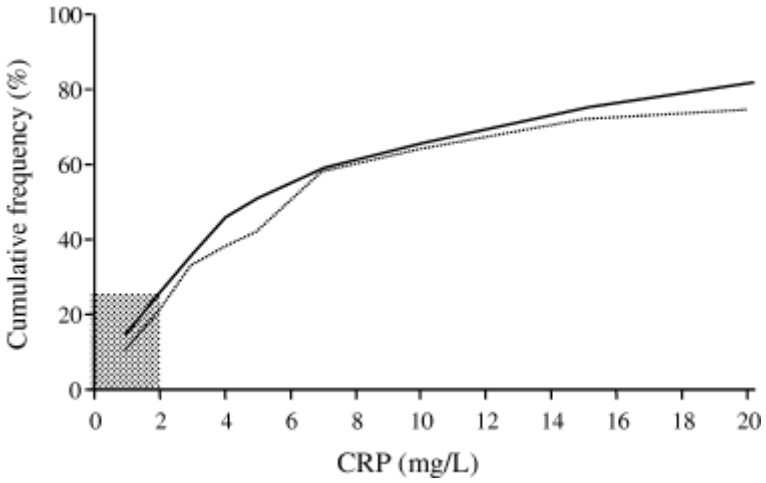


Figure 5. The cumulative frequency of elevated CRP in 304 incident end-stage renal disease patients (black line) starting dialysis therapy and 231 prevalent hemodialysis (dotted line) patients treated in Stockholm, Sweden, 2005. (With permission from Peter Stenvinkel "Inflammation in end-stage renal disease: the hidden enemy" (96)).

The prevalence of inflammation differs markedly between different regions of the world and the prevalence of increased CRP is lower in HD patients in Asia than in Europe, possibly caused by genetic and dietary differences (97). Treatment with nonspecific inhibitors of inflammation, such as aspirin and statin medication, is a possible option, but convincing evidence is lacking. To reduce the inflammatory burden of dialysis patients, it is beneficial to use ultra-pure dialysis fluid and biocompatible dialysis membranes as well as minimizing infections of access sites. The inflammatory reaction in HD patients is affected by the choice of dialyzer and modality (98). Pilot studies in a limited number of patients have reported that CRP levels are lower in patients treated with biocompatible membranes than when cuprophan membranes were used (99, 100). Fassett et al observed that HD patients with diabetes included in the 4D-study of atorvastatin treated with high-flux had a reduced mortality risk compared to those on low-flux membranes (101).

Hemodialysis patients treated for 3 hours 6 times a week (short daily HD) showed decreased plasma levels of inflammatory markers and a reduction of ventricular hypertrophy compared with HD patients on conventional dialysis regimens with treatment for 4 hours 3 times a week (102). Evidence that frequent dialysis regimens are beneficial is lacking. An observational study by Suri et al. (2013), comparing in-centre daily HD with conventional HD (15.7 and 11.9 weekly hours respectively), showed that patients with daily HD had a higher mortality rate than patients receiving conventional HD (103).

Concerning inflammation in HD patients, the purity of the dialysate is important. After a switch from conventional to online-produced ultrapure dialysate, lower circulating levels of CRP and IL6, improved nutritional status and lower cardiovascular morbidity have been observed (104, 105). Treatment with peritoneal dialysis (PD) also induced inflammation by impurity and/or bio-incompatibility of PD solutions (106).

1.6.3 Anti-inflammatory and anti-oxidative interventions

There is an interest in anti-inflammatory nutritional treatment and small studies have suggested that omega-3 fatty acids lower the inflammatory response in HD patients and HD patients eating fish have better survival (107, 108). Other nutritional factors of interest are soy, green tea and pomegranate juice, although evidence of protective effects is missing (109-111). Hemodialysis treatment with hydrogen-enriched solution is frequently used in Japan and may reduce oxidative stress during HD (112). A small pilot study of 28 patients with CKD stage 2 – 3 has shown that low-fructose diet reduces plasma levels of inflammatory markers and lowers the blood-pressure (113). In addition, a randomized study with supplementation of probiotics or placebo to 39 PD patients for one year, showed significant reduction of plasma inflammatory markers (114).

Convincing studies of beneficial effects of anti-inflammatory interventions as regards protecting CKD patients from CVD are lacking. In the 4D-study, a randomized, double-blind, prospective, multicenter study of 1,255 patients with type 2 diabetes mellitus and on maintenance HD treated with atorvastatin or placebo, no effect on CRP was observed. Atorvastatin had no significant effect either on cardiovascular death or all-cause mortality, or on non-fatal myocardial infarction or stroke in this population (115). Treatment with angiotensin converting enzyme inhibitor (ACEI) reduces plasma levels of PTX3 in diabetic CKD patients (116, 117). Sevelamer and vitamin D have also been suggested as drugs with anti-inflammatory effect in CKD patients but until now there is no established treatment recommendations (118).

2 THE AIMS OF THE STUDIES

2.1 OVERALL AIMS

The overall aim was to evaluate the association of selected biomarkers to the progression of CKD and cardiovascular risk in

1 The general population.

2 Patients with chronic kidney disease stage 2 – 5.

3 Dialysis patients.

2.2 SPECIFIC AIMS

1 To determine plasma reduced Hcy levels in patients with CKD (Paper I).

2 To determine the association between the inflammatory marker PTX3 and progression of chronic kidney disease in two community-based cohorts (Paper II).

3 To determine the variation and association to mortality of plasma PTX3, CRP, albumin and Hcy in hemodialysis patients, over a three-month period (Paper III).

4 To determine the role of PTX3 as a sensitive early marker of hemodialysis-induced inflammation (Paper IV).

3 METHODS

3.1 STUDY POPULATIONS

3.1.1 Study one

Seventy-eight patients treated at the department of renal medicine, Karolinska University Hospital, Stockholm, were included. Thirty-one patients were on dialysis (19 HD and 12 PD) and 47 patients non-dialyzed with CKD stage 3 – 5 with an estimated GFR ranging from 6 – 57 mL/min*1.73 m^2 (MDRD formula) (119). Fifteen healthy persons from the general population, matched with the patient groups with respect to sex and age, served as a control group. Of the HD patients, 17 had conventional HD treatment three times a week and 2 HD patients twice a week, altogether with a mean of 11.4 h/week with low-flux membrane (Polyflux 17L, Gambro AB, Lund, Sweden), while one patient had high-flux membrane (Polyflux 201H, Gambro AB, Lund, Sweden) and one patient had hemodiafiltration (HDF). Median single-pool Kt/V for urea was 1.6 (1.1 – 2.0).
Eleven PD patients were treated with continuous ambulatory PD (CAPD) and one patient with automated PD (APD); all but two CAPD patients used icodextrin (Extraneal, Baxter) at night. Median weekly Kt/V for PD patients was 2.2 ± 0.4.
All patients were on their regular medication and 50 % were prescribed vitamin B and C (Oralovite) including thiamine, riboflavin, pyridoxine chloride, nicotine amide and ascorbic acid.

3.1.2 Study two

3.1.2.1 The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS).

All men and women at 70 years of age living in Uppsala, Sweden 2001 to 2004 were invited to participate in the PIVUS study, described in detail on http://www.medsci.uu.se/pivus.htm (120). Of 2,025 invited individuals, 1,016 agreed to participate and 768 individuals had data on PTX3 levels and albumin creatinine ratio (ACR). A second examination cycle of PIVUS was performed from 2006 to 2009, when the participants were 75 years old. Of 964 invited individuals, 827 participated (86 %), and data on PTX-3 and estimated GFR were available in 768 individuals. There was no urine sample collected at the first examination at 70 years of age, therefore the second examination at 75 years of age was used for cross-sectional analyses of association between PTX-3 and eGFR and ACR respectively. The first examination, when the participants were 70 years old, was used as the baseline for longitudinal analyses of PTX3 and decline of eGFR.

3.1.2.2 The Uppsala Longitudinal Study of Adult Men (ULSAM).

The ULSAM study started in 1970 when all 50-year-old men, born 1920 – 24 and living in Uppsala, Sweden, were invited to participate in a health survey, described in detail on http://pubcare.uu.se/ULSAM (121). We used the 4th examination cycle, when the participants were 77 years old (1998 – 2001), at baseline. Of 1,398 invited men, 838 (60 %)

participated and for 651 individuals data on PTX3 and GFR was available. We used the 5th examination cycle of ULSAM (2003 – 2005), when the participants were 82 years old, to identify those who had progressed to CKD. At 82 years of age, all 952 men still living were invited; a total of 530 (56 %) men agreed to participate and 315 individuals who had data on PTX3 and eGFR were included.

Table 2. Baseline characteristics of PIVUS and ULSAM.

Variable	PIVUS	ULSAM
Number of subjects	768	651
Female, n	393 (51 %)	0 (0 %)
Age, (years)	75.3 ± 0.2	77.5 ± 0.8
CRP, (mg/L)	2.1 (2.8)	2.4 (1.5)
Pentraxin 3, (ng/ml)	1.8 (3.3)	2.1 (1.3)
Cardiovascular disease (CVD), n	157 (20 %)	175 (27 %)
Estimated glomerular filtration rate, (mL/min/1.73 m^2)	68 ± 19	74 ± 17
Urinary albumin/creatinine ratio, (mg/mmol)	1.3 (2)	0.8 (1.8)
Body mass index, (kg/m^2)	26.8 ± 4.3	26.3 ± 3.5
Systolic blood pressure, (mmHg)	149 ± 19	151 ± 21
Antihypertensive treatment, n	370 (48 %)	313 (48 %)
Cholesterol, (mmol/L)	5.5 ± 1.1	5.4 ± 1.0
High Density Lipoprotein, (mmol/L)	1.5 ± 0.5	1.3 ± 0.3
Lipid lowering treatment, n	206 (27 %)	118 (18 %)
Smoking, n	47 (6 %)	45 (7 %)
Diabetes, n	106 (14 %)	92 (14 %)

3.1.3 Study three

The cohort is a subgroup of the individuals from the Mapping of Inflammatory Markers in Chronic Kidney Disease (MIMICK), prevalent hemodialysis patients from 6 dialysis units in Stockholm and Uppsala (Sweden) (122). At start 254 patients were invited; after exclusions for reasons such as unwillingness to participate or contagious infections, 228 HD patients were included. Out of these, we included the 188 HD patients who had two consecutive measurements of CRP, PTX3, albumin and Hcy three months apart.

3.1.4 Study four

Twenty-two prevalent hemodialysis patients, who had been on dialysis for 3 months or more, were recruited. Twenty patients were treated at a limited-care HD center and two patients were dialyzed at a hospital unit. Patients with immunosuppressive therapy, ongoing infection or active inflammation were excluded. The patients were dialyzed for four hours three times a week or more, using bicarbonate dialysate and low-flux membranes (PolyFlux 21L, Gambro, Sweden) with blood flow (Qb) 250 – 330 mL/min. The control group was a selection of 61 population-based healthy subjects (42 male and 19 female, mean age 59 ± 14 years), used for comparative analyses.

3.2 STUDY PROCEDURE

3.2.1 Study one

In all patients and healthy subjects, blood samples were taken sometime between 7 and 9 am after an overnight fast. In HD patients, blood was taken before and after a single HD session and they were only allowed to have tea or coffee with bread, butter and jam during the HD treatment not to affect the methionine metabolism.

3.2.2 Study two

In both PIVUS and ULSAM cohorts, the investigations were performed with similar standardized methods, including blood sampling, blood pressure and questionnaires regarding medical history, medication, smoking habits, physical activity and socioeconomic status (120, 121)**.**

3.2.3 Study three

Data for comorbidity were classified in the same way as in Davies et al., on a 0 to 7 point scale including chronic and active conditions. The risk grading 1 – 3 was determined based

on number of comorbidities: low risk (no comorbidity), medium risk (score 1 – 2) or high risk (score ≥ 3) (123).
Nutritional status was categorized using the subjective global assessment (SGA), BMI, lean body mass index (LBMI) and fat body mass index (FBMI). In 188 HD patients, plasma samples for measurements of PTX3, CRP, albumin and Hcy were taken twice, before start of a dialysis session on the same weekday with three months in between. The samples were centrifuged at 2,500 g for 20 minutes at 4 °C and kept frozen at -70 °C unless immediately analyzed. During the follow-up period of 41 months, 88 individuals (47 %) died.

3.2.4 Study four

3.2.4.1 PTX3 during a HD session in 22 patients

During a midweek HD session, when the patients had been without dialysis treatment for one day, plasma samples were taken at 0, 30, 60, 120, 180 and 240 minutes during the HD treatment. The dialysis was standardized, all patients had a four hour dialysis treatment with blood flow 250 – 330 mL/min, dialysate flow rate 500 mL/min and as anticoagulant tinzaparin sodium (Innohep©, Leo Pharma, Sweden).

3.2.4.2 PTX3 during repeated HD sessions

In a subgroup of seven patients, we repeated the same schedule (samples at 0, 30, 60, 120, 180 and 240 min) during three HD sessions with low-flux filters, to evaluate if the total exposure of the inflammatory marker PTX3 would vary if the time from the previous HD treatment was 48 or 72 hours, respectively.

3.2.4.3 Impact of low-flux or high-flux membranes or hemodiafiltration (HDF) on inflammatory markers

In a subgroup of eleven patients, we repeated the same schedule (samples at 0, 30, 60, 120, 180 and 240 min) during three HD sessions with low-flux or high-flux membranes or HDF, to evaluate the effects of filters and dialysis modality on the measured inflammatory markers.

3.2.4.4 Impact of vascular access puncture on PTX3 release

In a subgroup of ten patients, we performed needle puncturing of the fistula one hour before the start of the dialysis treatment to see if the puncturing itself had an impact on PTX3 plasma levels. Plasma samples were obtained after insertion of the needles (- 60 min) and just before the HD start (0 min).
Result: The vascular puncture did not affect PTX3 levels during the following HD treatment.

3.2.4.5 Correction for ultrafiltration (UF)

Most HD patients gain weight between the HD sessions because of an inability to produce urine. During the HD treatment, aside from clearance of uremic toxins, excess fluid is removed from the patient and this induces hemo-concentration and increased concentrations in plasma. Therefore, we have corrected the concentrations of PTX3, IL6,

TNFα and hsCRP taken during HD for net UF using the formula:

Measured concentration / (1 + Δ BW/0.2 BW) = Concentration corrected for UF

The ΔBW is the change in bodyweight (BW) during HD and the extracellular volume is 20 % of the post-dialysis BW, assuming linear UF during HD (124).

3.3 BIOCHEMICAL ANALYSIS

3.3.1 Homocysteine

3.3.1.1 Study 1

Blood was collected for analysis of plasma concentrations of reduced, free (non-protein-bound disulfides) and protein-bound species. Total plasma concentration of Hcy (tHcy) is the sum of all three forms of Hcy. The procedure was to take blood samples in cooled EDTA tubes and centrifuge at +4 °C at 5,100 g for 5 minutes. Plasma was separated from red blood cells and analyzed fresh to determine free and reduced Hcy within 24 hours. Analysis of total Hcy in plasma could wait and samples were stored at -70 °C until analysis (Fig. 6). The techniques for separation and analysis of the different forms of Hcy are thoroughly described in paper 1 (125, 126).

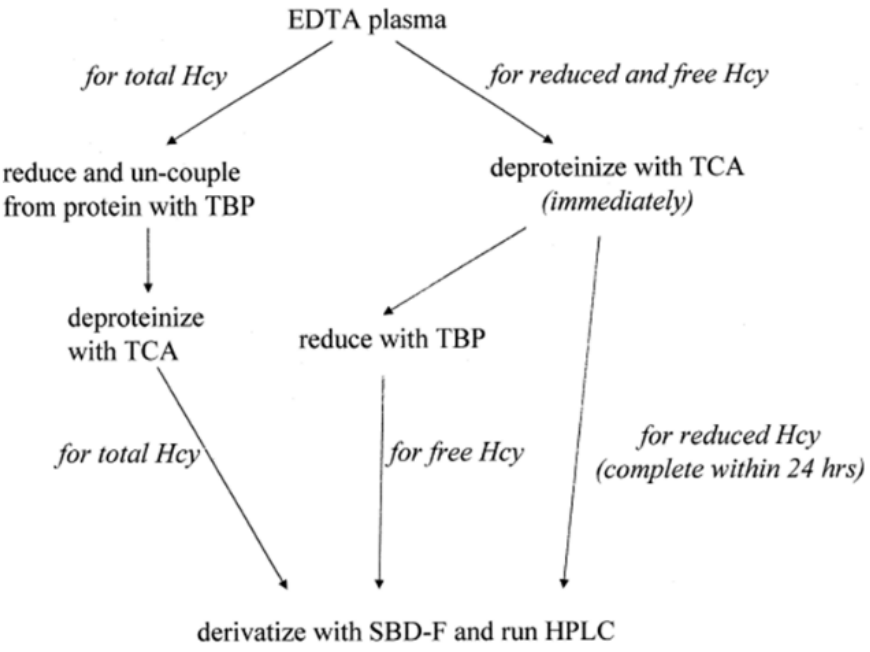


Figure 6. Techniques for separation and analysis of the different forms of Hcy. 4-fluoro-7-sulfobenzofurazan (SBDF), high-pressure liquid chromatography (HPLC), trichloroacetic acid (TCA), tri-n-butylphosphine (TBP).

3.3.1.2 Study 3

Total Hcy was analyzed using an immunometric assay on an Immulite© 1000 Analyzer (Siemens Healthcare Diagnostics, Los Angeles, CA, USA) according to the instructions of the manufacturers.

3.3.2 Pentraxin 3

3.3.2.1 Study 2

The plasma PTX3 concentration was analyzed using a commercial available enzyme-linked immunosorbent assay (ELISA) kit (DY1826, R&D Systems, Minneapolis, MN, USA).

3.3.2.2 Study 3

The plasma PTX3 concentration was measured using a commercially available ELISA kit (Perseus Proteomics Inc., Tokyo, Japan).

3.3.2.3 Study 4

The blood samples were taken just before the start of the dialysis treatment (0 minutes), during the dialysis treatment at 30, 60, 120 and 180 minutes, and at the termination of the dialysis at 240 minutes. The first sample was collected from the arterial needle before connecting to the dialyzer. The following samples were collected from the afferent sampling port without changing the blood flow.

The PTX-3 concentration was measured using the same type of ELISA kit as in study 3 (Perseus Proteomics Inc, Tokyo, Japan).

3.3.3 Cystatin C

3.3.3.1 Study 2

Cystatin C was measured with a latex-enhanced reagent (N Latex Cystatin C; Siemens, Deerfield, IL. USA) using a BN ProSpec analyzer (Siemens) and the data were used to estimate GFR in ULSAM with the formula eGFR = 77.24 * cystatin $C^{-1.26_2}$. In PIVUS, Gentian, Moss, Norway was used and eGFR was calculated using the formula eGFR = 79.901 *cystatin $C^{-1.43_9}$ (127, 128).

3.3.4 CRP and other inflammatory markers

3.3.4.1 Studies 1 and 3

hsCRP was measured using an immunometric assay from Immulite© 1000 Analyzer (Siemens Healthcare Diagnostics, Los Angeles, CA, USA).

3.3.4.2 Study 2

Measurements of hsCRP were performed with a latex enhanced reagent (Dade Behring, Deerfield, IL, USA) using a Behring BN ProSpec analyzer (Dade Behring).

3.3.4.3 Study 4

The serum levels of IL6, TNFα and hsCRP were quantified on the Immulite© automatic analyzer (Siemens Healthcare Diagnostics, Los Angeles, CA, USA).

3.3.5 Others

Laboratory analyses of creatinine, urea, albumin, hemoglobin, folate and vitamin B_{12} were determined using routine procedures at the local Department of Clinical Chemistry in papers I-IV. There is one exception; in article IV, albumin was determined with a fully automated routine method using bromcresol green on a Konelab 20XT centrifugal analyzer (Thermo Electron Corporation, Vantaa, Finland).

3.4 STATISTICAL ANALYSIS

3.4.1 Paper I

Data are given as mean ± standard deviation (SD) or median (range). One-way ANOVA or the Kruskal-Wallis one-way analysis of variance was used to evaluate differences between groups. The Student's t-test for paired comparisons was used to evaluate the differences between samples taken before and after HD. The association between variables was evaluated using Pearson's correlation coefficient or the Spearman rank-order correlation coefficient. Statistical significance was accepted at a p-value < 0.05 (two-tailed).

3.4.2 Paper II

Normally distributed continuous variables are presented as mean ± SD, skewed continuous variables as median (interquartile range) and categorical variables as n (%). To assess cross-sectional associations between PTX3 and GFR as well as ACR (expressed per 1 SD increase), we used multivariable linear regression models. The following multivariable models were used: **A** – Age- and gender-adjusted model (gender is only relevant in the PIVUS cohort). **B** – Inflammatory model (age, gender and CRP). **C** – Cardiovascular risk factor model (age, gender, CRP, smoking, BMI, systolic blood pressure, HDL cholesterol, total cholesterol, diabetes mellitus, antihypertensive treatment and lipid lowering treatment).

The longitudinal association between PTX3 levels at baseline and in patients with incident CKD (defined as GFR < 60 mL/min/1.73 m^2) at a re-examination after 5 years were investigated in both cohorts where the same multivariable models A-C were used. In these models, all individuals with GFR < 60 mL/min/1.73 m^2 at baseline were excluded. In both cohorts, we also investigated whether baseline PTX3 level predicted change in GFR

(ΔGFR) after 5 years, using multivariable regression models adjusted for age at baseline and follow-up and baseline GFR. In the PIVUS cohort, where data on PTX3 and GFR were available both at baseline and follow-up, we investigated the association between the change in PTX3 (ΔPTX3) and change in ΔGFR between baseline and follow-up after 5 years, using multivariable linear regression models adjusted for age at baseline and follow-up and baseline GFR (to avoid regression towards the mean). The statistical software package STATA 12.1 (Stata Corp, College Station, TX) was used.

3.4.3 Paper III

The patients were grouped based on which tertile (33rd and 66th percentiles) their baseline and 3-month levels of PTX3, CRP, albumin and Hcy fell in. For PTX3, the 33rd and 66th percentiles were 7.9 and 13.6 ng/mL for the baseline measurements and 6.9 and 12.1 ng/mL for the 3-month measurements.

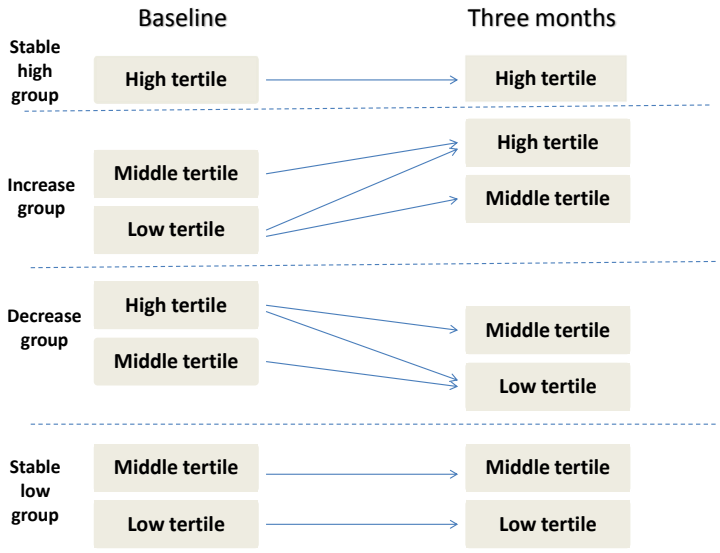


Figure 7. Classification of trimestral variation patterns for PTX3, CRP, albumin and Hcy.

At each time point, individuals were categorized based on each biomarker's tertiles of distribution (low, middle, high). The change in inflammatory markers was categorized based on the tertile of distribution of the studied biomarker at each time point. From the nine possible combinations, four groups were created by clustering patients with changes in the same direction: a) Individuals who showed a decrease in serum levels over the three-month period (from high to middle, middle to low, or high to low tertiles) were classified as a "decrease" group; b) Individuals showing an increase (from low to middle, middle to high and low to high tertiles) were classified as an "increase" group; c) Individuals with both values within the highest tertile of distribution were labeled as a "stable high" group; d) Individuals with both values within the lower or the middle tertile were labeled as a "stable low" group (Fig. 7). The same procedure was applied for each inflammatory mediator and for each cohort separately.

Normality for all variables was assessed by plotting the frequency distribution and taking into account the values of skewness and kurtosis. To test differences between the four groups, one-way ANOVA, Kruskal-Wallis and Chi square tests were applied for normally, non-normally distributed and categorical data respectively. Survival was assessed through the Kaplan-Meier analysis and the Cox proportional hazards model, including the above mentioned four fluctuation categories for each inflammatory mediator. Since age, sex, comorbidities and nutritional status are known to interact with inflammation and influence mortality, they were included as confounders in multivariate Cox models. To take into account the potential confounding effect of baseline inflammatory levels, the logarithmic transformed value was included in both crude and multivariate analyses. All variables satisfied the proportional hazards assumption, which was tested by correlating the Schoenfeld residuals of each covariate with the survival rank for a specific patient.

To assess the correspondence between changes of the different inflammatory markers, differences over the 3-month period were calculated (ΔCRP, ΔPTX3, Δalbumin, and Δhomocysteine) and correlated with each other using Pearson correlation tests. To study the within-subject range of variation for CRP, PTX3, homocysteine and albumin in hemodialysis patients, we calculated the intra-class correlation (ICC) from estimates of between-subject and within-subject variance, derived from mixed model (129). All the statistical analyses were performed in SAS version 9.3 (SAS institute, USA). For all hazard ratios, a 95 % confidence interval (95 % CI) not including 1, and for all other tests, a p-value < 0.05, were considered to be statistically significant.

3.4.4 Paper IV

All values are expressed as mean ± SD or median [25 – 75 percentiles], unless otherwise indicated. A p-value < 0.05 was considered to be statistically significant. Differences between time periods were analyzed by analysis of variance (ANOVA) using one-way ANOVA. Area under the curve (AUC) calculation of PTX3 levels was performed for each session and modality. The AUC was calculated using the trapezoidal method, and incremental AUC (IAUC) was calculated by subtraction of the basal PTX3 values. The baseline PTX3 was set to 1 in the AUC calculation. We used non-parametric analysis, Wilcoxon Signed Rank test, for comparison between two time periods. Because the p-values are not adjusted for multiple testing, they have to be considered descriptive. The statistical analysis was performed using statistical software SAS version 9.2 (SAS Campus Drive, Cary, NC, USA 27513).

3.5 ETHICAL APPROVALS

The studies in papers I, III and IV were approved by the Regional Ethical Review Board in Stockholm and the study in paper II was approved by the Regional Ethical Review Board in Uppsala.

4 RESULTS AND DISCUSSION

4.1 PLASMA REDUCED HOMOCYSTEINE IN PATIENTS WITH CKD (STUDY I)

Homocysteine (Hcy) is a sulfur-containing amino acid, derived from the methionine metabolism that is dependent on vitamin B and folic acid. Perturbation of the methionine metabolism results in accumulation of Hcy, particularly intracellularly (93, 130). Increased plasma concentration of tHcy is associated with CVD and progression of CKD in the general population (131, 132). Confusingly, there are discrepancies between earlier studies of Hcy levels and outcome in HD patients; one study shows that high levels of Hcy correlate with increased risk of vascular disease (132) and another study by Suliman et al. (2000) shows that low Hcy level is a risk factor for cardiovascular events (133). Interpretation of these contradictory findings is difficult, but wasting and hypoalbuminemia are suggested as confounders strongly associated with mortality and low tHcy levels. It is believed that the reduced form of Hcy, rHcy, is more atherogenic than either oxidized or protein-bound Hcy (134) and rHcy levels may therefore provide more information regarding Hcy toxicity than tHcy levels. The aim of this study was to examine the relation between the different forms of Hcy in dialysis patients (HD and PD) and in patients with CKD stage 3 – 5. In addition, the effect of hemodialysis on the different forms of Hcy was investigated.

In the present study and in agreement with previous studies, the highest levels of tHcy were found in PD patients where mean tHcy levels were 2.8 times higher, in HD patients 2.1 times higher and in CKD patients 1.9 times higher, compared with in controls. The same pattern was seen in fHcy and rHcy. The ratio rHcy/tHcy was significantly higher in dialysis patients than in healthy subjects or in CKD patients (Fig. 8).

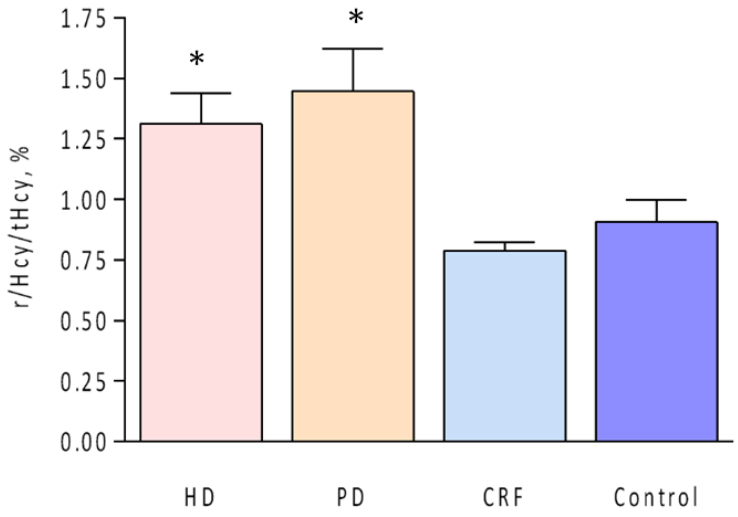


Figure 8. Ratios of reduced to total Hcy (rHcy/tHcy) in HD patients (n = 19), PD patients (n = 12) and CKD patients (n = 47). Age- and sex-matched healthy individuals is the control group (n = 15). * $p < 0.05$.

There were significant correlations between eGFR and tHcy, fHcy and rHcy and the same correlation was observed in the control group. There were no significant differences in plasma levels of tHcy, fHcy or rHcy in patients without or with supplementation of vitamin B_1, B_2 and B_6.

All three forms of Hcy were measured before and after a HD treatment in 19 patients. There was a 38 % decrease of tHcy level during one HD treatment. A greater decrease of fHcy was observed, as free disulfides (fHcy, Hcy-Hcy) are easily removed from plasma and the ratio fHcy/tHcy was 39 % before HD and 32 % after HD. Again, the harmful form of Hcy, rHcy showed a smaller decrease compared with tHcy and fHcy and the mean ratio rHcy/tHcy tended to increase (1.25 % before HD and 1.4 % after HD). The ratio rHcy/fHcy increases during HD and mirrors the redox change during HD with a relative increase of rHcy (Fig 9).

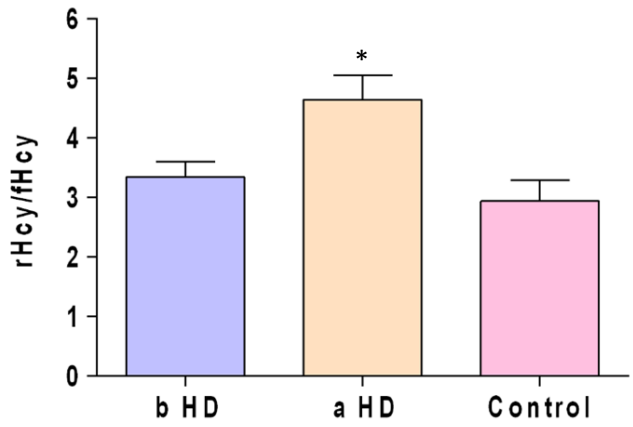


Figure 9. Plasma reduced to free Hcy (rHcy/fHcy) before and after one hemodialysis treatment (n = 19). Control group: Age- and sex-matched healthy subjects (n = 15). * $p < 0.05$.

This study shows that plasma rHcy concentrations are markedly elevated in CKD patients and strongly correlated with tHcy. Our finding of a smaller decrease of rHcy levels during a HD treatment, as compared with fHcy and tHcy levels, is of special interest. The greater variation in rHcy/tHcy in dialysis patients compared with in CKD patients and controls implies that changes in plasma rHcy concentrations do not only mirror changes in plasma tHcy levels in this population and that the harmful effect of rHcy may appear even when plasma level tHcy is low.

Two earlier studies of rHcy by Hultberg et al. and Himmelfarb et al. are at odds with our observations in the present study. Both groups found low concentrations of rHcy and decreasing ratios rHcy/tHcy during HD (135, 136). The study populations differ concerning smoking habits, supplementation of vitamin B and folic acid and biochemical methods of the different forms of Hcy. Our results are consistent with a study by Ueland et al., which showed

elevated plasma rHcy concentrations and also increased rHcy/tHcy ratios in HD and PD patients compared with controls (137).

Treatment with folic acid and vitamin B reduces the plasma concentration of tHcy, although several large vitamin intervention trials have shown that lowering the plasma concentration of tHcy does not reduce cardiovascular events in CVD patients with normal kidney function nor in CKD patients (88, 92, 138). In a large randomized controlled study by Ji et al. (2013), vitamin B supplementation for Hcy reduction significantly reduced stroke events (139). However, the new Cochrane review of 12 randomized controlled trials of Hcy-lowering intervention involving 47,000 individuals found no evidence of reduced risk of cardiovascular events through vitamin B treatment (89).

4.2 ASSOCIATION BETWEEN THE INFLAMMATORY MARKER PTX3, GLOMERULAR FILTRATION RATE AND CKD INCIDENCE IN TWO COMMUNITY-BASED COHORTS (STUDY II)

Routine laboratory tests, for example of creatinine and urea levels, are insensitive for identifying reduced kidney function, especially in early stages of CKD. During the last decade, many new biomarkers have been tested for early identification of kidney damage or kidney disease (140, 141). In a study from Copenhagen, elevated PTX3 level at admission is a strong predictor of short-term mortality in a community-based hospital setting (142).

In this study, plasma levels of the inflammatory marker PTX3 were analyzed in two large community-based cohorts of elderly in Uppsala, ULSAM and PIVUS. In these cohorts, higher PTX3 was associated with lower GFR in cross-sectional analyses.

Table 3. The cross-sectional association between PTX3 and GFR and ACR: Linear multivariate regression of the ULSAM and PIVUS cohorts.

ULSAM			
Model A		**Model B**	**Model C**
GFR	-0.12 (-0.19 – (-0.04))**	-0.10 (-0.18 – (-0.02))*	-0.09 (-0.16 – (-0.01)) *
ACR	0.08 (0.0 – 0.15)	0.05 (-0.02 – 0.12)	0.05 (-0.03 – 0.12)
PIVUS			
GFR	-0.15 (-0.22 – (-0.08))***	-0.14 (-0.21 – (-0.07))***	-0.16 (-0.23 – (-0.10)) ***
ACR	0.05 (-0.02 – 0.12)	0.04 (-0.03 – 0.11)	0.05 (-0.03 – 0.11)

Data are B-coefficients per standard deviation increment (95 % confidence intervals) ***p < 0.001, **p < 0.01, *p < 0.05. **GFR** = estimated glomerular filtration rate (Cystatin C); **ACR** = urinary albumin creatinine ratio **Models: A**- age and gender (PIVUS only), **B**- age, gender (PIVUS only) and CRP, **C**- age, gender (PIVUS only), CRP, smoking, BMI, systolic blood pressure, diabetes mellitus, HDL, cholesterol, antihypertensive and lipid treatment.

Our findings are in concordance with prior studies showing an association between PTX3 and advanced CKD (143, 144), but data on PTX3 and CKD in the general population is lacking. There is a large North American multi-ethnic study of a cohort of 2,824 men and women, with mean age 61 (45 – 84) years and without CVD or CKD (defined as Cystatin C estimated GFR > 60 mL/min/1.73 m^2), in which high PTX3 was associated with lower GFR, even after adjustments for demographics, comorbidities and IL6. When analyzing racial subgroups (26 % blacks, 19 % Hispanic, 34 % Chinese and 20 % whites), this

association was strong among blacks but non-significant among Hispanics, Chinese or whites (145).

Moreover, in longitudinal analyses, our study showed that higher PTX3 significantly predicted CKD incidence in both cohorts. We are not aware of any previous study reporting on the longitudinal association between PTX3 levels and CKD incidence in a community-based setting. In the ULSAM cohort, baseline PTX3 also predicted GFR decline and in the PIVUS cohort there was a close association between longitudinal changes in PTX3 and changes in GFR over 5 years. In contrast, no association was seen either between PTX3 levels and albuminuria in cross-sectional or in longitudinal analyses of ULSAM and PIVUS cohorts.

Table 4. Longitudinal analyses: Multivariate logistic regression of the association between PTX3 and the development of CKD (defined as GFR < 60 mL/min/1.73 m²) in the ULSAM (77 and 82 years, number of events/numbers at risk 206/315) and PIVUS (70 and 75 years, number of events/numbers at risk 229/746) cohorts, respectively.

Odds ratios with 95 % confidence intervals		
	ULSAM	**PIVUS**
Model A	1.33 (1.05 – 1.70) *	1.13 (0.96 – 1.34)
Model B	1.33 (1.04 – 1.69) *	1.13 (0.96 – 1.34)
Model C	1.37 (1.07 – 1.77) *	1.21 (1.01 – 1.45) *

Significance level * $p < 0.05$. **Model A**: Age (at the baseline and the follow-up examination) and gender (PIVUS) **Model B:** Age at baseline and follow-up, sex (PIVUS), and CRP **Model C**: Age at baseline and follow-up, sex (PIVUS), and CRP, BMI, smoking, systolic blood pressure, HDL, Cholesterol, diabetes, and antihypertensive and lipid.

The mechanism by which PTX3 is associated with CKD remains unclear, but higher PTX3 is related to vascular inflammation, indicating that inflammation and accordingly atherosclerosis is present already in mild and often undetected CKD. The properties of PTX3 may be helpful in early identification of patients at risk for CVD. In a recent study, circulating PTX3 seems to have the potential to be a marker of renal protective effects of atorvastatin medication. In patients with raised PTX3 at baseline the decline of GFR during the follow-up period of 2.5 years was significantly smaller in patients treated with atorvastatin than in those given a placebo (101).

Finally, PTX3 is a promising kidney damage biomarker already prior to the development of overt CKD.

4.3 THREE-MONTH VARIATION OF PLASMA PTX3 COMPARED WITH CRP, ALBUMIN AND HOMOCYSTEINE (STUDY III)

In this study we compared the four different biomarkers Hcy, albumin, CRP and PTX3 based on two measurements of trimestral variation and mortality risk in 188 prevalent HD patients. This is the first study to show repeated measurements of PTX3 in a dialysis cohort.

4.3.1 Variability

We found that PTX3 is highly variable within individuals, even more so than CRP, and that the trimestral variation of PTX3 between the two measurements was associated to mortality. From the variation of PTX3, CRP, albumin and Hcy, the intra-class correlation (ICC) was calculated and the within-subject variation was most pronounced for PTX3, having the lowest ICC (44 %); accordingly, repeated measurements of PTX3 over time in the same individuals will yield more disparate results. The ICC for Hcy is 81 %, indicating that most of the observed variability is explained by between-subject variation and consequently the within-subject variation is low, implying greater stability within individual patients upon repeated measurements over time (Fig. 10).

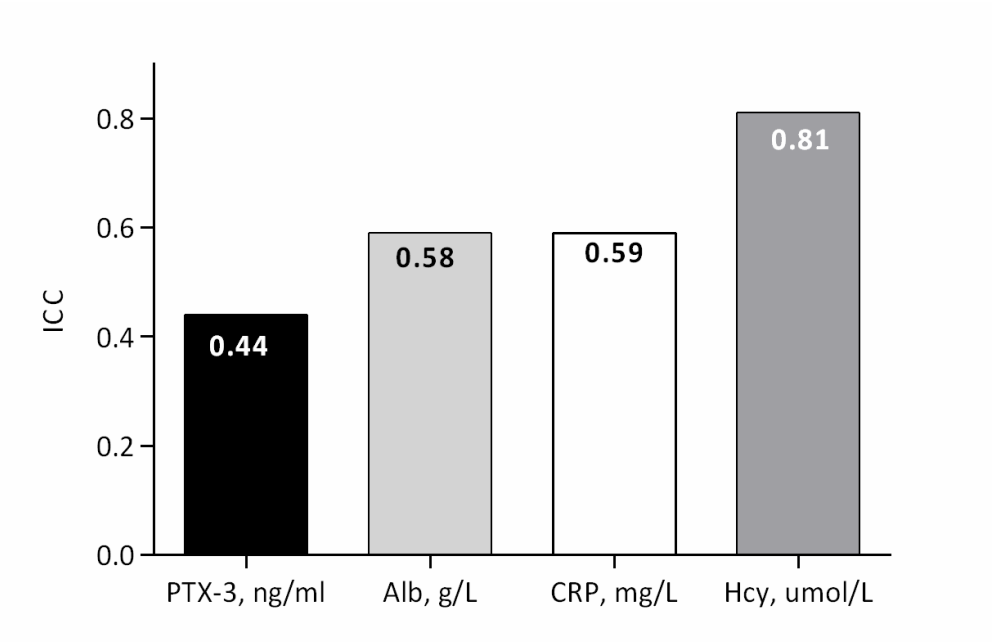
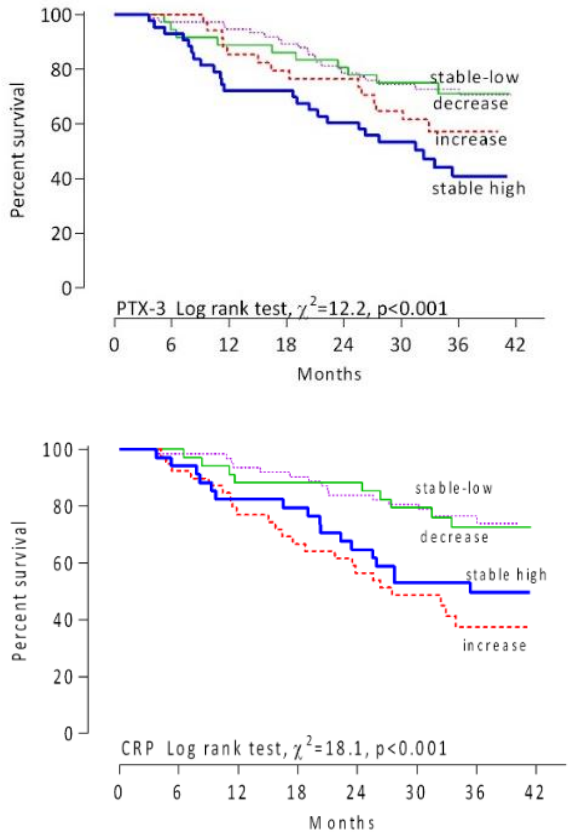


Figure 10. Variance components for PTX-3, albumin, CRP and Hcy in HD patients. The ICC was calculated from estimates of between-subject and within-subject derived from mixed models.

4.3.2 Mortality risk

4.3.2.1 PTX3 and CRP and mortality risk

The mortality risk for trimestral groups was assessed for PTX3, CRP, albumin and Hcy as crude and adjusted HRs. During the follow-up period of 41 months, 88 patients died (47 %). Patients in the "albumin stable high group" had the best survival rate. PTX3 and CRP were positively associated with a higher mortality risk, while albumin and Hcy were negatively associated with higher mortality risk. After adjustments for clinical factors the "PTX3 stable high group", the "CRP increase group" and "CRP stable high group" stayed significantly associated with high mortality risk compared with stable low groups of these markers (Fig 11). The variability of CRP (ICC = 0.59) in our study is in agreement with a recent study of healthy subjects, which showed an ICC level of 0.62 for CRP. The ICC for PTX3 was 0.44 and thus had a much higher intra-individual variation, which may reflect more rapid changes for PTX, produced locally in the vasculature, than for CRP, synthesized in the liver.



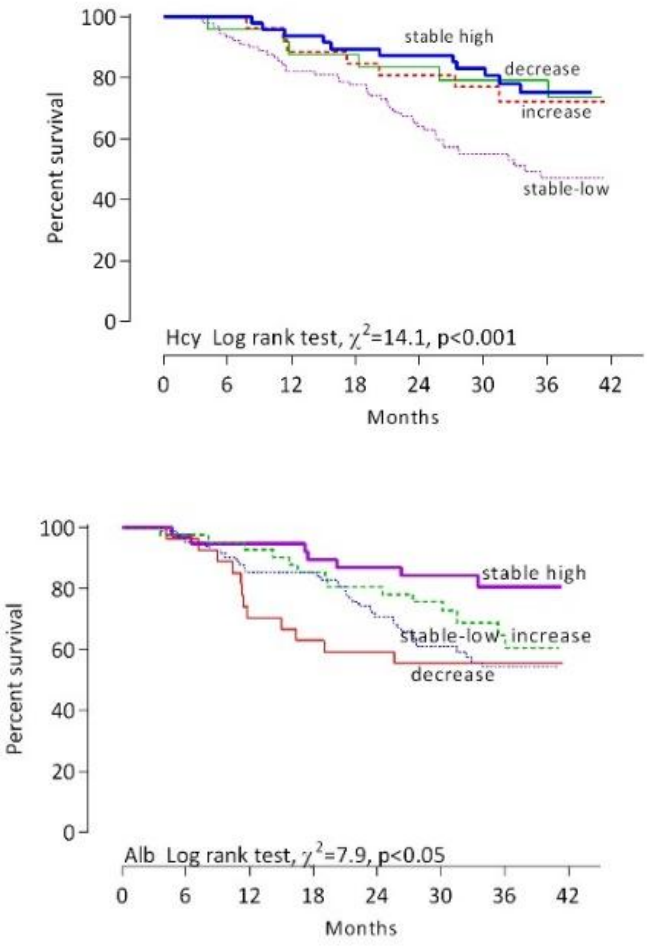


Figure 11. Kaplan–Meier survival curves for PTX-3, CRP, albumin and Hcy variation groups.

4.3.2.2 Albumin and mortality risk

Patients in the "albumin stable low group" and in the "albumin decrease group" had higher mortality risk compared with those in the "albumin stable high group". After adjustments for sex, age and comorbidity, only the "albumin decrease group" showed significantly higher mortality risk, demonstrating that a decreased albumin level is a clinical warning sign (Fig 11).

Low serum albumin is associated with poor outcome in dialysis patients (55, 146, 147). Albumin is a negative acute-phase reactant with a strong inverse association to inflammation in CKD patients and should be considered an inflammatory marker rather than a marker for nutritional status in this patient group (148).

4.3.2.3 Homocysteine and mortality risk

High homocysteine plasma levels are associated with worse cardiovascular outcome in the general population. However, in CKD patients, there is an inverse association between Hcy levels and CVD and mortality (87, 133). This is in accordance with our findings, as patients in the "stable low group" of Hcy had significantly increased mortality risk after adjustments

for age, sex and comorbidity compared with patients in the “stable high group” of Hcy. This inverse relation between low Hcy levels and worse outcome may be explained by inflammation and malnutrition in HD patients.

4.3.3 Summary

PTX3 measurements are less stable and show higher variation within patients (lower ICC value, ICC = 0.44) than albumin, CRP and Hcy (ICC = 0.58, 0.59 and 0.81 respectively). Persistently elevated PTX3 levels are significantly associated with high mortality and accordingly repeated measurements of PTX3 help in predicting mortality risk in HD patients.

4.4 PTX3, A SENSITIVE EARLY MARKER OF HEMODIALYSIS-INDUCED INFLAMMATION (STUDY IV)

PTX3 stands out as a rapid and sensitive marker of HD-induced inflammation and the pattern (149) of the individual PTX3 increase during HD is reproducible.

4.4.1 PTX3 during a HD session in 22 patients

The median plasma PTX3 of the 22 HD patients increased from 5.8 (3.9 – 8.1) to 8.4 (6.4 – 10.5) ng/ml, $p < 0.001$, during a standard HD treatment of 4 hours and the increase was significant already after 60 minutes (Fig. 12). The plasma concentration of TNFα showed a moderate decrease during the first 30 minutes of the HD treatment, but there were no statistically significant changes in IL6 or CRP levels. All values are corrected for UF using the Bergström formula: Measured concentration / (1 + Δ BW/0.2 BW) = Concentration corrected for UF ((124) and **3.2.4.5**).

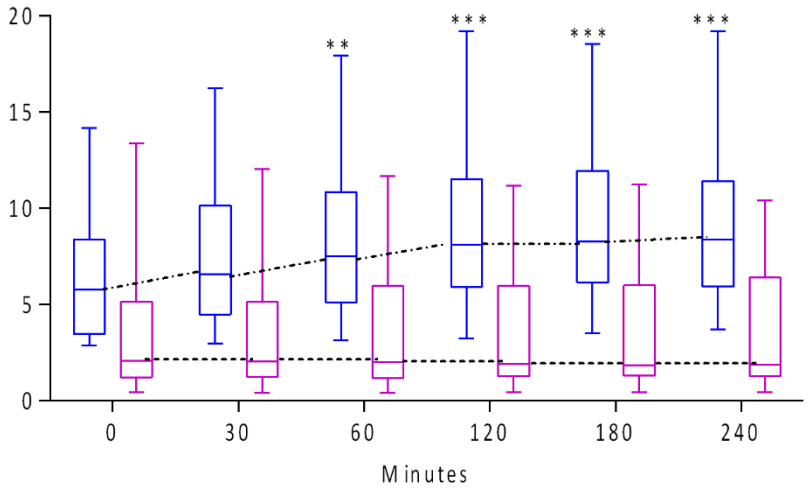


Figure 12. Box plot of **PTX3** (ng/mL); **CRP** (mg/L), during a 4 hour HD treatment. The values are corrected for UF (124). P-values are expressed as significant differences from the baseline value. **p < 0.01, ***p < 0.001.

4.4.2 PTX-3 during repeated HD sessions

In seven of the 22 patients, we repeated the study procedure in three HD treatments (Monday, Wednesday and Friday). The increase pattern of PTX3 was similar in all three HD treatments either there were two or three days in between the treatments. There was no significance when calculating AUC and IAUC to compare the three HD sessions.

4.4.3 Impact of low-flux membranes, high-flux membranes and hemodiafiltration (HDF) on inflammatory markers

There was no significant effect on the pattern of PTX3 levels by changing from low-flux to high-flux membranes or to HDF with high-flux membrane (Fig 13).

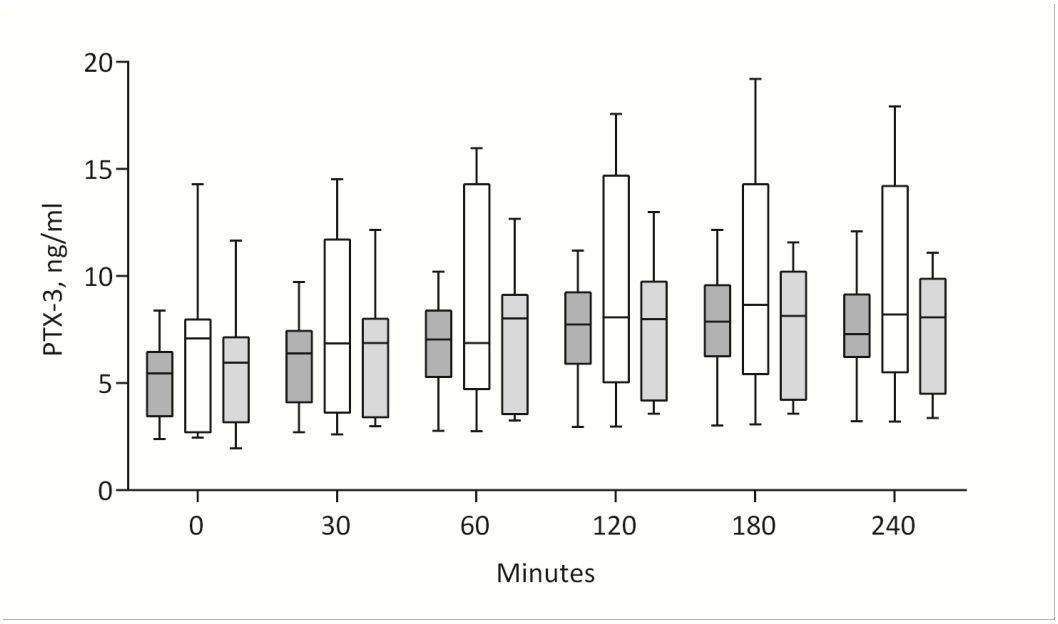


Figure 13. Box plot of PTX3 in 11 HD patients with different membrane and modalities; Low-flux (dark grey filled boxes), high flux (unfilled boxes) and HDF (light grey filled boxes), during a four-hour HD treatment. The values are corrected for UF (124).

The acute-phase protein PTX3 (42 kDa) is a rapid and sensitive inflammatory marker in response to dialysis-induced activation of the acute-phase immune system. The protein PTX3 is produced locally in the vasculature and can quickly be released from neutrophil granulae in response to pro-inflammatory cytokines (IL1, IL10 and TNFα). The acute-phase reactant CRP (21.5 kDa), a protein of the same family of highly conserved proteins as PTX3, both part of the innate immunity, is synthesized in the liver in response to the cytokine IL6. In this study, we show that a measurable systemic increase of plasma PTX3 can be detected in 60 minutes after stimuli (start of HD treatment); however, an increase of the systemic plasma level of CRP is not detectable during a standard HD treatment of 4 hours. The qualities of the protein PTX3 result in new possibilities to measure the inflammatory response in different constitutions of the HD treatment, tubing, filters, reverse transfer of dialysate contaminants (backflow), purity of water, different dialysis modalities, dialysis dose (Kt/V, frequency and weekly hours), needles, blood flow rate, ultrafiltration rate, anticoagulant treatment and many more factors.

To our knowledge, we were the first to study PTX3 levels during HD with different membranes and modalities, low-flux membranes, high-flux membranes and HDF with

high-flux membranes. We found no significant differences in the increase in PTX3 using different HD membranes or treatment modality in this study of 11 patients. The marked increase in PTX3 levels during HD sessions and modalities was seen in all patients, and the results are reproducible and consistent in all individuals which brings validity to the measurements of PTX3 during HD. Similar HD-induced effects on PTX-3 levels have been reported by Yamamoto et al (149). Our study cannot eliminate differences in HD modalities based on inflammatory activity without further studies with larger study groups. In a randomized controlled trial by den Hoedt et al (2014), the patients were treated with HD with low-flux filter or HDF for three years. The levels of CRP and IL6 decreased in patients with HDF and longtime HDF seems to reduce inflammatory activity compared to HD (150).

5 SUMMARY AND CONCLUSIONS

5.1 HOMOCYSTEINE

Homocysteine (Hcy) is a marker of disturbed metabolism in uremia and is suggested as a potential cardiovascular risk factor. The underlying effect of Hcy on the vasculature is thought to be exerted through the reactivity of the free sulfhydryl group in the reduced form of Hcy (rHcy). In our studies we found that

I rHcy is higher in patients with impaired renal function than in healthy controls. In dialysis patients, the ratio of reduced and total Hcy (rHcy/tHcy) is higher than in CKD patients and the ratio further increases during a HD treatment **(Study I)**.

II the intra-individual variation of Hcy in HD patients is low **(Study III)**.

III persistently low Hcy levels are associated with a high mortality risk in HD patients. The strong link between Hcy and hypoalbuminemia has previously been suggested to explain this association (151) **(Study III)**.

5.2 PENTRAXIN 3

The protein PTX3 is involved in the regulation of the innate immune system and is synthesized in the vasculature. It is quickly released from neutrophils after stimuli and subsequently detectable in plasma where PTX3 mirrors local inflammation. In our studies we have shown that

I PTX3 can predict the risk to develop CKD (defined as < 60 mL/min/1.73 m²) in the elderly (**study II**).

II PTX3 has higher variation within patients than CRP, albumin and Hcy according to the ICC analysis (**study III**).

III persistently high PTX3 levels are associated with a high mortality risk in HD patients (**study III**).

IV plasma PTX3 concentration shows a significant increase 60 minutes after the start of a HD treatment (**study IV**).

V the magnitude of the increase of PTX3 during HD did not significantly differ when the patients dialyzed with low-flux or high-flux membranes or with HDF (**study IV**).

5.3 STRENGTHS AND LIMITATIONS

Studies I–III are observational studies with the limitations associated with such studies and conclusions regarding causality cannot be drawn. **Study IV** is an interventional study where we measured inflammatory markers in response to different dialysis treatments such as HD with low-flux membrane, HD with high-flux membrane and HDF.

In **study I** we did map the plasma levels of different forms of Hcy in CKD and dialysis patients as the view at that time was that Hcy had a pathogenetic role in vascular disease and Hcy-lowering treatment was expected to give survival benefits. Later, large interventional studies ruled out advantages of such treatment in the general population as well as in CKD and dialysis patients.

In **study II** we have longitudinal data from two large cohorts of elderly in Uppsala with consistent results from both cohorts. A limitation is that there are only men in the ULSAM cohort.

In **study III** blood tests were taken in the morning or in the afternoon when patients came for their ordinary HD treatment and they were non-fasting. The variation was assessed based only on two measurements with three months in between.

Study IV contains four parts where 22 HD patients or subgroups of these patients were included. The results may not be representative for all HD patients as this cohort of HD patients was selected from a self-dialysis unit. Of note is the fact that the inflammatory burden of these patients is lower than for the general HD population. One of the strengths of this study is that we have measurements of PTX3, IL6, TNFα and CRP in parallel with achieved ultrafiltration (UF) volume at every 30 – 60 minutes during the HD treatments. All values are corrected for UF to avoid overestimation of circulating inflammatory markers due to changes in hemo-concentration.

5.4 FUTURE PERSPECTIVES

The protein PTX3 is a promising marker of inflammation in CKD patients. The role of PTX3 in the innate system is not fully understood, but recent studies indicate that PTX3 may protect against certain infections. Our results suggest an association between high PTX3 levels and the development of CKD. The clinical use of PTX3 assessment is not settled, but the protein may be a useful marker to monitor infections, complement activation and inflammatory diseases as well as vascular diseases and CKD. PTX3 appears to be one of the most sensitive markers to study hemodialysis-induced inflammation. In studies of new materials and hemodialysis devices, PTX3 has the potential to become a standard method for evaluating biocompatibility.

Plasma total Hcy, on the other hand, is not as yet a clinically useful biomarker. Several Hcy-lowering interventions with different combinations of B-vitamins and folic acid have failed to show any meaningful clinical benefit. Vascular effects of free reduced Hcy may still be worth exploring in prospective clinical studies, but the method is laborious and difficult to apply in large cohorts.

6 SVENSK SAMMANFATTNING

Personer med kronisk njursvikt har ökad risk för tidig död och majoriteten av dödsfallen är till följd av hjärt-kärlsjukdom. Förutom välkända riskfaktorer såsom rökning och förhöjda blodfetter är inflammation en riskfaktor för att utveckla hjärt-kärlsjukdom såväl hos befolkningen i stort som hos njursjuka. Många studier har gjorts för att närmare förstå bakgrunden till den komplexa folksjukdomen hjärt-kärlsjukdom och det har visat sig vara många faktorer som samverkar. Ett flertal proteiner är involverade i vårt immunförsvar och deras koncentration i blodet förändras vid en inflammatorisk process hos individen.

Syftet med min avhandling är att undersöka kärl- och inflammationsmarkörer hos njursjuka och relatera dessa till risk för död.

Pentraxin 3 (PTX3) är ett protein som härrör från samma familj som C-reaktivt protein (CRP) men har annorlunda egenskaper, bland annat bildas och utsöndras PTX3 direkt från kärlbädden och koncentrationen i blodet stiger därför snabbt efter inflammatorisk påverkan. Homocystein (Hcy) är en aminosyra som bildas när aminosyran metionin omsätts. Förhöjd serumkoncentration av Hcy associerar med ökad risk för död i hjärt-kärlsjukdom hos befolkningen i allmänhet. Vid sjunkande njurfunktion ses förhöjda nivåer av Hcy.

I **studie I** undersöker vi nivåerna av olika former av Hcy hos individer med kronisk njursvikt, med eller utan dialysbehandling. Vi visar att den reducerade formen av Hcy, som antas vara skadlig för kärlens insida, är förhöjd vid kronisk njursvikt och stiger ytterligare under en enskild bloddialysbehandling.

I **studie II** analyserar vi nivåerna av PTX3 hos äldre individer i två befolkningsgrupper (kohorter) i Uppsala; The Uppsala Longitudinal Study of Adult Men (ULSAM) och The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS). I den senare var hälften kvinnor. Resultaten visar att högre serumnivåer av PTX3 korrelerar med försämrad njurfunktion, dvs. sjunkande glomerulär filtration (GFR). Dessutom finner vi, genom uppföljning med nya prover efter fem år, att plasmanivån av PTX3 hos individer med normal njurfunktion vid första provtagningstillfället kan förutsäga utveckling av njursvikt.

I **studie III** undersöker vi fyra inflammationsmarkörer (PTX3, CRP, albumin och Hcy) hos patienter som behandlas med regelbunden bloddialys. Prover togs före dialys vid två tillfällen med tre månaders mellanrum. Vi analyserar variationen över tid och demonstrerar att PTX3 har störst individuell variation av de undersökta markörerna. Vi finner även att risken för död ökar hos individer med upprepade höga plasmanivåer av PTX3. Därutöver visar vi att stigande CRP, sjunkande albumin och upprepade låga värden av Hcy associerar med ökad dödlighet.

Slutligen undersöker vi i **studie IV** hur koncentrationen av proteinet PTX3 förändras under en bloddialysbehandling. Vi tog prover efter 0, 30, 60, 120, 180 minuter och vid dialysens slut efter 240 minuter. PTX3 är förhöjt redan vid dialysstart, men därutöver stiger PTX3-koncentrationen snabbt under själva dialysbehandlingen och ökningen är signifikant redan efter 60 minuter. Vi testar olika dialysfilter (låg- och högpermeabelt filter samt

hemodiafiltration (HDF) med högpermeabelt filter), men finner ingen signifikant skillnad i ökningen av PTX3.

Sammanfattningsvis så visar vi att PTX3 är en lovande markör för att påvisa inflammation och risk för död hos patienter med kronisk njursvikt.

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