Universitair Ziekenhuis Gent

UNIVERSITEIT GENT

Ghent University
Faculty of Medicine and Health Sciences
Department of Internal Medicine
Nephrology Division

MICRO-INFLAMMATION AND CARDIOVASCULAR DISEASE IN CHRONIC KIDNEY DISEASE: ROLE OF THE UREMIC PEPTIDES

Nathalie NEIRYNCK

Promotoren:

Prof. em. dr. Raymond Vanholder

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Thesis submitted in fulfillment of the requirements for the degree of
'Doctor in Medical Sciences'
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ABBREVIATION LIST

ACR	Albumin to creatinine ratio
ADMA	Asymmetric dimethyl arginine
AER	Albumin excretion ratio
B2M/β2M	β-2-microglobulin
BIS	Berlin Initiative Study
Ca	Calcium
CD	Cluster of differentiation
CI	Confidence interval
CGA	Cause, GFR, albuminuria
CKD	Chronic Kidney Disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CONTRAST	Convective Transport Study
Crea	Creatinine
^{51}Cr –EDTA	^{51}Cr-ethylenediaminetetraacetic acid
CRIC	Chronic Renal Insufficiency Cohort
CRP	C-reactive protein
CVD	Cardiovascular disease
CystC	Cystatin C
D/Da	Dalton
DDAH	Dimethylarginine dimethylaminohydrolase
DTPA	Diethylenediaminepentaacetic acid
DPBS	Dulbecco's phosphate buffered saline
eGFR	Estimated glomerular filtration rate
ESHOL	Estudio de Supervivencia de Hemodiafiltración On-Line
ESKD	End Stage Kidney Disease
EUTox	European Uremic Toxins Work Group
FDA	Food and Drug Administration
FGF-23	Fibroblast growth factor-23

fMLP	Formyl-methionine-leucine-phenylalanine
GC	Gas chromatography
GFR	Glomerular Filtration Rate
HEMO	Hemodialysis Study
HR	Hazard ratio
IDEAL	Initiation of Dialysis Early versus Late trial
IDMS	Isotope dilution mass spectrometry
Ig-λ	Immunoglobulin light chain lambda
Ig-κ	Immunoglobulin light chain kappa
IL1β	Interleukin 1-beta
IL6	Interleukin 6
IL18	Interleukin 18
KDIGO	Kidney Disease Improving Global Outcomes
KDOQI Initiative	National Kidney Foundation's Kidney Disease Outcomes Quality
LAL	Limulus Amebocyte Lysate
LC	Liquid chromatography
LMWP	Low molecular weight protein
LPS	Lipopolysaccharide
MDRD	Modification in Diet and Renal Disease
mGFR	Measured glomerular filtration rate
MPO	Membrane Permeability Outcome Study
MS	Mass spectrometry
mTNFR	Membrane tumor necrosis factor receptor
MW	Molecular weight
NAC	N-acetylcysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NIST	National Institute for Standard and Technology
NF-κB	Nuclear-factor kappa B
NO	Nitric oxide

PAD	Peripheral artery disease
PMA	Phorbol myristate acetate
PTH	Parathyroid hormone
R^2	Coefficient of determination
RCT	Randomized controlled trial
RbP	Retinol binding protein
ROS	Reactive oxygen species
SDMA	Symmetric dimethyl arginine
sTNFR	Soluble tumor necrosis factor receptor
^{99m}Tc –DTPA	^{99m}Tc-diethylenediaminepentaacetic acid
TLR	Toll like receptor
TNFα	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
VSMC	Vascular smooth muscle cell

CHAPTER 1
INTRODUCTION

CHAPTER 1.1

CHRONIC KIDNEY DISEASE AND ASSESSMENT OF KIDNEY FUNCTION

1.1.1 Definition and staging of Chronic Kidney Disease

In 2002, the National Kidney Foundation's Kidney Disease Outcomes Quality Initiative (KDOQI) defined chronic kidney disease (CKD) as abnormalities of kidney structure or decreased renal function, defined as a glomerular filtration rate (GFR) of < 60 ml/min/1.73m², present for > 3 months, irrespective of the underlying cause of renal disease. (table 1) CKD was classified into 5 stages based on GFR: ≥ 90 ml/min/1.73m² (stage 1), 60-89 ml/min/1.73m² (stage 2), 30-60 ml/min/1.73m² (stage 3), 15-30 ml/min/1.73m² (stage 4) and < 15 ml/min/1.73m² or renal replacement therapy (stage 5).[1] Stages 1 and 2 can as such be accepted only in the presence of concomitant kidney damage. (table 1)

Table 1: Criteria for the definition of chronic kidney disease (CKD) (either one of the following present for > 3 months) [1,2]

Markers of kidney damage (at least one)	- Albuminuria: defined as urinary albumin excretion ratio (AER) ≥ 30 mg/24 hours or albumin to creatinine ratio (ACR) ≥ 30 mg/g (normal < 10 mg/g) - Urine sediment abnormalities, such as hematuria, leukocyturia, casts, oval fat bodies - Electrolyte and other abnormalities due to tubular disorders, e.g. renal tubular acidosis, genetic tubular disorders, nephrogenic diabetes insipidus - Structural abnormalities detected by kidney imaging, e.g. cysts, masses, vascular abnormalities, hydronephrosis - Renal transplantation
Decreased GFR	GFR < 60 ml/min/1.73m². (normal: healthy young individual ~125 ml/min/1.73m²)

Mainly based on the GFR criterion, the prevalence of CKD in the general population is estimated around 5-10 %.[3-6] The aim of this conceptual model for CKD is earlier identification of patients at risk for complications, a poor prognosis or adverse outcomes.[7] In epidemiological studies following the 2002 guidelines, GFR as well as

albuminuria were found to be independently associated to mortality, adverse cardiovascular outcome and progression of kidney disease.[8-13] In 2012, an update of the guidelines regarding diagnosis and classification of CKD was published by Kidney Disease Improving Global Outcomes (KDIGO), adding albuminuria to GFR in the classification system, distinguishing CKD stage 3a (45-59 ml/min/1.73m²) and 3b (44-30 ml/min/1.73m²). (figure 1) In addition, the underlying cause of kidney disease received more emphasis, resulting in a classification system related to cause, GFR and albuminuria category (CGA), although the cause of kidney disease was not included in the grid which is proposed to be used for classification and prognosis. [2]

Figure 1: *CKD-stages based on GFR and albuminuria and associated risk score* [2]

Prognosis of CKD by GFR and Albuminuria Categories: KDIGO 2012				Persistent albuminuria categories Description and range		
				A1 Normal to mildly increased <30 mg/g <3 mg/mmol	A2 Moderately increased 30-300 mg/g 3-30 mg/mmol	A3 Severely increased >300 mg/g >30 mg/mmol
GFR categories (ml/min/ 1.73 m²) Description and range	G1	Normal or high	≥90			
	G2	Mildly decreased	60-89			
	G3a	Mildly to moderately decreased	45-59			
	G3b	Moderately to severely decreased	30-44			
	G4	Severely decreased	15-29			
	G5	Kidney failure	<15			

Green: low risk (if no other markers of kidney disease, no CKD); Yellow: moderately increased risk; Orange: high risk; Red, very high risk.

In chapter 2 and chapter 5.2 the association between estimated (e)GFR, as kidney function parameter, and uremic retention solutes was investigated. Therefore in this chapter, an overview on the value and the use of GFR and eGFR will be given.

1.1.2 Glomerular filtration rate

GFR is considered as the most relevant marker of kidney function, which is often believed to also grossly reflect tubular and endocrine functions of the kidney, and is defined as the volume of plasma filtered by the glomeruli per unit of time. The normal glomerular filtration rate in healthy young individuals is approximately 125 ml/min/1.73m² (or ~180 L/day) and declines gradually in the general population from the age of 40 years on at a rate of approximately 0.5-1 ml/min/1.73m² per year[2,14], reportedly ranging from 0.4 to 2.6 ml/min/year.[15]

1.1.2.1 Measurement of GFR (mGFR) via exogenous filtration markers

GFR can be measured by exogenous *filtration markers* which are ideally inert, freely filtered by glomerular filtration without tubular reabsorption, secretion or metabolization, and have no extra-renal clearance and no protein binding. Exogenous filtration markers are inulin (MW: 5200 Da), iothalamate (MW: 637 Da), iohexol (MW: 821 Da), ethylenediaminetetraacetic acid (EDTA) (MW: 292 Da) and diethylenediaminepentaacetic acid (DTPA) (MW: 393 Da). In practice some of them are mostly used as radio-isotopically labeled markers, being ^{125}I-iothalamate, ^{51}Cr-EDTA and ^{99}Tc-DTPA.[16,17]

GFR can be measured via an urinary or plasma clearance of one of these markers. Due to differences in analytical and physiological aspects of the different markers, such as limited tubular handling or minimal extra-renal clearance, there is however variability in accuracy between the different methods. Urinary inulin clearance via bladder catheterisation and continuous intravenous infusion was the first technique described and is considered as the absolute gold standard. This method is however only exceptionally applied due to the cumbersome procedure, the analytical difficulties and costs. The urinary clearance of inulin or another marker can be calculated as GFR = [Xu] x Vu/[Xpl] x t, with [Xu] being urinary concentration, Vu : urinary volume, [Xpl] plasma concentration and t : time. Incompleteness of the urine collection is the main source of bias.[16,17] Alternatively, plasma clearance after a single bolus administration can be performed by sampling at multiple time points to calculate GFR from the area under the curve (AUC) of concentration over time.[16-19]

Which technique is used largely depends on local expertise and availability of the marker.

In a meta-analysis comparing the different alternative exogenous filtration markers to urinary inulin clearance as the gold standard, urinary clearance of iothalamate and ^{51}Cr-EDTA and plasma clearance of iohexol and ^{51}Cr-EDTA appeared the methods that gave results that were the closest to those from inulin.[20] In clinical practice the use of these methods is usually reserved for specific indications, such as the evaluation of renal function of living kidney donors, before the administration of toxic drugs cleared by the kidneys (e.g carboplatin) or when a major influence of non-GFR determinants on endogenous markers is expected (see 1.1.2.2 and table 2) . [21]

1.1.2.2 Estimation of GFR (eGFR) via endogenous filtration markers

As already mentioned, measurement of GFR is not routinely done in clinical practice. Usually, GFR is estimated from the serum or plasma concentration of endogenous filtration markers by incorporating them into a formula together with correction factors for important and known non-GFR determinants.

a. Filtration markers

Creatinine (MW 113 Da) is the most widely used endogenous filtration marker, despite major limitations due to the influence of non-GFR determinants on its serum concentration and several analytical flaws. These non-GFR determinants essentially are related to muscle mass and nutritional factors. (table 2) Renal excretion occurs mainly via free glomerular filtration without tubular reabsorption, but with an additional tubular secretion that accounts for 5-10% of the urinary content and is different among individuals.[17,21,22] A small fraction is excreted via the intestine; the degree of removal via this route may increase in renal failure.[23]

Table 2: Major determinants influencing serum creatinine concentration

GENERATION	
Muscle mass	
Gender	Male ↑
Race	Black ↑
Age	Old ↓
Body composition	Amputation ↓ Muscular ↑
Chronic illness	e.g. inflammation, immobilization ↓
Diet	
Meat	↑
Vegetarian	↓
REMOVAL	
Renal excretion	
Glomerular filtration	Main excretion route
Tubular reabsorption	None
Tubular secretion	5-10%, can be blocked by medication e.g. cimetidine, trimetoprim
Intestinal excretion	
Limited, may be increased in uremia	

Although the analytical measurement of creatinine has much improved due to standardization to the international creatinine-reference material (National Institute for Standard and Technology, NIST 967) and calibration to an isotope dilution mass spectrometry (IDMS) traceable reference method, i.e. either gas chromatography (GC)/MS or liquid chromatography (LC)/MS, creatinine concentration determination remains challenging. In routine clinical laboratories the Jaffé method or enzymatic method are used. The traditional Jaffé method is based on the colorimetric reaction due to complex formation between picric acid and creatinine in alkaline milieu and

has the inherent problem of measuring also pseudo-creatinine chromogens, especially proteins and glucose. In the compensated Jaffé method, a mathematical compensation is performed for these pseudochromogens to approach the values of the enzymatic method to achieve IDMS-traceability. The enzymatic creatinine measurement is less prone to bias and does not measure these pseudochromogens, however inherent imprecision, which is present for every laboratory analysis remains. These differences in analysis can result in differences in reference values and are relevant when using creatinine in eGFR-formulae, especially for creatinine concentrations in the lower range (i.e. higher GFR-values).[24-26] Strictu sensu, the urinary creatinine clearance on an urine collection can be used to measure renal function, but the value obtained overestimated true GFR due to the tubular secretion of creatinine. In addition, the accuracy is further skewed by the risk for incomplete urine collection.[17,20]

Cystatin C (MW 13.3 kDa) is present in all nucleated cells and is an alternative endogenous filtration marker, which is entirely filtered by the glomeruli, and under normal conditions entirely reabsorbed and degraded in the tubuli, so that a renal clearance of cystatin C cannot be measured. Although cystatin C is much less influenced by muscle mass and was initially thought to be less dependent on non-GFR determinants compared to creatinine, it has been shown that age, gender, inflammation, hyperthyroidism, body mass index, proteinuria, diabetes and high dose corticosteroid use can influence cystatin C concentration.[27,28] The availability of a reference material for cystatin C (ERM-DA 471/IFCC) since 2011 is an important step towards the calibration of cystatin C, which is desirable before it is introduced as a routine clinical laboratory measurement. However, this standardization is not yet as uniform as for creatinine since a standardized analytical method is not yet available.[24]

Due to the limitations of creatinine and cystatin C as glomerular filtration markers, other markers are under investigation. Especially *beta-trace protein* (MW 19 kDa, 23 – 29 kDa, depending on degree of glycosylation) or L-type prostaglandin D2 synthase and *beta-2-microglobulin* (MW 11.8 kDa) are strongly associated to GFR, respectively with a coefficient of determination (R^2) around 0.756-0.842[29,30] and

0.853-0.938.[30,31] Their possible advantage in comparison to creatinine is the independence on muscle mass, although more and more other non-GFR determinants are recognized influencing their serum concentration such as age, gender and in the case of beta-2 microglobulin, inflammation and malignancy.[31,32] The insufficient knowledge on non-GFR determinants and the lack of analytical standards and validated eGFR-formulae, make them unsuitable for use in current clinical practice. Both are also associated to adverse outcome such as mortality in the general[33,34], CKD[35,36] and hemodialysis population.[36-39]

b. Commonly used eGFR-formulae

The performance of a GFR-formula is mainly dependent on a combination of bias (difference between eGFR and mGFR), precision (i.e. variability of eGFR around mGFR) and accuracy (combination of bias and precision). The 2002 KDOQI guidelines considered a $P_{30,}$ meaning an eGFR within 30% of mGFR, as clinically acceptable and recommended that P_{30} should be achieved in > 90% of the population in validation studies of an eGFR formula.[1] Although this goal has not yet been achieved for none of the available formulae, the use of eGFR is a generally accepted approach for the assessment of kidney function.

Due to the cheap and easily accessible analysis of creatinine, creatinine-based formulae are the most commonly used in clinical practice. The Cockroft-Gault formula was the first formula that was widely used for this purpose. It estimates creatinine clearance, but is not recommended anymore by nephrological societies, amongst others due to its poor accuracy and development based on non standardized creatinine and in a small study population.[40]

Nowadays, the Modification in Diet and Renal Disease-formula (MDRD)[41,42] and the creatinine-based Chronic Kidney Disease Epidemiology Collaboration-formula (CKD-EPI_{crea})[43] formulae are recommended and the most extensively validated. According to the 2012 KDIGO guidelines the use of the CKD-EPI_{crea} formula is preferred.[2] The overall performance of the CKD-EPI_{crea} formula is better compared to the MDRD (P_{30}: 84.1% vs. 80.6% respectively)[43], especially when eGFR is > 60 ml/min/1.73m², with MDRD tending to underestimate GFR in the higher range.[44] A systematic review including 12 studies[45] and comparing the performance of CKD-EPI_{crea} and MDRD

formula, found a P_{30} ranging from 59-95% for both formulae and confirmed the superior accuracy for the CKD-EPI_{crea} in the majority of the studies. However, when GFR was < 60 ml/min/1.73m², MDRD was slightly more accurate compared to the CKD-EPI_{crea}, with a tendency for the CKD-EPI_{crea} to overestimate GFR.[45] A possible explanation for this finding is the difference of mean GFR in the development population of both formulae, being 39 ml/min/1.73m² for the MDRD-formula[41,42] and 68 ml/min/1.73m² for the CKD-EPI formula.[43]

In advanced CKD, the use of eGFR is more debatable. Evans et al.[46] investigated the performance of different formulae in a large cohort of patients with advanced CKD (mGFR of < 30 ml/min/1.73m²). The accuracy of the formulae was lower compared to the higher GFR-range, with P_{30} being 66.8% for the CKD-EPI_{crea} and 65.2% for the MDRD. The best P_{30} was found for the revised Lund-Malmö formula[47] (75.6 %), a formula developed in a Swedisch cohort.[46] In CKD stage 5, the influence of non-GFR determinants on creatinine is more pronounced compared to the populations in which the formulae have been developed, for example due to malnutrition or reduced muscle mass.[48,49] Therefore the use of eGFR is not recommended in CKD stage 5.[50] In advanced CKD, a measured urinary creatinine clearance, especially when measuring an average creatinine and urea clearance can still be a useful alternative.[51]

In general CKD populations, Cystatin C-based formulae perform in most of the studies better compared to creatinine-based formulae, although the absolute increase in P_{30} is often limited to a few percentages. Especially combined creatinine- and cystatine C-formulae have shown to improve the performance of eGFR-formulae. The high analytical costs and lack of standardization of the analytical method limit its introduction in routine clinical practice. However, when eGFR is between 45-59 ml/min/1.73m² without any indication for kidney damage, the 2012 KDIGO-guidelines suggest using the CKD-EPI_{cystC} (P_{30}: 85.9%) or CKD-$EPI_{cystC\text{-}crea}$ (P_{30}: 91.5%) formulae as a confirmatory test.[52] (table 3)

Multiple other formulae based on creatinine and/or cystatin C have been developed, although most of these were not studied as thoroughly as the ones described above. Formulae used in specific populations will be discussed in the next section.

Table 3: Overview of eGFR-formulae discussed in the text

Reference	Formula	Development cohort	External validation cohort
MDRD-formula$_{(4variable)}$ [42]	175 x $Screa^{-1.154}$ x $age^{-0.203}$ [x 0.742 *if female*] [x 1.21 *if black*]	N= 1628 Mean GFR = 39 ml/min/1.73m²	Initially: none N=5504: P_{30}: 83% [53] N= 3896: P_{30}: 80.6% [43]
CKD-EPI$_{crea}$ [43]	eGFR= 141 x $\min(Screa/\kappa,1)^{\alpha}$ x $\max(Screa/\kappa,1)^{-1.209}$ x 0.993^{Age} [x 1.018 *if female*] [x 1.159 *if black*] (κ: 0.7 if female, 0.9 if male; α: -0.329 if female, -0.411 if male)	N= 5504 Mean GFR = 68 ml/min/1.73m²	N= 3896 P_{30}: 84.1%
CKD-EPI$_{cystC}$ [52]	133 × $\min(ScystC/0.8, 1)^{-0.499}$ × $\max(ScystC/0.8, 1)^{-1.328}$ × 0.996^{Age} [× 0.932 *if female*], where min indicates the minimum of Screa/κ or 1, and max indicates the maximum of Screa/κ or 1	N= 3522 Mean GFR = 68 ml/min/1.73m²	N = 1830 P_{30}: 85.9%
CKD-EPI$_{cystC\text{-}crea}$ [52]	135 × $\min(Screa/\kappa, 1)^{\alpha}$ × $\max(Screa/\kappa, 1)^{-0.601}$ × $\min(ScystC/0.8, 1)^{-0.375}$ × $\max(ScystC/0.8, 1)^{-0.711}$ × 0.995^{Age} [× 0.969 if female] [× 1.08 if black], where κ is 0.7 for females and 0.9 for males, α is −0.248 for females and −0.207 for males, min indicates the minimum of Screa/κ or 1, and max indicates the maximum of Screa/κ or 1	N= 3522 Mean GFR = 68 ml/min/1.73m²	N = 1830 P_{30}: 91.5%
Cockroft-Gault [40]	[(140-age) x weight] x [0.85 *if female*]/(Screa x 72)	N= 249 Creatinine clearance: 30-130 ml/min	Initially: none (Cohorts independent of investigators of development)
Lund-Malmö [47]	$\exp^{X - 0.0158 \times age + 0.438 \times \ln(age)}$ Female and Pcrea <150: X = 2.50 + 0.0121 × (150−Pcrea) Female and Pcrea ≥150: X = 2.50 − 0.926 × ln(Pcrea/150)	N = 850 Mean GFR = 55 ml/min/1.73m²	Initially none (Cohorts independent of investigators of development)

	Male and Pcrea <180: X = 2.56 + 0.00968 × (180−Pcrea) Male and Pcrea ≥180: X = 2.56 − 0.926 × ln(Pcrea/180) Pcrea in µmol/L, (conversion from mg/dl: x 88.4)	P_{30}: 85.8%	
$BIS1_{crea}$ (> 70 years) [54]	3736 x $Screa^{-0.87}$ x $age^{-0.95}$ [x 0.82 *if female*]	N= 570, > 70 years Mean GFR =60.3 ml/min/1.73m² P_{30}: 95.1%	Initially none (Cohorts independent of investigators of development: see text)
$BIS2_{cystC-crea}$ (>70years) [54]	767 x $ScystC^{-0.61}$ x $Screa^{-0.4}$ x $age^{-0.95}$ [x 0.87 if female]	N= 570, > 70 years Mean GFR =60.3 ml/min/1.73m² P_{30}: 96.1%	Initially none (Cohorts independent of investigators of development: see text)
Schwartz (revised) (children) [55]	0.413 x [height/Screa]	N= 349 Mean GFR = 41 ml/min/1.73m² P_{30}= 79.4%	Initially none

eGFR: estimated glomerular filtration rate, Screa: serum creatinine, SCystC: serum cystatin C, N: number of patients, P_{30}: accuracy P_{30}, Pcrea: plasma creatinine. The use of serum and plasma for the measurement of creatinine is equivalent.

1.1.2.3 Use of eGFR formulae in specific populations/conditions

a. Elderly

The number of patients older than 70 in the development population of the MDRD[41,42] and CKD-EPI formulae (creatinine and cystatin C-based)[43,52] was small, so that theoretically they should not be used in patients > 70-75 years. Some studies investigated the performance of MDRD and CKD-EPI in elderly and found a P_{30} accuracy ranging from 70 to 86%[56-60], comparable to results found in other populations, with superiority for CKD-EPI_{crea} (P_{30}: 74.7%-83%) and especially CKD-$EPI_{crea-cystC}$ (P_{30}: 85,3%-86%) compared to MDRD (P_{30}: 70.5%-81%), while the results with CKD-EPI_{cystC} (P30: 65.3%-86%) were inconsistent.[58,60]

Recently, the Berlin Initiative Study (BIS), consisting of a healthy cohort aged > 70 years, developed a specific creatinine-based (BIS 1) and creatinine-cystatin C- based (BIS 2) formula to be used in the elderly.[54](table 3) These formulae were validated in independent external cohorts of older patients with good performance (P_{30}: 75-88%)[56-59], which was superior [57,59]or equivalent [56,58] to the CKD-EPI .

b. Children

The Schwarz formula, introduced in 1979 and based on a constant, height and serum creatinine, is the most widely used formula in children. This formula was recently updated for enzymatically determined and IDMS traceable serum creatinine concentrations in a population of 349 children, aged between 1 and 16 years. [55] (table 3)

Also in children, cystatin C-based formulae generally perform better compared to creatinine-based formulae, probably partly due to the fact that cystatin C is less dependent on muscle mass. [61]

c. Race

In the MDRD[42] and CKD-EPI[43] formulae, ethnicity coefficients for blacks are incorporated, to compensate for higher creatinine values at similar GFR compared to whites probably due to a higher muscle mass. This is based on African-American subpopulations in the development cohorts of the formula. To estimate eGFR in

Africans living on the African continent, the use of MDRD or CKD-EPI without the ethnicity coefficients is more appropriate.[62,63]

Various ethnicity coefficients are suggested to be included in the MDRD or CKD-EPI in diverse Asian populations[64-67], although other investigators did not find an improvement in accuracy adding specific correction factors.[68,69]

d. Kidney transplant patients

In kidney transplant patients, the performance of the CKD-EPI$_{crea}$ and MDRD are comparable, although the MDRD performed better when GFR was < 60 ml/min/1.73m² and CKD-EPI$_{crea}$ when GFR was > 60 ml/min/1.73m².[70-73] In stable transplant patients, cystatin C-based formulae (P_{30}: ~80%) were superior compared to creatinine-based formulae (P_{30}: 68-75%). [70,74]

e. Drug dosing

Pharmacokinetic studies for drug dosing are generally based on creatinine clearance or the Cockcroft-Gault formula (table 3) as kidney function parameter. Although initially considered as inappropriate, it is now generally accepted to use eGFR with the same cut-off values for drug dosing, which was supported by the Food and Drug Administration (FDA) in 2010. It is however unlikely that a re-evaluation will be made for the majority of already registered drugs. In patients with extremes in body size, it is advisable to exclude the bias induced by an extrapolated body surface area (i.e. 1.73m²) by multiplying eGFR in ml/min/1.73m² with true individual body surface area to obtain an absolute eGFR in ml/min.[75]

1.1.3 Summary

Chronic kidney disease is classified based on the cause of underlying kidney disease, glomerular filtration rate and albuminuria, in order to stratify patients at increased risk. The CKD-EPI$_{crea}$ formula is acceptable to estimate GFR in the general and CKD populations, although the goal of an accuracy of P_{30} > 90% is not reached. On an individual patient level precision often is lower due to variable influences of non-GFR determinants on the creatinine concentration. Cystatin C- based formulae

or combined creatinine-cystatin C-based formulae often provide a superior performance compared to formulae based on creatinine, but are not yet used in routine clinical practice. mGFR using an exogenous marker can be a suitable alternative in specific indications.

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CHAPTER 1.2

AN UPDATE ON UREMIC TOXINS

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1.2.1 Abstract

In the last decade, uremic toxicity as a potential cause for the excess of cardiovascular disease and mortality observed in chronic kidney disease gained more and more interest. This review focuses on uremic toxins with known cardiovascular effects and their removal. For protein-bound solutes, for example, indoxylsulfate and the conjugates of p-cresol, and for small water-soluble solutes, for example, guanidines, such as ADMA and SDMA, there is a growing evidence for a role in cardiovascular toxicity in vitro (e.g., affecting leukocyte, endothelial, vascular smooth muscle cell function) and/or in vivo. Several middle molecules (e.g., beta-2-microglobulin, interleukin-6, TNF-alpha and FGF-23) were shown to be predictors for cardiovascular disease and/or mortality. Most of these solutes, however, are difficult to remove during dialysis, which is traditionally assessed by studying the removal of urea, which can be considered as a relatively inert uremic retention solute. However, even the effective removal of other small water-soluble toxins than urea can be hampered by their larger distribution volumes. Middle molecules (beta-2-microglobulin as prototype, but not necessarily representative for others) are cleared more efficiently when the pore size of the dialyzer membrane increases, convection is applied and dialysis time is prolonged. Only adding convection to diffusion improves the removal of protein-bound toxins. Therefore, alternative removal strategies, such as intestinal adsorption, drugs interfering with toxic biochemical pathways or decreasing toxin concentration, and extracorporeal plasma adsorption, as well as kinetic behaviour during dialysis need further investigation. Even more importantly, randomized clinical studies are required to demonstrate a survival advantage through these strategies.

1.2.2 Introduction

Knowledge on uremic toxicity has grown spectacularly over the past decades. Although barely discussed until late in the previous century, interest increased exponentially since then. Taking the example of one of the few compounds that have rarely been studied outside the context of uremia, indoxylsulfate, while in 1990 no single publication was devoted to this solute, in 2011 alone the number exceeded 60 (Fig. 1).

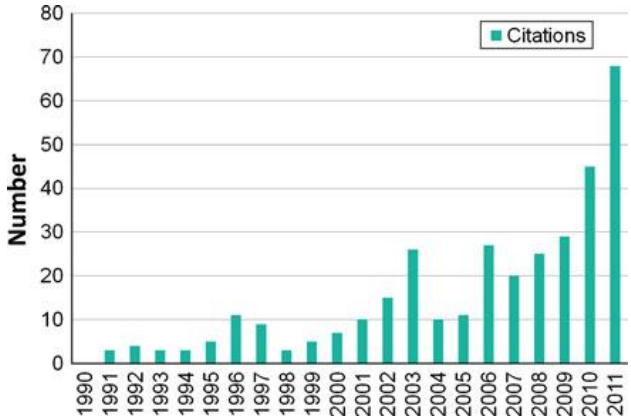

Fig. 1 Number of publications on indoxylsulfate, a prototype protein-bound uremic toxin, over the recent years. Whereas there were virtually no publications in the early nineties, last year over 60 papers were devoted to this issue

In this review, we summarize our view on the most relevant uremic toxins and their toxicity. Although the uremic syndrome affects almost every organ system and function (Table 1), we mainly focus on cardiovascular effects, one of the major sources of morbidity and mortality in uremia [1].

Based on the removal pattern during dialysis, uremic toxins are subdivided into three major classes [2]: (1) the small water-soluble compounds, with an arbitrary upper molecular weight limit of 500 D, easy to remove by any dialysis strategy; (2) the larger middle molecules (>500 D) only removed through dialyzer membranes with enhanced transport capacity and large enough pores (high flux); and (3) protein-bound molecules, mostly with a molecular weight <500 D, but a hampered removal pattern due to protein binding. Representative molecules will be discussed with regard to in vitro and in vivo toxicity, and clinical removal and outcome studies.

Table 1 The most relevant negative effects of uremic solute retention

Anorexia
Anemia
Cardiac failure
Coagulation disorders
Coordination disturbances
Fluid overload
Hyperparathyroidism
Hypertension
Immune dysfunction
Inflammation
Insulin resistance
Loss of strength
Malnutrition
Osteodystrophy
Pericarditis
Polyneuritis
Pruritus
Skin atrophy
Tremor
Vascular disease

The systems marked in italics contribute to cardiovascular morbidity and mortality

1.2.3 The small water-soluble compounds

Of the large number of known small water-soluble uremic compounds [2, 3], we will only discuss urea and the guanidines.

Although urea (60 D) is the prototype of this class, data corroborating its biochemical or biological effects are scanty. In a study from the Mayo Clinic, urea was added for three consecutive months to dialysate up to a concentration substantially exceeding that normally observed pre-dialysis [4]. While all other solutes were removed as usual, blood urea concentration markedly rose, however, without discriminate effects on uremic symptoms. Two randomized controlled trials (RCTs) increased urea removal above standard without improvement in survival rate [5, 6]. In two observational trials, next to urea removal, other factors such as length of dialysis or serum albumin were at least as much associated with outcome [7, 8]. In at least two studies, increasing solute removal without improving urea removal per se improved outcomes [9, 10]. Data in favor of a biochemical effect of urea were often obtained only at supra-physiologic concentrations, with the exception of a recent study, where urea at disease-relevant concentrations in vitro induced free radical production and insulin resistance in adipocytes [11].

The question may be raised whether other water-soluble compounds are characterized by a similar inertia as urea. An important group is composed of the guanidines (Table 2), which have been identified since many years as neurotoxins [12, 13]. Only recently, these compounds were also studied for their cardiovascular impact. Several guanidines were shown to be pro-inflammatory, by activating leukocyte function at the concentrations found in uremia [14, 15]. In addition, these data show that the different uremic toxin groups interfere with each other, as small water-soluble guanidines were responsible for the generation of TNF-α and IL-6, two middle molecules [15, 16].

Table 2 Some of the main guanidino compounds

α-Keto-δ-guanidinovaleric acid
α-*N*-acetylarginine
Asymmetric dimethylarginine (ADMA)
Argininic acid
β-Guanidinopropionic acid
Creatine
Creatinine
γ-Guanidinobutyric acid
Guanidine
Guanidino acetic acid
Guanidinosuccinic acid
Methylguanidine
Symmetric dimethylarginine (SDMA)
Taurocyamine

The list is not exhaustive

Since the guanidines are structurally similar to urea, also a comparable removal pattern could be expected. However, the distribution volume of several guanidines is significantly larger than that of urea, resulting in a decrease in effective removal [17]. Only guanidinosuccinic acid displays a smaller distribution volume than urea [17]. These calculated values were corroborated by direct experimental measurements, showing that guanidino compound concentrations during hemodialysis in the erythrocytes lagged behind versus plasma [18]. Mathematical simulations revealed that the most effective improvement in removal of the guanidines with a large distribution volume was obtained by increasing dialysis length [19]. For guanidinosuccinic acid, however, with its smaller distribution volume, removal was

more effective after increasing dialysis frequency [19]. For all compounds, the combination of frequent and long dialysis was optimal [19]. These differences in kinetic characteristics can only be explained by the low resistance imposed by the cellular wall to urea, which is almost unique for this molecule [20, 21].

Also, the dimethylarginines are guanidines; asymmetric dimethylarginine (ADMA) has since long been recognized as an inhibitor of nitric oxide synthase (NOS) causing endothelial dysfunction and vascular damage [22], a propensity that affects both the general and the uremic population [23–25]. Infusion of ADMA in healthy volunteers to a concentration as in uremia resulted in a decrease in cardiac output and a rise in vascular resistance [26]. In a dialysis population, ADMA concentration was correlated to intima-media thickness, an index of vascular damage [25].

Symmetric dimethylarginine (SDMA), a structural analogue of ADMA, has long been considered inert [22, 27]. Biologic activity was at first suggested by Bode-Boger et al. [28], showing a dose–responsive inhibition of NO synthesis by a mechanism different from that elicited by ADMA. SDMA plays as well a prominent role in leukocyte activation by enhancing generation of radical oxygen species (ROS), which is attributable to increased calcium influx via store-operated Ca^{2+} channels [29] and to activation of NF-κB resulting in cytokine production [16]. Inhibition of NF-κB by N-acetylcysteine (NAC) and of ROS production with SKF96365 and captopril prevented this leukocyte activation [16, 29]. A clinical study in 142 patients with different stages of chronic kidney disease (CKD) demonstrated a correlation of SDMA with TNF-α and interleukin-6 (IL-6) [16], which was markedly more significant than for ADMA [16].

The intradialytic kinetics of both ADMA and SDMA has not thoroughly been studied. Some data suggest that the removal of ADMA in standard dialysis is hampered, eliciting the hypothesis that the compound is protein bound [30], but more likely, removal is hindered by complex kinetics and distribution.

Especially for ADMA, alternative removal pathways have been assessed. In renal failure, ADMA is retained at least in part by the inhibition of the enzyme dimethylarginine dimethylaminohydrolase (DDAH) [31]. Increased expression of this enzyme decreases coronary damage in mice that received a cardiac allograft [32] and decreases angiotensin II-induced organ damage [33]. Vice versa, disruption of

DDAH impairs vascular homeostasis [34]. These studies offer proof of concept that ADMA concentration can be changed by modifying metabolism, with impact on the vascular status. As DDAH is inhibited by hyperhomocysteinemia, a more feasible approach for metabolic manipulation of DDAH and ADMA could be obtained by decreasing homocysteinemia. In a study by Koyama et al. [35], the combination of folate and methylcobalamin decreased ADMA in parallel with a decrease in homocysteinemia.

1.2.4 The middle molecules

The group of middle molecules, defined by a molecular weight > 500 D, is mainly composed of small peptides. Currently, more than 50 solutes comply with this definition [2, 3, 36] (Table 3); many of these are implied in cardiovascular disease, by causing inflammation, endothelial damage, smooth muscle cell proliferation, activation of coagulation or by interfering with calcium/phosphorus household. There is thus a pathophysiologic rationale for optimizing their removal. However, their effect on relevant cell mechanisms at the concentrations occurring in uremia has barely been studied. Data on the association of middle molecule concentrations with clinical outcome parameters are more elaborate.

The most used marker for middle molecule retention and removal is β2-microglobulin. In general, this molecule is, however, considered inert. Nevertheless, Wilson et al. [37] identified by proteomic analysis β2-microglobulin as the most adequate marker of severity of peripheral vascular disease in a population with no or moderate chronic kidney disease. In addition, β2-microglobulin has been associated with arterial stiffness in the general population [38] and bone remodeling in non-CKD postmenopausal women [39].

Table 3 Main middle molecules

Adiponectin	Cystatin C	Leptin
Adrenomedullin	DIP-I	Methionine-enkephalin
AGEs	Delta-sleep-inducing peptide	Motilin
AOPPs	Desacylghrelin	Myoglobin
α1-Acid glycoprotein	Dinucleoside polyphosphates	Neuropeptide Y
α1-Microglobulin	Endothelin	Orexin A
Angiogenin	FGF-23	Orexin B
Angiotensin A	Ghrelin	Osteocalcin
Atrial natriuretic peptide (ANP)	Glutathione	Parathyroid hormone (PTH)
Basic fibroblast growth factor	Guanylin	Prolactin
β-Endorphin	Hyaluronic acid	Resistin
β-Trace protein	Insulin growth factor-1 (IGF-1)	Retinol-binding protein (RBP)
β2-Microglobulin	Interleukin-1β (IL-1β)	SIAM-1
Calcitonin	Interleukin-6 (IL-6)	Substance P
Calcitonin gene-related peptide	Interleukin-10 (IL-10)	Tumor necrosis factor α (TNF-α)
Cholecystokinin	Interleukin-18 (IL-18)	Uroguanylin
Clara cell protein (CC16)	κ-Ig light chain	VEGF
Complement factor D	λ-Ig light chain	Vasoactive intestinal peptide

Data extracted from refs [2, 3, 36]; the list is not exhaustive

AGEs advanced glycation end products, *AOPPs* advanced oxidation protein products, *DIP-I* degranulation-inhibiting protein-I, *FGF-23* fibroblast growth factor-23, *SIAM-1* soluble intracellular adhesion molecule-1, *VEGF* vascular endothelial growth factor

With regard to outcome studies, in two secondary analyses of the HEMO study conducted in hemodialysis patients, β2-microglobulin was related to overall and infectious mortality [40, 41]. In a CKD population with several stages of CKD, interleukin-6 (IL-6) was related to mortality, whereas there was no association for tumor necrosis factor-alpha (TNF-α)[42]. In contrast, in a hemodialysis population, TNF-α was a stronger predictor of mortality than IL-6 [43]. Fibroblast growth factor-23 (FGF-23), a molecule essentially linked to bone mineral homeostasis, has been associated with progression of kidney failure [44], cardiac dysfunction [45] and overall mortality [46, 47]. Although merely seen as a marker, a recent study in animals showed a direct hypertrophic effect on the heart after chronic injection [48]. Also, these data thus suggest that middle molecule removal could favour outcome.

Increasing dialyzer pore size by applying high-flux membranes resulted in an increased removal of β2-microglobulin [49] and a decrease in pre-dialysis β2-microglobulin over time [50], with a further increase by adding convection [51–53] or by applying newer high-flux membranes with more homogeneous pores [54].

Removal of β2-microglobulin is not necessarily representative for that of other middle molecules. In a study by Ward et al. [55], the transition from high-flux hemodialysis to predilution online hemodiafiltration had a different impact on complement factor D

versus β2-microglobulin. In a study by Meert et al. [54], the change from first-to second-generation high-flux membranes resulted in more improvement in removal as the molecular weight of the studied molecules increased. All these data stress the need for thorough studies of the removal pattern of middle molecules at large, but kinetic analysis has up to now only been applied to β2-microglobulin. When evaluating the concentration pattern of β2-microglobulin during high-flux hemodialysis, Leypoldt et al. [56] demonstrated a ± 25 % decrease at the end of dialysis, which was, however, to a large extent neutralized by a postdialysis rebound phenomenon, pointing to a substantial multicompartmental distribution. Although the distribution volume of β2-microglobulin is 3–4 times smaller than that of urea, the shift from the extra-plasmatic to the plasmatic compartment is decreased almost 20-fold, hampering removal [57, 58].

The slow intercompartmental clearance of β2-microglobulin suggests that extended dialysis might benefit removal. Comparing 4-, 6-and 8-h dialyses in the same patients while dialyzer surface, total blood flow and dialysate flow per session were kept the same, β2-microglobulin removal into the dialysate increased by approximately 80 % with longer dialyses in spite of similar Kt/Vurea [17, 59].

In a series of secondary analyses of randomized controlled trials, increasing dialyzer pore size by applying high-flux membranes resulted in better outcomes compared to small-pore, low-flux dialysis [5, 60–63]. In the Membrane Permeability Outcomes (MPO) study, high-flux hemodialysis resulted in better survival outcomes in the subgroup with a serum albumin below 4 g/dL [10], which is the group targeted in the original study protocol [64]. Hypoalbuminemia is a feature of a large section of the current dialysis population [65].

When adding convection, RCTs showed a lower incidence of intradialytic hypotension [66], and at the borderline of significance in a small study, improved survival outcomes [9]. In two large RCTs, however, improved survival with online hemodiafiltration versus hemodialysis could not be demonstrated at primary analysis and was present only in the subgroups with the highest ultrafiltration volumes [67, 68].

1.2.5 The protein-bound molecules

Of the large group of protein-bound uremic substances [69] (Table 4), indoxylsulfate and the conjugates of p-cresol, p-cresylsulfate and p-cresylglucuronide have most extensively been studied; the section that follows is limited to those compounds.

Until some years ago, the study of the biochemical impact of the cresols had been restricted to the mother compound p-cresol [70], which, however, in reality is not present in the body. Its repeated registration in uremic samples was the result of an artifact, due to hydrolysis of the conjugates caused by acid deproteinization [70–72].

Whereas p-cresol is a known inhibitor of leukocyte function [73], p-cresylsulfate is pro-inflammatory by inducing leukocyte free radical production [74, 75]. Later studies indicated p-cresylsulfate also as a source of endothelial microparticle release, an indirect parameter of vascular damage [76], of renal fibrosis via epithelial to mesenchymal transition induced by the renin angiotensin system [77] and of transcriptional suppression of Klotho correlated with hypermethylation of the Klotho gene in renal tubular cells [78]. In a recent study, evaluating the cross talk between endothelium and leukocytes, p-cresylsulfate caused leukocyte recruitment [79].

While p-cresylsulfate shows the highest total concentration, p-cresylglucuronide is less protein bound, resulting in virtually the same free active concentration for both compounds [75]. Although p-cresylglucuronide per se is inactive toward leukocytes, it enhances the propensity of p-cresylsulfate to induce free radical generation [75], stressing the potential for synergism among uremic toxins. The association of p-cresylsulfate with p-cresylglucuronide also provoked endothelial albumin leakage, which was absent with p-cresylsulfate alone [79].

Many studies associated especially free p-cresol as surrogate of p-cresylsulfate with negative outcomes: propensity for infectious disease [80], uremic symptoms [81], cardiovascular disease [82, 83] and overall mortality [84]. More recently, studies analyzing p-cresylsulfate as such found associations with progression of kidney failure [85], coronary artery disease [86, 87], vascular calcification [88], and cardiovascular [89] and overall mortality [88, 89].

Table 4 The main protein-bound solutes

Advanced glycation end products (AGEs)
Carboxy methyl propyl furanpropionic acid (CMPF)
Cytokines
Interleukins
Tumor necrosis factor-α (TNFα)
Dimethylguanidines
Hippuric acid
Homocysteine
Indole-3-acetic acid
Indoxylglucuronide
Indoxylsulfate
Kynurenic acid
Kynurenine
Leptin
Phenolic compounds
P-cresylsulfate
P-cresylglucuronide
Phenolsulfate
Phenolglucuronide
Phenylacetic acid
Quinolinic acid
Retinol-binding protein

The list is not exhaustive; some of these protein-bound molecules have also a middle molecular weight range and therefore are also mentioned in Table 3

Many of the effects of indoxylsulfate are related to cardiovascular damage, such as PAI-1 activation as an index of free radical production [90], osteoblastic resistance against parathyroid hormone as a potential source of vascular calcification [91], endothelial micro-particle release [92], disruption of adherens junctions of endothelial cells [93], proliferation of smooth muscle cells [94] and renal [77] and cardiac fibrosis [95].

An interaction between leukocytes and endothelium was suggested in vitro by Ito et al. [96], showing endothelial NF-κB activation in association with leukocyte adhesion. To the best of our knowledge, up till now only one in vivo study showed a damaging effect of indoxylsulfate on the vascular structure as a whole: In salt-sensitive hypertensive Dahl rats, administration of indoxylsulfate up to uremic concentrations induced calcification of the vessel wall which was not present in wild-type rats and Dahl rats not receiving indoxylsulfate [97]. In a recent study, indoxylsulfate caused leukocyte recruitment to an extent comparable to that of lipopolysaccharide [79].

In clinical outcome studies, indoxylsulfate was associated with IL-6 concentration [98], coronary artery disease [87], vascular damage [99], progression of kidney failure [85] and mortality [99].

Removal of protein-bound solutes by dialysis strategies is less efficient than that of non-protein-bound solutes of similar molecular weight, due to the resistance induced by protein binding. Increasing pore size has no impact [100]. However, adding convection to diffusion increases reduction rate as well as clearance [51, 54], resulting in a longitudinal decrease in pre-dialysis concentrations [52, 53]; the question whether these decreases have clinical relevance remains unanswered.

Fractionated plasma separation and adsorption, an extracorporeal removal strategy usually applied in severe liver failure, approximately doubles the removal of protein-bound solutes compared to high-flux hemodialysis [101]; however, the study evaluating this setup was prematurely arrested because of serious clotting problems [102]. Nevertheless, this experiment offers proof of concept that extracorporeal adsorption might become a valuable tool in protein-bound solute removal.

With regard to peritoneal dialysis (PD), Evenepoel et al. [103] demonstrated significantly higher clearance of protein-bound compounds with high-flux hemodialysis compared to peritoneal dialysis even accounting for the better residual renal function with PD. Remarkably enough, however, plasma concentration of protein-bound compounds, which is conveying toxicity, is lower in PD [103–105], pointing to a role of other, possibly metabolic, factors in generating protein-bound compounds [106].

Generation of the precursors of protein-bound solutes largely occurs via amino acid metabolism by the intestinal flora [107], a process further accentuated in uremia [108]. The role of the intestine in uremic solute generation was definitely demonstrated by the finding of virtually absent protein-bound solute concentrations at metabolomic analysis of patients who had their colon removed [109]. A potential pathway to decrease protein-bound solutes might thus be via influencing these intestinal mechanisms [107]. However, protein restriction carries a risk of malnutrition. Metabolically more acceptable alternatives are the administration of prebiotics such as resistant starch [110] or oligofructose-enriched inulin [111], probiotics such as bifidobacterium [112] or intestinal sorbents such as AST-120

(Kremezin®)[113].

Historically, emphasis has always been on the removal of indoxylsulfate by AST-120, but it also likely adsorbs precursors from other protein-bound compounds. Already in 1993, Niwa et al. [114] demonstrated that in uremic rats, serum p-cresol was decreased after AST-120 administration. More recently, Kikuchi et al. [115] performed liquid chromatography/electrospray ionization-tandem mass spectrometry (LC/ESI-MS/ MS) to serum of uremic rats given AST-120 and showed a decrease versus untreated animals of at least 11 solutes, of which indoxylsulfate, hippuric acid, phenylsulfate and p-cresylsulfate were identified.

Apart from interfering with intestinal generation and metabolism, influence of renal tubular handling of toxins can decrease local toxicity to renal tubular cells. By inducing free radical production in renal tubular cells [90], a role for indoxylsulfate in the progression of renal failure due to inflammatory damage has been hypothesized. Indoxylsulfate enters into the renal tubular cells via organic acid pumps [116]; inhibiting these pumps, for example, by probenecid, protects against tubular necrosis induced by indoxylsulfate [117]. On the other hand, also stimulating organic acid pump systems (SLCO4C1) may help in removing organic acids from the tubule and so be protective [118]; statins activate SLCO4C1 [118], while uremic toxins inhibit efflux pumps [119].

In animal studies, indoxylsulfate or its precursors induced progression of renal failure [120, 121], whereas reduction in indoxylsulfate concentration by the intestinal sorbent AST-120 was nephroprotective [122] and prevented glomerulosclerosis [123].

Several trials in humans showed a benefit for AST-120 on progression of kidney disease. In a small population, AST-120 could postpone start of dialysis [124]. Based on a more elaborated randomized protocol, Akizawa et al. [125] demonstrated a slower progression of decrease of estimated glomerular filtration rate (eGFR) in non-dialyzed CKD patients on AST-120 versus placebo. In another RCT, Shoji et al. [126] showed a decrease in the slope of iothalamate clearance after the start of AST-120, which was not present in the untreated group. In diabetics with proteinuria and moderate CKD, a randomized study showed a less pronounced rise in serum creatinine in the AST-treated group [127]. Finally, AST-120 administered in the pre-dialysis stage also improved survival once hemodialysis was started [128].

Although it is conceivable that all protein-bound solutes and uremic toxins at large are removed by the kidneys, the question should be raised in how far our current marker of renal function, glomerular filtration rate (GFR), is related to concentration of these uremic solutes. The correlation between estimated GFR (eGFR) and several retention compounds was weak to nonexisting [129]. Although partly attributable to the use of eGFR rather than real measured GFR, the extreme differences in the correlation coefficients among compounds suggest that more than GFR, other elements, such as generation, metabolism, intestinal production or tubular clearance, have a crucial impact on uremic solute concentration [130]. Similar data were found for middle molecular peptidic compounds [131]. These findings may explain why patients in the Initiating Dialysis Early and Late (IDEAL) trial who were randomized to start dialysis with low eGFR did not reach this target, as there was a need to start treatment earlier because of clinical uremic symptoms that overrode the preset objective starting point based on calculations of GFR [132].

1.2.6 Conclusions

Although adequacy of dialysis is routinely defined by urea kinetics, uremia and the uremic syndrome are the consequences of the retention of more molecules than urea alone. Likewise, when analyzing and optimizing uremic solute removal, focus should be on more than urea removal alone. Even other small water-soluble compounds of similar metabolic origin like the guanidines, for which several findings point to toxic activity, show a different kinetic pattern from urea, which is relatively inert. The data with ADMA show that dialysis is not the only way to remove uremic toxins. The use of metabolic pathways to affect uremic toxin concentration has up till now insufficiently been explored. The studies with SDMA disclose another interesting therapeutic option, which is the potential to neutralize toxic effects by drugs countering the pathways causing this toxicity.

The relation of middle molecules with uremic toxicity has rather been evidenced in observational outcome trials than in vitro. Removal can be enhanced by increasing dialyzer pore size, which results in better patient outcomes, and can further be improved by adding convection, and using dialyzer membranes with more homogeneous pores, but also by prolonging dialysis. A positive impact of convection

on survival was only found in the subgroup with the highest exchange volumes.

Ample data suggest a biological impact and an association with outcomes of protein-bound uremic solutes. Removal by dialysis strategies remains deceiving compared to their non-bound counterparts of similar molecular weight. A better knowledge of the kinetics of these solutes is needed to develop strategies improving their removal. Adsorption is a promising option, both intestinally and extracorporeally. Whereas extracorporeal adsorption was not successful due to concomitant clotting problems, intestinal adsorption seems to protect against progression of kidney failure. These data need, however, confirmation in larger clinical trials.

1.2.7 References

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CHAPTER 1.3

LEUKOCYTE DYSFUNCTION IN UREMIA AS A CONTRIBUTOR TO THE PATHOPHYSIOLOGY OF CARDIOVASCULAR DISEASE IN CKD

Cardiovascular disease (40-50%) and infections (~25%) are the leading causes of death in CKD and especially end stage kidney disease (ESKD).[1-6] Increased cardiovascular risk in CKD is not entirely explained by the traditional risk factors such as age, gender, smoking, hypertension, dyslipidemia, family history and diabetes which are also common in CKD.[7,8] Non-traditional risk factors such as inflammation, oxidative stress, endothelial dysfunction, vascular calcification and malnutrition are common in CKD and could contribute to this increased risk.[9]

In the in vitro part of this thesis, the contribution of some uremic peptides to the induction of leukocyte oxidative stress was investigated. Therefore a global overview on leukocyte dysfunction in uremia and oxidative stress is presented in this chapter.

1.3.1 Uremia-related immune dysfunction: an overview

Leukocyte dysfunction in uremia is characterized on the one hand by a chronic activation of the immune system leading to oxidative stress and systemic micro-inflammation, which is involved in cardiovascular disease, atherosclerosis [10], malnutrition [11] and anemia.[12] On the other hand, there is an immune deficient state contributing to an increased infectious risk [6], decreased response towards vaccination [13] and malignancies.[14] (Figure 1) Merely all leukocyte subtypes of the innate and adaptive immune system are involved. [15-17] (Table 1)

Innate immune cells

In CKD, increased as well as impaired functional activity may be present in *monocytes* and *granulocytes*, contributing to the baseline activation of the immune system and to immune deficiency, respectively. Granulocyte counts are in general increased in kidney disease, while monocyte counts are unchanged or increased. (table 1) Monocytes and granulocytes are the main contributors to the chronic immune activation and increased reactive oxygen species (ROS) production in CKD leading to chronic micro-inflammation. Upon stimulation however, further increase in ROS production may be deficient, which possibly contributes to the impairment of host defense during infection.[16,18,19]

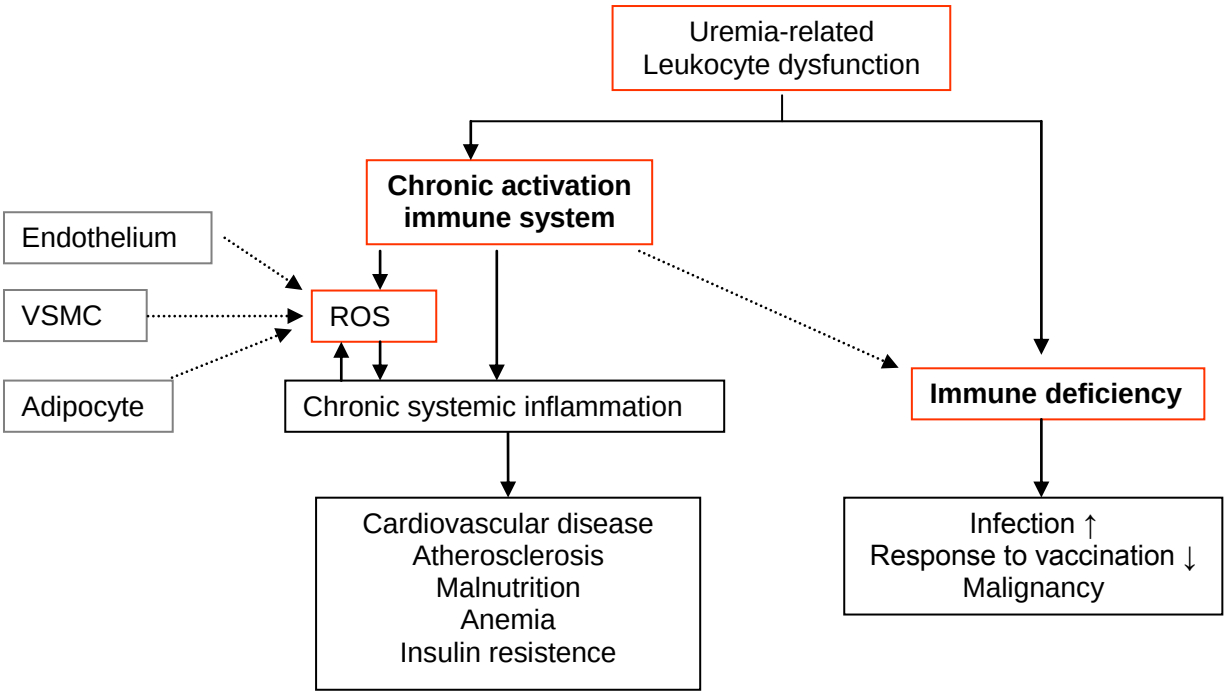

Figure 1: Schematic overview of uremia-related immune dysfunction
Abbreviations: ROS: reactive oxygen species, VSMC: vascular smooth muscle cell

Table 1: Main leukocyte types and alterations in uremia

Cell type	Healthy condition		Uremia
	%	function	counts
Granulocytes	40-75 %	First line antimicrobial activity Phagocytosis, Oxidative stress	↑
Monocytes	2-6 %	First line antimicrobial activity Phagocytosis, Oxidative stress Antigen presenting cell	↑ or ~
Dendritic cells	< 1%	Antigen presentation	↑
Lymphocytes	20-45%		
-Effector T cells		$CD4^+$: antigen presentation $CD8^+$: cytotoxic response against viruses, tumour cells	↓↓
- Regulatory T cells		Modulation of inflammatory and T cell response	↓
- B cells		Antigen presentation, antibody production	↓
- Natural killer cells		Killing of virus-infected cells, tumour cells	↓

The same duality is observed regarding the disturbed pro- and anti-apoptotic balance in granulocytes. In physiological conditions apoptosis is a tightly regulated process important for the resolution of inflammatory reactions. In general, the net effect of uremia is pro-apoptotic [18,20] increasing the susceptibility for infections. However, anti-apoptotic factors [21] have been also found which in turn increase the duration of the pro-inflammatory stimulus. Also the expression of toll like receptors (TLR), especially TLR4, was found to be upregulated in hemodialysis patients [22] while it was downregulated in predialysis CKD patients.[23] Furthermore, other factors contributing to an impaired anti-bacterial defense are decreased phagocytosis[24,25] and chemotaxis. [26]

Activated leukocytes in uremia interact with endothelium contributing to vascular damage.[27,28]

Three monocyte subtypes can be identified based on the cell surface expression of cluster of differentiation (CD)14 and CD16: classical monocytes ($CD14^{++}CD16^{-}$) and the more pro-inflammatory intermediate ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$). These intermediate and non-classical monocytes play an important role in the pathophysiology of cardiovascular disease. [29,30] In hemodialysis patients, the differentiation of monocytes is shifted towards higher counts of the pro-inflammatory $CD16^{+}$ monocyte subtypes [31,32], while this was not observed in peritoneal dialysis [31] or CKD patients. [33,34] The intermediate $CD14^{++}CD16^{+}$ monocyte subtype has been associated to adverse cardiovascular outcome in hemodialysis [31] and CKD patients. [33]

Dendritic cells are the most important antigen-presenting cells and consist of different subsets with specific functions mainly involved in host defense, response to vaccination as well as in atherosclerosis.[35,36] In comparison to healthy controls, the number of circulating plasmocytoid dendritic cells are decreased in CKD and in patients on hemodialysis.[37-39] Regarding myeloid dendritic cells, the results are less consistent: compared to healthy control the number was decreased in CKD stage 3 [38], unaltered in CKD (stage 3-5) and peritoneal dialysis and decreased [39] or unaltered in hemodialysis.[37] In addition, maturation and cytokine production of these

dendritic subsets was impaired.[40] Also the maturation of and endocytosis by monocytic derived dendritic cells was impaired in hemodialysis patients.[41]

Natural killer cells, a lymphocyte subtype with direct cytotoxic effects, are decreased in number, which may contribute to reduced tumor immune surveillance and increased susceptibility to viral infections.[42]

Adaptive immune system

Lymphocytopenia with a decrease in CD4, CD8 T cell and B cells is characteristic for CKD. In addition, T-cells have shown to have an increased apoptosis and shortened telomer length, mainly important in the host defense against infections.[15] There is also a decrease in regulatory CD4 T cells (T_{reg}) which play in physiological conditions a role in termination and modulation of an inflammatory stimulus and have shown to decrease inflammation in atherosclerotic plaques.[10] Their decreased number in uremia can lead to increased inflammation.

1.3.2 Oxidative stress

The *in vitro* part of this thesis investigated the role uremic peptides on the induction of leukocyte ROS by NADPH-oxidase, hence this review on the role of oxidative stress in CKD.

Oxidative stress is defined as an imbalance between the generation of free radicals and the lack of sufficient naturally present endogenous and dietary anti-oxidants. In physiological conditions, the main origin of free radicals is from mitochondrial origin and is crucial for the regulation of multiple enzyme systems.[43] In granulocytes, monocytes and macrophages, the increase in ROS is the first line defense against bacteria and fungi or other stress stimuli. An excess in reactive oxygen species (ROS) and reactive nitrogen species cause oxidation of macromolecules such as nucleid acids, lipids, carbohydrates and proteins, leading to tissue damage.[43-45] Furthermore ROS induces inflammation by activation of nuclear-factor kappa B (NFκB).[46]

The primary ROS is the superoxide anion ($O^{\cdot}{}_2^-$) resulting from the reduction of oxygen (O_2) by nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase

enzyme. $O^{\cdot}{}_2^-$ is a short-lived and transformed to hydogen peroxide (H_2O_2) by superoxide dismutase. $O^{\cdot}{}_2^-$ and H_2O_2 can be further reduced to hydroxyl radicals ($OH^{\cdot}$), considered as the strongest known free radical. This reaction is catalyzed by free metal ions such as iron (Fe^{2+}) or copper (Cu^{2+}). In the presence of Cl^-, myeloperoxidase catalyses H_2O_2 further to hypochlorous acid (HOCL) a major contributor in lipid peroxidation and production of oxidized low density lipoprotein (ox-LDL). Additionally, $O^{\cdot}{}_2^-$ forms reactive nitrogen species in the presence of nitric oxide ($ONOO^-$).[43,44,47,48]

Five different NADPH-oxidase isoforms (NOX 1-5) are expressed on different cell types involved in the cardiovascular system related to cardiovascular damage. They differ in their subunits and activation mechanism and each of them catalyze the formation of $O^{\cdot}{}_2^-$ as illustrated in figure 2.[45,48]

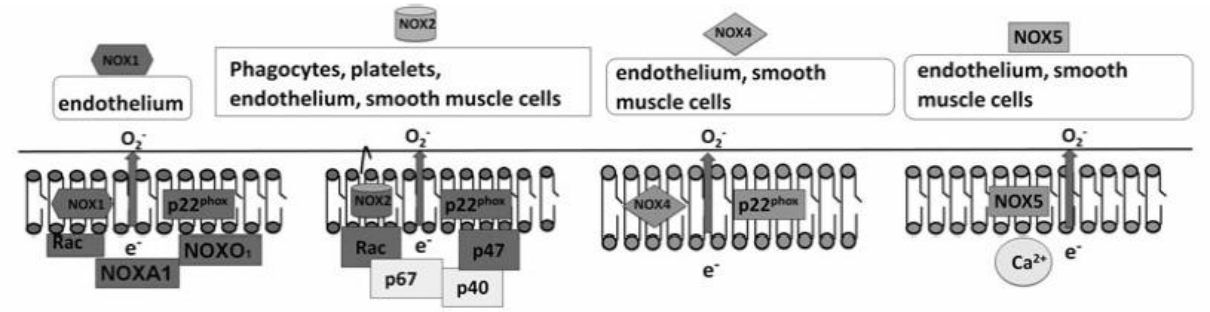

Figure 2: The members of the NOX-family involved in cardiovascular system: schematic structure and distribution on different cell types [45]

NADPH-oxidase in phagocytes is known as NOX2 and is in its active form a membrane enzyme constituted of 6 different subunits: p22phox, gp91phox/NOX2, p47phox, p67phox, p40phox and the GTP-binding protein Rac1(in monocytes)/2(in granulocytes). In resting cells, p47phox, p67phox, p40phox and the G-protein Rac 1/2 are located in the cytosol, whereas p22phox, gp91phox/NOX2 are located in the plasma membrane or membranes of granules where they form flavochrome b_{558}. The first step in the activation is the serine phosphorylation of the cytosolic subunit p47phox, followed by further phosphorylation of other cytosolic subunits, activation of Rac 1/2 and translocation of the cytosolic subunits to the membrane flavochrome

b_{558}.[45,47-49] (Figure 3) The activation of NADPH-oxidase can be induced by a large number of stimuli such as opsonized bacteria, the chemoattractant bacterial formylated peptides such as formyl-methionine-leucine-phenylalanine (fMLP) or directly by protein kinase C (PKC) activators, such as phorbol myristate acetate (PMA).[47,49]

Since ROS are unstable with a short half-life these are difficult to measure in practice. The products of lipid peroxidation such as oxidized-LDL and F_2-isoprostanes, of protein oxidation such as advanced glycated end products, advanced oxidation protein products, protein thiols, and malondialdehyde or of nucleid acid oxidation such as oxidized-DNA can be used as surrogates.[44]

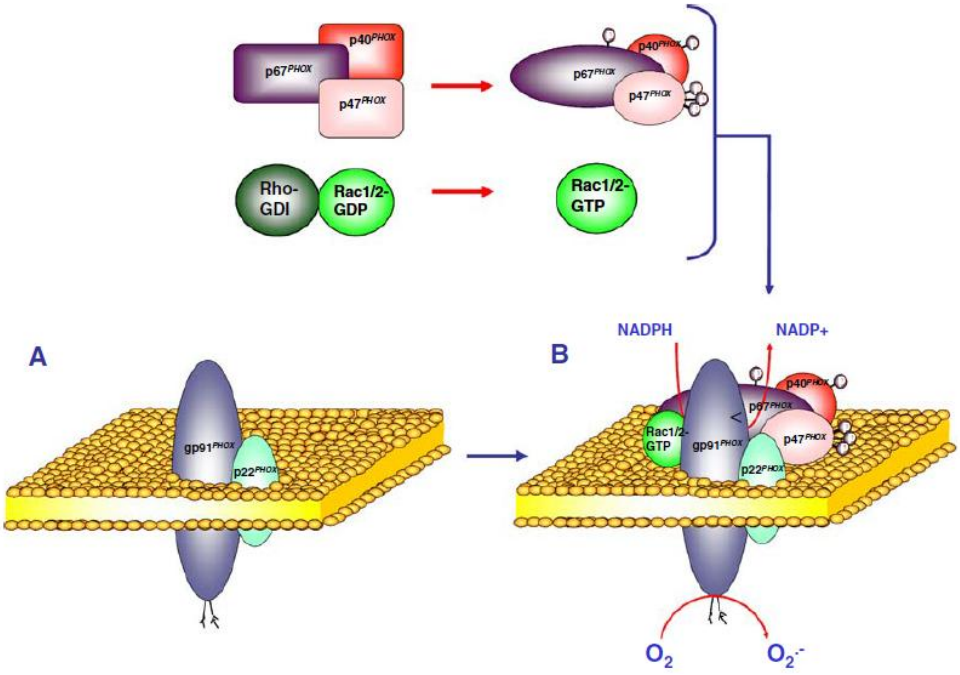

Figure 3: The NADPH-oxidase subunits in resting (A) en activated state (B) [49]

Oxidative stress in chronic kidney disease

CKD is a pro-oxidative state[50,51] to which multiple factors such as intravenous iron administration, bio-incompatible membranes, circulating endotoxins , malnutrition possibly related to a decreased anti-oxidant activity by decreased serum albumin,

decreased vitamin C and E, inflammation and uremic toxins can contribute.[52] In 2012, a Cochrane review was published on the use of anti-oxidant therapy in CKD and ESKD: no effects on cardiovascular disease or all-cause mortality could be found in CKD although a beneficial effect on intermediate outcomes in CKD patients could not be excluded. [53]

1.3.3 Summary

The chronic activation of the immune system and increase in oxidative stress in CKD can contribute to the pathophysiology of cardiovascular disease in CKD and are considered as non-traditional risk factors. NADPH-oxidase is one of the major enzymes in the production of ROS as a cause of oxidative stress.

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CHAPTER 1.4

OUTLINE AND AIMS

The general aim of this thesis is to reveal the duality/discrepancy between the biological activity of low molecular weight uremic peptides and their role as markers for/in CKD. Since oxidative stress is an important trigger in the uremic micro-inflammation, selected uremic peptides were investigated on their potential to induce leukocyte ROS. Furthermore the association between some uremic peptides and adverse outcome was investigated.

More specific aims are:

1) to study the association between eGFR, the common marker of kidney function and the serum concentration of different low molecular weight uremic peptides.

2) to evaluate the biological activity, assessed as the degree of induction of leukocyte oxidative stress, of beta-2-microglobulin, the prototype marker of the middle molecules.

3) to evaluate the contribution of cytokines, the typical markers for inflammation, to leukocyte oxidative stress, at concentrations relevant for CKD.

4) to evaluate the role of tumor necrosis factor receptors, more recently identified markers and members of the tumor necrosis factor-alpha (TNFα) system, in CKD by

- the investigation of their expression on leukocyte membranes and their association to eGFR

- the investigation of the association between their concentration and adverse outcome in advanced CKD.

CHAPTER 2

ESTIMATED GLOMERULAR FILTRATION RATE IS A POOR PREDICTOR OF THE CONCENTRATION OF MIDDLE MOLECULAR WEIGHT UREMIC SOLUTES IN CHRONIC KIDNEY DISEASE

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2.1 Abstract

***Background**:* Uremic solute concentration increases as Glomerular Filtration Rate (GFR) declines. Weak associations were demonstrated between estimated GFR (eGFR) and the concentrations of several small water-soluble and protein-bound uremic solutes (MW<500Da). Since also middle molecular weight proteins have been associated with mortality and cardiovascular damage in Chronic Kidney Disease (CKD), we investigated the association between several eGFR formulae and the concentration of Low Molecular Weight Proteins (LMWP) (MW>500Da).

***Material and Methods**:* In 95 CKD-patients (CKD-stage 2-5 not on dialysis), associations between different eGFR-formulae (creatinine, CystatinC-based or both) and the natural logarithm of the concentration of several LMWP's were analyzed: i.e. parathyroid hormone (PTH), Cystatin C (CystC), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), leptin, retinol binding protein (RbP), immunoglobin light chains kappa and lambda (Ig-κ and Ig-λ), beta-2-microglobulin (β_2M), myoglobin and fibroblast growth factor-23 (FGF-23)).

***Results**:* The regression coefficients (R^2) between eGFR, based on the CKD-EPI-Crea-CystC-formula as reference, and the examined LMWP's could be divided into three groups. Most of the LMWP's associated weakly ($R^2 < 0.2$) (FGF-23, leptin, IL-6, TNF-α, Ig-κ, Ig-λ) or intermediately (R^2: 0.2-0.7) (RbP, myoglobin, PTH). Only β_2M and CystC showed a strong association ($R^2 > 0.7$). Almost identical R^2-values were found per LMWP for all eGFR-formulae, with exception of CystC and β_2M which showed weaker associations with creatinine-based than with CystC-based eGFR.

***Conclusion**:* The association between eGFR and the concentration of several LMWP's is inconsistent, with in general low R^2-values. Thus, the use of eGFR to evaluate kidney function does not reflect the concentration of several LMWP's with proven toxic impact in CKD.

2.2 Introduction

Chronic Kidney Disease (CKD) is an independent risk factor for mortality and cardiovascular disease (CVD) [1]. As Framingham risk calculation cannot correctly predict this risk [2,3], other than traditional risk factors are at play. When kidney

function declines, retention of uremic solutes with potential to cause vessel damage and other toxic effects, conceivably plays a role in this [4,5].

Glomerular Filtration Rate (GFR) is used to express kidney function and this can accurately be measured by time-consuming and labor-intensive methods [6]. In clinical practice, serum creatinine (Crea) based formulae are used to calculate estimated GFR (eGFR), which offer an acceptable estimate of measured GFR (mGFR) [6–9]. However, if possible, mGFR is to be preferred as it may differ from eGRF especially in the lower GFR range in a CKD population or in patients with a body constitution that deviates from the average [6,10]. On the other hand, measuring GFR by one of these techniques is more costly and labor-intensive than to determine eGFR. Also, current guidelines classify CKD based on the Modification of Diet in Renal Disease study (MDRD) formula [11,12]. More recently, the CKD-EPI-Crea formula [13] has been proposed as a valid alternative, especially if eGFR is >60 ml/min/1.73m² [9], so that it possibly will be incorporated into the upcoming KDIGO guideline [14].

Since concentrations of uremic solutes rise when GFR deteriorates, it has been thought that GFR reflects the retention state of the patient and that the elevation of individual solute concentration of uremic toxins is closely related to the gradual deterioration of GFR. However, Eloot *et al*. [15] found very low regression coefficients between eGFR and several low molecular weight retention solutes in a CKD population.

The low molecular weight proteins (LMWP) are among the main representatives of the middle molecules, the third family of uremic retention solutes [16], and are interesting to study for their relationship with eGFR as with normal kidney function they are freely filtered through the glomerular basement membrane (GBM) and then mainly degraded into amino acids by the proximal tubules [17]. Furthermore the concentrations of several of the investigated LMWP's, such as inflammatory parameters and FGF-23, are already elevated in patients with a moderate reduction in GFR [18–21] or in more advanced CKD [22]. As a consequence, associations between these solutes and eGFR are often assumed. Assessing the predictive value of eGFR for their concentration is furthermore also relevant, because several LMWP's, such as interleukin-6 (IL-6) [23–26], tumor necrosis factor-alpha (TNF-α)

[27,28], beta-2-microglobulin (β_2M) [29,30], and fibroblast growth factor-23 (FGF-23) [31–33], have been linked to mortality or surrogate outcomes like vascular damage or progression of kidney failure. In addition, active removal of middle molecules by dialysis has been associated with better outcome [34].

Therefore, we investigated in a CKD population whether the concentration of several LMWP's would associate with eGFR, calculated by several eGFR formulae.

2.3 Material and methods

Ethics Statement

The study was approved by the local ethical committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale (CCPPRB) de Picardie, CHU Amiens, Amiens, France) and performed in accordance to the Declaration of Helsinki. Written informed consent was obtained from all patients.

Study population

This evaluation is a planned sub-analysis of a study undertaken over an 18-month period (January '06- June '07), which screened 150 Caucasian patients with prevalent CKD stage 2-5D from the Nephrology Department at Amiens University Hospital, in which uremic retention solutes in relation to clinical outcomes were analyzed [23,35–37].

All patients were over 40 years old and had a confirmed diagnosis of CKD (two previous eGFR measures of < 90 ml/min, calculated by the Cockcroft-Gault formula with an interval of 6-9 months) [38]. Exclusion criteria were chronic inflammatory disease, atrial fibrillation, complete heart block, abdominal aorta aneurysm, an aortic and/or femoral artery prosthesis, primary hyperparathyroidism, kidney transplantation, and any acute cardiovascular event in a 3 month period prior to screening for inclusion.

From the 140 patients who met the inclusion criteria, 45 were excluded from the current study because of hemodialysis treatment, which has an impact on solute

concentration and on eGFR. The 95 patients, included in this study, were classified in CKD stages according to the CKD-EPI-Crea-CystC formula for further analysis [8].

Sampling and laboratory methods

Blood samples of all patients were collected in the morning from 9 a.m. on, centrifuged, aliquoted, frozen and stored at -80°C. Cystatin C (CystC) (MW: 13.3 kDa) concentration was determined by immune-nephelometry (N latex Cystatin C®, Siemens Healthcare, Dade Behring, Marburg, Germany) and that of intact parathyroid hormone (PTH) (MW: 9.5kDa) with a chemiluminometric immunoassay (Liaison N-tact PTH CLIA®, Diasorin, Stillwater, MN, USA). The determination of retinol binding protein (RbP) (MW: 21kDa), beta-2-microglobulin (β_2M) (MW: 11.8kDa), myoglobin (MW: 17kDa) and total immunoglobulin light chains kappa (Ig-κ) and lambda (Ig-λ) (MW: 23kDa) was performed by laser nephelometry (BNProSpec®, Siemens Healthcare, Dade Behring, Marburg, Germany). ELISA's were used to determine the levels of interleukin-6 (IL-6) (MW: 23kDa), tumor necrosis factor-alpha (TNF-α) (MW: 17kDa) (R&D Systems, Wiesbaden, Germany), and leptin (MW: 16kDa) (DRG diagnostics, Marburg, Germany). Intact fibroblast growth factor-23 (FGF-23) (MW: 32kDa), was measured by a two-site (N-terminal and C-terminal) ELISA (Immunotopics, San Clemente, CA, USA). Serum creatinine (Crea) (MW: 113Da) was measured colorimetrically by standard laboratory methods.

eGFR- calculation

Six different formulae were used to estimate GFR: the CKD-EPI formula, based on Crea and CystC (CKD-EPI-Crea-CystC) $eGFR=177.6\cdot Crea^{-0.65}\cdot CystC^{-0.57}\cdot age^{-0.20}\cdot 0.82$ (if female) [8]; two formulae based on Crea alone: the MDRD $eGFR = 175\cdot Crea^{-1.154}\cdot age^{-0.203}\cdot(0.742 \text{ if female})\cdot(1.21 \text{ if black})$ [7] and the CKD-EPI creatinine (CKD-EPI-Crea) $eGFR=141\cdot \min(Crea/\kappa,1)^{\alpha}\cdot \max(Crea/\kappa,1)^{-1.209}\cdot 0.993^{Age}\cdot 1.018$ (if female)$\cdot$ 1.159 (if black) (κ: 0.7 if female, 0.9 if male; α: -0.329 if female, -0.411 if male) [13]; and three formulae based on CystC alone: Stevens $eGFR=127.7\cdot CystC^{-1.17}\cdot age^{-0.13}\cdot 0.91$ (if female)$\cdot$1.06 (if black) [8], Le Bricon $eGFR = [78\cdot(1/CystC)]+4$ [39] and Rule $eGFR=66.8\cdot(CystC)^{-1.3}$ [40].

Statistical analysis

The data are expressed as mean ± standard deviation and analysed by ANOVA if they were normally distributed. For data that were not normally distributed, median with interquartile range and Kruskall-Wallis test were used. Linear regressions and Pearson correlations were calculated on semi-logarithmic (LN) concentrations as a function of eGFR. Multifactorial analysis was performed to correct for well-known influencing factors for the concentration of several solutes. The regression model of CystC, β_2M, IL-6, TNF-α, Ig-κ and Ig-λ was adjusted for C-reactive protein (CRP), the one of FGF-23 and PTH for calcium, phosphorus and vitamin D-supplementation, the one of leptin for body mass index (BMI) and gender, and the one of RbP for BMI, 1/CRP and diabetes mellitus. A $P < 0.05$ was considered as statistically significant. All statistical analyses were performed using SPSS Statistics 19 (SPSS Inc, Chicago, IL) for Windows (Microsoft Corp, Redmond, WA).

2.4 Results

Ninety-five patients at different stages of CKD were included: 11.5% CKD stage 2, 39.0% CKD stage 3, 39.0% CKD stage 4, and 10.5% CKD stage 5 not on dialysis. Table 1 summarizes the demographic and clinical characteristics of the study population.

Table 1. Main demographic and clinical characteristics of the study population (n = 95).

	CKD stage					P
	Stage 2–5	stage 2	stage 3	stage 4	stage 5	
Number n (%)	95 (100)	11 (11.5)	37 (39.0)	37 (39.0)	10 (10.5)	
eGFR (ml/min/1.73m^2)	35±18	69±8	43±9	22±4	11±3	<0.001
Age (years)	68±12	65±8	69±12	65±13	66±15	0.07
Male gender n (%)	59 (62)	9 (82)	24 (65)	22 (60)	4 (40)	0.39
Diabetes Mellitus n (%)	45 (47)	4 (36)	19 (51)	18 (49)	4 (40)	0.50
BMI (kg/m^2)	29±7	26±5	29±6	31±7	28±7	0.28
Cholesterol (mmol/l)	5.0±1.1	5.4±0.7	4.6±1.1	5.3±1.1	4.6±0.5	0.02
Triglycerides (mmol/l)	1.9±1.4	1.7±0.9	1.6±0.7	2.4±1.9	2.2±1.2	0.06
CRP (mg/l)	3.11 [1.1–6.7]	2.3 [0.7–4.9]	2.8 [1.4–5.0]	3.7 [0.8–8.5]	4.1 [0.4–15.6]	0.696
Albumin (g/l)	38.9±6.4	40.6±8.8	38.4±5.7	39.9±5.8	33.8±6.7	0.07
Hemoglobin (g/l)	12.5±1.7	14.0±1.2	12.7±1.5	12.0±1.6	10.9±1.4	<0.001
Calcium (mmol/l)	2.3±0.1	2.3±0.1	2.3±0.1	2.3±0.2	2.3±0.2	0.96
Phosphate (mmol/l)	1.2±0.3	0.9±0.3	1.1±0.2	1.4±0.3	1.5±0.5	<0.001
Vit D supplement n (%)	17 (18)	1 (9)	5 (13)	6 (16)	5 (50)	0.06

CKD stages according to the CKD-EPI-Crea-CystC formula. Data are expressed as mean ± SD, median with interquartile range between square brackets or number for binary variables, with percentages between brackets per CKD class. CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; BMI, body mass index; Statistical analysis: ANOVA or Kruskall-Wallis; P-values comparing all stages.
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The concentrations of the studied LMWP's, except for the immunoglobulin light chains, increased progressively with declining kidney function (Table 2).

Table 2. Concentrations of uremic solutes ± standard deviation according to CKD-stage (CKD-EPI-Crea-CystC).

	CKD stage					P- value
	stage 2–5	stage 2	stage 3	stage 4	stage 5	
CystC (mg/l)	1.9±0.9	0.9±0.2	1.4±0.5	2.4±0.7	3.5±0.7	<0.001
β_2M (mg/l)	6.1 [3.2–9.3]	2.6 [2.1–4.6]	3.8 [3.0–5.3]	8.2 [6.7–10.4]	13.9 [12.8–16.4]	<0.001
PTH (pg/ml)	77.0 [42.5–135.5]	39.5 [25.5–44.0]	62.0 [42.0–83.5]	124.0 [74.0–196.0]	111.5 [22.0–173.0]	<0.001
RbP (mg/l)	82.0±32.8	52.9±15.2	67.0±21.8	102.6±32.2	95.9±31.5	<0.001
Myoglobin (mg/l)	82.7 [54.9–115.0]	49.9 [32.4–57.4]	72.3 [49.7–104.8]	98.8 [75.4–125.5]	170.5 [62.8–244.0]	<0.001
IL-6 (pg/ml)	2.6 [1.3–5.1]	1.1 [0.4–1.9]	2.2 [1.3–4.0]	3.0 [1.3–5.2]	7.0 [2.2–14.0]	0.001
TNF-α (pg/ml)	3.4 [2.2–4.6]	1.1 [1.1–2.5]	4.1 [2.2–4.6]	2.6 [2.2–5.1]	4.1 [3.1–7.2]	0.016
Leptin (ng/ml)	12.8 [2.0–43.5]	0.56 [<0.48–5.3]	8.5 [2.5–33.4]	21.4 [4.3–59.2]	26.3 [0.7–>105]	0.012
FGF-23 (pg/ml)	30.6 [26.8–34.4]	26.6 [25.8–27.3]	31.5 [27.6–34.5]	30.6 [27.6–34.9]	34.4 [33.1–35.0]	0.007
Ig-κ (g/l)	2.6 [2.2–3.0]	2.4 [2.3–2.8]	2.4 [2.1–4.8]	2.8 [2.4–3.0]	2.5 [2.1–3.1]	0.330
Ig-λ (g/l)	1.5 [1.3–1.8]	1.5 [1.3–1.7]	1.5 [1.3–1.7]	1.6 [1.4–1.8]	1.2 [1.2–1.8]	0.280

Concentrations are expressed as mean ± SD or median with interquartile range (between square brackets) as appropriate. CKD: chronic kidney disease, CystC: cystatin C, β_2M: beta-2-microglobulin, PTH: parathyroid hormone, RbP: retinol binding protein, IL-6: interleukin-6, TNF-α: tumor necrosis factor-alpha, Ig-κ: immunoglobulin light chain kappa, Ig-λ: immunoglobulin light chain lambda, FGF-23: fibroblast growth factor-23. Statistical analysis: ANOVA or Kruskall-Wallis; P comparing all stages.
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Our analysis primarily focused on the linear regression analysis with the natural logarithm (LN) of the concentration of each studied uremic retention solute concentration as dependent variable and the CKD-EPI-Crea-CystC eGFR as independent variable. This formula was chosen as reference because it is considered as one of the most accurate ones at this time while it incorporates both Crea and CystC, in contrast to all other studied formulae which are based on either Crea or CystC [8]. Associations between eGFR and LMWP's were expressed as regression coefficients (R^2) and are summarized in table 3 and figure 1.

The R^2-values per individual solute were divergent; according to these, associations could be arbitrarily divided into three groups: strong ($R^2 > 0.7$), moderate (R^2 0.2-0.7) and weak ($R^2 < 0.2$) (Figure 1). As expected, CystC ($R^2 = 0.828$) was strongly associated as it is one of the used parameters in the formula. Only β_2M showed a similar association ($R^2 = 0.770$). Retinol binding protein (RbP), myoglobin and parathyroid hormone (PTH) were moderately associated to eGFR with R^2-values of 0.423, 0.303 and 0.231, respectively. The association with eGFR was only weak for IL-6 ($R^2 = 0.117$), leptin ($R^2 = 0.084$), FGF-23 ($R^2 = 0.058$) and TNF-α ($R^2 = 0.056$).

There was even no association for immunoglobulin light chain kappa (Ig-κ) (R^2 = 0.021) and immunoglobulin light chain lambda (Ig-λ) (R^2 = 0) (P = N.S.).

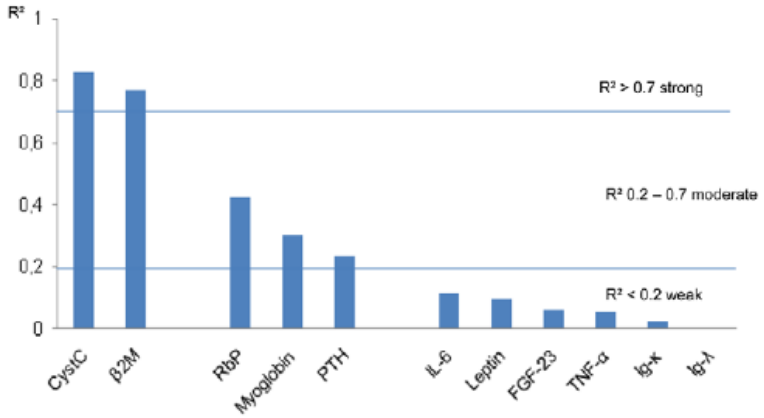

Figure 1. Regression coefficients between LN of studies LMWP's and eGFR. The coefficients of the linear regression analysis between the natural logarithm of the studied low molecular weight protein concentrations and estimated Glomerular Filtration Rate, according to CKD-EPI-Crea-CystC, can be divided into 3 groups: strong (R^2 >0.7), moderate (R^2 0.2–0.7) and weak (R^2 <0.2). The dashed lines indicate R^2 = 0.2 and 0.7. All correlations were significant except for Ig-κ and Ig-λ. LN: natural logarithm, LMWP: low molecular weight protein, eGFR estimated glomerular filtration rate, R^2: regression coefficient, Cyst C: Cystatin C, β_2M: beta-2-microglobulin, RbP: retinol binding protein, PTH: parathyroid hormone, IL-6: interleukin-6, FGF-23: fibroblast growth factor-23, TNF-α: tumor necrosis factor-alpha, Ig-κ: immunoglobulin light chain kappa, Ig-λ: immunoglobulin light chain lambda.
doi:10.1371/journal.pone.0044201.g001

Figure 2 shows the dot plots of solute concentrations as a function of eGFR. Whereas the relation is strong with little scatter around the linear regression line for β_2M (Panel A), the scatter is much larger for solutes with moderate to weak R^2-values, as illustrated for myoglobin (Panel B), IL-6 (Panel C) and especially Ig-λ (Panel D) for which there is no association at all. The large standard deviations or wide interquartile ranges of the individual solute concentrations per CKD-stage also illustrate the large inter-individual variability of LMWP concentration within the same eGFR-range (Table 2).

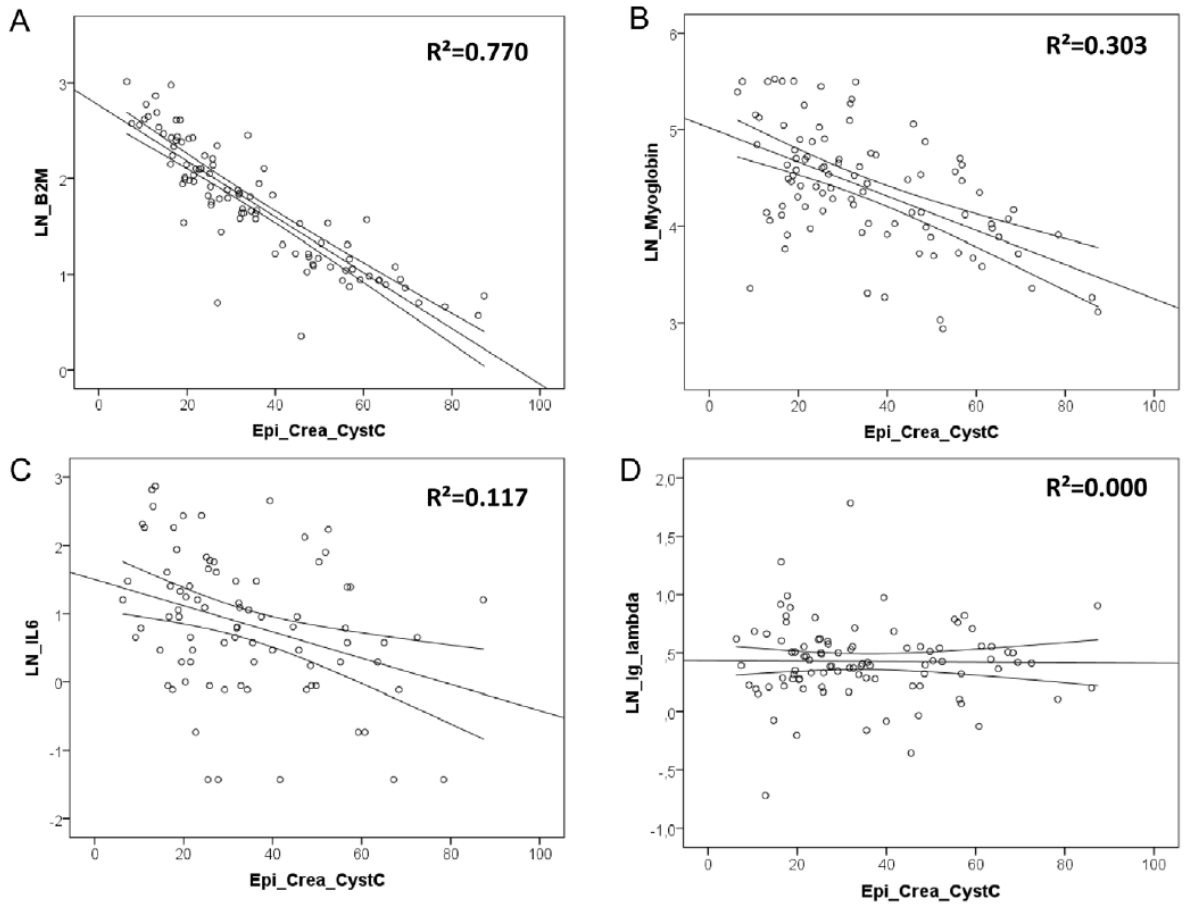

Figure 2. Dot plots with best fit linear regression lines for LN of LMWP's in function of eGFR. Dot plots with best fit linear regression lines for natural logarithms of β_2M, myoglobin, IL-6 and lg-λ, as examples of strongly, moderately and weakly correlating low molecular weight proteins, in function of estimated Glomerular Filtration Rate, calculated by CKD-EPI-Crea-CystC. The dots represent the individual concentrations and the lines the best fit linear regression line with the 95% confidence interval. LN: natural logarithm, LMWP: low molecular weight protein, β_2M : beta-2-microglobulin, IL-6: Interleukin-6, lg-λ: immunoglobulin light chain lambda, EPI-Crea-CystC: CKD-EPI formula based on serum creatinine and Cystatin C, R^2: regression coefficient, LN: natural logarithm.
doi:10.1371/journal.pone.0044201.g002

In addition we analyzed the correlation coefficients between the concentrations of the different LMWP and eGFR (CKD-EPI-Crea-CystC) in the group CKD stage 2-3 versus CKD stage 4-5. The correlation between RbP, PTH, myoglobin, FGF-23 and eGFR was significant in CKD-stage 2-3 while not in CKD stage 4-5. For the other investigated solutes, the R^2-values in CKD stage 2-3 and CKD stage 4-5 were more conform to each other. The respective R^2-values are summarized in table 4.

Table 3. Regression coefficients of LMWP's and different eGFR formulae.

R^2	CKD-EPI-Cr-CystC	MDRD	CKD-EPI-Cr	Stevens	Le Bricon	Rule
	R^2 >0.7					
Cyst C	0.828	0.572	0.569	0.920	0.939	0.902
β_2M	0.770	0.559	0.549	0.838	0.855	0.820
	R^2 0.2–0.7					
RbP	0.423	0.348	0.343	0.383	0.397	0.390
Myoglobin	0.303	0.246	0.262	0.287	0.297	0.293
PTH	0.231	0.130	0.132	0.279	0.274	0.276
	R^2 <0.2					
IL-6	0.117	0.090	0.097	0.126	0.127	0.123
Leptin	0.084	0.056	0.059	0.092	0.065	0.065
FGF-23	0.058	0.008	0.008	0.095	0.101	0.094
TNF-α	0.056	0.043	0.044	0.066	0.058	0.061
Ig-κ	0.021	0.021	0.020	0.016	0.015	0.011
Ig-λ	0.000	0.000	0.000	0.001	0.001	0.001

LMWP: Low Molecular Weight Protein, R^2: regression coefficient, eGFR: estimated glomerular filtration rate, CKD-EPI-Crea-CystC: CKD-EPI formula based on creatinine and cystatin C, MDRD: Modification of Diet in Renal Disease formula, CKD-EPI-Crea: CKD-EPI formula based on creatinine. CystC: cystatin C, β_2M: beta-2-microglobulin, RbP: retinol binding protein, PTH: parathyroid hormone, IL-6: interleukin-6, FGF-23: fibroblast growth factor-23, TNF-α: tumor necrosis factor-alpha, Ig-κ: immunoglobulin light chain kappa, Ig-λ: immunoglobulin light chain lambda.
doi:10.1371/journal.pone.0044201.t003

In a second step, the same analysis was performed with the other formulae under evaluation and compared to the results with the CKD-EPI-Crea-CystC-formula. The R^2-values between all eGFR formula and individual solutes were strikingly similar with only one exception (Table 3). β_2M was only moderately associated to Crea-based eGFR, with R^2-values of approximately 0.55, as compared to CystC-based eGFR ($R^2 > 0.8$). In this way β_2M followed the same pattern as CystC, for which this discrepancy could be attributed to whether CystC was included as a factor in the formula or not. Considering the other studied LMWP's, only PTH showed a moderately similar trend, with R^2 approximately 0.27 compared to R^2 approximately 0.13, with CystC- or Crea- based eGFR-formulae, respectively.

Table 4. Regression coefficients (R^2) of the concentration of LMWP's and eGFR (CKD-EPI-Crea-CystC) comparing CKD stage 2–3 versus CKD stage 4–5.

R^2	CKD stage 2–3 (n = 48)	CKD stage 4–5 (n = 47)
Cyst C	0.782	0.555
β_2M	0.619	0.549
RbP*	0.254	0.001
PTH*	0.195	0.000
Myoglobin*	0.257	0.059
IL-6	0.074	0.117
TNF-α	0.107	0.087
Leptin	0.060	0.001
FGF-23*	0.120	0.033
Ig-κ	0.013	0.016
Ig-λ	0.001	0.006

LMWP: Low Molecular Weight Protein, eGFR: estimated glomerular filtration rate, CKD-EPI-Crea-CystC: CKD-EPI formula based on creatinine and cystatin C. CystC: cystatin C, β_2M: beta-2-microglobulin, RbP: retinol binding protein, PTH: parathyroid hormone, IL-6: interleukin-6, TNF-α: tumor necrosis factor-alpha, FGF-23: fibroblast growth factor-23, Ig-κ: immunoglobulin light chain kappa, Ig-λ: immunoglobulin light chain lambda. *: LMWP's with a significant correlation in CKD stage 2–3, but no significant correlation in CKD stage 4–5.
doi:10.1371/journal.pone.0044201.t004

Finally, we performed multifactorial regression analysis for the different LMWP's with adjustment for several relevant variables. However, only two models induced a marked increase in R^2-value: for leptin the association rose from weak to moderate when BMI was added to the regression model (R^2 from 0.084 to 0.346), with BMI as an independent predictor for the leptin concentration. Likewise, after adjustment for CRP, IL-6 became moderately associated with eGFR (R^2 from 0.117 to 0.305 after adjustment). For all other solutes there was no change in R^2. (Data not shown)

2.5 Discussion

We analyzed the linear regression coefficients between the concentrations of several LMWP's retained in CKD and different eGFR-formulae in a CKD population, stage 2-5 not on dialysis. As a main finding, the R^2-values diverged considerably, ranging from high, $R^2 > 0.7$, to low, $R^2 < 0.2$. The majority of the evaluated LMWP's associated weakly ($R^2 < 0.2$ for IL-6, TNF-α, FGF-23 and leptin) or moderately (R^2: 0.2-0.7 for RbP, myoglobin and PTH). There was no correlation at all for the

immunoglobulin light chains. Only CystC and β_2M showed a strong association with eGFR ($R^2 > 0.7$) (Figure 1, Table 3). Although in some studies a correlation was sought for individual LMWP's and eGFR of mGFR, this present study seeked out the association of the concentration of several LMWP's and eGFR formulae together allowing their comparison.

The R^2-values for the weakly and moderately associating LMWP's did not differ substantially whether eGFR was calculated with the CKD-EPI-Crea-CystC-formula [8], the Crea-based formulae (MDRD [7] and CKD-EPI-Crea [13]), or the three different CystC-based formulae, (Stevens [8], Rule [40] and Le Bricon [39]) (Table 3). These low regression coefficients can partially be attributed to the known limitations of eGFR, as an index of mGFR [6,41]. However in at least four other studies, almost identical low regression coefficients were found between mGFR, assessed with different techniques, and the concentration of RbP (R^2 0.16) [42], myoglobin (R^2 0.38) [43], leptin (R^2 0.0004) [44] and FGF-23 (R^2 0.09) [45] as in our study, be it that transformation of the concentrations varied from study to study. In addition, the imperfect reflection of true GFR by eGFR can explain that regression coefficients are substantially lower than 1, but not that the range in between individual molecules is so discordant, whereas per molecule they are almost identical (Table 3, Figure 1). There was also an unpredictable and large variability in concentrations of different solutes within each eGFR stratum (Table 2). These data suggest another reason for the sometimes deceiving associations than a discrepancy among mGFR and eGFR, namely that uremic solute concentration depends on other factors than GFR as well. In this way, our study corroborates findings in an earlier study with small water-soluble and protein-bound compounds [15,46]**.**

These results are somewhat unexpected from a physiological point of view as the renal clearance of these LMWP's depends to a large extent on GFR alone. All these LMWP's are freely filtered through the GBM, followed, at normal physiological concentrations, by an almost entire uptake by the proximal tubules via a receptor-mediated process to be degraded subsequently into amino acids in the tubular lysosomes [17,47]. In this way, the proximal tubulus plays an important role in LMWP metabolism but without a direct contribution to their renal clearance; regarding the latter, GFR is the rate limiting step. This probably explains why we did not find any association between eGFR and the total (free plus bound) immunoglobulin light

chains, in contrast to Hutchison et al who evaluated only free light chains for their association to eGFR (free Ig-κ: R^2: 0.52; free Ig-λ: R^2: 0.44) [48], as only the free fraction passes the GBM. This is also in contrast to the small water-soluble and protein-bound uremic toxins, for which tubular secretion and/or reabsorption play an important role in renal clearance [15,46].

Table 5. Main factors influencing the concentrations of the studied LMWP's, other than GFR.

	Extra renal handling	Generation
CystC	–	Gender, age, hyperthyroidism, corticosteroid intake, malignancy, inflammation
β_2M	+ (~5%)	Inflammation, malignancy
RbP	?	Insulin resistance, obesity, DM, Zn-deficiency, liver dysfunction, infection
PTH	+	Hypocalcemia, hyperphosphatemia, hypo-VitD
Myoglobin	+ (in uremia?)	Different generation in uremia (?)
Leptin	+	Obesity, gender, low energy expenditure, insulin resistance
IL-6	+	Inflammation
TNF-α	+	Inflammation
FGF-23	+ ?	Hyperphosphatemia, regulation mineral metabolism
Ig-κ	+ ?	B-cell lymphoproliferative disorders, inflammation
Ig-λ	+ ?	B-cell lymphoproliferative disorders, inflammation

CystC: Cystatin C, β_2M: beta-2-microglobulin, RbP: retinol binding protein, PTH: parathyroid hormone, IL-6: interleukin-6, TNF-α: tumor necrosis factor-alpha, FGF-23: fibroblast growth factor-23, Ig-κ: immunoglobulin light chain kappa, Ig-λ: immunoglobulin light chain lambda, Zn: Zinc, DM: diabetes mellitus, Ca: Calcium, P: Phosphorus, VitD: Vitamin-D. For references: see Table S1.
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However, the concentration of small water-soluble and protein-bound solutes may be further influenced by many other factors as well, such as enzymatic metabolism, intestinal secretion/absorption, generation by intestinal flora, diet and changes in distribution volume [15,46]**.** It is conceivable that also the concentration of the weakly and moderately correlating LMWP's depends on other mechanisms than GFR, which even seem to have more important weight than GFR. Some known influencing factors such as changes in generation, homeostatic mechanisms and extra-renal clearance are summarized in table 5. Multifactorial regression analysis for the respective LMWP's including some of these parameters, increased the R^2-value as expected. E.g. for leptin, R^2 rose from 0.084 to 0.346 when corrected for BMI,which was an independent covariate for leptin concentration in a model with eGFR and BMI. The R^2-value between IL-6 and eGFR became 0.305 instead of 0.117, when adjusted for CRP, which, in contrast to BMI for leptin concentration, did however not independently predict IL-6 concentration in a model with eGFR and CRP. The majority of potentially influencing factors did however not importantly affect the R^2-

values. This suggests that other than well known mediators may influence these LMWP concentrations as well. In more advanced CKD, the influence of confounders, for example bone metabolism, is probably more important, which could explain partially that no significant associations were observed between PTH or FGF-23 and eGFR in CKD stage 4-5, while they were present in CKD stage 2-3 (table 4). Another contributing factor to this discrepancy in associations between CKD stage 2-3 versus CKD stage 4-5 might be purely mathematical, as the GFR-range in CKD 2-3 (30-90 ml/min/1.73m²) is much larger than CKD 4-5 (± 10-30 ml/min/1.73m²).

This study demonstrates that eGFR is not a reliable predictor of the concentration of most of the evaluated LMWP's, although several of them such as IL-6 [23–26], TNF-α [27,28] and FGF-23 [31–33,49] have been associated with mortality or with intermediate endpoints, such as vascular dysfunction or progression to end stage renal disease (ESRD) in CKD- or hemodialysis patients. Presumably, some of these solutes, especially if they would be representative for a cluster of other solutes, might by themselves become useful predictors of morbidity or mortality in CKD independently from eGFR. Based on the data collected in the present study, we investigated the mutual correlations between the concentrations of the different LMWP's; however, we could not identify such a marker, correlating strongly to other LMWP's without correlating to eGFR, among the investigated solutes (data not shown). This question, however, would be worthwhile to be investigated in larger populations.

In contrast to these weakly and moderately correlating LMWP's, there is a remarkable similarity in regression coefficients between CystC and β_2M. First, these molecules are the sole LMWP's studied that result in acceptably high associations with eGFR (Table 3). Second, they associate better with CystC-based eGFR formulae [8,39,40] than with Crea-based ones [7,13], the CKD-EPI-Crea-CystC [8] which contains both factors being intermediate (Table 3). Whereas this is no surprise for CystC which is included in some formulae and not in others, the pattern for β_2M seems to be identical. This suggests that the kinetics of both molecules during progression of CKD depend on similar factors or at least factors with a similar impact on solute concentration. Of note, some of the non-renal elements with impact on both concentrations [50,51], like chronic inflammatory disease or malignancy were among the exclusion criteria of this study. CystC was a superior marker of the association of

GFR with outcome in a study by Peralta *et al*., who showed that the predictive value for mortality or CVD of eGFR < 60ml/min/1.73m², based on a CystC-based eGFR, was better than eGFR based on the CKD-EPI-Crea-formula [45]. Recently, in a general population, CystC and β_2M were stronger predictors of mortality, CVD and evolution to ESRD than eGFR based on the CKD-EPI-Crea [52].

The present study has some shortcomings. First, the study population was rather small, with even smaller subgroups per CKD-stage. Second, we used eGFR which gives only an approximate value for glomerular filtration in comparison to more exact methods such as EDTA-clearance. We preferred to use methods which are applied on a day to day basis. As the differences in correlations are so striking, it is very likely that these findings can be extrapolated to GFR in general. The strengths of this study lie in the fact that several LMWP's are evaluated together in the same population for different eGFR formulae based on Crea, CystC or both.

Our present data, together with the previous ones [15], showing extremely variable associations between uremic retention solutes and a surrogate of GFR, suggests that eGFR per se is an inadequate indicator of the uremic status. This is also suggested by other studies. In a CKD population, Lilitkarntakul *et al.* [53] demonstrated that renal function did not independently predict arterial stiffness or endothelial dysfunction while the uremic retention solutes asymmetric dimethylarginine (ADMA), isoprostanes or endothelin-A did. In the Initiating Dialysis Early and Late (IDEAL) trial [54], approximately 75% of the patients randomized to start dialysis at low eGFR (5-7 ml/min/1.73m²), initiated dialysis earlier, mainly because of uremic symptoms.

In this study, the regression coefficients of different LMWP's in relation to eGFR are diverse and in general low. This shows that other factors than GFR are important for the development of the 'uremic status'. Further research is needed to evaluate whether these uremic toxins can be used as biomarkers for the risk stratification associated to uremic toxicity within the different CKD-stages and beyond eGFR.

2.6 Supporting Information

Table S1: Main factors influencing the concentrations of the studied LMWP's, other than GFR

	Extra renal handling	Generation	Reviewed in
CystC	-	Gender, age, hyperthyroidism, corticosteroid intake, malignancy, inflammation	[55]
β_2M	+ (~5%) [56]	Inflammation, malignancy	[57]
RbP	?	Insulin resistance, obesity, DM, Zn-deficiency, liver dysfunction, infection	[58–61]
PTH	+ [62,63]	Hypocalcemia, hyperphosphatemia, hypo-VitD	[64,65]
Myoglobin	+ (in uremia?) [56]	Different generation in uremia (?)	[66]
Leptin	+ [67,68]	Obesity, gender, low energy expenditure, insulin resistance	[69,70]
IL-6	+ [70–72]	Inflammation	[73]
TNF-α	+ [74]	Inflammation	[73,75]
FGF-23	+ ?	Hyperphosphatemia, regulation mineral metabolism	[65,76,77]
Ig-κ	+ ? [78]	B-cell lymphoproliferative disorders, inflammation	[79]
Ig-λ	+ ? [78]	B-cell lymphoproliferative disorders, inflammation	[79]

CystC: Cystatin C, β_2M: beta-2-microglobulin, RbP: retinol binding protein, PTH: parathyroid hormone, IL-6: interleukin-6, TNF-α: tumor necrosis factor-alpha, FGF-23: fibroblast growth factor-23, Ig-κ: immunoglobulin light chain kappa, Ig-λ: immunoglobulin light chain lambda, Zn: Zinc, DM: diabetes mellitus, Ca: Calcium, P: Phosphorus, VitD: Vitamin-D

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CHAPTER 3

UREMIA RELATED OXIDATIVE STRESS IN LEUKOCYTES IS NOT TRIGGERED BY BETA-2-MICROGLOBULIN

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3.1 Abstract

Background: Chronic kidney disease (CKD) is characterized by low-grade inflammation and increased risk for cardiovascular disease. Interest in beta-2-microglobulin (B2M) as a marker for cardiovascular outcome both with and without CKD has grown. Clinical studies suggested that B2M could be involved in the pathogenesis of vascular disease, for which chronic leukocyte activation is a pathogenic factor. We investigated whether B2M is pro-inflammatory by inducing oxidative burst in leukocytes.

Methods: Oxidative burst was measured at baseline and after stimulation with fMLP, E.coli or PMA (Phagoburst™) in whole blood of healthy volunteers in absence (saline) and presence of human B2M (hB2M) (10mg/l and 50mg/l) versus uremic whole blood. Due to suspicion of contamination, hB2M was dialyzed for purification and both purified B2M (dB2M) and dialysates were tested in the bursttest. As comparator, ROS in response to LPS was measured.

Results: Unpurified hB2M strongly enhanced ROS in monocytes and granulocytes after E.coli and PMA, and moderately after fMLP stimulation compared to control ($p<0.01$) and uremia ($p<0.01$), while at baseline hB2M only induced ROS in granulocytes ($p<0.05$). After purification, dB2M no longer increased burst activity, suggesting that contamination was responsible for the initial effect. An endotoxin concentration of <1.5EU/ml, as observed in hB2M, could not induce oxidative stress.

Conclusion: This study suggests that B2M, a traditional marker for middle molecule retention and a novel marker for cardiovascular outcome, may not by itself cause vascular damage by influencing inflammatory response due to induction of leukocyte free radical production. However, an effect on other cell types involved cannot be excluded. Our data further reveal that this type of research might be skewed by non-LPS contaminants, and that care should be taken to exclude this bias.

3.2 Introduction

Beta-2-microglobulin (B2M), a low molecular weight (MW) protein of 11.8 kDa, is more and more recognized as an independent marker for different outcomes linked to cardiovascular disease (CVD). Wilson et al. [1] identified B2M as a strong and

independent predictor for peripheral artery disease (PAD) in a population without known PAD or major chronic kidney disease (CKD). Others have associated B2M to intima media thickness [2] and pulse pressure [3] in hemodialysis patients, and to pulse wave velocity in the general population [4] and a population with known PAD[5]. Furthermore B2M was an independent predictor for cardiovascular events and mortality in the general population [6-8], in populations with underlying CVD [9-11] and also in patients with CKD and/or end-stage renal disease [12-14].

In addition to this, B2M is also frequently used as a biomarker of retention of the so-called middle molecules, the group of uremic retention solutes with MW > 500 Da. Its concentration gradually rises while CKD progresses and is strongly associated to measured [15] and estimated glomerular filtration rate (GFR) [16], reaching its highest concentrations in dialysis patients with mean concentrations up to 50 mg/l (normal 1-3 mg/l) [17, 18]. Due to the increased use of hemodiafiltration and high flux hemodialysis, there is a current trend for overall mean concentrations to decrease versus previously. However, whatever dialysis strategy used, the B2M concentrations generally increase with time [13, 19] essentially due to a loss of residual renal function[13], indicating that even with the current high efficient techniques, the weekly generation exceeds the possible weekly removal by dialysis. In addition, the concentration of B2M can also be influenced by non-renal factors such as inflammation or malignancy[20]. In parallel, there is also an association between progression of CKD and cardiovascular outcome[21].

As CKD is characterized by increased oxidative stress and low-grade inflammation, which are non-traditional risk factors for the excess of CVD in CKD [22], and as B2M is linked to cardiovascular events [7, 10, 11, 14], an active role for B2M in the pathogenesis of CVD in CKD could be hypothesized. In normal physiological conditions, B2M is part of and stabilizes the major histocompability I (MHC-I) complex in all nucleated cells. In dialysis patients, B2M plays an established role in the pathogenesis of dialysis related amyloidosis, mainly present in osteoarticular and cartilage tissue [23]. Other data on biological activity of circulating B2M on circulating leukocytes in CKD are scarce, although several studies mainly focusing on bone or fibroblasts and/or on AGE-modified B2M suggest pro-inflammatory properties [24-28].

Several uremic toxins have already been shown to induce free radical production in leukocytes [29-32] by which they may actively contribute to the inflammatory burden in CKD. Therefore, we investigated whether B2M, at concentrations as observed in CKD, could induce burst activation in leukocytes in vitro.

3.3 Materials and Methods

Sample collection

After informed consent, a sample of heparinized whole blood (NH, BD Vacutainer™, Becton Dickinson, Plymouth, UK) and serum (Venosafe™, Terumo Europe NV, Leuven, Belgium) was collected from non smoking healthy volunteers (n = 10). Additionally, predialysis heparinized whole blood samples were taken from stable hemodialysis patients (HD), as representative sample for the uremic condition (n = 10). Both, healthy volunteers and hemodialysis patients, did not have diabetes, concurrent infection, or malignancy and were not treated with immunosuppressive drugs.

Reagents

Commercial human beta-2-microglobulin (molecular weight 11.8 kDa), isolated from urine (Calbiochem®, Merck-Millipore, Nottingham, UK) (hB2M), was delivered as a solution in a phosphate buffered saline (PBS, 150 mM NaCl, 20 mM phosphate buffer, pH 7.3) at a stock concentration of 1000 mg/l. This was aliquoted in sterile tubes (Sartstedt, Nümbrecht, Germany) and stored at -20°C, according to manufacturer instructions. The manufacturer guaranteed more than 98 % purity on SDS-PAGE. E. coli lipopolysaccharide (LPS) (Ultrapure-LPS, E coli K12, Invivogen, San Diego, California) was reconstituted to a stock solution of 100 µg/ml in sterile saline (NaCl 0.9%, B. Braun, Melsungen, Germany). Pipetting was done with sterile tips (Biocert, Plastibrand®, Novolab, Belgium) and dilutions were made in sterile polystyrene tubes (BD Falcon™, Becton Dickinson, Erembodegem, Belgium), in a laminary flow hood.

Dialysis procedure

Due to suspicion of contamination of the hB2M, microdialysis of the solution was applied through a microdialysis unit, containing a membrane with a molecular weight (MW) cut-off of 10 kDa (Slide-A-Lyzer®, Thermo Scientific, Waltham, MA, USA) in order to purify the hB2M solution. Dulbecco's Phosphate Buffered Saline (DPBS) (1x, Gibco®, Life Technologies, UK) was used as dialysis fluid. 200µl hB2M (stock concentration 1000 mg/l) was added to the microdialysis cup against 1000 µl of DPBS. Under continuous rotation, at room temperature, the total dialysis time was 11h30. To maintain an optimal concentration gradient, nine dialysate fluid changes, with shorter time intervals in the beginning and longer time intervals in the end, were performed. At the end of the procedure the dialyzed hB2M solution (dB2M) was recovered, aliquoted and stored at -80°C for further analysis.

Dialysis fluid samples of the first (d1: 0-30 minutes) as well as of the final dialysis period (d2: 10 hours 30 minutes to11hours 30 minutes) were collected, aliquoted and stored at -80°C for further analysis.

Concentration determinations

The B2M concentration in hB2M, dB2M and in the dialysate samples was measured by ELISA (Orgentec Diagnostica GmbH®, Mainz, Germany). The test solutions were diluted 1/10 in serum of healthy volunteers to guarantee samples that were compatible for the assay and concentrations were calculated after the correction for the dilution factor.

Endotoxin determination

Endotoxin concentration was measured in hB2M and dB2M to assess which B2M concentration could be used without being skewed by the presence of LPS at a level inducing ROS production by itself. LPS was also measured in the different dialysis fluid samples by means of a kinetic chromogenic LAL (Limulus Amebocyte Lysate)-assay (Kinetic QCL™, Lonza ltd, Basel, Switzerland). The detection limit of the test is 0.005 endotoxin (EU) /ml.

Oxidative burst

Test solutions

Beta-2-microglobulin was tested at a relevant uremic concentration of 50 mg/l, which lies in the range of the highest reported mean uremic concentrations mainly observed in dialysis patients [17, 18] and a concentration of 10 mg/l, representative for CKD patients not on dialysis [14]. LPS at a concentration of 1.5 EU/ml, which was the maximum LPS concentration present in the hB2M solution (50 mg/l), and saline were used as controls. Furthermore LPS at a concentration of 1.5 and 3.0 EU/ml was compared.

Stock solutions of hB2M, dB2M and LPS were diluted in saline (NaCl 0.9%, B Braun, Melsungen, Germany) on the day of the experiment to a tenfold of the final test concentration (C 10 x), to be further diluted 1/10 in whole blood. The bursttest was also applied on the two different dialysate samples as test solution and on uremic whole blood from HD patients, as a comparator, relevant for the chronic uremic condition and the overall group of uremic toxins as present in vivo.

Oxidative Burst: test procedure

Saline (control), hB2M, dB2M (C 10x), LPS (C 10x), and dialysates, were diluted 1/10 in heparinized whole blood of healthy volunteers. Those samples, as well as uremic whole blood samples from HD patients were incubated for 10 min at 37°C, after which Reactive Oxygen Species (ROS) production in leukocytes was measured. For that purpose, the bursttest (Phagoburst™, Orpegen Pharma, Heidelberg, Germany) was applied according to manufacturer's instructions.

Burst activity in leukocytes was evaluated at baseline and after stimulation with N-formyl-methionine-leucine-phenylalanine (fMLP), a moderate stimulus, and stimulation with E. coli and phorbol-12-myristate-acetate (PMA), two strong stimuli. The generation of ROS was measured by assessing the oxidative modification of the fluorogenic substrate dihydrorhodamine-123 into rhodamine.

The samples were analyzed within 30 minutes using a FACScan (Becton Dickinson, Erembodegem, Belgium). Monocytes, granulocytes and lymphocytes were gated separately and the percentage of rhodamine positive cells (%) per gate was

measured at baseline and after fMLP stimulation. After E.coli and PMA stimulation the mean fluorescence intensity (MFI) was considered, as the majority of the cells were positive for ROS production under these conditions, except for lymphocytes, for which the percentage of positive cells after E. coli stimulation was also evaluated, due to a lower number of positive cells.

Statistical analysis

The data were not normally distributed and expressed as medians with interquartile range. A Kruskall-Wallis test or Friedman-Ranks test, in case of paired experiments, were used to analyse multiple conditions and followed by pairwise comparisons. In case of analysis of two groups a Mann-Whitney test was applied. A p-value of < 0.05 was considered as statistically significant. Statistical analysis was performed using SPSS statistics 20 (SPSS Inc, Chicago, IL, USA) for Windows (Microsoft Corp, Redmond, WA, USA). Graphs were made in GraphPad Prism 04 (GraphPad Software, La Jolla, CA, USA).

3.4 Results

Determination of endotoxin concentration

As endotoxin is a major source of contamination with stimulatory effects on burst activity, the endotoxin concentration was measured in the hB2M solution. Our test solution of hB2M (50 mg/l) contained a maximum of 1.5 EU/ml depending on the batch number, so that in the burst experiments a control of LPS at 1.5 EU/ml was used.

The endotoxin concentration was also measured in two other commercially available B2M-formulations but as they contained at least a concentration of 15 EU/ml (in 50 mg/l B2M), they were not used for further analysis (data not shown).

Effects of different endotoxin concentrations

In order to investigate at which concentration LPS could induce oxidative burst, different endotoxin concentrations were evaluated in the bursttest. While LPS did not induce burst activity at 1.5 EU/ml, a concentration of 3 EU/ml increased the

percentage of ROS producing monocytes and granulocytes at baseline (monocytes: 12.9% (3 EU/ml) vs. 2.7% (control), $p < 0.05$; granulocytes: 3.12% vs. 2.4%, $p < 0.05$) and after fMLP stimulation (monocytes: 28.8% vs. 10.4%, $p < 0.001$; granulocytes: 14.7% vs. 9.2%, $p < 0.001$). LPS had no effect on burst activation after stimulation with E. coli and PMA in all three leukocyte cell types.

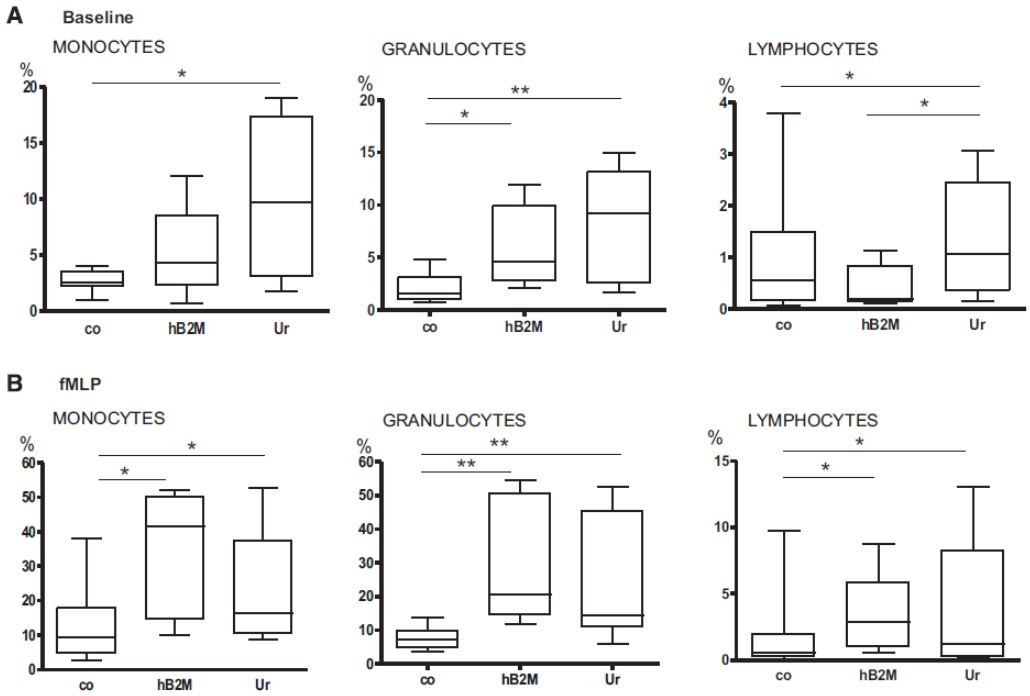

Figure 1. Effects of unpurified hB2M and uremic whole blood (hemodialysis patients) milieu on leukocyte burst activity in monocytes, granulocytes, and lymphocytes at (A) baseline and (B) after stimulation with fMLP. Co, control (saline); fMLP, *N*-formyl-methionine-leucine-phenylalanine; hB2M, human β2-microglobulin; Ur, uremia; %, percentage rhodamine-positive cells. *$P < .05$ and **$P < .01$ by Kruskall-Wallis + pairwise comparisons.

Effects of unpurified human beta-2-microglobulin on leukocyte oxidative burst

At baseline, hB2M (50 mg/l), increased the percentage of ROS-producing granulocytes ($p < 0.05$) compared to control, while no significant effects were observed in monocytes and lymphocytes (figure 1A). After fMLP stimulation, hB2M (50 mg/l) enhanced the percentage of rhodamine positive leukocytes compared to control (monocytes and lymphocytes, $p < 0.05$, granulocytes, $p < 0.01$) (figure 1B). More remarkably even, hB2M enhanced free radical production very strongly in all three leukocyte cell types after stimulation with E.coli ($p < 0.01$) (figure 2A) and PMA ($p < 0.01$) (figure 2B). This stimulation was more robust than expected and did not reflect burst activity as observed in whole blood from HD patients. Compared to control, in the whole blood samples of HD patients, the percentage of free radical

producing monocytes ($p < 0.05$), granulocytes ($p < 0.01$) and lymphocytes ($p < 0.05$), at baseline and after fMLP stimulation (figure 1) and the percentage of ROS-producing lymphocytes after stimulation with E. coli ($p < 0.01$) (figure 2A) was increased. After stimulation with PMA, burst activation was significantly blunted in granulocytes ($p < 0.05$) (figure 2B). Comparing the effects of hB2M to these of the uremic milieu, hB2M enhanced ROS-production after stimulation with E. coli and PMA in all leukocyte types more substantially ($p < 0.01$) (figure 2). These results led to the suspicion of contamination, other than endotoxin, as the endotoxin concentration in the B2M solution could not induce leukocyte activation.

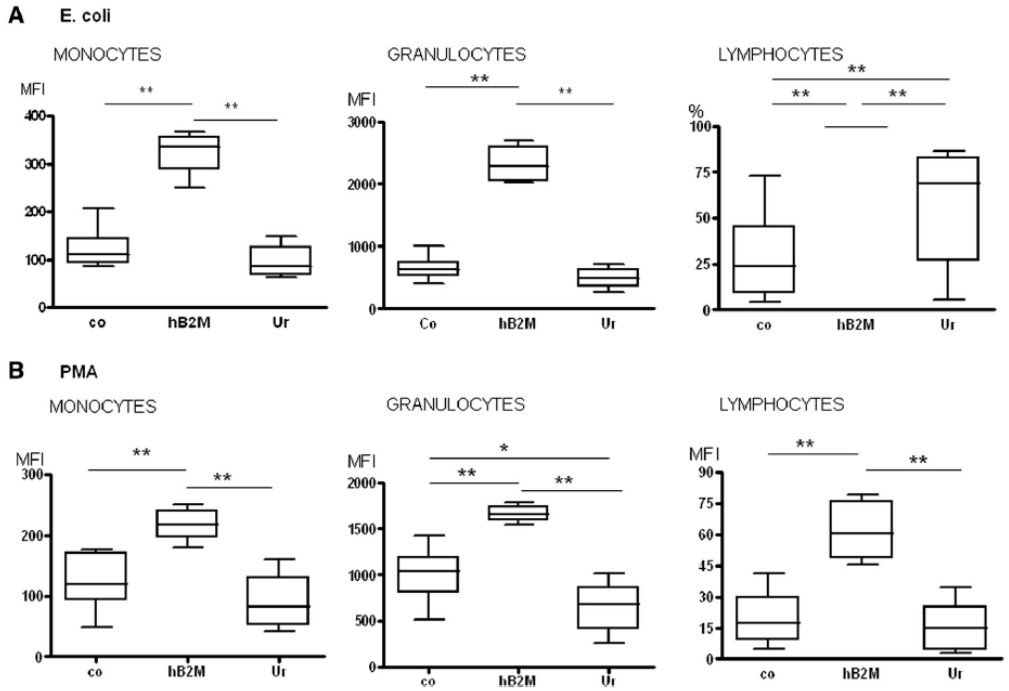

Figure 2. Effects of unpurified hB2M and uremic whole blood (hemodialysis patients) on leukocyte burst activity in monocytes, granulocytes, and lymphocytes after stimulation with (A) *E. coli* and (B) PMA. Co, control (saline); hB2M, human β2-microglobulin; MFI, mean fluorescence intensity; PMA, phorbol-12-myristate-acetate; Ur, Uremia; %, percentage rhodamine-positive cells. $^{*}P < .05$ and $^{**}P < .01$ by Kruskall-Wallis + pairwise comparisons.

Purification of human beta-2-microglobulin by dialysis

hB2M was purified by microdialysis to remove possible contaminants with MW <10 kDa, so that the effects of B2M per se on burst activation could be investigated. Before their assessment by the bursttest, the dB2M and dialysate samples were tested for their B2M- and endotoxin concentration (1/10 in healthy serum). In dB2M, the B2M concentration was adequately recovered and, as expected, similar endotoxin concentrations (1.6 EU/ml) as in the original solution were found, as LPS

did not pass the membrane. The dialysate contained, as expected, neither LPS (< 0.005 EU/ml) nor B2M.

Table 1. Results of the Bursttest After Purification of B2M (dB2M) and Dialysates at Baseline and After Stimulation With fMLP

Condition	Co (n = 10)	LPS 1.5 EU/mL (n = 10)	dB2M 10 mg/L (n = 4)	dB2M 50 mg/L (n = 4)	d1 (n = 3)	d2 (n = 4)	P Value*
Monocytes							
B (%)	3.1 (2.1-3.5)	4.3 (3.3-11.3)	4.5 (2.5-6.4)	7.6 (5.5-8.9)	10.2 (5.5-11.5)	6.3 (2.7-10.7)	.39
fMLP (%)	7.5 (5.6-9.9)	9.8 (9.3-27.6)	17.4 (13.6-24.3)	15.8 (12.4-18.6)	16.6 (13.1-30.8)	21.4 (6.7-35.4)	.23
Granulocytes							
B (%)	2.5 (1.7-3.4)	2.7 (2.4-4.1)	2.5 (1.5-3.5)	1.6 (1.5-2.4)	6.9 (3.9-11.6)	3.0 (1.2-3.8)	.80
fMLP (%)	7.6 (5.9-9.8)	10.9 (8.4-13.3)	10.0 (8.5-15.3)	6.6 (5.8-9.3)	20.8 (13.2-28.0)	14.5 (9.9-19.4)	.53
Lymphocytes							
B (%)	0.4 (0.2-0.5)	0.5 (0.2-0.8)	0.7 (0.5-1.5)	0.7 (0.5-1.1)	9.8 (4.9-11.0)	0.3 (0.3-1.3)	.67
fMLP (%)	0.5 (0.4-0.8)	0.7 (0.5-1.1)	2.2 (1.4-3.4)	0.7 (0.4-1.3)	2.8 (1.5-9.3)	1.0 (1.0-4.1)	.84

B, baseline; Co, control; d1, dialysate after first fluid change (0-30 min); d2, dialysate at end of dialysis procedure (10 h 30 min to 11 h 30 min, ninth fluid change); dB2M, dialysed β2-microglobulin; EU, endotoxin unit; fMLP, N-formyl-methionine-leucine-phenylalanine; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; %, percentage of free radical producing cells.
The results are expressed as medians with interquartile range in parentheses.
* P value of Kruskall-Wallis test.

Effects of purified beta-2-microglobulin on leukocyte oxidative burst

In contrast to hB2M (figure 1 and 2), dB2M (10 mg/l and 50 mg/l) had no effects on burst activity in leukocytes in any condition compared to controls (saline and endotoxin 1.5 EU/ml) (table 1, figure 3). Thus after purification, the stimulating effects of hB2M disappeared, indicating that one or more contaminants with MW < 10 kDa were responsible for the increased free radical production.

This was confirmed by the observations from the bursttest applied on the dialysates: d1, the sample of the first incubation period, in which the highest concentration of contaminant(s) could be expected, increased ROS production after E.coli and PMA stimulation in all leukocyte cell types, similar to the results observed with hB2M. In contrast, d2, at the end of the dialysis procedure, had no longer an effect on burst activity, which indicated that the contaminant(s) were entirely removed by dialysis during the earlier fluid changes (table 1, figure 3). The DPBS, used as dialysate, did not have any effect on the bursttest compared to saline (data not shown).

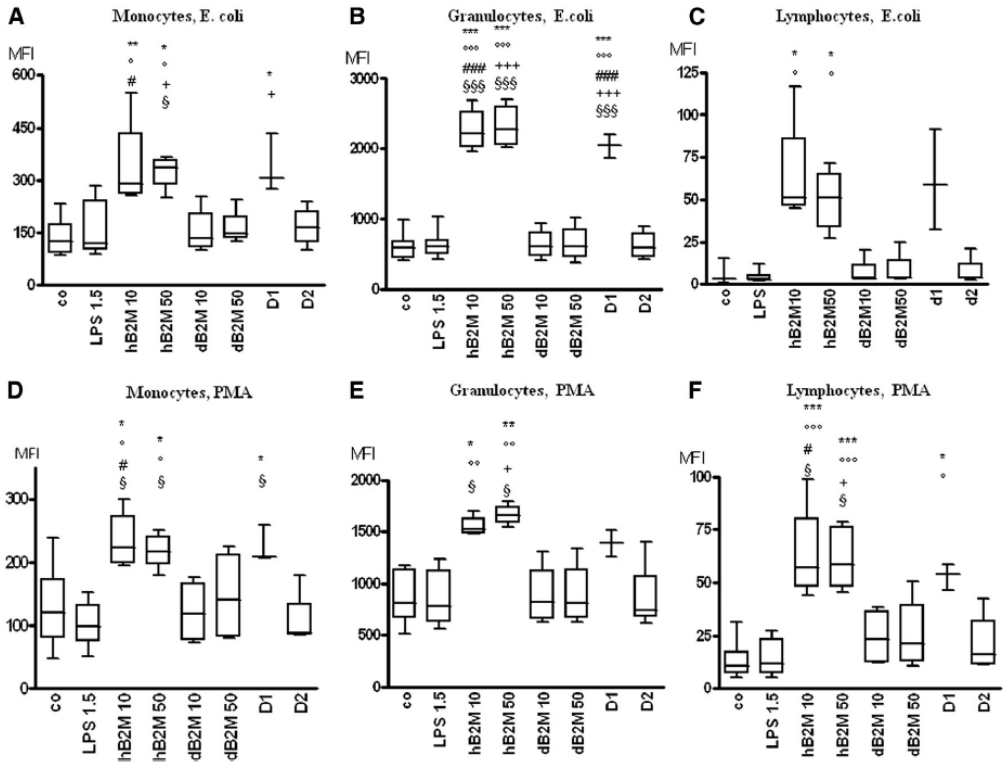

Figure 3. Effects of hB2M, dB2M, and dialysates on bursttest after stimulation with (A-C) *E. coli* and (D-F) with PMA show that dB2M had no longer an effect on free radical production whereas d1 had similar effects as hB2M. PMA, phorbol myristate acetate; co, control (saline); LPS 1.5, lipopolysaccharide 1.5 endotoxin units (EU)/mL; hB2M, human β2 microglobulin (unpurified); dB2M, dialyzed (purified) β2-microglobulin; 10, 10 mg/L; 50, 50 mg/L; d1, dialysate collection after 30 minutes (0-30 min); d2: dialysate at end (10h30-11h30), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs.co, ° $P < 0.05$, °° $P < 0.01$, °°° $P < 0.001$ vs LPS, # $P < 0.05$, ### $P < 0.001$ vs dB2M10, + $P < 0.05$, +++ $P < 0.001$ vs dB2M50, § $P < 0.05$, §§§ $P < 0.001$ vs d2; Kruskall-Wallis + pairwise comparisons.

3.5 Discussion

In this study we demonstrated that 1) after exclusion of effects of endotoxin and after purification, beta-2-microglobulin per se had no effect on oxidative burst in leukocytes in vitro and 2) extreme effects observed in a biological in vitro assay should be interpreted with caution and possible contamination should always be excluded. The initial B2M-solution (hB2M) increased very strongly free radical production in all three leukocyte cell types especially after stimulation with E. coli and PMA. After exclusion of effects of endotoxin and after purification of the hB2M, the effects on oxidative burst by the purified B2M disappeared. Although the present study for that reason in essence reports negative results, it is important to convey also this kind of data to the scientific community, given that the experiments are well described. This will save time and money for other researchers working in the same field.

CKD is an independent risk factor for CVD [33, 34]. Since B2M is one of the main uremic retention solutes, one could raise the hypothesis that it could be one of the solutes

playing an active role in the pathogenesis of CVD related to CKD. Our data suggest that B2M could not be considered as a culprit for CVD in CKD by inducing leukocyte burst activation, although B2M is related to mortality [6, 7, 9, 10, 12-14] and cardiovascular outcome [1-5, 7, 8, 10, 11, 14] in a multitude of studies. Effect on other vascular cell types, however, cannot be excluded.

Oxidative stress is one of the non-traditional risk factors and a pathogenic factor for CVD in CKD [22]. Our study demonstrated that whole blood samples from HD patients indeed are pro-oxidative in the experimental set-up of the bursttest, as free radical production was increased at baseline and after fMLP stimulation (figure 1). Several uremic retention solutes such as the small water soluble guanidine compounds [32, 35], the protein-bound compound p-cresylsulfate [30, 31] and the middle molecules, dinucleoside polyphosphates [36] have already been shown to stimulate leukocyte oxidative burst. However, based on our data, the oxidative effect of uremic serum on leukocytes cannot be attributed to the presence of B2M (table 1 and figure 3). This finding might also imply that at least in the context of uremic oxidative stress, specific removal of B2M as has been depicted in a number of occasions [37, 38] might not be very helpful, whereas its aspecific removal as a marker together with other compounds with similar MW of which a number has a pro-inflammatory potential [39, 40], might be a more logical approach. It should however be noticed that decreasing B2M levels in dialysis patients might remain important to reduce the occurrence of B2M related amyloidosis and its complications [41].

Other in vitro studies with B2M were mainly conducted in the context of dialysis related amyloidosis and suggested, somewhat in contradiction with the present data, that B2M or B2M variants had pro-inflammatory properties. But to the best of our knowledge, none of the studies combined genuine B2M with circulating leukocytes like the present study does. B2M per se induced osteoclastogenesis in cultured mice calvaria [42] and was biologically active on synovial fibroblasts by increasing the expression of VCAM-1 [25, 26] or matrix metalloproteïnase-3 [43]. B2M modified by advanced glycation end-products (AGE-B2M), induced monocyte chemotaxis and secretion of tumor necrosis factor-alpha and interleukin-1-beta in macrophages [24] and monocytes [27, 44]. Furthermore, AGE-B2M delayed monocyte apoptosis and induced monocyte to macrophage-like transition [44]. Whether these effects of AGE-B2M on monocyte cell cultures are representative for B2M per se in circulation

should be interpreted with caution. First of all, these effects were due to the interaction of AGE to its receptor (RAGE) and not due to a B2M effect per se [44, 45]. Second, in serum of dialysis patients only a small percentage of the total B2M is AGE-modified [25], so that the concentration in circulation is likely to be much lower than the used concentration in the experiments. To the best of our knowledge, it is not known whether there exists a strong correlation between B2M and AGE-B2M concentrations. Finally, the AGE-B2M in these studies was prepared in vitro, which does not necessarily reflect the in vivo present AGE-B2M. Only in the study by Miyata et al. [24], AGE-B2M isolated from urine from two long term dialysis patients was investigated next to AGE-B2M prepared in vitro, which demonstrated the same pro-inflammatory properties on monocytes for both. In our study, the in vitro set-up was as relevant as possible for the in vivo situation by studying B2M at appropriate uremic concentrations in whole blood.

In any way, this study also demonstrated that strong effects observed in biological in vitro assays should be interpreted with caution. A well known contaminant with effects on burst activation is LPS. However, we demonstrated that the maximal LPS concentration of 1.5 EU/ml in our test solutions did not induce burst activation neither at baseline nor after stimulation. Although LPS from different sources could have different biological effects [46], it is unlikely that LPS was responsible for the increased free radical production in the presence of hB2M in our experiments, since the purified B2M (dB2M) still contained LPS, but had no effects on leukocytes anymore (table 1, figure 3).

Molecules in solution that are added as buffer or preservative can also interfere with biological effects: if known, they can be included as controls [47]. The original PBS buffer of reconstituted B2M solution contained NaCl and a phosphate buffer. DPBS compared to saline however did not induce free radical production (data not shown). We performed a microdialysis with a membrane with MW cut off of 10 kDa in order to remove small solutes. The results of the bursttest on the dialysate samples showed that the responsible solute(s) could be easily removed: the dialysate sample of the first incubation period could induce similar ROS production as the original B2M-solution, indicating a rapid and easy diffusion over the membrane. The dialysate sample of the last incubation period did not have any effect anymore, which indicated

that substances with MW < 10 kDa, which induced oxidative burst, had in the meanwhile been cleared from the solution (table 1 and figure 3).

This study has some weaknesses. We only tested commercially available beta-2-microglobulin isolated from human urine. We did not test the structural variants of B2M occurring in dialysis patients. Although their concentration is only a fraction of total B2M [25, 48], biological activity in leukocytes is not excluded. Especially AGE-B2M could have pro-inflammatory properties, but probably rather due to the AGEs than due to B2M [44, 45]. Furthermore, we limited this study to a screening of the effect of B2M on leukocyte oxidative burst. One might argue that effects on other cell types, such as endothelium could be relevant. Those have not been excluded. However also at endothelial level, micro-inflammation and free radical production are essential damaging factors, and at least with regard to these key elements, B2M does not seem to play a major role, based on our data.

In conclusion, our study suggests that beta-2-microglobulin, an established marker for middle molecules and an emerging marker for cardiovascular outcome in CKD by itself, does not play a direct pathogenic role in cardiovascular disease by inducing free radical production in leukocytes. However effects on other vascular cell types cannot be excluded.

3.6 Practical application

This study provides data whether beta-2-microglobulin is only a biomarker or whether it could contribute to the pathogenesis of cardiovascular disease in chronic kidney disease by induction of leukocyte oxidative stress.

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CHAPTER 4

PRO-INFLAMMATORY CYTOKINES AND LEUKOCYTE OXIDATIVE BURST IN CHRONIC KIDNEY DISEASE:

CULPRITS OR INNOCENT BYSTANDERS?

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4.1 Abstract

Background: Pro-inflammatory cytokines are elevated in chronic kidney disease (CKD), a condition characterized by micro-inflammation with oxidative stress as key feature. However, their role in the inflammatory response at uremic concentrations has not yet been defined. In this study, the contribution of cytokines on induction of leukocyte oxidative stress was investigated.

Methods: Whole blood from healthy donors was incubated with 20-1400pg/mL TNFα, 5-102,8pg/mL IL6, 20-400pg/mL IL1β and 75-1200pg/mL IL18 separately or in combination. Oxidative burst was measured, at baseline and after stimulation with fMLP (Phagoburst™). The effect of the TNFα blocker, adalimumab (Ada), was evaluated on TNFα-induced ROS-production. Finally, the association between TNFα and the composite endpoint all-cause mortality or first cardiovascular event was analysed in a CKD population stage 4-5 (n= 121).

Results: While IL6, IL1β and IL18 alone induced no ROS-activation of normal leukocytes, irrespective of concentrations, TNFα induced ROS-activation at baseline ($p<0.01$) and after fMLP stimulation ($p<0.05$), but only at uremic concentrations in the high range (400 and 1400pg/ml). A similar pattern was observed with all cytokines in combination, but already at intermediate uremic concentrations (all $p<0.05$, except for monocytes after fMLP stimulation: n.s.), suggesting synergism between cytokines. ROS-production induced by TNFα (400pg/ml) and the cytokine combination was blocked with Ada. Uremia related oxidative stress in leukocytes of hemodialysis patients was however not blocked by Ada. In patients, TNFα was not associated to adverse events (HR: 1.52, 95% CI 0.81-2.85, $p = 0.13$).

Conclusion: Among several pro-inflammatory cytokines, TNFα alone was pro-oxidative but only at high range uremic concentrations. Adding a TNFα-blocker, adalimumab, blocked this ROS-production, but not the oxidative stress in blood samples from hemodialysis patients, suggesting that other uremic toxins than TNFα are more crucial in this process. This finding was corroborated by the lack of association between TNFα and mortality.

4.2 Introduction

The concentration of cytokines gradually increases in chronic kidney disease (CKD) [1], which is mainly attributed to an increased generation in response to uremic toxins [2-4] and reduced renal clearance [5,6]. In clinical studies in CKD, pro-inflammatory cytokines are used as a hallmark of micro-inflammation [7]. Both in pre-dialysis and dialyzed CKD, cytokines, especially interleukin (IL)-6, have been associated to multiple CKD-related adverse outcomes such as malnutrition [8], muscle wasting [9], atherosclerosis [8,10], anemia [11] and all-cause or cardiovascular mortality [12-20]. Surprisingly, data on the pathophysiological role of cytokines in leukocytes at concentrations as occurring in CKD are to the best of our knowledge not available.

Excessive oxidative stress is one of the key features of CKD-related micro-inflammation, to which a host of factors such as intra-venous iron administration, bio-incompatibility of dialysis membranes, endotoxemia and uremic solute retention, are contributing [21].

In vitro, IL6, tumor necrosis factor alpha (TNFα) and IL18 induce leukocyte oxidative stress at concentrations in the range or in excess of those observed in extreme clinical conditions, such as sepsis [22-25]. In contrast, whether cytokines at lower levels, as observed in CKD, could induce free radical production, has not yet been investigated.

A better understanding of the interaction between cytokines and oxidative stress in CKD could provide more insight in the mechanisms of uremic leukocyte activation, one of the triggers for CKD-related cardiovascular disease, and on potential therapeutic pathways to prevent these problems.

Therefore, the aim of the present study was to investigate the effects of four pro-inflammatory cytokines, at relevant uremic concentrations, separately and in a combination, on the induction of leukocyte oxidative burst in whole blood of healthy donors. The effect of a TNFα-blocker, adalimumab, on free radical production by TNFα was also evaluated. These findings were then compared to the impact of adalimumab on free radical production in leukocytes of hemodialysis patients. Finally, we analysed the *in vivo* association between TNFα and adverse outcome in a population with CKD stage 4-5.

4.3 Material and Methods

4.3.1 In vitro study

Samples

After informed consent, heparinized whole blood (NH, BD Vacutainer™, Becton Dickinson, Plymouth, UK) and coagulated blood (Venosafe™, Terumo Europe NV, Leuven, Belgium) samples were collected from non-smoking healthy volunteers and from stable, non-smoking hemodialysis patients before dialysis and heparinization. Patients with diabetes, concurrent infection, malignancy or treatment with immunosuppressive drugs were excluded. Patients underwent online-hemodiafiltration 3 times 4 hours a week. The quality of the dialysis fluid met the ultrapure standards (bacteria <0.1 CFU/ml, endotoxin <0.03 EU/ml) as checked on a regular basis. Blood was allowed to coagulate for 30 min and was then centrifuged (10 min, 3000rpm) in order to store the serum in aliquots at -80 °C. The protocol of the study was approved by the local ethics committee.

Reagents

The human recombinant cytokines interleukin (IL)-6, tumor necrosis factor alpha (TNFα) and IL1β (R&D Systems®, Abingdon, UK) were reconstituted in Dulbecco's phosphate buffered saline (DPBS) (1x, Gibco®, Life Technologies, UK) with 0.5% albumin (Sigma-Aldrich, St Louis, MO, USA) (DPBS-0.5%alb). Human recombinant IL18 (MBL International, Woburn, MA, USA), was reconstituted in sterile water (Sigma Aldrich, St Louis, MO, USA). The cytokine stock solutions were aliquoted and stored at -80°C. A commercially available TNFα-blocker, adalimumab (50mg/ml) (Abbvie, North Chicago, Illinois, USA), was stored at 4°C and diluted in sterile saline (NaCl 0.9%, B. Braun, Melsungen, Germany) immediately before use. The stock solutions of the appropriate reagents were further diluted immediately before addition to whole blood. The concentration (10, 20, 40 or 50 times of the final concentration) of the prepared stock solutions was such that, after addition, the dilution of the blood was similar for each condition and that solute concentrations were as described below.

Oxidative burst

Test solutions and experimental set-up

Controls

Saline and DPBS-0.5%alb were used as controls (co) for the experiments with respectively adalimumab and the cytokine solutions, as they were the solutions in which these compounds were diluted.

Dose response experiments of cytokines

The cytokines were tested individually in dose response at relevant uremic concentrations, varying from an average to high range [26-28]. The added test concentrations were: for TNFα 20; 70; 400 and 1400 pg/ml, for IL6: 5; 10; 21.5; 95.4 and 102.8 pg/ml, for IL1β: 20, 80 and 400 pg/ml and for IL18: 75; 150; 300; 600 and 1200 pg/ml. As the cytokines were added to whole blood of healthy donors, it should be noted that the final concentration in the blood was slightly higher than the aimed test concentration since these cytokines are present as such at very low concentrations in normal blood. It is highly unlikely that this would have influenced the results of the experiments, since the background concentration of cytokines in donor blood is much lower compared to the added concentrations of the cytokines.

Effects of a combination of pro-inflammatory cytokines

Also the combination of the four pro-inflammatory cytokines at a uremic concentration in the intermediate range was tested in whole blood of healthy donors. The combination contained TNFα at 70 pg/ml, IL6 at 10 pg/ml, IL1β at 20 pg/ml and IL18 at 150 pg/ml.

Effects of blocking TNFα with adalimumab in healthy controls

The effect of adalimumab (17.5 mg/l [29]) on burst activity was evaluated as such and in the presence of TNFα (400 pg/ml), which was the lowest TNFα concentration that induced burst activation per se or of the cytokine combination mentioned above.

Studies of oxidative burst activity in whole blood of hemodialysis patients

Leukocyte burst activation in heparinized whole blood of hemodialysis patients and healthy volunteers was compared. Furthermore, adalimumab was added to whole blood of hemodialysis patients to evaluate the possible influence of the TNFα-blocker on uremia-related oxidative stress.

Oxidative Burst test

Whole blood of healthy donors or hemodialysis patients was incubated for 10 min at 37°C with control solution or the different experimental solutions, after which Reactive Oxygen Species (ROS) production in monocytes and granulocytes was measured. For that purpose, the Burst test (Phagoburst™, Orpegen Pharma, Heidelberg, Germany) was applied according to manufacturer's instructions. Burst activity in leukocytes was evaluated at baseline and after stimulation with N-formyl-methionine-leucine-phenylalanine (fMLP), a moderate stimulus. The generation of ROS was measured by assessing the oxidative conversion of the fluorogenic substrate dihydrorhodamine-123 into rhodamine.

The samples were analyzed within 30 minutes by flow cytometry using a FACScan (Becton Dickinson, Erembodegem, Belgium). Monocytes and granulocytes were gated separately and the percentage of rhodamine positive cells (%) per gate was measured at baseline and after fMLP stimulation.

4.3.2 *In vivo* study

Study population

All non-transplanted chronic kidney disease (CKD) patients stage 4 and 5 not on dialysis, attending the Nephrology outpatient clinic, included in the biobank sample collection of the Nephrology Department of the Ghent University Hospital between January 2011 and July 2012, were eligible for this study (n = 121). Patients were sampled after written informed consent and outcomes were registered prospectively. Plasma samples were processed immediately after collection and stored at –80°C. The study was approved by the local ethical committee.

Baseline clinical parameters (age, gender, blood pressure, pulse, height and weight) were registered. Body mass index (BMI) was calculated as weight/height² (kg/m²), mean arterial pressure (MAP) as the sum of 1/3 of the systolic and 2/3 of the diastolic blood pressure and pulse pressure (PP) as the difference between systolic and diastolic blood pressure. Estimated glomerular filtration rate (eGFR) was calculated based on the creatinine-based CKD-EPI formula [30]. The following comorbidities were recorded: cardiovascular history when at least one of the following was present: arterial cardiovascular disease (coronary, cerebral or peripheral), atrial fibrillation or heart failure (requiring hospitalisation); malignancy; diabetes mellitus, defined as a history of diabetes or treatment with insulin or oral antidiabetic drugs; hypertension, defined as current hypertension (>140/90 mmHg) or the use of antihypertensive drugs; hypercholesterolemia, defined as history of or treatment with lipid lowering drugs, and smoking status (active versus no/former smoker).

Patients were followed until March 12th 2014 for the occurrence of cardiovascular events (acute coronary syndrome, de novo atrial fibrillation, acute heart failure, coronary artery bypass graft, percutaneous transluminal coronary angioplasty, cerebrovascular accident, percutaneous transluminal angioplasty) or all-cause mortality whichever came first.

4.3.3 Concentration determination

In the *in vitro* study, ELISA's for IL6, TNFα, IL1β (R&D Systems, Abingdon, UK) and IL18 (MBL International, Woburn, MA, USA) were used to verify the concentrations of the reconstituted recombinant cytokines in the stock solutions. Therefore they were diluted to a defined concentration within the standard curve of the ELISA. Furthermore, the TNFα concentration in the serum of the HD patients used for evaluation of oxidative burst activity as well as those in plasma samples of patients with CKD 4 and 5 in the *in vivo* study were quantified by ELISA (R&D Systems, Abingdon, United Kingdom). Serum creatinine and C-reactive protein (CRP) were measured by routine laboratory tests.

4.3.4 Statistical analysis

In vitro study

The data of the Burst-test were non-normally distributed and consequently expressed as medians with interquartile range and analyzed with Friedman-ranks test, Wilcoxon signed-rank test or Mann-Whitney test as appropriate..

For the *in vivo* study, Continuous data were expressed as mean with standard deviation or median with interquartile range depending on their distribution, and analysed by Student's t-test or Mann Whitney-test. Binary categorical data were expressed as frequencies and analysed with chi-square test. The association between TNFα concentration as continuous variable and outcome was analyzed by univariate Cox proportional hazards model. The primary outcome was the composite endpoint of mortality or first non-fatal cardiovascular event. The analysis was repeated with all-cause mortality as outcome. In a multivariate analysis, variables with a p-value of <0.1 in univariate analysis together with TNFα were included into the model. The results are reported as hazard ratios with their 95% confidence interval (CI). A p-value <0.05 was considered as significant. Kaplan Meier survival curves were made for both outcomes using the median TNFα as cut-off.

Statistical analysis was performed using SPSS statistics 22 (SPSS Inc, Chicago, IL, USA) for Windows (Microsoft Corp, Redmond, WA, USA). Graphs were made with GraphPad Prism 04 (GraphPad Software, La Jolla, CA, USA) or SPSS statistics 22. A p-value of < 0.05 was considered as statistically significant.

4.4 Results

4.4.1 In vitro study

Control solutions (n = 10)

The two control solutions, saline and DPBS-0.5%alb, had no effect on oxidative burst (data not shown).

Effect of cytokines on leukocyte oxidative burst in healthy donors.

Dose response of TNFα (n = 8)

TNFα increased ROS-production dose dependently in monocytes and granulocytes, at baseline and after stimulation with fMLP (figure 1). In monocytes, TNFα at 400 pg/ml and 1400 pg/ml induced free radical production at baseline and after fMLP-stimulation compared to control (all $p < 0.01$) and to 20 pg/ml (400 pg/ml: $p < 0.05$; 1400 pg/ml: baseline: $p < 0.05$, fMLP: $p < 0.01$) (figure 1A and 1C). In granulocytes, TNFα, at 400 pg/ml and 1400 pg/ml, induced ROS at baseline compared to control ($p < 0.01$) (figure 1B), while after fMLP stimulation, only TNFα at 1400 pg/ml increased free radical production compared to control ($p < 0.05$) (figure 1D).

Figure 1

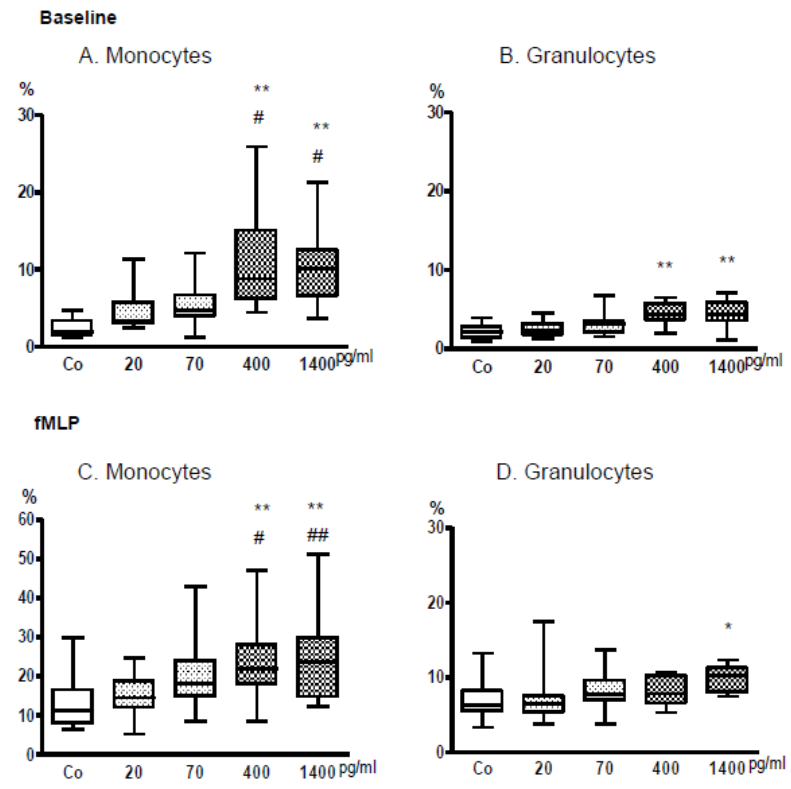

Figure 1: Dose response of oxidative burst in monocytes and granulocytes from healthy donors in dose response to TNFα at baseline and after stimulation with fMLP. Y-axis: percentage of rhodamine positive cells (%), X-axis: concentration of TNF-α in pg/ml; Co: control (DPBS-0.5%alb); *, **: $p < 0.05$, < 0.01 vs. co; #, ##: $p < 0.05$, < 0.01 vs. 20 pg/ml.

Dose response of other cytokines (n = 8)

The tested concentrations of IL6, IL1β and IL18 did neither induce nor inhibit free radical production in monocytes or granulocytes (data not shown).

Effect of the combination of the four cytokines (n = 8)

The combined cytokines had a stimulatory effect on leukocyte oxidative burst at baseline in monocytes and granulocytes (both $p < 0.05$) and after stimulation with fMLP in granulocytes ($p < 0.05$) (table 1). The pro-oxidative effects were similar to the ones observed with TNFα alone (400 pg/ml), although it is of note that the concentration of 70 pg/ml TNFα used in the combination solution did not induce ROS when added to normal blood by itself (figure 1).

Effects of adalimumab on leukocyte oxidative burst

Effects of adalimumab alone (n = 10)

Compared to control, adalimumab had neither an effect on free radical production at baseline (figure 2A-B) nor after stimulation with fMLP (figure 2C-D) in healthy donor blood.

Effects of adalimumab on oxidative burst induced by TNFα (n = 10)

Adalimumab blocked the free radical production induced by TNFα (400 pg/ml) in monocytes (6.9% vs. 3.3%, $p < 0.05$) (figure 2A) and granulocytes (5.5% vs. 2.6%, $p < 0.05$) (figure 2B), at baseline. In contrast, adalimumab did not completely inhibit the stimulatory effects of TNFα in monocytes (figure 2C) or granulocytes (figure 2D) after fMLP-stimulation.

Figure 2

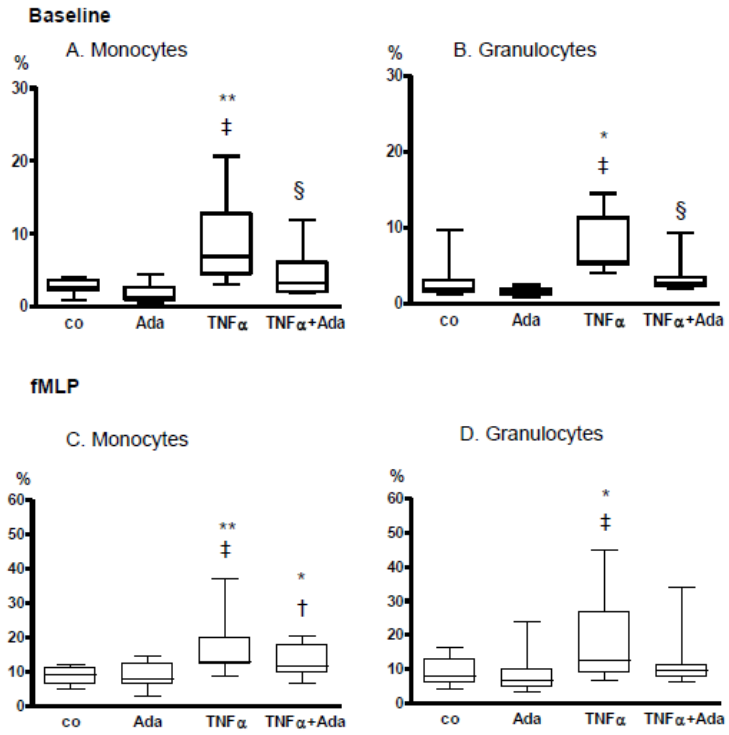

Figure 2: Effect of TNFα +/- adalimumab on basal and fMLP stimulated oxidative burst in healthy leukocytes.
%: percentage of rhodamine positive cells, co: control of healthy donor (DPBS-0.5%alb), Ada: adalimumab, TNFα: tumor necrosis factor alpha (400 pg/ml), TNFα+Ada: combination of TNFα and adalimumab, fMLP: N-formyl-methionine-leucine-phenylalanine. *: $p < 0.05$, **: $p < 0.01$ vs. co, †: $p < 0.05$, ‡: $p < 0.01$ vs. ada, §: $p < 0.05$ vs.TNFα.

Effect of adalimumab on oxidative burst induced by the cytokine combination (n = 8)

The pro-oxidative effects of the cytokine combination could be partially or entirely blocked by adalimumab at baseline in monocytes and granulocytes and after fMLP-stimulation in granulocytes (table 1). Despite the probable synergisms between the cytokines at this intermediate concentration (see above), the blockade with adalimumab suggests that TNFα plays a primordial role in these *in vitro* data.

Effects of adalimumab on uremia-related leukocyte oxidative burst in hemodialysis patients (n = 10)

Compared to healthy donors, monocytes and granulocytes from hemodialysis patients showed increased burst activaty at baseline ($p < 0.001$) and after fMLP stimulation ($p < 0.001$). Adalimumab, however, did not blunt this increased uremia-related oxidative stress present in blood from hemodialysis patients (figure 3).

Figure 3

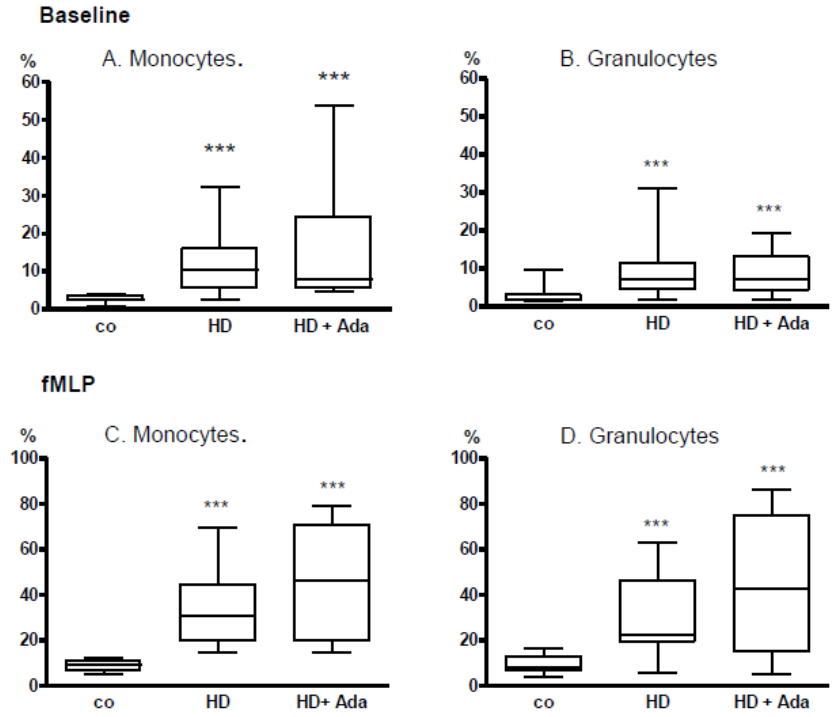

Figure 3: Effect of adalimumab on basal and fMLP stimulated oxidative burst in uremic leukocytes %: percentage of rhodamine positive cells, co: healthy control, HD: oxidative stress due to uremia in hemodialysis patient, HD + Ada: adalimumab added to whole blood of hemodialysis patients fMLP: N-formyl-methionine-leucine-phenylalanine.*** $p < 0.001$ versus co.

TNFα concentrations in samples of hemodialysis patients used for in vitro assessment

The serum concentration of TNFα in the samples of our hemodialysis patients was 9.9 ± 3.3 pg/ml which was lower than the concentrations reported in major reviews on uremic toxin concentrations (table 2) [26-28], be it close to the average mean reported by Duranton et al in 2013 (22 ± 25 pg/ml) [28] and within the range reported in clinical studies in hemodialysis patients between 2011 and 2014 (TNFα mean 13.6 ± 13.6 pg/ml, median of 6.4 pg/ml) (supplementary table 1). This observation, together with the lack of effect of adalimumab described above, point towards other factors than TNFα being at least as preponderant for causing leukocyte burst activity in dialysis patients treated according to today's standards. Synergistic effects, however, cannot be excluded.

4.4.2 *In vivo study*

Baseline clinical characteristics of the CKD 4-5 population (n = 121) divided according to the median of TNFα are presented in table 3. Patients with TNFα concentrations above the median, had a higher CRP, higher BMI and pulse pressure. There were no differences in age, gender or eGFR. After a total median [range] follow-up of 33 months [20-38 months], 41 patients reached the composite endpoint of mortality or non-fatal cardiovascular event (33.9%). Twenty-two patients died (18.2%) and 19 had a non-fatal cardiovascular event (15.7%), of which 6 patients died later on during follow-up, resulting in a total all-cause mortality of n = 28 (23.1%). In univariate analysis, TNFα was neither associated to the composite endpoint (HR: 1.52, 95% CI 0.81-2.85, p = 0.13), nor to all-cause mortality (HR: 1.01, 95% CI 0.93-1.10, p = 0.85). The Kaplan Meier curves for TNFα < or ≥ median (4.62 pg/ml) for the composite endpoint of death or first non-fatal cardiovascular event, as well as for all-cause mortality, show no difference in outcome according to TNFα concentration (figure 4). In univariate analysis, only higher age (HR: 1.05, 95% CI: 1.02-1.08), higher CRP (HR: 1.01, 95% CI: 1.01-1.01), a history of cardiovascular disease (HR: 2.90, 95% CI: 1.48-5.67) and diabetes (HR: 2.11, 95% CI: 1.14-3.91) were associated to increased risk for the composite endpoint. The same variables were also significantly associated to all-cause mortality in univariate analysis, except for a history of cardiovascular disease which showed only a trend (p = 0.09) (supplementary table 2). In a multivariate model, CRP and age remained significantly associated to the composite endpoint and CRP, age and gender were significantly associated to all-cause mortality (table 4).

Figure 4

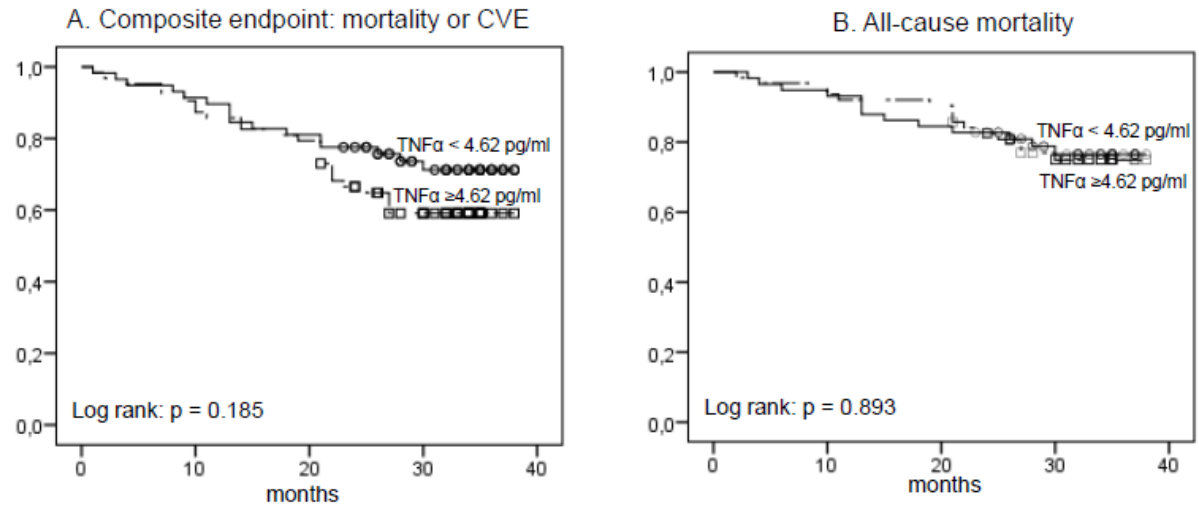

Figure 4: Kaplan-Meier survival curve for TNFα < or ≥ median (4.62 pg/ml) with the composite endpoint death or first non-fatal cardiovascular event in panel A and all-cause mortality in panel B. X-axis: follow-up in months, Y-axis: cumulative survival.

4.5 Discussion

The present study evaluated the role of four pro-inflammatory cytokines, IL6, TNFα, IL1β and IL18 in the context of CKD. The main findings of this study, indicating that the cytokines evaluated in this study at best are only ancillary contributors to inflammatory morbidity and mortality of uremia, are as follows: 1) At high uremic concentrations, only TNFα and not IL6, IL18 and IL1β increased the percentage of ROS producing monocytes and granulocytes at baseline and after fMLP-stimulation, in whole blood of healthy controls (figures 1 and 2); 2) The free radical production, induced by the combination of the four cytokines, at concentrations at which the individual cytokines induced no free radical production, showed a similar pro-oxidative pattern in monocytes and granulocytes as TNFα alone at higher concentrations (table 1); 3) The human monoclonal TNFα-antibody, adalimumab, could block partially or entirely the ROS production induced by TNFα alone (figure 2) and by the combination of cytokines (table 1); 4) In contrast, adalimumab could not blunt uremia-related oxidative stress in blood from hemodialysis patients (figure 3), suggesting that TNFα is not a main contributor to the ROS-production as observed

nowadays in uremia; 5) Finally, TNFα was not associated to adverse outcome in a population with CKD stage 4-5 (figure 4).

This study demonstrated for the first time that TNFα increased dose-dependently free radical production in monocytes and granulocytes, at baseline as well as after fMLP-stimulation at concentrations as observed in uremia, but only significantly at values in the high concentration range of what has been reported in uremia (400 and 1400 pg/ml) (figure 1); this is congruent with data obtained in other *in vitro* studies at concentrations at least tenfold above those used in the present study [22,23]. In combination with IL6, IL1β and IL18, TNFα induced already a similar increase in oxidative burst as 400 pg/ml TNFα alone at an intermediate uremic concentration of 70 pg/ml (table 1). Since TNFα at 70 pg/ml as well as the three other cytokines at any concentration did not induce ROS by themselves, this points to synergisms between the different cytokines. Synergism between uremic retention solutes was previously shown for p-cresylsulfate and p-cresylglucuronide on induction of leukocyte oxidative stress [31] and leukocyte rolling and capillary leakage in rat peritoneal vasculature [32].

Our data suggest that it is unlikely that TNFα by itself is one of the main responsible toxins in uremia-related leukocyte oxidative burst. Although the test concentrations in this study were within the range that is proposed as uremic (table 2) [26-28], the TNFα concentration (400 pg/ml) that provoked ROS production per se, is far above the concentrations that are found in studies in hemodialysis patients over the recent years; those ranged from 0.4-43 pg/ml with a mean and median concentration of 14 pg/ml and 6 pg/ml respectively (table 2, supplementary table 1). This concentration range is congruent with the mean TNFα concentration of only 9.9 pg/ml measured in our HD samples. Possible explanations for this trend in decreasing cytokine concentrations in HD patients are the use of ultrapure water for preparation of dialysis fluid [33], the better uremic toxin removal, the use of more biocompatible dialyzer membranes and the use of bicarbonate instead of acetate buffers [34]. Moreover, specifically for this *in vitro* study, in the hemodialysis patients, presence of comorbidities that could have caused additional inflammation, such as acute or chronic infection, smoking or malignancy were an exclusion criterion.

Furthermore, the TNFα-blocker, adalimumab, did not blunt the increased free radical production present in the whole blood of hemodialysis patients (figure 3). TNFα-blockade is clinically used in the treatment of rheumatoid arthritis, a condition which is also characterized by systemic micro-inflammation and associated to cardiovascular disease [35,36]. Also in studies in rheumatoid arthritis patients, TNFα-blockade could not attenuate the increased ROS production present in these patients [37,38], while for example chemotaxis could be decreased [37].

Finally, in our population with advanced CKD, which already have increased TNFα-concentrations, but are not yet affected by possible negative effects of dialysis therapy, the concentration of TNFα was not associated to adverse outcome (figure 4), as also shown earlier in a CKD population stage 2 to 5D [12]. This is in contrast to IL6 which has repeatedly been shown to be a strong predictor for outcome in CKD/dialysis [12,15-18,20] as well as in sepsis [39,40]. A possible explanation for this finding could be the difference in occurrence pattern of these two cytokines in the inflammatory cascade, with TNFα appearing earlier compared to IL6. TNFα initiates the inflammatory response, while IL6 is generated after NF-kB has been activated and is as such the result of the activated inflammatory system [41]. In the same vein, also CRP which is further downstream the cascade [42], was associated to adverse outcome in this (table 4) and other studies in CKD [13,15].

All arguments taken together, the results in the present study raise the question whether the reduction of cytokine activity by the administration of targeted monoclonals should be a goal in CKD, even if TNFα is pro-oxidative at high uremic concentrations. It is reasonable to accept, based on the present data, that TNFα concentration, as observed nowadays in most patients with CKD, are insufficiently high to expect a therapeutic benefit of TNFα blockage. It might be more important to prevent the sustained generation of TNFα and other cytokines by targeting first line uremic toxins such as intestinal derived protein-bound uremic toxins and SDMA which are pro-oxidative and pro-inflammatory [2,3,31,43]. Blocking TNFα in the context of uremia-related oxidative stress may happen too late in the cascade of effects to reduce ROS production. In line with this hypothesis, the results of a small randomized controlled trial evaluating the use of anti-TNFα therapy, etanercept, in hemodialysis patients, failed to reduce inflammatory markers after 44 weeks of treatment [44].

In summary, pro-inflammatory cytokines are not the main contributors to uremia-related oxidative stress. Out of the four investigated pro-inflammatory cytokines, only TNFα was pro-oxidative in normal monocytes and granulocytes at high uremic concentrations. This free-radical production could be blocked by a TNFα-blocker, adalimumab, but the uremia- related oxidative stress could not be blunted by this TNFα-blockade. Furthermore, TNFα concentration was not linked to hard clinical endpoints in a population with CKD stage 4-5. The present study suggests that preventing the rise in TNFα could be a more useful therapeutic strategy than targeting TNFα itself to reduce oxidative stress in uremia.

Disclosures: none

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Summary sentence:

Among several pro-inflammatory cytokines, only TNFα was pro-oxidative in normal leukocytes at high concentrations. In combination with the other pro-inflammatory cytokines, TNFα increased free radical production already at a lower but still intermediate concentration, suggesting synergism between the different cytokines. Although TNFα-blockade could block TNFα-related ROS-production in normal leukocytes, it did not blunt uremia-related oxidative stress, suggesting that other uremic toxins than TNFα are more crucial in this process. Furthermore, in a CKD-population stage 4-5, TNFα was not associated to all-cause mortality or cardiovascular events.

4.6 Tables

Table 1: Oxidative burst in leukocytes of healthy donors in the presence of a mixture of pro-inflammatory cytokines

Stimulus	**Control**	**Cytokine mix**	**Blocking with Ada**
Monocytes			
Baseline (%)	2.4 (2.1-2.9)	5.9 [a] (3.7-7.4)	3.1 [c] (2.4-5.8)
fMLP (%)	7.7 (5.1-11.1)	13.0 (8.0-16.1)	11.6 (10.4-21.0)
Granulocytes			
Baseline (%)	2.7 (2.5-3.0)	5.2 [a] (4.0-9.8)	3.1 [c] (1.2-4.2)
fMLP (%)	11.4 (8.0-17.9)	17.1 [a] (12.8-20.0)	11.1 [b] (9.7-13.4)

Medians with interquartile range between brackets. %: percentage of rhodamine positive cells, Ada: adalimumab. fMLP: N-formyl-methionine-leucine-phenylalanine. [a]: $p < 0.05$ vs. control; [b]: $p < 0.05$ vs. cytokine mixture; [c]: p 0.05-0.2 vs. cytokine mixture.

Table 2: Tumor necrosis factor alpha concentrations in uremia

Uremic TNFα concentration	Reference			
	Major reviews on uremic toxins concentrations			Literature search
	Vanholder et al., Kidney Int, 2003 [27]	Meert et al., Artif Organs, 2007 [26]	Duranton et al., J Am Soc Nephrol, 2013 [28]	Period 2011-2014
Maximum	408 pg/ml	1400 pg/ml	-	-
Mean	114 pg/ml	70 pg/ml		14 pg/ml*
- high mean			58 ± 10 pg/ml	range:
- average mean			22 ± 25 pg/ml	0.4 - 43 pg/ml

TNFα: tumor necrosis factor alpha. *The mean uremic concentration of the literature search is based on concentrations found in hemodialysis patients and is the calculated mean from the mean/median concentrations reported in the individual studies in the period 2011-1014 (based on 9 studies). For the original mean/median TNFα-concentrations, see supplementary table 1. The range is the minimum and maximum mean concentration of all individual studies. The time period of the literature search started from the year when the search of Duranton et al. stopped until 05/2014.

Table 3: Clinical characteristics of the entire study population and according to a TNFα concentration < or ≥ median of 4.62 pg/ml

Variable	Entire population	TNFα median		p-value
		< 4.62 pg/ml	≥ 4.62 pg/ml	
	N = 121	N = 58	N = 63	
Age (years)	74 [63-81]	73.5 [67.0-82.0]	74.0 [65.0-81.0]	0.919
Gender (M)	74 (61.2)	35 (60.3)	39 (61.9)	0.860
BMI (kg/m²)	**28.4 ± 5.8**	**27.1 ± 4.3**	**29.6 ± 6.7**	**<0.05**
MAP (mmHg)	100 ± 13	100 ± 14	98 ± 11	0.314
PP (mmHg)	**62 ± 19**	**66 ± 21**	**59 ±16**	**<0.05**
HR (/min)	69 ± 13	69 ± 13	70 ± 14	0.565
eGFR (ml/min/1.73m²)	23.0 [16.2-27.0]	21.1 [16.1-27.0]	23.9 [16.6-26.9]	0.705
CVH n (%)	60 (49.6)	24 (41.4)	36 (57.1)	0.083
DM n (%)	49 (40.5)	25 (43.1)	24 (38.1)	0.575
Malignancy n (%)	31 (25.6)	15 (25.9)	16 (25.4)	0.953
Cholesterol n (%)	83 (68.6)	39 (67.2)	44(69.8)	0.758
AHT n (%)	101 (83.5)	49 (84.5)	52 (82.5)	0.774
Smoking n (%)	10 (8.6)	3 (5.4)	7 (11.7)	0.226
TNFα (pg/ml)	**4.62 [3.68-6.00]**	**3.60 [2.62-4.03]**	**5.72 [4.89-7.97]**	**<0.001**
CRP (mg/l)	**3.0 [1.0-8.0]**	**2.0 [0.9-5.3]**	**4.0 [2.0-13.0]**	**<0.001**
Composite endpoint	41 (33.9)	16 (27.6)	25 (39.7)	0.160
All-cause mortality	28 (23.1)	13 (22.4)	15 (23.8)	0.856

Continuous data are presented as mean ± standard deviation or median with interquartile range between square brackets and categorical data as frequencies with percentages between brackets. TNFα: tumor necrosis factor alpha, BMI: body mass index, MAP: mean arterial pressure, PP: pulse pressure, HR: heart rate, eGFR: estimated glomerular filtration rate, CVH: history of cardiovascular disease, DM: diabetes mellitus, cholesterol: hypercholesterolemia, AHT: arterial hypertension, CRP: C-reactive protein, composite endpoint: death or non-fatal cardiovascular event. Variables in bold: p-value < 0.05

Table 4: Multivariate Cox proportional hazards model for the composite endpoint (Model A) and for all-cause mortality (Model B).

Variable	B	HR [95% CI]	p-value
***Model A**: Composite endpoint: death or first cardiovascular event*			
CRP (per mg/l)	0.014	1.01 [1.01-1.02]	0.001
Age (per year)	0.052	1.05 [1.02-1.09]	0.002
***Model B**: All-cause mortality*			
CRP (per mg/l)	0.022	1.02 [1.01-1.03]	< 0.001
Age (per year)	0.093	1.10 [1.04-1.16]	0.001
Gender (male)	1.187	3.28 [1.27-8.47]	0.014

Variables with a p-value in univariate analysis <0.1 (see supplementary table 2) and TNFα were included in the multivariate model. Included variables: Model A: age, CRP, diabetes mellitus, history of cardiovascular disease and TNFα. Model B: age, gender, CRP, history of cardiovascular disease, malignancy, pulse pressure and TNFα.

HR: hazard ratio, CI: confidence interval, CRP: C-reactive protein, TNFα: tumor necrosis factor alpha.

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4.8 Supplementary tables

Supplementary table 1: Tumor necrosis factor alpha concentrations in literature

Reference	TNFα-concentration	
Fontseré et al., Antimicr Agents Chemoth, 2014 [45]	3.1 pg/ml	Mean 13.6 ± 13.7 pg/ml Median 6.4 pg/ml, interquartile range: 4.3-25.7 pg/ml
Dukkipati et al., Sem Dial, 2013 [46]	5.4 pg/ml 6.3 pg/ml	
Kir et al., Clin Lab, 2012 [47]	24.3 pg/ml	
Izquierdo et al., BMC Nephrol, 2012 [48]	6.2 pg/ml	
Tayyebi-Khosroshahi et al., Saudi J Kidney Dis, 2012 [49]	6.9 pg/ml	
González-Espinoza et al., Nephrol Dial Transplant, 2012 [50]	0.4 pg/ml	
Guo et al., Clin Biochem, 2011 [51]	43.2 pg/ml	
Kuragano et al., Nephrol Dial Transplant, 2011 [52]	27.0 pg/ml	
Meuwese et al., Nephrol Dial Transplant, 2011 [53]	13.9 pg/ml	

TNFα: tumor necrosis factor alpha. The concentrations are the mean/median concentrations as reported in the original study.

Supplementary table 2: Univariate Cox proportional hazards model for the composite endpoint and all-cause mortality

Variable	Composite end-point: death or first non-fatal cardiovascular event		All-cause mortality	
	HR [95% CI]	p-value	HR [95% CI]	p-value
TNFα (per pg/ml)	1.52 [0.81-2.85]	0.192	1.01 [0.93-1.10]	0.850
CRP (per mg/l)	**1.01 [1.01-1.01]**	**<0.001**	**1.02 [1.01-1.03]**	**<0.001**
Gender (male)	0.86 [0.46-1.63]	0.648	2.14 [0.91-5.04]	0.081
Age (per year)	**1.05 [1.02-1.08]**	**0.001**	**1.08 [1.04-1.13]**	**0.001**
eGFR (per ml/min/1.73m²)	0.97 [0.92-1.02]	0.179	0.10 [0.91-1.02]	0.194
CVH (yes)	**2.90 [1.48-5.69]**	**0.002**	1.94 [0.90-4.21]	0.092
DM (yes)	**2.11 [1.14-3.91]**	**0.017**	1.18 [0.56-2.50]	0.668
Maligancy (yes)	1.38 [0.71-2.66]	0.343	2.03 [0.95-4.33]	0.068
AHT (yes)	0.72 [0.33-1.55]	0.398	0.51 [0.21-1.19]	0.118
Cholesterol (yes)	1.48 [0.73-3.03]	0.279	0.92 [0.41-2.03]	0.828
BMI (per kg/m²)	1.01 [0.96-1.06]	0.807	0.95 [0.95-1.08]	0.787
Smoking (yes)	0.26 [0.04-1.87]	0.179	0.41 [0.06-2.99]	0.376
PP (per mmHg)	1.01 [0.10-1.03]	0.107	**1.02 [1.00-1.04]**	**0.036**
MAP (per mmHg)	0.99 [0.97-1.02]	0.657	1.00 [0.97-1.03]	0.836

HR: hazards ratio, TNFα: tumor necrosis factor alpha, CRP: C-reactive protein, eGFR: estimated glomerular filtration rate, CVH: history of cardiovascaulr disease, DM: diabetes mellitus, AHT: arterial hypertension, cholerterol: hypercholesterolemia, BMI: body mass index, PP: pulse pressure, MAP: mean arterial pressure. P-value <0.05 is indicated in bold.

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CHAPTER 5

EVALUATION OF TUMOR NECROSIS FACTOR RECEPTORS IN CHRONIC KIDNEY DISEASE

CHAPTER 5.1.

SOLUBLE TUMOR NECROSIS FACTOR RECEPTOR 1 AND 2 PREDICT OUTCOMES IN ADVANCED CHRONIC KIDNEY DISEASE: A PROSPECTIVE COHORT STUDY

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5.1.1 Abstract

Background: Soluble tumor necrosis factor receptors 1 (sTNFR1) and 2 (sTNFR2) have been associated to progression of renal failure, end stage renal disease and mortality in early stages of chronic kidney disease (CKD), mostly in the context of diabetic nephropathy. The predictive value of these markers in advanced stages of CKD irrespective of the specific causes of kidney disease has not yet been defined. In this study, the relationship between sTNFR1 and sTNFR2 and the risk for cardiovascular events (CVE) and all-cause mortality was investigated in a population with CKD stage 4-5, not yet on dialysis, to minimize the confounding by renal function.

Patients and methods: In 131 patients, CKD stage 4-5, sTNFR1, sTNFR2 were analysed for their association to a composite endpoint of all-cause mortality or first non-fatal CVE by univariate and multivariate Cox proportional hazards models. In the multivariate models, age, gender, CRP, eGFR and significant comorbidities were included as covariates.

Results: During a median follow-up of 31 months, 42 events (32.1%) occurred of which 22 deaths (16.8%) and 20 (15.3%) first non-fatal CVE. In univariate analysis, the hazard ratios (HR) of sTNFR1 and sTNFR2 for negative outcome were 1.43 (95% confidence interval (CI): 1.23-1.73) and 1.13 (95% CI: 1.06-1.20) respectively. After adjustment for clinical covariables (age, CRP, eGFR, diabetes, a history of cardiovascular disease and pulse pressure) both TNFR remained independently associated to outcomes (HR: sTNFR1: 1.51, 95% CI: 1.31-1.75; sTNFR2: 1.13, 95% CI: 1.06-1.20). A subanalysis of the non-diabetic patients in the study population confirmed these findings.

Conclusion: sTNFR1 and sTNFR2 are independently associated to all-cause mortality or an increased risk for cardiovascular events in advanced CKD irrespective of the cause of kidney disease.

5.1.2 Introduction

Chronic kidney disease (CKD) has been linked to increased risk for cardiovascular disease and mortality independent of traditional cardiovascular risk factors [1]. This

has at least in part been attributed to micro-inflammation, since inflammatory markers were associated to cardiovascular disease or mortality in different CKD cohorts not on dialysis [2–5] as well as on dialysis [6–8].

Soluble tumor necrosis factor receptor 1 (sTNFR1) and soluble tumor necrosis factor receptor 2 (sTNFR2) are the circulating forms of their membrane bound counterparts (mTNFR1 and mTNFR2) which are essential for tumor necrosis factor alpha (TNFα)-signalling via different pathways. Interaction between TNFα and both mTNFR leads to a pro-inflammatory stimulus via activation of nuclear factor kappa B (NF-κB) or activator protein 1 (AP-1), while only mTNFR1 contains a death domain through which signalling leads to apoptosis [9]. The soluble receptors are released into circulation via shedding of membrane receptors, in exosomes or via alternative splicing of mRNA transcripts which leads to a loss of the transmembrane and cytoplasmic domains [10].

In contrast to TNFα, which failed to be associated to mortality or cardiovascular events in CKD [2], both circulating receptors are potential biomarkers in chronic kidney disease as predictors for outcome, be it in selected populations with diabetic nephropathy [11–13] or in early or moderate CKD [12,14]. Whereas diabetes can be seen as a pro-inflammatory stimulus per se and a broad range of GFR implies a higher risk for confounding because of the known relationship of sTNFRs with kidney function, the association of both sTNFRs to mortality and cardiovascular events has not yet been evaluated in a CKD population that was not selected on a specific cause nor in patients who suffer from advanced stages of disease.

In the present study, we evaluated the predictive value of sTNFR1 and sTNFR2 for the risk for all-cause mortality or cardiovascular events in a population with advanced CKD (stage 4-5 not on dialysis). Evaluating outcome in advanced CKD minimizes the influence of eGFR on outcomes and on the concentration of the evaluated marker. This may allow to generate hypotheses on the contribution of other important mechanisms in the pathophysiology of CKD, such as inflammation, while reducing the impact of their intrinsic association to kidney function.

5.1.3 Patients and Methods

Ethics statement

This study was approved by the local Ethics Committee (Ethical Committee, Ghent University Hospital, Ghent, Belgium) and performed in accordance to the Declaration of Helsinki. Written informed consent was obtained from all participants.

Study population

All non-transplanted CKD patients stage 4 and 5 not on dialysis, attending the Nephrology outpatient clinic and included in the biobank sample collection of the Nephrology Department of the Ghent University Hospital between January 2011 and August 2012, were included in this study (n = 131). Samples were processed immediately after collection and stored at –80°C. Outcomes were registered prospectively.

Baseline clinical parameters (age, gender, blood pressure, heart rate, height and weight) and etiology of the underlying kidney disease (vascular, diabetic nephropathy, glomerular disease/auto-immune, interstitial/postrenal, others and unknown) were registered. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2), mean arterial pressure (MAP) as the sum of 1/3 of the systolic and 2/3 of the diastolic blood pressure and pulse pressure (PP) as the difference between systolic and diastolic blood pressure. Estimated glomerular filtration rate (eGFR) was calculated based on the creatinine-based CKD-EPI formula [15]. The following comorbidities were recorded: cardiovascular history when at least one of the following was present: arterial cardiovascular disease (coronary, cerebral or peripheral), atrial fibrillation or heart failure (requiring hospitalisation); malignancy; diabetes mellitus, defined as a history of diabetes or treatment with insulin or oral antidiabetic drugs; hypertension, defined as current hypertension (>140/90 mmHg) or the use of antihypertensive drugs; hypercholesterolemia, defined as history of elevated serum cholesterol or treatment with lipid lowering drugs and smoking status (active versus no/former smoker).

Patients were followed and cardiovascular events (acute coronary syndrome, de novo atrial fibrillation, acute heart failure, coronary artery bypass graft, percutaneous transluminal coronary angioplasty, cerebrovascular accident, percutaneous

transluminal angioplasty) and all-cause mortality were registered until March 12th 2014. The composite endpoint in this study was all-cause mortality or a first non-fatal cardiovascular event, whichever occurred first.

Measurements

The concentrations of sTNFR1, sTNFR2 and TNFα, were determined on plasma samples by ELISA (R&D Systems, Abingdon, United Kingdom). Serum creatinine and C-reactive protein (CRP) were measured with routine laboratory methods.

Statistical analysis

Continuous data are expressed as mean with standard deviation or median with interquartile range depending on their distribution, and analysed by Student's t-test or Mann Whitney-test as appropriate. Binary categorical data are expressed as frequencies and analysed with chi-square test. Linear regression analysis was performed between sTNFR1 and sTNFR2 as dependent variables and the different clinical parameters as independent variables.

Univariate and multivariate analyses were performed by using Cox proportional hazards models to estimate the relationship between sTNFR1 and sTNFR2, as continuous variables and the risk for negative outcome defined as the composite endpoint of all-cause mortality or the occurrence of a first non-fatal cardiovascular event. Univariate analysis was also performed to assess the association between the baseline clinical variables and outcome.

In multivariate analysis, separate models were built for sTNFR1 and sTNFR2. Due to collinearity, sTNFR1 and sTNFR2 were not entered together in a model. First, age and gender were forced into a model with sTNFR1 or sTNFR2 (model 1). In the second model, possible confounders, which correlated significantly with sTNFR1 or sTNFR2, i.e. eGFR, CRP and TNFα (the latter only for sTNFR2), were added to model 1. The third model included the clinical covariables which reached a significance of $p < 0.2$ with outcome in univariate analysis (age, CRP, eGFR, history of cardiovascular disease, diabetes mellitus and pulse pressure). Model 2 and 3 were analysed by forward and backward regression procedures based on the likelihood

ratio test and gave similar results. In this publication, only the results of the stepwise forward procedure are reported as hazard ratio (HR) with a 95% confidence interval (CI).

The analysis described above was repeated in a subgroup analysis of patients with and without diabetes.

A p-value <0.05 was considered as statistically significant.

Finally, Receiver Operating Characteristic (ROC) curves were used to compare the discriminative power of sTNFR1, sTNFR2 as a single parameter to predict outcome. eGFR and age were assessed similarly as comparators.

All statistical analyses were performed with SPSS statistics 22 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft Corp, Redmond, WA, USA).

5.1.4 Results

Baseline clinical characteristics

In this study population including 131 patients with CKD stage 4-5 not on dialysis, the etiology of the underlying kidney disease was distributed as follows: renal vascular disease, mainly nephrangiosclerosis, n = 37 (28.2%); diabetic nephropathy, n = 28 (21.4%); glomerular/auto-immune disease, n = 20 (15.3%); interstitial/postrenal, n = 15 (11.5%); other, mainly autosomal dominant polycystic kidney disease, (unilateral) nephrectomy or use of calcineurin inhibitors in liver or heart transplants, n = 23 (17.5%); and unknown n = 8 (6.1%). Forty-two events (31.1%), defined as the composite endpoint of all-cause mortalitiy or first non-fatal cardiovascular event, occurred after a total median follow-up of 31 months [interquartile range 23-35 months]. Twenty-two patients (16.8%) died and 20 (15.3%) had a cardiovascular event, of which 6 patients died subsequently during follow-up. Baseline clinical characteristics and biochemical parameters of the entire population and those with and without event are presented in table 1. Patients who reached the composite endpoint, were older, had a higher C-reactive protein (CRP) and had more often a history of cardiovascular disease or diabetes. There was no difference in estimated glomerular filtration rate (eGFR) between both groups, complying with one of the aims of this study, i.e. to restrict the influence of kidney function on outcome among

subgroups by selecting a patient population with an eGFR over a narrow range. Both sTNFRs were significantly higher in the group who had an event.

sTNFR1 and sTNFR2 were strongly correlated (r = 0.76, p < 0.001) (Figure 1). Linear regression analysis with sTNFR1 and sTNFR2 as dependent variables showed only a correlation with eGFR (sTNFR1: r = -0.64, p <0.001; sTNFR2: r = -0.50, p < 0.001) and CRP (sTNFR1: r = 0.52, p < 0.001; sTNFR2: r = 0.54, p < 0.001). TNFα was only moderately correlated to sTNFR2 (r = 0.29, p < 0.01) and not to sTNFR1 (r = 0.17, p = 0.059). No significant correlations were found between both sTNFR and clinical variables listed in table 1.

Figure 1

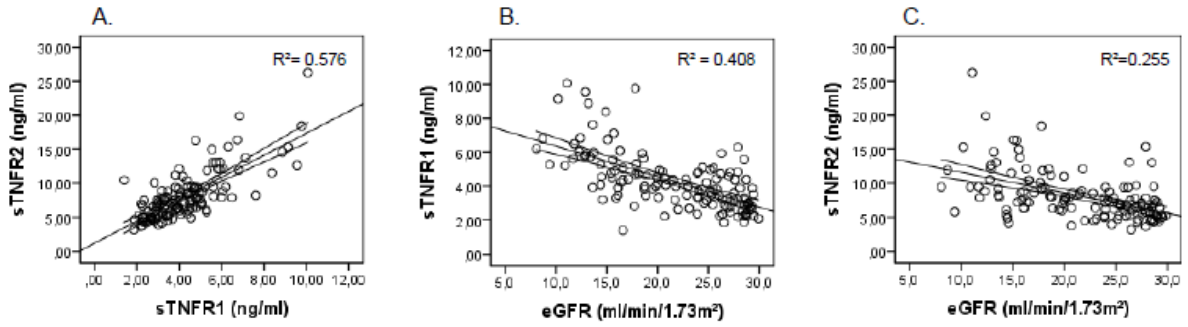

Figure 1: Dot plots showing the association between sTNFR1 and sTNFR2 (panel A), eGFR and sTNFR1 (panel B) and sTNFR2 (panel C). sTNFR1: soluble tumor necrosis factor receptor 1. sTNFR2: soluble tumor necrosis factor 2. eGFR: estimated glomerular filtration rate. R^2: coefficiënt of determination. The straight line represents the best fitted linear regression line with 95% confidence interval for the mean.

sTNFR1, sTNFR2 and outcome (death or first non-fatal cardiovascular event)

In univariate Cox proportional hazards analysis, sTNFR1 [Hazard ratio (HR): 1.43, 95% confidence interval (CI): 1.28-1.72] and sTNFR2 [HR: 1.13, 95% CI: 1.06-1.20] were associated to adverse outcomes. Older age, a higher CRP, a history of cardiovascular disease and diabetes mellitus also showed an association (Table 2). Kaplan Meier survival curves for both receptors are depicted in figure 2. For both sTNFRs, concentrations above the median were associated with higher event rates.

In multivariate models, sTNFR1 and to a smaller extent sTNFR2 remained significantly associated to outcome. In the first model (Table 3A) after adjustment for age and gender, both sTNFRs had an increased HR of 1.47, 95% CI 1.28-1.72 (sTNFR1) and 1.14, 95% CI 1.07-1.21 (sTNFR2). When further adjustments were made for possible confounders which were correlated to the sTNFRs in linear regression analysis (eGFR, CRP, TNFα), sTNFR1 and sTNFR2 together with age remained independently associated to outcome (Table 3B). In the third model after adjustment for age, eGFR, pulse pressure, CRP, a history of cardiovascular disease and diabetes mellitus, both receptors remained independently associated to outcome, the hazards ratio for sTNFR1 (HR: 1.51, 95% CI: 1.31-1.75) again being higher than for sTNFR2 (HR: 1.13, 95% CI: 1.06-1.20). Age and a history of cardiovascular disease were significant covariates in both models (Table 3C).

In summary, after adjustment for significant clinical covariables, sTNFR1 and sTNFR2 remained independently associated to increased risk of death or cardiovascular events. This association was stronger for sTNFR1 than for sTNFR2 (Table 3).

Figure 2

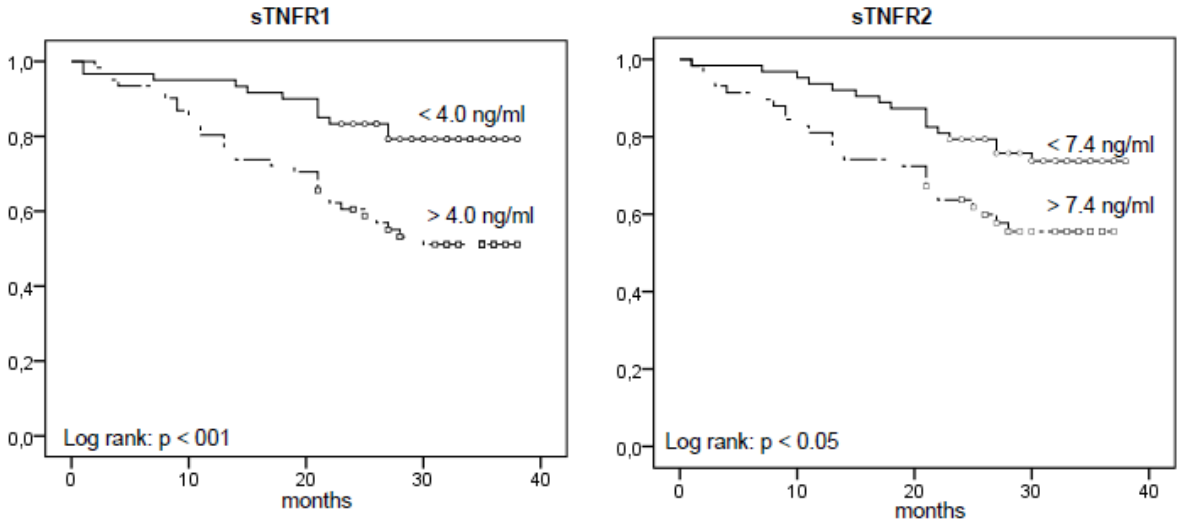

Figure 2: Kaplan Meier survival plots for sTNFR1 > or < than the median (4.0 ng/ml) and sTNFR2 > or < than the median (7.4 ng/nl). sTNFR1: soluble tumor necrosis factor receptor 1. sTNFR2: soluble tumor necrosis factor 2. Y-axis: survival rate

Receiver Operating Characteristic (ROC)-analysis

ROC-analysis showed that the discriminative power of sTNFR1 and sTNFR2 for all-cause mortality or cardiovascular event was the highest for sTNFR1 [AUC: 0.74, 95% CI 0.65-0.83], followed by sTNFR2 [AUC: 0.66, 95% CI 0.56-0.76] (Figure 3). These AUC values were in the same order as those for age [AUC: 0.70, 95% CI: 0.61-0.79], while eGFR was not discriminative [AUC: 0.57, 95% CI: 0.46-0.68].

Figure 3

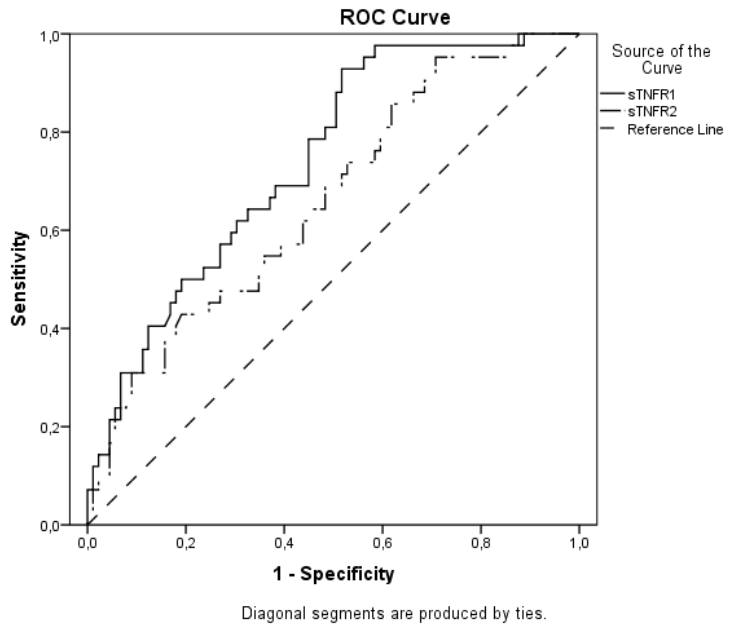

Figure 3: Receiver Operating Characteristics (ROC) curves of soluble tumor necrosis factor receptor 1 and soluble tumor necrosis factor receptor 2 for their discriminative power for all-cause mortality or cardiovascular event

Subgroup analysis: patients without and with diabetes

Twenty of the eighty patients without diabetes had an adverse event (25%). Patients, having an event, were older (80 vs. 67.5 years, $p < 0.001$), had a higher CRP (5.0 vs. 2.0 mg/l, $p < 0.001$), had more often a history of malignancy (45% vs. 20%, $p < 0.05$) and had a trend for more prevalent cardiovascular history (60.0% vs. 36.7%, $p = 0.068$). There were no differences for the other clinical variables listed in table 1. In univariate analysis sTNFR1 and sTNFR2 were significantly associated to adverse outcome, as well as age and CRP (data not shown). After adjustment for age, CRP, history of cardiovascular disease and malignancy, both sTNFRs remained significant

covariables in the model with a HR of 1.59, 95% CI 1.22-2.07 (sTNFR1) and 1.26, 95% CI 1.09-1.46 (sTNFR2) (Table 4).

In the subgroup of diabetes patients (n=51), only sTNFR1, and not sTNFR2 was associated to adverse outcome in univariate analysis. This significant association persisted after adjustment for age, gender and history of cardiovascular disease in multivariate analysis (sTNFR1: HR: 1.36, 95% CI, 1.44-1.67).

5.1.5 Discussion

This study evaluated the value of sTNFR1 and sTNFR2 as biomarkers for their association to the composite endpoint of all-cause mortality or first non-fatal cardiovascular event in a cohort with advanced CKD irrespective of the underlying etiology. The main finding of this study is that sTNFR1 and sTNFR2 are associated to adverse outcome, even after adjustment for clinical covariables, such as age, gender, eGFR, CRP. The association with outcome was stronger for sTNFR1 than for sTNFR2, a finding that was confirmed in the subgroup of patients without diabetes.

To the best of our knowledge, this is the first study to investigate associations between sTNFR1 and sTNFR2 and adverse outcomes in advanced CKD irrespective of the etiology. The study may be particularly relevant since by design, the narrow range of eGFR (<30 ml/min/1.73m²) minimized the impact of eGFR on outcome, which was confirmed by the lack of association to adverse outcomes (Table 2, Table 3) and there is also no influence of dialysis therapy. In spite of the small eGFR range, we found a relatively good correlation between eGFR and sTNFR1 (r = -0.64) and sTNFR2 (r = -0.50), underscoring that sTNFR1 and sTNFR2 with a respective molecular weight of approximately 30 and 40 kDa are probably mainly eliminated by renal clearance [16–18].

Also, in large cohort studies in the general population, sTNFR1 [19,20] and sTNFR2 [19,21] have been associated to an increased risk for major cardiovascular events [19,21] or mortality [20], even after adjustment for traditional risk factors [21]. However, in these cohorts, and in contrast to our study (table 3), no corrections were made for kidney function [21] or the association was only found when creatinine clearance ranged up to 75 ml/min, which implies a much broader range of eGFR than

in the present study [19]. As a consequence, it cannot be excluded that in these studies the sTNFR concentrations rather reflected the association of eGFR with adverse outcomes than that of sTNFRs itself. In the present study, sTNFRs were more informative for adverse outcome than what could be explained through their correlation with eGFR alone, since associations of sTNFRs to adverse outcome remained significant after adjustment for eGFR, while eGFR was not significant (Table 3). This observation could strengthen the hypothesis that sTNFRs are also markers for underlying pathophysiological mechanisms of cardiovascular risk in CKD. This is supported by findings in other populations with increased cardiovascular risk. sTNFR1 at admission for acute myocardial infarction was associated to infarct size and worse left ventricular function 4 months post-infarction [22], to the composite endpoint of death and new onset heart failure [23] and to long term cardiac and all-cause mortality post-infarction even when corrected for relevant covariates such as creatinine clearance [24] .

This is also consistent with the increased mortality risk associated to sTNFR1 in patients with diabetic nephropathy [12,13]. In the present study, the association between sTNFRs and outcomes was also confirmed in the subgroup of patients without diabetes (Table 4).

In our analysis, CRP was significantly correlated with sTNFR1 (r = 0.52) and sTNFR2 (r = 0.54). CRP was also significantly associated to a negative outcome in univariate analysis (Table 2), while in multivariate models including sTNFR1 or sTNFR2, the receptors were stronger covariates and remained independently associated to outcome while CRP lost its significance (Table 3).

Remarkably, although linked to each other in the same inflammatory TNFα–pathway, sTNFR1 and sTNFR2 do not entirely yield the same information, despite the strong mutual correlation between both receptors as observed in our study (r = 0.76) and also reported previously [11]. sTNFR1 did not correlate to sTNFα, while a significant but modest correlation was found for sTNFR2 (r = 0.29). In ROC analysis the discriminative power of sTNFR1 for adverse outcome was higher compared to sTNFR2 (Figure 3). In the subgroup of diabetes patients only sTNFR1 and not sTNFR2 was associated to outcome in univariate and multivariate analysis.

Constitutively, both sTNFRs are released from cell membranes by shedding [18,25] or as full-length receptors in exosomes [26,27]; the full-length sTNFR1 in exosomes being the most abundant form of sTNFR1 in serum of healthy controls [27]. Both forms are capable of binding TNFα [18,27]. Following acute pro-inflammatory stimuli, shedding is intensified, resulting in an increase in circulating sTNFR concentration [25,28] and in a possible modulation of immune response [29]. The function of these soluble receptors is debated: they are inhibitors for TNFα, especially in acute inflammatory settings [18,30], but, in more chronic conditions and in proportion to their concentration, they also increase the half-life of TNFα and may act as slow-release reservoirs of TNFα enhancing its cytotoxicity [31]. Which process prevails in CKD still needs to be elucidated, although the TNFR concentration range (3-12 ng/ml) in which Aderka et al. [31] found a prolonged activity of TNFα function, corresponds to concentrations found in CKD. Together with our findings of a relationship with adverse outcome, this could indicate that sTNFRs in CKD would rather increase the negative effects of TNFα than act as TNFα-inhibitors.

The limitations of the study are: the results cannot be generalized to all CKD stages, since we restricted the evaluation to CKD stage 4 and 5 intentionally to evaluate both receptors in a narrow range of eGFR, which also resulted in a rather small population. Nevertheless, the study unravelled convincing correlations between sTNFRs and outcomes even after adjustment for confounders such as eGFR or diabetes. Hence our data underscore the relationship of TNFRs, with hard clinical outcomes irrespective of renal function and the presence or absence of diabetes. The fact that especially sTNFR1 was consistently associated to different outcomes in advanced CKD as shown in this study as well as in community-based and specific high risk populations such as post-myocardial infarction, offers a strong argument in favour for its further evaluation in larger CKD cohorts.

Disclosure: none

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5.1.6 Tables

Table 1: Baseline characteristics of the entire study population and according to the occurrence or not of the studied events

Variable	Entire cohort	Death or first cardiovascular event		p-value
		No	Yes	
	N = 131	N = 89	N = 42	
Age (years)	73 [62-89]	70 [58-77]	78 [69-83]	<0.001
Gender (M)	83 (63.4)	56 (62.9)	27 (64.3)	0.88
BMI (kg/m²)	28.3 ± 5.7	28.2 ± 4.9	28.5 ± 7.2	0.80
MAP (mmHg)	99 ± 13	100 ± 13	99 ± 13	0.81
PP (mmHg)	61 ± 19	59 ± 17.7	65.2 ± 20.2	0.12
HR (/min)	69 ± 13	68 ± 13	72 ± 13	0.12
eGFR (ml/min/1.73m²)	22.8 [16.1-27.0]	23.0 [17.4-27.4]	20.1[15.4-26.3]	0.21
CVD	64 (48.1)	34 (38.2)	30 (71.4)	<0.001
DM	51 (38.9)	29 (32.6)	22 (52.6)	0.030
Malignancy	32 (24.4)	19 (21.3)	13 (31.0)	0.23
Cholesterol	90 (68.7)	58 (65.2)	32 (76.2)	0.20
AHT	108 (82.4)	74 (83.1)	34 (81.0)	0.76
Smoking (active)	12 (9.1)	10 (11.9)	2 (5.0)	0.22
CRP (mg/l)	3.0 [1.0-8.0]	2.0 [0.6-4.1]	6.5 [2.0-24.3]	<0.001
TNFα (pg/ml)	4.6 [3.7-6.0]	4.5 [3.3-5.5]	4.8 [3.8-6.8]	0.053
sTNFR1 (ng/ml)	4.0 [3.1-5.1]	3.7 [2.8-4.7]	4.8 [3.9-6.0]	<0.001
sTNFR2 (ng/ml)	7.4 [5.8-9.4]	6.9 [5.2-8.8]	7.9 [6.4-12.1]	<0.01

Data are presented as means ± standard deviation or medians with interquartile range between square brackets. For binary variables, frequencies with percentages between brackets are given. N= number of patients, M: male, BMI: body mass index, MAP: mean arterial pressure, PP: pulse pressure, HR: heart rate, eGFR: estimated glomerular filtration rate, CVD: history of cardiovascular disease, DM: diabetes mellitus, cholesterol: hypercholesterolemia, AHT: arterial hypertension, CRP: C-reactive protein, TNFα: tumor necrosis factor alpha, sTNFR1: soluble tumor necrosis factor receptor 1, sTNFR2: soluble tumor necrosis factor receptor 2. n.s.: not significant

Table 2: Univariate Cox proportional hazards analysis for outcome (death or first cardiovascular event)

Variable	B	HR [95% CI]
sTNFR1 (per ng/ml)	**0.391**	**1.43 [1.23-1.73]*** **
sTNFR2 (per ng/ml)	**0.119**	**1.13 [1.06-1.20]*** **
TNFα (per pg/ml)	0.035	1.04 [0.98-1.09]
CRP (per mg/l)	**0.015**	**1.02 [1.01-1.02]*** **
Gender (M)	0.111	1.12 [0.59-2.10]
Age (per year)	**0.052**	**1.05 [1.02-1.07]*** **
eGFR	**-0.033**	**0.97 [0.92-1.02]**
(per ml/min/1.73m²)		
MAP (per mmHg)	-0.004	1.00 [0.97-1.02]
PP (per mmHg)	**0.013**	**1.01 [1.00-1.03]**
BMI	0.005	1.01 [0.95-1.06]
CVD	**1.112**	**3.08 [1.58-6.03]*** **
DM	**0.726**	**2.07 [1.13-3.79]***
Malignancy	0.333	1.40 [0.73-2.68]
AHT	0.228	1.26 [0.58-2.71]
Cholesterol	0.432	1.54 [0.76-3.13]
Smoking	-0.703	0.50 [0.11-2.05]

HR: Hazard ratio, CI: confidence interval. In bold, variables with p-value < 0.2, included in the multivariate model (table 3, model 3). *: $p < 0.05$, ***: < 0.001. sTNFR1: soluble tumor necrosis factor receptor 1, sTNFR2: soluble tumor necrosis factor receptor 2, TNFα: tumor necrosis factor alpha, CRP: C-reactive protein, eGFR: estimated glomerular filtration rate, MAP: mean arterial pressure, PP: pulse pressure, BMI: body mass index, CVD: history of cardiovascular disease, DM: diabetes mellitus, AHT: arterial hypertension, cholesterol: hypercholesterolemia.

Table 3: Multivariate Cox proportional hazards models for death or cardiovascular event

Variable	B	HR [95% CI]	p-value
A. Model 1: sTNFR1 or sTNFR2, age, gender			
A1. sTNFR1			
sTNFR1 (per ng/ml)	0.396	1.49 [1.28-1.72]	< 0.001
age (per year)	0.015	1.06 [1.02-1.09]	< 0.001
gender (M)			n.s.
A2. sTNFR2			
sTNFR2 (per ng/ml)	0.129	1.14 [1.07-1.21]	< 0.001
age (per year)	0.058	1.07 [1.03-1.10]	< 0.001
gender (M)			n.s.
B. Model 2: sTNFR1 or sTNFR2, age, gender, eGFR, CRP, TNFα			
B1. sTNFR1			
sTNFR1 (per ng/ml)	0.395	1.49 [1.28-1.72]	<0.001
age (per year)	0.056	1.06 [1.02-1.09]	<0.01
gender, CRP, eGFR			n.s.
B2. sTNFR2			
sTNFR2 (per pg/ml)	0.136	1.15 [1.08-1.22]	<0.001
age	0.051	1.05 [1.02-1.09]	<0.01
gender, CRP, eGFR, TNFα			n.s.
C. Model 3: sTNFR1 or sTNFR2, age, eGFR, CRP, CVD, DM, PP			
C1. sTNFR1			
sTNFR1 (per ng/ml)	0.414	1.51 [1.31-1.75]	<0.001
age (per year)	0.044	1.05 [1.01-1.08]	<0.01
CVD	0.876	2.40 [1.19-4.85]	<0.05
eGFR, CRP, DM, PP			n.s.
C2. sTNFR2			
sTNFR2 (per ng/ml)	0.118	1.13 [1.06-1.20]	<0.001
age (per year)	0.045	1.05 [1.01-1.08]	<0.01
CVD	0.724	2.06 [1.02-4.19]	<0.05
eGFR, CRP, DM, PP			n.s.

HR: Hazard ratio, CI: confidence interval. In model 2: TNFα, only included in model with sTNFR2. sTNFR1: soluble tumor necrosis factor receptor 1, sTNFR2: soluble tumor necrosis factor receptor 2, TNFα: tumor necrosis factor alpha, eGFR: estimated glomerular filtration rate, CRP: C-reactive protein, TNFα: tumor necrosis factor alpha, CVD: history of cardiovasular disease, DM: diabetes mellitus, PP: pulse pressure, n.s.: not significant

Table 4: Multivariate Cox proportional hazards model for death or cardiovascular event in the subgroup of non diabetic patients

Variable	B	HR [95% CI]	p-value
Full model with sTNFR1			
sTNFR1 (per ng/ml)	0.463	1.59 [1.22-2.07]	<0.01
age (per year)	0.059	1.06 [1.01-1.11]	<0.05
gender, CRP, CVH, malignancy			n.s.
Full model with sTNFR2			
sTNFR2 (per ng/ml)	0.233	1.26 [1.09-1.46]	<0.01
age (per year)	0.063	1.07 [1.02-1.12]	<0.05
gender, CRP, CVH, malignancy			n.s.

HR: Hazard ratio, CI: confidence interval, sTNFR1: soluble tumor necrosis factor receptor 1, sTNFR2: soluble tumor necrosis factor receptor 2, TNFα: tumor necrosis factor alpha, eGFR: estimated glomerular filtration rate, CRP: C-reactive protein, TNFα: tumor necrosis factor alpha, CVD: history of cardiovasular disease, DM: diabetes mellitus, PP: pulse pressure, n.s.: not significant

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CHAPTER 5.2.

RENAL CLEARANCE VERSUS CHANGES IN LEUKOCYTE MEMBRANE EXPRESSION AS A CAUSE FOR ELEVATED PLASMA SOLUBLE TUMOR NECROSIS FACTOR RECEPTORS IN CKD

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5.2.1 Abstract

Introduction: Soluble tumor necrosis factor receptor 1 (sTNFR1) and 2 (sTNFR2) are associated to outcome in chronic kidney disease (CKD). Their origin and function in CKD are not entirely understood. Concentration changes in circulation as well as changes in leukocyte membrane expression could modulate TNFα signalling and function in CKD. The aims of the present study were to investigate the relationship between sTNFR concentrations on one hand and estimated glomerular filtration rate (eGFR) and changes in their membrane expression on leukocytes on the other.

Methods: Fifty patients with CKD stage 1-5 not on dialysis (ten patients in each stage), without diabetes, were matched per stage for age and gender. Linear regression analysis was performed between the reciprocals of sTNFR1, sTNFR2, measured with ELISA, and eGFR. In addition, we also explored whether an impact of the uremic status on leukocyte membrane expression of the TNFRs (mTNFR1, mTNFR2) and membrane (m)TNFα, could influence the soluble receptor concentrations by comparing the leukocyte membrane TNFR expression in hemodialysis patients (HD) (n=8) and controls (C) (n=8) by flow cytometry.

Results: The mean age of the CKD population was 49.9 ± 13.6 years. There was a strong correlation between eGFR and 1/sTNFR1 (r=0.932) and 1/sTNFR2 (r=0.889) in CKD stage 1-5 (both $p<0.001$), which remained significant in multivariable analysis after correction for covariables such as age and CRP. In HD, the concentrations of sTNFR1, sTNFR2 increased further compared to CKD stage 5 (sTNFR1: 9.8 vs. 5.8 ng/ml, $p<0.01$; sTNFR2: 18.2 vs. 8.7 ng/ml, $p<0.001$). In contrast and in spite of assessing subjects at the two extremes of kidney dysfunction, there was no difference in the global leukocyte membrane expression of mTNFR1, mTNFR2 and mTNFα between HD and C.

Conclusion: This study demonstrates that increased concentrations of sTNFR1 and sTNFR2 in CKD are mainly associated to decreased renal clearance but not to changes in leukocyte membrane expression.

5.2.2 Introduction

The concentrations of soluble tumor necrosis factor receptor 1 (sTNFR1) and soluble tumor necrosis factor receptor 2 (sTNFR2) increase in chronic kidney disease (CKD) [1–3] and dialysis patients [4,5]. Higher concentrations are associated to progression of kidney disease in patients with early or moderate CKD (CKD stage 1-3) [6–9] and to mortality in patients with diabetic nephropathy [7,10]. Although these molecules are members of the tumor necrosis factor alpha (TNFα)-system, they outperform circulating TNFα (sTNFα) in their predictive value for outcome [6,7].

The concentration of sTNFR1 and sTNFR2 in circulation is theoretically the sum of what is released from the cell surface and what is cleared from the body. In vitro studies [11–13] and animal studies in mice [14] have shown that sTNFRs are shed from the cell membrane mediated via TNFα converting enzyme (TACE) [11,14] and are present as full length receptors in circulating exosomes [12,13]. Animal studies in mice with normal renal function have shown that both sTNFRs are eliminated via the kidneys [14,15]. Acute inflammatory stimuli induce intensified shedding causing a rise in plasma concentration [14,16].

Progression of CKD at the same time leads to a decreased renal clearance but also to an enhanced inflammatory status, raising the question which of both factors prevails in the increased sTNFR levels in CKD and whether uremia has an influence on leukocyte membrane expression of sTNFRs. To the best of our knowledge, this question has not yet been assessed in renal failure conditions. Increased understanding of the origin of circulating TNFRs in CKD and the impact of uremia on membrane expression will extend the knowledge on their potential biological function and their possible influence on TNFα signalling in CKD.

Therefore, the aim of this study was to investigate the correlation between both sTNFRs and eGFR in a CKD population stage 1-5 in order to explore the contribution of glomerular filtration rate to sTNFR concentration in CKD. In parallel, the leukocyte membrane expression of mTNFRs in hemodialysis patients (HD) was compared to that of healthy controls (C) ex vivo, in order to unravel possible differences between the two most extreme stages of kidney function in receptor expression at baseline unstimulated conditions.

5.2.3 Material and Methods

Patient samples

Clinical correlation study

To investigate the correlation between sTNFR concentration and eGFR, fifty non-diabetic patients with CKD stage 1-5, classified according to the creatinine-based CKD-EPI formula [17], ten in each stage, matched for age and gender, were included in this cross-sectional study and selected from the sample collection of the Nephrology Department of the Ghent University Hospital. Every patient not dialyzed or transplanted, attending the Nephrology outpatient clinic was eligible for recruitment. Plasma samples were collected between January 2012 and August 2013, immediately centrifuged, aliquoted and stored at -80°C. The study was approved by the local ethics committee and written informed consent was obtained.

Baseline clinical parameters (age, gender, blood pressure, heart rate, height and weight) and etiology of underlying kidney disease (renovascular, autosomal dominant polycystic kidney disease (ADPKD), glomerular disease/auto-immune, interstitial/postrenal and others) were registered. Body mass index (BMI) was calculated as weight/height² (kg/m²), mean arterial pressure (MAP) as the sum of 1/3 of the systolic and 2/3 of the diastolic blood pressure and pulse pressure (PP) as the difference between systolic and diastolic blood pressure. To exclude acute infections or inflammatory syndromes, patients with a C-reactive protein (CRP) above 20 mg/l were excluded.

Samples for leukocyte membrane labelling study

For the labelling of leukocyte mTNFRs and mTNFα, whole blood samples were taken from healthy volunteers (C) (n = 8) and hemodialysis patients (HD) before the start of dialysis (n = 8) (3 men and 5 women in each group). Samples from C and HD patients were chosen to compare the two extremes with regard to kidney function. Controls had no known acute or chronic illnesses. HD patients were non-diabetics, had no history of malignancy, no acute or chronic infection and took no immunosuppressive drugs. Patients underwent online-hemodiafiltration 3 times 4 hours a week. The quality of the dialysis fluid met the ultra pure standards (bacteria <0.1 CFU/ml, endotoxin <0.03 EU/ml) as checked on a regular basis. Samples were

immediately put on ice, until the start of the membrane labelling (at maximum within 30 min after sampling).

Concentration Measurements

The concentrations of sTNFR1, sTNFR2 and sTNFα were determined on plasma samples of CKD patients of the correlation study and HD patients of the leukocyte membrane labelling study by ELISA (R&D Systems, Abingdon, United Kingdom) with a detection limit of 0.8 x 10^{-3} ng/ml, 0.6 x 10^{-3} ng/ml and 0.5 pg/ml respectively. Serum creatinine and CRP were measured by routine laboratory methods.

Membrane labelling study

Reagents

Fluorochrome labelled antibodies against membrane tumor necrosis factor receptor 1 (mTNFR1) (anti-mTNFR1-FITC, MBL International, Woburn, MA, USA), membrane tumor necrosis factor receptor 2 (mTNFR2) (anti-mTNFR2-AlexaFluor 700, R&D Systems, Abingdon, UK) and membrane tumor necrosis factor alpha (mTNFα) (anti-mTNFα-PE, R&D Systems, Abingdon, UK) were used for ex vivo labelling of leukocytes.

Protocol for membrane labelling of TNFα-receptors and membrane TNFα

Whole blood of C and HD was incubated with the fluorochrome labelled antibodies at 4°C to visualize the expression of mTNFRs and mTNFα on the leukocyte membrane at the time of sampling (E_{t0}). To assess whether the expression of the receptors on the leukocyte membrane was in steady state in an unstimulated condition, the samples were incubated ex vivo at 37°C for 1 hour before addition of the fluorochrome labelled antibodies and afterwards labelled at 4°C (E_{t1}).

After labelling, samples were lysed (lysing solution, R&D Systems, UK), centrifuged and washed with staining buffer (R&D Systems, UK). After resuspension in staining buffer, the samples were analysed immediately by flow cytometry (FACSCanto, Becton Dickenson, Erembodegem, Belgium). First, all leukocytes were gated together and the mean fluorescence intensity (MFI) of the entire leukocyte cluster

was assessed. Based on forward and side scatter, monocytes, granulocytes and lymphocytes were identified and the MFI of each cluster was measured separately.

Statistical analysis

Clinical correlation study

Continuous data were expressed as mean with standard deviation or median with interquartile range depending on their distribution, and analysed by Student's t-test, ANOVA with pairwise comparisons, Mann-Whitney test or Kruskal-Wallis test with pairwise comparisons as appropriate. Linear regression analysis was performed between the reciprocals of the concentrations of sTNFR1 and sTNFR2 as dependent variables and eGFR as independent variable. Univariate linear regression analysis was also performed between receptor concentration and the clinical and biochemical covariates. Covariates that were correlated to the sTNFRs with a p-value < 0.2 in univariate analysis were analyzed with multiple linear regression analysis by a stepwise forward procedure. Pearson correlation coefficients (r) with their p-value and coefficients of determination (R^2) were reported. A p-value < 0.05 was considered as statistically significant.

Membrane labelling study

Data were not normally distributed and as a consequence analysed with non-parametric tests. C and HD were compared with a Mann-Whitney test. The different test conditions in C and HD were compared with a Wilcoxon signed ranks test.

All statistical analyses were performed with SPSS statistics 22 (SPSS Inc., Chicago, IL, USA) for Windows (Microsoft Corp, Redmond, WA, USA). Graphs were made in GraphPad Prism 04 (GraphPad Software, LaJolla, CA, USA). A p-value of < 0.05 was considered as significant.

5.2.4 Results

Clinical Correlation study

The etiology of the underlying kidney disease in this non-diabetic population CKD stage 1-5 (n = 50) was distributed as follows: ADPKD: 18%, glomerular: 34%, renovascular: 12%, interstitial/postrenal: 18% and other: 20%. The overall mean age was 49.9 ± 13.6 years with an equal gender distribution. The mean MAPs and PPs were 96 ± 11 mmHg and 47 ± 1 mmHg, respectively and increased when kidney function deteriorated (table 1). The concentrations of sTNFR1, sTNFR2 and sTNFα increased gradually over the different stages of CKD with an earlier rise for both soluble receptors compared to sTNFα for which the concentration only increased in CKD stages 4-5 (table 1).

In univariate linear regression analysis, there was a strong correlation between eGFR and 1/sTNFR1 ($r = 0.939$, $p < 0.001$) and 1/sTNFR2 ($r = 0.889$, $p < 0.001$) (figure 1). Results of the univariate linear regression analysis between the receptors and other clinical and biochemical variables are shown in table 2. There was a strong mutual correlation between both receptors ($r = 0.945$, $p < 0.001$), and both receptors were also correlated to sTNFα, CRP, MAP and PP. sTNFR2 was correlated to age whereas only a trend was observed for sTNFR1.

In a multiple linear regression model for sTNFR1 with eGFR, age, CRP, sTNFα, MAP and PP included as covariables, only eGFR remained a significant determinant in the model (sTNFR1: $r = 0.930$, $p < 0.001$). Also, in a multiple linear regression model for sTNFR2 with eGFR, age, gender, CRP, sTNFα, MAP and PP as covariates, eGFR remained the strongest covariate, although CRP and gender were also significant covariables (table 3).

Figure 1

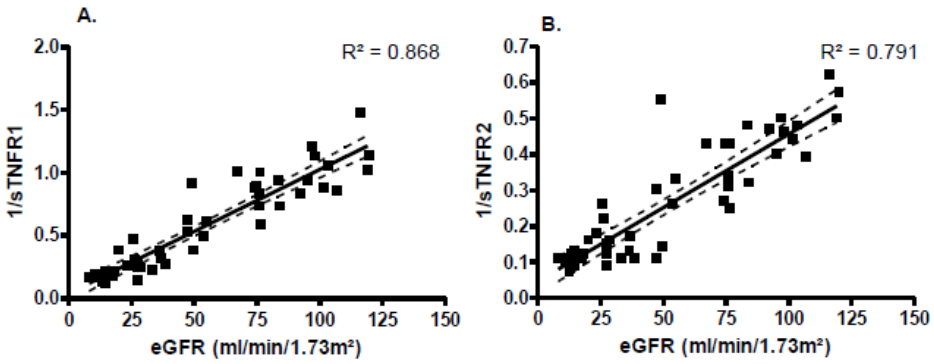

Figure 1: Dot plots with linear regression line between eGFR and 1/sTNFR1 (Panel A), 1/sTNFR2 (Panel B). eGFR: estimated glomerular filtration rate, 1/sTNFR1, 1/sTNFR2: reciprocal of the concentration of soluble tumor necrosis factor receptor 1 and 2, R^2: coefficient of determination.

Membrane labelling study

To evaluate the effect of uremia on the level of expression of receptors both in circulation and on the leukocyte membrane, the most extreme condition, being patients in CKD stage 5 on hemodialysis, was selected. The mean age of the HD patients was 68.6 ± 17.3 years. The HD patients had a mean urea and creatinine clearance of 1.8 ml/min) and the mean single pool Kt/V_{urea} was 1.75. The mean age of the controls was 41.4 ± 10.9 years.

In comparison to the concentrations in CKD stage 5 not on dialysis, the concentrations of sTNFR1, sTNFR2, sTNFα were further increased in HD patients (sTNFR1: 9.8 vs. 5.8 ng/ml, $p<0.01$; sTNFR2: 18.2 vs. 8.7 ng/ml, $p<0.001$; sTNFα 9.4 vs. 3.3 pg/ml, $p<0.01$). Despite this marked increase in plasma concentrations, the overall leukocyte membrane expression at baseline (E_{t0}) in HD was not different compared to C, with MFI for mTNFR1: 335.0 vs. 306.1 (p = n.s.), mTNFR2: 67.8 vs. 61.5 (p = n.s.) and mTNFα: 384.5 vs. 405.5 (p = n.s.) (figure 2). This was also observed for the different leukocyte subtypes separately, with the exception for a discrete difference for mTNFR2-expression on lymphocytes (HD: 11.0 vs. C: 17.5, $p < 0.01$) (table 4).

Furthermore, when comparing the overall leukocyte membrane expression between E_{t0} and E_{t1}, there was no difference between both conditions in HD and C, indicating

steady state (figure 2). Analysis of the membrane expression on the different leukocyte subtypes after 1 hour incubation at 37 °C (E_{t1}) revealed also no differences at all between HD and C. There were only some minor differences between E_{t1} and E_{t0} (mTNFR1, granulocytes in HD; mTNFR2, monocytes and granulocytes in controls; p=0.04 in all) (table 4). These findings make release of mTNFRs from the leukocyte membranes as an important mechanism for increased sTNFRs concentrations in HD patients less probable.

Figure 2

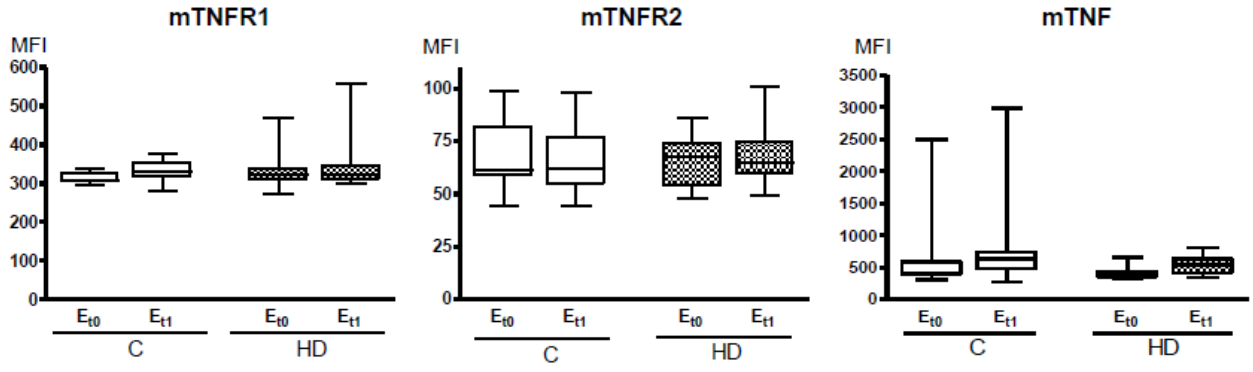

Figure 2: Overall expression of membrane tumor necrosis factor receptor 1, 2 and membrane tumor necrosis factor alpha on all leukocytes gated together indicating steady state in HD and in controls.
MFI: mean fluorescence intensity, mTNFR1: membrane tumor necrosis factor receptor 1, mTNFR2: membrane tumor necrosis factor receptor 2, mTNFα: membrane tumor necrosis factor receptor alpha. E_{t0}: baseline expression of receptors expressed on membranes, E_{t1}: Membrane expression of receptors after 1 hour incubation ex vivo at 37°C. C: healthy control, HD: hemodialysis patient. No differences between HD and C for E_{t0} and E_{t1}. No significant differences for the comparison between E_{t1} and E_{t0} in C and HD.

5.2.5 Discussion

In the present study, we investigated the correlation between sTNFRs and eGFR as well as the leukocyte membrane expression of both TNFRs and TNFα. The main findings of the study are: 1) In a stable CKD population stage 1 to 5, the concentrations of sTNFR1 and sTNFR2 are strongly correlated to eGFR, suggesting that renal clearance is one of the major determinants for TNFα-receptor concentration. 2) CRP is correlated to both receptors in univariate analysis, but remains only a significant covariate for sTNFR2 concentration in multiple regression

analysis. 3) The leukocyte membrane expression of both receptors is not different between healthy controls and hemodialysis patients.

In this study population with stable CKD-patients, sTNFR concentrations were largely dependent on eGFR (figure 1), which persisted after correction of covariates such as CRP, age, sTNFα, MAP, PP and gender. The concentration of both sTNFR further increased in HD. In contrast, the concentration of sTNFα (17kDa) rose only in the later stages of CKD (table 1), although also TNFα is at least partially cleared via the kidneys [18].

The soluble forms of TNFR1 and TNFR2 have a molecular weight of 30 kDa and 40 kDa respectively [19], meaning that they theoretically could be filtered by the glomeruli. They also appear in circulation in exosomes in their transmembrane form with a molecular weight of 55kDa and 75kDa, respectively [12,13]. Studies in mice with a normal kidney function have demonstrated that TNFRs are cleared via the kidney: they have been found in urine, intact and as small fragments indicating degradation in the kidney [14,15]. To the best of our knowledge it has not been clarified whether, in analogy to other peptides, they are reabsorbed after their appearance in the primary urine for example by the cubulin megalin receptor complex [20].

Although patients with a CRP > 20 mg/l were excluded from this study to limit the influence of acute inflammatory syndromes, both receptors were significantly correlated to CRP in univariate analysis (table 2), which remained significant for sTNFR2 in multiple linear regression analysis (table 3). This indicates that inflammation is another contributor to sTNFR concentrations, caused by release of sTNFR1 and sTNFR2 from the cell surface membrane, a process mediated by TNFα converting enzyme (TACE). In acute inflammation, this process reduces the membrane expression of TNFRs and is as such one of the regulating mechanisms of TNFα signalling [11,14,21,22]. Whether this process also plays a role in chronic inflammation is less clear. TACE was found in microparticles derived from atherosclerotic plaques and was able to induce release of sTNFR1, sTNFR2 and sTNFα from endothelial cells, hinting towards a possible role in chronic inflammation [23]. In our study, however, we did not find any differences in receptor expression on leukocyte membranes in HD patients compared to C and the membrane expression

was in steady state in both groups in an unstimulated condition (figure 2), making release as an important contributor to the plasma concentrations of sTNFR in stable CKD unlikely.

Hence in the view of the highly significant correlation found with eGFR, it seems that preserving kidney function will be the main approach to keep levels of sTNFR low. Still, release from the cell membrane is not entirely excluded and its possible contribution as a mechanism for increased sTNFR concentrations in CKD should be further elucidated, since this can provide additional information on the mechanisms regulating TNFα activity in CKD related micro-inflammation. The function of sTNFRs in circulation in CKD has not entirely been clarified: both receptors could block sTNFα activity [14,24] or prolong sTNFα half life [16,25], and thus act as a reservoir for sTNFα which could sustain its toxicity [26]. Which function is predominant in CKD is unclear and probably depends on receptor concentration [26].

The choice to match the patients for age and gender and exclude patients with markedly elevated CRP had mainly advantages but also some disadvantages. It allowed, especially in this small population, to isolate the effect of renal function on the different soluble receptors and to exclude the influence of other factors such as age, obesity, diabetes mellitus or inflammation which could be associated to TNFR concentration and CKD at the same time. The disadvantage might be that these findings cannot be generalized to e.g. older or diabetic populations. Due to the limitations in accuracy with the use of eGFR, one of the major limitations of this study is the lack of a measured GFR in the correlation study.

In summary, in stable CKD-patients, the concentrations of sTNFR1 and sTNFR2 are mainly dependent on glomerular filtration rate, while leukocyte membrane expression of sTNFRs is unaltered.

5.2.6 Tables

Table 1: Characteristics of the study population included in the correlation study according to CKD-stage.

	CKD 1 N= 10	CKD 2 N=10	CKD 3 N=10	CKD 4 N=10	CKD 5 N=10	p-value
Age (years)	43.0 ± 13.1	49.8 ± 10.6	49.5 ± 9.0	54.1 ± 10.8	55.8 ± 21.6	0.285
Gender n (M/F)	5/5	5/5	5/5	5/5	5/5	
eGFR (ml/min/1.73m²)	105.2 ± 10.2	76.5 ± 4.7	45.0 ± 7.8	24.5 ± 4.1	12.3 ± 2.1	< 0.001
MAP (mmHg)	90 ± 11	96 ± 11	94 ± 8	105 ± 10	99 ± 12	< 0.05
PP (mmHg)	39 ± 9	44 ± 8	46 ± 17	52 ± 12	56 ± 12	< 0.05
HR (/min)	68 ± 13	72 ± 22	63 ± 6	69 ± 11	65 ± 9	0.724
BMI (kg/m²)	23.8 [22.5-24.7]	25.9 [23.0-27.4]	23.9 [23.0-26.9]	30.5 [23.2-36.4]	25.9 [22.1-28.5]	0.211
sTNFR1 (ng/ml)	1.0 [0.9-1.1]	1.1 [1.1-1.3]	2.3 [1.7-3.1]	4.0 [3.4-4.6]	5.8 [5.5-6.3]	< 0.001
sTNFR2 (ng/ml)	2.1 [1.2-2.3]	3.1 [2.4-3.6]	6.6 [3.4-8.5]	6.5 [5.8-8.3]	9.7 [9.1-11.2]	< 0.001
sTNFα (pg/ml)	< 0.5	< 0.5	< 0.5	0.5 [<0.5-2.3]	3.3 [1.9-3.7]	< 0.001
CRP (mg/l)	0.9 [0.4-1.3]	1.3 [0.6-2.0]	1.0 [0.5-2.5]	2.0 [1.2-2.0]	3.0 [1.0-7.8]	0.123

CKD: chronic kidney disease, M: male, F: female, eGFR: estimated glomerular filtration rate, MAP: mean arterial pressure, PP: pulse pressure, HR: heart rate, BMI: body mass index, sTNFR1: soluble tumor necrosis factor receptor 1, sTNFR2: soluble tumor necrosis factor receptor 2, sTNFα: soluble tumor necrosis factor alpha, CRP: C-reactive protein. Data expressed as mean ± standard deviation or median with interquartile range between brackets. N=number.

Table 2: Univariate linear regression analysis between soluble receptors and possible covariates

Dependent variable	1/sTNFR1		1/sTNFR2	
Independent variable	r	p-value	r	p-value
eGFR (ml/min/1.73m²)	0.932	<0.001	0.889	<0.001
Age (years)	-0.266	0.062	-0.290	<0.05
Gender (F)	0.151	0.296	0.190	0.186
BMI (kg/m²)	-0.132	0.359	0.077	0.597
MAP (mmHg)	-0.362	<0.05	-0.422	<0.01
PP (mmHg)	-0.383	<0.01	-0.374	<0.01
HR (/min)	0.090	0.536	0.042	0.770
CRP (mg/l)	-0.290	<0.05	-0.325	<0.05
sTNFα (pg/ml)	-0.530	<0.001	-0.494	<0.001
1/sTNFR1	/	/	0.945	<0.001
1/sTNFR2	0.945	<0.001	/	/

R: correlation coefficient, sTNFR1: soluble tumor necrosis factor receptor 1, sTNFR2: soluble tumor necrosis factor receptor 2, eGFR: estimated glomerular filtration rate, BMI: body mass index, MAP: mean arterial pressure, PP: pulse pressure, HR: heart rate, CRP: C-reactive protein, sTNFα: soluble tumor necrosis factor alpha

Table 3: Multiple linear regression model for 1/sTNFR2

Model covariates	B [95% CI]	beta	p-value
constant	0.05 [0.002-0.097]		<0.05
eGFR (ml/min/1.73m²)	0.004 [0.003-0.004]	0.844	<0.001
Gender (F)	0.061 [0.020-0.102]	0.193	<0.05
CRP (mg/l)	-0.011 [-0.020- -0.001]	-0.147	<0.05

Excluded variables: age, tumor necrosis factor alpha, mean arterial pressure, pulse pressure**.**

sTNFR2: soluble tumor necrosis factor receptor 2, CI: confidence interval, eGFR: estimated glomerular filtration rate, F: female, CRP: C-reactive protein

Table 4: Expression of tumor necrosis factor receptor 1, 2 and membrane tumor necrosis factor alpha on different leukocyte subtypes in healthy controls and hemodialysis patients

Receptor	Baseline membrane expression at 4°C (E_{t0})			Membrane expression, when labelling after 1 hour incubation at 37 °C (E_{t1})		
MFI	C	HD	p	C	HD	p
mTNFR1						
Monocytes	344.5 [306.0-368.8]	331.5 [298.5-348.8]	0.35	347.5 [310.5-368.3]	324.0 [306.0-359.0]	0.71
Granulocytes	409.5 [398.8-452.8]	388.5 [307.5-433.5]	0.46	451.0 [413.3-469.0]	419.0* [402.0-440.5]	0.53
Lymphocytes	108.5 [99.8-114.3]	111.5 [101.0-120.8]	0.38	110.5 [106.3-116.3]	109.5 [100.8-115.0]	0.96
mTNFR2						
Monocytes	55.0 [46.8-98.0]	54.5 [49.8-60.3]	0.96	36.5* [32.0-42.8]	43.0 [34.5-47.8]	0.29
Granulocytes	83.0 [70.8-101.0]	77.0 [68.3-92.0]	0.79	90.5* [82.3-111.5]	92.5 [87.5-100.0]	0.83
Lymphocytes	17.5 [13.8-24.0]	11.0 [10.8-11.0]	<0.01	9.5 [8.8-16.3]	11.0 [10.0-14.8]	0.50
mTNFα						
Monocytes	683.1 [563.0-762.3]	549.0 [471.0-617.3]	0.07	856.5 [768.8-1034.0]	815.0 [558.5-915.3]	0.40
Granulocytes	629.5 [477.5-756.3]	430.0 [401.0-572.8]	0.05	710.5 [650.8-826.3]	632.0 [534.5-807.3]	0.40
Lymphocytes	125.0 [116.3-134.8]	120.0 [106.8-159.3]	0.96	129.0 [125.0-135.5]	121.5 [92.8-197.0]	0.88

MFI: mean fluorescence intensity, E_{t0}: baseline expression of receptors on membranes, E_{t1}: expression on leukocyte membranes after 1 hour of incubation at 37°C and labelling afterwards at 4°C. mTNFR1: membrane tumor necrosis factor receptor 1, mTNFR2: membrane tumor necrosis factor receptor 2, mTNFα: membrane tumor necrosis factor receptor alpha. p: p-value: comparison between C and HD; *: $p < 0.05$ when analyzing E_{t0} vs. E_{t1} in C and HD respectively.

5.2.7 References

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CHAPTER 6
DISCUSSION AND FUTURE PERSPECTIVES

In this thesis, we first investigated the association between eGFR, the commonly used index to evaluate kidney function, and the concentration of well-known low molecular weight peptides (Chapter 2). We then explored the potential biological activity of selected low molecular uremic weight peptides and focused on marker molecules like β-2-microglobulin (Chapter 3), a prototype molecule for the class of uremic toxins called middle molecules (MW > 500 Da)[1] and on pro-inflammatory cytokines, often used as markers of inflammation (Chapter 4). In chapter 5, tumor necrosis factor receptor 1 and 2, two peptides that recently gained more interest in the context of kidney disease and that are related to the TNFα-system, were investigated for their potential as markers for adverse outcome in CKD (Chapter 5.1), their biological role and the origin of their increased concentrations in uremia (Chapter 5.2).

In chapter 2, the association between the concentration of different uremic low molecular weight peptides and eGFR, estimated with different formulae, was investigated in a population with CKD stage 2-5 not on dialysis (n = 95). Apart from cystatin C and β-2-microglobulin which correlated strongly with eGFR, calculated according to the CKD-EPI$_{CystC\text{-}crea}$ formula, the association with the other evaluated uremic peptides (PTH, RbP, Myoglobin, IL6, TNFα, Leptin, FGF-23, Igκ and Igλ) was moderate to low ($R^2 < 0.5$). These results were similar irrespective of which other cystatin C-based or creatinine-based eGFR-formula was used, except for cystatin C and β-2-microglobulin, which were more strongly associated to cystatin-C based formulae than to creatinine-based formulae. Eloot et al.[2] also found comparable low associations between eGFR and several protein-bound and small-water soluble uremic solutes. Furthermore, regardless of the gradual increase in concentration of these uremic peptides during the course of CKD (except for total immunoglobulin light chains), there was a large inter-individual variability of solute concentration within each of the different CKD stages. These two observations point towards important non-GFR determinants influencing the investigated uremic peptides, such as inflammation (β-2-microglobulin, cytokines, cystatin C) or hormonal homeostasis mechanisms (PTH, FGF-23, leptin). In analogy, in hemodialysis patients, Kt/$V_{urea,}$ the

current marker assessing dialysis adequacy, was not associated to the concentration of several common uremic toxins. [3]

Despite the limitation that we did not assess a directly measured GFR, these findings challenge the use of GFR as single parameter for the evaluation of effects of renal function that have been linked to increased solute retention, which besides glomerular filtration also depends on renal tubular function, endocrine function, generation and metabolism. Findings from other studies also support this premise: in the Initiation of Dialysis Early versus Late (IDEAL) trial, a randomized controlled trial (RCT) investigating the timing of initiation of renal replacement therapy based on eGFR, 76% of the patients randomized to the late starting group (i.e. eGFR between 5-7 ml/min/1.73m²) had to start earlier due to the presence of uremic symptoms, which are believed to be caused at least partially by uremic toxins.[4] Likewise, Hsu et al. [5] found in a CKD population (CRIC cohort) that neither mGFR nor eGFR were associated to the prevalence of common CKD-complications (anemia, hyperkalemia, hyperphosphatemia and metabolic acidosis), which all showed an R^2 of approximately 0.1. Furthermore, β-2-microglobulin[6] and FGF-23[7] have been shown to be associated to mortality in a CKD population, independent of eGFR, suggesting that these markers are linked to or reflect additional patho-physiological mechanisms important to outcomes in CKD patients.

Further research is needed to investigate whether the concentration of one or a few uremic retention solutes might be representative for the concentration of other toxins, which would allow defining uremic retention with a limited number of markers. Therefore more knowledge is required on the relationship between GFR, the mechanisms of renal and extra-renal clearance of uremic retention solutes and their plasma concentrations. Whether uremic peptides could be useful markers in this context still needs to be determined.

It should be noted that with regard to the evaluation of certain aspects of kidney function, it could be more useful to measure the concentration of these uremic peptides in urine. Peptides with MW < 58000 Da and that are not protein-bound, are freely filtered through the glomerular membrane and reabsorbed via the cubulin/megalin complex in the proximal tubules. When kidney function is normal their concentration in urine is low.[8,9] It has been shown that the urinary

concentrations of β-trace-protein, β-2-microglobulin and cystatin C rise when GFR decreases. However, this is to a large extent an indicator of tubular dysfunction[10,11], whereas the tubules may be affected independently of the rest of the kidneys (e.g. with ingestion of nephrotoxic substances). Therefore, measuring urinary concentrations, does not necessarily provide information on uremic retention and potential systemic toxicity.

It is probably too optimistic to search for a unique marker which could cover all aspects of kidney function of interest, but it would be useful if a panel of markers could be defined covering the broad array of kidney functions. One could argue that adding new markers is in fact nothing else than a 'multimarker approach' which is already adopted in clinical practice by for instance measuring PTH, albuminuria and hemoglobin levels in the evaluation of patients with chronic kidney disease. However, these parameters are rather indicators of CKD complications and are not direct measures of uremic retention. Furthermore, regarding the evaluation of kidney function itself, markers which could distinguish patients at risk for progression or be informative on definite renal tubular damage would be a useful diagnostic addition to the current arsenal as well as a possible therapeutic target.

In chapters 3 and 4, we focused on the biological activity of β-2-microgloblin and pro-inflammatory cytokines, like TNFα, IL6, IL1β and IL18, which is screened in our laboratory by assessing their impact on the induction of leukocyte ROS via activation of NADPH-oxidase. This mechanism is relevant in uremia since increase in ROS contributes to the inflammatory burden in CKD and is considered as a non-traditional cardiovascular risk factor on one hand[12,13], while on the other, the continuous activation of the immune system can impair host defense upon activation by e.g. infectious stimuli.[14]

The *in vitro* assays on leukocyte oxidative stress to test the toxicity of individual uremic retention solutes were performed in whole blood of healthy donors. The advantage of this strategy is to allow interactions between different cell types and other plasma solutes during the experiment and to avoid possible activation of cells due to isolation procedure. It should however be noted that by doing this the final concentration of the added uremic retention solute in the donor blood is slightly

higher than the concentrations aimed for, since most of the uremic retention solutes are already present in low concentrations in healthy control sera. In our opinion, it is unlikely that this background concentration has a significant influence on the results, since the added test concentrations are several times above the expected background concentration.

In chapter 3, we demonstrated that unmodified β-2-microglobulin, the reference molecule for middle molecule/uremic peptide retention in uremia, did not have an effect on leukocyte oxidative burst activity. An evaluation on the potential patho-physiological role of β-2-microglobulin in other cell systems related to cardiovascular disease, such as endothelium, vascular smooth muscle cells, should be further encouraged since on the clinical level β-2-microglobulin is associated to adverse cardiovascular outcomes and/or mortality in the general[15-17] and high cardiovascular risk populations[18-20] as well as in CKD[6] and hemodialysis patients.[21,22] Also in kidney transplant patients, higher β-2-microglobulin concentrations post-transplantation were associated to higher mortality risk and graft loss.[23]

Although of course other cell systems are also of importance, the lack of biological impact on leukocytes could somehow be considered in line with the discrepancy between therapies which have shown to improve β-2-microglobulin removal and their effect on patient outcomes in large RCTs. Since many years we use the concentration of β-2-microglobulin as an index of removal of difficult to remove molecules. High-flux hemodialysis[24,25], hemodiafiltration[24,25] or longer dialysis sessions improve the removal of β-2-microglobulin[25-27] compared to standard dialysis. However, none of the large RCTs investigating these treatment strategies could demonstrate a convincing and undisputable impact on outcome.

Compared to low-flux hemodialysis, high-flux hemodialysis improved survival only in the subgroups of patients with a dialysis vintage of > 3.7 years in the Hemodialysis (HEMO)-trial[28], and in those with an albumin of < 4 g/dl or with diabetes in the Membrane Permeability Outcome (MPO)-trial[29] Online-hemodiafiltration did not reduce mortality risk compared to low-flux hemodialysis in the Convective Transport Study (CONTRAST)[30] or compared to high- flux hemodialysis in the Turkish Study.[31] The subgroups of patients in these studies treated with the highest convective

volumes had however a survival benefit.[30,31] Only the Online Hemodiafiltration Survival Study (ESHOL)[32] found a reduction in all-cause mortality in the patient group randomized to high volume post-dilution hemodiafiltration (HR 0.7, 95% CI 0.53-0.92). Importantly, patients who were randomized to the hemodiafiltration study group and who could not maintain the requested exchange volume were excluded from the study, which could have introduced bias in terms of patient characteristics between the two treatment groups, such as better vascular status in the hemodiafiltration arm.[32] This apparently weak to absent effect of hemo(dia)filtration with large pore filters was confirmed in recent meta-analyses.[33-36]

In the studies in which the control group was uniquely treated with low-flux dialysis, the β-2-microglobulin removal was indeed superior in the intervention arm, being high-flux dialysis in the HEMO-[22] and MPO-study[29] and online-hemodiafiltration in the CONTRAST-study.[30] In the two other RCTs investigating hemodiafiltration as intervention, the β-2-microglobulin levels were not different in both treatment arms, which is not surprising since the entire[31] or the majority[32] of the control groups in those studies were treated with high-flux hemodialysis. In longitudinal follow-up, the β-2-microglobulin concentrations rose in the majority of these RCTs (in all, except the Turkish Study) which was probably rather due to other factors, such as loss of residual renal function. It should also be taken into consideration that the removal pattern of other uremic peptides can be different from that of β-2-microglobulin, as was demonstrated for complement factor D by Ward et al.[37] Taken together, these data suggest that β-2-microglobulin is maybe not the only peptide that should be considered when evaluating the removal capacities of hemodialysis strategies, especially if the purpose would be to use β-2-microglobulin removal or concentration as a surrogate for hard endpoints.

Additionally, in chapter 3, the importance of a critical analysis of results of biological in vitro studies is illustrated. Our initial results obtained with β-2-microglobulin solutions, based on the products as they were purchased from commercial providers, revealed a much stronger effect on oxidative stress than expected. After purification of the commercial β-2-microglobulin solution by micro-dialysis, the stimulatory effects of β-2-microglobulin had disappeared while the dialysis fluid now induced ROS.

Unfortunately, we could not identify the contaminant responsible for the observed pro-oxidative effects.

In chapter 4, we studied the potential to exert pro-oxidative effects of four pro-inflammatory cytokines (TNFα, IL6, IL18, IL1β) of which the concentrations increase during the course of CKD. They have been shown to increase free radical production when applied at substantially higher concentrations than the ones found in uremia.[38-41] However, in a uremic concentration range, IL6, IL1β and IL18 did not increase leukocyte ROS at all. Only TNFα was pro-oxidative in monocytes and granulocytes at baseline and after fMLP-stimulation but at concentrations that were at the upper extreme of what has been observed in uremia (400 and 1400 pg/ml), and also way above the concentrations measured nowadays in the samples of our hemodialysis patients (approximately 10 pg/ml). Interestingly, a mixture of these cytokines already increased leukocyte oxidative stress at concentrations at which none of the individual cytokines had any effect, suggesting synergistic effects if individual uremic toxins are combined. In future experiments it would be of interest to combine currently observed uremic concentrations of cytokines with uremic toxins such as p-cresylsulfate[42] or phenyl acetic acid[43] which have already been shown to increase leukocyte ROS, or SDMA which both increased leukocyte ROS[44] and intracellular TNFα and IL6 concentrations.[45]

These findings also highlight the importance of the use of average uremic retention solute concentrations and of a dose response analysis when investigating them in biological assays. Testing only the highest reported uremic concentrations can be misleading since those do not necessarily represent the effect provoked at lower concentrations. Therefore it is important that the clinically relevant concentrations of uremic retention solutes are regularly reviewed and updated since therapies evolve leading to a reduction of uremic solute concentration with time, while also analytical techniques improve, resulting in more accurate concentration measurements. An updated list of uremic retention solutes, containing an addition of newly detected solutes but also corrections of reported solute concentrations compared to what was reported earlier[1], was recently published by the European Uremic Toxins Work Group

(EUTox)[46] in the period during which the experiments for this thesis were conducted. The authors recognize the relevance of mean uremic concentrations since they choose to report only the average and highest value of reported mean uremic concentrations as found in clinical studies. In contrast, in the first key publication by the same group on the classification of uremic retention solutes in 2003[1], mean concentrations and the maximum concentration as ever reported in individual patients, were listed. As a consequence, in the most recent overview[46], the concentrations suggested as relevant for most of the uremic retention solutes were lower than in the original one.[1] This evolution towards a decrease in concentration is certainly applicable to cytokines and explains the broad and variable range of concentrations that were tested in our dose response experiments, which were started before the update on uremic retention solutes [46] was published. We still based the choice of solute concentration on the classification of 2003[1], but since we noted that concentrations subsequently reported in more recent studies were substantially lower, we added experiments with lower cytokine concentrations, which were close to the concentrations published afterwards in the review of 2012.[46]

Furthermore, in chapter 4 we also investigated the effect of adalimumab, a commercially available human monoclonal antibody against TNFα which is used for the treatment of patients with rheumatoid arthritis and inflammatory bowel disease. Although this TNFα-blocker could neutralize TNFα-induced ROS in normal leukocytes, it had no effect on uremia-related ROS in samples of hemodialysis patients not treated with TNFα.

These results suggest that therapeutic interventions to reduce the inflammatory burden in CKD should probably not focus on targeted anti-cytokine therapy. This is also supported by the findings in a small RCT in hemodialysis patients by Don *et al.* [47], which found no effect on inflammatory markers such as CRP and IL6 after 44 weeks of treatment with the TNFα-blocker, etanercept. Due to the interaction between oxidative stress and inflammation, anti-oxidant therapy could be a more rational strategy to diminish oxidative stress and related inflammation in CKD on dialysis or not yet dialyzed. However, in a recent RCT in hemodialysis patients, the administration of a combination of tocopherols and α-lipoic acid failed to decrease

inflammatory markers, such as IL6 and hsCRP, and oxidative stress markers, such as F2 isoprostane and F2 isofurane.[48]

As several uremic toxins, such as indoxylsulfate, p-cresyl sulfate, ADMA and SMDA have shown to have a pro-inflammatory effect [49,50], future research to develop strategies to decrease inflammation in CKD should probably rather focus on reducing generation of uremic toxins with pro-inflammatory effect[51] for example at the intestinal level, on maintaining uremic toxin removal in the renal tubules[52,53] or on extracorporeal removal by dialysis or non-dialysis strategies such as adsorption.[54,55]

In the last section of this thesis containing original data (Chapter 5), we focused on tumor necrosis factor receptors (TNFR) 1 and 2, of which the soluble (sTNFR) as well as the membrane (mTNFR) bound forms possibly interact and modulate the effects of TNFα in the context of chronic inflammation in CKD. Until now, in the field of kidney disease, sTNFRs were mainly investigated for their potential role as predictive markers of progression of kidney disease or mortality, and predominantly in the context of diabetic nephropathy [56-58]. mTNFRs on the other hand were assessed for their role in local inflammation at the site of the kidneys.[59-61]

In the first study (Chapter 5.1), we investigated the association between both sTNFRs and the composite endpoint of mortality or first non-fatal cardiovascular event. To the best of our knowledge this is the first study evaluating the value of sTNFRs in predicting adverse outcomes in established CKD independent of the cause of underlying kidney disease. Although the eGFR-range was intentionally kept narrow (eGFR < 30 ml/min/1.73m²) in order to minimize the impact of eGFR on outcomes, we still found significant correlations between the concentrations of both receptors and eGFR (sTNFR1: r = -0.639, p<0.001; sTNFR2: r = -0.504, p<0.001). While, due to the design of the study, lower eGFR was not associated to worse outcome, higher concentrations of both sTNFRs were in multivariate analysis associated to the composite endpoint even after correction for relevant covariables such as age, eGFR, CRP, history of cardiovascular disease, diabetes mellitus and pulse pressure (HR: TNFR1 (per ng/ml): 1.51, 95% CI: 1.31-1.75; TNFR2 (per ng/ml): 1.13, 95% CI: 1.06-1.20). These results were confirmed in the non-diabetic subpopulation of this study, while in the subpopulation with diabetes we found a

significant association between sTNFR1 and outcome. This contrasts to TNFα, which was not associated to all-cause mortality and the composite endpoint (Chapter 4). The association between adverse outcome and sTNFR1 or sTNFR2 was already demonstrated in the general population and patients with diabetic nephropathy.[57,58,62-64]

In the second study (Chapter 5.2.) we further explored the cause of the increased concentrations sTNFRs in CKD in an attempt to gain information on their possible biological function. Theoretically their concentrations in circulation could increase due to decreased renal clearance and/or due to increased release from the cell surface, which has been demonstrated to occur in the context of inflammation.

The reciprocals of the concentrations of sTNFR1 and sTNFR2 were strongly correlated to eGFR in an age and gender matched non-diabetic CKD population stage 1 to 5, with r = 0.932 and r = 0.889 respectively. Based on these findings, especially sTNFR1 could be considered as a more than acceptable glomerular filtration marker. However, this should be interpreted with caution since this was a selected study population and even if patients with manifest inflammation (CRP > 20 mg/l) were excluded, there was still a significant correlation between both receptors and CRP (1/sTNFR1: -0.290 and 1/sTNFR2: -0.325). It is known that TNFRs are released from the cell membranes after a pro-inflammatory stimulus, resulting in an acute decrease of membrane expression.[65,66] Our results indicate however that the membrane expression of TNFR1 and TNFR2 is not different in hemodialysis patients vs. healthy controls and that mTNFRs are in steady state in both groups, making release of TNFRs from leukocyte membranes as a primary mechanism for the increased concentrations of sTNFRs in CKD less probable.

Together with the results of the last part of our study on sTNFRs (Chapter 5.2), the data of the outcome study (Chapter 5.1) suggests that sTNFRs are valuable potential markers in CKD and could be informative about kidney function as well as inflammatory status. However, further research is needed to determine whether these receptors are only markers in CKD or also biologically active. In view of these findings it would be also of interest to gain more knowledge on the biological function of these soluble receptors in the context of CKD. To investigate this, it will be necessary to perform in vitro experiments whereby relative concentrations of both

receptors and of TNFα as occurring in CKD are taken into account simultaneously. In a second step possibly also the concentrations of other uremic retention solutes should be considered in an attempt to mimic real-life conditions in the experimental set-up and to obtain relevant results. In this context, other cell types in close contact with circulation such as endothelium should be evaluated as well.

Summary

In summary, in this thesis we demonstrated a discrepancy between the presence of uremic peptidic markers or potential markers of CKD and biological impact. First, we have shown that eGFR, the widely used index of kidney function, is a weak indicator for the concentration of several low molecular weight peptides, except for the already known filtration markers (Cystatin C and β2-microglobulin). Second, β-2-microglobulin, the prototype molecule for the uremic middle molecules, has no effect on induction of leukocyte ROS production, an important trigger for micro-inflammation in CKD contributing to the patho-physiology of cardiovascular disease. Third, out of four pro-inflammatory cytokines of which the concentrations are moderately elevated in CKD and which are often used as markers to demonstrate inflammation, only TNFα was pro-oxidative but only at concentrations that were in the high upper range of those ever observed in uremia. We further demonstrated that TNFα blockade had no effect on uremia-related oxidative stress in vitro. However, when we assessed the role of sTNFR1 and sTNFR2 in CKD, two peptides that are part of the TNFα system, both were strongly correlated to eGFR in a CKD population stage 1-5 and were associated to adverse outcome in a CKD population stage 4-5, even after adjustment for eGFR and CRP. This makes them valuable candidates to be further evaluated as markers and for their patho-physiological role in CKD.

These data indicate that our knowledge on the role of middle molecules in uremia still is incomplete and that we cannot take their patho-physiological role as granted, even not that of molecules that are generally considered as valid markers of the uremic status. On one hand, current indices like eGFR or β2-microglobulin or cytokine concentrations do not automatically and indisputably result in the expected effects that would have proven their biological relevance. On the other hand however, our

research elucidated a number of novel findings about TNFα receptors which up to now have insufficiently been explored in CKD.

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HOOFDSTUK 6

DISCUSSIE EN TOEKOMSTPERSPECTIEVEN

In deze thesis, hebben we eerst de associatie onderzocht tussen eGFR, de courante parameter om nierfunctie te evalueren, en de concentratie van verschillende laag moleculair gewicht peptiden (Hoofdstuk 2). De biologische activiteit van enkele laag moleculair gewicht uremische peptiden werd gescreend, waarbij we ons geconcentreerd hebben op molecules die als merkers gebruikt worden, zoals β-2-microglobuline (Hoofdstuk 3), de prototype molecule van de groep van uremische toxines die tot de middelgrote moleculen behoren (MW > 500 Da) [1] en zoals cytokines, die gebruikt worden als merkers van inflammatie (Hoofdstuk 4). In hoofdstuk 5, onderzochten we de prognostische waarde van tumor necrosis factor receptor 1 en 2 voor mortaliteit en cardiovasculaire ziekte (Hoofdstuk 5.1), hun biologische functie en de origine van hun gestegen concentraties in uremie (Hoofdstuk 5.2).

In een CKD-populatie stadium 2-5 niet aan dialyse (n = 95) werd de associatie onderzocht tussen de concentraties van verschillende laag moleculair gewicht peptiden en eGFR, geschat op basis van verschillende formules (Hoofdstuk 2). Met uitzondering van cystatine C en β-2-microglobuline die een sterke correlatie vertoonden met eGFR (CKD-EPI$_{CystC-crea}$), was de associatie met de andere onderzochte molecules (PTH, RbP, myoglobin, IL6, TNFα, leptin, FGF-23, Ig-λ en Ig-κ) matig tot zwak ($R^2 < 0.5$). Ongeacht de gebruikte formule, of ze nu op cystatine C of creatinine gebaseerd was, waren de resultaten zeer gelijkaardig, behalve voor cystatine C en β-2-microglobuline die sterker geassocieerd waren met een op cystatine C in vergelijking met op creatinine gebaseerde formule. Eloot et al.[2] stelde eveneens lage associaties vast tussen eGFR en verschillende kleine wateroplosbare en proteïne gebonden uremische toxines. Daarenboven vertoonden de concentraties van de uremische peptiden een grote inter-individuele variabiliteit binnen eenzelfde CKD-stadium, ongeacht het feit dat hun concentraties gradueel stegen wanneer de nierfunctie achteruitging (behalve totaal Ig-λ en Ig-κ). Deze twee vaststellingen wijzen erop dat andere factoren dan eGFR de concentraties van de onderzochte uremische peptiden beïnvloeden, zoals inflammatie (β-2-microglobulin, cytokines, cystatine C) of hormonale homeostase mechanismen (PTH, FGF-23, leptin). In analogie met de bevindingen met eGFR, werd er geen correlatie gevonden tussen Kt/V_{urea}, de actuele

merker voor de evaluatie van dialyse-efficiëntie en de concentratie van verschillende uremische toxines.[3]

Ondanks de beperking dat we geen gemeten GFR hebben geanalyseerd in deze studie, stellen deze resultaten het gebruik van GFR als enige parameter om de effecten van nierfunctie te evalueren die gerelateerd zijn aan retentie van uremische toxines in vraag. Uremische retentie hangt namelijk ook af van renale tubulaire functie, endocriene functie, generatie en metabolisme. Data uit andere studies ondersteunen deze stelling: de Initiation of Dialysis Early versus Late (IDEAL) studie is een gerandomiseerde gecontroleerde studie (RCT) die de timing van het opstarten van nierfunctievervangende therapie onderzocht. Zesenzeventig procent van de patiënten die gerandomiseerd werden in de late starters groep (eGFR tussen 5 en 7 ml/min/1.73m²) dienden vroeger met dialyse te starten omwille van uremische symptomen, die gedeeltelijk veroorzaakt worden door uremische toxines.[4] Gelijkaardige bevindingen werden gedaan in een CKD-populatie (CRIC cohort) waarbij mGFR noch eGFR waren geassocieerd met de prevalentie van frequente uremische complicaties (anemie, hyperkaliëmie, hyperfosfatemie en metabole acidose) (R^2 bij benadering 0.1).[5] Daarenboven is aangetoond dat β-2-microglobuline[6] en FGF-23[7] geassocieerd zijn met mortaliteit in een CKD populatie, onafhankelijk van eGFR, wat erop wijst dat deze merkers gerelateerd zijn aan andere pathofysiologische mechanismen die de prognose van CKD patiënten beïnvloeden.

Verder onderzoek is nodig om na te gaan of de concentratie van één of enkele uremische retentiestoffen representatief zijn voor de concentratie van andere toxines, zodat uremische retentie met een beperkt aantal merkers zou kunnen gedefinieerd worden. Daarvoor is meer kennis nodig over de relatie tussen GFR, renale en extra-renale klaring van uremische retentiestoffen en hun plasmaconcentraties. De mogelijke bijdrage van uremische peptiden als merkers in deze context moet nog bepaald worden.

Hierbij dient ook opgemerkt te worden, dat om bepaalde aspecten van nierfunctie te evalueren, het meten van de urinaire concentratie van uremische peptiden misschien nuttiger zou kunnen zijn. Peptiden met een MW van < 58000 Da en die niet proteïne-gebonden zijn, worden vrij gefilterd via de glomerulaire basale membraan en gereabsorbeerd via cubuline/megaline receptoren in de proximale tubulus. Wanneer

de nierfunctie normaal is, is de concentratie van peptiden in urine laag.[8,9] Wanneer de GFR daalt, stijgen de urinaire concentraties van β-trace proteïne, β-2-microglobuline en cystatine C.[10,11] Dit is dus in grote mate een weerspiegeling van tubulaire functie die kan aangetast zijn onafhankelijk van andere functies van de nieren (b.v. door inname van nefrotoxische substanties). Daardoor biedt het meten van urinaire concentraties niet noodzakelijk informatie over uremische retentie en potentieel systemische toxiciteit.

Het is waarschijnlijk te optimistisch om ervan uit te gaan dat één enkele merker alle aspecten van nierfunctie kan omvatten, maar het zou een diagnostische aanwinst zijn, mocht een panel van merkers kunnen gedefinieerd worden die een brede waaier van aspecten van nierfunctie weerspiegelt. Men zou kunnen stellen dat er in de klinische praktijk al een soort 'multimarker aanpak' wordt toegepast door het meten van PTH, albuminurie en hemoglobine in de evaluatie van patiënten met CKD. Deze parameters zijn echter indicatoren van CKD complicaties en niet onmiddellijke maatstaven van uremische retentie. Daarenboven, voor wat betreft de evaluatie van nierfunctie op zich, zouden merkers die patiënten met een verhoogd risico op CKD progressie kunnen identificeren of die informatie over definitieve renale tubulaire schade zouden verschaffen, niet alleen nuttige diagnostische instrumenten kunnen zijn maar ook potentiële therapeutische targets.

In hoofdstuk 3 en 4 hebben we ons geconcentreerd op de biologische activiteit van β-2-microglobuline en pro-inflammatoire cytokines zoals TNFα, IL6, IL1β en IL18, die in ons laboratorium getest wordt door middel van inductie van vrije zuurstofradicalen in leukocyten. Dit mechanisme is belangrijk in de context van uremie aangezien een verhoogde oxidatieve stress langs de ene kant bijdraagt tot inflammatie in CKD en beschouwd wordt als een niet-traditionele cardiovasculaire risicofactor.[12,13] Langs de andere kant zorgt deze chronische immuunactivatie ervoor dat de immuunafweer verstoord is wanneer het immuunsysteem wordt geactiveerd door bv. infectieuze stimuli. [14]

In hoofdstuk 3 toonden we aan dat ongemodificeerd β-2-microglobuline, de referentiemolecule voor de middelgrote moleculen/uremische peptiden, geen effect had op leukocyt oxididatieve burst activiteit. Een verdere evaluatie van biologische activiteit van β-2-microglobuline in andere cardiovasculaire celsystemen, zoals endotheel en vasculaire gladde spiercellen, is aan te moedigen aangezien de concentratie van β-2-microglobuline geassocieerd is met een slechtere cardiovasculaire prognose en/of mortaliteit in de algemene bevolking [15,16,17] en populaties met een verhoogd cardiovasculair risico[18,19,20], alsook in patiënten met CKD [6] of aan hemodialyse.[21,22] Ook bij niertransplantpatiënten was een hogere β-2-microglobuline concentratie posttransplantatie geassocieerd aan een hoger mortaliteitsrisico of risico op transplantfalen.[23]

Uiteraard zijn ook andere celsystemen belangrijk, maar de afwezigheid van biologisch effect in leukocyten ondersteunt als pathofysiologische bevinding de discrepantie die er bestaat tussen therapieën die β-2-microglobuline verwijdering optimaliseren en hun effect op harde eindpunten bij patiënten. Beta-2-microglobuline wordt als referentie gebruikt om de verwijdering van de zogenaamde 'moeilijk te verwijderen uremische retentiestoffen' te evalueren. Het gebruik van een high-flux kunstnier [24,25], hemodiafiltratie[24,25] of langere hemodialyse-sessies[26,27,25] verbeteren de verwijdering van β-2-microglobuline in vergelijking met standaard hemodialyse. Desondanks kon geen enkele grote RCT die deze technieken onderzocht ontegensprekelijk en overtuigend een gunstig effect op prognose aantonen.

Vergeleken met low-flux hemodialyse, verbeterde high-flux dialyse de overleving enkel in subgroepen van patiënten die al > 3.7 jaar werden gedialyseerd (HEMO-studie) [28], die een albumine van < 4 g/dl of diabetes mellitus hadden (MPO-studie).[29] Online-hemodiafiltratie verminderde het mortaliteitsrisico niet vergeleken met low-flux dialyse (CONTRAST-studie)[30] of high-flux dialyse (Turkish HDF-studie).[31] Enkel de patiënten die behandeld werden met de hoogste convectievolumes hadden een overlevingsvoordeel.[30,31] Enkel in de ESHOL-studie[32] werd een reductie van de mortaliteit gevonden bij de patiënten die gerandomiseerd werden in de groep van hoog-volume postdilutie hemodiafiltratie (HR 0.7, 95% CI 0.53-0.92). Een belangrijke kanttekening hierbij is dat de patiënten die gerandomiseerd waren in de groep van hemodiafiltratie en die het gevraagde substitutievolume niet bereikten, geëxcludeerd werden uit de studie. Dit kan bias veroorzaakt hebben voor wat betreft de

patiëntenkarakteristieken tussen beide patiëntengroepen, zoals bv. een betere vasculaire status in de hemodiafiltratiegroep.[32] Recente meta-analyses die deze drie RCT's samen analyseerden met kleinere RCT's vonden evenmin een reductie in mortaliteit voor patiënten die behandeld werden met hemo(dia)filtratie [33,34,35,36], al konden Nistor et al.[34] een mogelijk gunstig effect van convectieve therapieën op cardiovasculaire mortaliteit niet uitsluiten.

In de studies waarin de controle groep enkel met low-flux dialyse werd behandeld, was de β-2-microglobuline verwijdering inderdaad superieur in de interventie-arm, zijnde high-flux dialyse in de HEMO-[22] en MPO-studie[29] en online-hemodiafiltratie in de CONTRAST-studie.[30] In de andere twee RCTs die hemodiafiltratie als interventie bestudeerden, waren de β-2-microglobuline concentraties niet verschillend tussen beide groepen. Dit is misschien niet zo verwonderlijk aangezien de volledige [31] of de meerderheid[32] van de controlegroep werd behandeld met high-flux dialyse. Tijdens de longitudinale follow-up steeg de β-2-microglobuline concentratie in de meerderheid van de RCTs (i.e. in alle behalve de 'Turkish Study') wat waarschijnlijk meer te wijten is aan andere factoren zoals het verlies van residuele nierfunctie. Een ander punt dat hierbij het vermelden waard is, is de vaststelling dat het eliminatiepatroon van andere uremische peptiden verschillend kan zijn dan dat van β-2-microglobuline, zoals werd aangetoond voor complement factor D [37]

Alles samen suggereren deze gegevens dat misschien niet alleen β-2-microglobuline moet geëvalueerd worden om dialysestrategieën voor wat betreft eliminatie van retentiestoffen te bestuderen. Dit voornamelijk als het de bedoeling is om β-2-microglobuline verwijdering of concentratie als een surrogaat te gebruiken voor harde klinische eindpunten.

Een andere vaststelling in hoofdstuk 3 illustreert het belang van een kritische evaluatie van resultaten van *in vitro* studies. Initieel vertoonde β-2-microglobuline, zoals aangekocht van de firma, een veel sterker effect of ROS productie in vergelijking met het verwachte resultaat. Na opzuivering van de oplossing door middel van micro-dialyse verdwenen de pro-oxidatieve effecten van β-2-microglobuline terwijl het dialysaat nu wel ROS induceerde. Jammer genoeg konden we de contaminant(en) niet identificeren.

In hoofdstuk 4 bestudeerden we de pro-oxidatieve effecten van vier pro-inflammatoire cytokines (TNFα, IL6, IL1β, IL18) waarvan de concentraties stijgen in het verloop van CKD. Van deze cytokines werd reeds aangetoond dat ze vrije radicalen productie konden induceren wanneer ze getest werden in veel hogere concentraties dan deze die in uremie worden vastgesteld.[38,39,40,41] In tegenstelling hiermee stelden we vast dat in een uremische concentratie range, IL6, IL1β en IL18 geen leukocyte ROS konden induceren. Alleen TNFα was pro-oxidatief in monocyten en granulocyten, maar enkel in extreem hoge uremische concentraties (400 en 1400 pg/ml), die meerdere malen hoger zijn dan de concentraties die actueel in hemodialysepatiënten worden vastgesteld. Ter vergelijking, in de stalen van de hemodialysepatiënten in onze studie bedroeg de gemiddelde TNFα concentratie 10 pg/ml. Opmerkelijk, een combinatie van deze cytokines verhoogden de burst activiteit in leukocyten al in concentraties waarbij de individuele cytokines nog geen effect hadden, wat synergisme tussen cytokines suggereert. Daarom is het interessant om in toekomstige experimenten, cytokines in uremische concentraties te combineren met uremische toxines zoals p-cresylsulfate[42] of phenylacetaat[43] waarvan al pro-oxidatieve effecten zijn aangetoond of SDMA waarvan is aangetoond dat het zowel ROS induceert[44] als dat het de intracellulaire concentraties van TNFα en IL6 verhoogt.[45]

Deze bevindingen wijzen eveneens op het belang van het gebruik van gemiddelde concentraties van uremische retentiestoffen en van een dosis antwoord analyse in biologische experimenten. Enkel de maximum uremische concentratie testen kan misleidend zijn aangezien dit niet noodzakelijk het effect van lagere concentraties weerspiegelt. Daarom is het ook belangrijk dat de klinisch relevante concentraties van uremische toxines regelmatig geüpdatet worden aangezien therapieën evolueren wat leidt tot een reductie van de toxine concentratie, terwijl tegelijkertijd de analytische technieken verbeteren waardoor een meer accurate concentratiebepaling mogelijk is. Een update van de lijst met uremische retentiestoffen werd recent gepubliceerd door de European Uremic Toxins Work Group (EUTox)[46] in de periode dat de experimenten van deze thesis werden uitgevoerd. Deze lijst bevat enerzijds nieuw gedetecteerde uremische retentiestoffen en anderzijds ook correcties van de concentraties van stoffen die al eerder werden gepubliceerd.[1] De auteurs erkennen

hierbij ook het belang van gemiddelde uremische concentraties aangezien ze ervoor kozen om gemiddelde concentraties op te lijsten zoals die in studiepopulaties werden vastgesteld. Dit is in tegenstelling tot de eerste publicatie door dezelfde auteurs in verband met de classificatie en concentratie van uremische retentiestoffen, waarbij een gemiddelde concentratie werd weergegeven en de maximale uremische concentratie die ooit werd gerapporteerd in een individuele patiënt.[1] Als een gevolg hiervan zijn de meeste concentraties die als relevant worden beschouwd, lager in de meest recente publicatie.[46] De trend dat de concentraties dalen is zeker ook toepasbaar op cytokines en verklaart de brede en variabele concentratie ranges die in onze dosis antwoord experimenten werden gebruikt, aangezien deze startten voor de update over uremische retentiestoffen[46] werd gepubliceerd. Wij hebben ons nog steeds gebaseerd op de concentraties zoals beschreven in de classificatie van 2003.[1] Aangezien we echter vaststelden dat de nadien gepubliceerde concentraties merkbaar lager waren, voegden we experimenten toe met lagere cytokine concentraties, die uiteindelijk dicht aanleunden bij de concentraties die nadien werden gepubliceerd in de review van 2012.[46]

In hoofdstuk 4 onderzochten we ook het effect van adalimumab, een commercieel beschikbaar monoclonaal antilichaam tegen TNFα, dat gebruikt wordt in de behandeling van patiënten met reumatoïde arthritis en inflammatoire darmziekten. Ook al kon deze TNFα-blocker TNFα-geïnduceerde ROS productie blokkeren in normale leukocyten, deze had geen effect op uremie-gerelateerde oxidatieve stress in stalen van hemodialysepatiënten.

Deze resultaten suggereren dat therapeutische interventies om inflammatie in CKD te reduceren, zich waarschijnlijk niet moeten richten op anti-cytokine therapie. Dit wordt ook ondersteund door de resultaten van een kleine RCT in hemodialyse patiënten, waarbij 44 weken behandeling met de TNFα-blocker, etanercept, geen effect had op inflammatoire merkers zoals CRP en IL6. Omwille van de interactie tussen inflammatie en oxidatieve stress, zou anti-oxidatieve therapie een andere strategie kunnen zijn om oxidatieve stress en gerelateerde inflammatie in CKD te kunnen verminderen. In een recente RCT echter, had de toediening van een combinatie van tocopherols en α-lipoidzuur geen effect op inflammatoire parameters

zoals IL6 en CRP, en oxidatieve stress markers zoals F2-isoprostaan en F2-isofuraan.[47]

Aangezien verschillende uremische toxines zoals indoxylsulfaat, p-cresylsulfaat, ADMA en SDMA, pro-inflammatoire eigenschappen hebben, zal toekomstig onderzoek dat zich toespitst op therapieën om inflammatie te verminderen, zich vermoedelijk eerder moeten richten op strategieën om de generatie van uremische toxines te verminderen vb. ter hoogte van de darm[48], om de eliminatie ervan in de niertubuli te behouden[49,50] of op de verdere ontwikkeling van extra-corporele technieken zoals dialyse of adsorptie.[51,52]

In het laatste deel van deze thesis met originale data (Hoofdstuk 5), concentreerden we ons op tumor necrosis factor receptor (TNFR) 1 en 2, waarvan zowel de receptoren in circulatie (sTNFR) als de membraangebonden receptoren (mTNFR) mogelijk de effecten van TNFα kunnen moduleren in de context van inflammatie in CKD. Tot hiertoe werden, in het domein van de nefrologie, de sTNFRs voornamelijk onderzocht voor hun prognostische waarde als merker voor progressie van CKD of mortaliteit, voornamelijk in populaties met diabetische nefropathie[53,54,55] en de mTNFRs voor hun functie bij lokale inflammatie ter hoogte van de nier. [56,57,58]

In de eerste studie (Hoofdstuk 5.1), werd de associatie onderzocht tussen beide sTNFRs en het samengestelde eindpunt mortaliteit of non-fatale cardiovasculaire ziekte. Voor zover we weten, is dit de eerste studie die de prognostische waarde van sTNFRs onderzocht in een populatie met gevorderd nierlijden onafhankelijk van de onderliggende oorzaak van de nierziekte. Ook al was er een nauwe eGFR-range (< 30ml/min/1.73m²), bedoeld om de invloed van eGFR op het onderzochte eindpunt te beperken, toch was er een significante correlatie tussen beide receptoren en eGFR (sTNFR1: r = -0.639, p<0.001; sTNFR2: r = -0.504, p<0.001). En terwijl, door de opzet van de studie, een lagere eGFR niet geassocieerd was met een hoger risico op het bereiken van het eindpunt, was een hogere concentratie van beide receptoren in multivariate analyse wel geassocieerd aan het samengestelde eindpunt, zelfs na correctie voor relevante covariabelen zoals leeftijd, eGFR, een voorgeschiedenis van cardiovasculaire ziekte, diabetes mellitus en polsdruk (HR: TNFR1 (per ng/ml): 1.51, 95% CI: 1.31-1.75; TNFR2 (per ng/ml): 1.13, 95% CI: 1.06-1.20). De resultaten

werden eveneens bevestigd in de subpopulatie van niet-diabetes patiënten. In de subgroep van diabetespatiënten was er enkel een significante associatie tussen sTNFR1 en het onderzochte eindpunt. Deze resultaten zijn in contrast met TNFα dat niet was geassocieerd met mortaliteit en het gecombineerde eindpunt (Chapter 4). De associatie tussen een slechtere prognose en sTNFRs was al gevonden in de algemene populatie en patiënten met diabetische nefropathie.[53,59,55,60,61]

In de tweede studie (Hoofdstuk 5.2) onderzochten we de oorzaak van de gestegen TNFR concentraties in CKD met de bedoeling informatie te vergaren over hun mogelijke biologische functie. Theoretisch kan hun concentratie in circulatie stijgen door verminderde renale klaring en/of ten gevolge van hun toegenomen vrijlating van de celmembraan, wat gelinkt is aan inflammatie en wat kan leiden tot een verandering in membraanexpressie van de receptoren. De reciproque van de sTNFR1 en sTNFR2 concentraties waren sterk gecorreleerd met eGFR in een leeftijd en geslacht gematchte niet-diabetes CKD populatie stadium 1 tot 5 (r = 0.932 en r= 0.889, respectievelijk). Deze bevindingen zouden kunnen doen uitschijnen dat vooral sTNFR1 een acceptabele glomerulaire filtratie marker is. Dit dient echter met enige voorzichtigheid geïnterpreteerd te worden omdat dit een sterk geselecteerde studiepopulatie was. Zelfs al werden patiënten met manifeste inflammatie (CRP > 20mg/l) geëxcludeerd, was er toch een significante correlatie tussen beide receptoren en CRP ((1/sTNFR1: -0.290 and 1/sTNFR2: -0.325). Het is gekend dat TNFR van het celmembraan wordt vrijgesteld ten gevolge van een pro-inflammatoire stimulus, hetgeen resulteert in een acute daling van de membraanexpressie.[62,63] Wij vonden echter geen verschil in de membraanexpressie van TNFR1 en TNFR2 tussen hemodialysepatiënten en gezonde controles. Daarenboven was de expressie van beide mTNFR in beide groepen in steady state. Het is dus onwaarschijnlijk dat vrijstelling van het celmembraan het primaire mechanisme is dat bijdraagt tot de verhoogde concentraties van sTNFR in CKD.

Samen met de resultaten van het laatste deel van onze studie naar TNFα-receptoren (Hoofdstuk 5.2), suggereren de bevindingen uit de eerste studie (Hoofdstuk 5.1) dat sTNFRs potentiële prognostische merkers kunnen zijn in CKD en dat ze zowel informatie zouden kunnen verschaffen over nierfunctie als inflammatoire status,. Verder onderzoek is echter nodig om uit te maken of deze receptoren enkel merkers zijn of dat ze ook een biologische functie hebben. In het kader van deze bevindingen,

zou het interessant zijn om de kennis betreffende hun biologische functie in de context van CKD uit te breiden. Om dit te doen zal het nodig zijn om experimenten op te stellen waarbij er rekening gehouden wordt met de relatieve concentraties van sTNFRs en TNFα zoals die voorkomen in CKD. In een volgende stap, zullen vermoedelijk ook de concentraties van andere uremische toxines in overweging moeten genomen worden in een poging om de reële situatie na te bootsen in een experimentele setting en aldus relevante resultaten te bekomen. Daarenboven zou het ook interessant zijn om hun effecten te bestuderen in andere celtypes zoals endotheelcellen aangezien deze cellen ook rechtstreeks in contact staan met bloed/plasmacomponenten.

Samenvatting

In deze thesis toonden we een discrepantie aan tussen de aanwezigheid van uremische peptide merkers of potentiële merkers van CKD en biologische impact. Ten eerste hebben we beschreven dat eGFR, de algemeen aanvaarde parameter van nierfunctie, zwak geassocieerd is met de concentratie van verschillende laag moleculair gewicht uremische peptiden, uitgezonderd voor al gekende filtratiemerkers (cystatine C en β-2-microglobuline). Ten tweede had β-2-microglobuline, het prototype van de uremische middelgrote molecules, geen effect op de inductie van leukocyt ROS productie, een proces dat een belangrijke trigger is van micro-inflammatie in CKD en bijdraagt tot de pathofysiologie van cardiovasculair lijden. Ten derde, van de vier onderzochte pro-inflammatoire cytokines, waarvan de concentratie licht gestegen is in CKD en die vaak gebruikt worden als merker voor inflammatie, was enkel TNFα pro-oxidatief, maar dan enkel in hoog uremische concentraties. Hierbij toonden we ook aan dat TNFα-blockade geen effect had op uremie-gerelateerde oxidatieve stress in vitro. Wanneer we tenslotte de rol van beide TNFα-receptoren, die deel uitmaken van het TNFα-systeem, in CKD exploreerden, vonden we dat beide sterk gecorreleerd waren met eGFR in een populatie met CKD stadium 1-5 en geassocieerd waren met een negatieve prognose in een CKD populatie stadium 4-5, zelfs na correctie voor eGFR en CRP. Dit maakt van hen kandidaten om hun pathofysiologische rol in CKD verder uit te diepen.

Deze data wijzen erop dat onze kennis omtrent de pathofysiologische rol van uremische peptiden in uremie nog steeds onvolledig is en dat we hun biologische impact niet zomaar kunnen voor waar aannemen, zelfs niet van molecules waarvan is aangenomen dat het valabele merkers zijn voor de uremische status. Aan de ene kant, kunnen we uit onze bevindingen niet ontegensprekelijk besluiten dat welgekende parameters zoals eGFR, β-2-microglobulin en cytokines hun biologische relevantie hebben aangetoond. Aan de andere kant, deden we enkele nieuwe vaststellingen in verband met TNFα-receptoren, die aangeven dat dit interessante molecules zijn om verder te onderzoeken in de context van CKD.

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	Selected courses on Statistics (Basic of Statistical Inference, Analysis of Variance, Applied Linear Regression);
	Clinical Studies: Study Design, Implementation, Reporting; Effective Scientific Communication; Personal Effectiveness en Project Management
2005	Radioprotection, diagnostic use of X-rays
2001	Electrocardiography

CLINICAL TRAINING

2007-2012	Assistant Specialist, Training in Internal Medicine and Nephrology, Ghent University Hospital, Belgium
2005-2007	Assistant Specialist, Training in Internal Medicine, AZ Sint-Jan, Brugge, Belgium

PUBLICATIONS

Articles (A1)

State-of-the-art non-targeted metabolomics in the study of chronic kidney disease
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Glorieux G, *Neirynck N*, Veys N, Vanholder R. Nephrol Dial Transplant 2012; 27: 4010-4021

An Obituary for GFR as the Main Marker for Kidney Function?
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Vanholder R, Schepers E, Pletinck A, *Neirynck N*, Glorieux G. J Ren Nutr 2012; 22: 90-94

Book Chapters

Classification and list of uremic toxins. *Nathalie Neirynck*, Rita De Smet, Eva Schepers, Raymond Vanholder and Griet Glorieux (2012) Uremic toxins. In Wiley Series on Mass Spectrometry p. 13-33. Ed Niwa T, DOI: 10.1002/9781118424032.

Indication to start kidney replacement therapy. In Management of Acute Kidney Problems.
N. Neirynck, A. De Vriese. Eds A. Jörres et al. Springer-Verlag Berlin Heidelberg, (2010), pp 471-479. ISBN 978-3-540-69441-0

Abstracts/Poster presentations

Role of monocyte membrane expression of tumor necrosis factor alpha (TNFα) receptors in altered immune response of hemodialysis patients. *Neirynck Nathalie*, Glorieux Griet, Schepers Eva, Dhondt Annemieke, Vanholder Raymond. ASN Kidney Week 2013, November 5-10, Atlanta, Georgia, USA

Elevated soluble tumor necrosis factor alpha receptor 1 and 2 concentrations in hemodialysis patients are not reflected by alterations in membrane expression. *Neirynck Nathalie,* Glorieux Griet, Schepers Eva, Dhondt Annemieke, Vanholder Raymond. ASN Kidney Week 2013, November 5-10, Atlanta, Georgia, USA

Effect of uremic toxins on leukocytes: the enigma of organic solute transport finally unraveled. Eva Schepers, Henricus Mutsaers, Annemieke Dhondt, *Nathalie Neirynck*, Rosalinde Masereeuw, Raymond Vanholder, Griet Glorieux. ASN Kidney Week 2013, November 5-10, Atlanta, Georgia, USA

Does beta-2-microglobulin exert a vascular damaging effect by activating leukocytes? *Neirynck N,* Glorieux G, Boelaert J, Liabeuf S, Massy Z, Vanholder R. ERA-EDTA Congress, 2013, May 18-21, Istanbul, Turkey

A pro-inflammatory change in numbers of monocyte and dendritic subtypes occurs early in the course of CKD. Eva Schepers, Griet Glorieux, Tim Van Den Abeele, *Nathalie Neirynck,* Raymond Vanholder. ERA-EDTA Congress, 2013, May 18-21, Istanbul, Turkey

Non-targeted metabolomics in the study of chronic kidney disease. Jente Boelaert,Ruben T' Kindt, Griet Glorieux, Eva Schepers, Lucie Jorge, *Nathalie Neirynck*, Frédéric Lynen, Pat Sandra, Koen Sandra, Raymond Vanholder. ERA-EDTA Congress, 2013, May 18-21, Istanbul, Turkey

Do cytokines at currently observed concentrations in CKD induce leukocyte activity? *Neirynck N.,* Glorieux G., Schepers E., Vanholder R. ERA-EDTA Congress, 2012, May, 24-27 Paris, France

Estimated glomerular filtration rate does not predict the concentration of low molecular weight proteins in chronic kidney disease. *Nathalie Neirynck,* Sunny Eloot, Griet Glorieux, Daniela V. Barreto, Fellype C. Barreto, Sophie Liabeuf, Aurélie Lenglet, Horst D. Lemke, Ziad Massy, Raymond Vanholder. ERA-EDTA Congress, 2012, May, 24-27 Paris, France

Oxidative burst is a sensitive screening tool in the search for pro-inflammatory capacity of uremic retention solutes in chronic kidney disease. *Neirynck N.,* Glorieux G., Vanholder R. Wetenschapsdag Faculteit Geneeskunde en Gezondheidswetenschappen, Universiteit Gent, 2012

Do cytokines at moderately elevated concentrations in CKD really induce leukocyte activity? *Neirynck N.,* Glorieux G., Schepers E., Vanholder R. ASN Kidney Week 2011, 2011, November, 8-13, Philadelphia, USA

The role of IL-6, TNF-α, IL-1β in the induction in the oxidative burst in leukocytes in CKD. *Neirynck N.,* Schepers E., Glorieux G., Vanholder R. 4th Meeting Uremic Toxins and Cardiovascular Disease, New Therapies. Groningen, The Netherlands, 2011

ORAL PRESENTATIONS

2014 Leukocyte Dysfunction and Vascular Disease. 5th Meeting Uremic Toxins and Cardiovascular Disease. 27-29/06/2014, Prague

Tumor necrosis factor α-receptors in CKD: Is there a link between membrane expression and plasma concentrations? EUTox Research Meeting, 2014, March, 29, Madrid, Spain

Uremic toxins. 12e Congres National De Néphrologie Maroc, 2014, March, 6-9, Agadir, Marokko

2013 Data on eGFR and uraemic toxin concentration. CME Course EUTox, ERA-EDTA Congress, 2013, May 18, Istanbul, Turkey

Pro-Con debate: What kills the dialysis patient? Uremic toxins. Annual Dialysis Conference, 2013, March 11, Seattle, USA.

The time for writing an obituary for GFR as a marker for dialysis initiation has arrived. Annual Dialysis Conference, 2013, March 10, Seattle, USA.

2012 Effect of the only moderately elevated TNF-alpha levels on oxidative burst of leukocytes in hemodialysis patients. Benelux Kidney Meeting, Joint meeting NfN and BVN-SBN, 2012, October 11, Eindhoven, The Netherlands

Water Quality in hemodialysis: more than endotoxin. CME course, Renal Sister Programme, ISN, 2012, October 5-6, Minsk, Belarus.

The use of beta-2-microglobulin in *in vitro* screening. EUTox-CME-course, Malaga, 2012, March 16, Malaga, Spain.

TNF-alpha and Adalimumab in uremia: effects on oxidative burst in leukocytes. EUTox-CME-course, Malaga, 2012, March 16, Malaga, Spain.

2011 The role of interleukin 6 in oxidative burst in leukocytes. EUTox Meeting Montpellier, 2011, March 18, Montpellier, France.

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