The potential of chemokines and trefoil factor peptides as biomarkers for the early detection of chronic kidney disease and for monitoring the course of renal diseases

Doctoral thesis at the Medical University of Vienna
for obtaining the academic degree

Doctor of Philosophy

Submitted by

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Vienna, 04/2017

Dedicated to my children
who are the most important people in my life

Acknowledgements

I would like to express my gratitude to Hendrik Jan Ankersmit, head of the Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, for giving me the opportunity to realize this project under his supervision and for his continuous support.

My special thanks go to Georg Roth and Claus Krenn at the Department of Anesthesia, General Intensive Care and Pain Medicine for their ongoing support and help in the realization of this project.

I would further like to thank my colleagues at the Medical University of Vienna and my colleagues at the Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration for their help and assistance in the conduction of the experiments.

The results of this thesis have been published in the “Translational Research” and the “PLOS ONE” journal and are given in the results section. For the published manuscripts as well as for all graphs, permission was obtained or graphs were taken from open source databases.

Declaration

This project was conducted at the Department of Surgery (Medical University of Vienna) in cooperation with the Department of Anesthesia, General Intensive Care and Pain Medicine (Medical University of Vienna) and the Department of Medicine III (Medical University of Vienna). Patients were recruited in cooperation with Thomas Reiter (Department of Medicine III, Medical University of Vienna) and Alexander Farr (Department of Urology, Medical University of Vienna). Experiments were performed with the help of Bianca Tudor and Daniel Klaus (Department of Anesthesia, General Intensive Care and Pain Medicine, Medical University of Vienna). Statistical analysis, data interpretation and manuscript preparation was performed under the supervision of Hendrik Jan Ankersmit (Department of Surgery, Medical University of Vienna), Georg Roth and Claus Krenn (Department of Anesthesia, General Intensive Care and Pain Medicine, Medical University of Vienna) with additional support of Walter Hörl (Department of Medicine III, Medical University of Vienna), Martin Haas (Department of Medicine III, Medical University of Vienna), Elisa Einwallner (Department of Laboratory Medicine, Medical University of Vienna) and Franziska Roth-Walter (Comparative Medicine, Messerli Research Institute, University of Veterinary Medicine Vienna, Medical University of Vienna and University of Vienna).

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Publications arising from this thesis

Increased chemokine excretion in patients suffering from chronic kidney disease. Translational Research. 2014 Dec;164(6):433-43.e1-2.156.

Trefoil Factor 1 Excretion Is Increased in Early Stages of Chronic Kidney Disease. PLoS One. 2015 Sep 21;10(9):e0138312.

Increased trefoil factor 2 levels in patients with chronic kidney disease. PLoS One. 2017 Mar 29;12(3):e0174551.

Abbreviations

ACE	Angiotensin-Converting Enzyme
AKI	Acute Kidney Injury
ANCA	Anti-neutrophil Cytoplasmic Antibody
CD95	Cluster of Differentiation 95, FAS Receptor
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CVD	Cardiovascular Disease
DM	Diabetes Mellitus
DKD	Diabetic Kidney Disease
eGFR	Estimated Glomerular Filtration Rate
ELISA	Enzyme-linked Immunosorbent Assay
ESRD	End-Stage Renal Disease
ESRF	End-Stage Renal Failure
FSGS	Focal Segmental Glomerulosclerosis
GFR	Glomerular Filtration Rate
HSP	Heat Shock Protein
IL	Interleukin
K/DOQI	Kidney Disease Outcomes Quality Initiative
MDRD	Modification of Diet in Renal Disease
MGN	Membranous Glomerulonephritis
MPGN	Membranoproliferative Glomerulonephritis
NKF	National Kidney foundation
ROC	Receiver Operating Characteristic
ROS	Reactive Oxygen Species
RPGN	Rapidly Progressive Glomerulonephritis
RPRF	Rapidly Progressive Renal Failure
TFF	Trefoil Factor
TGF-β	Transforming Growth Factor Beta
TH17	T Helper 17 Cells
TNF-α	Tumor necrosis factor alpha
TNFR1	Tumor necrosis factor receptor 1
TREG	Regulatory T Cells

Abstracts in English and German

Abstract

Patients suffering from chronic kidney disease (CKD) have high mortality rates and increased risks of serious complications, particularly of developing cardiovascular disease. Even a minimal deterioration in renal function can entail severe complications like anaemia, malnutrition, bone disease, or neuropathy. Moreover, CKD progression is silent. Consequently, many patients are identified shortly before the onset of symptomatic kidney failure. At that state, only a few therapeutic options are left to avert detrimental outcomes or progression to end-stage renal disease (ESRD), necessitating kidney replacement therapy. Therefore, the early identification of persons at risk would help to initiate an appropriate treatment on time and could help to slow the progression of renal failure to ESRD.

CKD is associated with ongoing inflammation, on the one hand, and initiation of repair mechanisms, on the other. Continuous inflammation is triggered among others by leukocyte activation and the migration of immunocompetent cells towards the site of damage. In doing so, chemokines are locally upregulated to facilitate leukocyte trafficking. An increase in chemokine expression has already been proven for different renal diseases. Therefore, one aim of this thesis was to measure chemokine levels in urine and serum of CKD patients, to calculate fractional chemokine expression, and to examine the potential of chemokine levels as biomarkers for CKD. Analyzed chemokines include CXCL11, CCL20, CCL22, and CCL17 which are essentially involved in the development and progression of CKD. During chronic inflammation, counterregulatory mechanisms are initiated to contain increased cell death and overcome continuous tissue destruction. One of these is the upregulation of Trefoil factor family (TFF) peptides, which are involved in the regeneration of mucus-containing epithelia. In the human urinary tract TFF peptides are secreted in a site-specific manner and have been shown to be upregulated in renal diseases. Therefore, the second aim of this thesis was to investigate the potential of TFF peptides as biomarker for CKD.

The serum and urinary concentrations of TFF peptides and the above mentioned chemokines have been measured in patients with CKD stages 1 - 5 by using the ELISA technique. The obtained concentration profiles were subsequently correlated with several clinical parameters. Furthermore, fractional chemokine and TFF peptide expression rates were calculated to evaluate protein excretion irrespective of glomerular function.

We found significantly elevated fractional chemokine expression rates in CKD patients as compared to healthy probands. Furthermore, fractional chemokine expression was able to predict various CKD stages, as depicted by ROC curve analysis. The TFF3 levels were significantly increased in later CKD stages, signifying the upregulation of counterregulatory processes during CKD progression. Comparable to TFF3, serum levels of TFF1 and TFF2 levels constantly increased with significantly elevated concentrations in higher CKD stages as compared to controls. In contrast, urinary levels of TFF1 and TFF2 were elevated with the onset of CKD and diminished with disease progression to levels comparable to healthy individuals, indicating the potential of these two proteins to identify patients with early CKD stages. The ROC curve analysis revealed that measurement of TFF peptide levels could help to predict different CKD stages and to identify individuals at risk.

In summary, we found a changed expression rate of chemokines and TFF peptides during CKD progression, with TFF1 and TFF2 being differently regulated to TFF3. However, further clinical studies are obligatory to unravel the role of chemokines and TFF peptides in CKD and to ultimately evaluate their potential as biomarkers for CKD.

Zusammenfassung

Patienten mit chronischem Nierenversagen (CNV) weisen eine hohe Mortalitätsrate und ein erhöhtes Risiko für weitere Erkrankungen auf. Sogar eine minimale Abnahme der Nierenfunktion kann dabei schwere Komplikationen wie Anämie, Mangelernährung, Knochenerkrankungen oder Neuropathie nach sich ziehen. CNV schreitet langsam voran und erste Symptome treten zumeist kurz vor einem manifesten Nierenversagen auf, weswegen viele Patienten erst im fortgeschrittenen Stadium als solche erkannt werden. Da jedoch ein möglichst frühzeitiger Therapiebeginn die besten klinischen Ergebnisse erzielt, ist die Identifizierung von Patienten mit eingeschränkter Nierenfunktion von großer Wichtigkeit, um das Voranschreiten der Erkrankung rechtzeitig einzudämmen.

Einerseits ist CNV mit einer anhaltenden Entzündungsreaktion assoziiert, andererseits jedoch auch mit einer erhöhten Aktivität zellulärer Reparaturmechanismen. Während der Entzündungsreaktion kommt es zu einer Aktivierung von Leukozyten sowie zu einer leukozytären Infiltration in das betroffene Gewebe. Dabei werden die Leukodiapedese und die weitere Migration der Leukozyten von sogenannten Chemokinen bestimmt. Für verschiedene Nierenerkrankungen konnte bereits ein Anstieg der Konzentration unterschiedlicher Chemokine im Nierengewebe selbst sowie im Serum und Harn nachgewiesen werden. Daher war ein Ziel dieser Dissertation, die Chemokine CXCL11, CCL20, CCL22, und CCL17 im Harn und Serum von Patienten mit chronischem Nierenversagen nachzuweisen, sowie deren fraktionelle Exkretion zu berechnen, um das Potential dieser Proteine als Biomarker für CNV zu ermitteln. Die analysierten Chemokine wurden aufgrund ihrer Beteiligung bei der Entstehung und beim Voranschreiten des CNV ausgewählt. Mit zunehmendem Verlust der Nierenfunktion werden verschiedene Regulationsmechanismen aktiviert, um die renale Entzündungsreaktion einzudämmen und dabei unnötigen Zelltod und Gewebedestruktion zu verhindern. Einer davon ist die Hochregulierung von Trefoil factor family (TFF) Peptiden, denen eine besondere Bedeutung in der Regenerierung von Schleimhäuten zukommt. In den menschlichen Harnwegen konnte bereits die Sekretion von TFF Peptiden nachgewiesen werden, sowie deren vermehrte Expremierung bei verschiedenen Nierenerkrankungen. Ein weiteres Ziel dieser Arbeit war daher, das Potential von TFF Peptiden als Biomarker zur Früherkennung von Patienten mit renalen Einschränkungen und zur Abschätzung des Voranschreitens des Nierenversagens zu ermitteln.

Die Konzentration von TFF Peptiden und den zuvor genannten Chemokinen wurde im Serum und im Harn von Patienten mit chronischem Nierenversagen der Stadien 1 bis 5

mittels ELISA gemessen. Gemessene Konzentrationen wurden anschließend mit verschiedenen klinischen Parametern korreliert sowie die fraktionelle Exkretion ermittelt, um die Proteinexkretion unabhängig von der glomerulären Funktion abschätzen zu können.
Bei Patienten mit CNV konnten wir eine signifikant erhöhte fraktionelle Chemokinexkretion im Vergleich zur gesunden Kontrollgruppe nachweisen. Die Analyse mittels ROC-Kurve ergab, dass durch die Ermittlung der fraktionellen Chemokinexkretion verschiedene Stadien des CNV abgeschätzt werden können. Des Weiteren hatten Patienten mit fortgeschrittenen CNV Stadien signifikant erhöhte TFF3-Konzentrationen in Serum und Harn, was auf eine Hochregulation von Reparaturmechanismen in höheren CNV Stadien schließen lässt. Vergleichbar zu TFF3 konnten wir höhere Serumkonzentrationen von TFF1 und TFF2 in Patienten mit fortgeschrittener Nierenerkrankung nachweisen. Deren Konzentration im Harn war jedoch am höchsten bei Patienten mit niedrigeren CNV Stadien was auf das Potential dieser Proteine hindeutet, frühzeitig gefährdete Personen zu identifizieren. Mit Voranschreiten der Erkrankung sanken die Harnkonzentrationen von TFF1 und TFF2 wieder auf Werte vergleichbar mit gesunden Kontrollen. Eine Analyse mittels ROC-Kurve zeigte, dass mittels der Messung von TFF-Peptidkonzentrationen verschiedene CNV Stadien ermittelt und Patienten in einem frühen Stadium diagnostiziert werden könnten.

Zusammenfassend konnten wir eine sich verändernde Expressionsrate von Chemokinen und TFF Peptiden während des Voranschreitens des CNV nachweisen, wobei TFF1 und TFF2 im Vergleich zu TFF3 unterschiedlich reguliert waren. Weiterführende Studien sind notwendig, um die Funktion von Chemokinen und TFF Peptiden im CNV letztendlich zu ergründen.

1. CHAPTER ONE: Introduction

1.1 The kidney

1.1.1 General Information

The kidneys are paired bean-shaped organs located in the abdomen near to the rear abdominal wall (1). In both kidneys fractions of blood with a specific molecular weight are filtered and excreted as urine, whereas vitally important molecules are reabsorbed (2). The kidneys also balance the water and electrolyte levels in the body. Hence, urine mainly consists of concentrated metabolic waste products, electrolytes, and if applicable of exogenous acquired toxic substances also including medicaments. Furthermore, the kidneys hold important endocrinal functions (2, 3). They produce erythropoietin, which controls erythropoiesis, and are important organs in blood pressure regulation by the production of the enzyme renin that participates in the renin-angiotensin aldosterone system. The kidneys are further involved in the calcium balance by the hydroxylation of a precursor molecule into active vitamin D_3 (2, 3).

The smallest functional unit of the kidney is the so-called nephron, which consists of the renal tubule and the renal corpuscle (4). The renal corpuscle contains the glomerulus that can be referred to as a capillary bundle and the Bowman's capsule surrounding the glomerulus (Fig. 1). Specialized cells wrapped around the capillaries, the so-called podocytes, form the visceral inner layer of the Bowman's capsule, simple squamous epithelium composes the parietal outer layer (4). Through the glomerular basement membrane and the visceral layer of podocytes, small blood molecules with a molecular weight up to about 10 kDa are filtered into the Bowman's space, generating the primary urine (2).

The main function of the renal tubule is the transformation of primary into final urine that is subsequently collected and excreted via the efferent urinary tract. For this purpose primary urine is concentrated in the renal tubule system by the reabsorption of water, ions, and further vitally molecules. At the same time, metabolic waste products and surplus electrolytes are excreted into urine (2). Specialized cells, lining the distal ascending limb of the tubule system and pending to the afferent arteriole of the glomerulus, compose the so-

called macula densa (1, 4). Those cells continuously measure the urine concentration in the distal tubule and regulate the afferent blood flow to maintain a constant filtration rate in the nephron. Thereby, the reabsorption of ions and water to the blood can be increased or decreased as needed. This autoregulatory feedback mechanism ensures the adjustment of renal function as reaction to changing systemic blood volume and blood pressure (2).

Renal blood flow is kept up at about 1.2 l per minute in a healthy adult individual (2, 3, 5). Approximately 180 l primary urine are filtered out of it by all nephrons of both kidneys every day. After concentration of primary urine in the tubule system, about 2 l final urine are excreted daily via the efferent urinary tract. Those amounts of filtered and reabsorbed water and molecules clearly illustrate the enormous filtering efficiency of the renal system.

1.2 Pathogenesis of renal diseases

1.2.1 General Information

The kidneys keep their function outside conscious control and for most people the kidneys' efficiency remains unknown their whole live long. Only when the kidney function declines and symptoms of renal failure become obvious, the kidneys' implication on physical health becomes apparent. But even when progressive loss of renal function occurs, the kidneys are able to produce sufficient primary urine over a long period (3). The adaptive hyperfiltration of the remaining nephrons can keep the liquid balance stable to some extent, resulting in the late occurrence of symptoms of renal failure (6). Consequently, renal diseases are detected late in the disease progression, resulting in a delayed start of therapy. However, especially the early detection of kidney diseases is of great importance to initiate an appropriate treatment on time and to slow down the progression of kidney failure (6).

Most kidney diseases are chronic conditions that can lead to chronic kidney disease (CKD) and the subsequent continuous loss of renal function (3, 5). Normally, the progression to end-stage renal disease (ESRD) expands over years, but also a rapid loss in kidney function within weeks might occur, resulting in life-threatening conditions.

The main causes of renal damage in industrial nations are diabetes and cardiovascular diseases, also including high blood pressure (Fig. 2) (7). Congenital renal diseases, interstitial nephritis, glomerulonephritis, systemic diseases with renal involvement, and occlusive diseases of the efferent urinary tract can be further origins of the development of CKD (Fig. 2).

The identification of the underlying renal disease is of great importancein order to choose the appropriate treatment to avert or delay further loss of kidney function (6). Knowledge of case history, physical examination, urine analysis, medical imaging and histologic analysis of renal biopsy are the main tools in the detection and identification of kidney diseases (8).

The following chapter should give a survey at the main causes of renal dysfunction and explains their pathogenesis, arising complications and therapy options. Described diseases are diabetic nephropathy, hypertensive nephrosclerosis, glomerulonephritis, and interstitial nephritis, which are the most frequent origins of the development of CKD (Fig. 2).

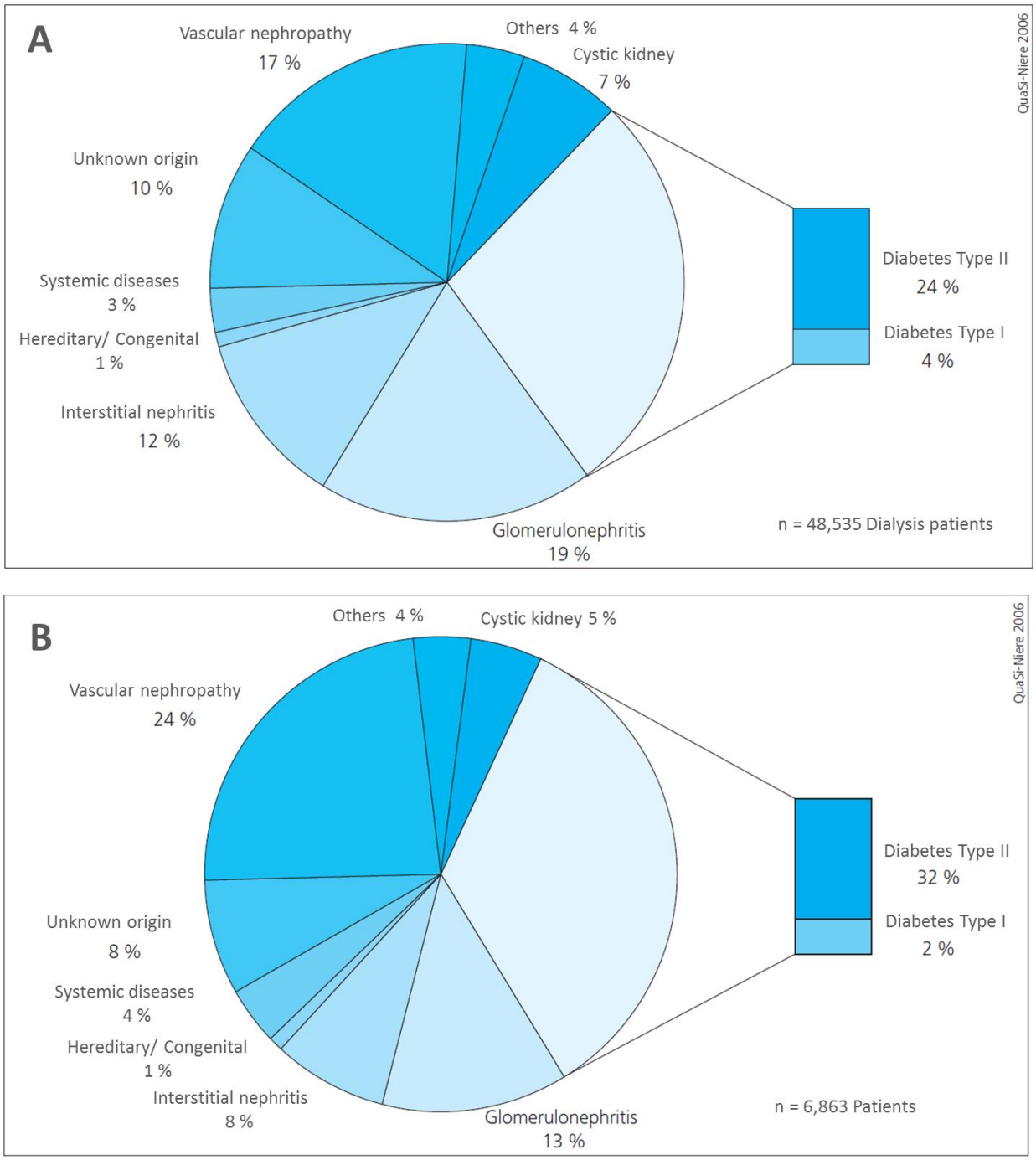

Figure 1: Pie chart A shows the incidences of renal diseases of all patients dependent on dialysis recorded in the German QuaSi database in 2006. Pie chart B shows the incidences of renal diseases at the time of diagnosis recorded in the German QuaSi database in 2006. (adopted from Frei and Schober-Halstenberg, 2008) (9)

1.2.2 Diabetic kidney disease

Diabetes mellitus (DM) is a main cause of continuously increasing morbidity and mortality worldwide (10). About 5.1 million deaths per year can be attributed to DM, most of them as a result of coronary heart disease (11). Additional complications of DM comprise further micro- and macrovascular diseases, also including diabetic kidney disease (DKD).

DKD affects 30 to 40 % of DM patients and is the foremost reason for end-stage renal disease (ESRD), necessitating kidney replacement therapy (12, 13) (Fig. 2). Originally DKD was defined as albuminuria and/or impaired renal function in the presence of DM type I or II and was considered the strongest predictor of mortality in patients with DM (14). Currently, besides diabetic nephropathy the diagnosis DKD also comprises ischemic nephropathy, atheroembolic renal disease and renal interstitial fibrosis, all occurring as a result of DM (15).

Symptoms of renal damage occur 5 to 10 years after the diagnosis of DM type I in most patients. The first clinical sign is hyperfiltration followed by albuminuria, manifest renal nephropathy and ESRD after 20 to 40 years (5). As DM type II remains clinically asymptomatic over a long period, most patients already have signs of renal damage at the time of diagnosis (5). Consequently, patients with DM type I should be screened for DKD after a maximum of 5 years after the diagnosis of DM; patients with DM type II should be screened immediately after the diagnosis (5). From then onwards, the annual examination of kidney function by blood and urine analysis should be obligatory. Abdominal sonography reveals echogenic, minimal enlarged kidneys even at advanced stages of DKD.

Kidney biopsy is normally omitted to confirm the diagnosis due to the high prevalence of DKD in patients with DM (5). However, initial histopathological changes include swelling of the basement membrane, podocyte damage and mesangial expansion caused by cell enlargement and elevated matrix secretion (14, 15). Podocyte hypertrophy and foot process effacement thereby result in functional changes such as albuminuria (15, 16). Glomerulosclerosis, tubular atrophy and nodular glomerulosclerosis (also known as Kimmelstiel-Wilson nodules) normally emerge in an advanced stage of DKD (17). In efferent and afferent arterioles intimal hyperplasia is predominant at first, eventually emerging into arterial hyalinosis (16).

DKD is a result of various metabolic and hemodynamic derangements during DM progression. The hyperglycaemia itself induces the synthesis of angiotensin II, which in turn causes hyperfiltration by efferent arteriolar vasoconstriction as well as an elevated release of reactive oxygen species (ROS) (15). ROS again triggers renal inflammation and fibrosis. Continuing inflammation and a chronically activated innate immune system further adds to renal damage in patients with DM (18). In addition, renin-angiotensin system genes

polymorphism is associated with increased risk for DKD development and progression to ESRD (19, 20).

The therapy of DKD includes glycaemic control (target value of $HbA1_c$ < 7 %) and blood pressure management, preferably by medication that blocks the renin-angiotensin system, such as angiotensin-converting enzyme (ACE) inhibitors or angiotensin receptor blockers (21).

1.2.3 Hypertensive nephrosclerosis

Arterial hypertension can lead to vascular impairment, which in turn can result in damage to organs like the eyes, the brain, and also the kidneys. Depending on the rapidity of the disease course, a distinction is made between the malignant nephrosclerosis and the (benigne) nephrosclerosis (5).

The malignant nephrosclerosis occurs as a result of excessive hypertension, but has become rare due to the broader acceptance of preventive medical examination (5). Consequently, the malignant nephrosclerosis is mostly diagnosed in young persons with previously unknown arterial hypertension. The disease is histologically characterized by a fibrinoid necrosis, myointimal proliferation, and thrombotic microangiopathy (5).

Though a relation between arterial hypertension and benigne nephrosclerosis is undeniable, hypertension as the solely source of nephropathy is lately questioned (22, 23). Correlations between high blood pressure and renal lesions are only moderate (24-26). Furthermore, renal histological findings in patients with nephropathy and arterial hypertension are consistent with changes due to normal aging, including hyalinosis of arterioles and intima thickening (26, 27). In addition, nephropathies associated with variants in the apolipoprotein L1 gene can cause secondarily increased blood pressure and are often wrongly attributed to primary hypertensive nephrosclerosis (22).

However, increased systemic blood pressure can aggravate nephropathy, due to the elevated pressure in the abnormal dilated afferent arterioles, eventually leading to glomerular hyperperfusion and hypertrophy (23). This in turn can result in a loss of glomerular autoregulation and can be examined in urine analysis as albuminuria or general proteinuria. Another reason for glomerulosclerosis is glomerular ischemia, leading to inflammation and tubular atrophy (23).

The diagnosis should include laboratory testing of blood and urine, as well as renal sonography. Renal biopsy is rarely performed in elderly patients with impaired renal function, but should be carried out generously in younger patients, due to the high incidence of

otherwise false positive diagnosis (5). Also genetic testing for variants in the apolipoprotein L1 gene are available and should be performed in patients with African ancestry, as it is the main cause of nephrosclerosis in this population (22).

The therapy of nephrosclerosis associated with hypertension comprises the control of blood pressure, preferentially with an ACE inhibitor, as well as the symptomatic treatment of renal failure and secondary disorders (5, 22).

1.2.4 Glomerulonephritis

The term glomerulonephritis, also known as glomerular nephritis, comprises several renal diseases, usually affecting both kidneys (3, 5). Though not all diseases hold an inflammatory component, glomerulonephritis is usually characterized by inflammation of the small renal blood vessels or by inflammation of the glomeruli, the main units involved in primary urine filtration. Initial findings are abnormal urine examinations; serious clinical manifestations are either the nephrotic syndrome, the nephritic syndrome, or rapidly progressive renal failure (RPRF) (3, 5). Chronic glomerular nephritis can result in CKD and, eventually, in ESRD.

Classification

Renal inflammatory diseases can be classified in primary and secondary glomerulonephritis. Primary glomerulonephritis affects the kidney directly, whereas during secondary glomerular nephritis, the kidney is damaged by systemic afflictions that can also harm other structures like the skin, the eyes, joints or internal organs (5). Based on histological findings, inflammatory diseases can also be subdivided into nonproliferative- and proliferative glomerulonephritis. In nonproliferative glomerulonephritis, the number of cells remains stable, whereas in proliferative glomerulonephritis an increased number of cells can be found in the glomeruli (3, 5). The most frequent forms of nonproliferative glomerular nephritis are the minimal change disease, the focal segmental glomerulosclerosis (FSGS), and the membranous glomerulonephritis (MGN). Proliferative glomerulonephritis comprises amongst others the IgA nephropathy, the membranoproliferative glomerulonephritis (MPGN), the post-infectious glomerulonephritis, and the rapidly progressive glomerulonephritis (RPGN) (3, 5). Nonproliferative glomerulonephritis usually results in the nephrotic syndrome, proliferative glomerulonephritis in the nephritic syndrome.

Clinical manifestations

Nephrotic syndrome

The nephrotic syndrome manifests itself with generalized edema, proteinuria (daily >3 g/1.73 m² body-surface), hypoalbuminemia (< 2.5 g/dl), and hyperlipidemia (3, 5). Due to ongoing renal inflammation, the filtration barrier becomes increasingly permeable for proteins, resulting in proteinuria and eventually in hypoalbuminemia, when protein loss exceeds the compensatory mechanisms of the liver (3, 5). This in turn can lead to edema if the intravascular oncotic pressure is unable to equalize the oncotic pressure in peripheral tissue. Furthermore, abnormal sodium and water retention in patients with nephrotic syndrome are adding to the risk of generalized edema. With the ongoing decline of the GFR, hypervolemic hyponatremia with low fractional sodium excretion can occur (28). The occurrence of hyperlipidemia is seen as a result of elevated liver activity and can lead to lipiduria in patients with nephrotic syndrome (29).

Nephritic syndrome

The nephritic syndrome is characterized by hematuria, decreased GFR, and systemic hypertension (3, 5). Continuous inflammatory damage to cells lining the filtration barrier leads to destruction of the epithelial barrier and eventually to hematuria. The histological examination of the urine sediment reveals red blood cell casts with dysmorphic red blood cells in particular. Systemic hypertension is the result of the activation of the renin-angiotensin-system (3).

Rapidly progressive renal failure

The RPRF is the most aggressive outcome of glomerulonephrits and is characterized by a rapid and continuous decrease in kidney function (5, 30). Within days till weeks about 50 % of the renal function can be lost. Patients with RPRF in the course of glomerulonephrits present a nephritic syndrome, including massive hematuria with acanthocytes and other dysmorphic red blood cells in the urine (5). Ultrasonography reveals normally sized kidneys, which can help to differentiate RPRF from a chronic course, in which kidneys are expected to be small, contracted, and echogenic (30). RPRF can occur as the clinical manifestation of a heterogeneous group of diseases, including primary renal diseases like glomerulonephritis, tubule-interstitial diseases, and vascular diseases, as well as systemic diseases affecting the kidneys, like systemic vasculitis, systemic lupus erythematosus, or thrombotic microangiopathy (30). Since the best therapy of RPRF is the treatment of the underlying disease, the early diagnosis and initiation of therapy is of great importance to avert or delay the progression to ESRD, needing life-long kidney replacement therapy. Consequently,

kidney biopsy is essential for a diagnosis on time and for understanding the chronicity of the disease process (30).

Most frequently occurring glomerulonephritis

Minimal change disease

The minimal change disease is named after inconspicuously appearing glomeruli in the light microscopic view. It is the most frequent cause of the nephrotic syndrome in children (about 90 % until an age of 10 years) and in about 20 % in adults, with a sudden occurrence of the nephrotic syndrome as the first clinical manifestation (3, 5). In adults, disease progression is more aggressive than in children and can lead more often to serious complications.

Since it is the most frequent cause of the nephrotic syndrome in infants, therapy with steroids can be started with the onset of symptoms without preceded renal biopsy in children. With positive response to therapy diagnoses can be assured. If no improvement is verified, a renal biopsy should be performed in order to differentiate a focal segmental glomerulonephritis. In adults the diagnosis is assured by kidney biopsy and the subsequent sample analysis by electron microscopy (3, 5).

Initial therapy includes high dosed steroids and if necessary Cyclosporin A and Cyclophosphamide. With response to treatment, a complete remission can be obtained within four weeks and the long-range prognosis of adult and infant patients is good if treated promptly. Nevertheless, the transformation into a FSGS and he progression to end stage renal disease (ESRD) are possible at all times (5).

Focal segmental glomerulosclerosis

FSGS is defined by the histologic finding of glomerular sclerosis, collapsed capillary, and adhesion between capillary loops and the Bowman’s capsules (31). It can emerge as primary FSGS, primarily affecting infants and adolescents, or as secondary FSGS in the course of different diseases with renal hyperfiltration and podocyte injury, e.g. adipositas, arterial hypertension, or reflux nephropathy (5, 31).

The first manifestation is mainly the nephrotic syndrome, but some patients only come down with proteinuria, hematuria, and hypertension. Diagnosis is normally assured by kidney biopsy (3, 5).

The initial therapy includes renoprotective treatments like normalization of weight, smoking cessation, and successful control of blood pressure preferably with angiotensin-converting (ACE) inhibitors. An immunosuppressive therapy is started with steroids, Cyclosporin A, or, in case of persistent proteinuria, with Tacrolimus or Mycophenolate (5). In

most patients, remission of FSGS can be induced if treated promptly. In adults about 2 % of patients with ESRD suffer from FSGS (7).

Membranous glomerulonephritis

MGN mostly affects adult men and often shows up with a nephrotic syndrome as the first clinical manifestation (5). In about 30 to 50 % of adults suffering from a nephrotic syndrome, a MGN can by verified via biopsy (7). In light microscopy, a swelling of the basement membrane without an inflammatory component or signs of proliferation assures the diagnosis (32). In most patients, MGN is of primary origin, though a secondary form is known and frequently associated with cancer diseases (5).

The course of MGN is in equal parts remissive, stable or progressive, eventually resulting in CKD. Therefore a symptomatic therapy of the nephrotic syndrome is started and the natural course of MGN is observed over 6 months. The symptomatic therapy includes control of blood pressure with target values < 130/80 mmHg, minimization of proteinuria with ACE inhibitors, lipid lowering, and restriction of salt intake. If spontaneous remission cannot be verified within 6 months and a secondary origin has been excluded, an immunomodulatory therapy is started, most frequently with steroids and Chlorambucil (3, 5).

IgA nephropathy

The IgA nephropathy is the most frequently occurring glomerulonephritis in adults, mostly affecting young men (33). Clinical manifestations include asymptomatic urine findings, hematuria, or the RPGN (5, 33). Diagnosis is assured by histology, showing mesangial IgA deposits. Those deposits lead to the secretion of pro-inflammatory cytokines, which in turn results in mesangial proliferative modifications, glomerulosclerosis, and diffuse interstitial changes. IgA glomerulonephritis is classified in 5 stages according to unfavorable prognostic findings. With higher stages the degree of inflammatory changes increases, resulting in a poorer prognosis (3).

The symptomatic therapy includes the control of blood pressure as well as the administration of ACE inhibitors and lipid-lowering medication. Therapy of inflammation with corticosteroids and other immunomodulatory drugs can limit proteinuria and can slow down the loss of GFR (33).

Between 20 and 40 % of the patients with IgA nephropathy develop a CKD and about 10 -20 % end up with kidney replacement therapy (7). The occurrence of acute kidney failure can point to the transformation of IgA glomerulonephritis into a rapidly progressive glomerular nephritis (33).

Membranoproliferative glomerulonephritis

The MPGN is a rare glomerulonephritis manly affecting infants and young adults. It accounts for about 10 % of the biopsy-confirmed glomerular nephritis (34, 35). The primary form of MPGN is a diagnosis by exclusion and arises only by deposits of immune complexes along the basement membrane. The secondary form is a complication of systemic diseases, also mainly due to deposits of immune complexes, but it can also emerge from a thrombotic microangiopathy or by deposits of myeloma proteins (3, 5).

In light microscopy, the swelling of the glomerular basement membrane and the diffuse mesangial proliferation are conspicuous (36). With the additional examination with electron microscopy, the differentiation between primary and secondary MPGN can be possible to some extent (36).

The clinical manifestation can include the nephrotic as well as the nephritic syndrome but also asymptomatic proteinuria and hematuria. Patients often also have arterial hypertension and a decrease in renal function. Laboratory testing can reveal the activation of the complement system (5).

Treatment of the underlying disease plays an important role in the improvement of the secondary form of MPGN. For primary MPGN therapy options are scarce and are limited to the treatment of symptoms like blood pressure normalization and intake of ACE inhibitors for the reduction of proteinuria. In patients with nephrotic syndrome steroids can help to limit the symptoms (3, 5). However, remission rates are low and within 10 years about half of the patients with primary MPGN suffer from ESRD, requiring kidney replacement therapy (3, 5).

Post-infectious glomerulonephritis

The pathophysiology of post-infectious glomerulonephritis still remains unclear. However, causative pathogens include streptococci, staphylococci, Escherichia coli, mycobacteria, parasites and rarely mycosis or viral infections (37). In developing countries post-infectious glomerulonephritis is frequent in infants, whereas in industrial nations older people are affected more often. Histologic findings are the diffuse proliferation of mesangial cells and the infiltration of leukocytes into capillary endothelial tissue (37).

Clinical manifestations of post-infectious glomerulonephritis occur about two weeks after infection and include the nephritic syndrome, edema, and arterial hypertension (37). Sometimes patients show symptoms of the nephrotic syndrome or of the progression of post-infectious glomerulonephritis to RPGN. These symptoms together with abnormal urine findings (acanthocytes and red blood cell casts) and an anamnesis of infection assure diagnosis and render kidney biopsy unnecessary (5). Laboratory analysis might reveal an

increased anti-streptolysin O and/or an elevated anti-DNase B titre after streptococci infection (3, 5).

The prevention of glomerulonephritis with an antibiotic therapy should be preferred; otherwise symptomatic therapy is initiated, including balanced liquid intake, restriction of salt consumption and medication to control arterial pressure. Symptoms of RPGN should result in renal biopsy and sometimes temporary dialysis is necessary to cope with decreased filtration rates (3, 5, 37).

Rapidly progressive glomerulonephritis

RPGN, also known as crescentic glomerulonephritis, is characterized by the rapid and continuous loss of renal function, becoming evident as RPRF (5, 30). Underlying diseases include primary renal as well as systemic vasculitis, and more seldom lupus nephritis, MPGN, IgA glomerulonephritis, and post-infectious glomerulonephritis (30).

Patients can complain about dark urine, generalized unspecific constitutional symptoms, flu-like syndrome, and purpura (30). Arterial pressure usually remains in the normal range or can be slightly raised. Seldom patients remain asymptomatic.

To find the exact diagnosis serum antineutrophilic cytoplasmic antibodies (ANCAs) are determined. The presence and also the concentration of p-ANCA (directed against myeloperoxidase) and c-ANCA (directed against Proteinase 3) in combination with immunofluorescence analysis of the kidney biopsy are thereby conclusive (30).

The therapy consists of treatment of the underlying disease and symptomatic therapy. Especially the fast identification and therapy of the causal affliction can help to limit renal damage and progression to ESRD (5).

1.2.5 Interstitial nephritis

Interstitial nephritis, also named tubule-interstitial nephritis, affects the interstitium and also the tubules of both kidneys. Due to the fact that tubular injury remains difficult to identify in light microscopy, whereas interstitial cellular infiltration and signs of interstitial inflammation can be easily spotted, the term interstitial nephritis is more often used in literature (38, 39). However, tubular damage appears as focal lesion penetrated by inflammatory cellular infiltrates. As a consequence, the tubular basement membrane becomes discontinued, leading to cellular injury of the surrounding epithelial cells (39). In the immunopathogenesis of interstitial nephritis the tubules hold important functions and their impairment finally results in a decreased filtration rate (39).

About 70 % of the interstitial nephritis cases occur as an adverse reaction to medication like antibiotics or analgetics (39). Signs of interstitial inflammation usually arise within days after drug exposure, but a disease development within weeks to months is also possible and has to be considered in the diagnostic process (39, 40). Other causes of interstitial nephritis include systemic diseases with renal involvement, toxins, and bacterial infections (39).

The normal course of interstitial nephritis is either acute or chronic and can finally lead to acute or chronic kidney diseases and the need of kidney replacement therapy. However, chronic interstitial nephritis usually occurs as a long-term consequence of a missed initial diagnosis after progression to irreversible fibrosis (39, 41).

Symptoms of interstitial nephritis are multifarious and mostly unspecific, preventing diagnosis. Patients can be affected amongst others by fever, fleeting skin rash, back pain, joint pain, and dysuria (39, 41). Laboratory analysis reveal eosinophilia in about 35 to 60 % of patients (39). Increased levels of serum creatinine and blood urea nitrogen are indicators of tubular dysfunction and are evident before a decline in filtration rate is obvious (39). Eosinophiluria can give a hint towards the diagnosis of interstitial nephritis, but it can also appear during other inflammatory processes of the kidney or the urinary tract (39). The abdominal sonography can reveal increased kidney size as a result of interstitial renal edema. In about 30 % of cases an abnormal gallium scan reflects the interstitial cellular infiltration, but lacks diagnostic specificity (42). In conclusion, early detection of interstitial nephritis can be managed by the detection of tubular dysfunction in non-oliguric patients, especially in the presence of a steady decline of the glomerular filtration rate (GFR) (39).

The most important therapy is the immediate withdrawal of the triggering agent (39, 41). When interstitial nephritis remains progressive even though the offending trigger has been eliminated, treatment with corticosteroids should result in an improvement of symptoms early after therapy initiation (39).

1.3 Chronic Kidney Disease

1.3.1 General Information

The term CKD subsumes the clinical manifestation of various renal diseases with comparable evolvement of symptoms of kidney failure. It is a crucial health problem with continuously increasing numbers of affected persons, poor outcome, and with an enormous impact on healthcare costs. To quote an example, in the US about 15 percent of the population is suffering from CKD with mortality rates about 50 percent higher than patients without renal afflictions (7) (Fig. 3). Also the number of patients dependent on kidney replacement therapy by either dialysis or transplantation almost doubled within ten years (7). US Medicare expenditures for all CKD stages sum up to total annual costs of $87 billion (7), illustrating the enormous burden on public health insurance systems in European countries.

CKD is defined by decreased renal function or by the presence of kidney damage for a minimum of three months or by a history of kidney transplantation (8). The natural course of CKD is the progression to end stage renal disease (ESRD) further triggered by secondary factors independent of the underlying renal disease, necessitating kidney replacement therapy by either dialysis or transplantation (43, 44). Further major outcomes include the development of complications caused by the impaired kidney function, and the increased risk for cardiovascular disease (43, 45, 46). Moreover, even a minimal decline of renal function can lead to serious complications like anaemia, malnutrition, neuropathy and bone disease (3).

Early identification and treatment of CKD could delay disease progression and prevent adverse outcomes (47). Unfortunately, the progression of CKD is silent. As a consequence, many patients are identified just prior to the onset of symptoms of renal failure, a state where few therapeutic options are left to impede CKD progression (6). Hence, the early identification of at-risk individuals would help to delay the progression of CKD to ESRD, requiring kidney replacement therapy.

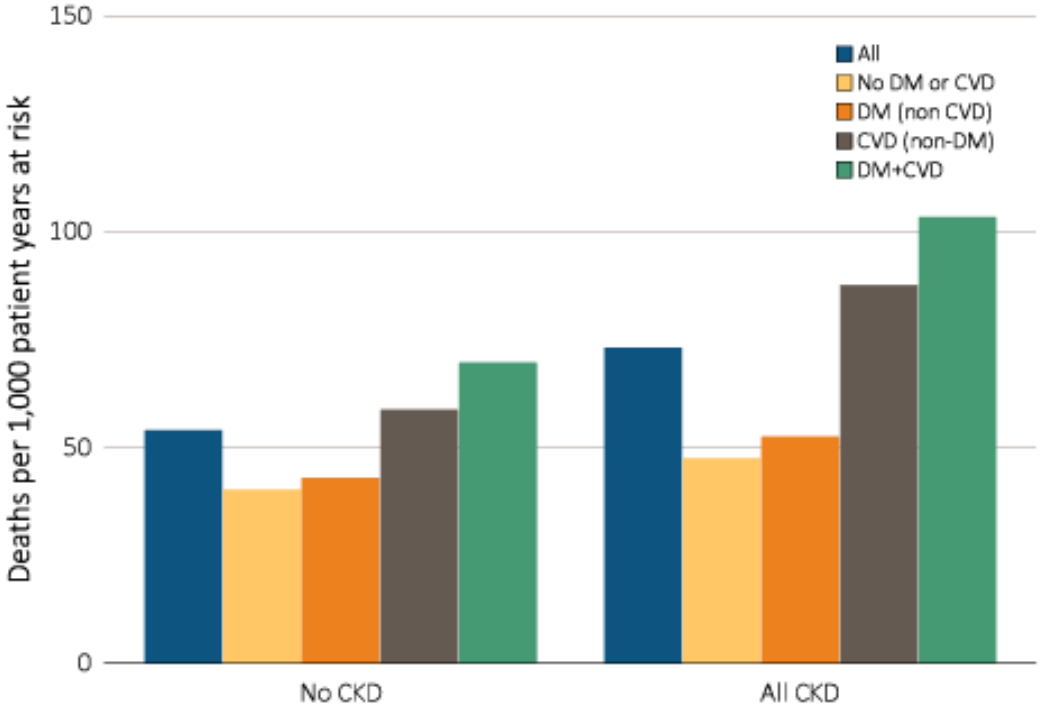

Figure 2: Adjusted mortality rates in US population aged over 65 years by cardiovascular disease (CVD), diabetes mellitus (DM), , and chronic kidney disease (CKD) in 2012. Data are adjusted for age, sex, race, prior year hospitalization, and comorbidities. (adopted from the USRDS, 2014 Annual Data Report) (7)

1.3.2 Detection and Assessment

CKD is defined as decreased kidney function (GFR < 60 ml/min/1.73 m² body-surface) or abnormalities of renal structure with implications on health for a minimum of three months (8). Markers of kidney impairment include urine sediment abnormalities, albuminuria, abnormalities due to tubular disorders, and/or structural abnormalities spotted by histology or imaging (8). Even though healthy individuals excrete a small amount of proteins, constant proteinuria is often the earliest indication of renal damage. Thus the most common screening test for kidney damage is the measurement of total urinary protein or only of urinary albumin (8). Independent of the renal function, proteinuria plays an important role in the pathogenesis of CKD progression and can be correlated with mortality and kidney outcome (48).

For the evaluation of total kidney function, the GFR is considered as the best overall measure (49). GFR is defined as the amount of primary urine filtered by both kidneys within a specific period and is specified as milliliter per minute per 1.73 m² body-surface. To

determine the GFR a 24-h hour urine isotope clearance is necessary (49), which is quite expensive in labor and costs. Therefore, in clinical practice the determination of creatinine clearance is used to approximately estimate the GFR. However, assessment of renal function via creatinine does not consider further physiological parameters influencing urinary protein concentration, such as muscle mass or increased meat intake. The Cockcroft-Gault equation, the Modification of Diet in Renal Disease (MDRD) study equation, and the CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) formula estimate GFR by also considering more parameters like body size, age, sex, and race (50-52). Application of those equations therefore minimizes the influence of other physiological processes on the assessment of overall kidney function. However, the MDRD study equation underestimates the GFR in individuals with a GFR > 60 ml/min/1.73 m² body-surface. The recently developed CKD-EPI formula is more accurate at high GFR and predicts adverse outcomes more exactly (53, 54).

Measurement of Cystatin C has a higher diagnostic sensitivity to estimate GFR in comparison to creatinine, since it is less affected by muscle mass or race (55). Especially higher GFRs can be estimated more precisely than by the assessment of serum creatinine. Furthermore, Cystatin C is more predictive of cardiovascular complications and mortality (56).

1.3.3 Classification

Based on the K/DOQI guidelines (Kidney Disease Outcomes Quality Initiative) of the National Kidney foundation (NKF), CKD patients are grouped within five CKD stages, based on their GFR (8). Stage 1 comprises patients with kidney damage but normal or increased GFR (≥ 90 ml/min/1.73 m² body-surface). Patients with mildly decreased GFR (60 – 89 ml/min/1.73 m² body-surface), moderately decreased GFR (30 – 59 ml/min/1.73 m² body-surface), or severely decreased GFR (15 – 29 ml/min/1.73 m² body-surface) are grouped within CKD stages 2, 3, or 4, respectively. In CKD stage 5 kidney failure is apparent by either a GFR < 15 ml/min/1.73 m² body-surface or the need of kidney replacement therapy. Due to differences in risks of mortality and other severe outcomes, CKD stage 3 is subdivided into stage 3a, comprising patients with mildly to moderately decreased GFR (45 – 59 ml/min/1.73 m² body-surface), and stage 3b, including patients with moderately to severely decreased GFR (30 – 44 ml/min/1.73 m² body-surface).

In most cases, the early stages of CKD are asymptomatic, can be reversible, and they are often chance findings in the assessment of comorbid afflictions.

In recent years, several studies have shown that both lower GFR and increased levels of albuminuria are independently associated with higher mortality, cardiovascular events, and the risk to progress to ESRD. At the same time, levels of albuminuria are predictive of disease progression and outcome at all CKD stages (57-61).

The combined examination of GFR and persistent albuminuria can help to estimate the patients' risk of CKD progression and can give a lead to the frequency of disease monitoring (Fig 4).

GFR categories (ml/min/1.73 m²) Description and range				Persistent albuminuria categories Description and range		
				A1	A2	A3
				Normal to mildly increased	Moderately increased	Severely increased
				<30 mg/g <3 mg/mmol	30–300 mg/g 3–30 mg/mmol	>300 mg/g >30mg/mmol
	G1	Normal or high	≥90	1 if CKD	1	2
	G2	Mildly decreased	60–89	1 if CKD	1	2
	G3a	Mildly to moderately decreased	45–59	1	2	3
	G3b	Moderately to severely decreased	30–44	2	3	3
	G4	Severely decreased	15–29	3	3	4+
	G5	Kidney failure	<15	4+	4+	4+

Figure 3: Prognosis of CKD and guide to monitoring frequency by GFR and Albuminuria Categories. Colors reflect the risk of disease progression: Green: low risk (if there are no other evident indicators of kidney disease, CKD is not proven); Yellow: moderately increased risk, Orange: high risk, Red: very high risk, Dark Red: ESRD. Numbers in the boxes give the recommended frequency of monitoring in number of times per year. (adopted form the KDIGO Clinical Practice Guideline for the Evaluation and Management of Chronic Kidney Disease, 2012) (8)

1.3.4 Course of CKD

Normal GFR values in healthy and young adults are about 120 to 130 ml/min/1.73 m² body-surface and continuously decline with aging (62-64). Though a decrease of GFR belongs to normal aging, impeded renal function also adds to the overall mortality in older people (65-67). Furthermore, the medication dosage has to be adjusted in elderly persons according to kidney function similar to other patients with CKD. As a consequence, the CKD definition also applies to older patients, resulting in an increased prevalence of CKD in the elderly.

Besides the natural decline in renal function with aging, the course of CKD can be progressive, stable, or even improve between disease stages (Fig. 5). In general, CKD is progressive or stable, whereas disease remission is rare (68-70). The decline in renal function is thereby further triggered by the increasing overload of the remaining nephrons, even though the underlying renal disease is properly treated (55). Thus, the adaptive hyperfiltration of the remaining nephrons results in intraglomerular hypertension and hypertrophy adding to renal glomerulosclerosis. Further factors of CKD progression include glomerular deposits of lipids and calcium phosphate as well as renal damages caused by increased blood glucoses or acidosis (3).

Specific therapy options for renal dysfunction are scarce, but CKD progression can be impeded by the early diagnosis and prevention of cardiovascular risk factors, such as increased blood glucose or hypertension (44). At the moment, treatment of hypertension is the most efficient therapy to slow CKD progression (44). In pre-dialysis patients, statin treatment can minimize the risk of all-cause as well as cardiovascular mortality and also reduces other non-fatal cardiovascular events (71), whereas in patients undergoing kidney replacement therapy, no reduction of cardiovascular death or other events could be proven (72-74). Blockade of the renin-angiotensin-system has been proven to especially recover proteinuric CKD (47). Although for all of those therapies an improvement in the patients' outcome has been confirmed, there are indications that they are underused in CKD patients, resulting in an increased risk for complications (75-77).

The most serious outcome of CKD is total kidney failure, also defined as ESRD. Symptoms of ESRD are normally caused by complications of reduced renal function, mostly resulting in the need of kidney replacement therapy by either dialysis or kidney transplantation. ESRD is thereby defined as a GFR of less than 15 ml/min/1.73 m² body-surface or the necessity of renal replacement therapy. Patients suffering from ESRD are grouped within CKD stage 5.

In conclusion, CKD remains often stable or progresses to ESRD. The early identification and treatment of complications can help to delay CKD progression. Though risk

of complications increases with higher CKD stages, complications can occur at any CKD stage and include the increased risk of cardiovascular disease, acute kidney injury (AKI), infections, malnutrition, anemia, neuropathy, cognitive deficiency, and impairment of physical function (43, 45, 46, 59-61).

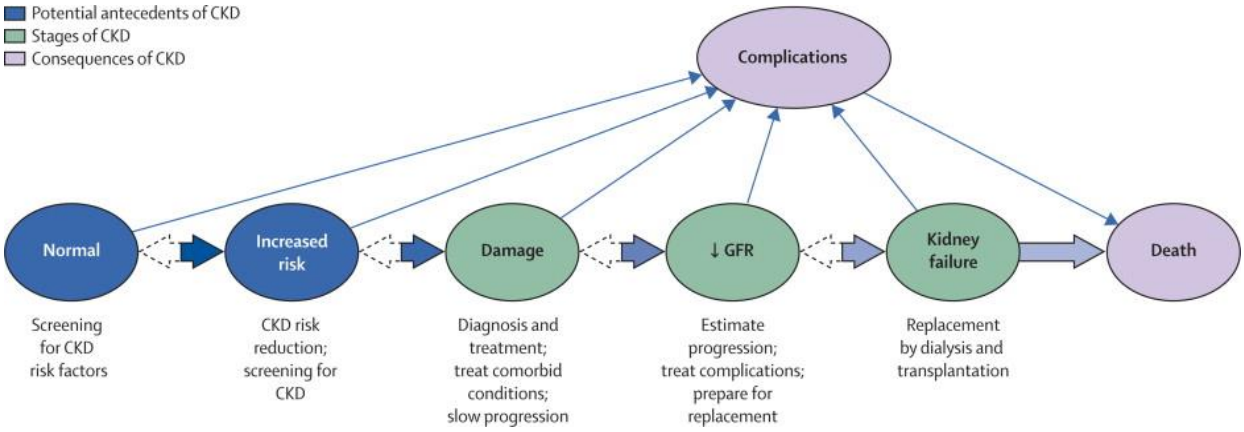

Figure 4: Conceptual model of CKD course and therapeutic strategies. Thick arrows between the ellipses represent progression (painted arrows) and remission (dotted arrows) of CKD. Complications comprise all complications of CKD and its treatment, including cardiovascular disease and complications due to decreased GFR. In later CKD stages, the risk of complications increases with the disease progression. (adopted from Levey and Coresh, 2012) (55)

1.3.5 End stage renal disease and dialysis treatment

The ultimate therapy of CKD is the replacement of renal function by dialysis. The dialysis should be initiated in patients with a GFR < 15 ml/min/1.73m^2 body-surface who additionally have signs of uremia, a progressive aggravation of their nutrition status, and/or who are no longer able to control their hydration status or blood pressure (78). Without clinical symptoms of renal failure, the kidney replacement therapy should be started before GFR has decreased to 6 ml/min/1.73m^2 body-surface (78). Consequently, the recommended cut-off point in patients without uremia or other symptoms of ESRD is a GFR between 8 and 10 ml/min/1.73m^2 body-surface (78).

Although mortality rates among ESRD patients have continuously declined over the last decades, they are still higher than in the general population or in patients with cancer, DM or cardiovascular diseases (13). Furthermore, the average yearly death rates among patients undergoing dialysis are higher than in patients after kidney transplantation (13).

Interestingly, the mortality in patients receiving hemodialysis is highest in the first year, it decreases significantly during the second year and slowly increases within five years after starting the dialysis (13). In contrast, average yearly death rates in patients with peritoneal dialysis continuously increases within five years after initiating the dialysis (13). Three years after the onset of ESRD, adjusted survival rates are only about 55 % for patients with hemodialysis and about 65 % for patients with peritoneal dialysis (13).

Besides cardiac arrest and myocardial infarction, further major causes of death in ESRD patients include sepsis and other infections (13). In patients needing dialysis treatment, the mortality secondary to sepsis is about 100 to 300 percent higher as compared to the general population and remains about 100 fold higher after stratification for age, race, and gender (79).

However, the increased risk for bacterial and viral infections in this patient collective can be attributed to the continuous disturbance of cell-mediated immunity, chronic inflammation, and high cell death rates accompanying ESRD and hemodialysis treatment (80-83). Uremia per se is associated with several metabolic disorders, leading to an imbalance between stimulation and suppression of immunological processes and resulting in an impaired function of granulocytes, lymphocytes, and monocytes (80, 84-87). Chronic inflammation in uremic patients is further related to increased mortality, a higher risk of developing a cardiovascular disease, malnutrition, and anemia (88). Tumor necrosis factor alpha (TNFα) is a pro-inflammatory cytokine and mediates among others the programmed cell death of various cells. Patients with ESRD show increased circulating TNFα levels (89), which in turn keep up a continuous inflammatory state. For patients undergoing dialysis, a positive association between TNFα serum levels and infection rates could be proven (90).

Our study group already showed the irregular state of T cell activation and elevated T cell death in ESRD patients with and without dialysis treatment (80, 91). T cells in those patient collectives show an increased Fas-receptor (CD95) expression on their surface and an augmented release of soluble CD95 and of soluble Tumor necrosis factor 1 (TNF1), the receptor of TNFα (80). Comparable to TNF1, the activation of the Fas-receptor pathway eventually results in programmed cell death. A subset of CD4+ Th1 helper cells is especially prone to CD95 induced apoptosis, whereas CD4+ Th2 helper cells appear insensible against this stimulus (92, 93). In patients with autoimmune diseases, increased T cell apoptosis was found together with diminished Th1 cytokines levels (IL-12, IL-12, and Interferon-c) but elevated concentrations of Th2 cytokines (IL-4 and IL-10) (94). In ESRD patients, our study group found elevated serum levels of the Th2 cytokine IL-10 and increased apoptosis rates of CD4+ T cells (80, 91). As IL-10 additionally inhibits Th1 cells and CD95 is increasingly

expressed on T cells in ESRD patients with and without dialysis, the findings of our group add to the pathophysiological concept of Th2 predominance in ESRD (91).

As the perturbation of the immune system and the continuous inflammation remain challenges during treatment planning, further research will be necessary to improve the patients' outcome and lower down the extreme morbidity in patients undergoing dialysis.

1.3.6 Biomarkers for CKD

The early diagnosis of CKD can help to initiate an appropriate treatment on time to prevent adverse outcomes (6). In this respect, biomarkers can simplify patient recognition in putative healthy individuals and would allow risk stratification in asymptomatic persons identified as having renal disease (49). However, the biomarker development for CKD is rather complex because the exact determination of the disease's onset is difficult. Most patients are asymptomatic over a long period and screening for different chronic renal diseases remains rather extensive. In contrast to AKI, which is mostly triggered by a defined event, the initiation and progression of CKD are silent and extend over an undefined time interval. These are all reasons why the exact timing and nature for CKD detection still remain difficult to identify (49).

The ideal biomarker for CKD should be inexpensive, easily accessible, and sensitive for early detection of renal malfunction (49). Furthermore, it should determine the site of the kidney damage, provide insight into the disease mechanism, depict the response to treatment planning, and determine the risk of complication from comorbid conditions like cardiovascular disease (49). Due to the complexity of CKD and the mutual appearance with other conditions, the identification of one biomarker possessing all of the ideal characteristics is very unlikely (49). Hence, the combined examination of multiple biomarkers in a panel might help to approximate the ideal test and increase test specificity. Profound knowledge about cellular mechanisms and pathophysiologic processes in disease progression are therefore necessary to identify and characterize potential biomarkers.

Currently, the best overall measure to estimate renal function is the 'real' GFR tracked by a 24 hour urine isotope clearance (49). In fact, this is quite extensive, leading to the estimation of GFR via serum creatinine clearance in clinical routine (49). As noted previously, the accuracy to estimate GFR via creatinine is dependent on several patient-dependent and –independent variables. In addition, serum creatinine can be diminished in later CKD stages, independent from renal creatinine clearance, resulting in deficient GFR measurements (95). Actually, serial creatinine measurement within 24 hours fails to estimate risk progression in

about 20 % of patients with CKD (96). Also a 24 hours creatinine clearance cannot quote an authentic prognosis of CKD progression (49).

As urine is easily accessible, proteinuria is currently used as the first diagnostic marker for renal damage in adults. However, it has some limitations for the initial detection of kidney disease. The excretion of proteins may arise long after renal damage and is occasionally absent in some kidney diseases, leading to false negative results and consequently to a delayed detection and treatment of CKD (95).

As the number of CKD patients continuously increases, it is of great importance to early identify persons at risk. Therefore, the identification of further biomarkers for the early detection of CKD should be in the main focus of research to improve the patients' outcome by the initiation of an adequate treatment early enough. Current research has spotted some more promising candidates, such as neutrophil gelatinase-associated lipocalin, cystatin C, asymmetric dimethylarginine, and liver-type fatty acid binding protein (97-100). However, larger longitudinal studies are necessary to estimate the potential of these proteins as biomarkers for CKD and more research in biomarker identification is necessary.

1.4 Chemokines

1.4.1 General Information

Chemokines are a large group of chemotactic cytokines, responsible for cell communication between different body cells, also including leukocytes (101). Their main functions are the directed recruitment and positioning of leukocytes at sites of inflammation via chemoattraction (102). To this end chemokines act jointly with further mediators, adhesion molecules and their receptors (103). To induce leukocyte migration, chemokines are presented on endothelial cells to intravasal rolling leukocytes, facilitating their extravasation, and guide the activated inflammatory cells to the site of tissue damage (102). As inflammatory cells express a defined pattern of chemokine receptors on their surface, the distinct attraction of needed leukocytes can be induced by accurate chemokine expression at the site of injury (104). Besides directional leukocyte trafficking, chemokines are further involved in angiogenesis, fibrogenesis, as well as in the proliferation and polarization of hematopoetic progenitor cells (105, 106).

Chemokines can be grouped according to the molecular structure of a conserved four-cysteine motif, which is present near the N-terminus, into four groups (CC, CXC, CX3C,

and XCL2) (107). Most chemokines act as ligands for multiple chemokine receptors of the same group. On the other hand, some chemokines are antagonists for chemokine receptors of other groups (102). Chemokine receptors themselves consist of seven transmembrane-spanning proteins that are linked with heterotrimeric G proteins and initiate several intracellular pathways when activated (108). After ligand binding, receptors are quickly internalized to stop cell movement and facilitate response to other paracrine factors (108). Chemokine receptors are grouped according to their ligands into four groups (CCR, CXCR, CX3CR1, and XCR1).

Chemokines can be further characterized as homeostatic or inflammatory chemokines. Members of the former group, which are also named lymphoid chemokines, are expressed at basal rate by a specialized subset of chemokine producing cells via specific stimuli but have also shown to additionally contribute to inflammatory processes (104, 109-111). Lymphoid chemokines organize the microarchitecture of secondary lymphoid organs at basal rate, regulate leukocyte homing, and facilitate the processes of cross talk between leukocytes (104, 112, 113). In contrast to lymphoid chemokines, inflammatory chemokines are rapidly upregulated and released only if required, to ensure prompt leukocyte trafficking to the site of injury. The pattern of chemokine expression varies between organs, resulting in a defined composition of inflammatory cells at different body sites (104). Some inflammatory chemokines bind to multiple receptors and, vice versa, most receptors bind more than one ligand usually with variable affinity. Furthermore, functionally different cell types can express the same chemokine receptors but still differ in the overall pattern of receptors (104). This complex multi-level interaction of chemokines and chemokine receptors is thought to be essential for the exact adjustment of immune response during inflammation (104).

1.4.2 Renal Inflammation

Inflammation describes the protective reaction of the body to dangerous stimuli, such as pathogens, damaged cells, or irritants (2). It is a process sustained by different immune cells, various molecular mediators, and blood vessels. Inflammation is initiated to remove the cause of primary cell injury and thus, of damaged cells and to induce subsequent tissue repair. Inflammatory reactions can be classified as acute or chronic dependent on the length of occurrence. In contrast to acute inflammation, which is self-limiting after tissue repair, chronic inflammation is characterized by a shift of invading immune cells and progressive tissue destruction, resulting in fibrosis (2). In chronic diseases, prolonged pathogenic

processes can maintain ongoing inflammation, resulting in fibrotic changes and dysfunction of the affected organ.

Also in CKD, continuous interstitial inflammation contributes to the persistent loss of renal function and further aggravates the disease progression in a vicious cycle (114). It is for this reason that the activation of different pro-inflammatory cellular pathways results in the upregulation of various cytokines and chemokines, in the increased expression of adhesion molecules, and in the elevated infiltration of inflammatory cells into renal tissue. Recruited inflammatory cells in turn trigger the response by cytokine liberation (102). Moreover, the release of reactive oxidative species (ROS) and local angiontensin II activity further aggravate renal fibrosis and myofibroblast formation by the disruption of the continuity between glomeruli and tubules, the development of pathogenic hypoxia, and the detraction of the renal autoregulation of glomerular blood flow (114, 115). Renal scarring finally occurs as a result of the mesenchymal differentiation of tubular epithelial cells and the generation of fibrosis by myofibroblast transformation of vascular pericytes (116, 117). Discontinuity of the glomerular filtration barrier results in proteinuria, which further aggravates renal damage, as proximal tubular cells produce pro-inflammatory and pro-fibrotic factors in response to increased protein exposure (114).

1.4.3 Renal chemokine expression

The chemokine expression in healthy kidneys remains low, but it is significantly upregulated during pathogenic conditions like inflammation, toxin exposure, or hypoxia (118).The main mediators triggering inflammatory chemokine expression are ROS and pro-inflammatory cytokines, such as Interleukin (IL) 1 beta and TNFα (118, 119). Furthermore, chemokine upregulation in the kidney can be induced under diabetic conditions or by further mediators including cyclic adenosine monophosphate, interferon gamma, low density lipoprotein, immunoglobulin complexes, vasoactive substances like angiotensin II, growth factors like platelet-derived growth factor or basic fibroblast growth factor, and pathogen associated molecules like lipopolysaccharides (119-121). During the development of CKD, elevated local chemokine concentration in turn results in the increased migration of T cells, macrophages, and dendritic cells into the kidneys (122).

In the acute phase of some renal diseases CXCL chemokines are upregulated and can be detected in the urine of patients (121, 123). Also in histologic samples of patients with glomerulonephritis, crescentic glomerulonephritis, and lupus nephritis, elevated numbers of CXCR1-positive neutrophils have been discovered in the glomeruli as well as in the

tubulointerstitium (123, 124). In the conversion of AKI into CKD, a change in chemokine expression from CXCL chemokines to CCL chemokines has been described resulting in a different pattern of renal leukocyte accumulation (Fig. 6) (125). Especially the involvement of various CCL and CX3CL chemokines has been described for different chronic kidney diseases during the progression but also during the remission of CKD (121). Urinary chemokine excretion can be positively correlated with the degree of inflammatory cell infiltration into the glomerulum and the tubulointerstitium. In doing so, urinary chemokine levels seem to reflect the chemokine production in the kidneys (126-129). However, the involvement of chemokines in chronic renal diseases is substantial and ongoing research still reveals new insights into immunologic processes and leukocyte trafficking via chemoattraction.

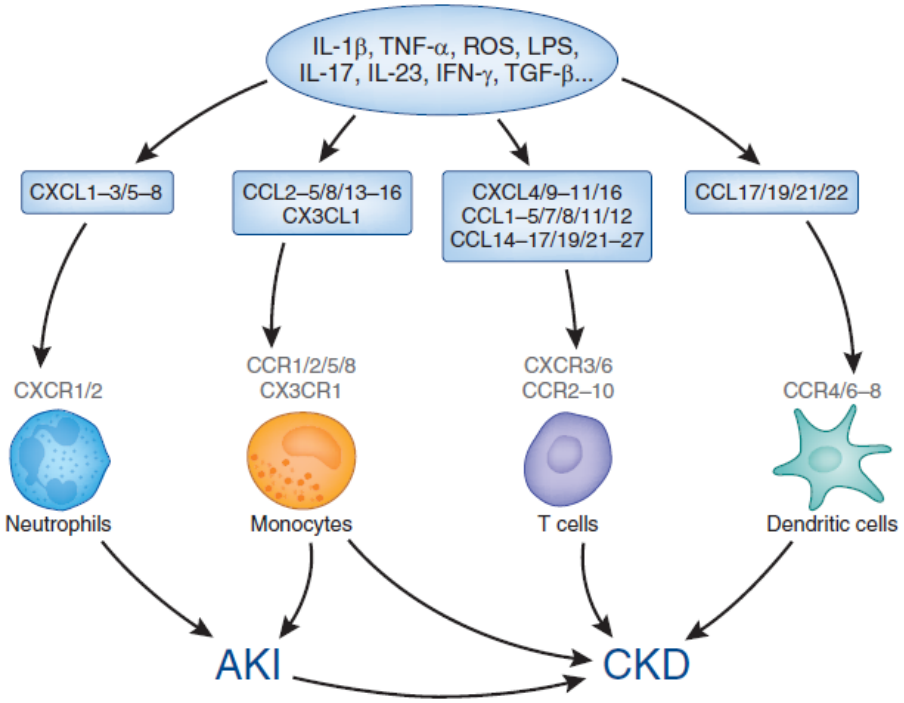

Figure 5: Illustration of chemokines and chemokine receptors and their impact on leukocyte attraction in acute kidney injury (AKI) and chronic kidney disease (CKD). As neutrophils and monocytes are especially involved in AKI, activated macrophages, T cells, and dendritic cells hold important functions during CKD progression in particular. (adopted from Chung and Lan, 2011) (121)

1.5 Trefoil Factor Family Peptides

1.5.1 General Information

Trefoil factor family (TFF) peptides are a small group of proteins that are involved in tissue repairing, especially of mucous-containing surfaces (130). They are secretory products of different mucous-producing cells and hold important functions during restitution and regeneration of mucous epithelia by the initiation of cell migration, angiogenesis, and the increase of cell resistance against pro-apoptotic stimuli (130, 131). Restitution describes the process in which mucosal coherence is restored via distension and migration of formerly anchored epithelial cells to repair uncovered areas of damage (Fig. 6). Consequently, restitution is of notable importance in tissues with frequent injuries of the mucous membrane such as in the digestive tract. Mainly there, damaged epithelium has to be covered within a short time, due to the high risk of inflammation by direct contact of the immune cells of the lamina propria with intestinal bacteria (132). Accordingly, the highest expression of TFF peptides can be found in the gastrointestinal tract (130).

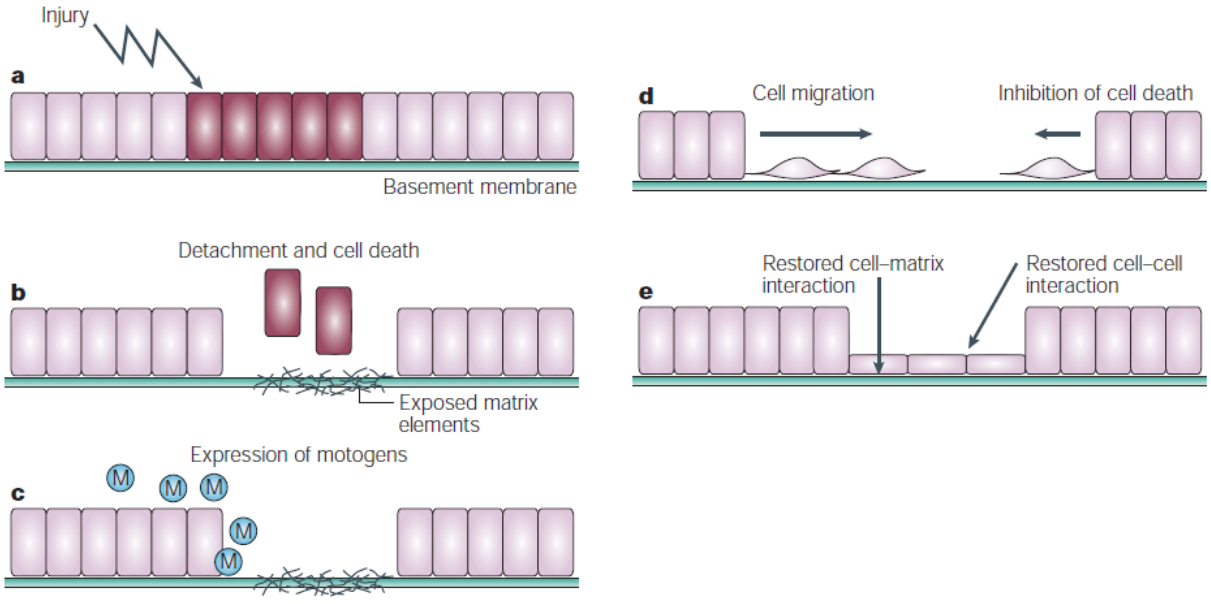

Figure 6: Schematic illustration of epithelial restitution. An injury (a) to mucous epithelium can result in cell detachment and cell death (b). Messenger substances speeding up repair mechanisms (motogens, M) are increasingly expressed and released from adjacent cells (c), resulting in cell migration and inhibition of detachment-induced cell death (d). Restitution finally is completed by restored cell-matrix and cell-cell interaction (e). (adopted from Taupin and Podolsky) (131)

TFF peptides are characterized by their distinctive three-leaved shaped pattern of disulphide bonds, the so-called trefoil domain (133). Three members are currently known: TFF1, TFF2, and TFF3. TFF2 was the first to be discovered and was formerly named pancreatic spasmolytic polypeptide as it showed inhibitory effects on gastric acid secretion and motility (134-136). TFF2 consists of 106 amino acids and has a molecular weight of approximately 12 kDa (137, 138). It holds two trefoil domains presumably originated by genomic duplication (135, 139). At first, TFF1 was detected in a breast cancer cell line and therefore initially named human breast cancer associated peptide 2 (140, 141). TFF1 contains 60 amino acids as well as one trefoil domain, it has a molecular weight of about 6.5 kDa, and can also be found as dimer with a molecular weight of approximately 14 kDa (133, 142). The last member of the trefoil factor family is TFF3, which was discovered in intestinal epithelial cells at first and was therefore initially named intestinal trefoil factor (143). It consists of 59 amino acids and one trefoil domain. The monomer has a molecular weight of about 6.6 kDa, the dimer of approximately 13 kDa (144, 145).

TFF peptides have been proven to be expressed in all tissues containing mucous-producing cells, but their highest expression rate is located in the gastrointestinal tract, where they can be found in a site specific distribution (130). In gastric tumor, the expression rate of TFF peptides can be diminished in about 50 % of the patients. However, aberrant expression rates have been further described for various intestinal cancers (146-150) as well as for cancers of the mammary gland (151, 152), the prostate (153), the pancreas (154), the liver, and gallbladder (155). Those findings especially intensified research effort in biomarker development for cancer focused on TFF peptides.

In chronic diseases, progressional inflammation is triggered by the constant exposure to pro-inflammatory stimuli, eventually resulting in fibrosis and organ dysfunction. To avert such outcomes, the affected cells initiate counterreactions to limit ongoing tissue damage and prevent further cell loss. Consequently, TFF expression is upregulated in mucous epithelial as one of many countermeasures to pro-inflammatory stimuli to stabilize and protect epithelial integrity in chronic diseases also including CKD (130).

1.5.2 Renal TFF peptide expression

In patients with carcinoma of the urinary tract, TFF peptide expression could be demonstrated in the kidney as well as in the urinary tract at basal rate, with TFF3 as the most abundant followed by TFF1 (156). In contrast, TFF2 expression was detected only in trace amounts in human tissue samples of the bladder (156). Examined samples were taken from

non-pathological regions without histologic traces of neoplastic changes or marks of inflammation and therefore might reflect basal TFF expression rates in the healthy human urinary tract. However, the influence of the cancer disease on TFF peptide expression cannot be finally excluded within this study, since invasive biopsy studies in healthy humans are difficult to conduct due to ethical reasons. In contrast to those findings, the same study revealed traceable concentrations of TFF2 but not of TFF1 or TFF3 in the urine of healthy individuals (156). As TFF3 was shown to be the most abundant in the urinary tract whereas TFF2 expression could not be verified in most histologic samples, these results are rather surprising although in accordance with current literature, describing higher urinary levels of TFF2 than of TFF3 (157).

However, TFF peptide expression differs from healthy individuals during pathogenic processes in order to overcome tissue destruction and limit cell death. To quote an example, AKI is highly associated with elevated levels of urinary TFF3. Therefore, urinary TFF3 concentration has already been accepted as biomarker by the US Food and Drug Administration for testing the acute renal toxicity of drugs in animal models (158). Also in patients with CKD, increased levels of TFF peptides have been detected in the patients' blood and urine (159, 160). Those elevated levels seem to reflect the organism's countermeasures to the high oxidative stress and the ongoing inflammation that accompanies CKD. However, it still remains unclear if the elevated serum and urine levels derive from the affected kidneys themselves or from other body cells that are reacting to the increased systemic stress, as substances that are normally excreted with urine accumulate in the body during CKD progression. Elevated urinary TFF peptide concentrations, in turn, might derive from increased serum levels and the accretive discontinuity of the glomerular filtration barrier that accompanies CKD.

As aberrant TFF peptide expression can be considered as direct counterreaction to pathogenic stimuli, ongoing research on TFF peptide regulation can help to understand cellular defense mechanisms and can give insights into repairing processes of mucous-containing tissues in particular.

1.6 Aims of the thesis

Patients with chronic renal impairment are currently detected late during disease progression, often only shortly before the onset of systemic comorbidities (6). Consequently, the early identification of persons at risk can help to delay disease progression to CKD due to the chance to start an adequate therapy on time. The consequent treatment of renal failure and occurring comorbidities can thereby prevent further loss of kidney function and can even delay or avert the ultimate need of kidney replacement therapy by kidney transplantation or dialysis (44, 47, 71). Therefore, the identification and evaluation of potential new biomarkers for the early detection of CKD and for the assessment of therapy response are of great importance and could further help to develop an individually adjusted treatment planning (49). As urine and serum are easily accessible and can be obtained non-invasively with no appreciable side-effects on the patients' wellbeing, systemically released molecules or excreted peptides are of special interest to serve as potential markers. In clinical routine, the measurement of serum creatinine is used to estimate overall kidney function (49, 95). Creatinine is a metabolite of muscle activity and is excreted via the urine in healthy individuals. With restricted kidney function, creatinine accumulates among other substances in the blood, allowing insights into renal health. However, in contrast to creatinine, which is systemically released as normal side product of muscle metabolism, the proteins that are upregulated either directly in the kidney and/or as a reaction to increased uremic stress are of special interest to serve as biomarkers for CKD due to their direct link to renal diseases.

As chronic renal afflictions are associated with inflammation and high systemic stress, the involvement of pro- and anti-inflammatory processes as well as cellular repairing mechanisms has been proven for various chronic kidney diseases, already discussed in previous chapters. With the progress of renal failure and systemic accumulation of usually excreted substances, the immune system gets more and more affected, which is obvious in the increased leukocyte aberration and the augmented cell death, which has already been investigated by our study group (80, 83, 91).

The aims of this thesis were to examine pro-inflammatory peptides and proteins involved in tissue repairing as possible biomarkers for CKD. Therefore, levels of specifically selected chemokines as well as TFF peptide concentrations were measured in blood and urine of patients suffering from CKD stages 1 – 5 and correlated with conventional kidney parameters. Furthermore, fractional excretion rates were calculated for every investigated protein to detect changes in renal excretion levels irrespective of the remaining glomerular filtration rate to gain insight into protein expression sites. Moreover, receiver operating characteristic (ROC) curves were calculated to examine the potential of all investigated

proteins to serve as biomarkers for the early detection of CKD and/or to monitor the course of chronic renal failure.

2. CHAPTER TWO: Results

2.1 Increased chemokine excretion in patients suffering from chronic kidney disease

To limit tissue damage after injury, inflammation is initiated to eliminate noxious agents and restore organ function. Also during the initiation and amplification phase of renal inflammation, leukocytes migrate into the interstitial space and the renal mesangium attracted by increasing local chemokine concentrations that are generated by stressed renal cells or already infiltrated leukocytes in response to various injuries (161-163). If the noxious agent cannot be eliminated, the inflammation usually becomes chronic resulting in kidney fibrosis and organ dysfunction.

Most kidney diseases are associated with chronic inflammation causing the continuous loss of renal function and the final need of kidney replacement therapy. During the progression of renal diseases to end-stage renal failure (ESRF), dendritic cells, macrophages, and T-cells are especially involved in the pathogenesis of CKD (122). Those leukocytes express receptors, whose ligands are - amongst others - CXCL11, CCL22, CCL20, and CCL17. For those chemokines and their receptors, their involvement in the progression of renal failure was proven in various animal models for kidney diseases (164-168). However, data in humans are rare and analyses about chemokine excretion during CKD progression hardly exist.

During our study “Increased chemokine excretion in patients suffering from chronic kidney disease” we detected changes in serum and urine levels of CXCL11, CCL22, CCL20, and CCL17 in patients suffering from CKD stages 1 – 5 (169). Furthermore, significant changes in fractional chemokine excretion could be detected between different CKD stages, pointing to the kidneys as the primary production sites of increased chemokine levels. ROC curve analyses identified chemokine excretion levels to be suitable to monitor the course of CKD and to differentiate between stages. Moreover, trends in varying chemokine expression in different CKD etiologies have been detected in this study.

The paper was published in the “Translational Research” journal.

FEATURED NEW INVESTIGATOR

Increased chemokine excretion in patients suffering from chronic kidney disease

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During chronic kidney disease (CKD) leukocytes attracted by chemokines can migrate into the kidney and further aggravate renal affliction by releasing proinflammatory and profibrotic factors. We therefore sought to investigate serum and urine chemokine levels of 114 patients with CKD and 21 healthy volunteers to examine their possible suitability as biomarkers for monitoring disease course and patient's risk assessment. Analyzed chemokines were CCL17, CCL20, CCL22, and CXCL11, which are especially involved in the development of chronic renal failure. Our results showed elevated fractional CCL22 excretion levels in patients with CKD stages 2–5 compared with healthy controls. Furthermore, fractional CCL22 excretion was increased in patients with CKD stages 4 and 5 compared with stages 1–3. Fractional CCL20 excretion showed a significant elevation in patients with CKD stage 5 compared with healthy individuals and patients with CKD stages 1–3. Fractional CXCL11 excretion was significantly elevated in patients with CKD stages 4 and 5 compared with healthy controls and patients with CKD stages 1–3. Moreover, receiver operating characteristic curve analysis showed the potential of chemokine excretion to predict various CKD stages (area under the curve (AUC) 0.835, $P < 0.0001$ for CCL22, stage 1 and higher; AUC 0.6887, $P = 0.0007$ for CCL20, stage 3 and higher; AUC 0.7549, $P = 0.0003$ for CXCL11, stage 3 and higher). Our results further uncovered trends in varying chemokine serum and excretion levels in different CKD etiologies. In conclusion, monitoring fractional chemokine excretion might be suitable for following CKD course and hence promoting individually adjusted treatment planning. (Translational Research 2014;164:433–443)

Abbreviations: AUC = area under the curve; CKD = chronic kidney disease; CRP = C-reactive protein; eGFR = estimated glomerular filtration rate; ELISA = enzyme-linked immunosorbent assay; GN = glomerulonephritis; PKD = polycystic kidney disease; ROC = receiver operating characteristic; Treg = regulatory T cell

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Submitted for publication April 23, 2014; revision submitted June 23, 2014; accepted for publication July 14, 2014.

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1931-5244/$ - see front matter

http://dx.doi.org/10.1016/j.trsl.2014.07.004

AT A GLANCE COMMENTARY

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Background

Because renal afflictions are currently detected late during disease progression, it is imperative that every effort is made to early identify patients at risk to prevent further loss of kidney function. Blood and urine are easy accessible and can be taken noninvasively. Therefore, new biomarkers detectable in blood and urine offer potential to monitor the course of renal diseases and the response to treatment, which could further promote the individual adjusted treatment planning.

Translational Significance

We identified urine and serum chemokines as potential biomarkers for the identification of patients at risk and to monitor the course of renal diseases.

INTRODUCTION

Chronic kidney disease (CKD) is associated with high morbidity and mortality rates because of an increased risk of cardiovascular disease and the development of other complications.[1-4] CKD is defined by an estimated glomerular filtration rate (eGFR) of <60 mL per minute per 1.73 m^2 body surface for at least 3 months.[2]

Many kidney diseases are associated with inflammation leading to a continuous decline of renal function. Because only a few renal afflictions are ameliorated after an acute phase, most nephropathies finally lead to end-stage renal failure with progressive tubulointerstitial injury and renal fibrosis.[5-7] During the disease progression leukocytes migrate into the renal mesangium or the interstitial space. Those leukocytes are attracted by chemokines, which are released by intrinsic renal cells in response to immunologic, toxic, ischemic, or mechanical injury during the initiation and amplification phase of renal inflammation.[8] Proteinuria and glomerular secreted chemokines further amplify the chemokine expression in tubular epithelial cells.[8] In addition, the attracted leukocytes release proinflammatory and profibrotic factors contributing to the renal damage. As a consequence, intrinsic renal cells secrete matrix components and chemokines.[9,10] The infiltrating leukocytes also secrete chemokines, which can attract even more immunocompetent cells, leading to a vicious circle.[11,12] These aforementioned processes lead to fibrotic changes in the kidney and to tubular atrophy, finally resulting in a continuous loss of renal function and cumulating in end-stage renal disease.[8]

Chemokines can be divided into 4 subfamilies classified by the spacing of their first 2 cysteine residues: CCL, CXCL, CX3CL, and CL.[13] They bind to G protein–coupled receptors, acting and attracting leukocytes toward the increasing concentration of the correspondent chemokine.

Acute kidney damage is primarily associated with the accumulation of neutrophils and monocytes,[14] whereas during the progression of renal failure to CKD, T cells, macrophages, and dendritic cells contribute to the pathogenesis.[15] T helper type 2 cells, regulatory T cells (Treg), and dendritic cells are attracted, among others, by CCL22 and CCL17 via binding to the CCR4 receptor,[13] whereas Tregs, some dendritic cells, and Th17 cells are known to express CCR6, whose ligand is the chemokine CCL20,[13] Th1 and Th17 cells, natural killer cells, as well as epithelial and endothelial cells express the receptor CXCR3, which binds to CXCL11.[13]

These chemokines contribute to the development and the progression of chronic renal failure in animal models and humans.[15] A lot more chemokines have been shown to be involved in the pathophysiology of CKD, such as CCL3, CXCL8, and CXCL16.[13] Because sample quantity is limited and little is known about changes in urine and serum levels of CCL17, CCL20, CCL22, and CXCL11 during CKD progression, we restricted our study to those chemokines covering ligands for most immunocompetent cells involved in the pathophysiology of CKD. We therefore sought to investigate serum and urine concentrations of the chemokines CCL17, CCL20, CCL22, and CXCL11 in patients suffering from CKD stages 1–5 and compared our results with healthy individuals to determine a possible potential of these chemokines as a biomarker for CKD. Furthermore, we analyzed chemokine levels with respect to the underlying CKD etiologies.

METHODS

Patients. The study was approved by the institutional ethics committee and is in accordance with the Helsinki Declaration of 1975. A total of 114 patients suffering from CKD stages 1–5 without dialyses were included in this study. All patients were screened and followed up in the outpatient clinic of the Division of Nephrology and Dialysis, Department of Medicine III, Medical University of Vienna. CKD was defined as the presence of kidney damage or decreased glomerular filtration rate according to the Kidney Disease Outcomes Quality Initiative criteria.[2] Twenty-one healthy volunteers served as controls. The patients' diagnoses, baseline demographics, and laboratory values, including

absolute and relative lymphocyte counts and their significant decrease during the progression of disease are shown in Table I. In patients with unknown entity no renal biopsy was performed, because proven diagnosis would not lead to a change in treatment planning. One additional patient with CKD stage 2 was suffering from hereditary angiomyolipoma and is not listed within CKD entities in Table I.

In a subset of 33 patients a 24-hour urine specimen was collected.

Laboratory data. Blood and urine samples were obtained from each patient and control. In addition, 24-hour urine samples were collected by 33 patients themselves. A small aliquot was taken before being further processed in the same way as the rest of the urine samples. Immediately after collection, blood was allowed to clot for up to 60 minutes at room temperature (22°C). Next, blood and urine were centrifuged at 2000 relative centrifugal force for 10 minutes at 4°C. Serum and urine aliquots were collected, snap frozen, and stored at −80°C until further use. CCL17, CCL20, CCL22, and CXCL11 were determined using enzyme-linked immunosorbent assay (ELISA) kits (DuoSet IC, R&D Systems, Minneapolis, MN, http://www.rndsystems.com) according to the manufacturer's instructions. As a substrate, tetramethylbenzidine (Sigma, St. Louis, MO, http://www.sigmaaldrich.com) was used, and color reaction was stopped with sulfuric acid. Subsequently, the optical density was measured at a wavelength of 450 nm using a microplate reader.

As absolute urine levels vary with water reabsorption and excretion, the fractional chemokine excretion was calculated by the formula, ([urine chemokine × serum creatinine]/[serum chemokine × urine creatinine]) × 100, to get an accurate survey of chemokine clearance.

Little is known about the proteins' stability in urine. Therefore, validation testings have been performed to specify the proteins' stability in urine by freeze-thawing. Known concentrations of chemokines were added to a total of 45 urine samples of 15 patients with CKD stages 1, 3, and 5, of which half of those samples underwent a freeze-thaw cycle. Freeze-thawing caused a median decrease of 6% for CCL17 and CCL22, 7% for CCL20, and 4% for CXCL11. To ascertain the reproducibility of the used ELISA kits, samples of 8 patients have been tested multiple times. The intra-assay variability was <10% for CCL17, CCL20, and CXCL11 and 11% for CCL22. The variability between assays was 13% for CCL20 and <10% for CCL17, CCL22, and CXCL11. Lower detection limits were 3.8 pg/mL for CXCL11, 7.9 pg/mL for CCL17, 2.0 pg/mL for CCL20, and 6.5 pg/mL for CCL22. To further overcome methodological aberrations, serum and urine samples of all stages and controls have been evenly distributed over every used ELISA plate.

Statistical analysis. Chemokine serum and urine levels as well as fractional excretion were compared between CKD stages 1–5 and the control group using the nonparametric Mann-Whitney test. Under the Bonferroni adjustment for multiple comparisons, an individual $P < 0.01$ was necessary to achieve statistical significance at the 5% level. Furthermore, chemokine serum and urine concentrations as well as fractional excretion were compared between CKD etiologies and controls for all CKD stages and separately for stages >2 with the nonparametric Mann-Whitney test. An individual $P < 0.006$ was necessary to reach statistical significance at the 5% level under the Bonferroni adjustment.

Correlation between chemokine serum and urine levels and various clinical parameters were assessed in all patients and controls by Spearman's correlation coefficient. Unless otherwise stated data are given as median and range.

RESULTS

Chemokine serum and urine levels in CKD stages. Only CCL20 serum concentrations were significantly elevated in patients with CKD stage 4 and CCL17 serum levels in patients with CKD stage 5 compared with healthy controls ($P = 0.0022$ for CCL20, $P = 0.0038$ for CCL17) (Table II). Sera concentrations of CCL22 were elevated in stages 4 and 5 compared with controls but did not reach statistical significance under the Bonferroni adjustment for multiple comparison ($P = 0.029$, $P = 0.0159$). The same applied for CCL20 serum levels in CKD stage 3 compared with healthy controls ($P = 0.012$) and for CCL17 in stage 5 in comparison with stage 2 ($P = 0.0274$).

Absolute CCL22 urine values were significantly elevated in CKD stage 2 compared with controls ($P = 0.0024$), stage 3 ($P = 0.0007$), and stage 5 ($P = 0.0021$). In stage 4, absolute CCL22 urine concentrations were increased in comparison with CKD stage 3 ($P = 0.0058$) and stage 5 ($P = 0.0045$). CCL20 urine levels were significantly elevated in patients suffering from CKD stage 5 compared with healthy controls ($P = 0.0001$), CKD stage 2 ($P = 0.0005$), and stage 3 ($P = 0.0028$). For absolute CXCL11 urine concentration a significant increase could be detected in stage 5 compared with controls ($P = 0.0092$).

Serum and absolute urine chemokine values are depicted in Table II and in the Supplementary Figs 1 and 2.

Chemokine serum and urine levels in various CKD etiologies. The values of P for chemokine levels analyzed with respect to different CKD etiologies with more than 5 patients are depicted in Table III.

Table I. Underlying kidney disease, baseline demographic data, and laboratory variables

Demographic parameters, CKD etiologies, laboratory values	All patients	CKD 1	CKD 2	CKD 3	CKD 4	CKD 5	Controls
N	114	10 (8.8%)	20 (17.5%)	40 (35.1%)	25 (21.9%)	19 (16.7%)	21
Age (y)	59 (19–88)	36 (19–61)	50 (19–80)	63 (23–78)	59 (29–88)	65 (20–81)	32 (21–67)
Gender (male/female)	66/48	7/3	8/12	27/13	15/10	9/10	14/7
Kidney disease							
Glomerulonephritis	33	3	8	9	6	7	
Vascular nephropathy	19	2	—	9	7	1	
Diabetic nephropathy	11	1	—	7	3	—	
Polycystic kidney disease	8	2	—	2	2	2	
Interstitial nephropathy	7	—	4	1	1	1	
Urine stasis	6	—	1	1	2	2	
Nephrectomy	4	—	2	—	—	2	
Carcinoma	4	—	1	2	1	—	
Unknown	21	2	3	9	3	4	
Serum creatinine (mg/dL)	1.86 (0.72–6.88)	0.90 (0.72–1.03)	1.01 (0.77–1.52)	1.65 (1.02–2.34)	2.69 (2.04–3.89)	5.00 (3.47–6.88)	0.99 (0.77–1.20)
Blood urea nitrogen (mg/dL)	31.45 (7.1–91.2)	12.6 (7.5–17.6)	14.3 (7.1–26.2)	30.5 (11.6–64.1)	51.2 (23.8–91.2)	63.3 (31.9–87.3)	13.3 (8.2–20)
Urine creatinine (mg/dL)	69.2 (12.7–294.5)	69.1 (22.7–252.9)	74.9 (14.8–243.5)	79.4 (12.7–294.5)	60.9 (29.3–172.1)	41.65 (17.7–108.2)	152.8 (41.8–418.6)
Urine urea (mg/dL)	843 (247–2557)	948 (336–1814)	1044 (247–2557)	931 (291–2464)	854 (267–1370)	647 (273–1381)	
Urine protein (g/L)	0.47 (<0.05–6.94)	0.06 (<0.05–0.21)	0.25 (<0.05–4.45)	0.51 (<0.05–2.79)	0.29 (<0.05–3.46)	1.07 (0.05–6.94)	
CRP (mg/dL)	0.29 (0.03–7.84)	0.165 (0.05–4.31)	0.14 (0.05–1.59)	0.51 (0.03–3.87)	0.23 (0.04–3.53)	0.56 (0.05–7.84)	
Absolute lymphocyte count	1.7 (0.4–16.5)	2.2 (1.9–3.6)	1.8 (1.3–3.4)	1.6* (0.4–16.5)	1.7† (0.8–2.4)	1.5† (0.9–2.2)	
Relative lymphocyte count (%)	26 (1–53)	29 (25–38)	36 (6–53)	25 (1–39)	21† (15–32)	21* (12–46)	

Abbreviations: CKD, chronic kidney disease; *CRP,* C-reactive protein.
Data are expressed as median with range. Patients specified with carcinoma were suffering from urothelial or renal cell carcinoma and underwent chemotherapy.
*Indicates significant decrease compared with CKD stage 1 in lymphocyte count ($P < 0.01$).
†Indicates significant decrease compared with CKD stage 1 in lymphocyte count ($P < 0.001$).

Table II. Chemokine serum and urine concentrations

Chemokines	Healthy	CKD 1	CKD 2	CKD 3	CKD 4	CKD 5
CCL22 serum	434 (201–958)	626 (276–1186)	530 (29–1612)	556 (75–1897)	664 (205–1899)	843 (169–1546)
CCL22 urine	44 (15–75)	39 (25–150)	68* (24–545)	35 (17–123)	50 (24–232)	31 (15–116)
Fractional CCL22 excretion	0.082 (0.008–0.319)	0.11 (0.013–0.247)	0.148* (0.033–10.92)	0.194† (0.016–1.042)	0.378† (0.069–2.27)	0.505† (0.022–2.633)
CCL20 serum	10 (0–37)	13 (8–53)	13 (0–176)	17 (1–442)	22* (5–131)	18 (4–244)
CCL20 urine	2/16 (0–10)	4/7 (0–14)	3/14 (0–8)	3/28 (0–1111)	8/18 (0–751)	13/18† (0–249)
Fractional CCL20 excretion	0.167 (0–1.452)	0.19 (0–1.52)	0.18 (0–3.034)	0.258 (0–55.702)	0.769 (0–244.327)	13.116† (0–420.322)
CCL17 serum	226 (56–495)	281 (84–620)	189 (1–834)	288 (40–1148)	268 (84–1118)	359* (143–1200)
CCL17 urine	0/12 (0–6)	0/5 (0–6)	0/7 (0–262)	0/15 (0–12)	0/7 (0–19)	0/7 (0–47)
Fractional CCL17 excretion	0 (0–0.056)	0.002 (0–0.044)	0 (0–112.865)	0 (0–0.244)	0 (0–0.119)	0.088 (0–0.116)
CXCL11 serum	17 (4–43)	21 (6–115)	18 (10–78)	29 (5–197)	23 (6–120)	25 (12–736)
CXCL11 urine	7 (3–14)	6 (3–16)	7 (2–77)	6 (1–13)	7 (3–24)	4* (0–60)
Fractional CXCL11 excretion	0.38 (0.168–3.612)	0.406 (0.007–2.893)	0.391 (0.148–4.434)	0.357 (0.042–5.15)	1.367† (0.125–5.139)	1.302† (0–8.016)

Abbreviation: CKD, chronic kidney disease.
Data are expressed as median with range (picograms per milliliter). Numbers behind slashes give the number of patients with CCL20 and CCL17 urine concentrations >0.
*Indicates significant elevation compared with healthy controls ($P < 0.01$).
†Indicates significant elevation compared with healthy controls ($P < 0.001$).

Table IV lists the P values for chemokine levels of healthy controls compared with CKD entities.

Furthermore, in all CKD stages, CCL20 serum concentrations were elevated in patients with carcinoma in comparison with patients with polycystic kidney disease (PKD) ($P = 0.0283$), glomerulonephritis (GN) ($P = 0.0077$), and vascular nephropathy ($P = 0.0482$).

In CKD stages >2, patients with carcinoma had higher CCL20 serum levels than patients with GN ($P = 0.0172$). CXCL11 urine concentrations were also increased in patients with carcinoma compared with patients with PKD for all stages ($P = 0.0485$).

CCL22 urine levels were decreased in all stages of CKD after nephrectomy compared with GN ($P = 0.0376$), interstitial nephropathy ($P = 0.0424$), and vascular nephropathy ($P = 0.0075$).

Fractional chemokine excretions increase progressively with CKD stages. Fractional CCL22 excretion was already significantly elevated in patients with CKD from stage 2 onward compared with healthy controls (Fig 1, *A*). In patients suffering from CKD stages 4 and 5, fractional CCL22 excretion was also significantly increased in comparison with CKD stage 1 ($P = 0.0003$ for stage 4, $P = 0.0001$ for stage 5), stage 2 ($P = 0.009$ for stage 4, $P = 0.0014$ for stage 5), and stage 3 ($P = 0.0006$ for stage 4, $P < 0.0001$ for stage 5).

On the contrary, fractional excretion of CCL20 was only significantly increased in stage 5, whereas fractional CXCL11 excretion reached statistical significance at stage 4 in comparison with healthy individuals (Fig 1, *B* and *C*). Furthermore, fractional CCL20 excretion was elevated in patients with CKD stage 5 compared with stage 1 ($P = 0.0018$), stage 2 ($P = 0.0004$), and stage 3 ($P = 0.0002$). In CKD stages 4 and 5, a significant increase in fractional CXCL11 excretion could be detected in comparison with CKD stage 1 ($P = 0.0069$ for stage 4, $P = 0.0033$ for stage 5), stage 2 ($P = 0.0023$ for stage 4, $P = 0.0003$ for stage 5), and stage 3 ($P = 0.0005$ for stage 4, $P = 0.0001$ for stage 5). Because CCL17 was hardly detected in urine samples, fractional CCL17 excretion equaled zero for most samples and did not reach statistical significance during the course of disease (Table II).

Fractional chemokine excretion in various CKD etiologies. The P values for fractional chemokine excretion analyzed with respect to different CKD etiologies are all depicted in Tables III and IV.

Correlations of chemokine serum and urine levels with clinical and kidney function parameters. The tested parameters were age, C-reactive protein (CRP), serum creatinine, eGFR, total urine protein, urine albumin, and absolute as well as relative lymphocyte count. In a subset of 33 patients, the correlation of chemokine

Table III. *P* values obtained by the comparison of chemokine levels in different CKD entities

CKD etiologies	Vascular nephropathy	Diabetic nephropathy	Polycystic kidney disease	Interstitial nephropathy
Glomerulonephritis	CCL17 serum: 0.0465 (0.0129); fractional CCL17 excretion: 0.0308	—	—	—
Diabetic nephropathy	CXCL11 urine: (0.0201)	—	CXCL11 urine: (0.0225)	—
Polycystic kidney disease	CCL20 urine: 0.0135 (0.0439); CCL17 serum: 0.0348; fractional CCL20 excretion: 0.0248 (0.043)	CCL20 urine: 0.0348	—	CCL17 serum: 0.014
Urine stasis	—	Fractional CCL22 excretion: (0.042)	—	—

Abbreviation: CKD, chronic kidney disease.
Only entities with a patient number >5 and *P* values <0.05 are specified. CKD etiologies show increased chemokine levels compared with the entity cited in the line. Values without brackets apply for all CKD stages and values in brackets for stages 3–5.

levels compared with the amount of excreted urine in 24 hours and to creatinine clearance was assessed.

No significant correlation could be found for serum or urine CXCL11 levels, or urine CCL17 concentrations within any of the tested parameters. A weak but significant correlation was found between CCL20 serum levels, CCL17 serum concentrations, absolute CCL22 urine levels, and CRP ($r = 0.1993$, $P = 0.032$ for CCL20; $r = 0.1825$, $P = 0.0499$ for CCL17; $r = -0.2054$, $P = 0.027$ for CCL22). However, the correlation coefficients were very weak ($r < 0.4$).

A significant positive correlation was found between absolute CCL20 urine concentrations and serum creatinine ($r = 0.2873$, $P = 0.0018$), CRP ($r = 0.2007$, $P = 0.0308$), urine protein levels ($r = 0.3654$, $P < 0.0001$), and urinary albumin ($r = 0.3257$, $P = 0.0018$). Moreover, there was a negative correlation between urine CCL20 and eGFR ($r = -0.3121$, $P = 0.0006$) and relative lymphocyte count ($r = -0.2807$, $P = 0.0033$).

Fractional CCL22 excretion negatively correlated with absolute ($r = -0.2271$, $P = 0.0253$) and relative lymphocyte count ($r = -0.1935$, $P = 0.0469$), whereas fractional CCL20 excretion showed a negative correlation with relative lymphocyte count ($r = -0.2916$, $P = 0.0025$).

No significant correlation between chemokine levels and the amount of excreted urine or creatinine clearance was found in the subgroup of patients who had a 24-hour urinary analysis.

Receiver operating characteristic curve analysis of fractional chemokine excretion. As depicted in Fig 2, *A*, receiver operating characteristic (ROC) curve analysis revealed an area under the curve (AUC) of 0.835 (0.7623–0.9388, $P < 0.0001$) indicating the possibility that fractional CCL22 excretion can predict CKD stages 1–5. Moreover, ROC curve analysis for fractional CCL22 excretion in patients with stages 3–5 display an AUC of 0.8722 (0.8057–0.9388, $P < 0.0001$). ROC curve analysis displayed an AUC of 0.6887 (0.5881–0.7893, $P = 0.0007$) for fractional CCL20 excretion and 0.7549 (0.6561–0.8538, $P = 0.0003$) for fractional CXCL11 excretion in patients with CKD stage 3 or higher (Fig 2, *B* and *C*).

DISCUSSION

We were able to demonstrate significantly elevated fractional CCL22 excretion levels in patients with CKD stages 2–5 compared with healthy controls. Moreover, fractional CCL22 excretion levels were significantly increased in patients with CKD stages 4 and 5 compared with patients with CKD stages 1–3. Fractional CCL20 excretion was significantly elevated in patients with CKD stage 5 compared with healthy controls and in patients with CKD stages 1–3. Fractional CXCL11 excretion was significantly increased in stages 4 and 5 in comparison with controls and CKD stages 1–3. Moreover, ROC curve analysis showed the potential of chemokine excretion to predict various CKD stages. We further found elevated chemokine levels in various CKD etiologies, but these differences did rarely reach statistical significance under the Bonferroni adjustment for multiple comparison. For completeness, chemokine expressions in CKD entities are discussed to gain an additional insight into chemokine expression during the pathophysiology of CKD.

During pathophysiological conditions like ischemia, local inflammation, or toxin exposure, the kidney secretes chemokines to attract leukocytes to perform

Table IV. *P* values obtained by the comparison of chemokine levels of healthy controls and different CKD entities

CKD etiologies	CXCL11	CCL17	CCL20	CCL22
Glomerulonephritis	Fractional excretion: 0.027 (0.038)	Serum: 0.046 (0.015)	Fractional excretion: 0.046 (0.023)	Fractional excretion: <0.001* (0.001*)
Vascular nephropathy	Urine: 0.008 (0.013)	Urine: (0.029); fractional excretion: (0.018)	—	Fractional excretion: <0.001* (<0.001*)
Diabetic nephropathy	Fractional excretion: (0.003)	—	—	Fractional excretion: 0.043 (0.003*)
Polycystic kidney disease	Urine: 0.008 (0.012); fractional excretion: (0.038)	Serum: 0.025	Urine: 0.011 (0.026); fractional excretion: 0.011 (0.017)	Fractional excretion: 0.008 (0.001*)
Interstitial nephropathy	—	—	Fractional excretion: 0.009 (0.01)	Fractional excretion: 0.044
Urine stasis	Fractional excretion: 0.026 (0.007)	—	—	Fractional excretion: <0.001* (<0.001*)
Cell carcinoma	—	—	Serum: 0.011 (0.037)	Fractional excretion: 0.022 (0.037)
Nephrectomy	—	—	—	Fractional excretion: 0.019

Abbreviation: CKD, chronic kidney disease.
Only *P* values <0.05 are specified. Healthy individuals show lower chemokine levels than patients with CKD irrespective of renal disease. Values without brackets apply for all CKD stages and values in brackets for stages 3–5.
*Indicates *P* values significant under the Bonferroni adjustment for multiple comparisons.

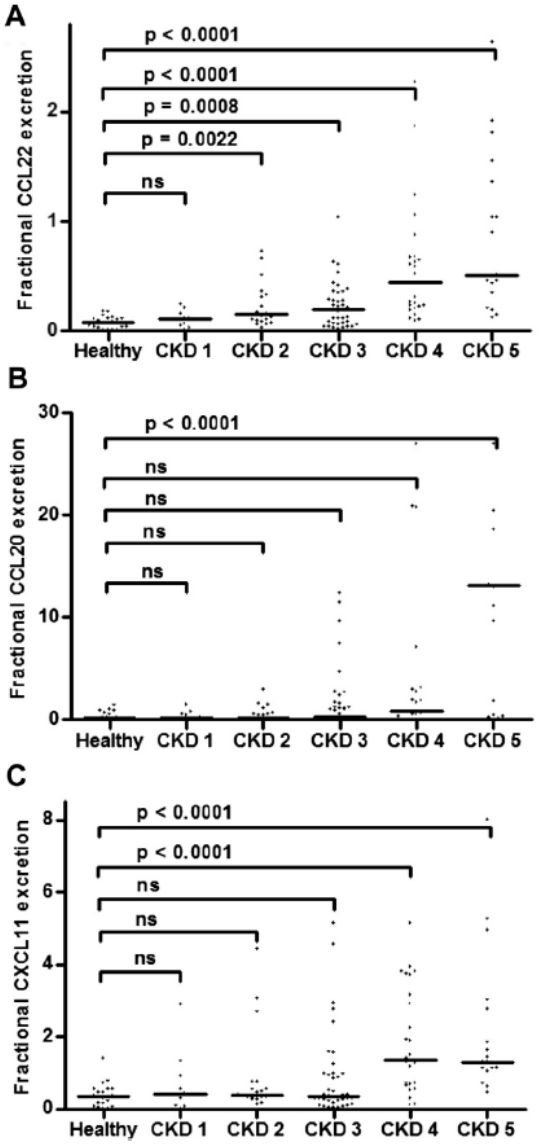

Fig 1. (**A**) Fractional CCL22 excretion, 1 data point outside the axis limits. (**B**) Fractional CCL20 excretion, 8 data points outside the axis limits. (**C**) Fractional CXCL11 excretion. Each dot represents an individual patient. The line indicates the median. CKD, chronic kidney disease.

damage control.[8,13] However, sustained inflammation or perpetual renal damage maintains local chemokine secretion and leukocyte recruitment and contributes to the progression of inflammatory kidney diseases by initiating fibrotic changes, finally resulting in end-stage renal disease.[8,13] This progress is accompanied by high systemic stress levels and increased cell death.[16-19]

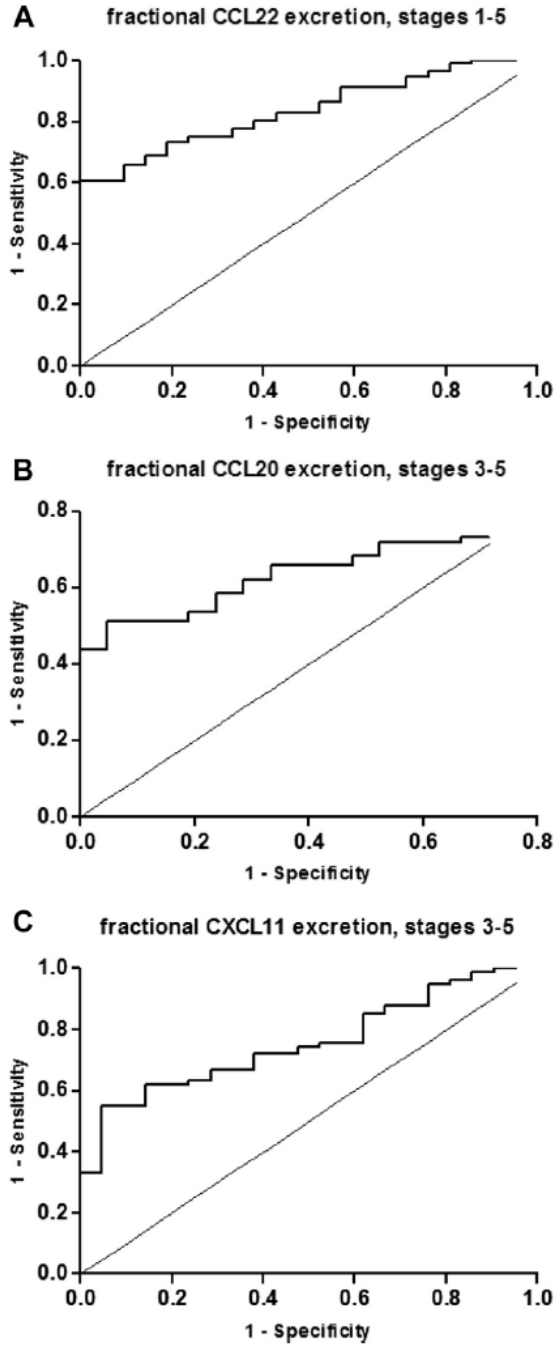

Fig 2. (**A**) ROC curve for fractional CCL22 excretion and CKD stage 1 and higher, AUC 0.835 (0.7623–0.9388, $P < 0.0001$). (**B**) ROC curve for fractional CCL20 excretion and CKD stage 3 and higher, AUC 0.6887 (0.5881–0.7893, $P = 0.0007$). (**C**) ROC curve for fractional CXCL11 excretion and stage 3 and higher, AUC 0.7549 (0.6561–0.8538, $P = 0.0003$). AUC, area under the curve; ROC, receiver operating characteristic.

Specifically, the continuous recruitment of T cells, macrophages, and dendritic cells with chemokines, such as CCL17, CCL20, CCL22, and CXCL11, seems to be detrimental to the kidney in CKD.[15] Their correspondent receptors are CCR4 (CCL17, CCL22), CCR6 (CCL20), and CXCR3 (CXCL11). In this respect, interstitial T cells and macrophages correlate with renal function and have a prognostic value for glomerular diseases.[9,20] Moreover, patients suffering from end-stage renal disease show impaired T-cell proliferation and elevated apoptotic turnover rates compared with controls.[21,22] In our study, we found significantly decreased lymphocyte counts (relative and absolute) in higher stages of CKD in comparison with stage 1. We further found a significant negative correlation between CCL22 as well as CCL20 excretion levels and lymphocyte counts. Thus, lymphopenia in end-stage renal disease might not only derive from an impaired cell proliferation or elevated apoptosis, but also from an increased migration of immunocompetent cells into the diseased kidney.

CXCL11. The main parts of the interstitial T-cell infiltrate are CXCR3 and CCR5 positive cells, whose number showed a positive correlation with renal function and proteinuria in glomerular diseases at the time of renal biopsy.[23] In animal models of crescentic GN and lupus, CXCR3-deficient mice showed a decrease in renal T-cell infiltration, especially in interferon γ–producing Th1 cells and interleukin 17–producing Th17 cells in comparison with wild-type animals.[24,25] Taking the data together, it is not surprising to find elevated CXCL11 urine concentrations in end-stage renal disease. Because there was no significant change in CXCL11 sera concentrations during the progression of disease, it is likely that urinary CXCL11 directly derives from the diseased kidney itself without having any effect on serum levels. Furthermore, fractional CXCL11 excretion was significantly elevated in CKD stages 4 and 5 compared with all other stages, indicating elevated chemokine expression by either renal cells or by infiltrated leukocytes in the damaged kidney. As depicted by ROC curve analysis, CXCL11 fractional excretion rates could provide an indication of changing tubular function during the course of disease or might reflect changes in chemokine production rates by cells within the tubulointerstitium.

We found further elevated CXCL11 urine levels in higher stages of patients with CKD with diabetic nephropathy compared with controls, PKD, and also vascular nephropathy, indicating a different chemokine expression in those etiologies. However, elevated chemokine expression levels have been detected both in diabetes types 2 and 1, and their possible application

as prognostic and diagnostic biomarkers has been discussed.[26,27]

CCL22 and CCL17. CCL22 and CCL17 attract macrophages and T cells via the CCR4 receptor. In a nephrotoxic nephritis model in rats, increased CCL22 messenger RNA and protein expressions could be detected in nephritic glomeruli throughout the course of the disease.[28] A blockade of CCL22 induction with a specific antibody had no effect in the early phase of disease, but suppressed the recruitment of macrophages into the glomeruli, prevented crescent formation, and reversed renal function impairment during later phases of the disease.[28] Therefore, CCL22 and its receptor CCR4 seem to play a critical role in the progression of GN to irreversible renal damage. Our data showed increased fractional CCL22 excretion in patients suffering from CKD compared with controls and lower stages of CKD. Additionally, ROC curve analyses identified fractional CCL22 excretion to distinguish even early stages of CKD. However, the increase in CCL22 serum levels in patients with CKD stages 4 and 5 did not reach statistical significance under the Bonferroni adjustment, indicating that CCL22 expression is favored in the kidney with hardly any impact on serum levels.

In patients who underwent nephrectomy, CCL22 urine concentrations are decreased compared with patients with other CKD etiologies, pointing toward a diminished CCL22 elimination by the remaining kidney. At the same time, we found significant increased fractional CCL22 excretion levels for most CKD entities compared with healthy controls, identifying CCL22 excretion as a potential biomarker irrespective of the underlying renal disease.

We further discovered elevated CCL17 serum levels in patients with GN compared with those with vascular or diabetic nephropathy and healthy individuals. But, because most examined patients in our study suffered from GN, vascular, or diabetic nephropathy, other CKD etiologies are under-represented, which might have weakened statistical power.

Urinary CCL17 was hardly detected in our samples, which could be either attributed to a lack of sensitivity based on the assay or because of the fact that CCL17 is not excreted during the progression of CKD. Despite that, an elevated fractional CCL17 excretion could also be demonstrated in patients with GN or vascular nephropathy. However, an increased renal elimination in GN patients was not obvious in higher stages of CKD. This might be a product of the low number of patients for which fractional excretion could be calculated, because CCL17 serum levels were not determinable for all patients because of methodological limitations.

CCL20. CCL20 upregulation contributes to T-cell recruitment, renal tissue injury, albuminuria, and loss of renal function.[29] Its receptor CCR6 is expressed on Treg and Th17 cells, but not on interferon ɣ–producing Th1 cells.[29] In this respect, CCR6 deficient mice show aggravated renal injury and higher mortality rates, suggesting that the reduced infiltration of Tregs and Th17 cells, but not of Th1 cells, aggravates renal damage. Therefore, CCL20 secretion may be an attempt to counter-regulate inflammatory processes. We also found significantly elevated CCL20 urinary samples of patients suffering from CKD. Furthermore, fractional CCL20 excretion was significantly elevated in end-stage renal disease in comparison with controls or lower stages of CKD, and ROC curve analysis was highly significant to predict CKD stages. Serum CCL20 levels remain largely unaffected by disease progression, again favoring the kidney as chemokine producing location. This theory is further supported by a study showing increased renal infiltration of CCR6-positive cells, especially in areas of nodular inflammatory cell accumulations in patients suffering from chronic renal inflammation.[30] Furthermore, expression of CCR6 in the inflamed kidney could be detected in tubular epithelial cells, whereas in healthy kidneys tubular CCR6 expression was only found occasionally.[30]

In renal cell carcinoma tissue CCL20 is overexpressed, leading to an accumulation of CCR6-positive tumor-infiltrating lymphocytes.[31] In clear-cell renal cancer the gene profile for CCR6 positively correlated with tumor size and stage.[32] Furthermore, chemotherapeutic agents increase CCL20/CCR6 expression in patients with epithelial cell carcinoma.[33] This upregulation can help metastatic cells to survive cell death caused by the chemotherapeutic substances by increasing cell proliferation and migration. In accordance with the literature, we found increased CCL20 serum concentrations in patients with renal or urothelial cell carcinoma who underwent chemotherapy in comparison with other etiologies of CKD and controls. This difference was obvious for all CKD stages and for stages 3–5. However, the etiology of renal damage can differ in patients suffering from carcinoma. It can be caused by dehydration through excessive vomiting, by the chemotherapeutic agents themselves, or by tumor lysis syndrome leading to massive renal damage.[34] As a consequence, the individual origin cannot always be defined as leading to a heterogeneous group regarding kidney failure. Nevertheless, CCL20 urine concentrations do not differ from other CKD etiologies or healthy individuals, indicating that increased CCL20 serum levels in patients with carcinoma derive from malignant processes but not from the injured kidney.

In patients with PKD, the epithelium inside renal cysts expresses chemokines resulting in fibrotic changes and progressing to end-stage renal disease.[35] In agreement with these findings, we were able to demonstrate increased CCL20 excretion levels in patients with PKD compared with controls or patients with diabetic or hypertensive nephropathy. This is suggesting the kidney as chemokine production site in those patients.

CONCLUSIONS

We were able to demonstrate elevated fractional chemokine excretion in patients suffering from CKD. Furthermore, ROC curve analysis showed potential of fractional chemokine excretion rates to predict various CKD stages. Moreover, chemokine levels appear to vary between the underlying etiologies.

Urinary chemokine levels seem to originate from the diseased kidney itself and not from the accretive disturbance of the glomerular filtration barrier that accompany CKD, because chemokine sera levels remain unchanged in most cases. The very weak correlation between urinary chemokine levels and eGFR, protein-, or albumin excretion further supports this assumption. In a baboon model of acute renal allograft rejection, urinary excretion of CXCR3 ligands was able to predict acute allograft rejection. Chemokine excretion was even increased before an elevation of serum creatinine could be detected.[36] Therefore, because serum and urine can be collected noninvasively, urine chemokine levels and especially fractional chemokine excretion offer potential use as a biomarker, not only to identify patients at an early stage of CKD, but also to monitor the course of disease and to predict acute allograft rejection early on. On the other hand, chemokine expression can be upregulated during acute diseases irrespective of renal afflictions. As a consequence, this would lead to a delay of blood withdrawal to examine chemokine levels for kidney function, during an acute phase of illness independent of kidney disease.

Initially, our study was planned to investigate chemokine concentrations during CKD progression and to evaluate their potential as biomarkers independent of the underlying renal disease. As a consequence to particularly elucidate the role of varying chemokine levels in different CKD etiologies, a greater number of patients per entity would be necessary to increase statistical power.

In summary, chemokine expression changes with the development of early- to end-stage CKD and seems to differ between the underlying etiologies of renal failure. However, our results warrant larger clinical studies with a higher number of patients and matched controls to assess the role of chemokines in CKD. Further examination and longitudinal investigation of chemokine profiles in CKD promise to identify potential biomarkers for the early detection of renal damage and to distinguish different CKD entities.

ACKNOWLEDGMENTS

Conflicts of Interest: There are no actual or potential conflicts of interest capable of influencing judgment on the part of any author relating to this work. All authors, except Prof. Walter Hörl, have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

We dedicate this work to our honored mentor and colleague Prof. Walter Hörl, who unfortunately passed away in June 2013.

All authors, except Prof. Walter Hörl, have reviewed and approved the manuscript. This is original work not previously published in any substantial part.

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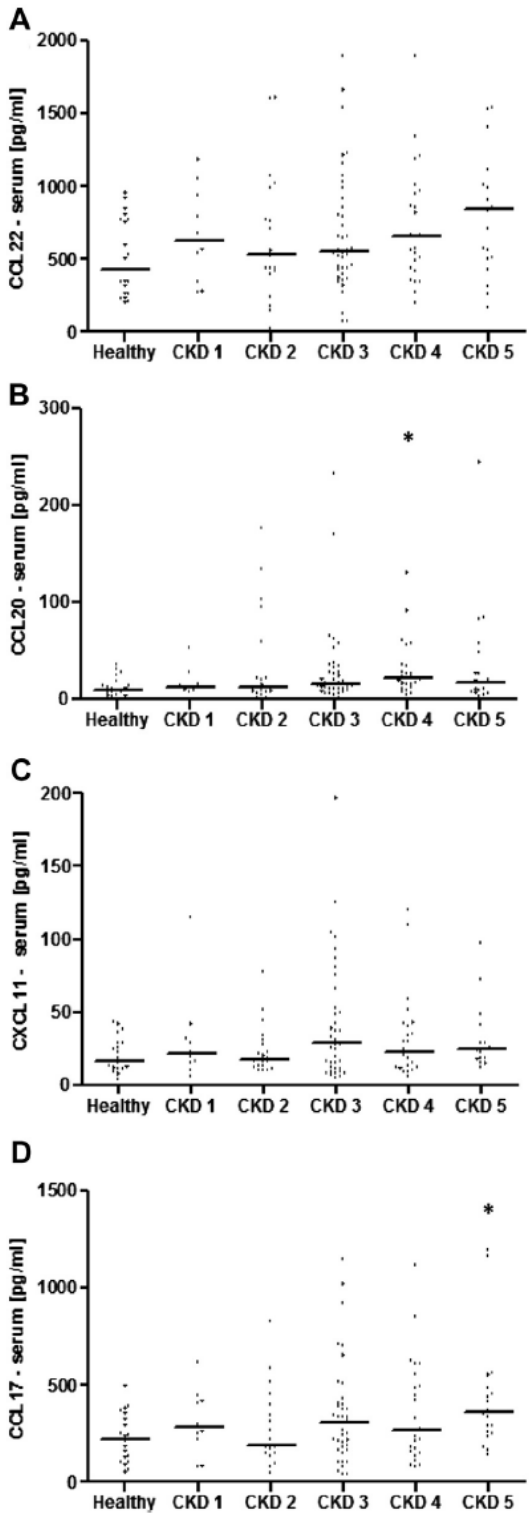
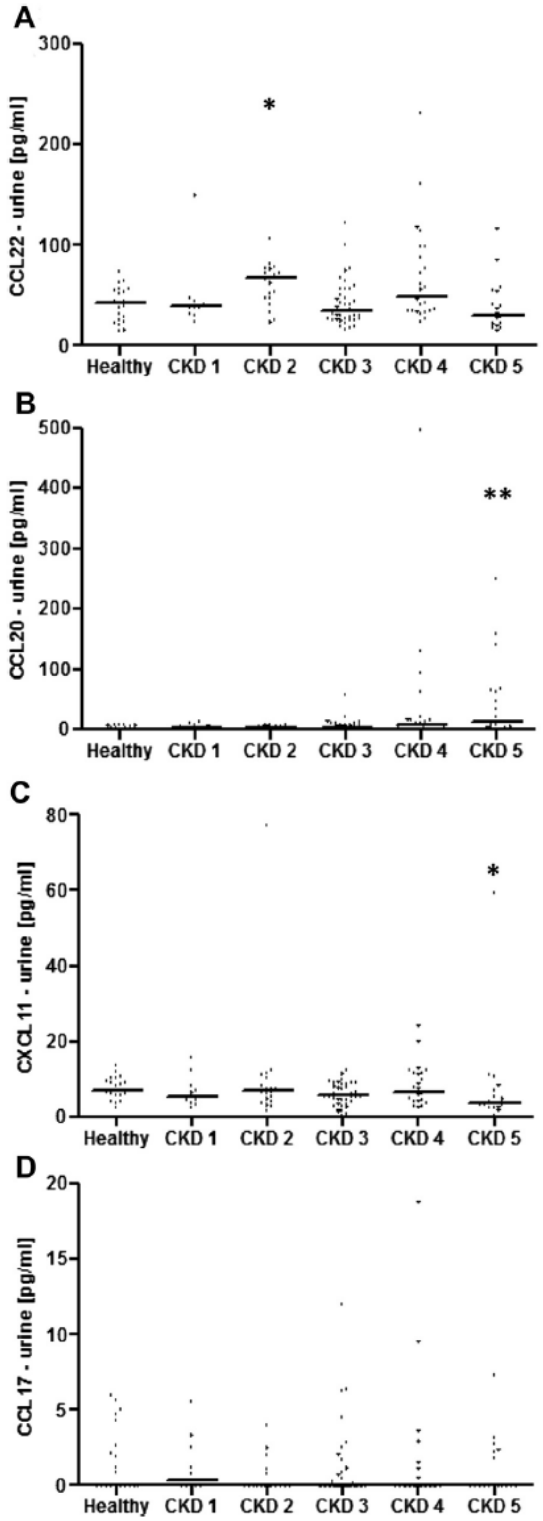


Supplementary Fig 1. (**A**) CCL22 serum levels. (**B**) CCL20 serum concentrations, 1 data point outside the axis limits in CKD stage 3. (**C**) CXCL11 serum levels, 1 data point outside the axis limits in CKD stage 5. (**D**) CCL17 serum concentrations. Each dot represents an individual patient. The line indicates the median and asterisks indicate a significant increase compared with healthy controls (*$P < 0.01$). CKD, chronic kidney disease.



Supplementary Fig 2. (**A**) CCL22 urine levels, 1 data point outside the axis limits in CKD stage 2. (**B**) CCL20 urine concentrations, 3 data points outside the axis limits in CKD stages 3 and 4. (**C**) CXCL11 urine levels. (**D**) CCL17 urine concentrations, 2 data points outside the axis limits in CKD stages 1 and 5. Each dot represents an individual patient. The line indicates the median and asterisks indicate a significant increase compared with healthy controls (*$P < 0.01$, **$P < 0.001$). CKD, chronic kidney disease.

2.2 Trefoil factor 1 excretion is increased in early stages of chronic kidney disease

To limit tissue damage and prevent ongoing cell death during CKD progression, counter mechanisms like the heat shock response are initiated (169-171). Also, TFF peptides are important proteins especially involved in repairing mucous-containing tissue. TFF peptides are secreted all along the urinary tract with TFF3 as the most abundant followed by TFF1 (156). Furthermore, TFF3 is already an established biomarker for renal damage in animal models (158) and is upregulated in patients with CKD (159, 160).

In the “Trefoil factor 1 excretion is increased in early stages of chronic kidney disease” study, elevated levels of TFF1 and TFF3 have been detected in the serum and urine of CKD patients stages 1 – 5 (172). TFF3 urine and serum levels as well as fractional TFF3 excretion gradually increased during progression of CKD with highest concentrations in CKD stage 5. Also TFF1 serum levels were elevated only in later CKD stages as compared to healthy controls (172). In contrast, urinary TFF1 concentrations were highest in early CKD stages and decreased during disease progression to levels comparable to healthy probands. ROC curve analyses reveal that TFF peptide concentrations can predict different CKD stages.
The paper was published in the “PLOS ONE” journal.

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RESEARCH ARTICLE

Trefoil Factor 1 Excretion Is Increased in Early Stages of Chronic Kidney Disease

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Citation: Lebherz-Eichinger D, Tudor B, Ankersmit HJ, Reiter T, Haas M, Roth-Walter F, et al. (2015) Trefoil Factor 1 Excretion Is Increased in Early Stages of Chronic Kidney Disease. PLoS ONE 10(9): e0138312. doi:10.1371/journal.pone.0138312

Editor: Nick Ashton, The University of Manchester, UNITED KINGDOM

Received: June 24, 2015

Accepted: August 28, 2015

Published: September 21, 2015

Data Availability Statement: All relevant data are within the paper.

Funding: There are no actual or potential conflicts of interest capable of influencing judgment on the part of any author relating to this work. The authors have no support or funding to report.

Competing Interests: The authors have declared that no competing interests exist.

Abstract

Chronic kidney disease (CKD) is associated with high morbidity and mortality. In many patients CKD is diagnosed late during disease progression. Therefore, the implementation of potential biomarkers may facilitate the early identification of individuals at risk. Trefoil factor family (TFF) peptides promote restitution processes of mucous epithelia and are abundant in the urinary tract. We therefore sought to investigate the TFF peptide levels in patients suffering from CKD and their potential as biomarkers for CKD. We analysed TFF1 and TFF3 in serum and urine of 115 patients with CKD stages 1–5 without dialysis by ELISA. 20 healthy volunteers served as controls. Our results showed, that urinary TFF1 levels were significantly increased with the onset of CKD in stages 1–4 as compared to controls and declined during disease progression ($p = 0.003$, < 0.001, 0.005, and 0.007. median concentrations: 3.5 pg/mL in controls vs 165.2, 61.1, 17.2, and 15.8 pg/mL in CKD 1–4). TFF1 and TFF3 serum levels were significantly elevated in stages 3–5 as compared to controls (TFF1: $p < 0.01$; median concentrations: 12.1, 39.7, and 34.5 pg/mL in CKD 3–5. TFF3: $p < 0.001$; median concentrations: 7.1 ng/mL in controls vs 26.1, 52.8, and 78.8 ng/mL in CKD 3–5). TFF3 excretion was increased in stages 4 and 5 ($p < 0.001$; median urinary levels: 65.2 ng/mL in controls vs 231.5 and 382.6 ng/mL in CKD 4/5; fractional TFF3 excretion: 6.4 in controls vs 19.6 and 44.1 in CKD 4/5). ROC curve analyses showed, that monitoring TFF peptide levels can predict various CKD stages (AUC urinary/serum TFF > 0.8). In conclusion our results show increased levels of TFF1 and TFF3 in CKD patients with a pronounced elevation of urinary TFF1 in lower CKD stages. Furthermore, TFF1 and TFF3 seems to be differently regulated and show potential to predict various CKD stages, as shown by ROC curve analysis.

Introduction

Chronic kidney disease (CKD) is associated with high morbidity and mortality and is thus an increasing health problem. Patients suffering from CKD have an elevated risk of cardiovascular diseases and the development of other serious complications [1–5]. CKD is defined either by an estimated glomerular filtration rate (eGFR) of less than 60 mL per minute per 1.73 m^2 body-surface or by the presence of kidney damage for at least 3 months [2]. Even a minimal decrease in GFR can lead to complications like anaemia or bone disease [2]. Since CKD progression is silent, many patients are identified shortly before the onset of symptomatic renal failure, at a stage where therapeutic options to prevent adverse outcomes are scarce [6]. Therefore, the early detection of individuals at risk is preferable, and can avert progression to total renal failure resulting in kidney replacement therapy by either dialysis or transplantation.

Worsening of kidney function is associated with an increased inflammatory response, apparent in the upregulation of pro-inflammatory cytokines, again triggering the continuous decline of renal function via this vicious circle [7–9]. To prevent further damage and limit cell death renal cells initialize counterreactions, like the initiation of the heat shock response [10–12]. Additionally, the trefoil factor family (TFF) peptides are important proteins involved in the regeneration and repair of the urinary tract. TFF peptides are secretory products of various mucine-producing epithelial cells and promote restitution and regeneration processes of mucous epithelia via induction of cell migration, resistance to proapoptotic stimuli, and angiogenesis [13, 14]. During restitution, mucosal continuity is restored by elongation and migration of epithelial cells to cover denuded areas of damage.

Though TFF peptides have mainy been investigated in the gastrointestinal tract, they were also detected in the urinary tract with TFF3 as the most abundant followed by TFF1 [15]. In preclinical studies TFF3 has already been established as a urinary biomarker for kidney toxicity in animal models [16] and has been successfully shown to be upregulated in CKD patients [17, 18].

To evaluate if TFF peptide levels change during progression of CKD, we investigated TFF1 in serum and urine of 115 patients suffering from CKD stage 1 to 5 in relation to TFF3 concentrations. Furthermore, we calculated fractional TFF1 and TFF3 excretion to detect changes in renal excretion levels independent from glomerular filtration rate.

Methods

Patients

The study was approved by the institutional ethics committee of the Medical University of Vienna and was performed in accordance with the Helsinki Declaration of 1975. All participiants have signed informed consent.

We included 115 patients with CKD stage 1 to 5 without dialysis or gastrointestinal diseases and all patients were screened and followed up in the out-patient clinic of the Division of Nephrology and Dialysis, Department of Medicine III, Medical University of Vienna.

CKD was defined as decreased glomerular filtration rate and/or the presence of kidney damage according to the K/DOQI criteria [2]. 20 healthy volunteers served as controls. In the control group kidney diseases, abdominal pain over the previous four weeks, and pregnancy were excluded. The patients' diagnoses, baseline demographics and laboratory values are shown in Table 1.

Laboratory data

A venous blood and urine sample was obtained from all patients and healthy volunteers. In addition, a 24 hour urine sample was collected from a subgroup of 33 patients. Serum and

Table 1. Underlying kidney disease, baseline demographic data and laboratory variables.

	All patients	CKD 1	CKD 2	CKD 3	CKD 4	CKD 5	Controls
N	115	10 (8.8 %)	20 (17.5 %)	40 (35.1 %)	26 (21.9 %)	19 (16.7 %)	20
Age (years)	59 (19-88)	36 (19-61)	50 (19-80)	63 (23-78)	59 (29-88)	65 (20-81)	31 (21-67)
Gender (male/female)	66/48	7/3	8/12	27/13	15/10	9/10	13/7
Kidney disease							
Glomerulonephritis	33	3	8	9	6	7	
Vascular nephropathy	20	2	-	9	8	1	
Diabetic nephropathy	11	1	-	7	3	-	
Polycystic kidney disease	8	2	-	2	2	2	
Hereditary angiomyolipoma	1	-	1	-	-	-	
Interstitial nephropathy	7	-	4	1	1	1	
Urine stasis	6	-	1	1	2	2	
Nephrectomy	4	-	2	-	-	2	
Carcinoma	4	-	1	2	1	-	
Unknown	21	2	3	9	3	4	
Serum creatinine	1.88	0.90	1.01	1.65	2.71	5.00	0.99
(mg dL^{-1})	(0.72-6.88)	(0.72-1.03)	(0.77-1.52)	(1.02-2.34)	(2.04-3.89)	(3.47-6.88)	(0.77-1.30)
Blood urea nitrogen	31.9	12.6	14.3	30.5	49	63.3	13.1
(mg dL^{-1})	(7.1-91.2)	(7.5-17.6)	(7.1-26.2)	(11.6-64.1)	(23.8-91.2)	(31.9-87.3)	(8.2-20)
Urine creatinine	69.9	69.1	74.9	79.4	66.4	41.65	131.7
(mg dL^{-1})	(12.7-294.5)	(22.7-252.9)	(14.8-243.5)	(12.7-294.5)	(29.3-172.1)	(17.7-108.2)	(31.4-316.1)
Urine urea	847	948	1044	931	855	647	
(mg dL^{-1})	(247-2557)	(336-1814)	(247-2557)	(291-2464)	(267-1370)	(273-1381)	
Urine protein (g L^{-1})	0.47	0.06	0.25	0.51	0.55	1.07	
	(< 0.05-6.94)	(< 0.05-0.21)	(< 0.05-4.45)	(< 0.05-2.79)	(<0.05-3.46)	(0.05-6.94)	
CRP (mg dL^{-1})	0.29	0.165	0.14	0.51	0.25	0.56	
	(0.03-8.53)	(0.05-4.31)	(0.05-1.59)	(0.03-3-87)	(0.04-8.53)	(0.05-7.84)	

Data are given as median with range; CKD, chronic kidney disease. Patients specified with carcinoma were suffering from urothelial or renal cell carcinoma and underwent chemotherapy. No renal biopsy was obtained from patients with unknown entity, since proven diagnosis would not have changed treatment planning.

doi:10.1371/journal.pone.0138312.t001

urine samples were centrifuged at 2000 RCF for 10 min at 4°C. Aliquots were transferred into tubes, snap frozen and stored at- 80°C until further use. TFF3 and TFF1 were determined using enzyme-linked immunosorbent assay (ELISA) kits (Human TFF3 Quantikine ELISA Kit and Human TFF1 DuoSet, R&D Systems, Minneapolis, Minnesota, www.rndsystems.com) according to the manufacturer's instructions. As a substrate, tetramethylbenzidin (TMB; Sigma, St. Louis, Missouri, www.sigmaaldrich.com) was used, and color reaction was stopped with 1% sulfuric acid solution. Optical density was measured with a Victor 3 microplate reader at a wavelength of 450 nm. To overcome methodological deviations serum and urine samples were randomized before analysis.

We performed spike/recovery and linearity testing according to the spike, recovery, and linearity protocol for validating untested samples of R&D systems. For this purpose, known concentrations of trefoil factor peptide were added to the used reagent buffer, urine, and serum samples. Linearity testing was performed by the serial dilution of spiked and unspiked samples. Recovery was obtained by the evaluation of neat and diluted spiked/unspiked samples. Average recovery for TFF1 was 95% for serum samples and 110% for urine samples. Average linearity was 98% for serum samples and 115% for urine samples. Average recovery for TFF3 was 100%

for serum samples and 98% for urine samples. Average linearity was 99% for all tested samples. Samples were tested multiple times on different plates and revealed an intra-assay variability of 1% for TFF1 and 2% for TFF3 and an inter-assay variability of 3% for TFF1 and 5% for TFF3, respectively. Testing specificity revealed no significant cross-reactivity between TFF1 and TFF3. The sensitivity was determined by the summation of two standard deviations to the mean optical density of twenty zero samples and by the subsequent calculation of the corresponding concentration. The minimum detectable dose was 3.3 pg/mL for TFF1 and 6.4 pg/mL for TFF3.

The fractional TFF peptide excretion was calculated using the formula: ((urinary TFF peptide x serum creatinine) / (serum TFF peptide x urinary creatinine)) x 100.

Statistical analysis

TFF protein concentrations in serum and urine as well as fractional excretion were analysed between CKD stage 1 to 5 and the control group. Gaussian distribution was assessed with the D'Agostino-Pearson normality test. Since Gaussian distribution could not be verified for all analyzed groups, the non-parametric Mann-Whitney-test (two-tailed) was used to compare trefoil factor concentrations. According to the Bonferroni adjustment for multiple comparisons, an individual $p < 0.01$ was necessary to achieve statistical significance at the 5% level.

Correlations between TFF peptide serum and urine levels and several clinical parameters were estimated for all patients and controls by Spearman's correlation coefficient. Unless otherwise specified, data are given as median with range. Statistical analysis was performed using GraphPad Prism Version 5.01 (GraphPad Software, Inc. California, US).

Results

Increased total TFF serum and urine levels

Total TFF1 serum concentration were significantly increased in patients with CKD stages 3 to 5 as compared to the control group (Fig 1A). Total urinary TFF1 levels were significantly higher in CKD stages 1 to 4, as depicted in Fig 1B. Calculation of fractional TFF1 excretion revealed no significant changes (Fig 1C). Total TFF3 serum levels were significantly elevated from stage 3 on, in comparison to the control group (Fig 2A). Total urinary TFF3 concentrations and fractional TFF3 excretion were significantly higher in CKD stages 4 and 5 as compared to controls (Fig 2B and 2C).

Correlations of total TFF serum and urine levels with clinical and kidney function parameters

The tested parameters were age, C—reactive protein (CRP), eGFR, serum creatinine, urinary albumin, and total urinary protein. In a subset of 33 patients, the correlation between TFF protein levels and the 24h creatinine clearance was evaluated.

There was a strong negative correlation ($r < -0.70$) between TFF3 serum levels and creatinine clearance or eGFR ($r = -0.71$, $p < 0.001$ and $r = -0.75$, $p < 0.001$, respectively; Table 2). TFF3 serum levels further show a strong positive correlation to serum creatinine ($r = 0.73$, $p < 0.001$) (Table 2). A negative correlation was found between creatinine clearance and urinary TFF3 in the subgroup of patients with a 24-hour urine analysis ($r = -0.55$, $p = 0.001$; Table 2).

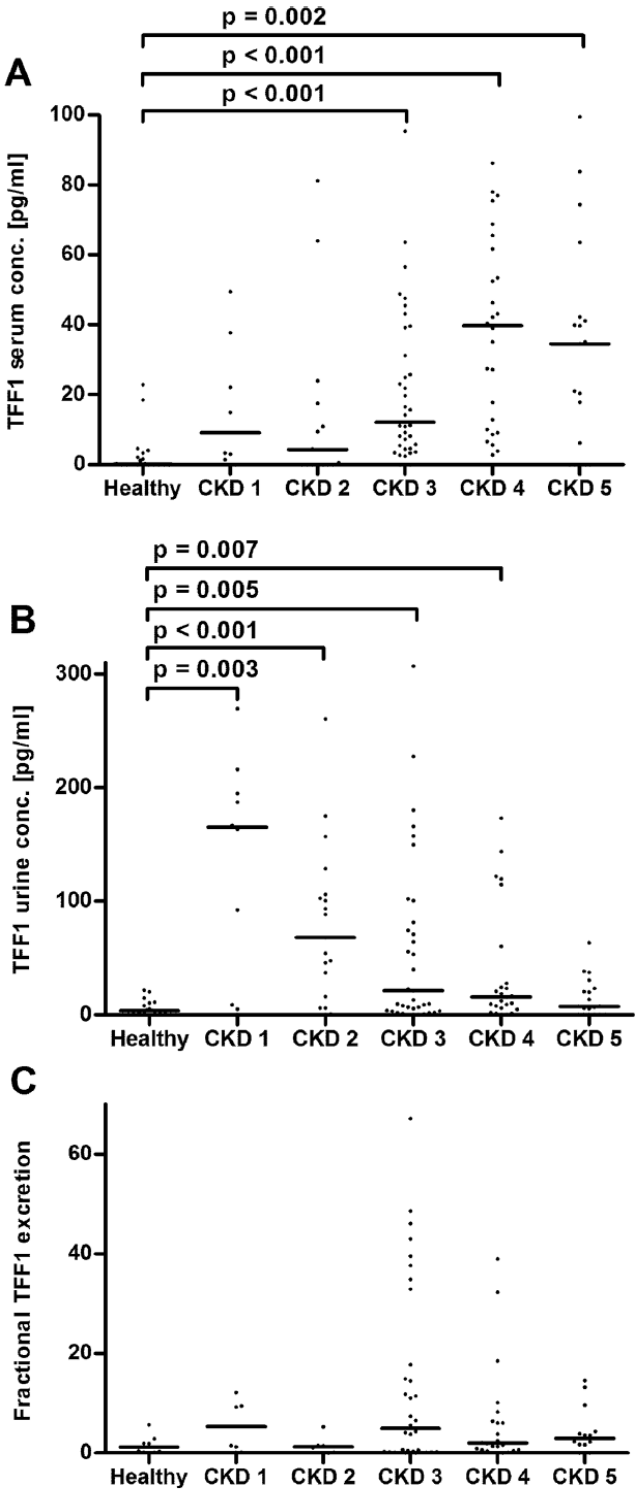
A
p = 0.002
p < 0.001
p < 0.001
TFF1 serum conc. [pg/ml]
100
80
60
40
20
0
Healthy
CKD 1
CKD 2
CKD 3
CKD 4
CKD 5
B
p = 0.007
p = 0.005
p < 0.001
p = 0.003
TFF1 urine conc. [pg/ml]
300
200
100
0
Healthy
CKD 1
CKD 2
CKD 3
CKD 4
CKD 5
C
Fractional TFF1 excretion
60
40
20
0
Healthy
CKD 1
CKD 2
CKD 3
CKD 4
CKD 5

Fig 1. TFF1 levels. Panel A: TFF1 serum concentrations, three data points outside the axis limits in CKD stages 1, 2, and 3. Panel B: Urinary TFF1 levels, three data points outside the axis limits in CKD stage 3. Panel C: Fractional TFF1 excretion, six data points outside the axis limits in controls and CKD stages 1–4. Each dot represents an individual patient. The line indicates the median. CKD, chronic kidney disease. Only significant p-values are given.

doi:10.1371/journal.pone.0138312.g001

ROC curve analysis of total TFF serum and urine levels

As depicted in Fig 3, ROC curve analysis displayed an area under the curve for the diagnosis of CKD stage 3 or higher of 0.88 (0.80–0.95, $p < 0.001$) for total serum TFF1 and 0.94 (0.84–1, $p < 0.001$) for total serum TFF3 concentrations. For fractional TFF3 excretion levels, ROC curve analysis revealed an area under the curve of 0.77 (0.67–0.87, $p < 0.001$; Fig 3D). Total urine TFF1 concentrations correlated with CKD stage 1 or 2 displayed an area under the curve of 0.83 (0.71–0.95, $p < 0.001$; Fig 3B).

Discussion

We were able to demonstrate a pronounced increase in TFF1 urine levels with the onset of CKD. Although the TFF1 urine concentrations abated as kidney function declined, they were still higher than in controls up to CKD stage 4. In contrast, TFF1 serum concentrations constantly increased during progression of chronic renal failure, reaching a significant difference compared to controls in CKD 3 to 5. Furthermore, we found elevated TFF3 peptide excretion levels in patients with CKD 4 and 5, and increased TFF3 serum concentrations from stage 3 on. ROC curve analysis showed the potential of TFF1 and TFF3 to predict various CKD stages.

CKD has a multifactorial origin and is associated with increased cell damage caused by uremic toxins, inflammation, and oxidative stress [19–21]. Persistent inflammation triggers sustained renal damage and contributes to the progression of kidney disease to end-stage renal failure. To minimize cell damage and limit ongoing cell death counterregulatory mechanisms are initialized in order to hold progression of renal dysfunction.

TFF peptides are evolutionarily a highly conserved group of proteins which participate in epithelial protection and restitution. They promote cell migration as well as angiogenesis, limit proapoptotic stimuli, and facilitate leukocyte migration [13, 22]. All TFF peptides are essential for epithelial restitution and can induce cell migration, but they differ in other accessory functions. An example is the tumour suppressive function of TFF1, which has been proven in an animal gastric cancer model [23]. TFF peptides are named after the three-looped structure of their cystein residues, the so called trefoil domain. TFF1 and TFF3 peptides have 60 and 59 amino acids respectively, and contain one trefoil domain each. The highest expression of TFF peptides can be found in the gastrointestinal tract [14]. Moreover, TFF peptides and especially TFF3 have been shown to be secreted by almost all epithelial tissues containing mucus-secreting cells, including the kidney and the urinary tract. Therefore, the involvement of TFF peptides in CKD progression seems likely and has already been proven for TFF3 [17, 18]. To the best of our knowledge, this is the first study evaluating TFF1 serum and urine concentration as well as fractional TFF excretion in patients suffering from CKD.

Our measurements of increased TFF3 serum and urine concentrations are in accordance with the findings from others [17, 18]. Due to ongoing epithelial damage, TFF3 expression is upregulated in chronically inflamed tissues to ensure epithelial restitution and regeneration. TFF peptides are synthesized all along the urinary tract, with TFF3 expression as the most pronounced [15]. Therefore, it is not surprising to find elevated TFF3 levels in patients suffering from CKD, an affliction associated with ongoing renal inflammation and epithelial damage. However, despite increased TFF3 peptide excretion, serum levels remained elevated, indicating

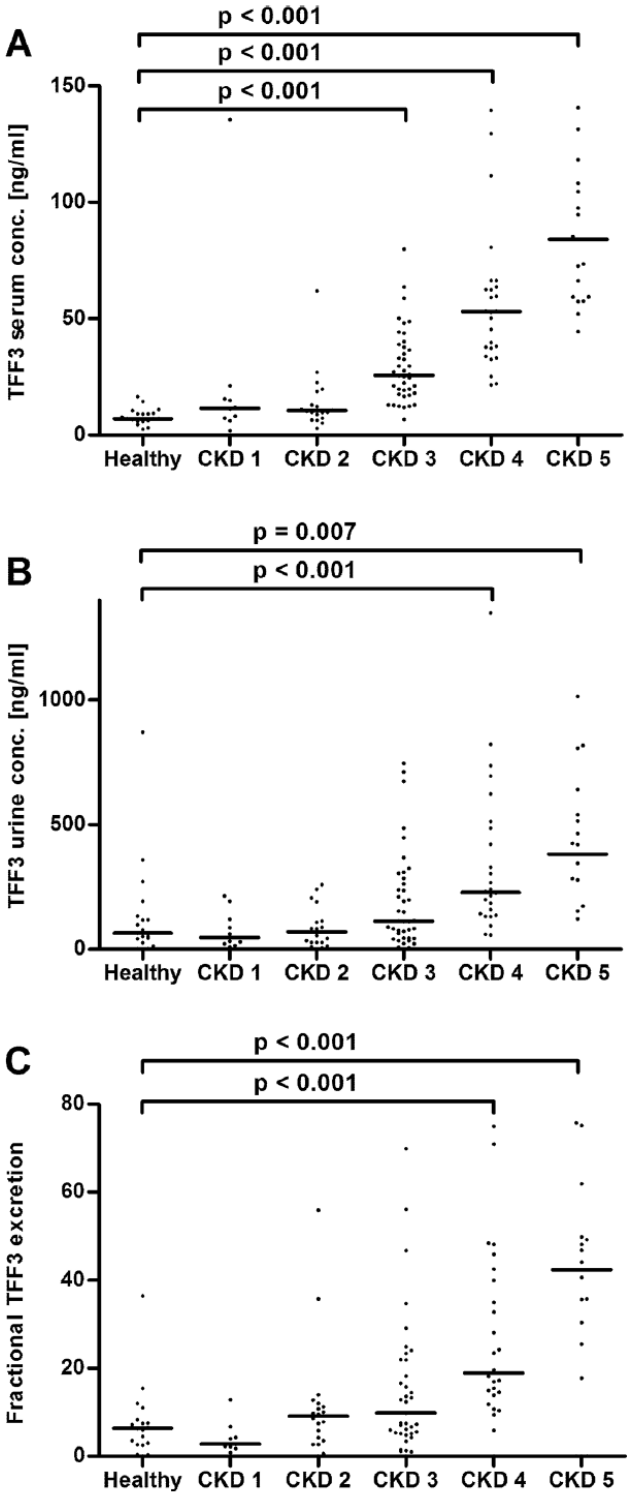
A
p < 0.001
p < 0.001
p < 0.001
TFF3 serum conc. [ng/ml]
150
100
50
0
Healthy
CKD 1
CKD 2
CKD 3
CKD 4
CKD 5
B
p = 0.007
p < 0.001
TFF3 urine conc. [ng/ml]
1000
500
0
Healthy
CKD 1
CKD 2
CKD 3
CKD 4
CKD 5
C
p < 0.001
p < 0.001
Fractional TFF3 excretion
80
60
40
20
0
Healthy
CKD 1
CKD 2
CKD 3
CKD 4
CKD 5

Fig 2. TFF3 levels. Panel A: TFF3 serum levels, two data points outside the axis limits in CKD stages 2 and 5. Panel B: Urinary TFF3 concentrations, one data point outside the axis limits in CKD stage 2. Panel C: Fractional TFF3 excretion, one data point outside the axis limits in CKD stage 5. Each dot represents an individual patient. The line indicates the median. CKD, chronic kidney disease. Only significant p-values are given.

doi:10.1371/journal.pone.0138312.g002

continuously increasing TFF3 expression rates to ensure epithelial integrity during CKD progression. On the other hand, sufficient TFF3 excretion might be impeded by diminished filtration due to the cumulative disturbance of the glomerular filtration barrier that accompanies CKD also leading to increased serum levels. However, ROC curve analysis showed the potential of TFF3 serum concentration and fractional TFF3 excretion levels to identify different CKD stages.

Similarly TFF1 is upregulated during inflammation to minimize epithelial damage and maintaining epithelial integrity [13]. Interestingly, we found elevated total TFF1 urine levels with the onset of renal failure. With CKD progression urinary TFF1 levels gradually decreased and returned to concentrations comparable with healthy individuals in CKD 5. In contrast, the elevated TFF1 serum concentrations reached significance only in higher CKD stages, pointing to the kidney as main production site of urinary TFF1 in early CKD. Hence, our findings demonstrate that TFF1 expression seems to be upregulated during the acute phase of kidney disease. Similar results were reported in a rat model of acid induced colitis, in which an elevated TFF1 expression in the distal colon was found during the acute phase of the disease [24]. In contrast, TFF3 expression was downregulated during the acute phase, but increased in the restitution phase [24]. In a methotrexate-induced mucositis model, TFF3 peptides were also minimized during acute phase, but reemerged during regeneration [25]. Those findings are in accordance with our results, showing a continuous increase in TFF3 peptide levels during progression of renal failure to end-stage renal disease. Hence, the initial increase in TFF1 expression and the subsequent secretion of TFF3 peptides in CKD patients might derive from a balanced interplay of TFF peptides trying to ensure mucosal protection during inflammatory and fibrotic processes. However, the observed decrease of total TFF1 urinary concentrations with the concurrent elevation of TFF1 serum levels during disease progression cannot be fully explained by our study. We hypothesize that in the acute phase of renal damage, TFF1 is secreted by the epithelial cells lining the kidney to overcome epithelial damage and is immediately excreted by normal renal clearance. Due to the diminished filtration as well as the increase

Table 2. Correlation of TFF serum and urine levels with clinical and kidney function parameters.

	TFF1 serum	TFF1 urine	TFF3 serum	TFF3 urine
Age (115 pairs)	r = 0.44, p < 0.001*	r = - 0.1, p = 0.3	r = 0.35, p < 0.001*	r = 0.17, p = 0.07
Serum creatinine (115 pairs)	r = 0.29, p = 0.002*	r = - 0.32, p < 0.001*	r = 0.73, p < 0.001*	r = 0.39, p < 0.001*
Estimated glomerular filtration rate (115 pairs)	r = - 0.32, p < 0.001*	r = 0.32, p < 0.001*	r = - 0.75, p < 0.001*	r = - 0.41, p < 0.001*
Total urine protein (114 pairs)	r = 0.24, p = 0.01*	r = 0.01, p = 0.9	r = 0.37, p < 0.001*	r = 0.35, p < 0.001*
Urine albumin (88 pairs)	r = 0.35, p < 0.001*	r = 0.02, p = 0.9	r = 0.37, p < 0.001*	r = 0.32, p = 0.003*
Creatinine clearance (33 pairs)	r = - 0.2, p = 0.3	r = 0.29, p = 0.1	r = - 0.71, p < 0.001*	r = - 0.55, p = 0.001*
CRP (115 pairs)	r = 0.13, p = 0.2	r = - 0.1, p = 0.3	r = 0.34, p < 0.001*	r = 0.16, p = 0.08

* indicates significance

doi:10.1371/journal.pone.0138312.t002

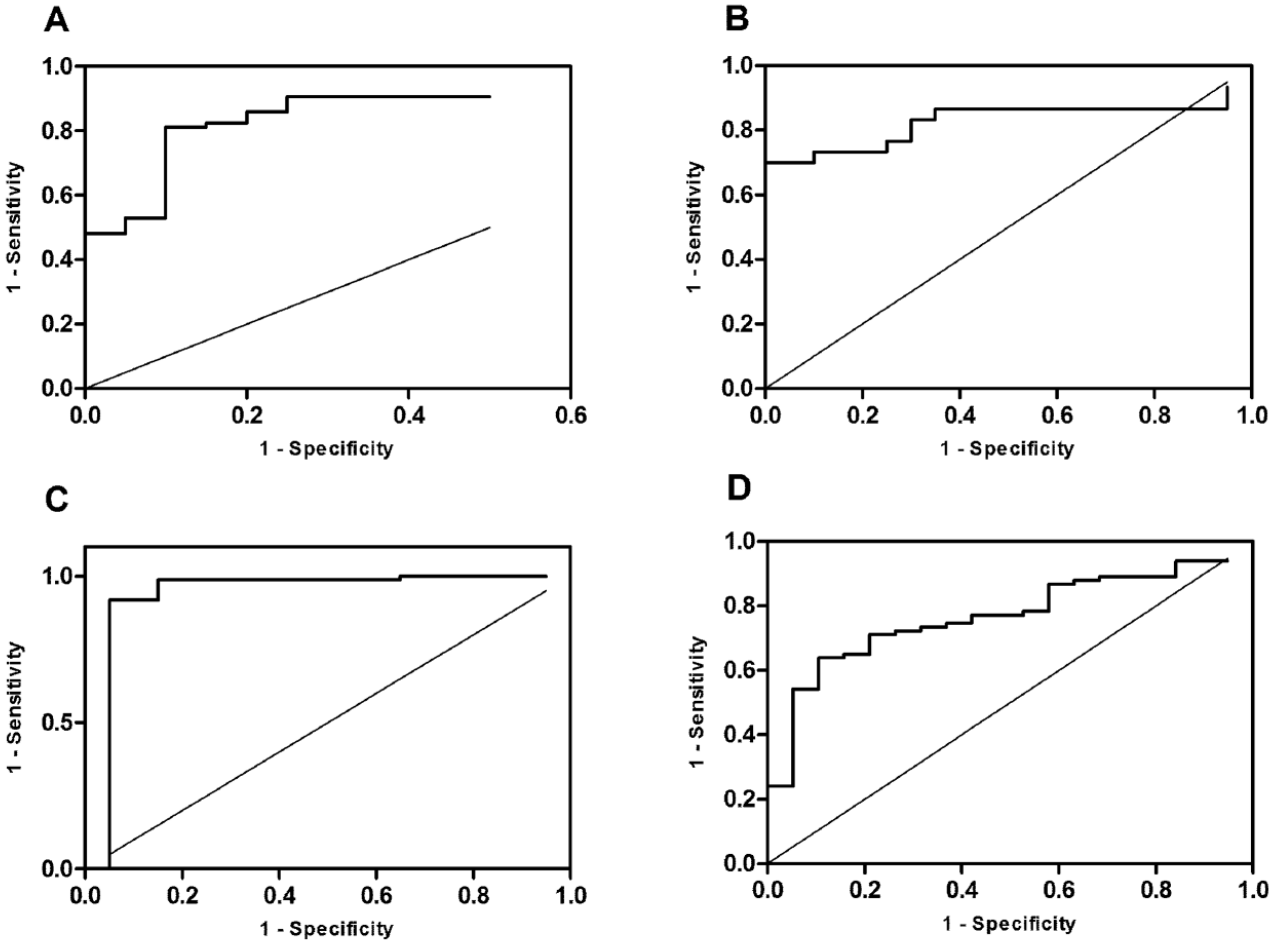

Fig 3. ROC curve analysis. Panel A: ROC curve for serum TFF1 and CKD stage 3 or higher, AUC 0.88 (0.80–0.95, $p < 0.001$). Panel B: ROC curve for urinary TFF1 and CKD stages 1 and 2, AUC 0.83 (0.71–0.95, $p < 0.001$). Panel C: ROC curve for serum TFF 3 and CKD stages 3 or higher, AUC 0.94 (0.84–1, $p < 0.001$). Panel D: ROC curve for fractional TFF3 excretion and CKD stages 3 or higher, AUC 0.77 (0.67–0.87, $p < 0.001$).

doi:10.1371/journal.pone.0138312.g003

in angiogenesis in the chronically inflamed kidney, TFF peptide concentration in the blood further increases, which is reflected in the observed rise in TFF1 and TFF3 serum levels at higher CKD stages. However, the initial increase of urinary TFF1 might show the potential of this peptide to early identify individuals at risk and to identify lower CKD stages, as depicted by ROC curve analysis. Furthermore, the combined examination of TFF1 and TFF3 in serum and urine might further improve diagnosis of various CKD stages.

Calculation of fractional TFF clearance revealed that TFF peptide excretion does not seem to depend solely on glomerular filtration. The fractional excretion rates for both proteins above 1, indicate that the source of the TFF peptides are also the epithelial renal cells, which seem to preferentially secrete TFF1 with disease onset followed by TFF3 during disease progression. Due to the decrease of urinary TFF1 in higher CKD stages, the calculation of fractional TFF1 excretion with a formula also including urinary and serum creatinine revealed no differences between patient and healthy probands.

Although this study has produced exciting results, we are aware of some methodological limitations. Even though ELISA testing revealed satisfactory results in recovery, linearity, sensitivity, and coefficient of variations, the kits are not designed for use in clinical testing and thus may remain a source of data bias, possibly influencing results. Moreover, due to the low number of patients not all groups were normally distributed. Consequently, a nonparametric statistical test was applied to detect differences between CKD patients and controls, which might have weakened statistical power. The inclusion of a greater number of patients in early CKD stages may be necessary to evaluate the relevance of TFF1 in the acute phase of kidney injury in future studies. Additionally, this study was planned as a cross-sectional analysis to evaluate TFF peptide levels independent of the underlying renal disease. Our results indicate that TFF1 has the potential to early identify individuals at risk, and that changes in TFF peptide expression might predict disease progression independent of renal affliction. However, we are aware of the limitations of our study by its descriptive design. Finally, the simultaneous evaluation of patients with different causes of CKD might conceal important findings in certain afflictions. Therefore, further clinical testing and longitudinal surveys with more patients are necessary to unravel the role of TFF peptides during progression to end stage renal disease.

Conclusion

Elevated levels of TFF1 and TFF3 were found in patients suffering from CKD, with TFF1 and TFF3 being differently regulated. Changes in TFF peptide levels might identify aberrations in glomerular filtration and may provide information on disease progression. In particular, the pronounced increase of urinary TFF1 concentration with the onset of CKD indicates that TFF1 might be suitable as a biomarker for the early detection of individuals at risk. However, larger clinical studies and longitudinal surveys are needed to assess the role of TFF peptides in renal failure and their potential as biomarkers.

Author Contributions

Conceived and designed the experiments: DL GAR. Performed the experiments: DL BT TR. Analyzed the data: DL FR GAR. Contributed reagents/materials/analysis tools: HA MH CGK. Wrote the paper: MH FR CGK GAR.

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2.3 Increased TFF2 levels in patients suffering from chronic kidney disease

In TFF1 knock-out mice an additional reduced TFF2 expression was found, pointing towards a genetic co-regulation of those two proteins (173). In accordance to this finding, in the “Increased TFF2 levels in patients suffering from chronic kidney disease” study, elevated TFF2 concentrations were found in the urine of patients with early stages of CKD (174), which has also been described for TFF1 within this thesis (172). Detected TFF2 urine concentrations decreased with the progression of CKD, whereas TFF2 serum levels gradually increased from early to later CKD stages, which is comparable to changes described for TFF1 levels in the same patient collective (172). Fractional TFF2 excretion revealed no significant differences between CKD stages, but ROC curve analysis identified serum and urine TFF2 concentrations to identify different CKD stages.
The paper was published in the “PLOS ONE” journal.

PLOS ONE

RESEARCH ARTICLE

Increased trefoil factor 2 levels in patients with chronic kidney disease

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Citation: Lebherz-Eichinger D, Tudor B, Ankersmit HJ, Reiter T, Haas M, Einwallner E, et al. (2017) Increased trefoil factor 2 levels in patients with chronic kidney disease. PLoS ONE 12(3): e0174551. https://doi.org/10.1371/journal.pone.0174551

Editor: Tatsuo Shimosawa, The University of Tokyo, JAPAN

Received: October 7, 2016

Accepted: March 11, 2017

Published: March 29, 2017

Data Availability Statement: All relevant data are within the paper.

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Abstract

In chronically damaged tissue, trefoil factor family (TFF) peptides ensure epithelial protection and restitution. In chronic kidney disease (CKD), TFF1 and TFF2 are reported to be upregulated. Especially in the early phase, CKD is associated with silently ongoing renal damage and inflammation. Moreover, many patients are diagnosed late during disease progression. We therefore sought to investigate the potential of TFF2 as biomarker for CKD. We followed 118 patients suffering from predialysis CKD and 23 healthy volunteers. TFF2 concentrations were measured using ELISA. Our results showed, that median TFF2 serum levels were significantly higher in patients with later CKD stages as compared to healthy controls ($p < 0.001$) or early stages ($p < 0.001$). In patients with mid CKD stages TFF2 serum levels were significantly higher than in healthy controls ($p = 0.002$). Patients with early or mid CKD stages had significantly higher TFF2 urine concentrations than later CKD stages ($p < 0.001$ and $p = 0.009$, respectively). Fractional TFF2 excretion differed significantly between early CKD stages and healthy controls ($p = 0.01$). ROC curve showed that TFF2 levels can predict different CKD stages (AUC > 0.75). In conclusion, urine and serum TFF2 levels of CKD patients show a different profile dependent on CKD stages. Whereas TFF2 urine levels continuously decreased with disease progression, TFF2 serum concentrations progressively increased from the early to later CKD stages, indicating changes in renal function and offering the potential to examine the course of CKD.

Introduction

Chronic kidney disease (CKD) describes the steady loss of kidney function and is defined by kidney damage or by an estimated glomerular filtration rate (eGFR) of less than 60 mL per

minute per 1.73 m^2 body-surface for a minimum of three months [1]. CKD patients are at increased risk of developing serious complications like cardiovascular diseases and even a slight decrease in GFR can result in anaemia or bone disease [1–3]. The progression of CKD proceeds silently, resulting in patients diagnosed at a state where most therapeutic options to prevent adverse outcomes are insufficient [4]. Consequently, the early detection of individuals at risk is highly desirable to initiate a timely treatment to prevent disease progression.

The decline of kidney function is associated with ongoig inflammatory response and increased cell death [5–7]. Trefoil factor (TFF) peptides have been shown to be upregulated in the damaged kidney, obviously to ameliorate epithelial destruction [8–10]. These proteins are secreted by several mucine-producing epithelial cells and are involved in mucousal healing. TFF peptides facilitate epithelial restitution and regeneration processes by the induction of cell migration, angiogenesis, and by raising cell resistance to proapoptotic stimuli [11–13]. Restitution describes the recovery of mucosal continuity via elongation and cell migration to cover damaged denuded areas.

TFF peptides are evolutionally a highly conserved group of proteins and are named after the so called trefoil domain, which consists of a three-looped structure of cysteine residues. The protein family comprises three members: TFF1, TFF2, and TFF3, with TFF2 to be discovered first of all [11]. TFF peptides are secreted by most epithelial tissues that contain mucus-secreting cells including renal tubular epithelial cells in the kidney. In animal models for kidney toxicity, TFF3 is already an accepted biomarker for the estimation of renal damage [14]. Increased levels of TFF3 and TFF1 have also been detected in patients with CKD and closely correlated with renal function [9,10,15].

To evaluate if TFF2 levels also change during progression of CKD we investigated TFF2 levels in serum and urine of 118 patients suffering from early, mid or later stages of CKD in comparison to healthy controls. Furthermore, we analysed the potential of TFF2 as biomarker for renal damage and calculated fractional TFF2 excretion to unravel changes in renal excretion irrespective of the glomerular filtration rate.

Methods

Patients

This study has been approved by the ethics committee of the Medical University of Vienna and was conducted according to the Helsinki Declaration of 1975.

CKD patients were diagnosed, screened and followed up at the Division of Nephrology and Dialysis, Department of Medicine III in the Medical University of Vienna. CKD was defined as decreased glomerular filtration rate and/or the presence of kidney damage in accordance with the K/DOQI criteria [1]. For calculating eGFR the formular described in the 2002 guidelines was used to allow comparisons to already published studies dealing about TFF levels in CKD. 118 patients with CKD stage 1 to 5 were included into the study during a follow-up appointment at the outpatient clinic of the Division of Nephrology and Dialysis. 23 healthy volunteers served as controls. In the control group, abdominal pain over the last four weeks, kidney diseases, and pregnancy were excluded via anamnesis. All participiants signed an informed consent before inclusion into the study.

Table 1 shows the patients' diagnoses, baseline demographics and laboratory values.

Laboratory data

Blood and spontaneous urine samples were obtained from all patients and healthy volunteers. Additionally, 24-hour urine samples were obtained in a subgroup of 34 patients. Serum samples were collected in a 9 mL Z Serum Clot Activator Tube and the urine in Vacuette® Urine

Table 1. Patients baseline demographic and laboratory data. Underlying kidney diseases.

	All patients	Early CKD stages (stage 1, stage 2)	Mid CKD stages(stage 3)	Later CKD stages(stage 4, stage 5)	Controls
N	118	33 (28%)	39 (33%)	46 (39%)	23
Age (years)	56 (19–88)	44 (19–80)	61 (23–78)	61 (20–88)	39 (21–67)
Male/Female (%)	57/43	48/52	69/31	52/48	65/35
Weight (kg)	75 (36–120)	80 (54–120)	77.5 (50–118)	72.5 (36–116)	
Height (cm)	172 (120–198)	172 (152–198)	174 (147–185)	166 (120–190)	
BMI	25.3 (15.4–39)	26 (21.8–39)	25.6 (18.3–33.3)	25.7 (15.4–36)	
Smoker/Non-smoker (%)	60/40	67/33	65/35	54/46	
Kidney disease					
Glomerulonephritis	34	12	9	13	
Vascular nephropathy	20	3	8	9	
Diabetic nephropathy	12	2	7	3	
Polycystic kidney disease	10	3	2	5	
Hereditary angiomyolipoma	1	1	-	-	
Interstitial nephropathy	7	4	1	2	
Urine stasis	6	1	1	4	
Nephrectomy	4	1	-	3	
Urothelial/Renal cell carcinoma	3	1	2	-	
Unknown	21	5	9	7	
Creatinine (mg dL^{-1})	1.86(0.72–6.88)	0.98(0.72–1.52)	1.68(1.02–2.34)	3.54(3.14–6.88)	0.99(0.77–1.3)
Urea(mg dL^{-1})	31.9(7.1–91.2)	12.9(7.1–33.4)	30.5(11.6–64.1)	55.6(23.8–91.2)	13.1(0–20)
Urine creatinine (mg dL^{-1})	70.9(12.7–294.5)	72.4(69.9–252.9)	81.1(12.7–294.5)	56.6(17.7–172.1)	138.5(31.4–418.6)
Urine urea Nitrogen(mg dL^{-1})	844(247–2557)	976(247–2557)	939(291–2464)	676(267–1381)	
Urine protein (g L^{-1})	0.47(< 0.05–6.94)	0.21(< 0.05–4.45)	0.47(< 0.05–2.79)	0.74(< 0.05–6.94)	
Protein:creatinine ratio (g $gCrea^{-1}$)	0.29 (0–8.99)	0.07 (0–4.47)	0.13 (0–7.72)	1.09 (0–8.99)	
CRP (mg dL^{-1})	0.28(0.02–8.53)	0.15(0.02–4.31)	0.5(0.03–3.87)	0.3(0.04–8.53)	

Patients with urothelial or renal cell carcinoma underwent chemotherapy. Drug abuse could be verified in two patients diagnosed with mid CKD and in one patient with ESRD. Data are given as median with range. CKD, chronic kidney disease.

https://doi.org/10.1371/journal.pone.0174551.t001

Tubes (both provided by Greiner Bio-One International GmbH, Austria). Serum and urine samples were centrifuged at 2000 RCF for 10 min at 4˚C. Aliquots were then transferred in tubes, snap frozen and stored at—80˚C.

TFF2 concentrations were measured by an enzyme-linked immunosorbent assay (ELISA) kit (Human TFF2 DuoSet, R&D Systems, Minneapolis, Minnesota), based on Vestergaard *et al* [16]. The measurement was performed according to the manufacturer's instructions. Serum samples were diluted 1:5 and urine samples 1:125 to obtain protein concentrations within the standard curve. Tetramethylbenzidin (TMB; Sigma, St. Louis, Missouri) was used as substrate and color reaction was stopped by adding 1 N sulfuric acid solution (in aqua bidistilled diluted 95–98% sulfuric acid, Merck KGaA, Darmstadt, Germany). The optical density was immediately measured with a Victor 3 microplate reader at a 450 nm wavelength. To overcome potential methodological deviations, samples were randomized before analysis. Spike/recovery and linearity testing was performed in accordance with the spike, recovery, and linearity protocol for validating untested samples of R&D systems. Therefore, known concentrations of TFF2 were admixed to urine and serum samples and the used buffer. Additionally, serial dilution of spiked and unspiked samples were performed for linearity testing. By the evaluation of pure and diluted samples (spiked and unspiked) the recovery was obtained. The average recovery was 94% and the average linearity was 105%. Multiple testing on different plates revealed a coefficient of variation of 3% for intra-assay precision and 9% for inter-assay precision. The

kit's sensitivity was obtained by the summation of two standard deviations to the mean optical density of twenty-two zero samples and by the following calculation of the corresponding concentration. The minimum detectable concentration was 23 pg/mL. No cross-reactivity to TFF1 or TFF3 could be detected by testing the kit's specificity.

The fractional TFF2 peptide excretion was calculated using the formula: ((urinary TFF2 peptide x serum creatinine) / (serum TFF2 peptide x urinary creatinine)) x 100.

Statistical analysis

TFF2 concentrations in serum and urine and fractional TFF2 excretion were analysed in all groups (early, mid, and later CKD stages as well as in the control group). Gaussian distribution was assessed with the D'Agostino-Pearson normality test. Normal distribution could not be verified for all analyzed groups. Consequently, the non-parametric Mann-Whitney-test (two-tailed) was used to compare TFF2 levels. TFF2 levels within CKD entities diagnosed in more then 10 patients (glomerulonephritis, vascular nephropathy, diabetic nephropathy) were analysed with the Mann-Whitney-test (two-tailed). After Bonferroni correction for multiple comparisons, an individual $p < 0.016$ was necessary to achieve statistical significance at the 5% level.

The correlation analysis between the TFF2 serum and urine concentrations and several clinical parameters (age, serum creatinine, urinary protein, creatinine clearance, urinary protein:creatinine ratio, eGFR, CRP) were performed by Spearman's correlation coefficient. Unless otherwise specified, data are given as median and range. All statistical analysis was performed with the GraphPad Prism Version 5.01 (GraphPad Software, Inc. California, US).

Results

Absolute and fractional TFF2 levels in CKD stages

Total TFF2 serum concentrations were significantly higher in mid and later CKD stages as compared to healthy controls (Fig 1A). Furthermore, TFF2 serum levels in later CKD stages differed significantly from early stages (Fig 1A). Urine TFF2 levels were significantly higher in early and mid CKD stages as compared to later stages (Fig 1B). The fractional TFF2 excretion was higher in early stages as compared to healthy controls, but not in mid and later stages of CKD (Fig 1C)

Absolute and fractional TFF2 levels in CKD entities

TFF2 serum levels were significantly higher in patients with vascular nephropathy or diabetic nephropathy as compared to patients suffering from glomerulonephritis (Table 2). Fractional TFF2 excretion was significantly elevated in patients with glomerulonephritis as compared to patients with vascular nephropathy (Table 2). No significant differences in TFF2 levels were found between vascular and diabetic nephropathy (Table 2).

Correlations of total TFF serum and urine levels with clinical and kidney function parameters

There was a significant negative correlation between TFF2 serum levels and creatinine clearance in the subgroup of patients with a 24-hour urine analysis (Table 3). Age positively correlated with serum TFF2 levels, but the correlation coefficient was only moderate (< 0.4) (Table 3). There was a significant correlation with TFF2 concentrations and eGFR (Table 3). Serum TFF2 positively correlated with serum creatinine, as depicted in Fig 2A. Urine TFF2

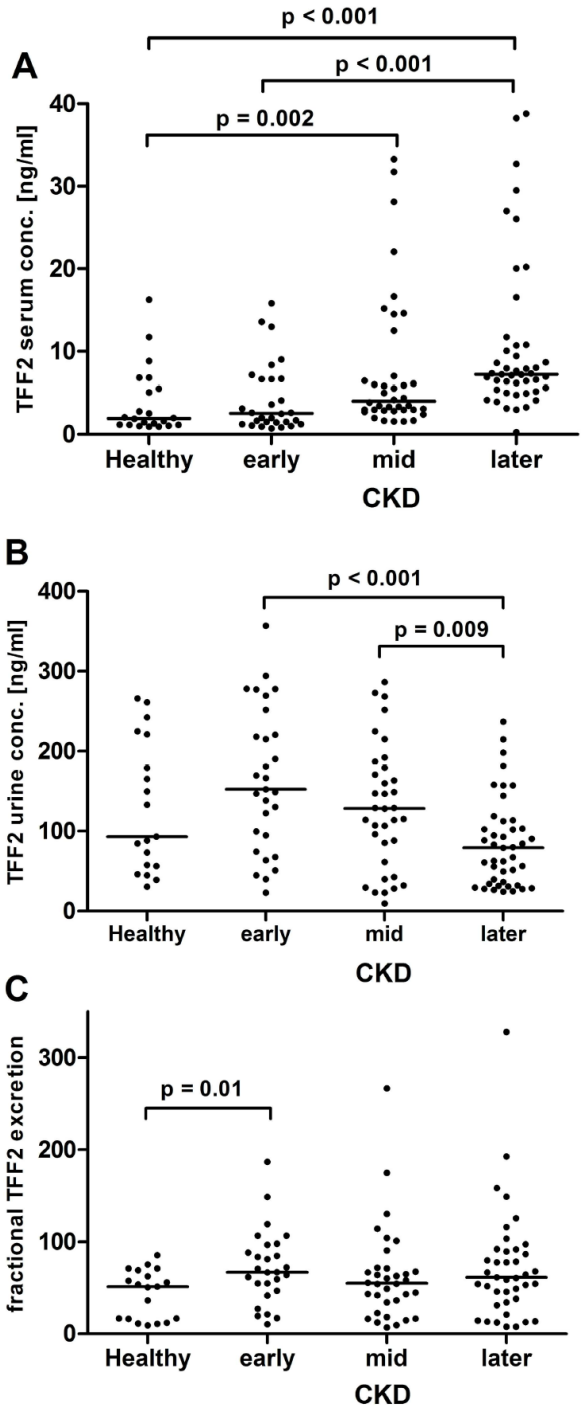

Fig 1. TFF2 levels. Panel A: TFF2 serum levels, one data point outside the axis limits in early CKD stages. Panel B: TFF2 urine concentrations. Panel C: Fractional TFF2 excretion, one data point outside the axis limits in later CKD stages. Each dot represents an individual patient. The line indicates the median. CKD, chronic kidney disease. Only significant p-values are given.

https://doi.org/10.1371/journal.pone.0174551.g001

Table 2. TFF2 serum and urine concentrations as well as fract TFF2 excretion analysed within nephropathies diagnosed in more than 10 patients.

	Glomerulonephritis	Vasc. nephropathy	Diabetic nephropathy
TFF2 serum conc., (ng ml^{-1})	3.6 (1–10)	9.5 (2–33.3)*	13.5 (4.2–28.1)*
TFF2 urine conc., (ng ml^{-1})	114.2 (9.5–272.7)	86.2 (23.3–277.3)	90.4 (29.5–286.6)
fract. TFF2 excretion	67.1 (9.6–328)	16.1 (6.8–77.7)*	38.2 (16.6–130.3)

All patients with the given disease were included, Asterisks indicate significant differences in values between vascular nephropathy or diabetic nephropathy as compared to glomerulonephritis ($p < 0.01$). No significant differences between vascular nephropathy and diabetic nephropathy or within urine concentrations could be found.

https://doi.org/10.1371/journal.pone.0174551.t002

concentrations significantly correlated with serum creatinine (Table 3, Fig 2B). All correlations are given in Table 3 and Fig 2.

ROC curve analysis of total TFF2 serum and urine levels

For ROC analysis data from patients in the early and the later CKD group were included (n = 33 and 46, respectively). As depicted in Fig 3, ROC curve analysis displayed an area under the curve for serum TFF2 of 0.79 (0.70–0.88, $p < 0.001$; Fig 3A) and one optimum cut-off value at 2.986 for the diagnosis of later CKD stages. For the differentiation of early vs. later CKD stages by TFF2 urine concentration, ROC curve analysis revealed an area under the curve of 0.75 (0.63–0.87, $p < 0.001$; Fig 3B) and one best cut-off value at 122.391.

Discussion

We were able to demonstrate significantly higher TFF2 serum levels in patients with mid and later CKD stages as compared to healthy controls. Additionally, early CKD stages differed significantly from later stages. In contrast, TFF2 urine levels declined during CKD progression with significant lower levels in later CKD stages as compared to early or mid stages. TFF2 urine levels in early stages tended to be higher compared to controls, but did not reach statistical significance under the Bonferroni adjustment for multiple comparison. However, fractional TFF2 excretion was significantly higher in early CKD stages as compared to healthy probands. Significantly, the ROC curve analysis showed that TFF2 urine and serum levels may predict different CKD stages. Furthermore, patients suffering from vascular or diabetic nephropathy had significantly higher TFF2 serum levels as compared to patients with glomerulonephritis.

During the progression from kidney disease to end-stage renal failure, persistent inflammation triggers cell damage and tissue degeneration [5,6], often resulting in the need of kidney

Table 3. Correlation of TFF2 serum and urine concentrations with clinical and kidney function parameters.

	Serum TFF2	Urine TFF2
Age (113 pairs)	r = 0.34, p < 0.001*	r = 0.05, p = 0.6
Urine protein (111 pairs)	r = 0.22, p = 0.02	r = 0.05, p = 0.6
Creatinine clearance (32 pairs)	r = - 0.64, p < 0.001*	r = 0.18, p = 0.3
Protein:creatinine ratio(111 pairs)	r = 0.21, p = 0.03	r = - 0.1, p = 0.3
eGFR (113 pairs)	r = - 0.43, p < 0.001*	r = 0.37, p < 0.001*
CRP (113 pairs)	r = 0.22, p = 0.2	r = - 0.18, p = 0.9

Asterisks indicate significance (* $p < 0.001$).

https://doi.org/10.1371/journal.pone.0174551.t003

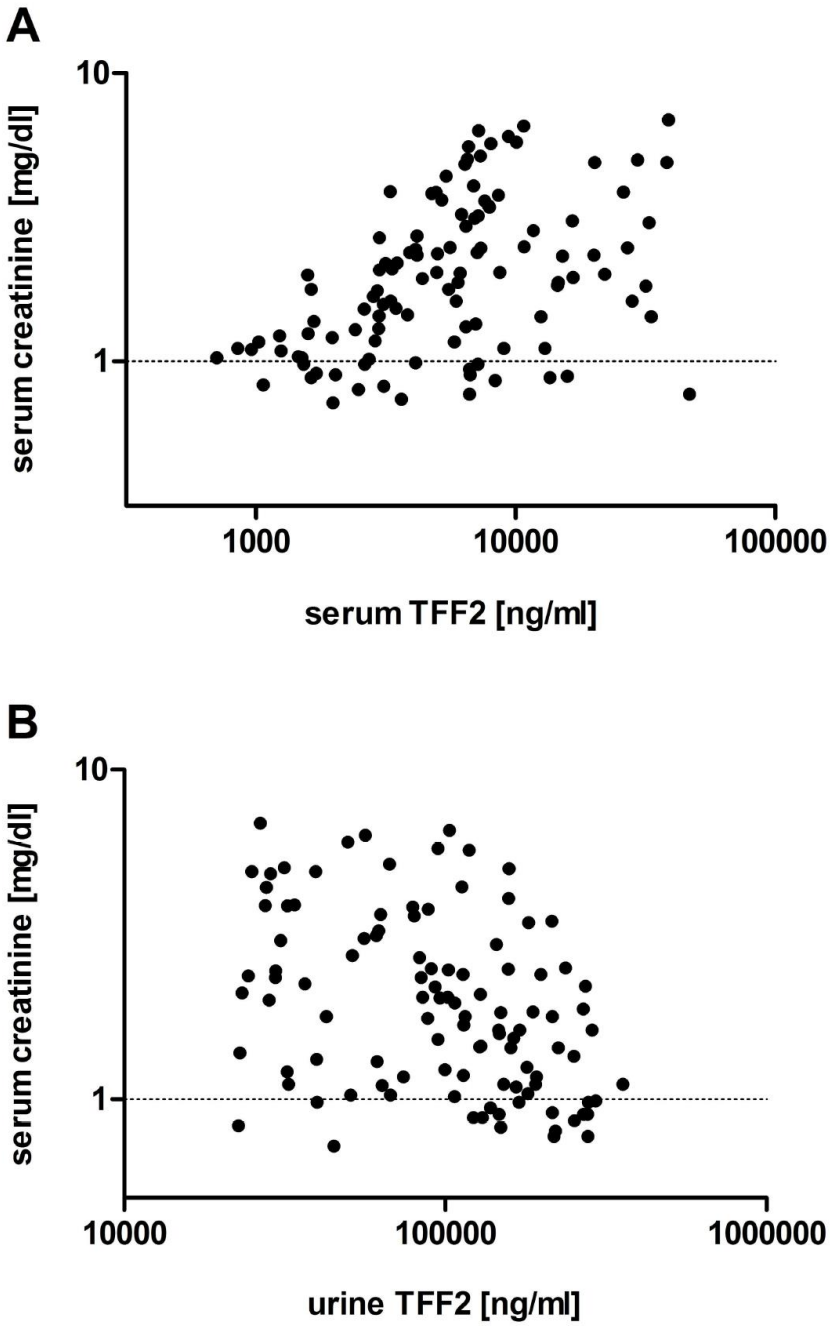

Fig 2. Correlations. Panel A: Serum TFF2 and serum creatinine correlated significantly using Spearman's rank correlation coefficient (Spearman's r = 0.44, p < 0.001, 113 pairs). The X and the Y-axis are given as log scale. Panel B: Urine TFF2 and serum creatinine negatively correlated using Spearman's rank correlation coefficient (Spearman's r = 0.4, p < 0.001, 111 pairs). The X and the Y-axis are given as log scale.

https://doi.org/10.1371/journal.pone.0174551.g002

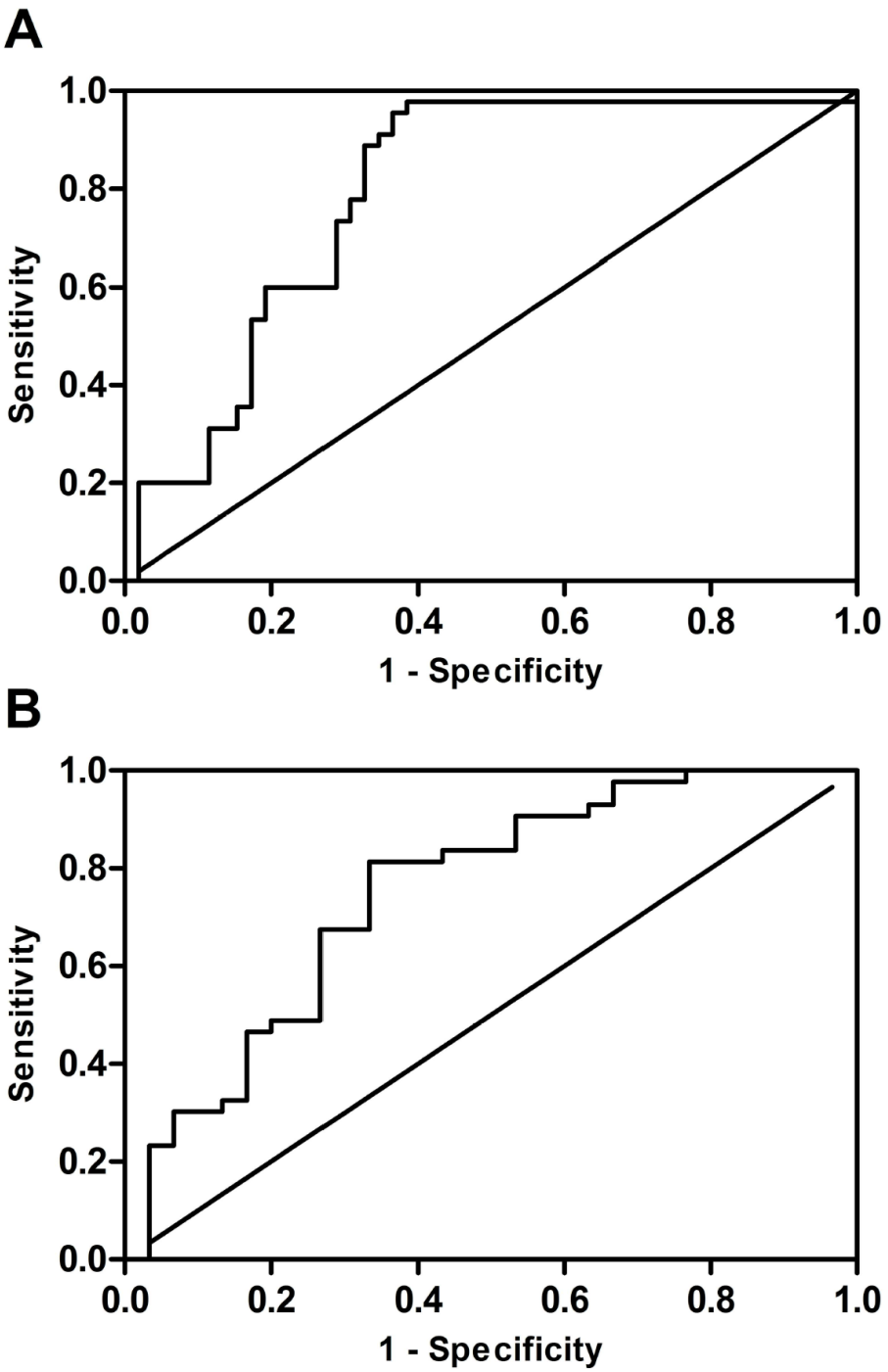

Fig 3. ROC curve analysis. Panel A: ROC curve for serum TFF2 and later CKD stages, AUC 0.79 (0.70–0.88, $p < 0.001$). Panel B: ROC curve for urine TFF2 and early CKD stages, AUC 0.75 (0.63–0.87, $p < 0.001$).

https://doi.org/10.1371/journal.pone.0174551.g003

replacement therapy. To contain the progression of renal dysfunction, counter-regulations like the initiation of a heat-shock response are mounted to prevent cell damage and limit renal cell death [17,18]. TFF peptides hold important functions during epithelial protection and restitution. They facilitate epithelial cell migration to cover damaged areas, constrain proapoptotic signals and promote angiogenesis and leukocyte migration. TFF peptipes have been mostly studied in the gastrointestinal tract and its associated diseases of chronic inflammation such as inflammatory bowel disease, where upregulation of TFF peptides has been demonstrated [16,19].

Lately multiple studies have emphasized the importance of TFF peptipes also in other systems and associated diseases, such as the kidney and the urinary tract [9,10,15,20,21]. In patients with renal cell carcinoma expression of TFF peptides are highly upregulated along the urinary tract with TFF3 being the most prominent one [20]. Analyzed specimens were taken from non-pathological regions and signs of inflammation or neoplastic changes were exclusion criteria. However, the influence of carcinoma diseases on renal peptide expression cannot be finally excluded in non-pathological regions. The same study described increased urinary TFF2 levels in patients suffering from nephrolithiasis and associated TFF2 upregulation during urinary tract infection by so far unpublished results [20]. In patients with CKD increased levels of TFF1 and TFF3 in urine and serum were found and their expression correlated with renal function [9,10]. Consequently, also the involvement of TFF2 in CKD is very likely. To the best of our knowledge, this is the first study evaluating TFF2 levels in chronic renal injury.

Indeed, we were able to demonstrate higher TFF2 urine levels during early kidney diseases and that normalization of TFF2 serum levels is facilitated by increased fractional excretion. Over time, however, as kidney function further declines, the compensatory increase of TFF2 excretion is exhausted, which in turn leads to a successive increase of serum TFF2 levels. A non-significant trend towards increased urinary TFF2 levels in early CKD stages compared to controls was noticed and may reflect the described initial repair response and immediate clearance. In the gastrointestinal tract higher TFF peptide levels are associated with ulceration and inflammation and can be correlated with disease activity [16,19]. Similarly, TFF2 upregulation in chronic renal failure might occur to limit cell death and epithelial damage. The main production site of urinary TFF2 seems to be the kidney, as fractional TFF2 excretion rates above 1 point towards active tubular excretion.

Recently increased urinary TFF1 levels have been detected with the onset of CKD, which normalized with disease progression to levels comparable to that of healthy controls [10]. In contrast TFF3 urine excretion was low in healthy controls and early CKD stages but increased during disease progression. Again, TFF1 and TFF3 serum concentrations only increased with later CKD stages. Interestingly, studies in TFF1 knock-out mice revealed a reduced expression of TFF2, indicating a genetic co-regulation of TFF1 and TFF2 [22]. Their findings are in line with our data collected from urine and serum samples of CKD patients, strengthening the assumption of a TFF1 and TFF2 co-regulation. During the initial phase of renal diseases, TFF1 secretion is increased obviously by elevated urinary levels when compared to healthy probands or later CKD stages [10]. Consequently, higher urinary TFF1 levels and the potentially co-regulated TFF2 might reflect the initial acute phase of renal diseases. In animal models TFF1 and TFF2, but not TFF3 have been shown to be upregulated during the acute phase of gastrointestinal diseases, while TFF3 reemerged during the restitution phase [23–25]. Comparably, TFF3 levels have been shown to constantly increase during CKD progression to end-stage renal failure [9, 10].

ROC curve analysis revealed that by measurement of urine or serum TFF2, different CKD stages could be estimated. Moreover, TFF2 concentrations significantly correlated with serum creatinine, which is the most common routine clinical biomarker used to assess renal function.

Furthermore, the contrary elevation of TFF2 in serum and urine could indicate changes in renal function and might offer potential to examine CKD course and treatment progression, as TFF2 levels significantly correlated with eGFR. This in turn could help to guide the individually adjusted treatment plan.

Besides varying TFF2 levels in different CKD stages, we found significantly increased TFF2 serum levels in patients with vascular or diabetic nephropathy as compared to patients with glomerulonephritis. Even though patient number within renal diseases is low, this finding allows a first insight into differently regulated TFF2 expression rates within various CKD entities.

Though this study generated promising results, we are aware of some methodological limitations. Although the used ELISA kit was reliable in recovery, sensitivity, linearity, and coefficient of variations, it was not designed for use in clinical testing. Samples had to be diluted to obtain TFF2 concentrations within the standard curve, which might have obliterated potential changes in protein levels. By testing undiluted samples a significant change in TFF2 urine concentrations between healthy probands and early CKD stages might be detectable. This adds to the importance of further clinical testing of TFF2 as biomarker for the early detection of CKD. Our study was initially designed to evaluate TFF2 levels irrespective of the underlying kidney disease. As a consequence, the number of patients suffering from rare CKD entities is low and might conceal important findings in those diseases. Therefore, the inclusion of additional patients with different renal diseases and CKD stages would increase statistical power and might help to unravel the role of TFF2 in the acute phase of renal failure and during progression to end-stage renal disease. Furthermore, histological examinations could identify the source of TFF2 expression in the affected kidney which in turn could help to understand the regulation of repair mechanisms within kidney diseases.

Conclusion

We provide evidence of a differential TFF2 concentration pattern in urine and serum of patients suffering from CKD. Whereas TFF2 urine levels abated in mid and later stages as compared to early stages, serum TFF2 concentrations increased progressively in later stages. The disparate TFF2 concentration profile in urin and serum indicates changes in renal function and might offer potential to identify different CKD stages. However, larger clinical studies and longitudinal surveys will be necessary to reveal the role of TFF2 as biomarker in CKD and during progression to end-stage renal disease in different renal diseases.

Author Contributions

Conceptualization: DL GR.

Formal analysis: DL EE FR CK.

Methodology: DL BT.

Project administration: DL GR.

Resources: HA MH TR.

Supervision: HA CK.

Validation: DL BT GR CK.

Writing – original draft: DL GR.

Writing – review & editing: HA MH EE FR GR.

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3. CHAPTER THREE: Discussion

3.1 General Discussion

3.1.1 Chemokine levels in patients with CKD

Inflammation is the body's defense mechanism against invading pathogens and malignant cells while limiting additional collateral injury. Various pathogenic processes, such as infection, ischemia-reperfusion, complement pathway dysregulation, and local immune-complex formation or deposits can induce local inflammation (186). Acute inflammation proceeds until elimination of the inducing origin and is terminated amongst others by leukotrienes, prostaglandins, lipoxins, and neutrophils. Neutrophils thereby restrict inflammation via apoptosis and cytokine clearance (187). The dysregulation of any of these processes can lead to chronic inflammation and might result in chronic diseases with inflammatory components.

Also most renal diseases are associated with a pro-inflammatory state and can be life-threatening conditions such as AKI. In case of chronic renal inflammation, most diseases eventually end up in kidney fibrosis and the need of kidney replacement therapy. CKD is associated with continuous inflammation, adding substantially to the morbidity and mortality of CKD patients (187). The progression of chronic renal failure is further triggered by cytokine release and the elevated production and activity of adhesion molecules, resulting in interstitial T cell invasion and sustained inflammation (186, 187). Ongoing inflammation finally leads to tissue destruction and organ dysfunction even though counter-regulatory mechanisms are induced, obvious by the up-regulation of various mediators for tissue repairing, such as IL- 4, IL-10, tumor growth factor beta, and inhibitors of pro-inflammatory cytokines (18, 187). Inflammation further adds to the mortality of cardiovascular disease that accompanies CKD by promoting vascular calcification and endothelial dysfunction (77, 187).

Leukocytes hold important functions for progression and upkeep of inflammatory processes in renal diseases. Chemokines are thereby essential for the recruitment of immunocompetent cells by generating a chemotactic gradient towards the site of injury. For the exact adjustment of the defined pattern of leukocytes needed at inflammatory sites, subsets of immunocompetent cells express a distinct pattern of chemokine receptors on their surface (104). Furthermore, various chemokines bind to several chemokine receptors in different affinity, allowing altogether the fine-tuning of the immune response to numerous

injuries (104). In the conversion from acute to chronic renal failure, a change in chemokine expression from CXCL chemokines to CCL chemokines has been observed (125). As neutrophils and monocytes promote AKI, the progression of CKD is mainly mediated by the infiltration and activation of macrophages, dendritic cells, and T cells (121). Especially the number of interstitial T cells and macrophages correlates with the renal function and can be seen as prognostic value for several glomerular diseases (188, 189). Patients with ESRF further show decreased relative and absolute lymphocyte counts as well as impaired T-cell proliferation and increased apoptotic turnover rates of immunocompetent cells, adding to the risk of lymphopenia in these patients (80, 91, 169). As serum lymphocyte counts could be negatively correlated with chemokine excretion levels, the increased leukocyte migration into the diseased kidney further seem to contribute to general lymphopenia in patients with ESRD (169).

The trafficking of macrophages, dendritic cells and T cells, which are especially involved in CKD progression, is mediated by chemokines binding to the corresponding receptors on the cells' surface. T helper 2 cells, regulatory T cells (Treg) and dendritic cells express amongst others the chemokine receptor CCR4 on their surface (121) (Fig. 7). As Tregs have anti-inflammatory properties, their increased recruitment into renal tissue can help limiting renal injury (190-192). Tregs further protect against macrophage-dependent damage by suppressing the production of the pro-inflammatory chemokine CCL3 by macrophages (191). In addition, Tregs, some dendritic cells, as well as T helper 17 (Th17) cells also carry the receptor CCR6 (Fig. 7). The renal upregulation of CCL20, which is the ligand of CCR6, resulted in increased T cell recruitment and in general loss of renal function (168). At the same time, the deletion of CCR6 also produced kidney injury and increased mortality in a nephritic mouse model; this is due to the diminished recruitment of Tregs and Th17 cells with no impact on the infiltrating Th1 cells that trigger macrophage-dependent injury by the production of interferon gamma (168). However, the reduction of Treg recruitment on one hand and the functional Th1 response on the other hand is thereby considered to worsen overall kidney function (168). Besides CCR6, Th17 cells also carry the receptor CXCR3 (Fig. 7). Furthermore, the receptor CXCR3 is expressed on the surface of natural killer cells, T helper 1 (Th1) cells, as well as epithelial and endothelial cells (Fig. 7). In CXCR3-knockout mice, glomerular injury and renal T cell infiltration was reduced in lupus nephritis and crescentic glomerulonephritis (167, 193). As CXCR3 is expressed by IL-17-producing Th17 cells and by interferon gamma-producing Th1 cells, CXCR3 seem to be a promising target for therapy of renal damage mediated by T cells (121).

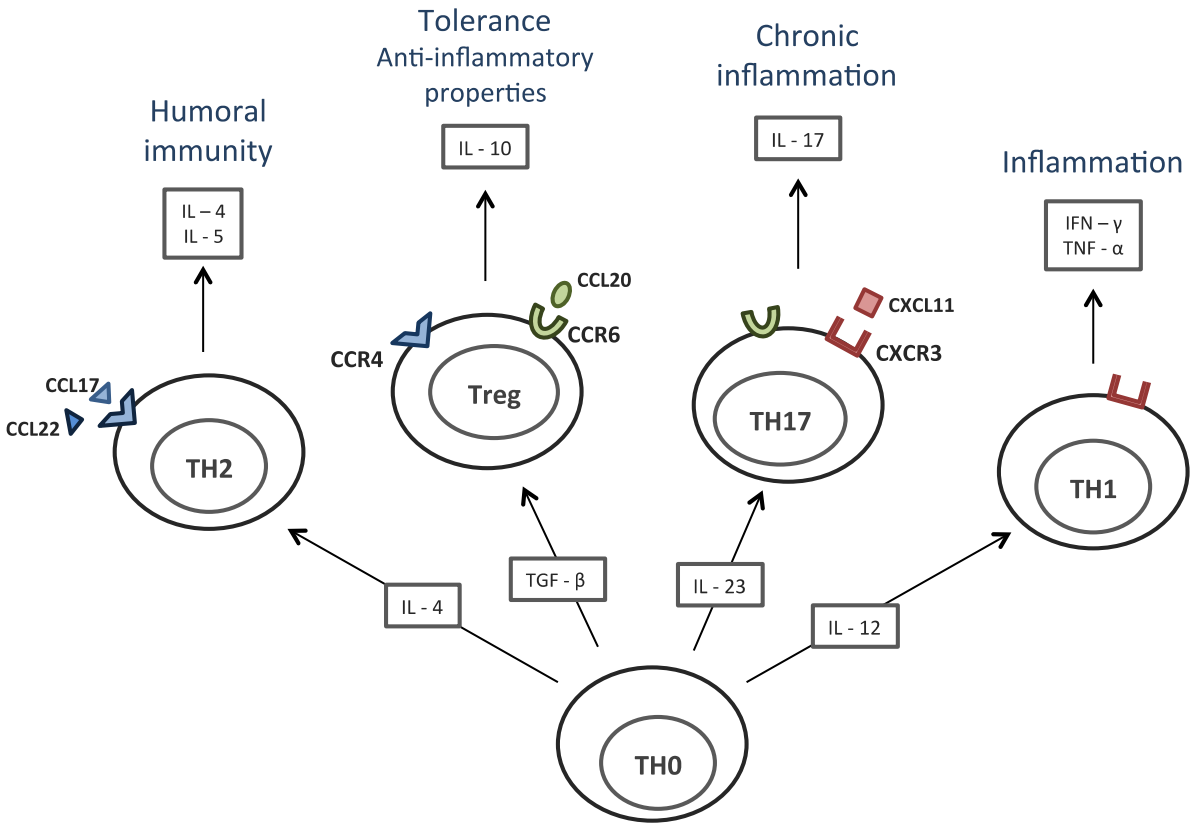

Figure 7: Illustration of analyzed chemokines (CCL17, CCL20, CCL22, CXCL11) and their corresponding receptors (CCR4, CCR6, CXCR3) expressed on T helper cells. Increased CCL20 was associated with elevated T cell recruitment and general loss of renal function. At the same time, in CCR6- knockout mice, kidney injury increased, due to the diminished recruitment of Tregs and Th17 cells with no impact on the infiltrating Th1 cells. Deletion of CXCR3 decreased renal inflammation and renal infiltration of Th17 and Th1 cells.

As before mentioned leukocytes are especially involved in the development and progression of CKD, as well as in the improvement of renal injury, we examined the concentration of the corresponding ligands of the aforementioned chemokine receptors in serum and urine of patients suffering from CKD stages 1 - 5. The analyzed ligands include the chemokines CXCL11, CCL22, CCL20, and CCL17 (Fig 6.). However, we only found selectively significant changes in urinary and serum chemokine levels in patients suffering from CKD when compared to healthy controls (169). In contrast, fractional chemokine excretion revealed continuously increasing chemokine excretion levels for CXCL11, CCL20, and CCL22 during CKD progression, with significant changes in later CKD stages when compared to healthy probands or early stages. As serum chemokine levels mostly remained unaffected by the progression of renal failure, the kidney seems to be the main production site of urinary

chemokines (169). Consequently, elevated fractional chemokine excretion rates did not seem to derive from the accretive disruption of the glomerular filtration barrier that accompanies CKD, but rather reflect a change in tubular function or alterations in chemokine production rates by tubulointerstitial cells during disease progression. The renal source of urinary chemokine levels was further confirmed by the very weak correlation between urinary chemokine concentrations and eGFR, protein or albumin excretion (169). Also current literature supports the assumption of a renal chemokine production by reporting a positive correlation between urinary chemokine concentrations and the degree of inflammatory cell infiltration into the glomerulum and tubulointerstitium (126-128, 194-197).

However, urinary chemokine levels have been shown to predict the progression of different renal afflictions and the outcome of kidney transplants. To mention an example, in patients suffering from lupus nephritis, urinary chemokine excretion reliably predicted disease activity (198). In our study we detected differing chemokine levels mainly in the urine of patients with various CKD etiologies, but we could not verify statistical significance for different afflictions under the Bonferroni adjustment for multiple comparison (169). Apart from varying chemokine levels in renal diseases, the chemokine expression has been shown to increase during allograft rejection (165, 199-201). In a baboon model, urinary levels of CXCR3 ligands predicted acute allograft rejection even before an increase in serum creatinine was evident (165). In patients who underwent kidney transplantation, urinary chemokine levels measured 6 months post operation, effectively predicted late graft outcomes up to 24 months after transplantation and could be correlated with diminishing GFR and graft loss (199-201). In our study, ROC curve analyses revealed the potential of fractional chemokine excretion to identify CKD patients at an early stage and to predict different stages (169).

Taken the data together, chemokine excretion shows promising results to predict and monitor the progression of various kidney diseases as well as acute allograft rejection after kidney transplantation. Furthermore, the analysis of chemokine levels in urine and serum has no side effects on the patients' health, as blood and urine can be collected non-invasively. On the other hand, the chemokine expression can be upregulated during any other acute disease independent of renal failure, leading to false positive findings in view of diagnosis and/or monitoring of kidney disease. Despite those promising results, more investigation is needed to unravel the suitability of chemokine levels as biomarkers for renal diseases and outcome parameters after kidney transplantation.

3.1.2 TFF peptide levels in patients with CKD

Persistent inflammation triggers tissue damage and promotes continuous loss of kidney function creating a vicious circle. Furthermore, increased uremic toxins and oxidative stress are adding to the overall mortality in CKD patients (202). The progression of renal fibrosis eventually ends up in ESRD necessitating kidney replacement therapy. In order to hold the progression of kidney dysfunction, counterregulatory mechanisms are induced to limit cell damage and cell death via apoptosis and necrosis. Anti-inflammatory chemokines, like IL-10, are compensatorily upregulated during CKD progression as a response to the pro-inflammatory milieu (203). Interestingly, higher IL-10 levels were associated with an increased risk of cardiovascular events during the follow-up but, as patients with advanced stages of CKD had higher IL-10 serum levels than patients with lower stages, the authors speculate that IL-10 upregulation signify an anti-inflammatory response to increased inflammatory stress rather than a detrimental effect of IL-10 (203). However, immunomodulatory therapies are in the focus of ongoing research to slow or even hold the progression of chronic renal failure. Antibodies against pro-fibrotic cytokines, such as transforming growth factor beta (TGF-β), and their receptors are tested to slow-down fibrotic changes and avoid tissue loss (204, 205). Local immunotherapy with either IL-10 or TGF-β antagonists resulted in reduced local macrophage infiltration, less apoptosis, and diminished fibrotic changes in an unilateral ureteral obstruction disease model (206). However, as TGF-β is involved in further physiological processes, its inhibition can lead to serious dysfunctions. Consequently, anti-TGF-β therapies are discussed ambivalently in literature, leading to increased research to inhibit solely the pro-fibrotic properties of TGF-β (207, 208).

Beside the direct influence on immunologic processes by the compensatory upregulation of anti-inflammatory chemokines, further cellular mechanisms are initiated to diminish ongoing cell damage or even restore the organ's integrity. One example is the heat shock response, which is induced as a response to various stressful conditions that accompany CKD such as increased oxidative stress or elevated urea concentrations (209, 210). Heat shock proteins (HSP) are intracellularly upregulated when needed to assure cell protection by reassembling protein homeostasis, limit cellular damage, and minimize apoptosis by interfering with apoptotic pathways (209, 210). However, CKD is associated with elevated cell death rates, obvious by increased serum levels of cell death markers (83, 91, 211, 212), which leads to a release of intracellular repair proteins like HSP into extracellular space (213, 214). Thus, the potential of urinary and serum HSP levels as biomarker for CKD has already been described (171, 214).

Besides intracellular defense mechanisms, which are important to limit continuous cell damage during chronic inflammation, some repair proteins, such as TFF peptides, are actively secreted into the extracellular space to restore tissue integrity (Fig. 8). Mucous-producing cells secrete TFF peptides in order to stabilize and protect epithelial integrity and to induce epithelial restitution by promoting cell migration and angiogenesis (130, 131) (Fig. 8). Thus, all TFF members are involved in epithelial restitution and are able to restrict pro-apoptotic stimuli, but they vary in further accessory functions. To quote an example, TFF1 has tumor suppressive properties, which has been demonstrated in a murine gastric cancer model (173). TFF peptides in general, and especially TFF3, are secreted by almost all tissues that contain mucus-secreting cells, but their highest expression rate is located in the gastrointestinal tract (131). In the kidney and the urinary tract, TFF peptides are expressed in a site-specific manner with TFF3 as the most frequent followed by TFF1 (156). In preclinical studies urinary TFF3 is already used as biomarker for renal toxicity in animal models for AKI (158). However, in patients suffering from CKD an upregulation of TFF3 in urine and serum signifies the counterregulation to increased epithelial damage during disease progression (159, 160).

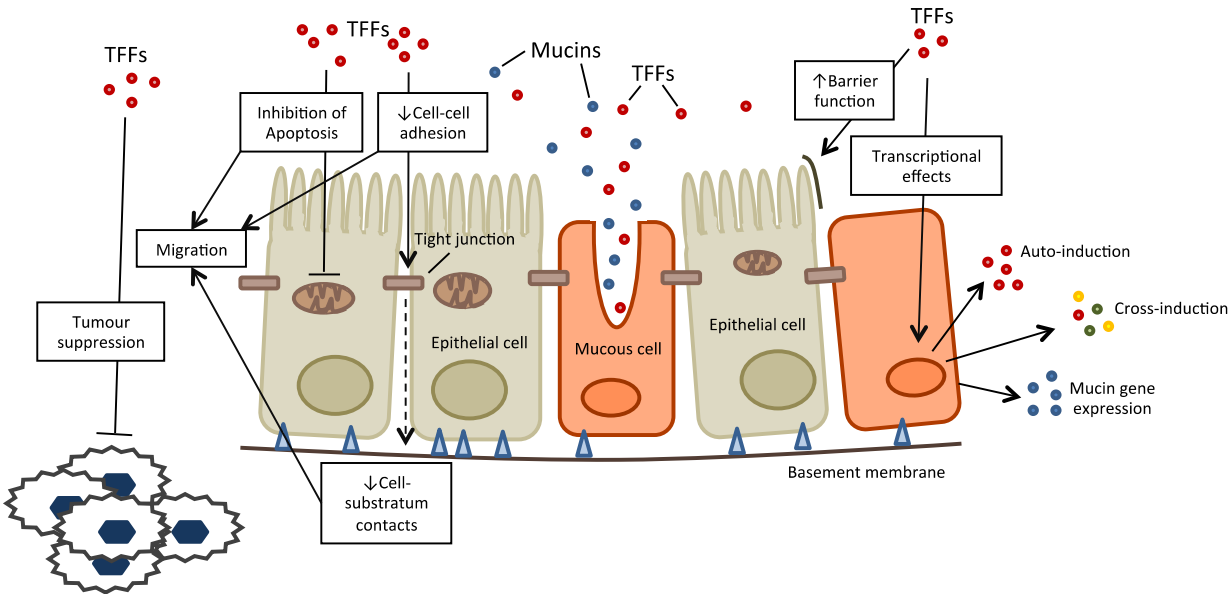

Figure 8: Illustration of TFF peptide functions. Mucous producing cells additionally secret TFF peptides to promote epithelial restitution by limiting apoptosis and minimizing cell contacts. Transcriptional effects of TFF peptides include tumour suppression (TFF1) and induction of mucine gene expression as well as TFF peptide expression through auto- and cross-induction of other TFF peptides. (graph adapted from Taupin and Podolsky, 2005) (131)

In our studies we investigated TFF peptide levels in serum and urine of CKD patients and calculated the fractional TFF peptide excretion to examine renal excretion levels independent from glomerular function. In accordance with literature, we found significantly increased TFF3 serum and urine levels as well as elevated fractional TFF3 excretion rates in higher CKD stages as compared to healthy probands (160, 172). In doing so, TFF3 levels gradually increased during CKD progression with significantly elevated serum levels from stage 3 on and significantly increased excretion levels from stage 4 on (172). Rinnert *et al.* showed that TFF peptides are secreted all along the healthy urinary tract with TFF3 as the most abundant (156). Consequently, the compensatory upregulation of TFF3 during progression of CKD was likely and could be proven within our study and by the study of Du *et al.* (160, 172). However, serum TFF3 levels remained elevated in spite of increased excretion rates, possibly signifying constant upregulation of TFF3 to curtail epithelial damage during disease progression (172).

Comparable to TFF3, also the other members of the TFF peptides are upregulated during inflammation to restore epithelial integrity and limit pro-apoptotic stimuli (130). Thus, we found significantly increased TFF1 and TFF2 serum levels in patients suffering from higher CKD stages (172, 174). In contrast, total urine levels of TFF1 and TFF2 were increased only in early CKD stages and diminished with disease progression to levels comparable to healthy probands. Those findings are surprising and point towards the kidneys as primary production sites of urinary TFF peptides. Fractional TFF peptide excretion rates above 1 are further supporting this assumption, signifying renal cells as main sources of urinary TFF peptides. However, our findings showed that the renal secretion of TFF1 and TFF2 seems to increase during the early phase of kidney diseases (172, 174). This is in accordance with literature, describing an upregulation of TFF2 in patients with acute inflammatory conditions of the urinary tract, such as nephrolithiasis or acute urinary tract infection (156). Also in animal models of inflammatory gastric diseases, an elevation in TFF1 and TFF2 expression could be detected especially during the acute phase of disease (183, 184). Meanwhile, TFF3 was downregulated but reemerged during restitution and chronification (183, 185). Furthermore, murine studies indicate a genetic co-regulation of TFF1 and TFF2, since a diminished TFF2 expression has been demonstrated in TFF1 knock-out mice (173). Within our studies, we also detected a comparable trend of TFF1 and TFF2 levels in CKD patients, strengthening the assumption of a genetic co-regulation of the two peptides.

Taking the data together, we hypothesize that during the acute phase of kidney disease, TFF1 and the co-regulated TFF2 are intensively secreted by epithelial renal cells in order to overcome epithelial destruction. Thereby, TFF1 and TFF2 are immediately excreted

by so far normal renal function and thus can be traceable in the urine of patients with early CKD stages. With ongoing renal inflammation and the chronic manifestation of kidney disease, the TFF3 expression is compensatorily upregulated to additionally limit epithelial cell death and induce restitution. With disease progression glomerular filtration rates continuously decline, possibly constraining the urinary TFF peptide excretion which in turn might add to increasing TFF peptide serum levels.

However, the ROC curve analysis demonstrates the potential of serum and urine TFF peptides to estimate different CKD stages. Furthermore, TFF peptide levels significantly correlated with serum creatinine, the most frequently used biomarker to assess renal function in clinical routine. Therefore, TFF peptides in serum and urine show potential to serve as biomarkers for CKD and could give an insight into disease progression. Especially the disparate trends of TFF1 and TFF2 in serum and urine and the assessment of the differently regulated TFF3 might offer potential to early detect changes in renal function and to identify different CKD stages. Furthermore, the initial increase of urinary TFF1 and TFF2 with the onset of CKD might offer the possibility to detect patients with early CKD stages on time. However, larger clinical studies are obligatory to determine the role of TFF peptides during CKD progression and their applicability as biomarkers for chronic renal failure.

3.2 Conclusion and Outlook

In conclusion, we found a change in the expression rate of pro-inflammatory chemokines during CKD progression on one hand, but also an upregulation of repair proteins on the other.

CKD is associated with ongoing inflammation, which is triggered among others by leukocyte activation via the release of chemokines. During chronic inflammation, chemokines are differently expressed in order to facilitate leukocyte migration towards inflammatory sites. In patients suffering from CKD we were able to demonstrate elevated fractional chemokine expression rates as compared to healthy volunteers. In addition, fractional chemokine expression showed potential to assess different CKD stages, as shown by the ROC curve analysis. In order to overcome inflammatory epithelial destruction and limit cell death, TFF peptides are secreted by mucus-secreting cells lining the kidney. In our studies we detected elevated TFF peptide levels, with TFF1 and TFF2 being differently regulated to TFF3. Especially the initial increase of urinary levels of TFF1 and TFF2 might help to early identify individuals at risk, as shown by the ROC curve analysis.

However, chemokines and the TFF peptides measurement revealed promising results in predicting the course of CKD, but their expression can also be upregulated during inflammatory afflictions independent from renal diseases. To quote an example, increased serum levels of chemokines and TFF peptides have already been detected in patients with inflammatory gastric diseases (180, 181, 215). Therefore, blood withdrawal to examine renal function would have no diagnostic benefit in patients suffering from any inflammatory affliction irrespective of kidney disease.

However, our studies on preclinical biomarker identification have generated promising results, though methodological limitations might have weakened statistical power. Used ELISA kits showed reliable results when tested on sensitivity, recovery and coefficient of variation, but still were not designed for use in clinical testing. Thus, they pose a possible risk for data bias. Furthermore, due to the low number of patients in early CKD stages, some tested groups were not normally distributed. This in turn prompted us to apply a nonparametric statistical test, which again could have weakened statistical power. Moreover, our studies were initially planned to detect differences in protein expression rates during CKD progression and to evaluate the potential of chemokine and TFF peptide levels as biomarkers for CKD regardless of the underlying kidney disease. Consequently, the inclusion of patients with different renal afflictions might have concealed crucial findings in certain diseases.

In summary, chemokine and TFF peptide expression change during the progression of renal afflictions to ESRF. Moreover, all tested proteins show potential to serve as biomarkers for CKD in either identifying patients at an early stage of CKD and/or monitoring the course of the disease. According to Bennet and Devarajan, who classified biomarker development into five phases, our studies fall within phase one, the preclinical discovery (49, 216). Phase one is characterized by the discovery of potential biomarkers in body fluids or tissues and by the prioritization of promising candidates. Phases 2 – 5 include biomarker translation and validation testing (49, 216). Consequently, pending tests to evaluate chemokine and TFF peptide levels as biomarkers for CKD include assay development, retrospective studies, prospective screenings and disease control. Larger clinical studies and longitudinal surveys with a higher number of included patients are therefore obligatory to ultimately disclose the role of chemokines and TFF peptides in chronic renal diseases.

4. CHAPTER FOUR: Materials and Methods

4.1 Subjects

The patients included in this study were diagnosed with CKD stages 1 to 5 in accordance with the criteria of CKD of the NKF (44). All patients were in the outpatient treatment of the Division of Nephrology and Dialysis, Department of Medicine III in the Medical University of Vienna. The control group consisted of healthy probands, who were negating abdominal pain within the last month, pregnancy, or kidney diseases.

4.2 Laboratory analysis

Serum and urine samples were acquired from all study participants; additionally, urine was collected over 24 hours by a subgroup of CKD patients. Samples were collected either in a 9 ml Z Serum Clot Activator Tube or in Urine Tubes. Both tubes were obtained from Greiner-Bio-One International GmbH, Austria. Samples were centrifuged for 10 minutes at 2000 relative centrifugal force. Subsequently, the supernatat was immediately snap frozen in aliquots and stored at – 80 °C.

Serum and urine protein concentrations were measured by using DuoSet ELISA kits of R&D Systems, following the manufacturer's instructions. To minimize methodological aberrations, samples were randomized before analysis. Dilution experiments were performed to evaluate ideal protein concentrations within the standard range. Color reaction was started by adding tetramethylbenzidin, provided by Sigma, and stopped by adding 1 N sulfuric acid solution. Optical density was subsequently measured with a Victor 3 microplate reader at a wavelength setting of 450 nm.

ELISA testing on recovery and linearity were performed in accordance with the instructions of the spike, recovery, and linearity protocol of R&D systems for validating untested samples. In doing so, the defined concentrations of proteins to be tested were added to the urine and serum samples and to the buffer solution, required to perform ELISA analysis. Furthermore, freeze-thawing experiments were conducted to determine the proteins' stability in urine by adding known protein concentrations to urine samples, whereof half were snap frozen and subsequently thawed. For linearity testing pure as well as spiked samples were serially diluted. After measuring the optical density, the diluted, unvalidated

samples had to be parallel to the standard curve. In detail, linearity was assessed by using the formula: % Recovery = (Observed value of dilution / Expected value of dilution) x 100. Concentrations thus obtained were also used for recovery testing by the comparison to undiluted samples. Recovery was estimated with the following formular: % Recovery = ((spiked sample value – unspiked sample value)/ amount spiked into sample) x 100. The acceptance range for linearity and recovery recommended by R &D systems lies between 80 and 120 %. The sensitivity of used ELISA kits was determined by the addition of two standard variances to the mean optical density of a minimum of twenty zero samples.

The fractional protein excretion was calculated to determine the excretion levels irrespective of the glomerular function and was obtained by the application of the following formula: ((urinary protein x serum creatinine) / (serum protein x urinary creatinine)) x 100.

Plasma and serum as well as urine analytes were routinely determined at the Department of Laboratory Medicine at the Medical University of Vienna. The corresponding values were extracted from the electronic case documentation systems routinely used at the Medical University of Vienna.

4.3 Statistical analysis

The total serum and urine protein levels as well as the fractional protein excretion were analyzed between defined groups. For normality testing, the Gaussian distribution was assessed with the D'Agostino-Pearson test. As normality testing revealed no Gaussian distribution within all tested groups, the two-tailed Mann-Whitney-test was used for further analysis. The Bonferroni adjustment for multiple comparisons was applied to avoid the accumulation of alpha-error.

Correlation testing between serum/urine protein levels and extracted clinical parameters was performed for patients as well as for healthy controls using the Spearman's correlation coefficient.

Data are given as median with range, unless otherwise declared.

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Curriculum Vitae

Lebherz-Eichinger Diana, MSc, MD

PERSONAL BACKGROUND

Date of Birth:	January 13th, 1983, Vienna, Austria
Citizenship:	Austria
Family Status:	married since 2007
	three children; born 2008, 2012 and 2016

EDUCATION

2013/04	University Course in Laboratory Animal Science, FELASA class B, GV-SOLAS certified, University of Veterinary Medicine, Vienna, Austria
2011/10 – Present	PhD Student at the Christian Doppler Laboratory for Cardiac and Thoracic Diagnosis and Regeneration, Department of Surgery, Medical University of Vienna, Vienna, Austria
2011/06	Graduation Dr.med.univ. (MD), Medical University of Vienna, Vienna, Austria
2010/02	Graduation Mag.rer.nat. (MSc), University of Vienna, Vienna, Austria
2009/09 – Present	Research Fellow at the RAIC Laboratory 13C1, Department of Anesthesiology, General Intensive Care, and Pain Medicine, Medical University of Vienna, Vienna, Austria
2007/01 - 2009/11	Diploma thesis at the Department of Microbial Ecology (Supervisor: Prof. Michael Wagner), University of Vienna, Vienna, Austria,
2005/10 – 2011/07	Medical Student at the Medical University of Vienna, Vienna, Austria
2005/02 - 2005/07	Student of Molecular- and Marine Biology at the “Vrije Universiteit Brussels”, Brussels, Belgium
2001/10 - 2005/01	Student of Biology and Ecology at the University of Vienna, Vienna, Austria, (supplementary optional subjects: 76 ECTS-credits)

2001/06	High School Graduation
1993 – 2001	High School “Wiedner Gymnasium”, with emphasis on modern languages, Vienna, Austria,
1989 – 1993	Elementary School “Piaristen St. Thekla”, Vienna, Austria

CLINICAL TRAINING

2014/04	Course in Regional Anesthesia
2013/06 – Present	Resident at the Department of Anesthesiology, General Intensive Care and Pain Medicine, Medical University Vienna, Vienna, Austria
2005 – 2011	80 weeks of clinical training as medical student

LABORATORY TECHNIQUES

Clonation and Cultivation of human and bacterial cell lines

DNA and RNA isolation and reprocessing

Enzyme-linked Immunosorbent Assay (ELISA)

Fluorescence activated cell sorting (FACS)

Fluoreszenz-in-situ-Hybridization (FISH)

Histological Practice

Practice in large and small animal testing

(burn model – pig, CLP model – mouse, acute liver failure – rat)

Microarray

Microbial phylogenetic analysis with ARB and other programs

PCR and qPCR

Southern- and Western blot

Transendothelial electrical resistance (TEER)

Utilization of Good Clinical and Scientific Practice including protection of human and animal rights and implementation of international quality standards and operating procedures

REVIEW ACTIVITY

Biomarkers, Cell Stress and Chaperones, Clinical and Experimental Medicine

CONGRESSES AND MEETINGS

2010/09	LICAGE Meeting of the Liver Intensive Care Group - Pisa
2010/12	2nd EACTS Meeting on Cardiac and Pulmonary Regeneration - Vienna
2011/05	ESBACC 1st European Symposium on Bariatric Anaesthesia and Critical Care - Vienna
2011/05	EAROC International Symposium: The morbidly obese adolescent bariatric surgery - Vienna
2011/09	AIC - Annual Conference of the Austrian Organisation of
2013/09	Anaesthesiology, Reanimation and Intensive Care - Vienna
2014/01	18th Conference of the Austrian Organisation of Maxillofacial Surgery - Mayrhofen
2014/10	27th ESICM Annual Congress
2014/11	AIC - Annual Meeting of the Austrian Society of Anesthesia, Reanimation and Intensive Care Medicine

MISCELLANEOUS ACTIVITIES

2008/10	Investigation of patient satisfaction at the Department of
2010/09	Anaesthesiology and Intensive Care Medicine,
	"Krankenanstalt Rudolfstiftung", Vienna, Austria
2004/06 - 2006/02	Employment at the Zoo-pedagogic Department, Zoological Garden of Vienna, Vienna, Austria
2004/08	Internship at the Department of Technical Quality Management, Laboratory, OMV Refining & Marketing GmbH
2003/07	Internship at the Zoological Garden of Vienna, Vienna, Austria
1999/07	Assistant of Ibolya Crepaz, MD, and Manfred Crepaz, MD,
	Dental surgeons

MENTORED DIPLOMA STUDENTS

Bianca Tudor, Camille Fournier, Antonia Frank, Oliver Overheu,

- Medical University of Vienna, Vienna, Austria

PUBLICATIONS

All original articles: 17

First authorship: 8

Roth GA, **Lebherz-Eichinger D**, Ankersmit HJ, Hacker S, Hetz H, Vukovich T, Perne A, Reiter T, Farr A, Hörl WH, Haas M, Krenn CG. **Increased total cytokeratin-18 serum and urine levels in chronic kidney disease.** *Clin Chim Acta. 2011 Apr 11;412(9-10):713-7.*

Mangold A, **Lebherz D**, Papay P, Liepert J, Hlavin G, Lichtenberger C, Adami A, Zimmermann M, Klaus D, Reinisch W, Ankersmit HJ. **Anti-gal titers in healthy adults and inflammatory bowel disease patients.** *Transplant Proc. 2011 Dec;43(10):3964-8.*

Lebherz-Eichinger D, Ankersmit HJ, Hacker S, Hetz H, Kimberger O, Schmidt EM, Reiter T, Hörl WH, Haas M, Krenn CG, Roth GA. **HSP27 and HSP70 serum and urine levels in patients suffering from chronic kidney disease**. *Clin Chim Acta. 2012 Jan 18;413(1-2):282-6.*

Sulyok I, Fleischmann E, Stift A, Roth G**, Lebherz-Eichinger D**, Kasper D, Spittler A, Kimberger O**. Effect of preoperative fever-range whole-body hyperthermia on immunological markers in patients undergoing colorectal cancer surgery.** *Br. J. Anaesth. 2012 Nov; 109(5):754-61.*

Roth GA, Nickl S, **Lebherz-Eichinger D**, Schmidt EM, Ankersmit HJ, Faybik P, Hetz H, Krenn CG. **Lipocalin-2 serum levels are increased in acute hepatic failure.** *Transplant Proc. 2013 Jan; 45(1):241-4.*

Klaus DA, Motal MC, Burger-Klepp U, Marschalek C, Schmidt EM**, Lebherz-Eichinger D**, Krenn CG, Roth GA. **Increased plasma zonulin in patients with sepsis.** *Biochem Med (Zagreb). 2013;23(1):107-11.*

Lebherz-Eichinger D, Krenn CG, Roth GA. **Keratin 18 and Heat shock proteins in chronic kidney disease.** *Advances in Clinical Chemistry 2013; 62:123-149*

Lebherz-Eichinger D, Klaus DA, Reiter T, Hörl WH, Haas M, Ankersmit HJ, Krenn CG, Roth GA**. Increased chemokine excretion in patients suffering from chronic kidney disease.** *Transl Res. 2014 Dec;164(6):433-43.*

Motal MC, Klaus DA, **Lebherz-Eichinger D**, Tudor B, Hamp T, Wiegele M, Seemann R, Krenn CG, Roth GA. **Increased plasma vaspin concentration in patients with sepsis: an exploratory examination.** *Biochem Med (Zagreb). 2015;25(1):90-6.*

Schiefer J, **Lebherz-Eichinger D**, Erdoes G, Berlakovich G, Bacher A, Krenn CG, Faybik P. **Alterations of Endothelial Glycocalyx During Orthotopic Liver Transplantation in Patients With End-Stage Liver Disease.** *Transplantation. 2015 Mar 9.*

Lebherz-Eichinger D, Gutenbrunner B, Reiter A, Roth GA, Herold CJ, Crenn K. **Omitting routine chest radiographs in intensive care units: the economic impact.** *ICU Management, 2015;15(2):54-8.*

Lebherz-Eichinger D, Schwarzer R, Motal MC, Klaus DA, Mangold A, Ankersmit HJ, Berlakovich GA, Krenn CG, Roth GA. **Liver transplantation reverses hypergammaglobulinemia in patients with chronic hepatic failure.** *Biochem Med (Zagreb). 2015 Jun 5;25(2):252-61.*

Lebherz-Eichinger D, Tudor C, Ankersmit HJ, Reiter T, Haas M, Roth-Walter F, Krenn CG, Roth GA. **Trefoil factor 1 is increased in early stages of chronic kidney disease.** *PLoS One. 2015 Sep 21;10(9):e0138312.*

Klaus DA, Seemann R, Roth-Walter F, Einwallner E, Motal MC, Tudor B, **Lebherz-Eichinger** D, Wiegele M, Krenn CG, Roth GA. **Plasma levels of chemokine ligand 20 and chemokine receptor 6 in patients with sepsis: A case control study.** *Eur J Anaesthesiol. 2016 May;33(5):348-55.*

Lebherz-Eichinger D, Tudor B, Krenn CG, Roth GA, Seemann R. **Impact of different sedation protocols and perioperative procedures on patients admitted to the intensive care unit after maxillofacial tumor surgery of the lower jaw: A retrospective study.** *J Craniomaxillofac Surg. 2016 Apr;44(4):506-11.*

Hacker S, Mittermayr R, Nickl S, Haider T, **Lebherz-Eichinger D**, Beer L, Mitterbauer A, Leiss H, Zimmermann M, Schweiger T, Keibl C, Hofbauer H, Gabriel C, Pavone-Gyöngyösi M, Redl H, Tschachler E, Mildner M, Ankersmit HJ. **Paracrine Factors from Irradiated Peripheral Blood Mononuclear Cells Improve Skin Regeneration and Angiogenesis in a Porcine Burn Model.** *Sci Rep. 2016 Apr 29;6:25168*

Lebherz-Eichinger D, Tudor B, Ankersmit HJ, Reiter T, Haas M, Einwallner E, Roth-Walter F, Krenn CG, Roth GA**. Increased trefoil factor 2 levels in patients with chronic kidney disease**. PLoS One. 2017 Mar 29;12(3):e0174551

SUBMITTED MANUSCRIPTS (1)

Lebherz-Eichinger D, Tudor B, Krenn CG, Roth GA, Seemann R. **Impact of different sedation protocols and perioperative procedures on patients admitted to ICU after maxillofacial tumor surgery of the upper jaw: a retrospective study.**

ABSTRACTS (14)

Mitterbauer A, Wechselauer J, Hacker S, Mangold A, Nickl S, **Lebherz-Eichinger D**, Werba G, Hoetzenecker K, Janig F, Kortüm B, Liepert J, Ankersmit HJ, Lichtenauer M. **Stability of Chemokine Levels in Serum and Plasma: Influence of Temperature and Time of Measurement**, *ÖKG-Jahrestagung 2010*

Lebherz-Eichinger D, Roth GA, Zimmermann M, Schmidt EM, Faybik P, Hetz H, Ankersmit HJ, Krenn CG. **Elevated Interleukin 33 and soluble ST2 serum levels in patients with liver failure**, *LICAGE Meeting 2010*

Roth GA, Sipos W, Böhmdorfer M, Schmidt EM, Hetz H**, Lebherz-Eichinger D**, Ritzmann M, Jäger W, Krenn CG. **The effect of the molecular adsorbent recirculating system (MARS) on Moxifloxacin clearance**, *LICAGE Meeting 2010*

Roth GA, **Lebherz-Eichinger D**, Ankersmit HJ, Hacker S, Vukovich T, Perne A, Reiter T, Farr A, Hörl WH, Haas M, Krenn CG. **Increased total Cytokeratin-18 serum and urine levels in chronic kidney disease**, *AIC 2010*

Lebherz-Eichinger D, Schmidt EM, Klaus D, Haas M, Roth GA, Krenn CG. Heat shock protein levels in patients suffering from chronic kidney disease*, AIC 2011*

Schmidt EM, **Lebherz-Eichinger D**, Hetz H, Klaus D, Krenn CG, Roth GA. **Effect of hypernatremia on the permeability of human vascular endothelial cells**, *AIC 2011*

Klaus DA, Roth GA, Motal MC, Schmidt EM, **Lebherz-Eichinger D**, Krenn CG. **Zonulin – a novel marker of gut wall integrity during sepsis,** *AIC 2011*

Lebherz-Eichinger D, Schwarzer R, Schmidt EM, Motal M, Klaus D, Ankersmit HJ, Krenn CG, Roth GA. **The effect of therapy options on immunoglobulin levels in patients with liver failure,** *8th YSA-PhD Symposium 2012, ESICM 2012*

Hacker S, Mittermayr R, Mildner M, Haider T, Nickl S, Zimmermann M, Beer L, **Lebherz-Eichinger D**, Schweiger T, Mitterbauer A, Keibl C, Werba G, Frey M, Ankersmit HJ. **Regenerative effects of secreted factors derived from peripheral blood mononuclear cells in cutaneous wound healing after full-thickness skin defects, burn and skin grafting: results of animal studies**. *10th IQUAM congress and consensus conference 2012, Österreichischer Chirurgenkongress 2013*

Lebherz-Eichinger D, Roth GA, Tudor B, Krenn CG, Seemann R. **Auswirkung verschiedener Sedierungsschemata und chirurgischer Vorgehensweisen auf die Länge des postoperativen Intensivaufenthaltes nach tumorchirurgischen Eingriffen des Kiefer-Gesichtsbereiches**, *18th Conference of the Austrian Organisation of Maxillofacial Surgery – Mayrhofen, 2014*

Lebherz-Eichinger D, Gutenbrunner B, Reiter A, Tudor B, Roth GA, Krenn CG. **Differences in economic effects of abandoning routine chest radiographs in academic and regional care Intensive Care Units**. *ESICM Annual Congress 2014*

Lebherz-Eichinger D, Roth GA, Tudor B, Krenn CG, Seemann R. **Impact of different sedation protocols and perioperative procedures on patients admitted to an ICU after maxillofacial tumour surgery.** *ESICM Annual Congress 2014, AIC 2014*

Tudor B, Wodack KH, **Lebherz-Eichinger D,** Trepte CJ, Roth GA, Reuter DA, Krenn CG. **Altered electrical activity of the heart in pigs during apnoe**. *ESICM Annual Congress 2014, AIC 2014*

Tudor B, Lahner M, **Lebherz-Eichinger D**, Roth GA, Krenn CG. **Unasyn® causes QT prolongation during treatment of intensive care patients**, *ISICEM 2016*

PRESENTATIONS (7)

Lebherz-Eichinger D, Roth GA, Zimmermann M, Schmidt EM, Faybik P, Hetz H, Ankersmit HJ, Krenn CG. **Elevated Interleukin 33 and soluble ST2 serum levels in patients with liver failure**. *LICAGE Meeting 2010*

Lebherz-Eichinger D, Schmidt EM, Klaus D, Haas M, Roth GA, Krenn CG. Heat shock protein levels in patients suffering from chronic kidney disease. *AIC 2011*

Schmidt EM, **Lebherz-Eichinger D**, Hetz H, Klaus D, Krenn CG, Roth GA. **Effect of hypernatremia on the permeability of human vascular endothelial cells.** *AIC 2011*

Lebherz-Eichinger D, Schwarzer R, Schmidt EM, Motal M, Klaus D, Ankersmit HJ, Krenn CG, Roth GA. **The effect of therapy options on immunoglobulin levels in patients with liver failure.** *ESICM 2012*

Lebherz-Eichinger D, Roth GA, Tudor B, Krenn CG, Seemann R. **Auswirkung verschiedener Sedierungsschemata und chirurgischer Vorgehensweisen auf die Länge des postoperativen Intensivaufenthaltes nach tumorchirurgischen Eingriffen des Kiefer-Gesichtsbereiches**. *18th Conference of the Austrian Organisation of Maxillofacial Surgery – Mayrhofen, 2014*

Lebherz-Eichinger D, Gutenbrunner B, Reiter A, Tudor B, Roth GA, Krenn CG. **Differences in economic effects of abandoning routine chest radiographs in academic and regional care Intensive Care Units.** *ESICM 2014*

Lebherz-Eichinger D, Roth GA, Tudor B, Krenn CG, Seemann R. **Impact of different sedation protocols and perioperative procedures on patients admitted to an ICU after maxillofacial tumour surgery.** *ESICM 2014, AIC 2014*