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INNATE IMMUNITY AND CARDIAC STRUCTURE IN CHRONIC KIDNEY DISEASE

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INNATE IMMUNOLOGY AND CARDIAC STRUCTURE IN CHRONIC KIDNEY DISEASE

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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”The world is full of magical things
patiently waiting for our wits to grow sharper”

- Bertrand Russell

ABSTRACT

The inflammatory state of chronic kidney disease (CKD) leads to increased susceptibility to infections and cardiovascular complications. In this thesis we aimed to investigate development of inflammatory properties at different stages of CKD and changes in innate immunity as well as cardiac structure. Fibroblast growth factor 23 (FGF23), a phosphaturic hormone known to impact cardiovascular outcome and inflammatory markers in CKD was also analyzed.

In the **first paper**[1] a cross sectional study of transmigrated monocytes in patients with advanced CKD was performed. Patients with CKD had an increased percentage of $CD16^+$ monocytes, distorted TNF-α and IL-10 levels and a significantly higher level of fractalkine ($CX_3CL_{1)}$. This inflammatory profile may in part mediate the altered immune response in CKD.
The aim of the **second paper**[2], was to investigate end organ damage to the heart of the pro-inflammatory state of CKD by evaluating cardiac structure and function in patients with CKD stages 2-5, compared with healthy controls in the PROGRESS cohort. Transthoracic echocardiography and Tissue Doppler Imaging (TDI) were performed to describe cardiac dimensions such as left ventricular mass, wall thickness and diastolic and systolic function. CKD patients had a higher prevalence of left ventricular hypertrophy (LVH) and alterations in systolic and diastolic myocardial function compared to the healthy controls.
In the **third paper**, inflammatory changes and altered monocyte function in patients with CKD stage 2-3 was compared to healthy controls in the PROGRESS cohort at baseline and at follow up after 3 and 5 years. Monocytes from CKD patients showed early functional abrasions, with altered adhesion molecule expression and significantly lower fMLP-induced upregulation of CD11b and decreased level of L-selectin (CD62L). CKD patients also had lower oxidative burst in response to fMLP over time as well as elevated pro-inflammatory cytokines; TNF-α, RANTES and IL-12. These findings suggest that a transformation of monocyte function occurs at an early phase of renal impairment and may together with increased plasma levels of pro-inflammatory cytokines contribute to the higher vulnerability of CKD patients to comorbidities.
Our primary objective of the **fourth paper** was to characterize the altered chemokine profile and leukocyte function at CKD stages 2-5 and investigate correlations between these markers and levels of FGF23. Elevated levels of FGF23 in CKD are associated to worse outcome and cardiovascular complications. FGF23 has also been described to interact in inflammatory processes. FGF23 was significantly elevated in the CKD group, and correlated to GFR, PTH, urinary albumin excretion (UAE) and phosphate as well as to the expression of IL-12 and RANTES. *In vitro* incubation of leukocytes with FGF23 reduced CD11b expression in resting as well as in fMLP-stimulated granulocytes. Together this indicates an influence of FGF23 on leukocyte transmigration and an interference with chemokine signaling in CKD.

In Summary; several factors are involved in inflammation in CKD. Better understanding of immunologic mechanisms and altered cellular function at different stages of CKD might help to explain the enhanced risk of cardiovascular disease as well as the increased susceptibility to infections. With improved knowledge of the inflammatory processes accompanying CKD we might obtain diagnostic and prognostic tools to improve clinical outcome.

LIST OF SCIENTIFIC PAPERS

This thesis is based on the following papers which will be referred to in the text by their Roman numerals

I Increased Accumulation of CD16+ Monocytes at Local Sites of Inflammation in Patients with Chronic Kidney Disease.
Wallquist C, Paulson JM, Hylander B, Jacobson SH and Lundahl J. *Scand. J. Immunol. 2013; 78: 538–544.*

II Cardiac remodelling and functional alterations in mild-to-moderate renal dysfunction: comparison with healthy subjects.
Asp AM, **Wallquist C**, Rickenlund A, Hylander B, Jacobson SH and Lundahl J, Eriksson M. *Clin. Physiol. Funct. Imaging 2015; **35**: 223–230.*

III Early changes in monocyte adhesion molecule expression and TNF-α levels in chronic kidney disease - a 5 year prospective study.
Wallquist C, Mansouri L, Norrbäck M, Hylander B, Jacobson SH and Lundahl J. *Am J Nephrol 2016;**44**:268-275*

IV Associations of fibroblast growth factor-23 with markers of inflammation and leukocyte function in chronic kidney disease.
Wallquist C, Mansouri L, Norrbäck M, Hylander B, Larsson T Jacobson SH, and Lundahl J. *Submitted for publication*

Other publication based on the cohort, not included in this thesis:

Carotid remodeling and intima thickness in mild-to-moderate chronic kidney disease: A comparison with healthy subjects and advanced chronic kidney disease.
Asp AM, **Wallquist C**, Rickenlund A, Hylander B, Jacobson SH and Lundahl J, Eriksson M. *Submitted for publication*

CONTENTS

LIST OF ABBREVIATIONS

ACEI	Angiotensinogen Converting Enzyme Inhibitor
ARB	Angiotensin Receptor Blocker
aHTs	Antihypertensives
BMI	Body Mass Index
BP	Blood Pressure
CAPD	Continuous Ambulatory Peritoneal Dialysis
CKD	Chronic Kidney Disease
CKDEPI	Chronic Kidney Disease Epidemiology Collaboration
CRP	C Reactive Protein
CX_3CL_1	Fractalkine
DCFH-DA	DiChloroFluorescin DiAcetate
ELISA	Enzyme Linked Immunosorbent Assay
ESRD	End-Stage Renal Disease (stage of renal function when RRT is needed)
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein IsoThylCyanate
FGF23	Fibroblast Growth Factor 23
fMLP	N- formyl-Methionyl-Leucyl-Phenylalanine
GFR	Glomerular Filtration Rate
HDL	High Density Lipoprotein
HPT	Hyperparathyroidism
HR	Hazard Ratio
ICAM1	Intra Cellular Adhesion Molecule 1
IFN	Interferon
IL	Interleukin
KDOQI	Kidney Disease Outcomes Quality Initiative
LDL	Low Density Lipoprotein
LM-rev	Lund Malmö revised formula

LPS	Lipopolysaccharide
LVH	Left Ventricular Hypertrophy
LVMI	Left Ventricular Mass Index
MAP	Mean Arterial Pressure
MDRD	Modification of Diet in Renal Disease (study and equation)
MFI	Mean Fluorescence Intensity
NADPH	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NS	Non Significant
PBS	Phosphate Buffered Saline
PMA	Phorbol-12-Myriastate-7-Acetate
PMN	Polymorphonuclear
PTH	Parathyroid Hormone
RAAS	Renin Angiotensin Aldosterone System
RANTES	Regulation on Activation, Normal T and Secreted
ROS	Reactive Oxygen Species
RRT	Renal Replacement Therapy
SD	Standard Deviation
SOD	Superoxide Dismutase
TNF	Tumor Necrosis Factor
U-ACR	Urine-albumin/creatinine ratio
UAE	Urinary Albumin Excretion
VCAM	Vascular cell adhesion molecule

1 GENERAL INTRODUCTION - CHRONIC KIDNEY DISEASE

1.1 EPIDEMIOLOGY AND ETIOLOGY

Chronic kidney disease (CKD) is a cumbersome and life threatening disorder. Renal failure exposes the patients to an accelerating degree of inflammation and disabling uremia. Furthermore this condition brings additional sickness and death through higher incidence of cardiovascular morbidity and mortality[3–5] as well as increased susceptibility to infections[6]. Treatment of end-stage renal disease (ESRD) with renal replacement therapy (RRT), achieved by hemodialysis, peritoneal dialysis or kidney transplantation, can be lifesaving. RRT is expensive and not readily offered outside the industrialized world. Demand and costs of RRT are increasing each year[7,8]. In Sweden, the number of dialysis patients has increased by 35 % since the millennium[9]. In the industrialized world the plethoric geriatric group mediates a steadily growing incidence of RRT in the population[10,11]. Patients with CKD are a heterogenic group with the declining renal function due to many different etiologies[9], where diabetic nephropathy, nephrosclerosis and glomerulonephritis constitute the largest diagnosis groups.

1.2 CLASSIFICATION OF CHRONIC KIDNEY DISEASE

National kidney foundation and the Kidney Disease Outcomes Quality Initiative (NKF/ KDOQI) published an unifying classification and definition of CKD in 2002[12], and in 2013 an updated version was launched. Based on glomerular filtration rate (GFR) there are currently five stages or grades (G) where grade 3 is subdivided into 3a and 3b (Table 1). Structural or functional abnormalities of the kidney with a duration exceeding a time frame of three months is required to meet the definition. In the absence of evidence of kidney damage, GFR category G1 and G2 do not fulfill the criteria for CKD.

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

Table 1. GFR categories according to KDIGO guidelines [12,13]

1.3 GLOMERULAR FILTRATION RATE ESTIMATIONS

Inulin, a fructose polysaccharide, is considered the gold standard for GFR measurement[14]. Other frequently used methods to measure GFR are ^{125}I-iothalamate, ^{51}Cr-EDTA, and iohexol[15]. For every day clinical use however, these methods are too costly, time consuming and not without adverse effects. A more convenient and less expensive substitute to estimate GFR are endogenous substances such as creatinine, urea and cystatin C measured in urine or blood[16]. Serum creatinine (S-Cr) is the cheapest and most widely used alternative, but has multiple disadvantages. The S-Cr level is affected by the individual muscle mass, proportion of recently ingested meat and the body's fluid homeostasis[17].

Over the years, several attempts to develop a reliable equation to estimate GFR from the S-Cr have been made. The Cockcroft-Gault formula[18] estimates the creatinine clearance, which is not corrected by body surface area, and thus the absolute value of the filtration rate. Due to the increased creatinine secretion, the creatinine clearance usually overestimates GFR when the GFR is low. With these limitations taken in account the Swedish agency for health technology assessments workgroup stated in their report on estimating renal function from 2014 a general recommendation to discard this method[19,20]

The MDRD formula was launched 1999 and revised 2006[21], standardizing the GFR to body surface area (ml/min/1.73m^2) and based on the Modification of Diet in Renal Disease (MDRD) study[22]. The CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) formula is another more recently developed equation and obtains more accurate eGFR values in the high eGFR range (>60 ml/min/1.73 m^2) as well as eGFR values in the lowest range[23]. In research, the MDRD equation is more widely established and believed to give a more accurate estimate of the GFR, particularly for overweight and geriatric individuals[16,21]. Both the MDRD and the CKD-EPI equations include variables for age, gender and race and have in direct comparison to Cockcroft-Gault[23] and creatinine clearance measured from 24-hour urine collections demonstrated superiority[24]. The revised Lund-Malmö GFR estimating equation that combine creatinine and cystatin C and has in a large Swedish cohort outperformed MDRD and CKD-EPI[25,26].

Cystatin C, a proteinase inhibitor produced by all human cells and dependent on glomerular filtration has become an increasingly popular endogenous marker for GFR. It offers better precision among patients with mild CKD and is less sensitive to diet, gender, age and body configuration[27]. However, cystatin C eGFR should not be used on patients on medication with glucocorticoids or with hyperthyroidism in whom the levels are significantly increased.

At extremities in age or body configuration, severe malnutrition or obesity, in pregnancy, in vegetarian or vegan diet, muscle diseases or rapidly changing renal function all eGFR methods have their limitations.

2 BACKGROUND

2.1 RISK FACTORS FOR CKD

Already at the age of 25 years the GFR starts to decline with a pace of about 1 ml/min per year. There is a considerable inter-individual variation in the progression rate but midst patients with CKD, the progression rate is usually more rapid than in the general population[28,29]. By targeting blood pressure control, lowering albuminuria and optimizing body fluid volume and electrolyte balance, nephrologists try to delay or halt the progression rate, preventing patients from developing uremia and premature start of dialysis. The kidneys are exposed to multiple potentially damage inflicting causes ranging from exogenous toxins to life style factors and metabolic or inflammatory diseases. With these repetitive strikes at renal function over time, continuous loss of nephrons follow and glomerular hypertension develops in the remaining nephrons that try to compensate the overall loss of function by hyperfiltrating. This hyperfiltrative state leads to increased urine albumin excretion and enhanced mesangial cell proliferation as well as an elevated pro-inflammatory cytokine production[30,31]. Consequentially, renal fibrosis and nephrosclerosis develops.

2.2 CARDIOVASCULAR COMPLICATIONS IN CKD

CKD generally aggravates arteriosclerosis and already at modest level of CKD (stage 1 – 2) cardiovascular risk increases[32,33]. Cardiovascular disease (CVD) poses the greatest risk of premature death seen among patients with CKD, leading to 40% of all deaths among European dialysis patients[34]. The incidence of left ventricular hypertrophy as well as congestive heart disease and coronary heart disease increase with subsequent loss of renal function[35]. Risk of cardiovascular death is up to 30 times higher in dialysis patients than in age- and sex-matched controls in the general population[36]. Diabetes mellitus, hypertension, hypercholesterolemia, smoking and physical inactivity are well established traditional risk factors for CVD and are highly prevalent in patients with mild CKD (stage 1 – 3)[37]. Patients with CKD are challenged with additional pathological processes such as endothelial dysfunction, oxidative stress, low grade chronic inflammation, fluid overload, acidosis, AV-fistula complications, anemia and alterations in calcium and phosphate levels, as well as vascular calcification and secondary hyperparathyroidism[38,39]. In spite of all these striking data, CVD is frequently underdiagnosed and undertreated in patients with CKD[40]. These patients also display a combined atherosclerotic pathophysiology with both damaging intima located plaques and smooth muscle cell hyperplasia that in time develop extensive calcifications in the media layer of the arteries[41–43]. The entire vascular apparatus from coronary arteries to aorta and heart valves get widely infested with calcified plaques.

In CKD patients, lipid abnormalities are found early on and changes character as progression of renal failure continues[44].

Dyslipidemia in renal disease commonly exhibit an atherogenic profile with elevated LDL, reduced HDL and high triglycerides (TG). Increased glomerular permeability leads to loss of lipoprotein lipase and albuminuria stimulates hepatic synthesis of lipoproteins[45–47]. Inflammation suppresses the anti-oxidative action of HDL which leads to higher amounts of oxidized LDL[48]. Lipid-lowering medication has shown benefits in the early to moderate CKD population, probably related to the degree of LDL reduction, rather than the pleotropic effects of statins[49]. Treatment with lipid-lowering agents only moderately reduce atherosclerotic events in individuals in late CKD without being proven to alter mortality[50]. Left ventricular dysfunction (LVD) predicts congestive heart failure (CHD) and the presence of CHD predicts an exceedingly high mortality rate in dialysis patients[51]. Risk factors of LVD are multiple in CKD; ranging from volume overload, hypertension, inflammation, anemia and AV-fistulas with high blood flow that increases cardiac output[52–54]. LVH can be subdivided in concentric hypertrophy, concentric remodeling and eccentric hypertrophy. Eccentric hypertrophy may be a consequence of increased preload triggered by anemia and/or intra vascular volume expansion (salt and fluid loading) while concentric hypertrophy frequently results from afterload augmented by hypertension or increased systemic arterial resistance (secondary effect to RAS-activation or vessel calcifying HPT)[55]. The adjusted relative risk (RR) of death in a dialysis population is greatest for patients with eccentric hypertrophy[56]. N-terminal pro-brain natriuretic peptide (NT-proBNP) is used as a marker of CHF in non-renal patients where an increase leads to inhibition of the renin-angiotensin-system, vasodilatation and natriuresis[57–59]. With loss of renal function proBNP clearance is reduced, which leads to rapidly increasing levels. However even after correcting for volume overload, NT-proBNP is a prognostic factor of LVD and death in CKD[60–62] just like other newer cardiovascular prognostic biomarkers such as cardiotrophin and GAL-3 also have been shown to, after adjustment, be applicable in CKD[63,64]. Combined heart and kidney failure has been named the cardiorenal syndrome (CRS), a troublesome symptomatic condition requiring high hospitalization frequency and displaying a discouraging mortality rate[65,66] An inflammatory etiology to CRS has been proposed with enhancing inflammatory burden from oxidative stress on the endothelium[67].

2.3 ECHOCARDIOGRAPHY IN CKD PATIENTS

Cardiac imaging in CKD can monitor clinical progress, provide prognostic data and stratify risk as well as assess cardiac effects of therapies such as fluid management, dialysis and renal transplantation. 74% of patients with CKD stage 5 display evidence of LVH at the initiation of dialysis treatment[68]. Intrinsic cardiac disease can nevertheless be complicated to diagnose in CKD due to comorbidities as diabetes, hypertension or old age, all making symptom patterns less obvious. Additional CKD specific circumstances with volume overload or anemia further confound the clinical evaluation. Accurate diagnosis is essential for initiation of appropriate therapy. 2D transthoracic echocardiography is inexpensive, non-invasive and widely available, hence frequently regarded as the first-line investigative tool for determining cardiac function and structure. It classifies systolic function through evaluation of ejection fraction (EF) and fractional shortening (FS), as well as registers change in LV geometry according to LV end-diastolic volume, wall thickness and left ventricular mass index (LVMI). 2D echocardiography can identify structural changes associated with poor prognosis[69] and differentiate between type of hypertrophy. However, this method can be prone to inaccuracy as some measurements are derived rather than actual measures, it has low sensitivity in detecting subtle alterations in left ventricular function and is undermined by low reproducibility. Impaired relaxation and compliance of the left ventricle generally result in diastolic dysfunction. Diastolic function can be estimated by 2D echocardiography with comparison of blood flow across the mitral valve during early diastole (E, passive filling) and late diastole (A, atrial contraction). Under normal conditions, passive filling is greater than filling during atrial contraction, giving an E:A ratio of 1-2. A ratio < 1 signifies impaired relaxation whereas E:A > 2 indicates a restrictive filling (limited filling under late diastole). Elevated left atrial volume (LAV) predicts all-cause mortality and is a surrogate marker for diastolic dysfunction in CKD[70].

Tissue Doppler imaging (TDI) has the ability to detect myocardium wall motion abnormalities even when EF is in the normal range. These segmental contraction-relaxation disturbances of the myocardium affect the three-dimensional myocardium distortion under systole (longitudinal shortening, radial twist and circumferential contraction). TDI is especially reliable in detecting longitudinal and radial strain, amplifying the high-velocity signals from heart valve and myocardium describing systolic and diastolic function from tissue velocity. An altered longitudinal strain inclines worse prognosis despite preserved EF[71,72]. Furthermore, TDI can assess the vertical motion of the mitral annulus; an E/e´ ratio (i.e. mean early diastolic transmitral velocity (E) to mitral annulus diastolic velocity (e´)), ≥13 is indicative of diastolic dysfunction. Less available but somewhat superior; 3D transthoracic echocardiography produces assessments of ventricular volume and mass from full LV geometry data comparable to MRI results, not risking the overestimations of EF or underestimation of diastolic volumes like the 2D technique.

2.4 KIDNEY-BONE-AXIS AND FGF23

CKD-MBD or chronic kidney disease – mineral bone disorder, is a condition characterized by vascular calcification, biochemical abnormalities, fractures and increased mortality risk. Derived from osteocytes and osteoblasts, levels of fibroblast growth factor 23 (FGF23) are elevated at an early stage of renal failure[73]. FGF23 is a phosphaturic hormone which reduces the synthesis of active 1,25-dihydroxy vitamin D_3[74]. In CKD renal handling of phosphate is compromised leading to a compensatory rise in FGF23 and development of secondary hyperparathyroidism[75–78]. FGF23 appears to be a more sensitive biomarker of early kidney disease than creatinine and enhances phosphate secretion through binding to the tubular FGF-receptor 1c and the Klotho co-receptor in CKD stages 2-3. In addition, FGF23 simultaneously inhibits 1α-hydroxylase and stimulates 24-hydroxylase causing a decrease in circulating 1,25-dihydroxy vitamin D levels and also appears to reduce parathyroid hormone (PTH) secretion.

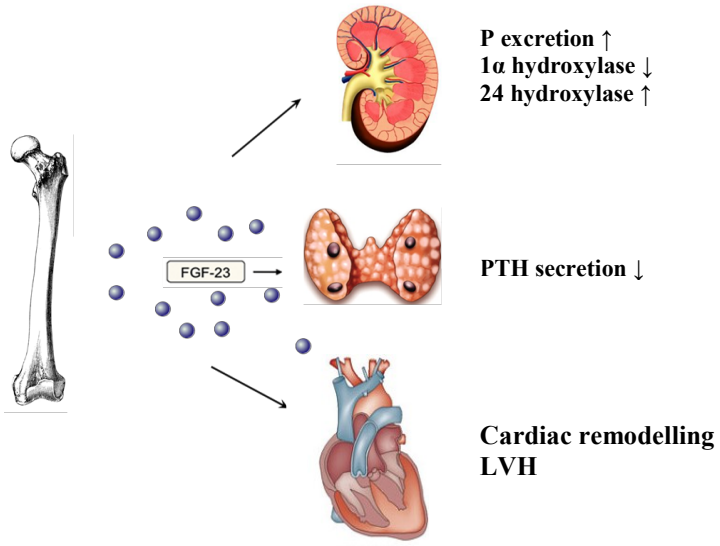

Figure 1 The effect of FGF23 on target organs.

Moderate changes of FGF23 are commonly seen in CKD 2-3 but a markedly elevated level is found in ESRD[79]. Changes in the levels of FGF23 in chronic renal failure have been shown to independently predict CKD progression in renal disease[78]. Adverse outcomes including cardiovascular disease (CVD) and mortality have been linked to higher systemic levels of FGF-23[80–83]. FGF-23 has been shown to enhance cardiovascular dysfunction, through diverse mechanisms ranging from impaired vasodilatation, enhanced dyslipidemia, heart valve and coronary vessel calcification, direct myocyte damage and LVH[84–89].

Circulating FGF23 levels are increased in heart failure and cardiomyocytes have been found to produce FGF23 which theoretically might have a paracrine effect that mediates adverse cardiac remodeling in the setting of heart disease[90–92].

Development of LVH can be abrogated by blocking of FGF23 binding to Klotho-independent FGFR4 activation despite presence of severe hypertension[93,94]. Klotho deficiency in CKD is in part related to systemic and renal inflammation and vascular calcification[95,96]. FGF23 stimulates the RAAS-system[97] and alter calcium trafficking in cardiomyocytes resulting in increased contractility, hypertrophy and arrythmogenesis[98,99]. FGF23 has been described to interact in metabolic processes such as inflammation, obesity, insulin resistance and iron homeostasis[83,100–107]. Recent studies have implied diverse effects of FGF23 on inflammation in CKD[101,108]. FGF23 associations to inflammatory markers have been described in both CKD and non CKD cohorts[100–103,109] The relationship between FGF23 and innate immunity appear to be more complex than previously assumed and would benefit from further investigations including influence of FGF23 on effector cell function.

2.5 INNATE IMMUNITY

The initial phase of a immune reaction to invading pathogens is often referred to as the innate immune response and largely depends on complement activation and neutrophil mobilization[110]. Exposure to bacterial peptides activates the complement cascade which surges a proteolytic reaction targeting the microorganism and co-stimulates the key effector cells; the neutrophils. Neutrophils have receptors that directly recognize both complement (C3b, C5a) and bacterial peptides. Circulating neutrophils that are exposed to microbes transform to a primed state, leave the circulation and enter the site of microbiologic invasion, where they develop more specific properties, such as phagocytosis and ability to discharge inflammatory mediators[111]. Neutrophils orchestra the innate but also the adaptive immune response by releasing pro-inflammatory substances[112]. A subsequent step in the innate immune response is the recruitment of other inflammatory cells. A chemotactic gradient is developed with peak concentrations of chemokines closest to the inflamed area. This gradient leads the way for the, due to their slower onset of extravasation, casually late entering monocytes. Monocytes have a longer survival than neutrophils and rapidly develops into macrophages on arriving at sites of inflammation[113] where they participate in phagocytosis and generates oxidative burst[114]. Stimulated macrophages release IL-12 and TNFα, both mediating sustained leukocyte transmigration and provoke the liver to produce systemic C-reactive protein (CRP)[115].

2.6 LEUKOCYTE EXTRAVASATION AND TRANSMIGRATION

Leukocytes exercise their physiological and pathophysiological effect extravascular after adhering to the endothelium and transmigrating into the intima or the subendothelial space. In order to practice their local defense mechanisms they need to get recruited and leave the circulation through several consecutive steps: rolling, activation, firm adhesion and transmigration (Figure 1). The first step is dependent on adhesion molecule interaction between the neutrophil and the endothelium of the vessel wall. This process, called tethering, is mediated by selectins, allowing the neutrophil to scan the endothelium for the presence of G-protein coupled receptors that activate a second phase of integrin-mediated adhesion. Once firmly adhered to the endothelium, the neutrophil squeezes through diapedesis into the extracellular matrix and onward subsequent transmigration guided by a chemoattractant gradient directing them to the distinct inflammatory target zone[113,116].

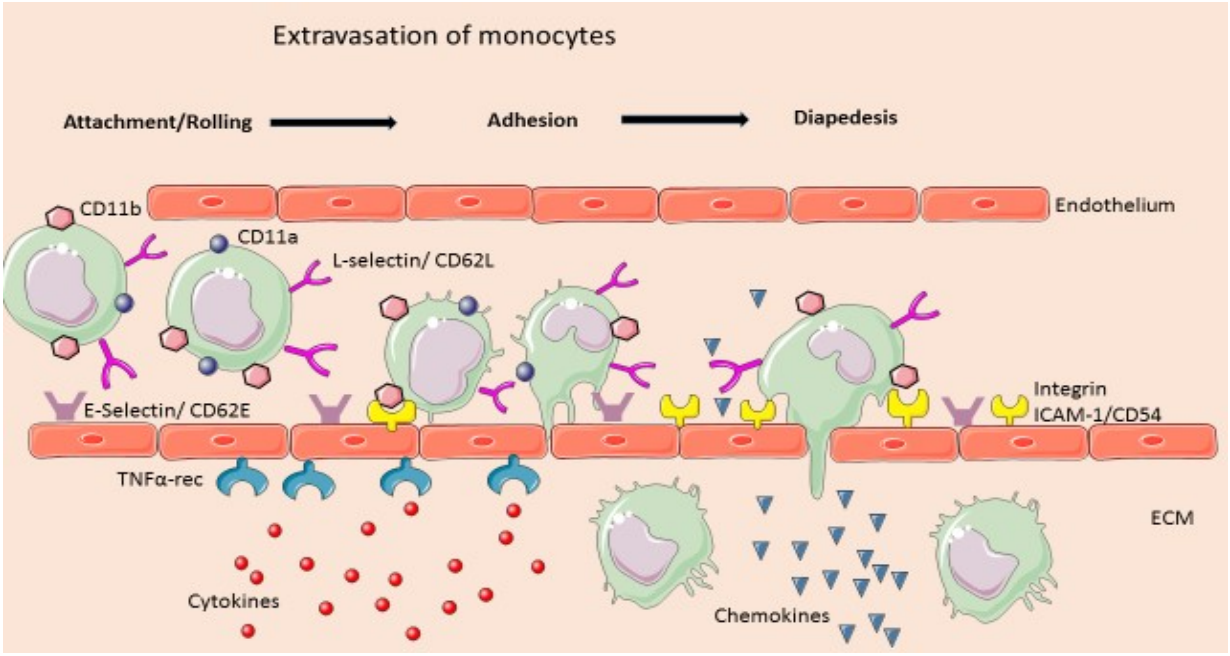

Figure 2 Extravasation and transmigration of monocytes.

2.7 ADHESION MOLECULES: INTEGRINS AND SELECTINS

The integrin family members are membrane receptors that anchor the extracellular environment (matrix or other cells) with the intracellular cytoskeleton. There are three subtypes of integrins grouped according to their type of β subunit[117]. The most abundant integrin found on leucocytes is β2 (CD18). At least three different α subunits have been described to bind to the β2-integrin. The CD11b/CD18 combination is predominantly expressed on monocytes, granulocytes and macrophages. Under non inflammatory circumstances peripheral neutrophils and monocytes express a low amount of surface CD11b/CD18 and keep the molecules stored in intracellular granulae. Activation of cells rapidly recruit CD11b/CD18 to the surface, making CD11b/CD18 a reliable marker of initiated adhesion leading up to transmigration of neutrophils[117–120]. The first interactions between leukocytes and endothelial cells are mediated by selectins. Selectin family members, L-, P- and E-Selectin, capture passing leucocytes in the bloodstream and initiate their deceleration along the vessel wall. This adhesion is initiated by weak interactions that produce a characteristic "rolling" motion of the leukocytes on the endothelial surface. P-Selectin and L-Selectin, also called CD62, act in concert and are essential for these initial interactions. Different endothelial stimuli induce an up-regulation of adhesion molecules[117,120]. Since they mediate cell adhesion, intergrins and selectins participate in cell development, proliferation, migration and apoptosis. Deficit or aberrant expression of specific adhesion molecules might contribute to inflammatory disease as well as to tumor development[117,121].

2.8 OXIDATIVE METABOLISM AND ROS PRODUCTION

At microbial invasions, phagocytic cells produce reactive oxygen species (ROS) as a defense mechanism for intracellular killing of bacteria[67,122]. Macrophages and neutrophils generate ROS through activating the NADPH oxidase enzyme complex, thus reduce oxygen O_2 to reactive superoxide (O_2^-). This process is referred to as the respiratory burst or oxidative metabolism. Further processed by SOD2, glutathione peroxidase and catalase, superoxide is converted to highly reactive hydrogen peroxide (H_2O_2)[123].

2.9 CHEMOATTRACTANTS, CHEMOKINES AND CYTOKINES

Chemoattractants are molecules signaling an ongoing inflammatory event in the body. Products from the complement cascade (C3a, C5a), bacterial fragments (fMLP, LPS) and chemokines are all considered chemoattractants.[118]

Chemokines are small either soluble or membrane bound messenger molecules produced by most leukocytes as an inflammatory response after encounters with microorganisms or pro-inflammatory cytokines such as TNFα or IL-1. They mediate up-regulation of adhesion molecules, serve as chemoattractant factors and promote recruitment of inflammatory cells. By binding to sugar residents in the extracellular matrix they gradually get captured at the inflammatory site[124].

Cytokines are soluble secreted proteins produced in response to an antigen and participate in cell growth, activation and differentiation. Based on cysteine residue positioning, chemokines divide into two subfamilies, CXC and CC chemokines. Neutrophils respond strongly to CXC chemokines while many CC chemokines specifically attract macrophages and T-cells[110].

Cytokines are produced by practically all cells involved in innate immunity and a disturbed balance between pro- and anti-inflammatory cytokines can result in tissue damage[125]. Since cytokines can stimulate a cascade of other cytokines from a variety of cell types they rarely act alone and are reliant on binding proteins that both protect them from degradation and serve as an extracellular cytokine reservoir. Some cytokines have predominantly paracrine local effects while others operate systemically[126].

The chemokine fractalkine or **CX_3CL_1**, displays a unique transmembraneous domain that keeps it anchored to the endothelial cell. Proteolysis generates a soluble circulating chemotactic active form of fractalkine. Both the membrane bound and soluble type mediates selectin and integrin independent adhesion and chemotaxis[127,128]. $CD16^+$ monocytes particularly rely on the fractalkine pathway to endeavor extravasation[129]. There are data suggesting that fractalkine exhibit a monocyte supporting function resulting in increased cell survival in inflammation as well as a capability to protect plaque organized monocytes from apoptosis. This might imply a role of fractalkine in the development of vascular disease[130].

The chemokine **RANTES** (Regulation on activation, normal T and secreted), also called CCL5, orchestra the recruitment of inflammatory cells such as monocytes, dendritic cells, neutrophils and macrophages. With the help of cytokines (in particular, IL-12 and IFNγ), RANTES also induces the proliferation and activation of certain natural-killer cells and enhances the histamine release from eosinophils as well as affects migration of T-cells and monocytes[131]. RANTES induce expression of integrins and metalloproteinases that are involved in movement through the endothelial basement membranes and tissues[131]. High levels of RANTES is associated with a wide range of immune-mediated diseases including glomerulonephritis and interstitial nephritis[132,133]. Blocking of RANTES during early phases of chronic ischemia has been shown to mediate decreased neutrophil and macrophage recruitment to infarcted tissue leading to improved cardiac function and survival in murine studies[134].

Pro-inflammatory cytokine **TNFα** has been regarded as a superior regulator of the cytokine cascade that provides a rapid form of host defense against infection but is fatal in excess. Although produced by monocytes, T-lymphocytes, fibroblasts and neutrophils in acute and chronic inflammation the major cellular origin of TNFα is activated macrophages[135,136]. TNFα activates neutrophils and mediates neutrophil adherence, chemotaxis, degranulation and oxidative burst[137]. Inducing vasodilatation, increased vascular permeability and promoting intravascular coagulation, TNFα play a central role in sepsis and organ failure[112,113,]. TNFα exhibit a pivotal role in regulating both pro- and anti-inflammatory mediators. TNFα is highly pleotrophic with effects on insulin resistance, lipid metabolism, coagulation and endothelial dysfunction[138]. It should be noted however, that the association between TNFα and CRP is rather weak[139]. Hence, circulating levels may be affected by a number of different factors and that circulating TNFα levels may not reflect biologic activity at the tissue level.

Systemically acting Interleukin **(IL)-6** has a dual role, both as a pro-inflammatory cytokine associated with development of atherosclerosis and progression of ERSD[80,138] but also as an anti-inflammatory cytokine downregulating IL-1 and TNFα as well as increasing glucocorticoid synthesis and reduces IFNγ with the net result pointing towards a superior anti-inflammatory role[140].

Produced early in the infectious process predominantly by monocytes and dendritic cells**, IL-12** is a pro-inflammatory cytokine that has a central function in the initiation and regulation of the induction of cell-mediated immunity. IL-12 is an important regulator of the differentiation of native T cells into Th1 cells, which is crucial in determining resistance and the type of reaction that will be elicited in response to a particular pathogen[141].

Because of their synergistic roles in stimulating inflammation, IL-12, IFNγ - and TNFα are considered to be major pro-inflammatory cytokines [141].

IL-10 is a cytokine produced by monocytes and T-lymphocytes. It inhibits monocyte, macrophage and NK cells production of pro-inflammatory cytokines. IL-10 has an anti-inflammatory profile and it also enhances B-cell survival, proliferation, and antibody production[142].

IL-10 can block NF-κB activity and is capable of inhibiting synthesis of pro-inflammatory cytokines such as IFN-γ, IL-2, IL-3, TNFα and GM-CSF made by macrophages and regulatory T-cells. Decrease in IL-10 expression results in inadequately regulated TNFα levels as IL-10 regulates the TNFα-converting enzyme. As a result, TNFα levels rise and drive inflammation[143].

Bacterial peptides triggers extravasation of leukocytes[144] and the bacterial tripeptide N-formyl-methionyl-leucyl-phenylalanine (**fMLP**) is regularly used as a chemoattractant in studies of leukocyte activation and chemotaxis. By docking to G-protein coupled receptors several reactive pathways are initiated, inducing many different inflammatory responsive cellular functions including migration and cytokine production[122,145,146].

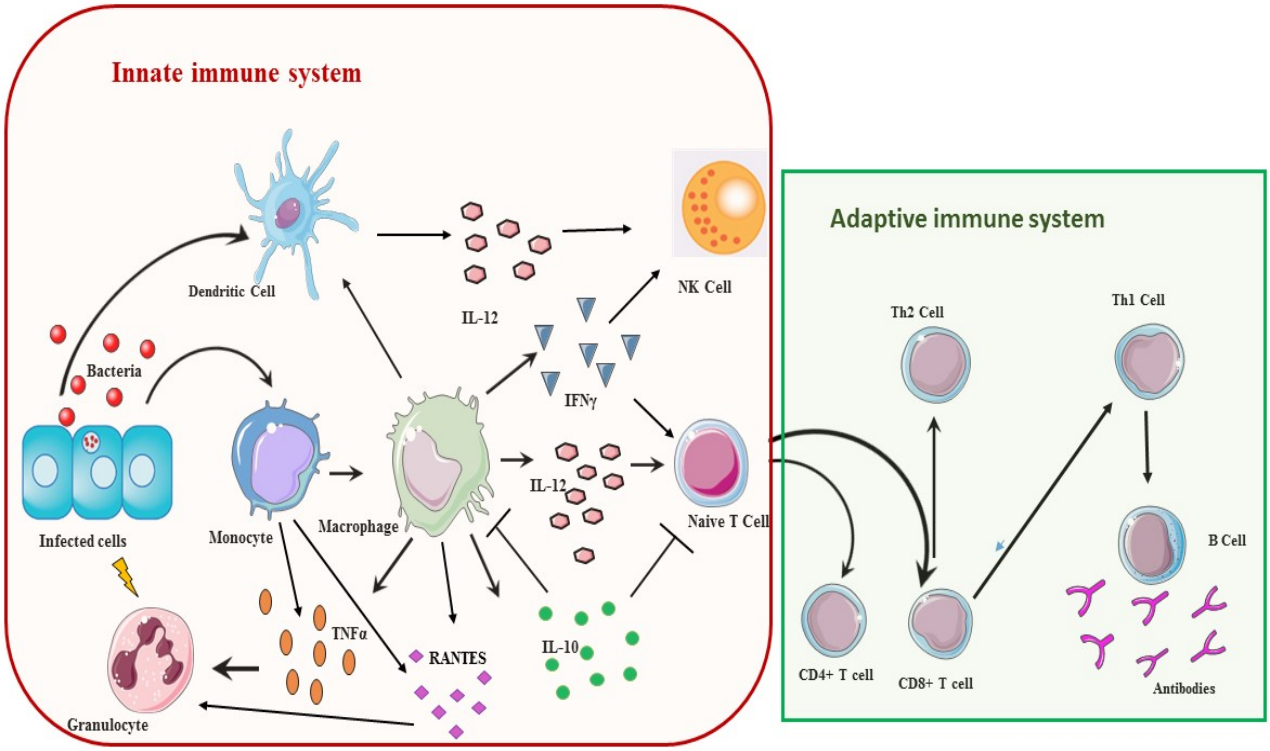

Figure 3 The innate and adaptive immune systems

2.10 INFLAMMATORY CELLS IN CKD

Uremic toxins affect leukocytes in CKD patients. Altered leukocyte adherence to the endothelium, impaired chemotaxis, weakened phagocytosis and decreased activity of inflammatory cells all occur in CKD[147–150]. The persistently activated immune system in CKD is mirrored by a maladaptive cytokine expression pattern[151–153]. Activated monocytes have been suggested to play an important role in the development of atherosclerosis. Pro-inflammatory subgroup $CD16^+$ monocytes have been independently associated with cardiovascular events in a non-dialysis CKD cohort which might imply this subpopulations involvement in human arteriosclerotic development[154] Inflammatory triggered monocytes enhance their intracellular production of oxygen radicals as well as up-regulate adhesion molecules that enables them to infiltrate the endothelial wall[155,156]. Neutrophils and monocytes from patients with advanced CKD have impaired expression of CD11b/CD18[157,158] There is increasing evidence for a causative connection between inflammatory markers with oxidative stress and cardiovascular disease and progression of renal failure[67,80,159].

2.11 CHEMOKINES AND CYTOKINES IN CKD

Without showing overt clinical symptoms of infection or visible active inflammation, CKD patients display a plasma profile with elevated levels of IL-1, IL-6, TNFα and CRP[138]. This subclinical persistent state of inflammation increase mortality risk and incident cardiovascular complications[160–162]. Research has pinpointed significant alterations in both the adaptive and innate immune responses in CKD[138,163,164]. Cytokine overflow induce fibrosis and incite monocyte infiltration eventually causing apoptosis and glomerulosclerosis[165]. In CKD, presence of renal tissue invading macrophages and myofibroblasts is associated with the degree of renal function. Macrophages produce pro-inflammatory cytokines such as IL-6 and TNFα [166]. During the interdialytic interval, the cytokine production from monocytes in dialysis patients is normal, although these cells release large amounts of pro-inflammatory cytokines under stimulation. In general, IL-12 levels are increased in CKD patients with or without dialysis therapy, the elevated levels are probably reflecting a reduced renal clearance but also an increased production from DCs that are stimulated by uremic toxins and oxLDL. Elevated serum levels of IL-12 have been shown in the sera of chronic HD patients, and the overproduction of IL-12 has been associated with accelerated apoptosis of monocytes and T cells[167,168]. Nevertheless, increased IL-12 levels were associated with improved survival in a large cohort of patients on dialysis[169,170].

3 AIMS

The overall objective of this thesis was to gain knowledge about changes in innate immunity and cardiac structure in patients with different stages of chronic kidney disease and investigate if FGF23-levels and the pro-inflammatory state of CKD in part catalyze the adverse clinical outcomes seen in renal insufficiency.

The specific objectives were to:

Paper I

Investigate the expression of $CD16^{+}$ and CX_3CR_1 on peripheral and *in vivo* extravasated monocytes in patients with CKD stage 4-5 and in parallel measure levels of inflammatory cytokines in peripheral blood and in the interstitium.

Paper II

Investigate if mild-to-moderate CKD patients exhibit alterations in cardiac structure and systolic and diastolic function in comparison to patients in advanced CKD as well as to healthy controls.

Paper III

Investigate monocyte function in terms of adhesion molecule expression and oxidative metabolism in patients with mild-to-moderate CKD as compared to healthy controls and follow the disease progress over 5 years.

Paper IV

Investigate the inflammatory profile in a cross-sectional study of CKD stage 2-5 and examine the relationship between FGF23 and immune modulators as well as if FGF23 exposure *in vitro* directly modifies the phenotype of granulocytes and monocytes.

4 METHODS

This is a general overview of the methods used in the present thesis. For detailed descriptions please refer to each individual article.

4.1 COHORT CHARACTERISTICS

Patients with CKD in all studies were recruited from the Department of Nephrology at the Karolinska University Hospital, Solna, Sweden. Informed consent was obtained from all patients. All participants gave informed written consent and the study was approved by the local ethical committee at the Karolinska University Hospital, Stockholm, Sweden.

4.1.1 Paper I – The skin chamber cohort

Included 12 patients with CKD stage 4-5ND (MDRD eGFR < 20 ml/min x 1.73 m^2) with a mean age of 61 ± 6.2 years. The aetiology of renal impairment ranged from glomerulonephritis, interstitial nephritis, adult polycystic kidney disease, amyloidosis and nephrosclerosis. Patients with known active systemic inflammatory disease, infectious disease, diabetes mellitus as well as those prescribed antibiotics, corticosteroids, non-steroid anti-inflammatory drugs, statins, warfarin or immunosuppressive agents were excluded.
12 age- and gender-matched healthy controls with a mean age was 60 ± 8 years and eGFR > 80 ml / min x 1.73 m^2 were included as a control group.

4.1.2 Paper II – IV The PROGRESS cohort

Paper II-IV included patients from the PROGRESS cohort, a prospective observational study originally designed to detect markers of progression of renal failure. Data from Group 1 was only collected for year 0.

Table 1 Inclusion and exclusion criteria of the PROGRESS cohort.

	Group 1 (n= 49) Advanced CKD	Group 2 (n= 54) Mild-to-Moderate CKD	Group 3 (n= 54) Healthy controls
Inclusion criteria	GFR < 20ml/min	GFR 50-70ml/min	GFR > 80ml/min
Exclusion criteria	Previous kidney transplant, kidney donor or blood-borne disease		Known heart disease. Current treatment for hypertension, hyperlipidemia or diabetes. Blood- borne disease.

Exclusion criteria for all groups: Active infections. Current immunosuppressive therapy with steroids or cytotoxic drugs. Age criteria: 18-62 years.

Table 2 Demographic characteristics of the PROGRESS study at inclusion

	Mild-Moderate CKD (stage 2-3)	**Advanced CKD (stage 4-5)**	**Controls**	p-value n
GFR ml/min x 1.73m^2	60.1 ± 5.2	15.3 ± 3.9	99.3 ± 12.0	<0.001
Age, years	47 ± 11	49 ± 12	48 ± 11	0.60
Male, n (%)	33 (61)*	29 (59)*	33 (61)	0.97
Height, m	1.74 ± 0.09	1.73 ± 0.10	1.76 ± 0.09	0.3
Weight kg	78.7 (18.7)	77.7 (16.6)	77.1 (12.9)	0.88
BMI	25.7 ± 4.9	26.0 ± 4.2	24.9 ± 3.5	0.42
Heart rate (beats/min)	65 ± 13	65 ± 13	65 ± 10	0.9
SBP (mmHg)	123 ± 15	130 ± 20	117 ± 12	<0.001
DBP (mmHg)	77 ± 10	78 ± 10	73 ± 9	0.03
Current or past smoker, n (%)	27 (50.9)	25 (51.0)	20 (37.7)	0.29
Diabetes, n (%)	11 (20.4) IDDM 6 NIDDM 5	7 (14.3) IDDM 1 NIDDM 6	-	0.42

CKD = chronic kidney disease; GFR = glomerular filtration rate; n = number; BMI = body mass index; BSA = body surface area Values reported as number (percentage), or mean ± standard deviation or median (interquartile range) for skewed variables. p value: analysis of variance (ANOVA) or Kruskal–Wallis test (continuous values), chi-square (categorical values). *=matched for age and sex

Available clinical data for association studies and parameters ranging from inflammatory markers, biochemical markers, nutritional status, general health status, bone density status (DEXA) and cardiovascular investigations with blood pressure, effort test, echocardiography, carotid Doppler, 24-hour blood pressure registration and mortality data were collected.
The final participant was enrolled in 2009 and group 2 and 3 of the cohort were monitored during a follow-up time of 5 years. Of the 54 healthy controls, 30 were randomly selected from the Swedish Total Population Register and 24 were recruited through the website of the regional university hospital. Interviews were performed with potential healthy controls concerning prior health history and medication. One included patient did not complete the baseline echocardiography and was excluded from the echocardiography study (paper II) together with her control.

Table 3 Cause of CKD and pharmacological treatment in the PROGRESS cohort

Diagnosis of CKD, n (%)	CKD 2 – 3 (n = 54)	CKD 4 – 5 (n = 49)	p value, n
Hereditary/ congenital diseases	14 (26)	13 (27)	0.94
Primary glomerulonephritis	17 (32)	12 (25)	0.43
Secondary glomerular/systemic disease	9 (17)	10 (20)	0.63
Miscellaneous/ unknown	14 (26)	14 (29)	0.76
Medication, n (%)			
Diuretics	12 (22)	34 (69)	< 0.001
ACE inhibitors	23 (43)	29 (59)	0.093
Angiotensin II receptor blockers	22 (40)	23 (47)	0.53
Beta-blockers	11 (20)	20 (41)	0.024
Calcium channel blockers	10 (19)	28 (57)	< 0.001
Statins	13 (24)	32 (65)	< 0.001

ACE = angiotensin converting enzyme; Values reported as number (percentage), or mean ± standard deviation or median(interquartile range) for skewed variables. p value: analysis with Mann- Whitney U-test or t-test

The category "Systemic disease" included nefroangiosclerosis, diabetic nephropathy and two cases of SLE but no case of vasculitis in group 2. None of the Lupus patients expressed an elevated CRP-level at any of the measuring points (hsCRP year 0 – year 3 - year 5: 0.8 -0.87 - 0.83 mg/l and 0.8 - 0.6 - 0.46 mg/l respectively). Nor did the only SLE diagnosed participant in group 1 have an elevated CRP at baseline (hsCRP 0.98 mg/l).

Table 4 DEXA and lipid data from the PROGRESS cohort, all groups at baseline

Variable	Controls GFR 99.3 ± 12.0 (n = 54)	CKD 2 – 3 GFR 60.1 ± 5.2 (n = 54)	CKD 4 – 5 GFR 15.3 ± 3.9 (n = 49)	p value, n
BSA, m^2	1.92 ± 0.19	1.92 ± 0.24	1.91 ± 0.23	n.s
Body fat percentage	23(8)	27(9)	27(11)	n.s
BMC g	2570 (413)	2537 (660)	2380 (434)	n.s
BMD g/cm^2	1.2 (0.1)	1.2 (0.2)	1.1 (0.1)	0.02
T-score	0.3 (1.0)	0.4 (1.4)	-0.3 (1.3)	0.01
Z-score	0.8 (1.1)	1.0 (1.4)	0.4 (1.3)	n.s
Total Cholesterol	5.14 ± 1.20	4.98 ± 1.04	4.63 ± 1.02	n.s
LDL	3.36 ± 1.12	3.15 ± 0.93	2.64 ± 0.93	0.002
HDL	1.33 ± 0.41	1.26 ± 0.40	1.19 ± 0.37	n.s
Triglyceride	1.0 ± 0.62	1.38 ± 0.84	1.7 ± 0.84	< 0.001
hsCRP	0.90 (0.46 – 2.30)	2.30 (1.10 – 4.10)	1.60 (0.96 – 3.00)	0.009

DEXA variables all reflect total body measurement., BSA- Boy Surface Area m^2 BMC = Bone Mineral Content g/cm, BMD = Bone Mineral Density T-score= number of SD above or below the mean for a healthy 30-year old with the same sex and ethnicity, Z-score = score number of SD above or below the mean for the same sex, age and ethnicity as the patient. Lipids measured in mmol/L. LDL = low-density lipoprotein; HDL = high-density lipoprotein; hs-CRP = high-sensitive C-reactive protein in mg/l; Mean (SD) Differences in mean values analyzed with ANOVA-test (F-test). p>0.05 = n.s

Paper II

All 103 patients from both CKD groups (stages 2-3 / mild-to-moderate dysfunction or stage 4-5 / advanced renal failure) in the Progress 2002 cohort were included in a baseline study of echocardiographic variables compared with the healthy controls.

Paper III

Included the patients from Group 2 (mild-moderate CKD) and the healthy controls from the PROGRESS cohort and collected data at baseline, year 3 and year 5 in a prospective follow up study.

Paper IV

All 103 CKD 2-5 patients and 53 healthy controls from the PROGRESS cohort were examined for FGF-23 levels, biochemical profile, inflammatory markers and cellular data in a cross-sectional study at year 0 (baseline).

4.2 ROUTINE LABORATORY AND CLINICAL MESUREMENTS

Generally, plasma and serum samples were spun and stored at -70° C. Laboratory results and clinical data were gathered from the patients' records at the time of inclusion and at follow up. Analyses for creatinine were executed using routine methods.

All participants in the PROGRESS cohort had glomerular filtration rate *(*GFR) measured by iohexol at inclusion but only the CKD stage 2-3 patients had iohexol analysis repeated at 3^{rd} and 5^{th} year. Filtration capacity in healthy controls at 3^{rd} and 5^{th} year was calculated with creatinine based formulas. In paper II-IV we used the CKD- EPI formula since this has been shown to prove a more exact estimate of kidney function in the range of mild CKD.

4.3 THE SKIN CHAMBER METHOD

A fundamental step in the host defense mechanism is when polymorphonuclear leukocytes migrate into the tissue. The transmigration process is intricate and includes several consecutive steps. The skin chamber technique is a well-documented method providing means to study extravasated leukocytes in a local exudation without systemic inflammatory responses[171]. A local inflammatory reaction provokes the leukocytes to leave the blood stream and wander to sites where they can be gathered. Skin blisters are induced by suction and a gentle heating loosens the epidermal layer from the underlying dermis on the volar surface of the forearm[172]. The eruptions are produced without harming the capillaries or dermal tissue. Blister roofs are removed and plastic chambers containing a chemoattractant, autologous serum or PBS, are mounted over the raw wound surfaces[173]. Analysis of temporal changes of the cell population in the chamber has shown that mononuclear cells appear early but are soon outnumbered by polymorphonuclear cells that constitute 90-98% of the cells after 10-24 hours. Transmigrated cells in our studies were collected and analyzed after an incubation time of 10 hours.

Figure 4 Skin chamber technique with mounted plastic chambers

4.4 LUMINEX METHOD

Soluble inflammatory mediators; such as cytokines and chemokines in serum and chamber fluid in paper I, III and IV were performed on the Luminex-100 system and assessed by Milliplex 26-plex (Millipore Corp). This method has the advantage of being performable on very small sample volumes, with a lower detection limit of 3 pg/ml. Briefly, distinctly dyed microspheres are coated with capture antibodies that will catch the specific analyte of the investigated sample. Added fluorescent detection antibodies are then distinguished by a laser that discriminate both the colour of the microsphere and each tagged detection antibody.

4.5 FLOW CYTOMETRIC ANALYSIS

FACS (fluorescence-activated cell sorting) or flow cytometry measure cell characteristics by laser-scanning cell suspensions flowing through the instrument. Data on cell size and granularity are provided by the instrument recognizing different leukocyte cell populations by their light-scattering properties. The cells ability to refract and reflect light respectively differ between different types of leukocytes.

A two-parameter scatter plot histogram is produced by the computer presenting the different populations of white blood cells. Cell size is expressed as forward scatter (FSC) on the y-axis whereas granularity and membrane dimension condensed as density are expressed as side scatter (SSC) on the x-axis. With flouro-chrome-marked antibodies more precise details about the cells can be revealed when the laser excitation makes them emit light of different wave length (represented with a specific color).

In paper I, III and IV monocytes and granulocytes were selected by flow cytometry and their adhesion molecules expression identified in addition to their oxidative metabolism (hydrogen peroxide formation), all measured and quantified as mean fluorescence intensity (MFI).

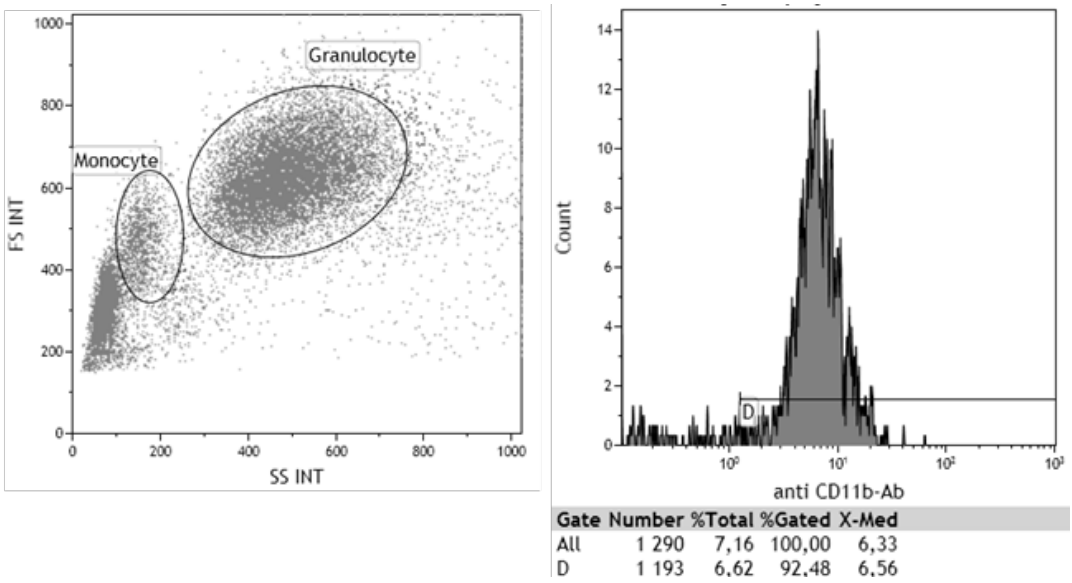

Figure 5 Principles of cell separation by cytometry

4.6 TRANSTHORACIC ECHOCARDIOGRAPHY

Transthoracic echocardiography is used to evaluate cardiac function through measuring dimensions such as left ventricular mass, wall thickness and diastolic and systolic function. TDI – Tissue Doppler Imaging describe tissue movement and velocity.
Left ventricular ejection fraction (LVEF) and AV plane measurement have traditionally been used to assess left ventricular systolic function estimating diagnostic and prognostic outcome[174–176]. Global left ventricular longitudinal strain (GLS) obtained by using two-dimensional speckle tracking analysis or TDI assessing longitudinal LV function by s´(s′ = peak systolic mitral annular velocity) have been shown to be more reproducible and superior to LVEF in predicting cardiac events and all-cause mortality[177].
Left ventricular diastolic function and filling pressure can be measured using Doppler recordings of transmitral and pulmonary venous flow velocities[178]. TDI measurement of early passive transmitral inflow velocity (E) and the rate of the atrial component of LV filling (A) joined in the E/A ratio show a reduced value with declining diastolic function[179]. A ratio between diastolic flow and wall velocity, called the E/e′ ratio (the early diastolic filling rate (E) to pulsed tissue doppler velocity of the septal mitral annulus during passive filling (e′)), has been demonstrated to effectively, and with high reproducibility, assess diastolic function[180–182]. Moreover, in diabetic patients, the E/e′ ratio has been shown to independently envisage cardiac failure and mortality[182,183]

4.7 **ADDITIONAL METHODS** (featured Paper)

Analysis of surface molecules (**I, III-IV**)	¤ Performed by labeling **leukocytes** with flourochrome conjugated antibodies.
Cell counts (**I, III-IV**)	¤ Determines total cell count and distribution of individual cell types.
Oxidative Metabolism (**IIII-IV**)	¤ Examination of intracellular H_2O_2 production after fMLP stimulation by DCFH-DA system and flow cytometry.
Immunoassay (**I**)	¤ Determines the concentration of pro-inflammatory molecules in serum and chamber exudates by commercially available assays (ELISA)
In vitro activation (**I, III-IV**)	¤ Studies of adhesion molecule expression on leukocytes after fMLP or PMA stimulation
In vitro incubation with FGF23 (**IV**)	¤ Studies of adhesion molecule expression on leukocytes after FGF23 treatment

5 STATISTICAL ANALYSIS

Descriptive statistics were utilized to characterize the study populations. Categorical variables were expressed as frequencies (%) and proportions. Continuous variables were summarized as mean (±SD) for normally distributed data or median values (IQR) for non-normally distributed values. Categorical variables were stated as percentages. P-values were calculated from Chi_2-tests (missing observations omitted) and Student's t-test for the categorical variables and continuous variables, respectively.
A P value <0.05 for a two-tailed test was considered significant.

5.1 PAPER I

Results were expressed as mean ± standard deviation for the normally distributed data; age, BMI, creatinine, eGFR, CRP, hemoglobin, PTH, phosphorus and albumin. Non-parametric data; cellcount, $CD16^+$, CX_3CR_1 and soluble factors were presented as median and 25-75% interquartile range. Box plots represent 25-75% interquartile range with a line at the median and bars at the non-outlier values. Statistical analysis and comparison between groups were performed using Mann Whitney U-test.

5.2 PAPER II

Results are presented as number, percentage, mean and standard deviation (SD). Group comparisons were performed using one-way analysis of variance (ANOVA), Tukey's post hoc test and chi-square test (v2) where applicable.

5.3 PAPER III-IV

Normally or non-normal distributed biochemical data, cytokine/chemokine levels (Luminex), adhesion molecule expression and respiratory burst cell data, were assessed graphically by histogram plots with associated kurtosis and skewness tests, and by Shapiro-Wilk test. Potential outlier- values were examined graphically by box plotting the data. An outlier was defined as an absolute value bigger than 3.5 x SDs above or below its calculated mean, and an absolute value bigger than 10 x iqr (the inter-quartile range) from its calculated median.
In Paper III, data from year 0 (baseline), year 3, and year 5, statistically significant differences between the groups were assessed by Student's t-test and Mann Whitney U test. Delta values (Paper III), were calculated for Luminex, and cell data according to the formula:
$Y_i^{delta} = X_i^{t=5} - X_i^{t=0}$, where X is the measured value at time *t* for individual *i*.
Additionally, generalized linear regression models were calculated to compare and quantify differences between the study groups. Comparisons of mean and median differences between all groups simultaneously (multiple comparisons testing) were done by ANOVA, and Dunn's testing, an extension to the Kruskal-Wallis test respectively. P-values were adjusted by Bonferroni correction or Benjamini-Hochberg correction [184] . The overall significance level α was set at 0.05. Analyses of correlations between FGF-23 levels and biochemical, Luminex, and cell data in Paper IV, were performed by Spearman's rank and Kendall's correlation.

6 RESULTS AND DISCUSSION

6.1 EXPRESSION OF ADHESION MOLECULES

6.1.1 CD16 on transmigrated and peripheral monocytes (I)

Patients with CKD stages 4-5 had an increased percentage of $CD16^+$ monocytes both in the peripheral circulation and at sites of induced interstitial inflammation. The ratio of the percentage of $CD16^+$ monocytes between the peripheral circulation and the interstitial inflammatory site in the two studied groups were comparable, indicating similar transmigration capacity of the $CD16^+$ monocytes in CKD patients and healthy controls. The higher accumulation rate at the interstitial inflammation in CKD patients might be a consequence of the increased peripheral pro-inflammatory $CD16^+$ pool.

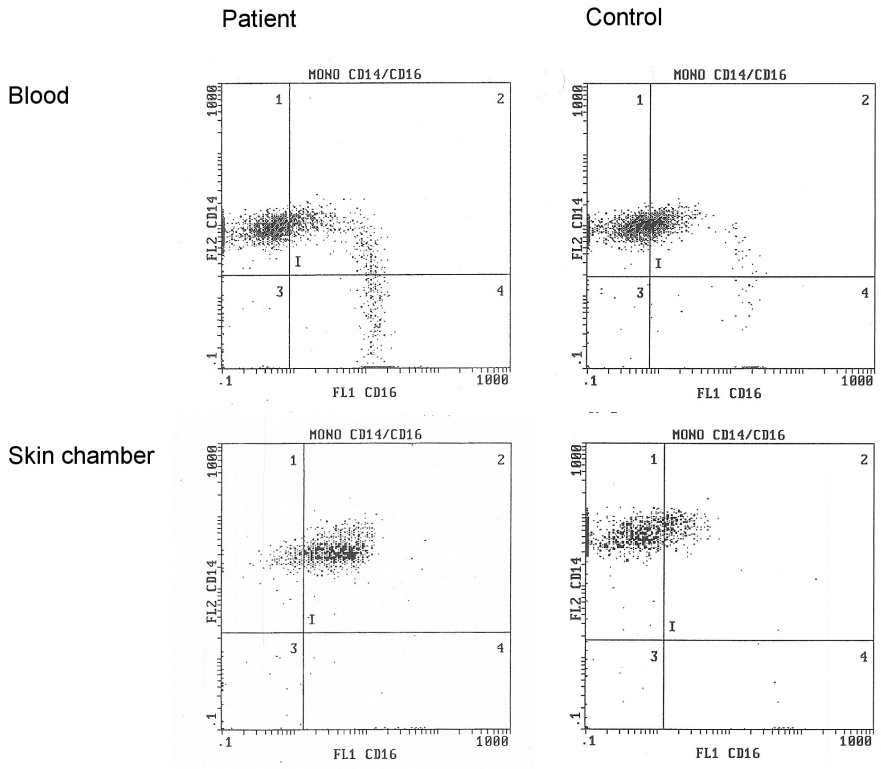

Figure 6 Flow cytometric charts of $CD16^+$ expression on monocytes in circulation and chamber fluid in CKD patients and controls respectively

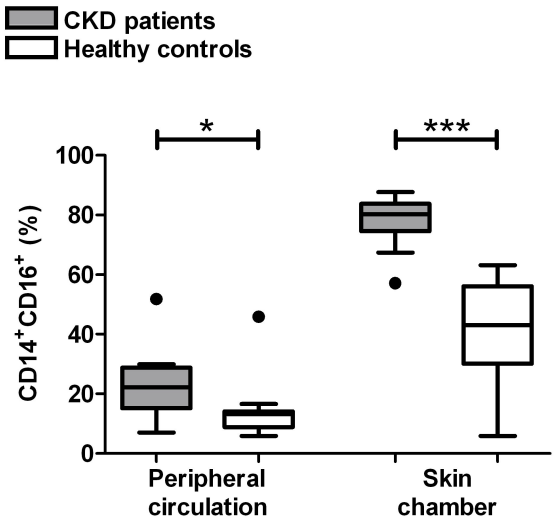

Figure 7 Boxplot showing the elevation of $CD16^+$ monocytes in CKD patients compared to healthy controls. * = p value < 0.05 and *** = p value < 0.001.

Several studies have reported an increase of circulating $CD16^+$ monocytes in CKD[185,186] and $CD16^+$ monocytes have been found to associate to CV events hence believed to be involved in human atherosclerosis[154,187]. HDF treatment on the other hand, has been shown to reduce the percentage of $CD16^+$ cells[188]. This study demonstrates an increased percentage of pro-inflammatory $CD16^+$ monocytes both in blood and in the inflammatory interstitial site in patients with advanced CKD.

6.1.2 CX_3CL_1 levels and CX_3CR_1 expression on monocytes (Paper I)

Fractalkine, CX_3CL_1, is a membrane anchored chemokine released from the cell surface by proteolysis. It binds the fractalkine receptor CX_3CR_1 and through these dual forms, functions as a chemokine as well as an adhesion molecule for a wide variety of immune regulatory cells[130,189,190]. CX_3CL_1 has a highly antiapoptotic effect on human monocytes[191]. A balance between pro-apoptotic and apoptosis-inhibiting factors is necessary for the maintenance of an effective immune response without harmful side effects of an excessive neutrophil activation. $CD16^+$ monocytes have a high expression of CX_3CR_1 which enhances accumulation of these cells at sites of overexpressed CX_3CL_1[192]. In study I we found an expression of CX_3CR_1 of above 90 % on circulating monocytes in both CKD and healthy controls and the expression decreased substantially following extravasation, probably as a result of shedding. Patients with CKD had a significantly higher concentration of CX_3CL_1 in both blood and in the interstitial fluid, compared to healthy controls. This indicates a facilitated transmigration and tissue retention mechanism for the pro-inflammatory $CD16^+$ subtype of monocytes in CKD patients. Once the pro-inflammatory monocytes have left the circulation and entered the local inflammatory site, they can further stimulate neutrophil recruitment by means of producing chemokines and perpetuate inflammation and tissue injury.

6.1.3 Expression of CD11b and CD62L on leukocytes (Paper III and IV)

Adhesion molecule CD11b has an important role in leukocyte transmigration, directing innate immunity cells to act on invading microbes. In our prospective follow up study (Paper III) on mild-to-moderate CKD there were no significant differences in monocyte expression of CD11b or CD62L at baseline (year 0), in resting state, comparing patients and controls. These findings suggest that monocytes are in a restored state at earlier phases of CKD, reflected in a normal expression of CD11b. Interestingly, following stimulation with fMLP, we observed a higher expression of CD11b on the surface of patients' monocytes at baseline. This may suggest an inclination of these cells to translocate the intracellular stored CD11b to the surface, possibly due to a higher responsiveness of primed cells in the pro-inflammatory milieu. In contrast to the baseline value, a significant decline in the CD11b expression occurred following stimulation with fMLP, both at 3rd and 5th year. This may reflect an inability to mobilize CD11b from the intracellular vesicles or a refractoriness of monocytes exhausted by the constant inflammatory burden of renal insufficiency [1,147,157,158,193,194]. How a lower CD11b expression evolves over time supports our previous studies where we found that peripheral resting monocytes from patients with advanced renal disease express a lower level of CD11b, when compared to healthy controls[158]. A preserved response of monocytes to fMLP stimulation was previously found in patients treated with high flux hemodialysis[147], perhaps indicating a reversibility of this cellular refractory effect by hemodialysis. A low response to fMLP may contribute to the elevated susceptibility to infections in CKD patients. In Paper IV inactive granulocytes and monocytes from CKD stage 2-5 patients showed an elevated expression of CD11b which might reflect an impact of amplified activity of TNFα and RANTES on these cells, exposing cells to a higher inflammatory elementary level. However, no difference between patients and controls was detected in terms of CD11b expression on fMLP stimulated granulocytes while the expression on monocytes was increased in patients. Monocytes are in general more inflammatory active and cytokine responsive whereas granulocytes exhibit greater impact on host defense and direct immunity. There was also a greater interpersonal variability in CD11b expression on monocytes in patients as compared to controls, perhaps reflecting fluctuations of cytokine levels.
CD62L (L-selectin) plays an important role in adhesion of cells to the inflamed endothelium. Following adhesion and activation of monocytes, CD62L is rapidly enzymatically cleaved and shed from the cell surface[195,196]. In a previous study we have shown that monocytes from patients with severe renal failure express a lower CD62L in periphery[158].

In Paper III, CD62L expression on monocytes in patients with mild-to-moderate CKD was comparable to healthy controls at baseline but had a significantly lower expression both at 3rd year and at 5th year of the study. This could imply a better feature of monocytes at an earlier phase of the disease, but a lower translocation, or higher shedding of CD62L over time, probably due to enhanced production of inflammatory mediators. For instance pro-inflammatory TNFα enhances shedding of CD62L[195–197]. The significant increase of TNFα over the study years with a simultaneous decrease in CD62L levels, may support this hypothesis.

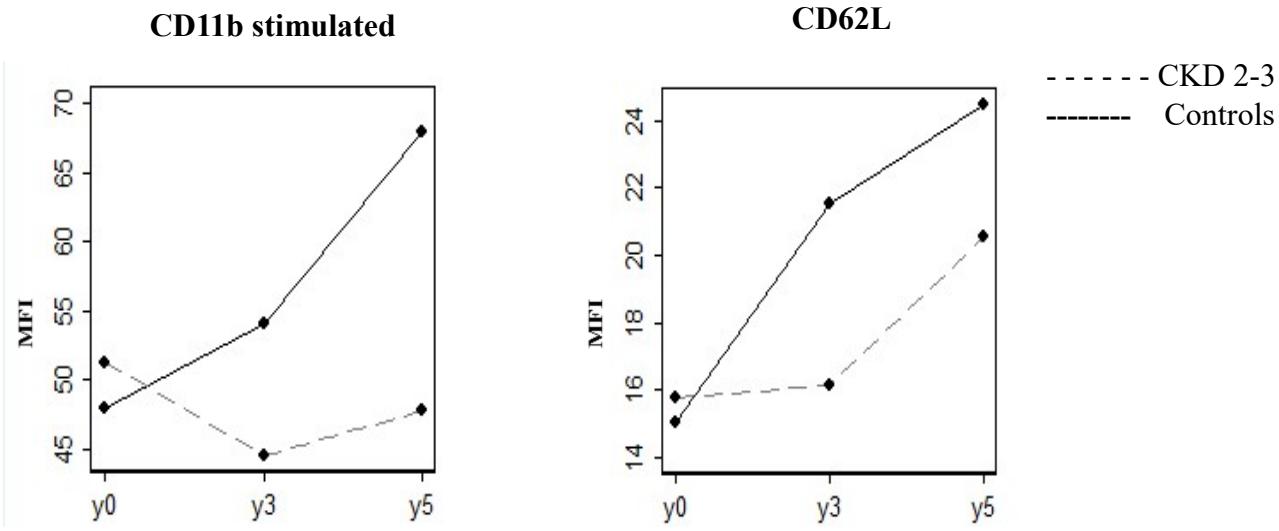

Figure 8 Expression of CD11b and CD62L in CKD patients and Controls at baseline (y0), year 3 and year 5 (Paper III).

The counterbalance act of the innate immunity is crucial to avoid tissue damage. The altered monocyte function and excessive pro-inflammatory cytokine expression in our CKD 2-3 cohort might imply consequences of the distorted innate immune system. Alterations in chemokine levels as well as in adhesion molecule expression might potentially act either harmful or protective. Perhaps there is a dysregulation rather than a dysfunction of the innate immunity network, inducing the inflammation in CKD.

6.2 CHEMOKINES (I, III and IV)

CKD patients are subjected to a state of chronic inflammation[198], a condition reflected in consequential imbalance of pro-inflammatory and anti-inflammatory cytokines[112,152,153,199]. Alterations occur in both innate and adaptive immune responses in CKD due to inflammation, uremia, dialysis procedures and oxidative stress.

In Paper I, CKD stage 4-5 patients had a significantly higher concentration of TNFα in the peripheral circulation compared to healthy controls. However, in the skin chamber fluid, concentrations of TNFα and IL-10 were significantly lower in CKD patients compared to in healthy controls. In addition, the TNFα/ IL-10 ratio was significantly higher in serum from CKD patients compared to in controls.

The impaired TNFα concentration gradient between the circulation and the interstitial space has previously been associated to an increased risk of septicaemia as well as adverse outcome[200–203]. An impaired cytokine gradient might also contribute to decreased leukocyte trafficking to secondary inflammatory sites, potentially contributing to the impaired immune response and increased susceptibility to infections in CKD.

Experimental apheresis studies have been shown to effectively restore chemokine gradients, leading leukocyte trafficking toward infected tissue and away from healthy organs[204] However, attempts to acute remove TNFα and IL-6 with hemoadsorption have not achieved decreased levels post treatment, even though treated subjects had an improved survival. Hence, blood purification must instigate some other beneficial immuno-modulating effect, with a so far unknown mechanism[205].

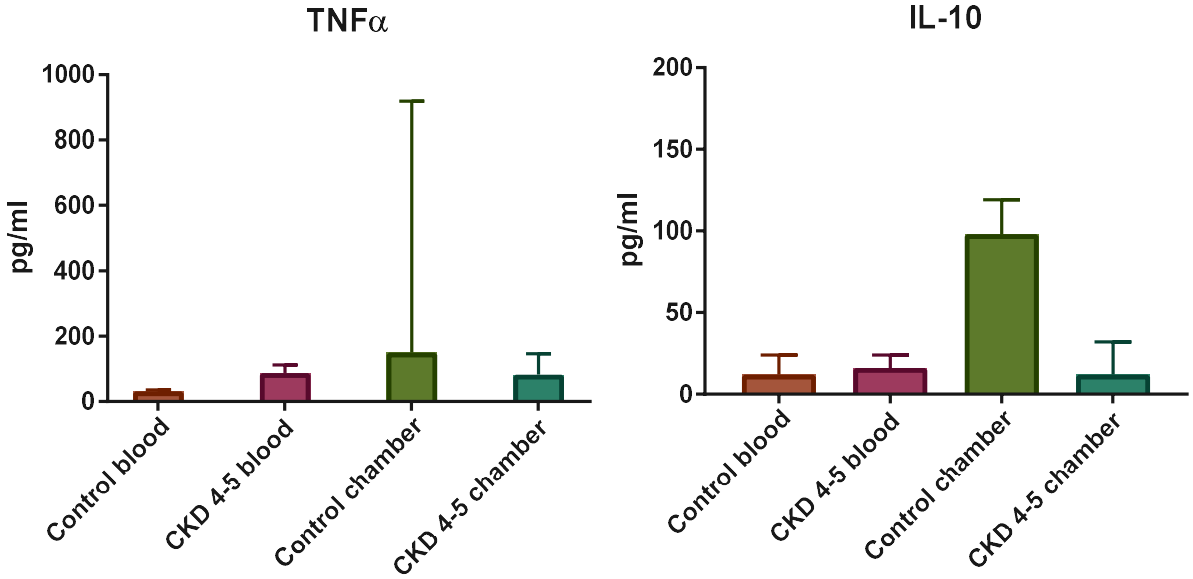

Figure 9 TNFα and IL-10 levels in CKD vs Controls in peripheral circulation and chamber fluid from Paper I**.**

In Paper III and IV, there were significantly higher levels of TNF- α, IL-12 and RANTES in plasma from patients, compared to in healthy controls. Notably, in paper III, levels of chemokines in general tended to fluctuate over time which most likely reflects a variability of the inflammatory process. Elevated cytokine levels in advanced CKD are partly due to a reduced renal clearance but may also be a result of an increased production from dendritic cells that are stimulated by retained toxins[153]. However, at early stages of renal disease an accumulation is less plausible and elevated levels would probably result from an actual pathogenic mechanism rather than from a decline in filtration rate.
Augmented production of pro-inflammatory cytokines such as TNF-α, IL-1, IL-8, IL-15 and IL-12 has been shown in both pre-dialysis and in hemodialysis groups, indicating a role of the uremic milieu in this process, independent of dialysis treatment[199]. Elevated levels of pro-inflammatory cytokines might contribute to enhanced vascular plaque formation as well as changes in cardiac function and structure, leading to cardiovascular morbidity.

RANTES modulates migration of monocytes by inducing expression of CD11b/CD18[206]. Elevated levels of RANTES have been associated with sustained inflammation and multiple immune-mediated diseases[132,207]. Moreover, TNFα is known to induce production of RANTES under inflammatory conditions[131]. Aberrant IL-12 expression has been reported in infectious, autoimmune, inflammatory conditions and atherosclerosis, modulating the adaptive immune response[208].

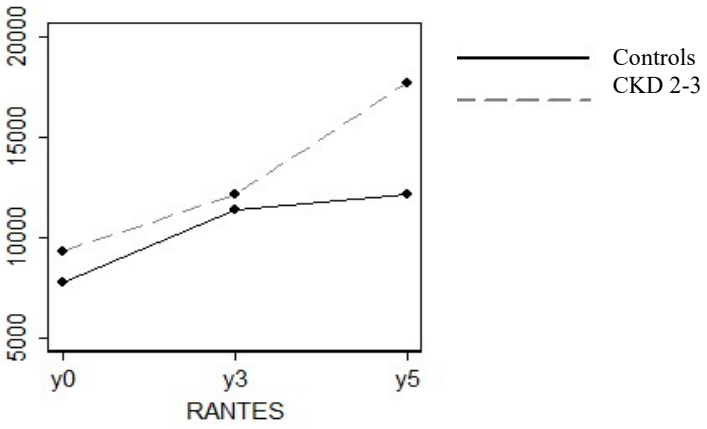

Figure 10 Concentration (pg/ml) of RANTES (CCL5) in plasma from CKD patients and controls at baseline, year 3 and year 5 from Paper III.

High levels of RANTES and TNFα are evidence of a high inflammatory state together with a poor opposed anti-inflammatory regulation in CKD patients[142]. These cytokines might serve as potential surveillance biomarkers to monitor development of inflammation in kidney failure.

6.3 OXIDATIVE METABOLISM

There was no significant difference in H_2O_2 production at baseline (Paper III and IV) or at follow up, between patients and healthy controls in Paper III, but stimulation with fMLP resulted in a significantly lower oxidative burst response in monocytes over time in CKD patients.

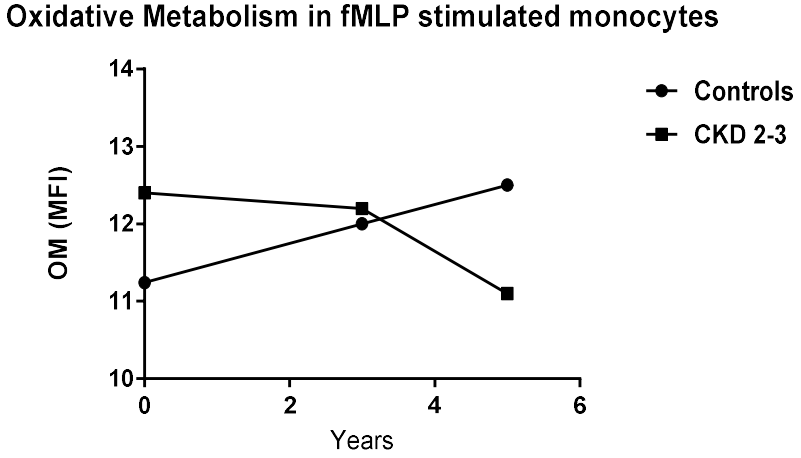

Figure 11 H_2O_2-production measured as Oxidative Metabolism (OM) (MFI) median in (fMLP)-stimulated cells in CKD patients and controls at baseline, year 3 and year 5.

6.4 FGF23 (Paper IV)

6.4.1 Levels of FGF23

An elevation of FGF23 is the earliest detectable serum abnormality in patients with CKD-MBD (mineral and bone disorder). As kidney function decreases FGF23 increases gradually, reaching more than 200 times the normal levels at advanced CKD[209]. Accordingly, the FGF23 concentration in Paper **IV** varied markedly among CKD patients and data confirmed a strong inverse correlation between FGF23 and kidney function. As anticipated, there were significantly higher levels among CKD patients as compared with healthy controls.
The FGF-23 levels were right-skewed and were natural log-transformed for further analysis. Median FGF-23 level in subjects with normal kidney function (controls) was 17.5 RU/ml (IQR 11.1), in patients with mild-to-moderate CKD 26. 3 RU/ml (IQR 26.2) and in severe CKD 175.8 RU/ml (IQR 439.7).

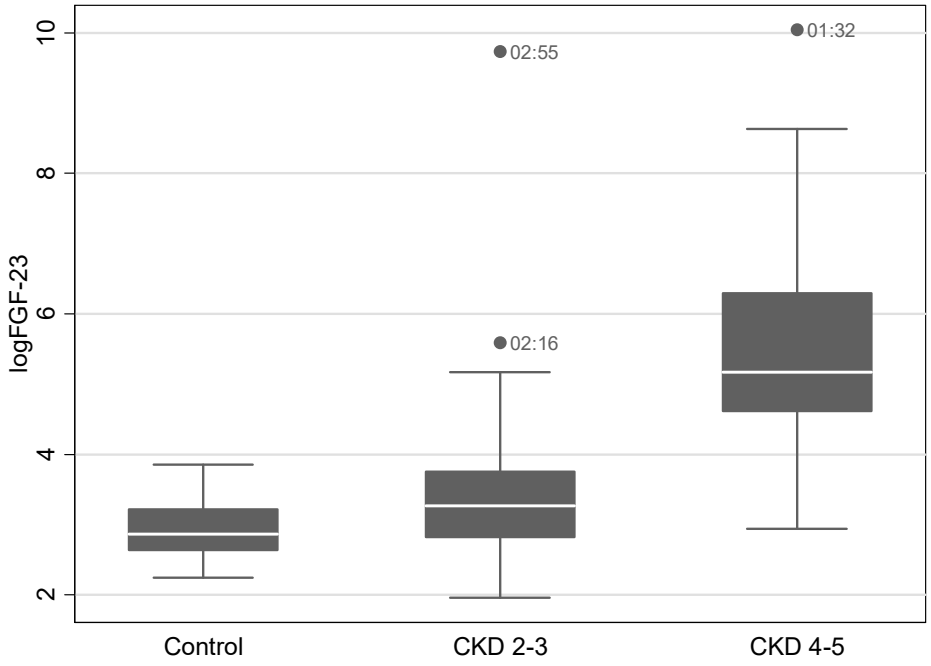

Figure 12 Diagram of the FGF23 distribution depending on kidney function in a sub-study from Paper IV.

6.4.2 FGF23-correlations

The relationships between FGF23 and a broad set of inflammatory and non-inflammatory biomarkers were investigated. Importantly, the established relationship between FGF23, renal function and markers of mineral metabolism was corroborated. Association studies between FGF23 and non-inflammatory biomarkers showed significant correlations between FGF23 and phosphate, PTH and calcium but not with CRP or fibrinogen. In addition, FGF23 level was associated with albuminuria (UAE/urinary albumin excretion), a prognostic marker for disease progression[210–212]. This is interesting since recent studies may indicate presence of the relevant FGF23 receptor(s) in podocytes[213]. The pro-inflammatory cytokine TNF-α was significantly increased in CKD patients as compared to in healthy controls, but did not correlate to FGF23 level. Moreover, significantly higher levels of transmigration triggering chemokine RANTES and immune response initiating IL-12 were found in CKD patients as compared to in controls and were also associated to rising FGF23 level.

FGF23 was associated with CD11b expression on resting granulocytes and monocytes in the CKD group, but not with CD11b on fMLP activated cells. This might reflect how FGF23 in a cohort including various stages of CKD predominantly exhibits an immune-modulating effect through augmented impact on pro-inflammatory cytokines and cell priming which perhaps precedes a more direct cell targeting effect on active granulocytes that might develop as kidney function deteriorates and FGF23 levels increases.

Thus, FGF23 levels were associated to elevated chemokines IL-12 and RANTES in CKD as well as to leukocyte altered adhesion molecule expression on unstimulated cells.

FGF23 linkage to inflammatory markers has been described in several studies in both CKD and non CKD cohorts[100–103]. These studies have delivered pieces of information on the relationship to inflammation but none have previously analyzed different aspects of innate immunity with multiple soluble markers and effector cells simultaneously.

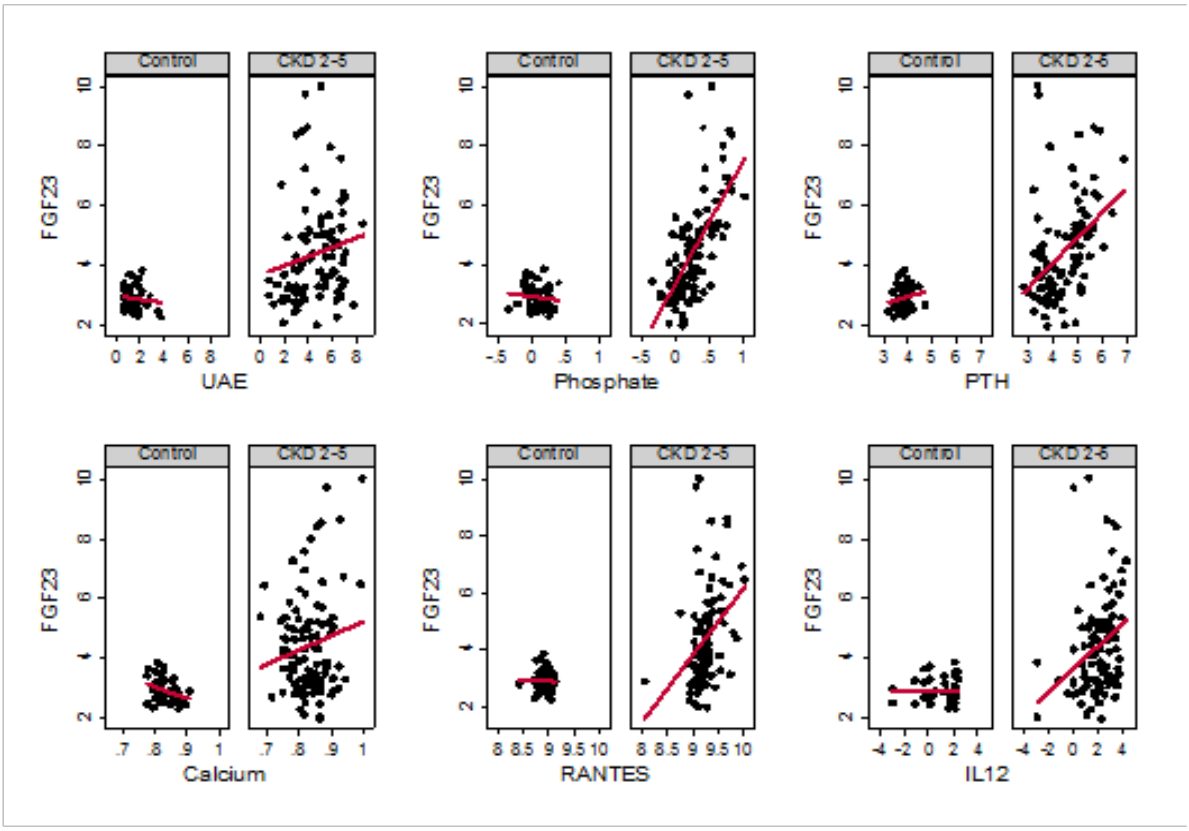

Figure 13 FGF23 Correlation plots. Illustrating log-log correlation plots between FGF23 and biochemical markers. Six specific plots where we found a statistically significant ($p<0.05$) difference of the FGF23-marker correlation between groups. Scale values show orders of magnitude instead of units. For example, considering the FGF23-Phosphate plot, an observation (individual) with the highest number on the x-axis had the highest measured phosphate level.

6.4.3. Cell incubation with FGF23 protein

Given a potential dual effect of FGF23 the aim was to analyze the direct effect on neutrophil function related to transmigration, focusing on expression and mobilization of CD11b. In incubation experiments *in vitro*, leukocytes from healthy volunteers were exposed to different levels of FGF23, simulating conditions of gradually worsening renal function. Granulocytes exposed to high levels of FGF23 showed significantly lower expression of CD11b both in resting as well as in fMLP stimulated cells when compared with cells unexposed to FGF23. An impaired granulocyte response to fMLP can theoretically result in either a diminished granulocyte recruitment subsiding host defense, but perhaps also trigger an anti-inflammatory effect mediated by a down regulated cell response.
One might speculate that FGF23 possibly affects innate immunity in a dose dependent manner. Initially at moderately elevated levels, FGF23 may induce production of pro-inflammatory chemokines. As a result, host defense would be strengthened with chemokine endorsed CD11b expression, priming resting granulocytes and monocytes as well as activated monocytes. Conversely, exposure to high concentrations of FGF23, equivalent to levels in advanced CKD, may operate directly through a receptor dependent pathway on granulocytes to downregulate CD11b and hereby result in impaired host defense mechanisms. Granulocyte recruitment appears to be compromised by high FGF23 exposure. However, FGF23 incubated monocytes showed no difference in CD11b expression. This cellular discrepancy in response might indicate diverging principle target molecule of FGF23. Neutrophils predominantly express the migration mediating FGF receptor FGFR2[214] whereas monocytes display high expression of FGFR1[215]. Thus an exposure to high concentrations of FGF23, equivalent to levels in advanced CKD, may operate directly through a receptor dependent pathway on granulocytes to downregulate CD11b and hereby result in impaired host defense mechanisms.

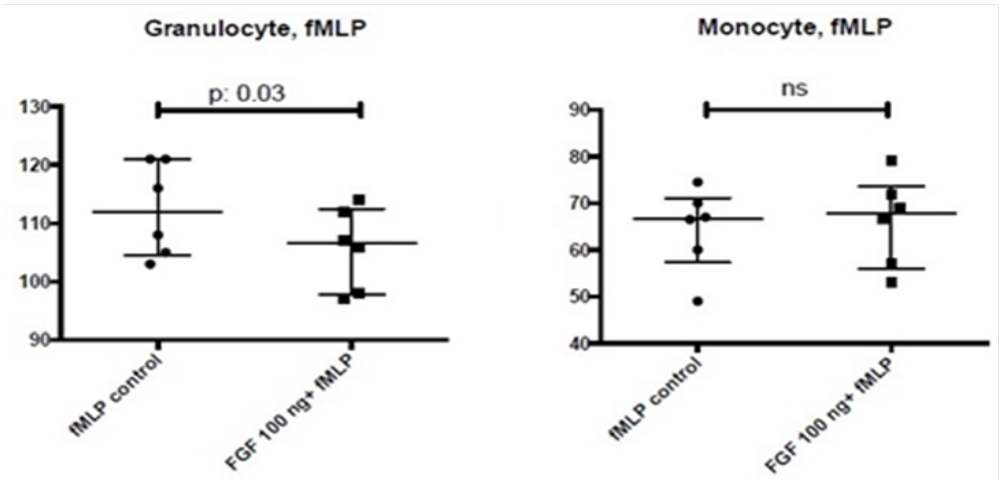

Figure 14 Granulocyte and monocyte fMLP response in CD11b expression (MFI) with and without FGF23 exposure *in vitro*.

6.5 CARDIAC STRUCTURE IN CKD (Paper II)

It is clear that there is a close relationship between CKD and increased risk of cardiovascular disease. From early stages to end stage renal disease (ESRD); coronary artery disease, arrhythmias, congestive heart failure and sudden cardiac death represent the main causes of morbidity and mortality in CKD. With progressive CKD a more compensatory hypertrophy, dilation and dysfunction of the heart (uremic cardiomyopathy) might occur as a consequence of myocardial apoptosis, decreased myocardial capillary density and intermyocardial fibrosis[216]. Echocardiographic abnormalities such as LVH, impaired EF and increased end-systolic and end diastolic LV volumes have been reported from early to severe stages of CKD[52,53,217]. However, the exact pathophysiological mechanisms behind the high prevalence of cardiovascular disease in earlier stages of renal impairment remain insufficiently investigated. In our study (Paper II) of cardiac structure at different stages of CKD we found alterations even in the mild-to-moderate CKD group.

6.5.1 Systolic function

CKD patients had a higher prevalence of LVH compared with the controls; 30% in CKD 2-3, and 37% in stage 4-5 as compared to 13% in controls. There was however no significant difference between the groups in LVEF calculated with Teichholz method but systolic dysfunction in terms of impairment of longitudinal systolic movement measured by AV-plane method with TDI showed lower longitudinal systolic function in CKD patients as compared to controls, as assessed by atrio-ventricular plane displacement and s´.

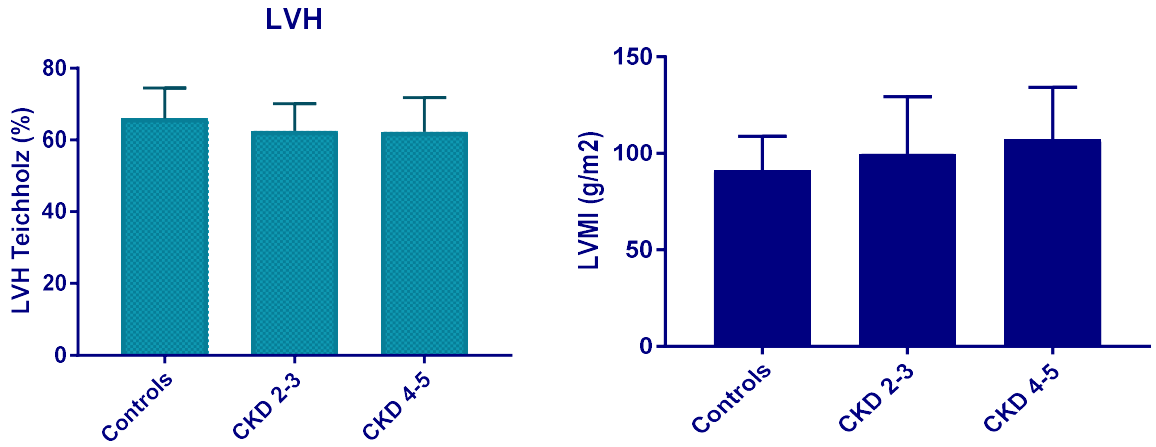

Figure 15 LVH prevalence with Teichholz 2D echocardiography showed no significant difference between the groups whereas LVMI were higher in CKD patients as compared to controls.

6.5.2 Diastolic function

There was no significant difference in traditional characteristic measurements of diastolic dysfunction, such as transmitral inflow pattern (E/A ratio) or left atrial size. With TDI method however, CKD patients had significantly lower septal diastolic velocity (e´) and higher mitral mean E/e´ compared to controls, indicating altered diastolic function in the patients. This indicates an impairment of diastolic function in the patients with CKD, although the majority had preserved LVEF. These changes in diastolic variables may be precursors of clinical heart failure.

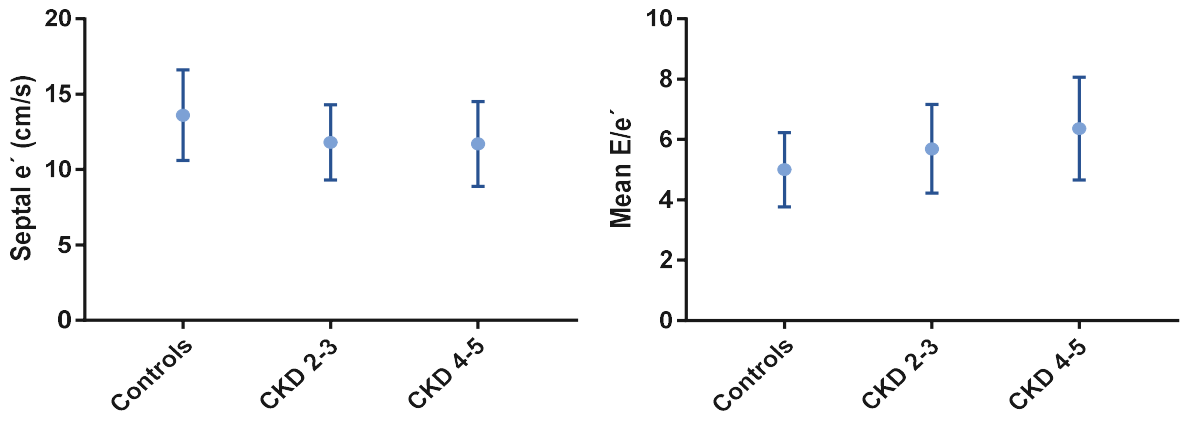

Figure 16 TDI data showed had significantly lower septal diastolic velocity (e´) and higher mitral mean E/e´ compared to controls, indicating altered diastolic function in the patients.

Systolic dysfunction in late stages of CKD is associated with increased mortality and CV events even in asymptomatic patients [218]. In non-dialysis CKD diastolic dysfunction has a prevalence of 29% whereas numbers up to 80 % have been found in the dialysis population.[219] With TDI, we found alterations in systolic and diastolic myocardial function in the CKD patients compared to the healthy controls. Several previous studies have demonstrated changes in LV geometry in patients with CKD but association between kidney function and impaired global systolic function (LVEF) measured by traditional echocardiographic methods has not been established. After adjusting for potential confounders the Chronic Renal insufficiency Cohort (CRIC) study showed association between reduced renal function and abnormal cardiac structure, but not to systolic or diastolic function[219]. Our findings are consistent with previous studies showing that TDI is a more sensitive tool than conventional echocardiography for the detection of impaired diastolic function in the patients with CKD[72,220]. Echocardiographic diagnosis and assessment of alterations in cardiac structure in early CKD have clinical implications on when to initiate optimization of blood pressure control, renal anemia, treat secondary hyperparathyroidism and volume overload. These medical interventions can to some extent reverse LVH and thereby save lives and suffering in CKD patients.

6.6 DEVELOPMENT OF GFR IN THE PROGRESS COHORT (Paper III)

The healthy control group (Group 3) in the PROGRESS study decreased in GFR over the five years of study to a greater extent than anticipated, while the mild to moderate CKD patients lost GFR to a lower degree than expected. Both groups lost the filtration capacity at the same rate; a mean GFR loss of 10 ml/min/1.73m^2 over 5 years.

The frequent therapeutic interventions in the CKD-group with more regular follow up and aggressive treatment of hypertension, hyperlipidemia and proteinuria, might very well have halted the progression rate. There is also a problem with predicting the outcome of GFR development in a relative kidney competent group. For example, some participants were included at, as it would turn out, an uncharacteristically low GFR, only to recover renal function and not progress any further.

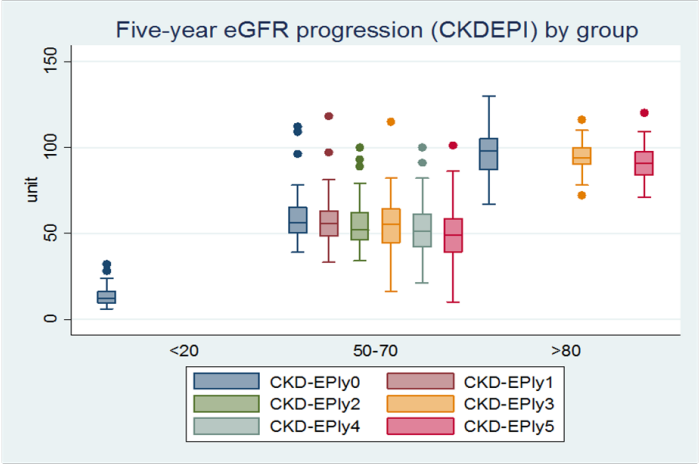

Figure 17 Progress in eGFR in CKDEPI CKD 2-3 (yearly) and controls (year 0, 3 and 5). CKD 4-5 at baseline (year 0).

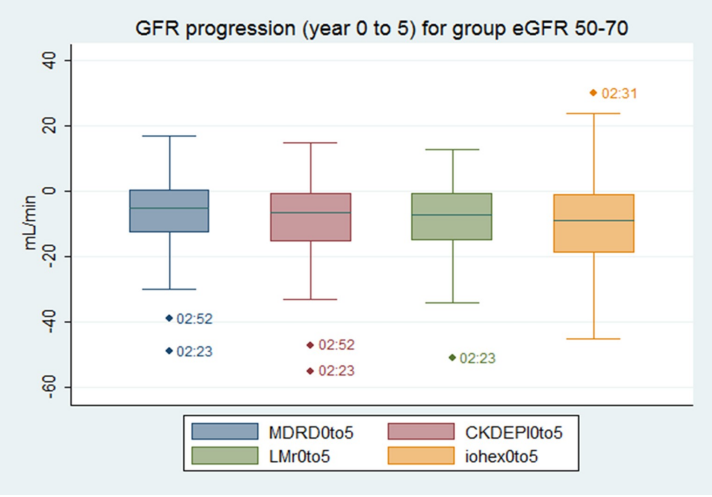

Figure 18 Progress of GFR (ml/min) in the mild-to-moderate CKD-group of the PROGRESS cohort. Data displayed both with 3 types of eGFR methods (MDRD, CKDEPI and LundMalmöRevised formula) as well as iohexol clearance. Presented in delta value (value year 5 minus value at baseline year 0).

Patients with diabetes as CKD diagnose were relatively few in the cohort which also can have slowed down the median progression rate of the CKD group, since patients with diabetic nephropathy generally loose kidney function faster than other CKD groups.

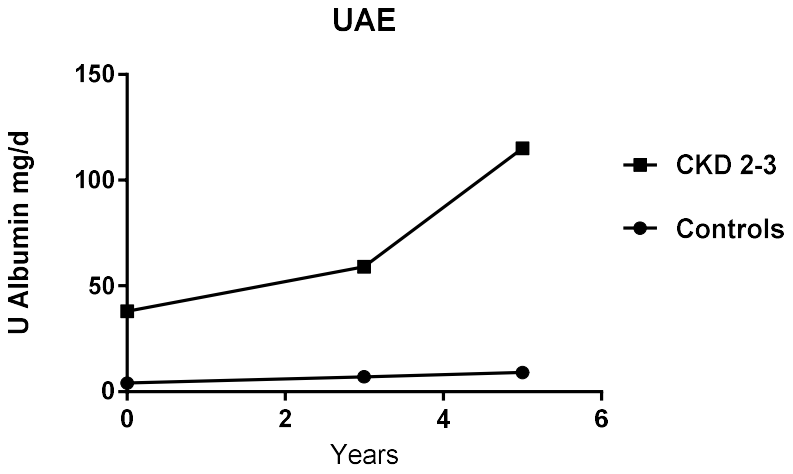

Figure 19 Development of Urinary Albumin Excretion (UAE) in controls and patients CKD 2-3 over the 5 study years in the PROGRESS cohort.

7 GENERAL DISCUSSION AND METHODOLOGICAL CONSIDERATIONS

7.1 STUDY DESIGN

All the studies we performed were cohort studies but in Paper I we used a case control design. Paper II and IV were based on a cross sectional study of baseline data from the observational single-center PROGRESS study. In paper III we used prospective observational data with 5 years follow up from the PROGRESS cohort.
Observational studies investigate events that take place within a population without an experimental design. According to this set up exposed and non-exposed subjects are most likely different. Adjustment for differences between the divers groups in an observational study is needed. Case-control studies are usually performed when the studied disease is rare but the events fairly common. The method is to select cases that have developed the disease and compare them with healthy controls from the same study base. Differences between patients and controls have to be adjusted for by logistic regression analysis. Since we wanted to obtain the same sex and age distribution, the controls were matched on these variables.

7.1.1 Selection of cases and controls

Incident CKD cases would by definition imply newly diagnosed, which is problematic since CKD show so few early signs. This is resolved by using a set predefined GFR level or range and by this define incident as when a patient passed the predefined upper inclusion value. Prevalent CKD cases includes all patients living with CKD within a defined area, which makes the group more heterogeneous since there is an over-representation of patients with milder and slower progressing disease. This is why incident CKD more precisely gives a picture over how progression rate develops.
An advantage of our study cohort is the high attendance of the participants and the fact that the non-selective referral policy concerning disease severity, makes it possible to follow patients during many years which reduces the patients lost to follow up.
Participants in both cohorts were predominantly white Caucasian upper middle aged patients without inflammatory disease and great heterogeneity concerning etiology to CKD, why generalization of our results to other patient groups must be done with caution.
There are of course some study limitations. The sample size of the PROGRESS study of 49 + 54 patients and 54 controls may have been underpowered to demonstrate potential associations. Also, we were not able to ascertain the duration of comorbid conditions, such as hypertension and diabetes.
In the skin chamber cohort, 12 patients were included, since several previous experiences in the field have shown that small patient samples demonstrate significant differences.

7.1.2 Cohort studies

In our third paper the PROGRESS group was used as a prospective cohort study where we followed the individuals prospectively and registered events occurring along the way. The difficulty with cohort studies is that they generally demand a large number of participants to secure that enough events occur. In addition, they are time consuming and expensive to manage. Research on CKD has its obstacles with the large individual variability in disease causes and manifestations as well as consequential differentiating set point of diagnosis at different CKD stages. Circumstances leading to insufficient data on duration and progression rate from the actual disease onset. Furthermore, as we became aware of with the PROGRESS cohort, in the majority of CKD cases, disease progresses slowly, obliging for a long follow-up time in order to correctly describe the development and to identify biomarkers that predict further progress. In the PROGRESS cohort there was a considerable diversity in disease progression rate and for group 2 being at such an early stage of CKD mortality was low and few patients progressed to renal replacement therapy (RRT).
The number of measurements varied between individuals in the research follow up resulting in cases lost under the follow up time. At inclusion we had 54 patients with mild to moderate CKD and 53 controls, but a reduced turn up rate delivered data from 47 CKD patients and 42 healthy controls year 3 and somewhat improved figures at year 5, with 49 versus 45 persons.

7.2 VALIDITY

Defined as results adhering as close to the true value as possible, validity can either be described as internal (inferences) or external (generality – do results apply to settings other than those studied?). In general, there is always the question of generalizability of the results to the investigated population. How representable are the participants of the intended investigated population – for example - patients who start on dialysis have a better outcome then those who do not. There are several different systematic errors, biases which need to be considered when designing a case-control or cohort study. In paper IV we aimed to strengthen our data by a validating strategy. We analyzed correlations between FGF23 and biochemical markers that previously have been confirmed in larger studies to have associate with the FGF23 levels. We thereafter carried on with association analysis between FGF23 and the less well described inflammatory markers. Other potential validity concerns are the technical issues in the measurement of inflammatory biomarkers with the Miliplex method. Due to the fluctuating nature of cytokine levels, the expression is depending on inflammatory effector cell activity. On the other hand, using the Miliplex technique enabled us to analyze inflammatory markers portraying different aspects of the inflammatory immune system from very modest sample volumes.

7.2.1 Confounding

Confounding may occur if a related factor affects the compared groups in a way which relates to and interferes with the studied outcome. A true confounder is not a consequence of the studied exposure but has to be associated to both the exposure and disease. Paper II – is perhaps high blood pressure and not CKD/ inflammation behind altered diastolic function? In Paper II there are confounders as anemia, hyperparathyroidism and hypertension to take in to account since they might affect outcome echocardiography data on LVMI.
In observational studies one needs to be observant of confounding by indication – meaning sicker patients are treated more actively than healthier patients. This needs to be adjusted for by regression or stratification. However, it is impossible to adjust for everything a nephrologist decides and tries in her or his clinic. Moreover, there might be residual confounding due to insufficient clinical information on pharmacological interventions or co-morbidities in addition to unaccounted laboratory parameters. In addition, there were missing or incomplete data in our studies on other potentially relevant confounders such as dietary intake or timing of prandial factors. Impaired kidney function alters several hormonal pathways but it is not fully understood whether these changes play any significant role in systemic inflammation or if these enhance kidney injury [221].

7.2.2 Selection bias

Differences might exist between groups already before a study is carried out rendering a problem called selection bias, where a preferential recruitment of individuals to the study groups might be liable for the observed effect. Socio-economic groups might be more or to a lower extent represented at some clinics raising the question of selection bias. However, in Sweden this kind of selection bias in unlikely since the national health insurance gives basically equal access to health care regardless of income or habitat. Failed or insufficient response to treatment with blockers of the RAAS-system with regard to outcome of blood pressure and albuminuria most probably reflects pre-existing renal damage, providing, at least to some extent, the high predictive value of follow-up measurements for theses variables on disease outcome. Lead time bias, refers to how early detection and diagnosis gives appearance of longer survival. This however, is probably not a relevant concern in our cohort where a predetermined GFR level determined inclusion.

7.2.3 Misclassification

Differential misclassification can be prohibited by the use of standardized protocols, interviews and registry data. Trained professionals at the renal clinic were involved in the diagnostic work why misclassifications seem unlikely. The PROGRESS study has several strengths in this respect. The demographic data set was robustly characterized. GFR was determined by iohexol providing an optimal CKD classification which reduces the probability of misclassification and slightly improved the power of the statistical analysis.
Insufficient exploration of covariates and exposure might lead to non-differential misclassifications where both groups are equally affected driving the results towards the null-hypothesis.

7.2.4 Precision

When random sampling results in point estimates that are close, statistical precision is obtained. To verify precision one needs to analyze the confidence intervals as we did in Paper IV. Even results with high precision might be biased and therefore inaccurate. Sample size is crucial to size of standard error. The number of individuals who develop the measured outcome and distribution of events across the population are critical factors to obtain precision. In small cohorts measuring exact biomolecular markers the sample size is less of incidental importance than in prospective case-control studies where sample size is vital. Having stated this we are aware of the modest amount of individuals included in the PROGRESS. This has made us refrain to certain analyses concerning progress of disease and end target organ damage. This would imply a greater number of participants to achieve precision.

8 CONCLUSIONS AND FUTURE PERSPECTIVES

Irrespective of etiology, renal diseases have inflammation and immune system activation as common underlying pathophysiological generators. The findings from the present work contribute to the conception that there is persistent activation of pro-inflammatory elements of the innate immunity at different stages of CKD. The thesis provide a deeper knowledge of the mechanisms behind changed leukocyte function and altered cytokine expression in CKD. Our findings with alterations in systolic and diastolic myocardial function in patients with even at early stages of CKD, indicate that cardiac involvement is already present in mild-to-moderate CKD and may be a precursor of premature cardiac morbidity. Whether the pathogenic mechanisms behind the cardiovascular structural and functional effects of CKD also has an association to FGF23 remains to be proven.

Specifically, based on the different study results, we conclude that:

- In advanced stage of CKD the level of pro-inflammatory $CD16^+$ monocytes increase both at local sites of inflammation as well as in the blood. Hence, extravasated monocytes may enhance the pro-inflammatory milieu.

- There are alterations in diastolic heart function in mild to moderate CKD.

- Patients with CKD have early adhesion molecule alterations and increased levels of pro-inflammatory cytokines as well as an impaired ability to induce oxidative metabolism. This implies an impaired cell transmigration and a weakened response to invading microorganisms.

- FGF23 levels were associated to elevated chemokines IL-12 and RANTES in CKD as well as to leukocyte altered adhesion molecule expression at unstimulated state but not to oxidative metabolism markers.

- Systemic FGF23 levels are associated with multiple markers of the innate immune system and high levels suppress the transmigration factor CD11b in granulocytes.

Patients with CKD have increased risk for infections and this is an important field of further investigation, with the aim to minimize the consequences of secondary blows on an already heavily burdened group of patients.
In continuous analysis of leukocyte function in CKD special efforts should be made to reveal the connection to uremic toxins and how they affect the immune system.
We need to further study the mechanisms behind the refractoriness of neutrophils when they encounter inflammatory stimuli, since this reaction most likely is essential for the altered leukocyte function in CKD. If we figure out where to suppress inflammation we might ameliorate the preservation of target organ function.
CKD implicates a substantial risk of premature cardiovascular disease that potentially get enhanced by pro-inflammatory cells and cytokines as well as by pleotropic acting FGF23 with vascular calcification and cardiac remodeling as results. The risk of diastolic and systolic impairment in early stages of CKD needs to be recognized and brought to the attention of both cardiologists and nephrologists so that we can act on this information and tailor treatment according to individual conditions in order to prevent or halt further progress.

In summary, we found increased levels of pro-inflammatory chemokines and $CD16^{+}$ monocytes in advanced CKD while patients with mild-to-moderate CKD displayed early alterations in adhesion molecules in addition to the increased cytokine levels. Altered cardiac structure and function were found early in CKD and FGF23 levels were associated with multiple markers of the innate immunity.

9 SUMMARY IN SWEDISH / Populärvetenskaplig sammanfattning

Leukocyterna – kroppens vita blodkroppar, har som främsta uppgift att patrullera i blodcirkulationen och uppmärksamma samt oskadliggöra invaderande mikroorganismer eller andra potentiella hot. Vävnadsskada eller infektion triggar igång det så kallade naiva immunförsvaret direkt. De två viktigaste leukocyterna för detta immunsvar är monocyter respektive neutrofiler. Monocyter aktiveras i blodbanan och tar sig sedan till skadad eller inflammerad vävnad. Celltransporten från blodet ut i vävnaden kallas för transmigration och är en viktig process som innefattar flera kronologiska förändringar i cellfunktionen och regleras av vidhäftningsmolekyler på cellytan (adhesionsmolekyler) som CD11b och CD62L samt genom svar på signalmolekyler (kemokiner).

Vi vet sedan tidigare att patienter med njursvikt har en ökad infektionskänslighet och även en ökad risk för kardiovaskulär sjukdom. Det har visat sig att leukocyter hos njursjuka fungerar sämre med en nedsatt förmåga att hantera invaderande mikroorganismer.

I **arbete I**, undersökte vi monocyter både i blodcirkulationen och lokalt i inflammerad vävnad. Vi använde oss av en hudkammarmodell som gör det möjligt att studera celler som tagit sig ut i vävnaden. Syftet var att studera de utvandrade monocyterna hos patienter med kronisk njursvikt och jämföra dem med celler från friska kontroller. Vi kunde påvisa en ökad förekomst av en subgrupp av proinflammatoriska monocyter, sk $CD16^{+}$ celler, hos patienter med avancerad njursvikt. Vi fann även en ökad nivå av de proinflammatoriska signalmolekylerna TNFα i blodet samt av fraktalkin i blod och blåsvätska hos de njursjuka. I blåsvätskan var både TNFα och IL-10 lägre hos patienterna, som tecken på att immuncellerna inte förmådde att fungera optimalt.

I våra tre följande arbeten har vi undersökt leukocytfunktion, olika inflammationsparametrar och hjärtfunktionsmarkörer i en studiepopulation som kallas PROGRESS-studien. Det är en prospektiv observationsstudie som sträcker sig över 5 år och som följer utvecklingen av en rad kliniska och laboratorieparametrar i en grupp individer med lätt till måttligt nedsatt njurfunktion. Dessa jämförs med en grupp ålders och könsmatchade icke njursjuka personer. Vid år noll inkluderade vi även data från patienter med avancerad njursvikt vilket gav oss möjlighet att utföra tvärsnittsstudier mellan tre grupper (arbete II) och i en större bredare njursviktgrupp (arbete IV) år 0.

I **arbete II** jämfördes alla tre grupper vid år 0 avseende hjärtfunktion undersökt med hjärtultraljud (ekokardiografi) samt med en teknik för att bedöma hjärtvävnades rörelsedynamik (Tissue Doppler imaging - TDI). Vi kunde konstatera att det fanns förändringar i hjärtats struktur och fyllnadsmekanik både hos patienter med avancerad njursjukdom och ibland de med måttligt nedsatt njurfunktion.

Arbete III koncentrerades på att följa monocytfunktion och inflammatoriska markörer över tid hos gruppen patienter med mild till måttlig kronisk njursvikt jämfört med de friska kontrollerna. Vi fann förändringar i uttryck av vidhäftningsmarkörer på cellytan och nedsatt förmåga till produktion av oxidativa syremetaboliter (vilka cellen använder som bakteriedödande vapen). Därutöver var nivåerna av inflammations underhållande cytokiner såsom TNFα, RANTES och IL-12 högre hos patienterna jämfört med de friska kontrollerna. I **arbete IV** analyserade vi nivåer av hormonet FGF23 och korrelerade dem till inflammatoriska markörer och cell transmigrationsmolekyler hos samtliga patienter (njursvikt grad 2-5) och jämförde det med friska kontroller. FGF23 ökade med avtagande njurfunktion. Vi fann att FGF23 nivåer var korrelerade till uttryck av chemokiner som RANTES och IL-12 samt till högre urinutsöndring av albumin, parathormon och fosfat hos CKD-patienterna jämfört med hos de friska kontrollerna. Inkubering av celler från friska donatorer med FGF23 protein i provrör nedreglerade ytmolekyler för transmigration på neutrofila celler.

Sammanfattningsvis råder ett kroniskt tillstånd med rubbad immunjämvikt vid njursvikt. Balansen mellan pro och anti-inflammatoriska faktorer och immunceller snarare än den exakta nivån av en enskild markör är av överordnad betydelse för den inflammatoriska processen (t.ex. ateroskleros, njursviktsprogress mm). Ytterligare forskning behövs för att öka kunskapen om de komplexa mekanismerna bakom hur kronisk njursvikt påverkar immunförsvaret och om kopplingarna mellan inflammation, FGF23 och de kardiovaskulära konsekvenser som drabbar patienter med njursjukdom.

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Ethical approvals / permissions regarding the thesis

1. Dnr 02-052. KI research committee Nord at Karolinska University hospital processed application stated below at the committee meeting on february 4th 2002
Title: Factors with impact on progression of renal failure.
APPROVED 2002-05-24

2. Dnr 02-052. Additional application 2003-03-24
Title: Factors with impact on progression of renal failure.
COMPLETION APPROVED 2003-04-02.

3. Dnr 2007/763-31/3 KI research committee Nord at Karolinska University hospital processed application stated below at the committee meeting on August 15 2007
Title: The function of white blood cells in patients with chronic kidney disease
APPROVED 2007-08-15

4. Dnr 2007/763-31/3 Additional application 2007-10-153-03-24
Title: The function of white blood cells in patients with chronic kidney disease
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