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Validation and evaluation of novel biomarkers for early renal damage in the clinical setting

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a-GST	a-glutathione-S-transferase
A1M-U	Urinary alpha1-microglobulin
ACE	Angiotensin-converting enzyme
ADH	Antidiuretic hormone
ADQI	Acute Dialysis Quality Initiative
AKI	Acute kidney injury
AKIN	Acute Kidney Injury Network
ATP	Adenosine triphosphate
AUC	Area Under the Curve
B2M-U	Urinary beta2-microglobulin
BDW	Biomarker definition group
BMI	Body mass index
BUN	Blood urea nitrogen
CC-16-U	Urinary clara cell protein 16
Cd	Cadmium
CI	Confidence interval
CKD	Chronic kidney disease
CKD-EPI	Chronic kidney disease epidemiology collaboration
Cl	Chloride
CPB	Cardiopulmonary bypass
Cr-EDTA	Chromium-ethylenediaminetetraacetic acid
CRT	Creatinine
EMEA	European Medicine Agency
eGFR	Estimated glomerular filtration rate
FDA	Food and Drug Administration
Fe	Iron
G^2	Goodness-of-fit
GFR	Glomerular filtration rate
H_2O_2	Hydrogen peroxide
HAVCR-1	Hepatitis A virus cellular receptor 1
HCl	Hydrochloric acid

HRP	Horseradish peroxidase
Hg	Mercury
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
ICP-MS	Inductively coupled plasma mass spectrometry
ICU	Intensive care unit
IDMS	Isotope dilution mass spectrophotometry
IL-18	Interleukin 18
KDIGO	Kidney Disease: Improving Global Outcomes
KIM-1	Kidney Injury Molecule 1
L-FABP	Liver-type fatty acid binding protein
MDRD	Modification of diat and renal disease
MT	Metallothionein
Na	Sodium
NaCl	Sodiumchloride
NAG	N-acetyl-D-glucosamidase
NA/K-ATPase	Sodium-potassium adenosine triphosphatase
NaOH	sodium hydroxide
NGAL	Neutrophil gelatinase-associated lipocalin
NO	Nitric oxide
Pb	Lead
RIFLE	Risk-Injury-Failure-Loss-Endstage
ROC	Receiver operating characteristics
ROS	Reactive oxygen species
RRT	Renal replacement therapy
s-CRT	Serum creatinine
s-Cystatin C	Serum Cystatin C
s-NGAL	Serum neutrophil gelatinase-associated lipocalin
SD	Standard deviation
SES	Socio-economic status
TAL	Thick ascending limb
TIM-1	T-cell immunoglobulin mucin 1
u-Cd	Urinary cadmium

u-CRT	Urinary creatinine
u-Cystatin C	Urinary Cystatin C
u-KIM-1	Urinary kidney injury molecule 1
u-NAG	Urinary N-acetyl-D-glucosamidase
u-NGAL	Urinary neutrophil gelatinase-associated lipocalin
UO	Urinary output
USG	Urinary specific gravity
ZOL	Ziekenhuis Oost-Limburg

1 General introduction and aims.

1.1 The healthy kidney

1.1.1 Functional anatomy

The kidneys are bean shaped, paired organs located along the posterior muscular wall of the abdominal cavity (**Figure 1.1**). They are important in the body homeostasis and perform a wide range of vital functions in the healthy body, such as removing wastes and excess water from the blood, balancing chemicals in the body, releasing hormones, helping control blood pressure, helping to produce red blood cells, producing vitamin D. They play a major role in maintaining a constant body pH, through the net excretion of hydrogen ions when the blood is too acidic and the net excretion of bicarbonate ions when the blood is too alkaline.

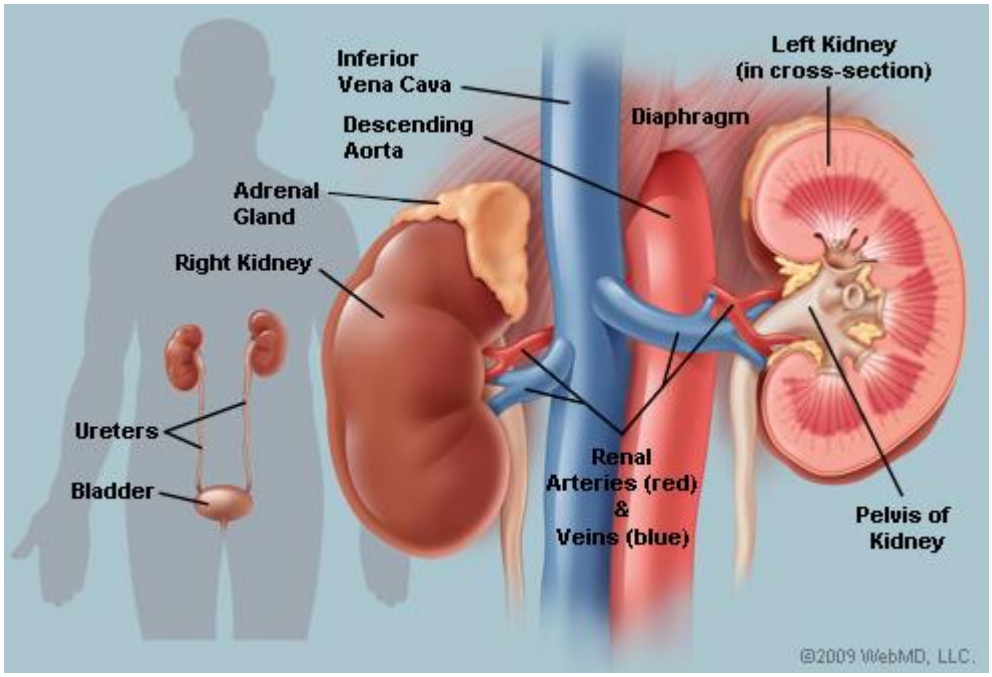


Figure 1.1 Location of the kidney in the human body. "©2009, WebMD, LLC. All rights reserved"

The kidneys consist actually of a multitude of over a million functional units called the nephrons (**Figure 1.2**). Every day about 190 liters of blood reaches the kidneys via the renal arteries. The blood enters the nephron through a

specialized filtration unit, called the glomerulus. The glomerulus is a bundled network of capillaries that increases the surface area of blood in contact with the blood vessel walls. It forms a thin filter which allows water and small solutes to enter the nephrons. Posterior to the glomerulus initiates the proximal tubule, which can be divided into an initial convoluted portion (pars convoluta) and a straight portion (pars recta). Epithelial cells lining the proximal tubule have the capacity to transport valuable molecules such as glucose, amino acids and ions from the filtrate back to the blood. Next to this reabsorption, several organic anion and cation transporters are found in the epithelial cells, which can secrete (transport from the blood into the proximal tubule lumen) a variety of endogenous and exogenous solutes (such as ammonia and creatinine (CRT)) that failed to enter the nephrons through the glomerulus (Giebisch and Windhager 2009a).

The proximal tubule is followed by the loop of Henle, a U-shaped tubule located in the inner part of the kidney, the medulla. It consists of a descending limb and an ascending limb. Through a highly specific countercurrent system (the active removal of sodium (Na) and chloride (Cl) ions in the ascending limb and the passive uptake of these ions in the descending limb, creating an osmotic gradient over the length of the loop of Henle), water will be reabsorbed through osmosis in the descending limb, and passively taken up by vessels surrounding the loop of Henle. In this part of the kidney, the concentration/dilution processes of the urine will take place. These processes are driven by the differences in osmolarity in the different layers of the kidney.

In the most outer part (the cortex) the osmolarity on the one hand, and Na, Cl and urea concentrations in the interstitial fluid on the other hand, are constant and similar to those of the extracellular fluid in other parts of the body. In the outer medulla an increase of sodium chloride (NaCl) concentration is observed, while in the inner medulla, on top of that, the urea concentration increases. These differences are caused by a combination of active transport of NaCl and the passive permeability of water, urea and NaCl, which all differ between the different segments of the tubulus.

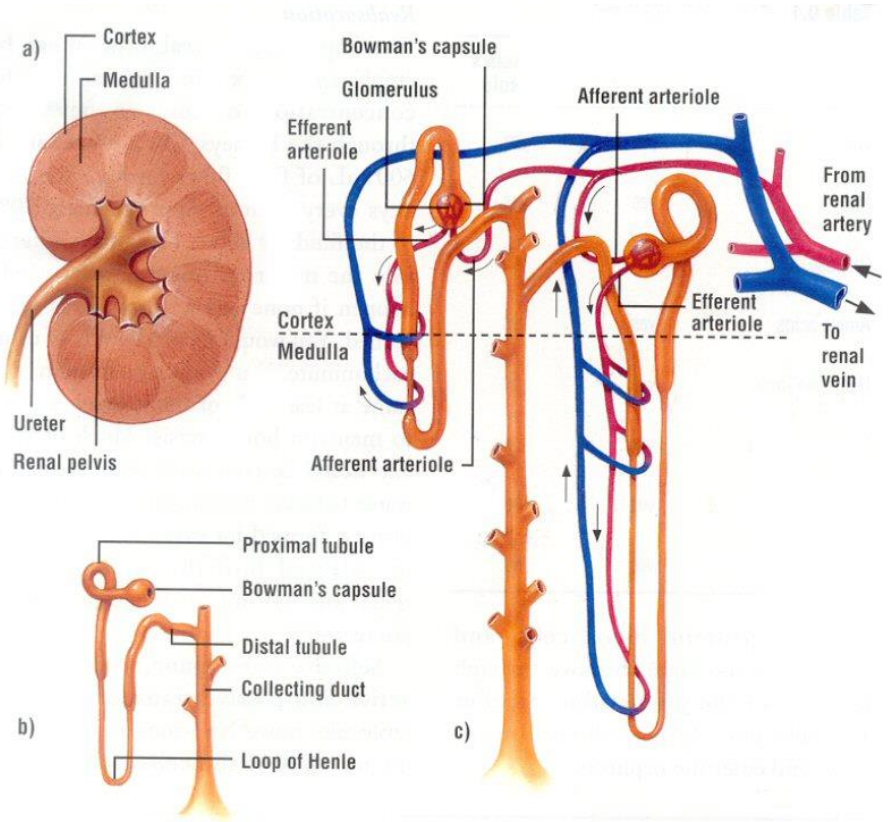


Figure 1.2 Anatomy of the nephron. A) A coronal section of the human multipapillate, B) An isolated nephron tubule, C) Renal nephrons in relation to the surrounding circulation system. http://kvhs.nbed.nb.ca/gallant/biology/nephron_structure.html

The descending tubule of the loop of Henle is highly permeable for water but not for Na, Cl and urea. Inside the tubule an osmolarity of 300 mOsm/l is observed, while the osmolarity in the surrounding interstitium is much higher. This causes water to move from the lumen to the surrounding interstitium.

As a consequence, the fluid inside the lumen is concentrated when it enters the ascending limb of the loop of Henle, with higher Na and Cl concentrations inside the lumen compared to the surrounding extracellular fluid. As this part of the tubule is highly permeable for NaCl but not for water, NaCl will passively diffuse from the lumen to the interstitium.

In the more distal part of the ascending limb (the thick ascending limb, TAL), NaCl will further be extracted from the lumen through active transport. In this distal part, the permeability for water is still very low, with little water movement as a result. Due to this passive and active extraction of NaCl from the luminal fluid, the osmolarity of the luminal fluid is lower than the osmolarity of the surrounding extracellular environment at the ending of the distal tubule, when entering the collecting duct.

In the collecting duct, NaCl continues to be actively withdrawn from the lumen. The collecting duct is the main site of action of Arginine Vasopressin (also known as antidiuretic hormone (ADH)). ADH, which is produced by the hypothalamus and released by the posterior pituitary, is responsible for the regulation of the dilution/concentration of urine. In the absence of ADH (during diuresis), the collecting tubules and ducts are virtually impermeable for water. In this case, water will remain in the lumen of the collecting duct and the urine will stay diluted. If ADH is released (during antidiuresis), the water permeability of the collecting tubules and ducts will be enhanced (by the insertion of aquaporin2 water channels into the membranes), which causes passive water transport out of the lumen. This will result in the generation of concentrated urine. Besides the effect on water permeability, ADH will also increase urea permeability of the collecting duct, resulting in an increased urea concentration in the inner medulla. Moreover, it will increase the active NaCl reabsorption in the TAL. These two latter mechanisms will further benefit the generation of concentrated urine, by enhancing the osmotic gradient in the inner medulla, favoring water reabsorption in the collecting duct (Giebisch and Windhager, 2009b).

1.2 Renal damage

As mentioned previously, the kidneys filter about 190 l of plasma every day in a male adult. It is therefore self-evident that they come in contact with a wide range of hazardous chemicals and toxins that can damage the different parts of the nephrons. Moreover, the kidney can also be subject to a sudden reduction in blood flow, leading to renal ischemia. A short overview of the different causes of renal damage will be described below. The different parts of the nephron, with their specific functional and anatomical properties, can all be subject to these insults. However because nephrons are so architecturally complex and

functionally integrated, damage to one portion of the nephron can affect the function of the entire nephron (Robertson 1998) .

First of all the glomerulus can be damaged. Observational studies in hypertensive humans have shown that sustained hypertension can lead to progressive glomerulosclerosis due to damage to the delicate glomerular capillary beds. Next to this indirect damage to the epithelial cells in the glomerulus, these cells can also be directly damaged by several chemicals and toxins. The epithelial damage of the glomerulus will cause a loss of charge- and size-selectivity of the glomerulus, causing proteins and red blood cells (that are not filtered in healthy glomeruli) to enter the renal lumen and end up in the urine, thus causing proteinuria and/or hematuria. This loss of proteins and red blood cells can have major health consequences (Brenner et al. 2000), which we will not further discuss, since it is not the focus of this thesis.

Next to the glomerulus, the tubules can be affected by toxic as well as ischemic insults. The proximal tubule is highly vulnerable for several reasons: first of all it exhibits a high oxygen and metabolic substrate requirement; secondly, as a considerable amount of water gets reabsorbed at that segment of the nephron, the concentration of the filtered substrates rises steeply. This effect is even exacerbated by active secretion of substrates into the lumen by the proximal tubule cells. Thirdly, the percentage of blood perfusing the kidney is disproportionally high. Although they only account for 0,5% of total body weight, the kidneys receive 20-25% of the resting cardiac output. Of this renal blood flow, 90% is distributed in the cortex, where mainly proximal tubule cells are located (Valtin 1983). The first part of the proximal tubule (S1 and S2) exhibits a very active endocytosis/lysosomal apparatus, which makes this part of the tubule especially vulnerable to injury related to lysosomal overload and protein bound toxic substances. The more distal part (S3) is more susceptible for injury via transporter- associated accumulation of harmful substances in the epithelial cells and hypoxia reperfusion related damage (Khan and Alden 2002).Impairment of the tubule will lead to an altered transport of filtered solutes across the brush border membrane and a disruption of secretory processes at the basolateral membrane. These altered transport mechanisms will lead to a reduced reabsorption of proteins and water, causing proteinuria

and polyuria. Tubular damage can either be acute (f.e. after cardiac surgery) or chronic (f.e. after long term cadmium (Cd) exposure).

1.3 Acute Kidney Injury (AKI): definition and staging classification

The damage caused to one or more parts of the nephron, described in the previous section, can lead to AKI, formerly known as acute renal failure. AKI is characterized by the rapid loss of excretion in the nephron. As discussed in the following sections, there is a general consensus on the characteristics of AKI and on the use of CRT and urinary output (UO) as diagnostic tools for AKI. However, this syndrome remained a rather artificial concept and the scientific world needed to consent on a clear definition of the syndrome in order to be able to describe and understand epidemiology, to randomize patients in controlled trials, to test therapies in similar groups of patients, to develop animal models and to validate diagnostic tests (Ricci et al. 2008).

A first effort in reaching this consensus was done by the Acute Dialysis Quality Initiative (ADQI) by publishing their consensus definition for AKI, the Risk–Injury–Failure–Loss–Endstage renal disease (RIFLE) classification. The RIFLE definition is intended not only to determine whether AKI is present or not in a patient, but also to define the level of the severity of the condition. The classification system is based upon a change in serum creatinine (s-CRT) or glomerular filtration rate (GFR) from a baseline value, and urine flow rate per body weight over a specified time period. Patients who exhibit an 1,5 x increase in s-CRT compared to baseline values OR an UO below 0,5 ml/kg/h for more than 6h, are classified as being 'at Risk' for AKI. If there is a 2 x increase in s-CRT or an UO below 0,5 ml/kg/h for more than 6h they are classified in the 'Injury group'. The 'Failure group' represents patients with even higher increases (3x or absolute values over 4 mg/dl) in s-CRT or lower UO (less than 0,3 ml/kg/h for more than 24h or anuria for 12h), finally the criterion for the 'Loss group' is a loss of renal function for more than 4 weeks. Patients who suffer from endstage renal damage for more than 3 months are classified in the 'endstage group' (Bellomo et al. 2004) (see fig. 1.3).

In 2007 a modified version of the RIFLE classification was published, also known as the Acute Kidney Injury Network (AKIN) classification (Mehta et al. 2007). In short, in the AKIN classification stage 1,2, and 3 replace "Risk", "Injury", and

"Failure", respectively; an absolute increase in s-CRT of at least 0.3 mg/dl has been added to Stage 1; patients starting renal replacement therapy (RRT) are automatically classified as Stage 3, regardless of their s-CRT and UO; and the outcome categories "Loss" and "Endstage Renal Disease" have been eliminated. An overview of the criteria used in the RIFLE and the AKIN classification system for AKI are given in **Figure 1.3**.

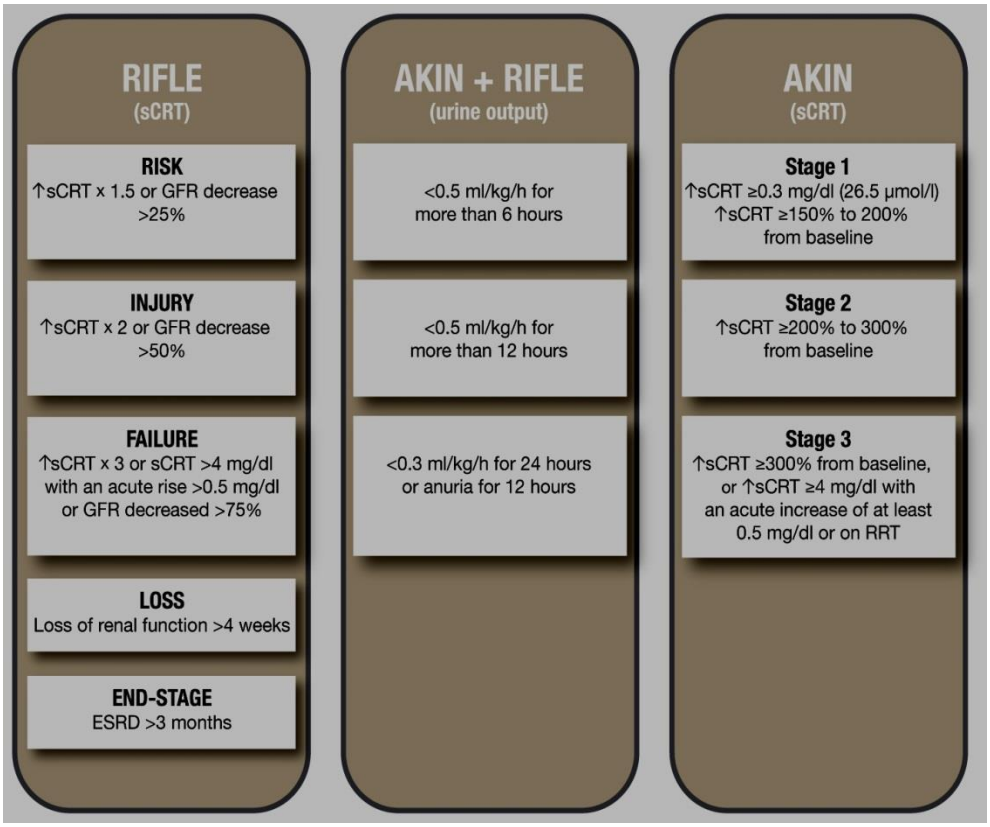


Figure 1.3 Overview of the criteria for the Risk–Injury–Failure–Loss–Endstage renal disease (RIFLE) classification (left and middle panel) and the Acute Kidney Injury Network (AKIN) classification (right and middle panel). Adapted from Bellomo et al. 2004 and Mehta et al. 2008. sCRT: serum creatinine.

In 2012 the KDIGO (Kidney Disease: Improving Global Outcomes), another international initiative to develop and implement clinical practice guidelines for patients with kidney disease, published guidelines for the evaluation and management of AKI (KDIGO 2012). In **Figure 1.4** the definition and staging of AKI according to the 2012 KDIGO guidelines are provided.

AKI is defined as any of the following (*Not Graded*):

- Increase in s-CRT by ≥0.3 mg/dL within 48 hours; OR
- Increase in s-CRT to ≥1.5 times baseline, which is known or presumed to have occurred within the prior 7 days; OR
- Urine volume <0.5 mL/kg/h for 6 hours.

AKI is staged for severity according to the following criteria.(*Not Graded*)

Stage 1: Increase in s-CRT by 1.5-1.9 times baseline; OR
Increase in s-CRT by ≥0.3 mg/dL; OR
Urine output <0.5 mL/kg/h for 6-12 hours
Stage 2: Increase in s-CRT by 2.0-2.9 times baseline; OR
Urine output <0.5 mL/kg/h for ≥12 hours
Stage 3: Increase in s-CRT by 3.0 times baseline; OR
Increase in s-CRT to 4.0 mg/dL; OR
Initiation of renal replacement therapy; OR
In patients <18 years, decrease in eGFR to 35 mL/min/1.73 m²;
Urine output <0.3 mL/kg/h for ≥24 hours; OR
Anuria for ≥12 hours

Figure 1.4 Definition and staging of Acute Kidney Injury according to the 2012 KDIGO guidelines. Adapted from KDIGO 2012.
AKI: Acute Kidney Injury; s-CRT: serum creatinine; eGFR: estimated glomerular filtration rate.

In 2013, the National Kidney Foundation–Kidney Disease Outcomes Quality Initiative (NKF-KDOQI) workgroup discussed the new KDIGO guidelines. They stated that the KDIGO guidelines are a merging and adaptation of the RIFLE and AKIN guidelines and could be beneficial in the early detection of AKI. They did have some significant reservations regarding the applicability of the KDIGO guidelines. In general they mention that at the low stages of AKI, the sensitivity is very high, but the specificity is low. Although this allows the early

implementation of simple interventions such as avoiding potential nephrotoxic medications or diagnostic or therapeutic procedures, chances are that the low specificity of the AKI definition could result in an unnecessary increase in nephrology consults and therapeutic costs, with uncertain benefit. More specific comments were made regarding (i) the use of UO, as there is little evidence on the correlation between UO and adverse clinical outcomes and changes in UO might be responses to diuretics or volume depletion, without any injury to the kidney, (ii) the use of small changes in s-CRT, as they can be attributed to extrarenal causes such as diet, CRT generation, medication or variability in Na and volume homeostasis, (iii) the independent role of duration of AKI and (iv) the applicability of the guidelines in a pediatric setting (Palevsky et al. 2013).

1.4 Causes of renal damage

As mentioned before, the kidneys are subject to a range of damaging influences. In this section an overview of the different causes of renal damage is presented. Although the different causes are described separately, from the clinical viewpoint it is acknowledged that renal damage is frequently multifactorial, with concomitant components of the causes mentioned below, and with overlapping pathogenetic mechanisms.

1.4.1 Toxic insults

Nephrotoxicity (renal damage due to toxic insults) may occur when the kidneys are exposed to a variety of nephrotoxic drugs (both in therapeutic and in diagnostic settings) or after environmental or occupational exposure to certain heavy metals (Cd, lead (Pb), mercury (Hg),…) and solvents (f.e. hydrocarbons). The most common nephrotoxic therapeutic drugs are some antibiotics (such as gentamycin and vancomycin); chemotherapeutics (f.e. cisplatin); analgesics (NSAID'S), immunosuppresives (f.e. calcineurin inhibitors), angiotensin-converting Enzyme (ACE)-inhibitors,… In diagnostics, intravenous radiocontrast dyes are known to exert nephrotoxic effects (Perazelli 2009).

A review, published in 2009 in the Clinical Journal of the American Society of Nephrology by Perazelli, provides an overview of the important nephrotoxins that the kidney is exposed to, the factors that increase vulnerability of the kidney to potential toxins, the renal compartments afflicted by toxins and the

clinical renal syndromes promoted by these agents (Perazelli 2009). However in the light of this thesis only the nephrotoxic effect of Cd exposure will be further discussed.

1.4.1.1 Cd nephrotoxicity

Cd is a heavy metal that, due to historical industrial activities and mining, is a widespread environmental and occupational pollutant. Despite recent efforts by many countries and international agencies to reduce the usage of Cd, the Cd footprint remains high, due to its zero degradability and its long half-life in the body (10-30 years) (Nawrot et al. 2010). Next to other organs, such as the liver, the lungs and the bone, the main target organ of Cd toxicity is the kidney (Jarüp 2002). The main exposure route for humans is either on the work floor (industrial exposure), through the ingestion of contaminated water or food, due to environmental accumulation of Cd or through cigarette smoking (Saturag et al. 2010).

Regardless of the route of exposure, once absorbed into the bloodstream, it will be taken up by the hepatocytes, where it gets bound to methallothionein (MT). These Cd-MT complexes can be released in the bloodstream. After they get filtered by the glomerulus of the kidney, they can be absorbed by the epithelial cells of the proximal tubules (Klaassen et al. 2009) (**Figure 1.5**). When the Cd-Mt complexes accumulate in the epithelial cells , they cause changes in cell-cell adhesion, cellular signaling pathways, and autophagical responses and can even lead to cell death through apoptosis and necrosis (Prozialeck and Edwards 2012), resulting in a generalized reabsorptive dysfunction characterized by polyuria and proteinuria. Long term exposure to Cd, even in low concentrations, can therefore lead to chronic kidney damage.

There is a strong evidence that the altered transport in the proximale tubule due to Cd intoxication is caused by mechanisms affecting the Na-K-ATPase (Sodium-potassium adenosine triphosphatase) transport systems. One mechanism through which these transport systems can be affected is explained by Gonick (2008). The author states that Cd is initially segregated in a nontoxic complexed form with Mt in the cytoplasm of the proximal tubule, but when the organism's capacity to synthesize new Mt is exhausted, the Cd, now less firmly bound to

high molecular weight proteins, moves to other cell organelles (such as the mitochondria and the microsomes), where they affect the Na-K-ATPase.

A second explanation is provided by Thévenod (2003). According to this researcher, Cd induces oxidative damage in cells, through the stimulation of reactive oxygen species, which can further decrease the Na-K-ATPase activity.

In an industrially exposed population, the proteinuria appeared to be a mixed type, of both high and low molecular weight proteins, indicating both glomerular protein loss as well as a decreased tubular reabsorption (Bernard et al. 1978).

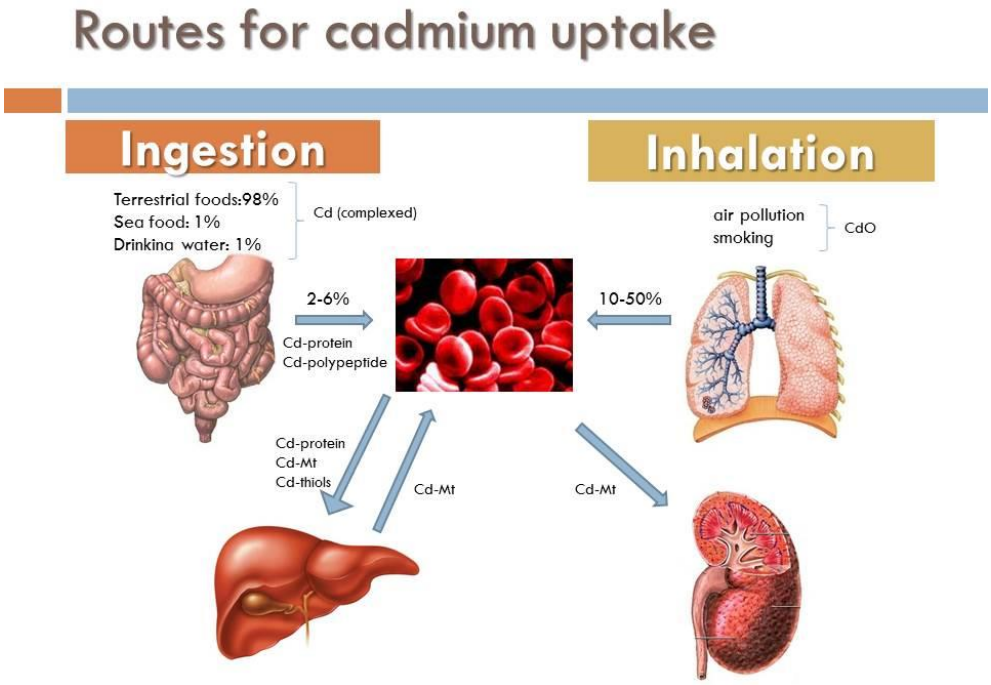


Figure 1.5 Routes for cadmium uptake.

After ingestion or inhalation, the different forms of cadmium will be absorbed into the bloodstream and taken up by liver cells, where they get bound to methallothionein. When released back into the bloodstream, these cadmium-methallothionein complexes get filterted by the glomeruli of the kidney.

Cd: cadmium; CD-Mt: cadmium-methallothionein.

The threshold of u-Cd at which tubular proteinuria appears, was initially set at u-Cd values of 10µg/g creatinine, but has been gradually reduced over the years, due to the development of more sensitive tests (Gonick, 2008). Roels et al. stated that early biochemical alterations (e.g. hypercalciuria) can be observed at a threshold value of 2.4 µg Cd/g CRT (Roels et al. 1999).

Although u-Cd has long been believed to be a good measure for the chronic accumulation level of Cd in kidney tissue, this has recently been questioned by Chaumont et al. They believe the relationship between the amount of Cd accumulated in the kidney and the u-Cd concentrations, as it was established in industrial workers (with high Cd exposures) was extended to the general population, without taking into account the route, level and duration of exposure. Moreover, they state that in the general population, subject to low doses of Cd exposure, the concentrations of u-Cd are merely based on physiological variations, unrelated to metal accumulation and toxicity (Chaumont et al. 2013).

1.4.2 Ischemia-reperfusion injury

The initial phase of ischemia reperfusion injury (the ischemia phase) is typified by a profound adenosine triphosphate (ATP) depletion. This leads to an increase in hypoxanthine, activating the xanthine oxidase system, which in turn leads to the generation of reactive oxygen species (ROS). Another consequence of ATP depletion, is the impaired function of ATP-dependent ion-pumps, leading to an increase of mitochondrial and cytosolic calcium concentrations (Green et al. 1989). As a consequence, phospholipase A gets activated, initiating the arachidonic acid cascade, with a further generation of free radicals as a result (Freeman and Crapo 1982). Another pathway, in the ischemic phase, is the Fenton reaction (Green et al. 1989). In this reaction, free radicals are produced through the reaction of iron (Fe) ions and hydrogen peroxide (H_2O_2) (Kremer 1999).

The ROS, generated in the ischemic phase, will lead to leucocyte attraction and activation in the epithelial cells, mediated through adhesion cells. The effect of leucocyte attraction and activation is threefold: (1) a further production of free radicals will occur; (2) leucocytes will release intrinsic enzymes that have

destructive effects on the epithelial cells); (3) leucocytes can physically obstruct renal capillaries with inevitable impairment of the microcirculation and extension of the ischemic insult (Weight et al. 1996).

In healthy kidney tissue a balanced interaction between endothelin and nitric oxide (NO) regulates the renal microvascular homeostasis, with NO counteracting the vasoconstrictive effect of endothelin. In ischemia-reperfusion injury, the endothelin/NO ratio is altered. Not only will endothelin be upregulated, but the post-ischemic kidney tissue becomes refractory to the vasodilatory action of NO. This imbalance leads to an increase in renal vascular resistance with a decreased renal blood flow and GFR as a result (Weight et al. 1996).

In the tubular cells, all these effects together lead to the inversion of polarity, loss of adhesion to the basement membrane and induction of apoptosis (Bellomo et al. 2012).

1.4.3 Cardiovascular surgery

Cardiovascular surgery involving cardio-pulmonary bypass (CPB) (also known as extra-corporal circulation) is often associated with AKI. Incidences of AKI after cardiac surgery with CPB have been reported to be between 1 and 30% in different patient groups (Conlon et al. 1999). Patients who develop AKI have increased risk of mortality, a longer length of hospital stay and higher medical costs across a broad spectrum of conditions (Chertow et al. 2005).

Although the etiology of AKI after cardiac surgery remains far from fully understood, researchers believe that the condition develops due to a combination of ischemic injury, which is caused by poor renal perfusion associated with the use of CPB, and nephrotoxic injury, which is caused by both endogenous toxins, such as myoglobin, and surgery-related exogenous agents, such as anesthetic agents, contrast media, diuretics and antibiotics (Endre 1995). Another pathophysiological pathway that is involved in the development of AKI after CPB is inflammation. CPB provokes a systemic inflammatory response syndrome. During CPB, the activation of neutrophils and vascular endothelium in combination with an upregulation of adhesion molecules is observed. Moreover, platelets also undergo activation, degranulation and adherence to vascular endothelium. The combination of these events causes the

elaboration of cytotoxic oxygen-derived free radicals, proteases, cytokines and chemokines. This inflammatory response is further driven by diffuse end-organ ischemia and the activation of factor XII (Hageman factor) (Rosner and Okusa 2006).

1.4.4 Other causes

Critically ill patients at the intensive care unit (ICU) form another group that is highly susceptible to develop AKI. Underlying triggers in these patients are sepsis, recent cardiac surgery (*cfr supra*), rhabdomyolysis, major bleedings and acute decompensated heart failure (e.g. cardiorenal syndrome). In patients with severe liver dysfunction, AKI can arise, which is known as the hepatorenal syndrome. As the underlying mechanisms of these renal problems are multi-organ in nature, the pathophysiology of these forms of AKI are too elaborate for this introduction. A very comprehensive review providing an overview of the underlying mechanisms in these forms of AKI was written by Bellomo in 2012 (Bellomo et al. 2012).

1.5 Biomarkers of renal damage

1.5.1 What is a good biomarker?

In 2001 the term biomarker was defined by the Biomarkers Definitions Working Group (BDW) of the National Institute of Health as 'a characteristic that can be objectively measured and evaluated as an indicator of normal biological or pathogenic processes, of pharmacological responses to a therapeutical intervention or other health care interventions' (Biomarkers Definitions Working group 2001). Examples of biomarkers are proteins, lipids, imaging patterns, electrical signals, genomic, metabolomic or proteomic patterns and cells present on urinalysis (Ferguson and Vaidya 2010). Thus, identifying biomarkers includes all diagnostic tests, imaging technologies and any other objective measures of a person's health status. The biomarker can either be produced by the diseased organ itself (e.g. tumour) or by the body in response to the disease or insult. Biomarkers are potentially useful along the whole spectrum of the disease process. Before diagnosis, markers could be used for screening and risk assessment. During diagnosis, markers can determine staging, grading, and selection of initial therapy. Later, they can be used to monitor therapy, select

additional therapy or monitor recurrent diseases (Biomarkers Definitions Working group 2001).

Although any characteristic of the body could hypothetically be taken into account as a possible biomarker, the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) provided some criteria that help distinguish good biomarkers. (1) A biomarker should be **specific**. It should preferably (or exclusively) be produced in the target tissue on the one hand and be able to differentiate between different pathologies on the other hand. (2) The biomarker should be **sensitive**. It should be able to provide an early indication of the disease (before other clinical symptoms develop). It should be virtually undetectable in controls but increase significantly after injury or disease and moreover have a long half-life. (3) It should be **proportional to the degree of severity** of pathology. (4) It should be able to **predict disease progression**. (5) It should be **robust**, not be confounded by unrelated conditions, be rapid, simple, accurate, and inexpensive. (6) The biomarker should be **translatable,** allowing the comparison between pre-clinical and clinical results. (7) The biomarker should be measurable in a **non-invasive** way. To achieve this, it should be released from the tissue into accessible fluid samples (ICH 2008 and 2011). As seen before, the kidney consists of different entities all performing their proper function. An additional criterium when describing the ideal biomarker specifically for renal damage is therefore the ability to distinguish between the **site of damage within the kidney**, enabling more precise pharmaceutical interventions.

1.5.2 CRT clearance as the current golden standard for the assessment of renal function

Renal function is quantified by the GFR (ml filtered/min). This is the flow rate of filtered fluid through the kidney. As the GFR can not be measured directly, it is determined by the clearance rate of a solute that is freely filtered from the blood by the glomerulus and is neither reabsorbed, secreted nor metabolized in the tubulus. The reference method to determine GFR is the urinary clearance of inulin or chromium-ethylenediaminetetraacetic acid (Cr-EDTA) during continuous intravenous infusion (Delanghe 2009). By measuring the urinary and plasma

concentrations of these solutes, as well as the urine flow, the GFR can be calculated through the following formula:

$$GFR = \frac{\text{Urine concentration x Urine flow}}{\text{Plasma concentration}}$$

As the injection of exogenous solutes is rather cumbersome, the GFR can also be clinically identified by monitoring the endogenous s-CRT in patients (Lameire and Hoste 2004). CRT is a small 113 Da molecule. It is derived from the metabolism of creatine in skeletal muscle and from dietary meat intake. Creatine is required for the utilization of ATP-derived energy at sites of high energy utilization (muscle, brain, heart). In the muscle, creatine and its phosphorylated form, break down to CRT that is released in the plasma (Longo et al. 2011). CRT generation from the muscles is proportional to the total muscle mass and muscle catabolism. CRT is released in the plasma at a relative constant rate and is freely filtered by the glomerulus. It is, analogous to inulin, theoretically not secreted nor reabsorbed in the tubules. Therefore a decrease in GFR will be reflected by a decreased CRT clearance. The CRT clearance is the volume of blood plasma that is cleared of CRT per unit time (Stevens et al. 2006).
To measure CRT clearance directly, a 24h urine sample has to be collected, as well as a plasma sample. The CRT clearance can then be calculated as followed:

$$Clearance_{CRT} = \frac{\text{u-CRT x 24-hour volume}}{\text{s-CRT x 24 x 60mins}}$$

As the collection of 24h urine sample is a rather time consuming procedure, often offering inaccurate and incomplete samples, estimation equations were developed, by which the CRT clearance can be estimated, without the need of a 24h urine sample. The first equation that was developed to estimate CRT clearance was the Cockcroft-Gault equation in 1976 (Cockroft and Gault 1976). This equation takes into account age and weight, as surrogate for muscle mass. It is given as:

$$eC_{CRT} = \frac{(140 - \text{age}) \text{ x Mass (in kg)x } [0{,}85 \text{ if female}]}{72 \text{ x s} - \text{CRT } (\text{in} \frac{\text{mg}}{\text{dl}})}$$

In 1999, a formula to estimate the GFR was developed by the Modification of Diet in Renal Disease (MDRD) study groups. Other than the Cockroft-Gold formula, it takes into account the body surface and race (Levey et al. 1999):

$$eGFR = 186 \text{ x s-CRT}^{-1.154} \text{ x Age}^{-0{,}203} \text{ x } [1{,}212 \text{ if black}] \text{ x } [0{,}742 \text{ if female}]$$

This equation was re-expressed in 2006 for use with an Isotope Dilution Mass Spectrophotometry (IDMS) traceable standardized s-CRT assay, which yields s-CRT values that are 5 percent lower (Levey et al. 2006).

$$eGFR_{(mL/min/1.73\ m^2)} = 175 \times \text{s-CRT}^{-1.154} \times \text{Age}^{-0.203} \times [0.742 \text{ if female}] \times [1.212 \text{ if black}]$$

In an attempt to diminish the bias, especially at higher levels of estimated GFR (eGFR), in 2009 the Chronic Kidney Disease Epidemiology Collaboration (CKD—EPI) equation for estimation of GFR was developed as follows (Levey et al. 2009):

Female	s-CRT ≤62	GFR = 144 × (s-CRT/0.7)$^{-0.329}$ × (0.993)^{Age}
	s-CRT >62	GFR = 144 × (s-CRT/0.7)$^{-1.209}$ × (0.993)^{Age}
Male	s-CRT ≤80	GFR = 141 × (s-CRT/0.9)$^{-0.411}$ × (0.993)^{Age}
	s-CRT >80	GFR = 141 × (s-CRT/0.9)$^{-1.209}$ × (0.993)^{Age}

It has to be taken into account that the former equations should not be used in patients under 18 years old (not validated), in patients with instable CRT values (f.e. pregnant women), in patients that exhibit extreme muscle or dietary characteristics (f.e. bodybuilders, after amputation, in extreme obese patients, in case of malnutrition, …).

Although a wide range of comparative studies have been published, trying to elucidate which of the previously described formulae performs the best in estimating the GFR, results are inconclusive (Early et al. 2012) and the different estimation equations are still used in clinical research, often compromising proper comparison between research results. It can be stated however that the CKD-EPI equation has lower bias, especially at eGFR greater than 60 mL/min/1.73m (Levey et al. 2009).

1.5.3 Pitfalls s-CRT

Although s-CRT is still widely used to assess the renal function, this biomarker appears to be far from the ideal renal biomarker because of several reasons: First of all the GFR is overestimated, as 10% to 25% of CRT is secreted by the tubules into the urine and is not filtered by the glomeruli. Moreover, in case the kidney is impaired, the proportion of CRT secretion by the tubules is further increased, which contributes to an even greater overestimation of the GFR. The tubular secretion of CRT varies among and within individual persons, especially in those with mild to moderate reduction in GFR (Levey 1990).

Secondly, although the production and release of CRT in the plasma is considered to be reasonably stable, it is variable depending on sex, age, race, dietary intake, muscle mass and disease-associated loss of muscle mass. This results in a significant variation of baseline s-CRT levels (Jones et al. 1998). For these reasons, there are substantial inter- and intra-individual variabilities in the relationship between the levels of s-CRT and GFR .

Thirdly, as CRT needs time to reach a new steady state, rapid changes in GFR are not reflected by simultaneous changes in s-CRT levels, and the diagnosis of impaired renal status is delayed by hours to days (Moran and Myers 1985).

On top of the above mentioned factors compromising the sensitivity and specificity of CRT clearance as a marker for GFR, an increase in s-CRT clinically does not distinguish among prerenal (decreased perfusion), postrenal (outflow

obstruction) and intrinsic (glomerular or tubular defects) renal causes (Coca et al. 2008).

Finally, the major shortcoming of s-CRT as a biomarker for renal damage, lies in the fact that the levels do not rise until the glomerular filtration rate has fallen by about 50%, giving the so-called CRT blind range (Martensson et al. 2012). This results in a delayed diagnosis of AKI, by which crucial hours/days are lost before appropriate therapeutics actions can be undertaken. This will be further discussed in the general discussion of this thesis (see chapter 7).

These shortcomings highlight the urgent need for the discovery and validation of novel sensitive and specific biomarkers of early renal damage, which can detect real-time renal impairment, allowing the initiation of interventions that can prevent and/or treat renal impairment early in the disease course.

1.5.4 Novel biomarkers

Due to the unsatisfactory performance of s-CRT as a marker for early kidney damage, scientific groups all over the world and in all subdivisions of renal research have made the detection and validation of novel biomarkers one of their priorities. Recently a broad range of novel potent biomarkers has been discovered through omics technologies. These techniques make it possible to observe global changes of transcripts (genomics), proteins (proteomics) or metabolites (metabolomics) in cellular systems or tissues, at the onset of AKI or renal damage, after ischemia, toxic insults or the combination of both.

After the discovery phase, also called phase I, the novel biomarkers have to be further validated through 4 more phases of validation, as prescribed by 'the US National Cancer Institute's 'Early Detection Research Network' in 2001. In short: phase II consists of the validation of the reproducibility, sensitivity and specificity of the marker. In phase III the sensitivity and specificity for the detection of different disease conditions are validated. In phase IV the specificity and sensitivity is further evaluated on prospective cohorts. This makes it possible to estimate false referral rates and the ability of the biomarker to describe the extent and characteristics of the disease. Phase V focusses on the evaluation of the overall benefits and risks of the new diagnostic test (Sullivan Pepe 2001).

Although a large amount of biomarkers, that are discovered in phase I, fail to pass the evaluation of the clinical opportunities of the biomarker in the following phases of the validation process, a few of them showed promising results. An overview is provided in **Figure 1.6**. Four of them will be discussed in depth: Kidney injury molecule 1 (KIM-1), Neutrophil gelatinase-associated lipocalin (NGAL), N-Acetyl-D-glucosaminidase (NAG) and cystatin C, as these are the biomarkers that will be further investigated throughout this thesis.

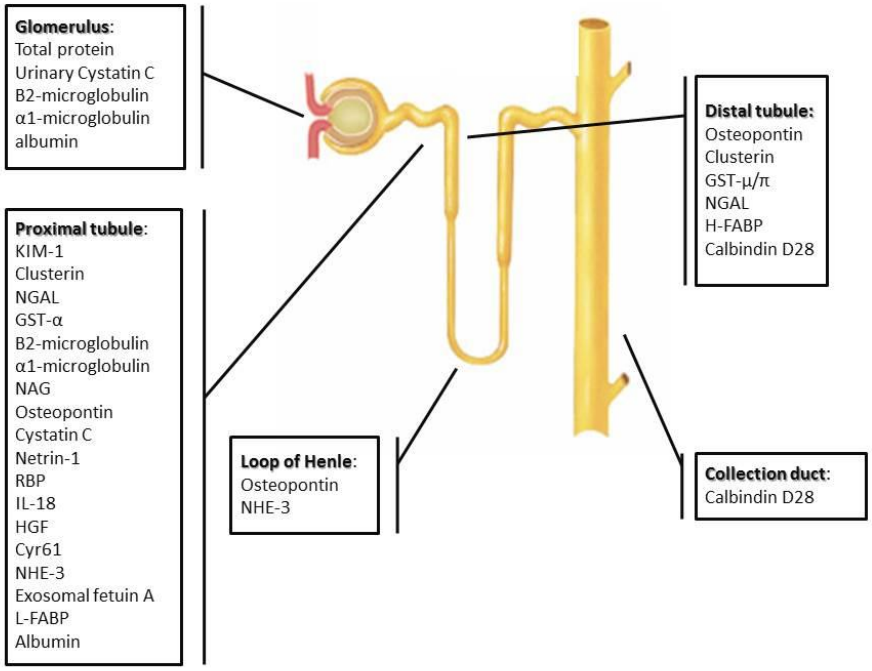


Figure 1.6 Overview of novel promising nephron segment-specific biomarkers for the detection of Acute Kidney Injury. Adapted from Bonventre et al. 2010.

1.5.4.1 KIM-1

KIM-1, also referred to as T-cell immunoglobulin mucin-1 (TIM-1) and Hepatitis A virus cellular receptor-1 (HAVCR-1), is a glycosylated type I cell transmembrane glycoprotein with a six-cystein immunoglobulin-like domain and

two N-glycosylation sites and a domain rich in threonine/serine and proline (Waanders et al. 2010) (**Figure 1.7**). It was first described by Ichimura et al in 1998 (Ichimura et al. 1998).

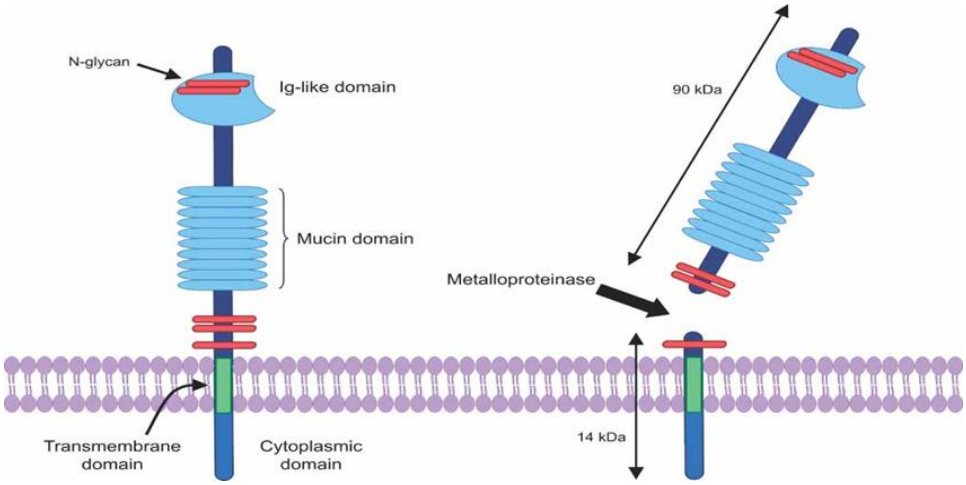


Figure 1.7 Structure of KIM-1. The protein is a type-1 membrane protein with most of the protein made up of an extracellular domain that consists of a signal peptide, an Ig domain and a mucin domain. There is one transmembrane domain and a short intracellular domain with at least one important tyrosine phosphorylation domain. The protein can be cleaved by a metalloproteinase, after which the ectodomain (90 kDa) appears in the urine, leaving a 14 kDa membrane-bound fragment that is tyrosine-phosphorylated (Tyr-P) (Waanders et al. 2010; with permission).

In normal physiological conditions, KIM-1 is expressed in very low levels in the kidney. However, in case of **proximal tubule damage**, KIM-1 gets highly upregulated during dedifferentiation of the proximal tubule epithelial cells. Moreover the ectodomain gets cleaved by metalloproteinases and is shedded in the urine (Zhang et al. 2007). Recent research has unraveled the functional role of the upregulation of KIM-1 after renal injury: KIM-1 can recognize and induce phagocytosis of dead cells, which could otherwise obstruct the tubule lumen, one of the characteristics of AKI. As KIM-1 is a phosphatidylserine receptor, it is able to recognize apoptotic cells. The apoptotic cells and necrotic debris will bind the Ig-domains of KIM-1, which are located on fagocytotic cups at the apical surface

of surrounding surviving epithelial cells, after which they will get internalized into fagosomes. Moreover, it is also a receptor for oxidized lipoproteins, recognizing the oxidized phospholipids on the outer leaflet plasma membrane of dying or necrotic cells (Ichimura et al. 2008). Due to these characteristics, KIM-1 can transform epithelial cells into semi-professional phagocytes (Bonventre 2009).

Although the function of the shedded KIM-1 ectodomain in the tubule lumen is unknown, the presence of the ectodomain in the urine makes it easy to measure the upregulation of KIM-1 in a non-invasive way, meeting one of the criteria of a good biomarker. In animal studies , the utility of KIM-1 as a biomarker to detect kidney injury has been demonstrated in a variety of pathological conditions such as protein-overload nephropathy (van Timmeren et al. 2006), ischemia-induced renal injury (Ichimura et al. 1998), polycystic kidney disease (Kuehn et al. 2002) and kidney injury induced by various nephrotoxicants such as Cd (Prozialeck et al. 2007b and 2009b), cisplatin (Wadey et al. 2013) , folic acid (Ichimura et al. 2004), contrast agents (Jost et al. 2009), gentamicin, Hg and chromium (Zhou et al. 2008).

In humans the possible role for KIM-1 as a biomarker for early damage has been shown in several clinical settings. Both in pediatric (Krawczeski et al. 2011) and in adult patients (Liangos et al. 2009), KIM-1 has shown to have potential as a biomarker for early kidney damage after cardiac surgery involving CPB. The utility of KIM-1 as a biomarker has also been studied in several other clinical settings such as: renal cell carcinoma (Zhang et al. 2013), in preeclampsia (Xiao et al. 2013), in urinary tract infection (Petrovic et al. 2013), in kidney transplants (Jin et al. 2013) and in AKI caused by sepsis (Takasu et al. 2013).

1.5.4.2 NGAL

NGAL is a 25-kDa protein that is bound covalently to gelatinase from neutrophils. It is a ubiquitous protein that can be found in low concentrations throughout the body, which is expressed and secreted by immune cells, hepatocytes, and renal tubular cells in various pathologic states. Siderophores, small iron binding molecules are the major ligands for NGAL. Siderophores are synthesized by bacteria, to provide them with Fe. By binding to siderophores, NGAL can exert a bacteriostatic effect. Secondly, NGAL acts as a growth and

differentiation factor in multiple cell types, including developing and mature renal epithelia, and some of this activity is enhanced in the presence of siderophore-Fe complexes. Moreover some of these cells might produce siderophore-like molecules that can bind NGAL, causing NGAL-mediated Fe-shuttling, which is critical to various cellular responses like proliferation and dedifferentiation (Schmidt-ott et al. 2007). In case of renal ischemia, NGAL is upregulated in **the distal parts of the damaged tubule** (Paragas et al. 2011).

Although this upregulation of NGAL in the distal nephron segments comprises the major fraction of urinary NGAL (u-NGAL) (Schmidt-ott et al. 2007), diminished reabsorption of NGAL in the **proximal tubule**, f.e. through epithelial damage or saturation of the megalin-dependent endocytosis caused by proteinuria, can also account for higher u-NGAL concentrations. Increases in serum NGAL (s-NGAL) concentrations are also observed after AKI and can be explained by several mechanisms: first of all it is known that AKI leads to an increased NGAL mRNA expression in distal organs (e.g. longs and liver) with a release of over-expressed NGAL in the systemic circulation as a result (Grigoryev et al. 2008); secondly, NGAL is an acute phase reactant and may be released from neutrophils, macrophages and other immune cells, which are activated during AKI; finally, AKI can cause a **decline in GFR**, resulting in a decrease in renal clearance of NGAL, resulting in an accumulation of NGAL ion the systemic circulation (Devarajan et al. 2010).
Both preclinical transcriptome profiling studies and downstream proteomic analyses in the kidney after ischemic or nephrotoxic AKI in animal models have identified NGAL to be one of the most upregulated proteins (Devarajan et al. 2008). These findings have led to the testing of NGAL as a possible biomarker for early kidney damage in a variety of renal disorders (such as AKI after cardiac surgery, renal carcinoma, AKI in the critically ill,…) in different patient groups, with positive results (Mishra et al. 2005 and Di Carlo et al. 2013).

1.5.4.3 NAG

NAG is a lysosomal brush-border enzyme with two isoforms (A and B). It is mainly expressed in the proximal tubule, where it is responsible for the breakdown of glycoproteins. Due to its molecular weight of 140 KDa it cannot be

filtered through the glomerular basal membrane. Therefore, its excretion in the urine correlates with increased tubular lysosomal activity and tubular cell injury (Skalova et al. 2005).

NAG is stable in urine and due to its specific localization in **the proximal tubule**, its utility as a biomarker for early tubular damage in a variety of renal disorders has been studied. For example in renal injury or dysfunction due to diabetes mellitus (Kato et al. 1997); after administration of nephrotoxic drugs, such as aminoglycosides (Wiland and Szechcinski 2003), after environmental exposure to nephrotoxicants such as Cd (Satarug et al. 2004), after kidney transplantation (Kontanko et al. 1996); in hypertension (Agirbasli et al. 1996) and after cardiac surgery (Han et al. 2009).

Although NAG meets some of the characteristics of a good biomarker (for early renal damage) there are also some downsides: there are several conditions such as exposure to nephrotoxicants and increased urinary urea that inhibit the NAG urinary isoenzymes in the urine, compromising precise measurement of urinary concentrations of NAG (u-NAG) (Bondiou et al. 1985). Moreover, in a variety of conditions without renal injury such as rheumatoid arthritis (Iqbal et al. 1998) and hyperthyroidism (Tominaga et al. 1989), without clinically significant renal injury, elevated levels of NAG are reported.

1.5.4.4 Cystatin C

Cystatin C, a member of the cystatin superfamily of cysteine protease inhibitors, is a non-glycosylated 13 kDa basic protein. It is produced by all nucleated cells in the body. In normal physiological conditions it is filtered by the glomerulus and virtually completely reabsorbed by the proximal tubule. Therefore it is virtually undetectable in urine of healthy subjects (Uchida and Gotoh 2002).

As Cystatin C is filtered freely by the glomeruli and reabsorbed by the proximal tubule, the serum concentrations of Cystatin C (s-Cystatin C) are useful in the estimation of the GFR. Moreover, in contrast to s-CRT, s-Cystatin C is independent of muscle mass. This has led to the development of estimation equations for GFR, based on s-Cystatin C (analogous to the equations based on s-CRT) (Newman 2002) or even based on a combination of s-Cystatin C and s-CRT levels (Ma et al. 2007). However, it has to be taken into account that despite its independency of muscle mass, the production of Cystatin C is

dependent on physiological determinants and hormonal, humeral or anthropometric factors (Séronie-Vivien et al. 2008).

Besides the use of s-Cystatin C as a biomarker for **glomerular function**, urinary Cystatin C (u-Cystatin C) is believed to be a good estimate of **proximal tubular damage**. Proximal tubule damage will lead to an impaired reabsorption of Cystatin C, causing a rise in u-Cystatin C levels (Conti et al. 2006). The non-invasive measurement of u-Cystatin C will therefore inform clinicians on the proximal tubular state of the patient.

1.6 The aim of this work

To further validate the use of KIM-1, in combination with NGAL, NAG and Cystatin C, as a biomarker for early kidney damage, the first goal of this study is to investigate the optimal collection, sample handling and storage conditions for the measurement of these biomarkers in urine. Furthermore we want to establish reference values for these urinary biomarkers in a healthy population, taking into account possible effects of age and gender. The next part of this study will focus on the possible association of urinary KIM-1 (u-KIM-1) with environmental long time, low-dose Cd exposure in humans. Finally, in a multicenter prospective setting, we will investigate the predictive value of pre- and postoperative u-KIM-1, u-NGAL, u-Cystatin C and s-Cystatin C (both individually and combined) as biomarkers for postoperative AKI after cardiac surgery involving CPB in an adult population.

2 Effect of pH on the stability of kidney injury molecule 1 (KIM-1) and on the accuracy of its measurement in human urine.

This chapter is based on:

Effect of pH on the stability of kidney injury molecule 1 (KIM-1) and on the accuracy of its measurement in human urine.
Pennemans, V., De Winter, L.M., Faes, C., Van Kerkhove, E., Reynders, C., Rigo, J.M., Swennen, Q., Penders, J.
Clin Chim Acta, 2010. 411: p. 2083 – 6.

2.1 Abstract

U-KIM-1 is a novel biomarker for tubular kidney damage, however little is known about its stability. The goal of this study is to examine the effect of urinary pH on the stability of u-KIM-1. Urine samples were collected from 45 volunteers. Samples were aliquoted, adapted to different pH values (range 4 to 9) and stored at −80°C. After thawing, each aliquot was divided into two, of which one was used to measure u-KIM-1 (human tim-1/kim-1/Havcr Elisa kit; R&D systems) at the same pH at which it was stored, while the other was readapted to pH 7 before measurement. U-KIM-1 values of aliquots of the same sample are stable when stored at pH 6, 7 and 8 whereas at lower and higher storage pH, u-KIM-1 levels decrease significantly. When samples are readjusted to a neutral pH just before u-KIM-1 measurement, there are no longer significant differences between u-KIM-1 in aliquots stored at different pH values. No effect of urinary pH on the stability of u-KIM-1 was seen. However, at the time the experiments were performed, the only commercially available human tim-1/kim-1/Havcr Elisa kit of RD systems is pH dependent and we therefore suggest samples should be adjusted to neutral pH before measurement.

2.2 Introduction

In 1998 Ichimura et al. discovered KIM-1 as a type 1 transmembrane glycoprotein. It can be found in renal tubular epithelial cells in ischemic rat kidneys (Ichimura et al. 1998). In healthy kidney tissue KIM-1 is virtually undetectable, while it is highly upregulated in tubular epithelial cells that are being damaged by ischemic (Ichimura et al. 1998) and toxic (Ichimura et al. 2004) insults. The ectodomain contains a six cysteine immunoglobulin-like domain and a mucin domain. When upregulated, the ectodomain is cleaved and released into the urine (Han et al. 2002).

As u-KIM-1 is hardly found in urine of healthy people, its quantification can be used as a biomarker for renal tubular damage. The upregulation of KIM-1, which is suggested to be an epithelial cell adhesion molecule, is associated with renal tubular cell dedifferentiation and proliferation (Ichimura et al. 1998). In kidney tissue and/or in urine of rodents, KIM-1 upregulations were detected after ischemia-reperfusion (Park et al. 2003; Zahedi et al. 2003; Kieran et al. 2003; Vaidya et al. 2006), toxic injury caused by Cd (prozialeck et al. 2007b, 2009a,

2009b), cisplatin, S-(1,1,2,2-tertrafluoroethyl)-1-cysteine and folic acid (Ichimura et al. 2004), vancomycin (Dieterich et al. 2009), gentamycin (Amin et al. 2004; Sieber et al. 2009), Hg and chromium (Zhou et al. 2008), in polycystic kidney disease (Kuehn et al. 2002), in proteinoverload nephropathy (van Timmeren et al. 2006), RAS-mediated renal damage (de borst et al. 2007), in 5/6 nephrectomized (Horiba et al. 2004) and brain dead (Schuurs et al. 2004) rats. In humans, upregulation of KIM-1 is found in patients with renal cell carcinoma (Han et al. 2005), urate nephropathy (Nepal et al. 2008), in AKI (Vaidya et al. 2008a) and chronic tubular injury (Han et al. 2002, Reeves et al. 2008, van Timmmeren et al. 2007a), in allograft nephropathy (van Timmeren et al. 2007b; Zhang et al. 2008) and in AKI after cardiac surgery (Han et al. 2009). To facilitate the detection of u-KIM-1 in human and in rodent urine samples, a sandwich ELISA was developed and adapted to a commercially available microsphere-based Luminex xMAP technology with polyclonal antibodies raised against the human KIM-1 (RD Systems Europe, Abingdon UK). A first assay validation study on the human KIM-1 kit was conducted by Chaturvedi et al. The assay's linearity, intra-run precision, inter-run precision, lower limit of quantification, recovery, dilutional verification, reference range, stability and length of run were validated (Chaturvedi et al. 2009). To our knowledge, however, little is known about the effect of the urinary pH, which can vary widely inter- and intra-individually, on the stability of u-KIM-1. In the present study, we examine the effect of urinary pH on the stability of u-KIM-1 and the accuracy of its measurements in urine samples with normal and elevated u-KIM-1 values using the commercial human KIM-1 kit (RD Systems Europe, Abingdon UK).

2.3 Materials and methods

2.3.1. Study population

For the group with normal u-KIM-1 values (group N) 24 second-morning urine samples of healthy, non-smoking volunteers (12 females and 12 males; mean age 43 yr; range 22 to 87 yr) were collected. For the group with the elevated u-KIM-1 values (group E), 21 urine samples of ICU patients and 65-plus volunteers (10 females and 11 males; mean age 63 yr; range 27 to 85 yr) that had u-KIM-1 concentrations >1000 pg/ml were selected for the study. Written

informed consent was obtained from all participants. The study was approved by the ethics committee of the Ziekenhuis Oost Limburg (ZOL), Belgium.

2.3.2 Urine collection and handling

From each volunteer, a sample of second-morning urine was collected. Immediately after collection, samples were allowed to sediment at room temperature for 30 min. pH values of the samples were measured. Mean pH value was 6.06 with a range of pH 4.90 to 7.75. Aliquots of 600 µl of the supernatant of each sample were adjusted to pH-values of 4, 5, 6, 7, 8 and 9 respectively (covering the physiological range of urine pH) by adding droplets of 1 mol/l sodium hydroxide (NaOH) or 1 mol/l hydrochloric acid (HCl). pH was measured with an Ankersmit 420A pH meter (Orion, Boston, MA, USA). Adjusted samples were stored at −80°C, within maximum 4h after voiding. Samples were kept frozen for a maximum of three weeks. After thawing pH values were checked to assess the pH stability during the freezing episode. One defrosted aliquot per subject was vortexed and divided into two aliquots of 300 µl each. One aliquot was kept at the modified pH, while the other aliquot was readjusted to a neutral pH of 7 (6.8 to 7.2), just before u-KIM-1 measurement.

2.3.3 u-KIM-1 measurements

u-KIM-1 concentrations were determined using the human tim-1/ kim-1/Havcr Elisa kit (RD systems Europe, Abingdon, UK). The assay procedure was executed according to the prescriptions of the manufacturer. Briefly, the day before measurement, the 96-well microplate was coated with PBS diluted Capture Antibodies (100 µl per well). After overnight incubation, the plate was washed three times with 300 µl wash buffer. This was followed by blocking the wells with 300 µl reagent diluent during one hour at room temperature. After a second washing cycle (3×), 100 µl of the samples and standard solutions were added to the wells for a 2h incubation. The following steps, all preceded by another washing cycle (3x), consisted of the addition of 100 µl detection antibody (incubation time of 2h), the addition of 100 µl streptavidin-HRP (horseradish peroxidase) (incubation time of 20 min, protected from light), the addition of 100 µl substrate solution (incubation time of 20 min, protected from light) and the addition of 50 µl stop solution. Immediately after the addition of the stop

solution, the optical density of the wells was determined using a BMG LABTECH FLUOstar OPTIMA plate reader (Isogen life sciences, De Meern, The Netherlands) set to 450 nm with a wavelength correction set to 540 nm. All solutions were delivered by RD systems Europe (Abingdon, UK). Each sample was assayed once after thawing and all measurements were performed in duplicate. During every incubation period, the plate was covered with an adhesive strip. Mean intra-assay variation of the u-KIM-1 measurements was 5.27% (SD 4.04%), while mean inter-assay variation was 9.49% (SD 6.50%).

2.3.4 Statistical analysis

For the different groups, mean percentage recovery is calculated as the mean of the differences (%) between the u-KIM-1 values at the modified storage pH and the KIM-1 value at storage pH 7. A linear mixed model is used to test whether there is a significant change as compared with the pH 7 value, while accounting for the association in the data. The u-KIM-1 values are log-transformed in order to deal with the skewness in the data. Dummy variables corresponding to the different pH values are used in the model, with the pH 7 value as the baseline value. Data are shown with standard deviation. SAS software was used for the statistical analyses (SAS Institute Inc., Cary, NC, USA).

2.4 Results

2.4.1 Detection of u-KIM-1 when stored and measured at different pH-values

Mean u-KIM-1 value of group N (normal KIM-1 values) stored and measured at pH 7 was 438 pg/ml (SD= 249 pg/ml), while group E (elevated u-KIM-1 values) had a mean u-KIM-1 value of 2659 pg/ml (SD= 1813 pg/ml). In the first part of this study, aliquots of the same sample were stored at different pH values, ranging from pH 4 to 9, and measured at the same pH values as they were stored. Under these conditions, u-KIM-1 values, measured with the human tim-1/kim-1/Havcr Elisa kit, are relatively stable at pH 6, pH 7 and pH 8. Mean percentage recovery for both groups (group N and group E) was more than 90% for pH 6 and 8 compared to pH 7 (**Figure 2.1**; upper panel). However, at pH 5 the measured u-KIM-1 concentration fell below 50% compared to pH 7 ($p<0.001$). A further decline of percent recovery below 10% was observed at pH

4 ($p<0.001$). In group N, at pH 9 the percent recovery was significantly declined to 81% ($p<0.001$). In group E, the percent recovery at pH 9 was 84.7%.

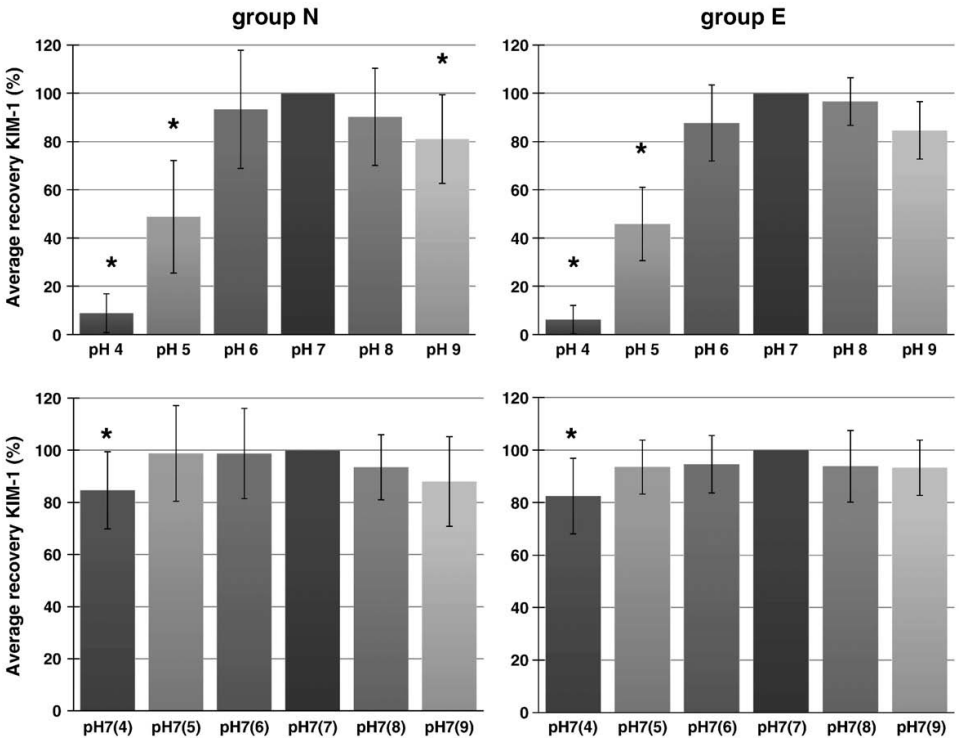


Figure 2.1 (Upper panel) Average percent recovery of u-KIM-1 in aliquots of urinary samples with normal (left panel, n=24 individuals) and elevated (right panel, n=21 individuals) values. The aliquots were measured and stored at different pH values (4, 5, 6, 8 and 9) and compared to u-KIM-1 in aliquots stored and measured at pH 7. (Lower panel) Average percent recovery of u-KIM-1 in aliquots of urinary samples with normal (left panel) and elevated (right panel) values stored at different pH values (4, 5, 6, 8 and 9) but measured at pH 7, compared to u-KIM-1 stored and measured at pH 7. * $p<0.001$.

2.4.2 Detection of u-KIM-1 when stored at different pH values but measured at pH 7

In the second part of the study, the samples that were stored at different pH values (4, 5, 6, 8, and 9) were adapted to pH 7 just before measurement. Under these conditions, there are no significant differences between u-KIM-1 values of aliquots of a sample previously stored at pH 5, 6, 8 and 9 compared to u-KIM-1 values of aliquots stored at pH 7. In both groups, percent recovery at those

storage pH values is above 90%. However, a small though significant ($p<0.001$) decline in percent recovery (85% and 83% in group N and group E respectively) was observed in samples that had a pH value of 4 prior to neutralization to pH 7 before measurement (**Figure 2.1**; lower panel).

2.5 Discussion

KIM-1 is a biomarker for tubular kidney damage that was discovered only about 15 years ago (Ichimura et al. 1998). Recent research mainly focuses on its usefulness as an early biomarker for kidney injury, but little is known about the stability of u-KIM-1 and the collection and measurement requirements. The influence of some collection conditions have already been tested in human urine samples. For u-KIM-1, centrifugation, treatment with protease inhibitor tablets or 5 freeze–thaw cycles did not induce detectable degradation. No significant degradation of u-KIM-1 was observed either when stored at 4°C and at room temperature up to 2 h, or after prolonged storage (up to two years) at −80°C with repeated freeze and thaw cycles (Waikar et al. 2007; Han et al. 2009). In a study of Chaturvedi (Chaturvedi et al. 2009), u-KIM-1 was analytically validated in human urine and the clinical accuracy of the ELISA kit from the R&D duo-set, which is used in this study as well, was validated. More specifically they validated the following parameters: assay linearity, intrarun precision, inter-run precision, lower limit of quantification, recovery, dilutional verification, reference range and length of run. They found that u-KIM-1 in the different samples (n= 6) recovered within 99–114% when subjected to 3 freeze–thaw cycles and when stored at −20°C and −70°C for a maximal period of 14 days (Chaturvedi et al. 2009). In the present study, we evaluated whether the pH of the urine has an effect on the stability of u-KIM-1 measurements using the human tim-1/kim-1/Havcr Elisa kit from R&D. As shown in **Figure 2.1**, we found that the measurement of u-KIM-1 was accurate near neutral pH (6, 7 and 8). However, u-KIM-1 values were underestimated at low (pH 4 and 5) or high pH (pH 9). In the two groups (n= 45) a total of 24 subjects had a pH value lower than 6. This means that over 50% of the u-KIM-1 values would have been underestimated due to pH dependency of the ELISA measurement. We therefore suggest that, when the human tim-1/kim-1/Havcr Elisa kit (R&D systems) is used, urine samples be adapted to a neutral pH before measurement.

To make a distinction between normal and elevated u-KIM-1 values in urine samples, we used a rather arbitrary cut-off score for u-KIM-1 of 1000 pg/ml. Since no information is available in the existing literature on the range of u-KIM-1 values that can be considered healthy or pathological, we based this cut-off score on the values of u-KIM-1 in healthy subjects that were found in the study of Chaturvedi et al. and ranged from 60 – 837 pg/ml (Chaturvedi et al. 2009). In the recently developed dipsticks for the early detection of u-KIM-1, a cut-off score of 800 pg/ml is used (Vaidya et al. 2009). To differentiate between an effect of pH on the stability of u-KIM-1 during storage and an effect of pH on the measurement of u-KIM-1 using the ELISA, we performed two types of measurements. First, we measured the u-KIM-1 values in the aliquots of each sample that were modified to pH 4, 5, 6, 7, 8 and 9. Second, we also appreciated u-KIM-1 values after re-adapting to pH 7 just before the ELISA measurement. Our data clearly demonstrate that pH rather affects the ELISA measurement than u-KIM-1 itself. Percent recovery of aliquots with neutralized pH was above 90%. Except for pH 4, where a significantly lower percent recovery of 83% was observed (**Figure 2.1**, lower panel). This decline can possibly be attributed to slight dilution effects due to the manipulation of the pH of these samples, since higher pH differences have to be overcome than in the other samples. A possible explanation for the pH dependency of the ELISA measurement may be that the activity of HRP in the presence of the substrate 3,3',5,5'-tetra-methylbenzidine is believed to be optimal in the range of pH 6 to 6.5 (Straus 1964). However the fixation itself can also have an influence on this optimal pH (Hundgen 1977). This can explain that a pH of 7 was optimal as was seen in this study.

In conclusion, we can state that there is no effect of the urinary pH value on the stability of u-KIM-1, stored at−80°C for a period of maximum three weeks. However the commercially available human tim-1/kim-1/Havcr Elisa kit using the duo-set reagents from R&D to develop the ELISA is pH dependent. When using this kit, we therefore suggest neutralizing the pH of the human urine samples before KIM-1 measurement.

3 Collection and storage requirements for urinary kidney injury molecule 1 (KIM-1) measurements in humans.

This chapter is based on:

Collection and storage requirements for urinary kidney injury molecule-1 (KIM-1) measurements in humans.
Pennemans, V., Rigo, JM., Penders, J., Swennen, Q.,
Clin Chem Lab Med, 2011. 50(3): p. 539-43.

3.1 Abstract

U-KIM-1 is a recently discovered biomarker for early renal damage. However, little is known about the collection and storage requirements prior to its measurement in human urine. Samples of healthy volunteers were collected and aliquoted. The effect of pre-freezing time, thawing, addition of protease inhibitors, centrifugation, storage time (up to 1.5 years) and temperature (4°C, –20°C and –80°C) was tested. Addition of protease inhibitors and centrifugation prior to freezing did not affect the u-KIM-1 measurements. When samples were kept at room temperature for longer than 3 h before freezing or defrosted more than 1h before measurement, mean u-KIM-1 values differed significantly compared to aliquots with minimal pre-freezing and thawing time. Samples frozen at –80°C were stable for up to 1.5 years; however an increasing number of freeze-thaw cycles adversely affected u-KIM-1 measurements. When stored at 4°C and –20°C, samples were less stable compared to those stored at –80°C. This study recommends that urine samples collected for u-KIM-1 measurements are frozen within 3h after voiding and only be defrosted immediately prior to measurement. Addition of protease inhibitor and centrifugation prior to measurement is not necessary. Samples are preferably stored at –80°C and freeze-thaw cycles should be avoided.

3.2 Introduction

U-KIM-1 is one of the seven novel kidney-damage biomarkers that have been approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) for pre-clinical safety studies of novel drug candidates (FDA 2008; EMEA 2008). KIM-1 is a type I transmembrane glycoprotein that can be found in the proximal tubule of the kidney. Its ectodomain contains a cysteine-rich immunoglobulin-like domain, two N-glycosylation sites and a domain rich in threonine/serine and proline (Waanders et al. 2010) .

In healthy kidney tissue KIM-1 is virtually undetectable, while it is highly up-regulated in tubular epithelial cells that are damaged by ischemia and toxic insults (van Timmeren et al. 2007a, Ichimura et al. 1998). When upregulated, the ectodomain is cleaved by metalloproteinases and shed into the urine, which enables the quantification of KIM-1 levels without any invasive techniques (Waanders et al. 2010) .

In rodents, KIM-1 is up-regulated in the proximal tubule during dedifferentiation of the kidney epithelium, which is an early manifestation in response to damage (Prozialeck et al. 2009b). An upregulation of KIM-1 is found after ischemia-reperfusion (Park et al. 2003; Zahedi et al. 2003; Kieran et al. 2003; Vaidya et al. 2006), toxic injury caused by Cd (Prozialeck et al. 2007b, 2009a, 2009b), cisplatin, S-(1,1,2,2-tertrafluoroethyl)-1-cysteine and folic acid, vancomycin (Dieterich et al. 2009), gentamycin (Amin et al. 2004, Sieber et al. 2009), Hg and chromium (Zhou et al. 2008), in polycystic kidney disease (Kuehn et al. 2002), in protein-overload nephropathy (van Timmeren et al. 2006), RAS-mediated renal damage (de Borst et al. 2007), in 5/6 nephrectomized (Horiba et al. 2004) and brain dead (Schuurs et al. 2004) rats.

In humans, up-regulation of KIM-1, which is suggested to be an epithelial cell adhesion molecule, is found in patients with renal cell carcinoma (Han et al. 2005), urate nephropathy (Nepal et al. 2008), in AKI (Vaidya et al. 2008a) and chronic tubular injury (van Timmeren et al. 2006, Han et al. 2008, Reeves et al. 2008), in allograft nephropathy (van Timmeren et al. 2007b, Zhang et al. 2008) and in AKI after cardiac surgery (Han et al. 2009).

In order to define collection and storage requirements to optimize u-KIM-1 measurements, in this study the stability of u-KIM-1 of healthy men and women was tested with various collection and storage conditions. We investigated possible influences of freezing time, protease inhibitors, centrifugation, thawing time and storage temperature and duration.

3.3 Materials and methods

3.3.1 Sample collection

3.3.1.1 Collection requirements

Second morning urine samples were obtained from 20 non-smoking healthy men (n= 9) and women (n= 11). Written informed consent was obtained from all participants and the study conforms to the provisions of the Declaration of Helsinki. Mean age of the participants was 26.7 years (range 21 – 34 years). pH of each sample was measured with an Ankersmit 420A pH meter (Orion, Boston, MA, USA) and, if necessary, adapted to a value between 6 and 8 by adding droplets of 1 mol/L NaOH or 1 mol/L HCl (Sigma-Aldrich Chemie Gmbh, Munich, Germany) (Pennemans et al. 2010). Samples were then divided into 13 aliquots

of 1 mL. Five aliquots were frozen at –80°C immediately without further treatment. In one aliquot, protease inhibitor was added (Complete mini, Roche Diagnostics, Mannheim, Germany) to investigate the possible effect of serine, cysteine and metalloproteases on the stability of u-KIM-1 in stored urine samples. One aliquot was set 1h to sediment at room temperature and 1 mL of the supernatant was frozen at –80°C. One aliquot was centrifuged at 2554 g for 5 min after which 1 mL of the supernatant was frozen at –80°C. Four aliquots were kept at room temperature before freezing for 1, 3, 24 and 48h, respectively. All samples were kept frozen at –80°C for a maximum of 3 weeks after collection. From the five aliquots that were frozen at –80°C immediately after voiding, one aliquot was thawed 24h before measurement, one aliquot 3h before measurement and one aliquot 1h before measurement. After thawing, samples were kept at room temperature until analytical testing was performed.

3.3.1.2 Storage requirements (short-term)

Second morning urine samples were obtained from 30 non-smoking healthy male (n= 7) and female (n= 23) volunteers. Written consent was obtained from all participants or from participants' parents if the participant was under 18 years old. The average age of the volunteers was 35.7 years, ranging from 3 to 60 years. If necessary, the pH of the urine samples were adapted to a value between 6 and 8 by adding droplets of 1 mol/L NaOH or 1 mol/L HCl (Pennemans et al. 2010). Samples were then divided into eight aliquots. One aliquot was used to measure the u-KIM-1 values immediately within a maximum of 4 h after voiding. A second aliquot was used to measure urinary CRT (u-CRT) values, using an automated analyzer (Modular ® P800-ISE900 System, Roche Diagnostics, Mannheim, Germany), according to the kinetic Jaffe method (compensated, rate blanked). From the remaining six aliquots, two aliquots each were stored at 4°C, –20°C and –80°C within a maximum of 4h after voiding. One aliquot per storage temperature was used to measure u-KIM-1 values after 24h, 1 week, 3 weeks, 7 weeks and 12 weeks of storage. After each measurement the aliquot was stored again at 4°C, –20°C and –80°C, respectively, resulting in a total of five freeze-thaw cycles in the –20°C and –80°C condition. The second aliquot, stored at –20°C and at –80°C, was measured after 12 weeks of storage without previous freeze-thaw cycles.

3.3.1.3 Storage requirements (long-term)

One hundred and twenty-six samples were used, which had been collected within the framework of an environmental study conducted in autumn 2009 [for details see Pennemans et al. (2001). They were frozen at –80°C within 4h after voiding. If necessary, pH was adapted to neutral values prior to freezing. U-KIM-1 was measured in these samples the first time within 2 months after collection. A second u-KIM-1 measurement was conducted after a freezing period of 19 – 22 months at –80°C.

3.3.2 u-KIM-1 measurements

u-KIM-1 concentrations were determined using the human tim-1/kim-1/Havcr ELISA kit (RD systems Europe, Abingdon, UK). The assay procedure was executed according to the prescriptions of the manufacturer. Briefly, the day before measurement, the 96-well microplate was coated with PBS diluted Capture Antibodies (100 µL per well). After overnight incubation, the plate was washed three times with 300 µL wash buffer. This was followed by blocking the wells with 300 µL reagent diluent during 1h at room temperature. After a second washing cycle (3×), 100 µL of the samples and standard solutions were added to the wells for a 2h incubation. The following steps, all preceded by another washing cycle (3×), consisted of the addition of 100 µL detection antibody (incubation time of 2 h), the addition of 100 µL streptavidin-HRP (incubation time of 20 min, protected from light), the addition of 100 µL substrate solution (incubation time of 20 min, protected from light) and the addition of 50 µL stop solution. Immediately after the addition of the stop solution, the absorbance of the wells was determined using a BMG LABTECH FLUOstar OPTIMA plate reader (Isogen life sciences, De Meern, The Netherlands) set to 450 nm with a wavelength correction set to 540 nm. All solutions were delivered by RD systems Europe. All measurements were performed in duplicate. During every incubation period, the plate was covered with an adhesive strip.

3.3.3 Statistical analysis

Data are given as mean u-KIM-1 values (pg/mL) and standard deviation (SD). To compare the mean u-KIM-1 values of the different collection and storage requirements, mean percentage differences (%) are calculated as the mean of

the individual differences (given as %) between the matched samples of different collection and storage conditions.

Normal distribution of the data is tested by means of the D' Agostino and Pearson omnibus normality test. Since data were not normally distributed, mean values of different collection and/or storage conditions are compared by means of the Wilcoxon matched pairs test. Two-tailed p-values < 0.05 were considered statistically significant. Statistical analyses were performed using Graphpad Prism 5 (GraphPad Software, La Jolla, CA, USA).

3.4 Results

3.4.1 Collection requirements

3.4.1.1 Sample handling

Mean u-KIM-1 value of the samples that did not undergo any treatment prior to freezing or measurement was 440 pg/mL (SD= 295.4 pg/mL). The addition of protease inhibitor did not affect the u-KIM-1 measurements. Furthermore, there was no significant difference between the samples that were centrifuged, the samples that were set for 1h to sediment and the samples that did not undergo any treatment to remove sediment.

3.4.1.2 Time before freezing

Figure 3.1 shows the effect of the time at room temperature before freezing on the u-KIM-1 values. When comparing the samples that were frozen after 1h and after 3h, no statistically significant difference was observed with samples that were frozen immediately after collection. However, when samples were kept at room temperature for 24h before freezing, mean % recovery of u-KIM-1 was 122.3% (p= 0.007). This mean % recovery became even higher (134.7%) when samples were kept at room temperature for 48h before freezing. When conducting a Wilcoxon paired test, the latter two conditions show a statistically significant difference with samples that were frozen immediately (p= 0.007 and p= 0.0002, respectively).

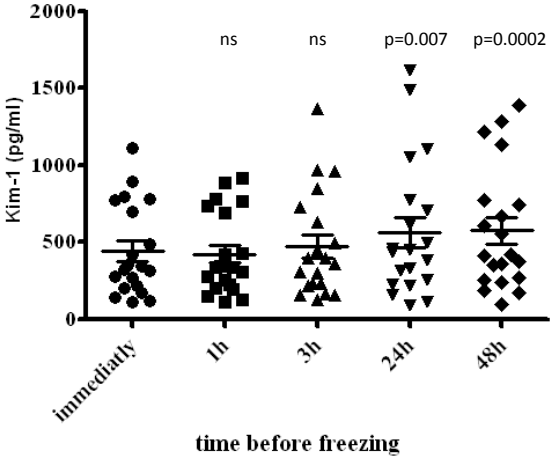


Figure 3.1 Effect of time at room temperature before freezing. Comparison of u-KIM-1 values (n= 20) measured in matched samples that were frozen (–80°C) immediately, 1h, 3h, 24h and 48h after voiding. The p-values were calculated using the Wilcoxon matched pairs test. Horizontal bars represent mean values with 95% confidence intervals. Abbreviations: Kim-1: kidney injury molecule 1; ns: not signifant)

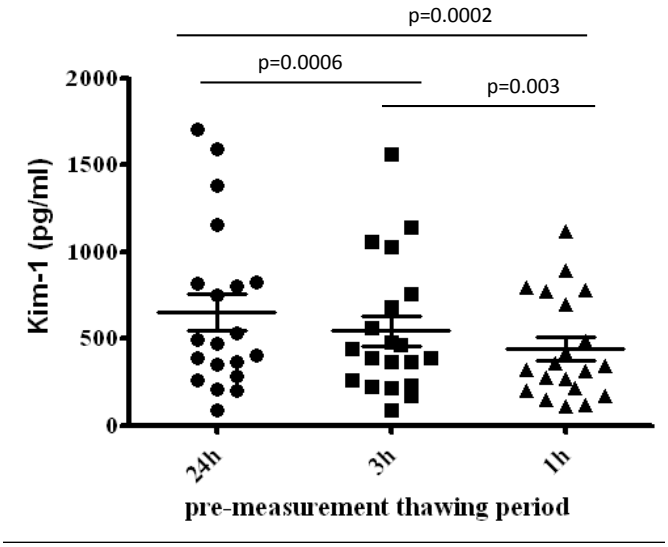


Figure 3.2 Effect of pre-measurement thawing period at room temperature. Comparison of u-KIM-1 values (n= 20) measured in matched samples that were thawed 24h, 3h and 1h prior to measurement, after storage at – 80°C. The p-values were calculated using the Wilcoxon matched pairs test. Horizontal bars represent mean values with 95% confidence intervals.

3.4.1.3 Thawing period

As shown in **Figure 3.2** the duration between thawing and the u-KIM-1 measurement has an effect on the u-KIM-1 values measured. Both the u-KIM-1 values at the 3h thawing period ($p < 0.01$) as the ones at the 24h thawing period ($p < 0.01$) differ significantly of the u-KIM-1 values measured in samples that were thawed 1h before measurement. Indeed, when samples were thawed 24h before measurement, an average % recovery of u-KIM-1 of 152% (SD= 60%) was seen and after a thawing period of 3h before measurement this % recovery was 125% (SD= 31%) compared to samples that were thawed 1h before measurement.

3.4.2 Storage requirements

3.4.2.1 Short-term storage

Urine samples were measured freshly and subsequently aliquots were stored at 4°C, –20°C and –80°C and measured after 24h, 1 week, 3 weeks, 7 weeks and 12 weeks. At 12 weeks, aliquots, which had not been thawed before, were measured as well. Mean u-KIM-1 values of freshly measured aliquots was 834.2 pg/mL (SD= 637.6 pg/mL). Mean percentage difference and 95% confidence intervals are shown in **Table 3.1**. Data show a degradation of mean u-KIM-1 values of about 10% per freeze-thaw cycle, with slightly bigger declines when stored at –20°C. When measured after 12 weeks storage at –20°C without previous thawing, percentage recovery is 78.58% (SD= 23.31%); when stored for 12 weeks at –80°C without previous thawing, percentage recovery is 96.52% (SD= 26.09%). CRT levels were not significantly correlated with the % recovery of the different storage conditions (data not shown).

3.4.2.2 Long-term storage

The mean u-KIM-1 values that was measured within 1 month after collection was 694.8 pg/mL. When measured again at 1.5 years after collection, stored at -80°C, mean u-KIM-1 value was 696.9 pg/mL. Mean difference was 2.2 pg/mL with a SD of 332.7 pg/mL, which was not significant (p= 0.512).

Table 3.1 Stability of u-KIM-1 at different storage conditions.

storage time (freeze-thaw cyles)	Mean percentage difference (95% confidence interval of mean % difference) when compared to freshly measured samples		
	4°C	-20°C	-80°C
24 hours (1x)	89 (83 – 96)	80 (70 - 90)	86 (79 – 92)
1 week (2x)	99 (90 – 108)	72 (60 – 84)	68 (61 – 76)
3 weeks (3x)	131 (120 – 143)	58 (51 – 65)	72 (62 – 81)
7 weeks (4x)	106 (91 – 120)	48 (40 – 58)	72 (63 – 81)
12 weeks (5x)	127 (105 – 150)	69 (63 – 77)	78 (70 - 87)
12 weeks without freeze-thaw	-	79 (70 – 88)	97 (87 – 106)

Data (n=30) are given as mean percentage difference (95% confidence interval of mean % difference) when compared to freshly measured samples. Mean percentage difference is calculated as the mean of the individual differences (given in %, with freshly measured u-KIM-1 values set at 100%) of the matched samples of the different storage conditions.

3.5 Discussion

In the current study we evaluated different collection, handling and storage conditions prior to u-KIM-1 measurements. In order to further validate u-KIM-1 as a biomarker for AKI it is critical to understand the effect of those different conditions. We found no effect of addition of protease inhibitors and sedimentation or centrifugation of samples prior to freezing. The time between collection and freezing did affect the u-KIM-1 measurements. When samples were frozen at 1h and at 3h no effect was seen on u-KIM-1 measurements, when compared to immediate storage at –80°C after collection. However, when samples were kept at room temperature for 24h and for 48h before freezing, a mean % recovery of 122.3% and 134.7% , respectively, was observed, indicating an over-estimation of u-KIM-1 values. No effect of u-CRT levels on the % recovery was observed in these groups.

In 2009, Han et al. (Han et al. 2009) conducted a similar study on a small group (n= 6) of AKI patients and observed no significant degradation of u-KIM-1 values up to 24h at 4°C and at room temperature. When the thawing period prior to measurement is prolonged, a similar rise in % recovery, i.e. over-estimation of u-KIM-1, is observed. When thawed 3h before measurement % recovery is 125% (p= 0.003) compared to thawing 1h prior to measurement. When thawing period takes 24h, a % recovery of 152% (p= 0.0002) is observed. Different storage duration and temperature conditions were tested as well. As shown in **Table 3.1**, each freeze-thaw cycle (both at –20°C and at -80°C storage temperature) causes a decline in mean u-KIM-1 values of about 10%. When stored for 12 weeks without freeze-thaw cycles prior to measurement, the % recovery of u-KIM-1 is 78.58% (SD= 23.31) and 96.52% (SD= 26.09) at –20°C and –80°C, respectively. When u-KIM-1 was measured in aliquots of urine samples which were stored for 1.5 years at –80°C and compared to aliquots that were measured within 1 month after voiding, no significant difference in mean u-KIM-1 values was found.

In a previous study of Han et al. (Han et al. 2009) no degradation was observed in six urine samples of AKI patients that were stored for 1 year with three repeat freeze-thaw cycles. Differences in these outcomes may be due to different patient groups (AKI patients vs. healthy subjects), to the sample size (6 vs. 30)

or to methodological differences. In a comparable sample size (n= 6) Chaturvedi et al. (Chaturvedi et al. 2009) did not find any significant degradation of u-KIM-1 either when stored up to 7 days at room temperature, at –20°C or at –80°C, nor after three repeated freeze-thaw cycles. When considering an acceptable inter-assay variation of 15%, it can be concluded that samples should be stored at – 80°C. **Table 3.1** shows that long-term storage does not cause degradation of u-KIM-1 in urinary samples; however, freeze-thaw cycles need to be avoided. U-KIM-1 values in samples that are stored at 4°C are rather stable up to 1 week. However, when stored for a longer period, u-KIM-1 values start to rise significantly.

As studied previously, u-KIM-1 is stable in urine at different pH values (Pennemans et al. 2010). However, adaptation of the pH to values between 6 and 8 is necessary due to the pH dependency of the ELISA kit used to measure u-KIM-1 values. In the urine samples that are used for the current study, adaptation was necessary in 39% of the samples.

3.6 Conclusions

In conclusion we suggest that urine samples collected for the purpose of u-KIM-1 measurements are measured freshly or frozen at –80°C within maximum 4h after voiding. Addition of protease inhibitors or centrifugation is not necessary. When frozen at –80°C it is recommended to restrict freeze-thaw cycles to a minimum since a decline of 10% in u-KIM-1 values is observed per freeze-thaw cycle. Samples can be stored at –80°C for at least 1.5 years without any decrease in u-KIM-1.

4 Establishment of reference values for novel urinary biomarkers for renal damage in the healthy population: are age and gender an issue?

This chapter is based on:

Establishment of reference values for novel urinary biomarkers for renal damage in the healthy population: are age and gender an issue?
Pennemans, V., Rigo, JM., Faes, C., Reynders, C., Penders, J., Swennen, Q.
Clin Chem Lab Med., 2013. 51(9): p. 1795-802.

4.1 Abstract

Recently, a lot of research has focused on the discovery of novel renal biomarkers. Among others, u-KIM-1 and u-NGAL have been proven to be promising biomarkers in a wide variety of renal pathologies. However, little is known about the normal concentrations in urine of healthy subjects. Therefore, the goal of our study is to establish reference values for u-KIM-1, u-NGAL, u-NAG and u-cystatin C in a healthy population, taking into account possible effects of age and gender. We collected urine samples from 338 healthy, nonsmoking subjects between 0 and 95 years old. Subjects with elevated α-1-microglobulin (A1M-U) values were excluded. Next to the urinary concentrations of KIM-1, NGAL, NAG, and cystatin C, we measured u-CRT and urinary specific gravity (USG) to correct for urinary dilution. The possible effect of age and gender on the four urinary biomarkers was investigated, and reference values were established. For the absolute urinary concentrations of the biomarkers, age had a significant effect on all the biomarkers, except for cystatin C, whereas gender significantly affected all four of them, except for NAG. The normalization of biomarkers for u-CRT and USG had an effect on the correlation between the biomarkers on one hand and age and gender on the other. In conclusion, age and gender had different effects on u-KIM-1, u-NGAL, u-NAG, and u-cystatin C. Based on this knowledge, age- and gender-specific reference values for u-KIM-1, u-NGAL, u-NAG, and u-cystatin C were established.

4.2 Introduction

Renal biomarkers do not only play an emerging role in the detection of acute drug-induced kidney toxicity in nonclinical drug development but are also a crucial tool for the detection of possible renal damage in clinical settings, such as admittance at ICU after major surgery. Although s-CRT, which is a measure for the GFR, is the most widely used parameter to assess renal function, some major shortcomings concerning specificity and sensitivity fed the urge to search for more accurate tools to assess renal function in different settings (Tomlanovich et al. 1986; Parikh and Devarajan 2008).

Recent proteomic studies led to the discovery of several novel biomarkers. KIM-1, for example, was discovered by Ichimura et al. in 1998 (Ichimura et al. 1998). They reported that this putative epithelial cell adhesion molecule,

containing a novel immunoglobulin domain, is upregulated in the renal proximal tubule cells after injury (Ichimura et al. 1998). Subsequent research has not only investigated the utility of KIM-1 in the diagnosis and prognosis of AKI (Huang and Don-Wauchope 2011) but also demonstrated that KIM-1 was upregulated after the administration of a broad array of nephrotoxicants in rats (Waanders et al. 2010; Prozialeck 2009b). When KIM-1 is upregulated in the kidney due to ischemic or toxic insults, the ectodomain is cleaved in the urine (Han et al. 2002), which makes it possible to measure KIM-1 in a noninvasive way. In 1993, Kjeldsen et al. (Kjeldsen et al. 1993) isolated NGAL. NGAL is a 25-kDa lipocalin, bound to gelatinase in specific neutrophil granules and can be found in a broad range of healthy and pathologic tissues (Gagneux-Brunon et al. 2012). In case of renal ischemia, NGAL is upregulated in the distal parts of the damaged tubule (Paragas et al. 2011), and it has been proven to be a promising biomarker for AKI in a broad range of patient populations (Mishra et al. 2005; Bennett et al. 2008; Makris et al. 2009; Tuladhar et al. 2009; Chakraboty et al. 2012), which can be especially useful for the very early assessment of AKI in the ICU and emergency department (Clerico et al. 2012).

Despite the promising role of these novel urinary biomarkers in the early detection of AKI, little is known about the normal values of these biomarkers in healthy persons. To our knowledge, only Cullen et al. (Cullen et al. 2012) have established a 95% cutoff for u-NGAL in a healthy population of 174 men and women aged between 19 and 88 years (Cullen et al. 2012), and very recently, Cangemi et al. (Cangemi et al. 2013) reported reference values for u-NGAL in a pediatric population.

The goal of our study is therefore to investigate the normal values of u-KIM-1 and u-NGAL, together with two other renal biomarkers [u-cystatin C and u-NAG] in a large healthy population ranging between 0 and 95 years old. We will investigate the possible influences of age and gender and establish reference values for the absolute urinary concentrations as well as for the urinary concentrations, normalized either to u-CRT or USG, to take into account the urinary dilution.

4.3 Materials and methods

Between March 2011 and February 2012, a total of 338 urine samples were randomly collected from nonsmoking healthy volunteers (199 women and 139 men). Healthy subjects were selected based on their medical history (only subjects with no previous episodes of renal problems were selected) and on their current A1M-U levels. Inclusion of subjects occurred only when A1M-U levels were below 12 mg/L (the cutoff score used in the clinic as a reference value) to exclude patients with chronic kindey disease (CKD). In total, 12% of the tested persons were excluded. Subjects were between 0 and 95 years, with an average of 45.8 years. A stratified sampling design was used, assuming that every age stratum (0 – 10, 11 – 20 years old, and so forth) contains at least 30 samples. A written consent was obtained from all participants (or the participants' parents in case of underaged participants). The study conforms to the provisions of the Declaration of Helsinki.

The pH of each sample was measured upon voiding with an Ankersmit 420A pH meter (Orion, Boston, MA, USA) and, if necessary, adapted to a value between 6 and 8 by adding droplets of 1 mol/L NaOH or 1 mol/L HCl (Sigma- Aldrich Chemie GmbH, Munich, Germany) (Pennemans et al. 2010). Samples were then left for sedimentation (without the addition of protease inhibitors) for a period of 30 min and were then aliquoted and stored at −80°C within 4h after collection. Aliquots were kept at −80°C for a maximum duration of 6 months before measurement, without freeze-thaw cycles.

U-KIM-1 and u-NGAL concentrations were determined using the human TIM-1/KIM-1/Havcr ELISA kit and the NGAL kit, respectively (RD Systems Europe, Abingdon, UK). The assay procedure was executed according to the prescriptions of the manufacturer. Briefly, the day before measurement, the 96-well microplate was coated with PBS-diluted Capture antibodies (100 µL per well). After overnight incubation, the plate was washed three times with 300 µL of wash buffer. This was followed by blocking the wells with a 300 µL reagent diluent for 1h at room temperature. After a second washing cycle (3×), 100 µL of the samples and standard solutions were added to the wells for a 2h incubation. The following steps, all preceded by another washing cycle (3×), consisted of the addition of 100 µL of detection antibody (incubation time, 2h),

100 µL of streptavidin-HRP (incubation time, 20 min, protected from light), 100 µL of substrate solution (incubation time, 20 min, protected from light), and 50 µL of stop solution. Immediately after the addition of the stop solution, the absorbance of the wells was determined using a BMG LABTECH FLUOstar OPTIMA plate reader (Isogen Life Sciences, De Meern, The Netherlands) set to 450 nm, with a wavelength correction set to 540 nm. All solutions were delivered by RD Systems Europe. All measurements were performed in duplicate. During every incubation period, the plate was covered with an adhesive strip.

U-cystatin C concentrations were determined by means of the sandwich ELISA method using the Cystatin C human ELISA kit (Biovendor R&D Products, Heidelberg, Germany). As recommended by the manufacturer, urine samples were diluted 20× before measurement. 100 µL of diluted standards, quality controls, dilution buffer (= blank), and samples were pipetted in duplicate on the antibody-coated 96-well plate. The plate was incubated 30 min at room temperature while shaking at 300 rpm on an orbital microplate shaker. After a threefold washing cycle and addition of 100 µL of conjugate solution, the plate was again incubated at room temperature for 30 min, shaking at 300 rpm on an orbital microplate shaker. The following step was again preceded by a threefold washing step consisting of the addition of 100 µL substrate solution (incubation time of 10 min, covered with aluminum foil to prevent exposure to direct sunlight). To stop the color development, 100 µL of the stop solution was added, and absorbance was determined using the BMG LABTECH FLUOstar OPTIMA plate reader (Isogen Life Sciences, De Meern, The Netherlands) set to 450 nm, with the reference wavelength set to 630 nm.

The u-NAG was measured colorimetrically using the NAG assay kit (BQ Kits, San Diego, CA, USA). 50 µL of blank, calibrator, and samples was pipetted in duplicate on a 96-well plate. 75 µL of the reagent solution mix (a 5:1 MNP-G1c-NAc/HCl and citric acid/potassium phosphate solution) was added to the wells. The plate was incubated for 5 min at 37°C, after which 250 µL of Na carbonate buffer was added. Absorbance reading at 505 nm ware conducted with the BMG LABTECH FLUOstar OPTIMA plate reader set at 505 nm, and the Δ-OD was determined by subtracting the blank value.

U-CRT values and A1M-U levels were measured using an automated analyzer (Modular ® P800-ISE900 System; Roche Diagnostics, Mannheim, Germany), according to the kinetic Jaffe method (compensated, rate blanked) and immunological agglutination, respectively. USG was tested by refractometry using a urinalysis system (Urisys 2400; Roche Diagnostics, Germany). The system analyzes the USG in a flow cell filled with urine sample during the aspiration of the sample.

Statistical analysis starts with a normalization of the response variable through Box-Cox transformation whenever a deviation of normality is seen. Multiple linear and quadratic regression analyses were conducted to study the possible association among age, gender and the renal biomarkers. A stepwise selection criterion was applied to select the best fitting model to the response value. To avoid bias through differences in urine concentration, the possible effect of age and gender on the biomarkers was analyzed both before and after correcting for u-CRT or USG. The mean values of the four biomarkers were calculated for the different age/gender groups. To establish reference values, the lower and upper values of the 95% prediction intervals of the Box-Cox-transformed variables were calculated. Note that a prediction interval for a new individual resembles a confidence interval (CI), but it is wider to ensure that the prediction error is taken into account. A back-transformation is then used to calculate the prediction interval at the level of the original scale of the variables. In case of a significant correlation between the biomarker and age/gender, the reference values are calculated for the different age and gender groups.

4.4 Results

The frequency distribution for the different biomarkers (both absolute urinary concentrations and the concentrations normalized for u-CRT and USG) for the reference population (n= 338) are given in **Figures 4.1 – 4.3**, respectively.

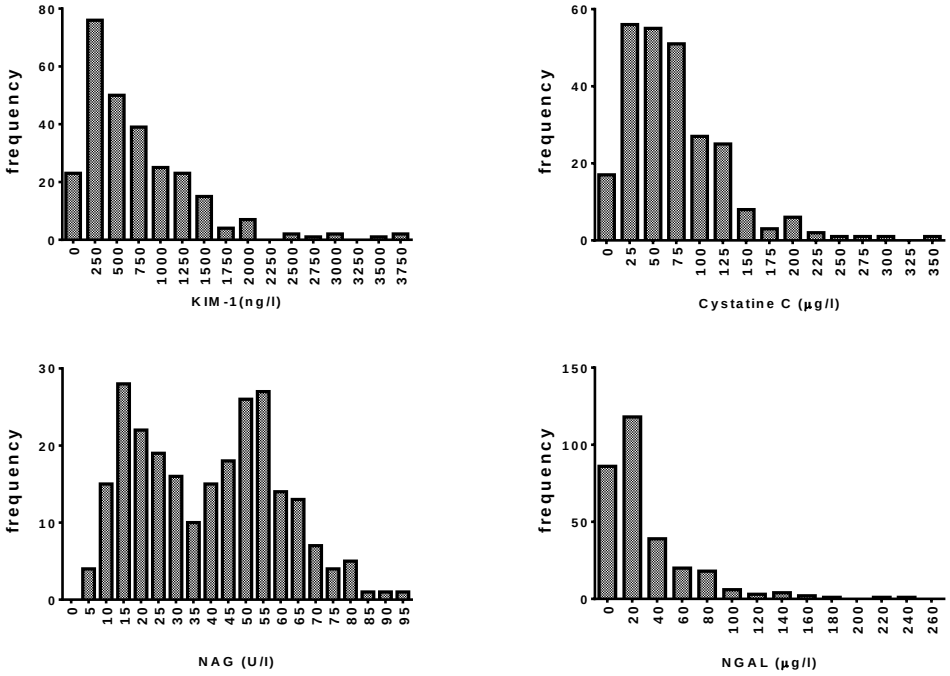


Figure 4.1 Frequency distributions of the absolute urinary concentrations of KIM-1, cystatin C, NAG, and NGAL for the entire study population (n= 338), ranging in age from 0 to 95 years.

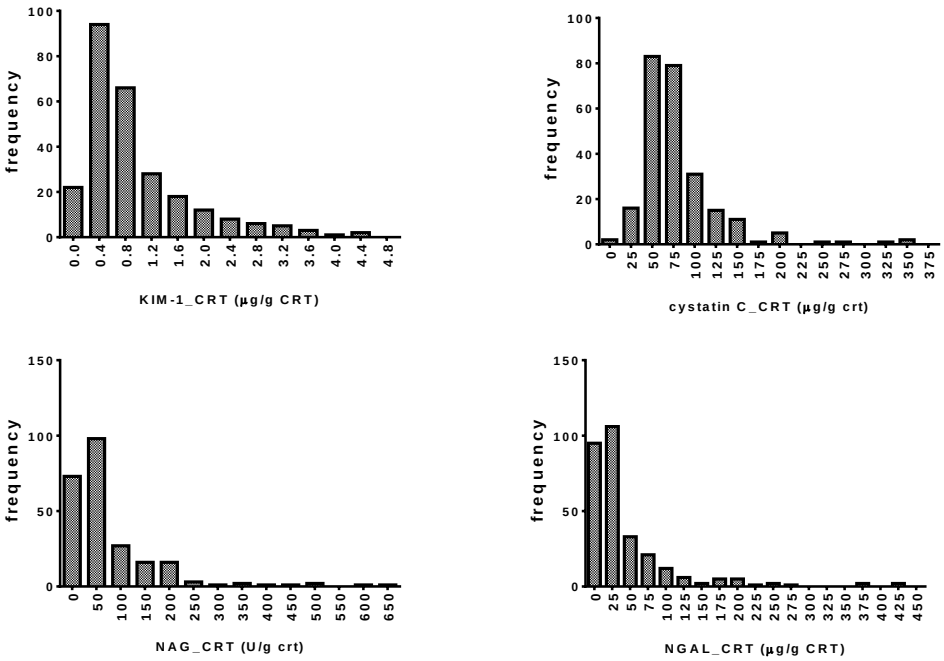


Figure 4.2 Frequency distributions of urinary concentrations of KIM-1, cystatin C, NAG, and NGAL, normalized to u-CRT, for the entire study population (n= 338), ranging in age from 0 to 95 years.

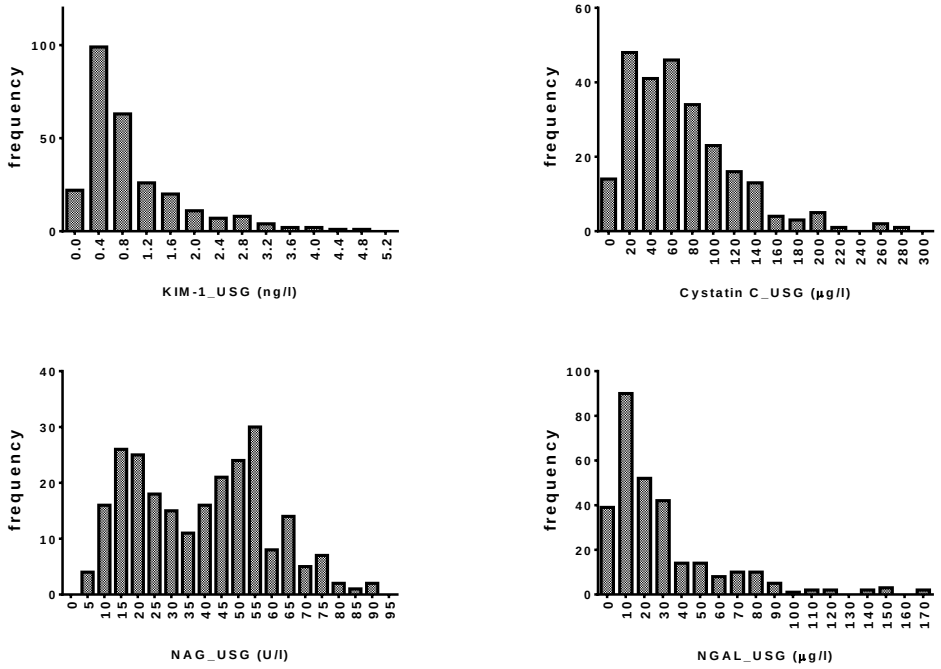


Figure 4.3 Frequency distributions of urinary concentrations of KIM-1, cystatin C, NAG, and NGAL, normalized to USG, for the entire study population (n= 338), ranging in age from 0 to 95 years.

Table 4.1 shows an overview of the gender and age effects on the different biomarkers, both for absolute concentrations and for concentrations corrected for u-CRT and USG. For the absolute urinary values, a significant positive linear association was observed between u-KIM-1 and both age ($p < 0.0001$) and gender ($p = 0.02$). However, when normalized for u-CRT and USG, a quadratic trend in age ($p < 0.0001$) is observed, which is due to the quadratic relationship between u-CRT and age. No significant effect of gender is observed for the normalized concentrations. For u-cystatin C, there was only a significant linear association with sex ($p = 0.01$). When corrected for u-CRT, the correlation with age became significantly quadratic again ($p < 0.0001$). When corrected for USG, however, only a significant linear correlation with gender is present ($p = 0.01$). u-NAG shows a significant linear trend in age for both the absolute concentration

(p= 0.003) and the values normalized for USG (p= 0.001) and no correlation with sex. The age effect became quadratic when normalized for u-CRT (p< 0.0001), and a significant linear correlation with gender became apparent (p= 0.02). Finally, there is a significant gender effect on u-NGAL (p< 0.0001) as well as a significant interaction between age and gender (p= 0.002), indicating that the age effect on u-NGAL is different in male and female subjects. Age and sex are significantly correlated with u-NGAL when normalized for u-CRT (p< 0.0001); however, when normalized for USG, only age has a significant quadratic effect (p= 0.002).

In Table 4.2, gender- and age-related mean and upper limit reference values for the different biomarkers (both in mass as normalized for u-CRT and USG) are given. The reference values are based on the 95% prediction intervals. If no gender or age effects were observed, the general reference values for that biomarker are calculated.

Table 4.1 Gender and age effects (with p-value) on the different biomarkers (both absolute concentrations and corrected for u-CRT and USG)

	Age effect (p-value)	gender effect (p-value)	interactional effect (p-value)
u-KIM-1	linear (<0.0001)	linear (0.02)	NS
u-KIM-1/CRT	quadratic (<0.0001)	NS	NS
u-KIM-1/USG	quadratic (<0.0001)	NS	NS
u-Cystatin C	NS	linear (0.01)	NS
u-Cystatin C/CRT	quadratic (<0.0001)	NS	NS
u-Cystatin C/USG	NS	linear (0.01)	NS
u-NAG	linear (0.003)	NS	NS
u-NAG/CRT	quadratic (<0.0001)	linear (0.02)	NS
u-NAG/USG	linear (0.001)	NS	NS
u-NGAL	NS	linear (<0.0001)	linear (0.002)
u-NGAL/CRT	quadratic (0.001)	linear (<0.0001)	NS
u-NGAL/USG	quadratic (0.002)	NS	NS

Association (with p-values) between age and gender on the one hand and the different biomarkers (u-KIM-1, u-Cystatin-C, u-NAG and u-NGAL; both absolute urinary values, as values normalized for u-CRT and USG) on the other, are determined using multiple linear and quadratic regression analyses in 338 healthy non-smoking men and women (aged 0-95). A stepwise selection criterium was applied to select the best fitting model to the response value.

Abbreviations: NS (no significant); u-KIM-1 (urinary kidney injury molecule 1); CRT (urinary creatinine); USG (urinary specific gravity); u-NAG (N-acetyl-β-D-glucosamidase); u-NGAL (neutrophil gelatinase associated lipocalin).

Table 4.2 Reference values for u-KIM-1, u-Cystatin C, u-NAG and u-NGAL for a healthy population, ranging from 0 to 80+years old.

	0yr	10yr	20yr	30yr	40yr	50yr	60yr	70yr	80yr
u-KIM-1 (ng/l)									
Male	1502	1673	1860	2063	2284	2523	2783	3064	3367
	(314)	(369)	(432)	(502)	(580)	(667)	(763)	(870)	(987)
Female	1249	1399	1562	1741	1936	2147	2378	2628	2898
	(239)	(284)	(335)	(394)	(459)	(532)	(614)	(705)	(805)
u-KIM-1/CRT (µg/g CRT)									
Both sexes	3,61	2,70	2,28	2,14	2,24	2,61	3,38	4,88	7,88
	(0,71)	(0,54)	(0,46)	(0,43)	(0,45)	(0,52)	(0,68)	(0,98)	(1,58)
u-KIM-1/USG (ng/l)									
Male	1679	1680	1787	2002	2342	2855	3632	4856	5901
	(272)	(279)	(298)	(334)	(389)	(476)	(607)	(810)	(1129)
Female	1114	1334	1606	1925	2283	2671	3080	3512	3982
	(180)	(221)	(269)	(323)	(382)	(448)	(516)	(588)	(661)
u-Cyst-C (µg/l)*									
Male	208,2	-	-	-	-	-	-	-	-
Female	180,4	-	-	-	-	-	-	-	-
	(55,60)								
u-Cyst-C/CRT (µg/g CRT)									

Both sexes	424	283	220	191	183	193	223	289	434
	(95)	(73)	(62)	(56)	(54)	(57)	(63)	(75)	(97)
u-Cyst-C/USG (µg/l)*									
Male	204,6	-	-	-	-	-	-	-	-
	(69,3)								
Female	179,1	-	-	-	-	-	-	-	-
	(54,9)								
u-NAG (U/l)									
Both sexes	80,62	76,91	75,13	75,05	76,54	79,60	84,31	90,83	99,47
	(34,83)	(32,52)	(31,36)	(31,30)	(32,34)	(34,53)	(37,94)	(42,71)	(49,01)
u-NAG/CRT (U/mg CRT)									
Male	63,18	35,78	23,98	18,89	17,40	18,73	23,53	34,62	59,97
	(8,24)	(4,75)	(3,20)	(2,52)	(2,32)	(2,49)	(3,14)	(4,62)	(7,95)
Female	84,76	47,96	32,12	25,29	23,31	25,08	31,53	46,41	80,40
	(11,08)	(6,38)	(4,30)	(3,38)	(3,11)	(3,35)	(4,22)	(6,21)	10,68)
u-NAG/USG (U/I)									
Both sexes	81,15	76,89	74,82	74,66	76,29	79,69	85,00	92,47	102,51
	(33,80)	(31,38)	(30,16)	(30,06)	(31,08)	(33,27)	(36,75)	(41,70)	(48,36)
u-NGAL (µg/l)									
Male	52,97	62,50	73,88	87,54	103,95	123,70	147,52	176,31	211,16

	(6,67)	(7,94)	(9,45)	(11,25)	(13,39)	(15,94)	(18,97)	(22,57)	(26,87)
Female	141,80	145,43	149,26	153,60	158,37	163,62	169,38	175,68	182,58
	(17,94)	(18,54)	(19,15)	(19,78)	(20,44)	(21,12)	(21,82)	(22,54)	(23,29)
u-NGAL/CRT (µg/g CRT)									
Male	16,39	14,09	12,84	12,49	13,03	14,60	17,57	22,66	31,15
	(1,65)	(1,39)	(1,26)	(1,23)	(1,28)	(1,44)	(1,73)	(2,23)	(3,09)
Female	31,84	27,42	25,03	24,37	25,43	28,49	34,27	44,14	60,57
	(3,21)	(2,72)	(2,46)	(2,40)	(2,50)	(2,80)	(3,37)	(4,35)	(6,02)
u-NGAL/USG (µg/l)									
Both sexes	102,16	93,37	89,25	88,88	91,95	98,70	109,94	127,30	153,67
	(30,77)	(28,42)	(27,26)	(27,14)	(28,06)	(30,13)	(33,58)	(38,87)	(46,71)

Upper limit of 95% prediction interval (and mean value between brackets) of the different biomarkers (both absolute values as values normalized for creatinine and specific gravity), for the different age groups. * no age effect was observed (upper limit can be used for all the age groups)

In case a sex difference was observed, the data are represented separately for men and women, otherwise data are represented for the two sexes together.

4.5 Discussion

Although a number of research has focused on the promising role of some novel urinary biomarkers in the early detection of kidney damage in a broad range of pathologies and intoxications, thus far, only a few studies have focused on establishing reference values in healthy populations, and more specifically, whether there is an age or gender effect. It is generally recognized that kidney function diminishes with increasing age. This decrease is due to hemodynamic (decrease in GFR and arteriolar resistance) and structural changes (such as loss of renal mass, hyalinization of afferent arterioles, increase of sclerotic glomeruli and tubulointerstitial fibrosis) (Weinstein and Anderson 2010). These age-associated changes in renal function have to be taken into account when establishing the reference values of biomarkers for the general population. Therefore, the goal of this study was to establish age- and gender-associated reference values for a panel of four novel renal biomarkers (i.e., u-KIM-1, u-NGAL, u-cystatin C, and u-NAG) for healthy males and females aged 0 – 95 years old. To our knowledge, this is the first study to propose gender- and age-related reference values for this panel of renal biomarkers based on the 95% prediction intervals in a large population ranging from 0 to 95 years old, both for the absolute urinary concentrations and for values normalized for u-CRT and USG.

We found an effect of age on the absolute values of u-KIM-1, u-NAG, and u-NGAL. For u-KIM-1 and u-NGAL, this effect was linear: concentrations increased with increasing age. For u-NAG, this trend was quadratic, with higher concentrations in both the children and the elderly, when compared with 20 – 50-year-old subjects. No age effect was observed for u-cystatin C. When the urinary concentrations are normalized for u-CRT, the correlations between the urinary biomarkers and age are quadratic. However, this is likely due to the quadratic correlation between u-CRT and age. u-CRT is low in children, increases during adulthood, and decreases again in the elderly group, due to the lower amount of lean body mass (and hence the production of u-CRT) in children and elderly (Barr et al. 2005). Next to the age dependency of u-CRT, sex also influences u-CRT concentrations (Mage et al. 2004), which can affect the correlation between gender and the urinary biomarker concentrations, when

normalized for u-CRT. Another problem with u-CRT normalization is that patients should be in steady state, which is not the case in some clinical settings. Therefore, it remains a point of debate whether urinary kidney injury biomarkers should be normalized for u-CRT or not (Goldstein 2010). To counter for these possible misleading values, we also provided the reference values for the urinary biomarker concentration normalized for USG, which is another commonly used method to remove variance due to urinary dilution.

For u-KIM-1, no study has thus far focused on the measurement of u-KIM-1 values in healthy subjects. When comparing the mean concentrations of u-KIM-1 found in the control subjects in other recent studies (Sarafidis et al. 2012; Kim et al. 2012; Krawczeski et al. 2011) with the mean urinary concentration of KIM-1 for the corresponding age group in our study, values of the same magnitude are observed. U-NGAL levels are positively correlated with the female gender in young children and adults. This gender effect is also observed in a study in newborns (Askenazi et al. 2011; Huynh et al. 2009) and in a healthy adult population (Cullen et al. 2012), but this conflicts with the data observed in a large population of neonates and children (Cangemi et al. 2012). A possible explanation for these conflicting results is the rather low number of samples in the pediatric age studied in this study or the difference in preanalytical procedure. However, in our study, the gender effect disappears with increasing age. To our knowledge, Cullen et al. are the only ones to have established reference values in a healthy adult population. Although the mean values for comparable age groups between the two studies are from the same order of magnitude (with a mean value around 100 µg/L), we observed a linear relationship between age and u-NGAL, contrary to the quadratic effect seen in the study of Cullen et al. (Cullen et al. 2012).

Although we tried to perform this research in the best possible conditions, there are some shortcomings that need to be discussed. First, no blood samples were collected, which impedes the possibility of conforming the healthy renal status of the subject through the measurement of s-CRT levels (and calculation of the eGFR). Secondly, the manual methods used to measure the different biomarkers are less useful in clinical settings because their long turnover time would impede the early diagnostic advantages of the biomarkers. However, as for some biomarkers (u-KIM-1), no automatic assays were available at the start of the

study, we used manual methods for all the biomarkers to ensure a more precise comparison between the different biomarkers. Although for u-NGAL, concentrations of the same magnitude were observed compared with the urinary values found using an automated platform (Cangemi et al. 2012); improved analytical performance may affect clinical performance.

Finally, it would have been useful to measure the levels of proteinuria and albuminuria, which have been shown by Nejat et al. (Nejat et al. 2012) to influence the u-cystatin C and u-NGAL levels. In conclusion, we provide a set of reference values (both absolute urinary concentrations and values normalized to u-CRT and USG) for a panel of four novel biomarkers for healthty subjects, taking into account possible age and gender effects.

5 The association between urinary kidney injury molecule 1 (KIM-1) and urinary cadmium in elderly during long-term, low-dose cadmium (Cd) exposure: a pilot study.

This chapter is based on:

The association between urinary kidney injury molecule 1 and urinary cadmium in elderly during long-term, low-dose cadmium exposure: a pilot study.
Pennemans, V., De Winter, L.M., Munters, E., Nawrot, T.S., Van Kerkhove, E., Rigo, JM., Reynders, C., Dewitte, H., Carleer, R., Penders, J., Swennen, Q. Environ Health, 2011. 10: p. 77.

5.1 Abstract

U-KIM-1 is a recently discovered early biomarker for renal damage that has been proven to be correlated to urinary Cd (u-Cd) in rats. However, so far the association between u-Cd and u-KIM-1 in humans after long-term, low-dose Cd exposure has not been studied. We collected urine and blood samples from 153 nonsmoking men and women aged 60+, living in an area with moderate Cd pollution from a non-ferrous metal plant for a significant period. U-Cd and u-KIM-1 as well as other renal biomarkers (A1M-U), beta2-microglobulin (b2M-U), blood urea nitrogen (BUN), urinary proteins and microalbumin) were assessed. Both before ($r= 0.20$; $p= 0.01$) and after (partial $r= 0.32$; $p< 0.0001$) adjustment for u-CRT, age, sex, past smoking, socio-economic status (SES) and body mass index (BMI), u-KIM-1 correlated with u-Cd concentrations. No significant association was found between the other studied renal biomarkers and u-Cd. We showed that u-KIM-1 levels are positively correlated with u-Cd concentration in an elderly population after long-term, low-dose exposure to Cd, while other classical markers do not show an association. Therefore, u-KIM-1 might be considered as a biomarker for early-stage metal-induced kidney injury by Cd.

5.2 Introduction

Cd is an ever-present and global environmental pollutant (Nawrot et al. 2010). Current Cd emission has been drastically reduced, but Cd continues to be a health hazard, because historically accumulated Cd cannot be degraded and its half-life in the body is long (10-30 years) (Bernard 2008). Next to the bone, the liver and the longs (Staessen et al. 1999), a main target for chronic, low-level Cd exposure is the kidney, leading to proximal tubule dysfunction (Bernard 2008; Sabolic 2006; Jarüp 2002). Tubular dysfunction causes polyuria and low molecular weight proteinuria and some of these urinary proteins, e.g. A1M-U (Ikeda et al. 2004), b2M-U (Staessen et al. 1994; Nordberg et al. 2009) and clara cell protein-16 (CC-16-U) (Bernard et al. 1994) are used as urinary biomarkers of the early stages of Cd nephrotoxicity. In other studies, Cd toxicity is monitored by the Cd-binding protein metallothionein in urine (Nordberg et al. 2009; Shaikh et al. 1990; Chen et al. 2006), u-NAG (Staessen et al. 1994; Nordberg et al. 2009) or even u-Cd itself (Staessen et al. 1994; Chen et al.

2006; Bernard et al. 1992; Roels et al. 1999). Although these renal biomarkers are widely used, questions arose concerning specificity and sensitivity (Ikeda et al. 2004; Bernard et al. 1994; Chen et al. 2006; Nakajima et al. 2005; Vincent et al. 1989; Porstmann et al. 1990; Shikimi et al. 2001). There clearly is a need for an early and stable biomarker for proximal tubule damage caused by Cd.

KIM-1, originally discovered by Ichimura et al., is a type 1 membrane glycoprotein found on renal proximal tubule epithelial cells. It contains in its extracellular portion a unique 6-cysteine immunoglobulin-like domain and a mucin-domain (Ichimura et al. 1998). An intracellular highly conserved tyrosine kinase phosphorylation motif is a strong indicator that KIM-1 is a cell signaling molecule (Bailly et al. 2002). KIM-1 expression is induced in a variety of renal diseases, whereas in healthy kidney tissue KIM-1 is virtually undetectable (Ichimura et al. 1998; van Timmeren et al. 2007a). In the case of kidney damage, KIM-1 is expressed on the apical membrane followed by cleavage of the ectodomain (90 kDa) which is released in the urine in rodents (Ichimura et al. 1998; Bailly et al. 2002, Vaidya et al. 2006; Amin et al. 2004; Prozialeck et al. 2007b; Zhou et al. 2008) and in humans (Liangos et al. 2007; Vaidya et al. 2008a; Han et al. 2002; van Timmeren 2007b). KIM-1 is upregulated in the proximal tubule during dedifferentiation of the kidney epithelium, an early manifestation in response to damage (Prozialeck et al. 2009b). In rats, Prozialeck et al. showed that KIM-1 is a very early urinary marker for Cd-induced kidney injury (Prozialeck et al. 2007b]. They showed that u-KIM-1 was elevated before other urinary biomarkers of Cd nephrotoxicity, such as metallothionein, CC-16-U, proteinuria, a-glutathione-S-transferase (a-GST), u-NAG and u-Cd itself (Prozialeck et al. 2007b, 2009a). Moreover they showed that the Cd-induced increase in KIM-1 expression can be detected before signs of necrosis appear and when there is only a modest level of apoptosis in the proximal tubule (Prozialeck et al. 2009b). It has been well established in humans that KIM-1 appears in the urine at an early stage in kidney damage and also, that Cd affects proximal tubule function when the Cd burden is high. Cd triggers the expression of KIM-1 at a very early stage in animal models (Prozialeck and Edwards, 2010).

The aim of the present pilot study is to assess the appearance of u-KIM-1 after long-term, low-dose Cd exposure in humans, because, to our knowledge, this has not been investigated in the population.

5.3 Methods

5.3.1 Study population and sample collection

The total population (n 3069) of the general practice in Genk is registered in the framework of a registration network for family practices in Flanders (INTEGO) (Bartholomeeusen 2005). The study area is representative of the total population. Non-smoking men and women, 60 to 80 years old, with no acute infection at enrolment and no history of malignancies, were selected in the southern region of Genk from a quarter adjacent to an industrial area where a non-ferrous metal plant, a major motor company and a power station are located and which is crossed by multiple highroads. Sampling was combined with the annual influenza vaccination at a local doctor's practice. Eligible people were notified in advance by letter. Of those that routinely are advised for influenza vaccination, approximately 86% joined the vaccination program. Of those that were eligible, 154 were recruited, and 99% agreed to participate in our study. Of the 153 persons that agreed to participate (79 women; mean age 71 yr and 74 men; mean age 70 yr), for one person no urinary sample was collected and this person was not included in the analyses. Personal information was processed anonymously in conformity with privacy policy. Informed consent was obtained from all participants and the study was approved by the ethics committee of the ZOL, Belgium. Questionnaires were administered to assess lifestyle, profession, education, past smoking status, as well as data on age, weight and gender. Family income was given as net monthly overall family income and subdivided into low (<1500€), medium (1500€ - 3000€) and high (>3000€) family income. Education was coded as low (primary school), medium (high school) and high (university). Past smoking was quantified as pack years by multiplying the number of packs of cigarettes smoked per day by the number of years the person has smoked. Finally, individual medical backgrounds were used to determine possible interference of drug administration or diseases, with kidney function. Second morning urine samples and blood samples were

collected from all participants. Urine samples were aliquoted (6 × 2 ml), stored on ice for a maximum of four hours and subsequently frozen at -80°C.

5.3.2 Routine analyses and renal biomarker measurements

Routine analyses of the urine samples were performed in the clinical laboratory of the regional hospital ZOL in Genk. Using an automated analyzer (Modular® P800- ISE900 System, Roche Diagnostics; Mannheim, Germany), the following urinary analyses were performed, according to manufacturer's instructions: CRT according to the kinetic Jaffe method (compensated, rate blanked), total protein by a colorimetric biuret test and A1M-U based on immunological agglutination. b2M-U was determined by particle-enhanced immunonephelometry using the BN ProSpec (Siemens Healthcare Diagnostics; Marburg, Germany). Microalbumin was nephelometrically determined (Immage® Immunochemistry System, Beckman Coulter; Suarlee, Belgium). Blood urea and s-CRT were measured following the same assays as with the urinary analyses. BUN was determined as blood urea times two (covering the molar mass of the two nitrogens). U-KIM-1 was analyzed by a commercially available sandwich ELISA: Human TIM-1/KIM-1/HAVCR Duoset (R&D Systems; Abingdon, U.K.), validated by Chaturvedi et al. (2009). The assay procedure was performed according to the prescriptions of the manufacturer. When necessary, samples were adjusted to pH 7.0 before measurement (Pennemans et al. 2010). The optical density was determined with a fluorescence microplate reader (FLUOstar OPTIMA, BMG Labtechnologies; Offenburg, Germany), set to 450 nm with a wavelength correction at 540 nm. All samples were measured in duplicate.

5.3.3 U-Cd analyses

Cd concentrations in urine were analyzed by means of inductively coupled plasma mass spectrometry (ICP-MS) using the ELAN® DRC-e (Axial Field™ Technology, Perkin Elmer SCIEX; Zaventem, Belgium). Urine samples and standards were diluted 1:10 in 1% nitric acid.

5.3.4 Statistical analyses

For database management and statistical analyses, SAS Software version 9.1 (version 9.1, SAS Institute Inc, Cary (NC), USA) and GraphPad Prism 5.01 (GraphPad Software Inc, La Jolla (CA), USA) were used. Non-normally distributed data were log transformed. For comparison of means and proportions, we applied Student's t-test and the c2-statistic, respectively. We investigated associations between markers of kidney function and u-Cd using Pearson's correlation and multiple linear regression. Estimated effect sizes and 95% CI were calculated from linear regression coefficients for a two-fold increase in u-Cd. A priori three models were chosen: model 1 shows unadjusted data, in model 2, results are adjusted for u-CRT, sex, age, past smoking, BMI and SES (based on educational degree and monthly family income), while in model 3 data are adjusted for sex, age, past smoking, BMI and SES and given as function of u-CRT. When residuals are calculated (figures), we adjusted the different parameters for u-CRT, sex, age, past smoking, BMI and SES, in order to remove these potential confounding factors from the association. Correlations were considered significant when $p < 0.05$. All tests were two-sided.

5.4 Results

The study population consists of 153 participants (52% women) with a mean age of 71 years. Patient characteristics can be found in **Table 5.1**. From all the participants, of the 54% that have ever smoked there was a significant difference between men and women (75% and 35% respectively, $p< 0.0001$). Those who had smoked in the past had an average of 18 pack years. The average distance between the subject's residence and the heavy metal industrial zone was 2743 m, while the mean distance to the two main roads was 294 m and 562 m. Participants have lived at their current addresses for a mean period of 36 years (range: 3 to 75 years).

Table 5.1 Participants characteristics

Characteristics	**Total group (n=153)**
Anthropometrics	
Sex, female	79 (52%)
Age, years	71 ± 4.5
BMI, kg/m²	27.2 ± 4.3
Socio-economic status*°	
Low	62 (41%)
Median	59 (39%)
High	29 (19%)
Familial income, per month°	
<1500€	71 (47%)
1500-3000€	77 (51%)
>3000€	2 (1%)
Smoking status	
Ex-smoker†	81 (54%)
Never smoked†	68 (46%)
Exposure to environm tobacco smoke¶	61 (48%)
Use of medication§	
Antiplatelet medication	13 (9%)
Statins	81 (53%)
ACE inhibitor	27 (18%)
Insulin	5 (3%)
Antidiabetic medication	19 (13%)
NSAID	23 (15%)
Blood analyses	
Hemoglobin, g/dl	14.17 ± 1.24
Red blood cells, 10^6/µl‖	4.75 (4.68 – 4.82)
White blood cells, 10^3/ µl‖	6.58 (5.76 – 7.53)
Neutrophils, %‖	55.10 (53.60 – 56.66)
Lymphocytes, %‖	29.70 (28.35 – 31.12)
Monocytes, %‖	6.52 (5.76 – 7.53)
Eosinophils, %‖	2.79 (2.39 – 3.25)

Ferritin, ng/ml[ǁ]	121.9 (108.0 – 137.6)
CRP, mg/dl	
s-CRT, mg/dl[ǁ]	0.86 (0.83 – 0.89)
Glucose, mg/dl[ǁ]	99.95 (96.50 – 103.50)
Cholesterol, mg/dl	200.7 ± 37.26
HDL, mg/dl[ǁ]	56.64 (54.04 – 59.37)
LDL, mg/dl	114.4 ± 34.48
Triglycerides, mg/dl[ǁ]	122.1 (112.8 – 132.1)

Data are arithmetic mean ± standard deviation or absolute number (percentage of study population);

[ǁ] data which are not normally distributed and for which geometric mean (95% confidence interval) is given.

*Based on educational degree; °n=150; [†]n=149; [§]n=152;[¶] n=126

Geometric mean u-Cd level was 0.76 µg/g CRT. Geometric mean of the u-KIM-1 concentrations as well as the mean concentrations of other renal biomarkers (b2M-U, A1M-U, BUN, urinary proteins, microalbuminuria) and u-CRT are given in table 5.2.

U-KIM-1 was not influenced by gender (p= 0.83), age (p= 0.08), distance between housing and industrial zone (p= 0.57), SES (p= 0.40), past smoking (p= 0.14) and BMI (p= 0.83). Both before (**Table 5.3** and **Figure 5.1**) and after adjustment (**Table 5.3**) for sex, age, past smoking, BMI and SES (including education and income) variables, u-KIM-1 correlated positively and significantly with the u-Cd concentration.

Table 5.2 Mean urinary cadmium and renal biomarker values.

	Mean (95% CI)
Cadmium, µg/l†	0.80 (0.73 – 0.88)
Cadmium/CRT, µg/g CRT†	0.76 (0.70 – 0.84)
u-KIM-1, pg/ml*	569 (498 – 651)
u-KIM-1/CRT, µg/g CRT*	0.55 (0.49 – 0.62)
α1-microglobulin, mg/l†	3.19 (2.58 – 3.91)
α1-microglobulin /CRT, mg/g CRT†	2.97 (2.42 – 3.64)
Proteins, mg/l*	71.80 (63.81 – 80.80)
Proteins/CRT, mg/g CRT*	66.61 (59.99 – 73.95)
Albumin, mg/dl*	8.73 (7.46 – 10.21)
Albumin/CRT, mg/g CRT*	8.43 (7.32 – 9.70)
β2-microglobulin, mg/l‡	0.12 (0.11 – 0.13)
β2-microglobulin/CRT, mg/g CRT‡	0.12 (0.11 – 0.13)
BUN, mg/dl†	35.94 (34.47 – 37.46)
Urinary CRT, mg/dl*	104.9 (96.55 – 114.0)

Geometric mean (95% confidence interval) of urinary cadmium and renal biomarkers. All parameters were measured in urine; except for blood urea nitrogen, which was measured in blood. † n=152; * n=153; ‡ n=140. Abbreviations: BUN: blood urea nitrogen; CI: confidence interval; CRT: urinary creatinine.

For the other biomarkers (BUN, microalbuminuria and urinary proteins) unadjusted and adjusted multiple linear regression models showed no significant correlation between these biomarkers of kidney function and u-Cd (**Figure 5.1 and Table 5.3**).

Table 5.3 Estimated change (%) in urinary biomarkers calculated for a two-fold increase in u- Cd concentration

	Estimated effect size (%)	95%CI(%)	R^2	p-value
Model 1				
u-KIM-1	23.73	6.92 to 43.18	0.05	0.005
Microalbumin	4.40	-13.42 to 25.89	0.001	0.65
Proteins	5.66	-3.52 to 15.71	0.01	0.24
BUN	-5.65	-10.53 to -0.49	0.04	0.03
Model 2				
u-KIM-1	39.54	18.26 to 64.65	0.27	0.0001
Microalbumin	4.85	-14.14 to 28.03	0.27	0.64
Proteins	3.15	-6.26 to 13.52	0.49	0.53
BUN	-4.66	-9.83 to 0.80	0.08	0.10
Model 3				
u-KIM-1	26.59	7.82 to 48.62	0.13	0.005
Microalbumin	-2.80	-20.42 to 18.78	0.09	0.78
Proteins	0.66	-8.65 to 10.92	0.11	0.89
BUN	-3.69	-8.99 to 1.91	0.07	0.19

All data were log-transformed and estimated effect size is given as % estimated effect size calculated for a two-fold increase in urinary cadmium concentrations (µg/g creatinine) with the corresponding 95% confidence interval, R^2 gives the explained variance.

Abbreviations:u-CD, urinary cadmium; u-KIM-1, urinary kidney injury molecule 1; BUN, blood urea nitrogen; CI, confidence interval.

Model 1: unadjusted but given as function of creatinine.

Model 2: adjusted for creatinine (except for blood urea nitrogen), sex, age, past smoking, body mass index and socio-economic status

Model 3: adjusted for sex, age, past smoking, body mass index and socio-economic status; given as a function of creatinine.

Both for b2M-U and A1M-U a considerable amount of urine samples that were tested (84% and 28% respectively) were below the limit of detection, suggesting the assessments that were used were not sensitive enough. Therefore, no analysis was conducted for b2M-U and A1M-U in association with Cd.

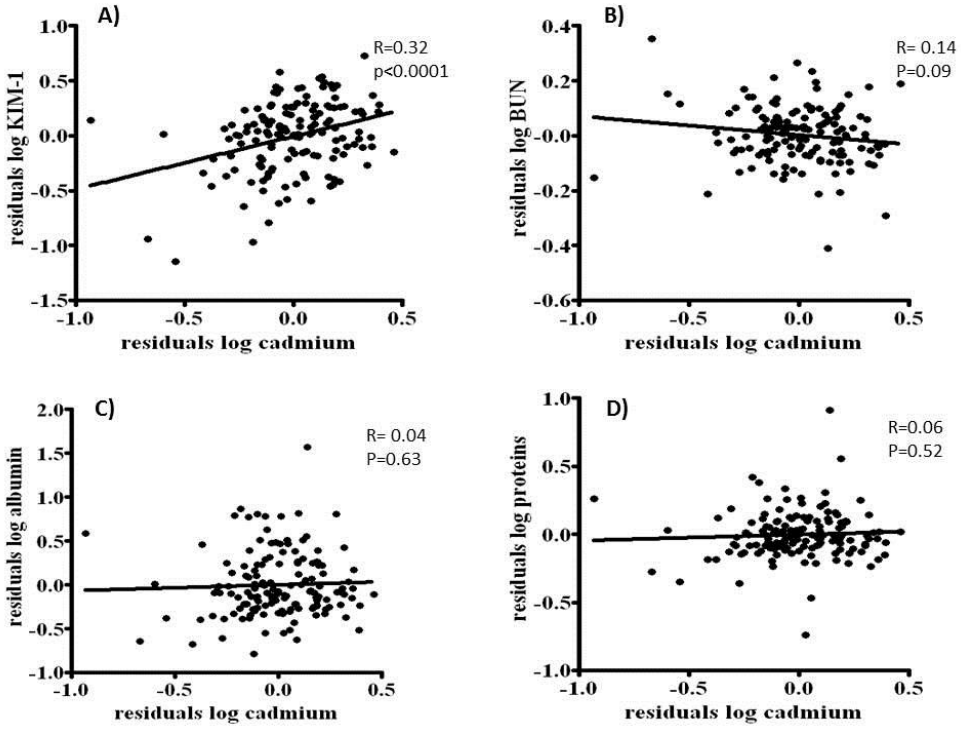


Figure 5.1 Correlations of the residuals of urinary KIM-1 and the other biomarkers with the residuals of urinary cadmium. Correlations of the residuals of A) log KIM-1, B) log blood urea nitrogen, C) log albumin and D) log proteins with the residuals of log cadmium. All parameters were measured in urine, except for blood urea nitrogen. Residuals were computed to remove the variance by age, sex, socio-economic status, body mass index, urinary creatinine (except for blood urea nitrogen) and past smoking. Data on KIM-1, cadmium, protein, blood urea nitrogen and albumin concentrations were log-transformed to obtain a normal distribution. Abbreviations:KIM-1: kidney injury molecule 1; BUN, blood urea nitrogen.

5.5 Discussion

About a decade ago, KIM-1 was discovered in the search for molecules involved in the pathogenesis of AKI. We demonstrated among elderly an association between u-KIM-1 and u-Cd. Depending on the biomarker of nephrotoxicity thresholds of u-Cd can range from about 2.4 µg Cd/g CRT for the onset of early biochemical alterations (e.g. hypercalciuria) to 10 µg Cd/g CRT for the development of the classic tubular microproteinuria (Roels et al. 1999). Here, we showed biochemical changes at u-Cd levels below 1 µg Cd/g CRT.

Ichimura et al. were the first to describe KIM-1 as a type 1 membrane glycoprotein, which contains a 6- cystein immunoglobulin-like domain in its extracellular portion, and a Thr/Ser-Pro rich domain characteristic of mucin-like O-glycosylated proteins (Ichimura et al. 1998). KIM-1 also has a transmembrane domain and a cytoplasmic domain, which contains a conservative tyrosine kinase phosphorylation site, indicating that KIM-1 may be a signaling molecule (Bailly et al. 2002). In healthy kidney tissue, KIM-1 is virtually undetectable whereas in the injured kidney, KIM-1 expression is rapidly upregulated at the apical side of the proximal tubule (Ichimura et al. 1998; vaidya et al. 2006). This process is accompanied by the shedding of the extracellular domain of KIM-1 into the urine (Bailly et al. 2002). The ectodomain is stable in urine (Pennemans et al. 2010) and has shown to be a sensitive biomarker of renal injury induced by a variety of agents including the heavy metal Cd: Prozialeck et al. have proven KIM-1 to be a putative early biomarker for proximal tubule damage caused by high doses of Cd in rats, outperforming the classic biomarkers such as MT, CC-16, a-GST and urinary proteins [Prozialeck et al. 2007b, 2009a, 2009b). In addition, KIM-1 appears before any lethal injury is detected in the proximal tubule epithelial cells (Prozialeck et al. 2009b). Thus, using this biomarker, very early detection of cell stress may be possible, which would allow for the reversal and/or the treatment of Cd-induced kidney injury (Wu and Parikh 2008).

To our knowledge however, no research has ever focused on the correlation of the Cd burden and the u-KIM-1 concentrations in humans. Since we were mainly interested in the possible role for KIM-1 as a biomarker after long term environmental Cd intoxication, we chose a non-smoking elderly population living

near a non-ferrous metal industrial zone in Genk for a longer period. In this region, levels of Cd have been reported to be higher than in other Belgian gauging regions according to the Flemish environmental agency (VMM: Mira-T indicator rapport 2008). Both blood Cd and u-Cd are indicators of Cd body burden; however u-Cd correlates better with the duration of exposure than does blood Cd (Bernard et al. 1992; Ikeda et al. 2010; WHO 2000), which makes it a better indicator for long-term Cd exposure. Therefore, we compared the u-Cd concentrations with KIM-1 and other biomarkers of nephrotoxicity.

As shown in **Figure 5.1**, after adjustment for CRT, sex, age, past smoking, BMI and SES, only u-KIM-1 was significantly correlated with Cd levels. U-Cd concentration averaged below 1 µg/g CRT among our population. This may explain why albuminuria, proteinuria and BUN did not correlate with u-Cd. These markers mainly identify later stages of Cd-induced kidney injury (Kobayashi et al. 2006; Vaidya et al. 2010). Although A1M-U is stable across physiological pH (Payn et al. 2002), its specificity is undermined by the influence of several conditions such as liver disease (Vincent et al. 1989), HIV (Porstmann et al. 1990), mood disorders (Shikimi et al. 2001) and other environmental influences, for example Pb exposure (Endo et al. 1993). In contrast to b2M-U, which is degraded rapidly in acidic urine (Bernard 2004), u-KIM-1 has been proven to be stable over the physiological range of urinary pH values (Pennemans et al. 2010). Moreover, it originates from proximal tubule cells (van Timmeren et al. 2007; Vaidya et al. 2008), which makes it a much more specific renal biomarker than proteins originating from other parts of the body. Prozialeck et al. found in rats that u-KIM-1 starts to increase significantly earlier and at lower doses of Cd than MT, CC-16, proteins and a-GST (Prozialeck 2007a, 2009a, 2009b)

The present study corroborates this high sensitivity for human subjects. Since KIM-1 has a high cysteine content and Cd is known for its high binding capacity with cysteine complexes (Bottari et Festa 1997), future research should focus on whether this influences the correlation between u-KIM-1 and u-Cd.

5.6 Conclusions

In conclusion, this pilot study shows that u-KIM-1 levels are significantly correlated with u-Cd levels in an elderly population after long-term, low-dose exposure to Cd, probably indicating beginning metal-induced kidney injury. To further elucidate the exact role KIM-1 can play as a biomarker for early cadmium-induced renal damage, future research should concentrate on the comparison of KIM-1 with other biomarkers by using the most sensitive and reliable measuring techniques, with inclusion of a paired control group, living in unpolluted areas.

6 Validation of novel urinary biomarkers of acute kidney injury (AKI) after cardiac surgery requiring cardio-pulmonary bypass (CPB).

6.1 Abstract

Post-operative AKI is a common complication after cardiac surgery involving CPB. Due to a lack of reliable early detection methods, prognosis of AKI patients is rather poor. New promising biomarkers for AKI need to be validated in this clinical setting.

In a multi-center prospective cohort of 259 adult patients undergoing cardiac surgery involving CPB, we collected blood and urine samples at different time points (pre-op, 3h, 6h, 12h and 24h post-op) for the measurement of u-KIM-1, u-NGAL, u-Cystatin C and s-Cystatin C. Patients were divided into 2 groups, AKI and non-AKI, based on the AKIN criteria. When comparing the concentrations of these biomarkers between the AKI and the non-AKI group, there were significant differences for all biomarkers at at least one time point, but s-Cystatin C was the only biomarker that was significantly upregulated at all time points measured. In a simple regression model only u-NGAL, u-Cystatin C and s-Cystatin C were significant predictors for AKI at one or more time points. In the multiple regression model however, only s-Cystatin C remained a significant predictor at all time points except at 24h post-op. Moreover, only s-Cystatin C significantly added discriminative power to a clinical model (consisting of BMI, eGFR and CPB duration) at 3h post-op, 6h post-op and 12h post-op.

In conclusion, the discriminative power of the biomarkers u-KIM-1, u-NGAL, u-Cystatin C and s-Cystatin C was rather moderate in the prediction of AKI in a routine clinical setting after cardiac surgery with CPB, with only s-Cystatin C adding discriminative power to a clinical model.

6.2 Introduction

Postoperative AKI is one of the most serious and frequent complications after cardiac surgery involving CPB (Conlon et al. 1999). It is associated with significantly increased mortality, length of stay and costs (Chertow et al. 2005). Due to the rather late, insensitive and nonspecific biomarker that is used to detect AKI, i.e. s-CRT (Bolignano et al., 2012), the diagnosis of AKI in patients undergoing cardiac surgery is nowadays often delayed, impairing early intervention, prevention of morbidity and reduction in mortality. Therefore the discovery and validation of new biomarkers for the early detection of AKI in these patients is a hot research topic. Recently a range of novel potent

biomarkers for the early detection of AKI were discovered. Among others, some promising biomarkers such as u-KIM-1 (Han et al. 2008), u-NGAL (Mishra et al. 2005; Parikh et al. 2006; Bennett et al. 2008), u-Cystatin C and s-Cystatin C (Koyner et al. 2008) merit special attention. In the last decade, several research groups have been focusing on the clinical relevance and validation of those novel biomarkers in the early detection of AKI after cardiac surgery. Vanmassenhove et al. summarized the findings in an in-depth review in 2013 (Vanmassenhove et al. 2013). Because the clinical application of each of those single biomarkers shows a rather disappointing picture, focus should be shifted to the use of a panel of biomarkers to optimize sensitivity and specificity. Recently, some promising results were found in the setting of children undergoing cardiac surgery (Krawczeski et al. 2011; Zappitelli et al. 2011; Peco-Antic et al. 2013, Parikh et al. 2011, 2013). These pediatric cardiac patients, a fairly homogenous group without co-morbid conditions, such as diabetis mellitus, hypertension and older age, are a good starting point for understanding and optimizing the utility of biomarkers of AKI after cardiac surgery. However, since the majority of patients undergoing cardiac surgery with CPB, is generally older in age, there is a need to extrapolate research findings to more heterogeneous age groups. To date, a handful of studies have been published on the combined clinical relevance and predictive value of more than 2 urinary biomarkers in the early detection of AKI after cardiac surgery with CPB (Liangos et al. 2009; Koyner et al. 2010, 2012a; Han et al. 2009). However, these studies were either focusing on biomarkers other than u-KIM-1, u-NGAL and u-Cystatin C, were single center studies or were conducted on a rather small group of patients. The aim of the present multicenter prospective study is therefore to examine the performance characteristics of pre- and postoperative u-KIM-1, u-NGAL, u-Cystatin C and s-Cystatin C in a cohort of adults undergoing cardiac surgery with CPB and to study whether these novel biomarkers -both individually and combined- can add predictive value to the currently used but imperfect s-CRT in the early detection of postoperative AKI.

6.3 Material and methods

Prior to cardiac surgery requiring CPB, 259 patients were enrolled in a prospective study at ZOL (n= 175) and Jessa Ziekenhuis (Hasselt, Belgium) (n= 84), both large (830 and 988 beds resp.) regional secondary- and tertiary-care hospitals, located in Belgium, between July 2012 and June 2013. The study was approved by internal ethical committees at both sites, as well as at Hasselt University (Belgium). Written informed consent was obtained before enrollment. Patients with severe pre-existing renal insufficiency were excluded (eGFR <15). Urine and blood samples were obtained immediately before and 3, 6, 12 and 24 hours after initiation of CPB. Samples were aliquoted. One aliquot was immediately sent to the Clinical laboratory of ZOL for routine analyses (including s-CRT and u-CRT) performed according to the standard laboratory methods using an automated analyzer (compensated IDMS-calibrated, rate-blanked Jaffe method, Modular® P800-ISE900 System, Roche Diagnostics; Mannheim, Germany). The other aliquots were stored at -80°C at Hasselt University for future biomarker analyses. Before storage, pH of each urine sample was measured with an Ankersmit 420A pH meter (Orion, Boston, MA, USA) and, if necessary, adapted to a value between 6 and 8 by adding droplets of 1 mol/L NaOH or 1 mol/L HCl (Sigma-Aldrich Chemie Gmbh, Munich, Germany) (Pennemans et al. 2010).

AKI was defined by the AKIN criteria: an elevation of s-CRT of at least 0.3 mg/dl, an increase of 50% compared to baseline within 48h or a reduction in UO defined as <0.5 ml/kg/h for more than 6h after open heart surgery (Mehta et al. 2008).

Prior to and after surgery, clinical and demographic data, as well as details of the hospitalization and the surgery were obtained for each patient.

U-KIM-1, u-NGAL and u-cystatin C concentrations were determined in duplo, using the commercially available human tim-1/kim-1/Havcr ELISA kit, the Human Lipocalin-2/NGAL Elisa kit and the Human Cystatin C ELISA kit, respectively (RD systems Europe, Abingdon, UK). The assay procedures were executed according to the prescriptions of the manufacturer. For details see material and methods of previous chapters. S-Cystatin C levels were determined immunoturbidimetrically using the Cystatin C Tina-quant kit for automated

measurements at the modular® P800-ISE900 (both kit and analyser by Roche Diagnostics; Mannheim, Germany).

As patient characteristics, described in table 6.1, were not normally distributed (tested using the D'agostino-Pearson test), non-parametric Mann-Whitney U test and Fisher exact test were used to compare the characteristics between the AKI and non-AKI group.

To study the longitudinal evolution of a biomarker for patients with and without AKI a linear mixed model was used. The fixed effect part of the model contains the variables AKI and a polynomial of degree 3 for time (5 time points) as well as the interaction between AKI and the polynomial for time to test whether the change over time is different between patients with and without AKI. U-KIM-1, u-NGAL and u-Cystatin C are normalized per gram CRT to account for urinary dilution.

A random intercept accounts for each subject's deviation from the population mean. The response variable contains the 5 measurements per subject of the biomarker. The response was log-transformed to normalize the data.

The group differences at each time point were tested through the linear mixed model by specifying linear combinations of the estimated parameters for the fixed effects. To adjust for the multiple comparisons at each time point, the Benjamini-Hochberg method was used. Each biomarker was analyzed separately. The R-package nmle (Pinheiro et al. 2013) was used to fit the linear mixed models.

To assess the discriminative power of the biomarkers at a specific time point separately, a simple logistic regression was used. The discriminative ability of all biomarkers at a specific time point simultaneously was assessed with multiple logistic regression. Receiver operating characteristcs (ROC) curves were generated and their corresponding area under the curves (AUC) were calculated. The AUC's were compared using DeLong's method. The Benjamini-Hochberg method was used to adjust for the multiple comparisons. The R-package pROC (Robin et al. 2011) was used to calculate sensitivity, specificity, AUC and to compare ROC curves.

To take into account the effect of possible confounding factors, we identified 9 potential clinical predictor variables: sex, age, BMI, duration of procedure, duration of CPB, the administration of vasopression/inotropy medication for

more than 24h, the administration of packed cells, pre-operative eGFR, and the duration of intubation). To identify the predictor variables among the clinical factors mentioned above, a multiple logistic regression model was fitted with the backwards elimination selection method. Next, each of the 4 biomarkers per time point were added individually to the obtained clinical model. ROC curves for each model were generated and their corresponding AUCs were calculated.

The models combining the biomarkers and the clinical predictors were compared to the clinical model to assess improvements in the model performance using the likelihood ratio test (LRT). The LRT uses the difference in the goodness-of-fit (G^2) statistics for the two models. The AUCs of the two models were compared using DeLong's method. The R-package pROC (Robin et al. 2011) was used to calculate sensitivity, specificity AUC and to compare ROC curves.

The significance level is set to 5%.

6.4 results

In total 259 patients (of which 175 men) were enrolled in the study. Patients' characteristics can be found in **Table 6.1** for the entire patient group and for the subgroups AKI and non-AKI separately. P-values of the comparison of the patients' characteristics between the AKI and non-AKI groups are provided.

Table 6.1 Patient characteristics

Characteristic	total (n= 259)	AKI (n= 84)	no-AKI (n= 175)	p-value
Male, n (%)	175 (67%)	62 (74%)	113 (65%)	NS
Age, years	68 (10)	70 (9)	67 (11)	0.0433
BMI	27.0 (4.5)	27.7 (4.8)	26.7 (4.3)	0.0363
Surgery time, min	348 (281)	381 (115)	332 (332)	<0.0001
CPB time, min	135 (58)	163 (63)	121 (51)	<0.0001
Intubation time, min	1660 (3984)	2512 (5662)	1258 (2808)	0.0021
ICU stay, days	4.2 (5.3)	5.8 (8.4)	3.4 (2.3)	0.009
Packed cells administered,n	1.2 (2.3)	1.8 (2.6)	0.9 (2.0)	<0.0001

vasopression/ inotropy >24h, n (%)	125 (48%)	45 (54%)	73 (42%)	0.083
RRT, n (%)	7 (2%)	7 (8%)	0 (0%)	0.0003
Baseline u-CRT, mg/dl	121 (64)	117 (56)	123 (68)	0.73
Baseline s-CRT, **mg/dl**	0.98 (0.34)	1.08 (0.41)	0.93 (0.29)	0.0028
Baseline eGFR (CKD-EPI)	74.12 (17.24)	69.49 (20.30)	76.45 (15.01)	0.0240
Baseline u-KIM-1 µg/l	846 (753)	926 (723)	807 (766)	0.1185
Baseline u-KIM-1_CRT, µg/g CRT	0.82 (0.92)	0.91 (0.75)	0.785 (0.10)	0.062
Baseline u-NGAL, **µg/l**	901 (1479)	1211 (2172)	749 (946)	0.0196
Baseline, **u-NGAL_CRT, µg/g CRT**	0.96 (1.77)	1.31 (2.47)	0.79 (1.28)	0.1218
Baseline u-Cystatin C,µg/l	103 (80)	109 (85)	100 (77)	0.2670
Baseline u-Cystatin C_CRT,µg/g CRT	96 (131)	118 (203)	86 (71)	0.1484
Baseline s-Cystatin C, mg/L	0.90 (0.38)	0.98 (0.39)	0.86 (0.36)	0.0175

Unless stated differently, mean values (standard deviation) are reported for continuous variables. Patients are divided in AKI and non-AKI subgroups according to the Acute Kidney Injury Network (AKIN) criteria (Mehta 2008). Abbreviations: AKI: acute kidney injury; BMI: body mass index; CPB: cardio-pulmonary bypass; ICU: intensive care unit; RRT: renal replacement therapy; u-CRT: urinary creatinine; s-CRT: serum creatinine; eGFR: estimated glomerular filtration rate according to the CKD-EPI guidelines; u-KIM-1: urinary kidney injury molecule 1 ;u-KIM-1_CRT: urinary kidney injury molecule 1 normalized for urinary creatinine; u-NGAL: urinary neutrophil gelatinase-associated lipocalin; u-NGAL_CRT: urinary neutrophil gelatinase-associated lipocalin normalized for urinary creatinine; u-Cystatin C: urinary cystatin C; u-Cystatin C_CRT: urinary cystatin C normalized for urinary creatinine; s-Cystatin C: serum cystatin C

In **Figure 6.1** the mean log urinary (for KIM-1, NGAL and cystatin C, normalized for u-CRT) and log serum (for cystatin C) concentrations for the different biomarkers are plotted at different time points both for AKI and non-AKI patients. For u-KIM-1_CRT a significant difference was detected between both patient groups at 3h post-op (i.e. time point 2); u-NGAL_CRT differed significantly at 3h, 6h and 12h post-op (i.e. time point 2,3,4), while u-Cystatin C_CRT differed significantly at 6h, 12h and 24h post-op (i.e. time point 3, 4,5). Finally a significant difference between AKI and non-AKI patients was detected for s-Cystatin C at all time points (pre-op, 3h, 6h, 12h and 24h post-op).

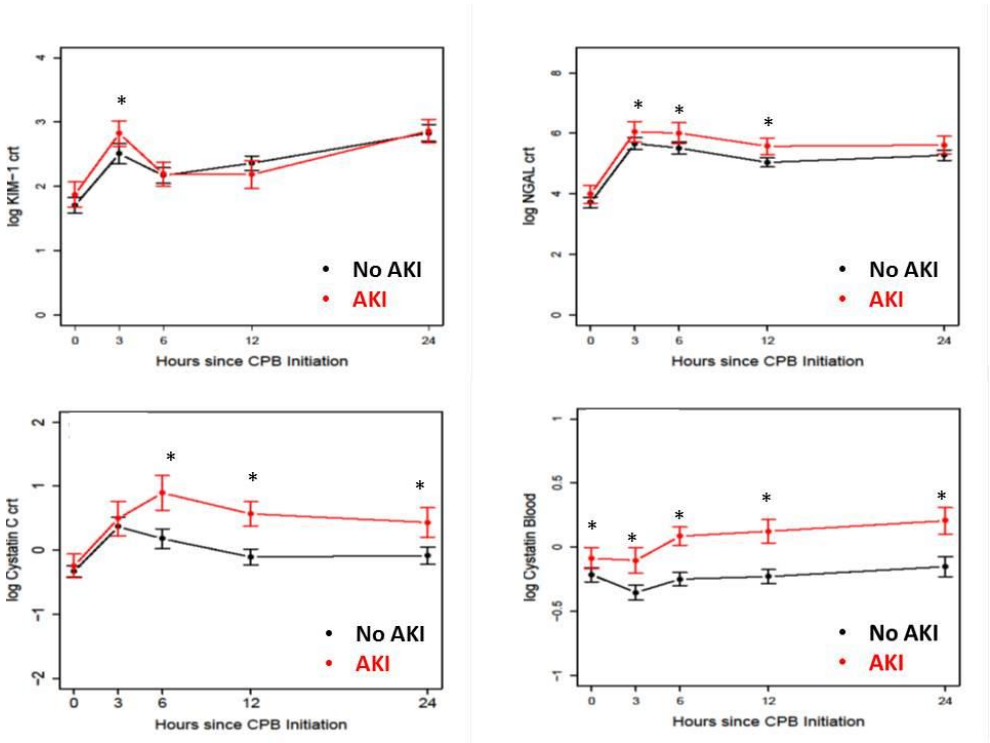
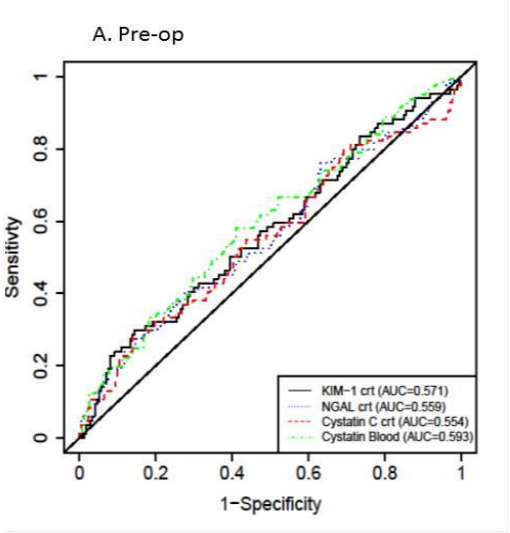
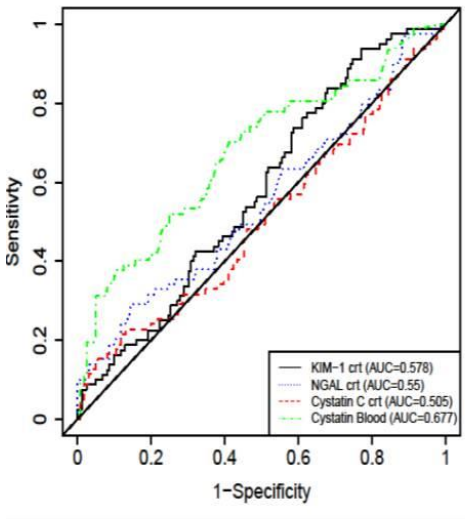


Figure 6.1 Estimated group profiles (AKI and non-AKI) with their 95% confidence intervals for (A) log u-KIM-1_CRT, (B) log u-NGAL_CRT, (C) log u-Cystatin C_CRT, (D) log s-Cystatin C. * $p<0.05$. Abbreviations: KIM-1 crt: urinary kidney injury molecule 1 normalized for urinary creatinine; NGAL crt: urinary neutrophil gelatinase-associated lipocalin normalized for urinary creatinine; cystatin C crt: urinary cystatin C normalized for urinary creatinine; cystatin blood: serum cystatin C; AKI: acute kidney injury; CPB: cardiopulmonary bypass.

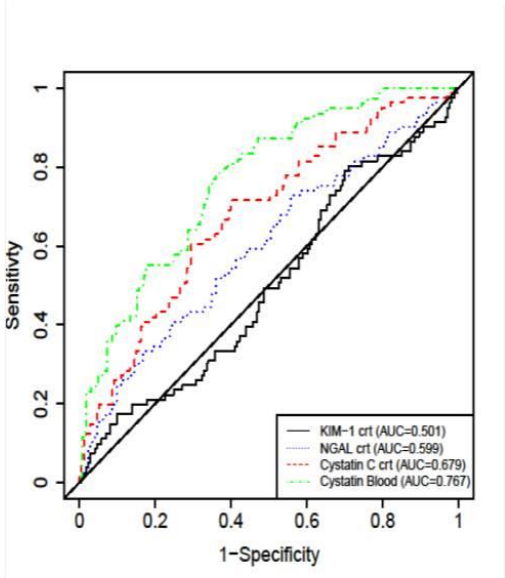
The discriminative power of the biomarkers to predict AKI at the different time points was investigated through simple (one biomarker) and multiple (all biomarkers combined) logistic regression models. In the simple regression model only u-NGAL_CRT and s-Cystatin C were significant predictors at time points pre-op (with AUC values of 0.559 and 0.593 respectively) and 3h post-op (with AUC values of 0.55 and 0.677, respectively). At 6h and 12h post-op, u-NGAL_CRT (AUC= 0.599 and 0.627, respectively), u-Cystatin C (AUC=0.679 and 0.723, respectively) and s-Cystatin C (AUC= 0.767 and 0.755, respectively) were significant predictors for AKI. In the multiple regression model only s-Cystatin C was a significant predictor for AKI at time points pre-op, 3h post-op, 6h post-op and 12h post-op. AUC values of the multiple model were 0.612 at pre-op, 0.669 at 3h post-op, 0.787 at 6h post-op and 0.767 at 12h post-op. At 24h post-op, none of the biomarkers were significant predictors, neither in the single regression model, nor in the multiple regression model. The ROC curves for the prediction of AKI at the different time points for each biomarker separately are given in **Figure 6.2**.



B. 3u post-op



C. 6u post-op



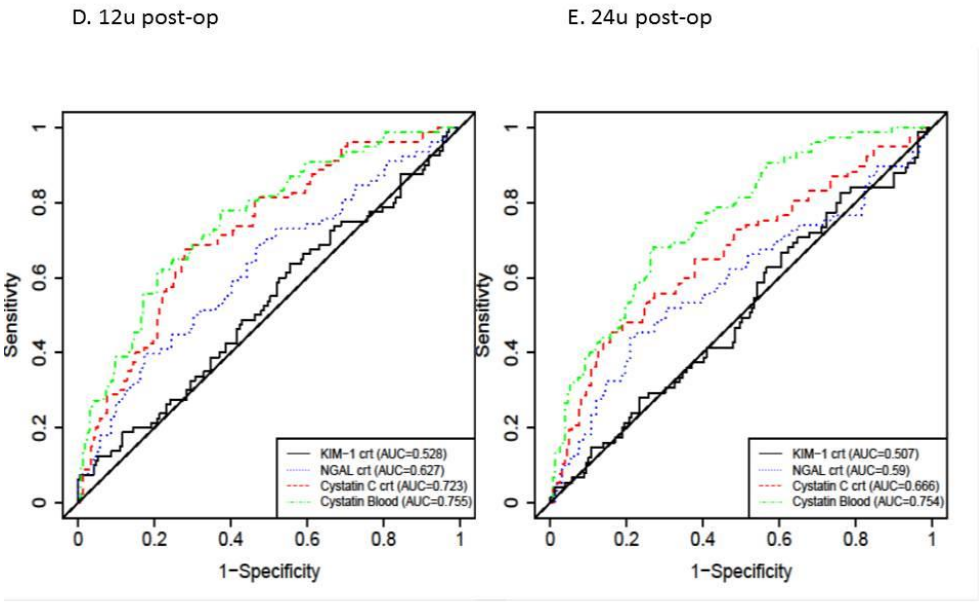


Figure 6.2 ROC curves with corresponding Area Under the Curve (AUC) scores for the prediction of AKI for u-KIM-1_CRT (in black), u-NGAL_CRT (in blue), u-Cystatin C_CRT (in red) and s-Cystatin C (cystatin blood in green) at time point pre-op (A), 3h post-op (B), 6h post-op (C), 12h post-op (D) and 24h post-op (E). Abbreviations: KIM-1 crt: urinary kidney injury molecule 1 normalized for urinary creatinine, NGAL crt: urinary neutrophil gelatinase-associated lipocalin normalized for creatinine; cystatin C crt: urinary cystatin C normalized for urinary creatinine; cystatin blood: serum cystatin C; AUC: area under the curve; AKI: acute kidney injury.

From the nine possible confounding factors identified (i.e. sex, age, BMI, duration of the procedure, duration of CPB, vasopressin/inotropy administration longer than 24h, number of packed cells administered, pre-op eGFR and duration of intubation), only duration of CPB, BMI and pre-op eGFR are significant predictor factors, identified through a multiple regression model with the backwards elimination selection method. The clinical model, composed of these three predictive values has AUC scores of 0.737 (3h post-op), 0.767 (6h post-op), 0.745 (12h post-op) and 0.755 (24h post-op). When adding the individual biomarkers to this model, s-Cystatin C increased the AUC significantly at 3h, 6h and 12h post-op. u-Cystatin C only increases the AUC significantly at

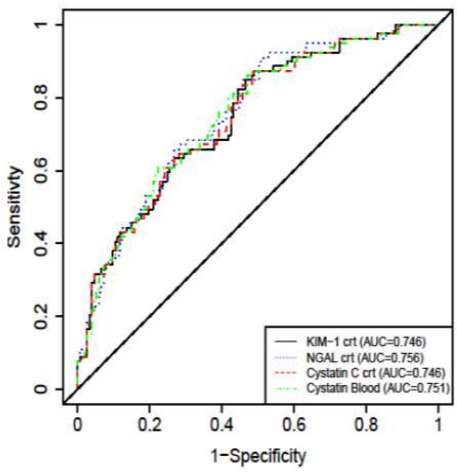
12h post-op. At pre-op and at 24h post-op none of the individual biomarkers added a significant increase to the AUC of the clinical model. The different G^2 and AUC scores of the clinical model and the combination of the clinical model with the separate biomarkers for 3h post-op, 6h post-op and 12h post-op are provided in **Table 6.2**. When using the LRT a significant difference in G^2 can also be seen when adding u-NGAL_CRT (3h post-op and 12h post-op) and u-cystatin (12h post-op) (**Table 6.2**). The ROC curves of the clinical model combined with a biomarker at the different time points are given in **Figure 6.3**.

Table 6.2 Evaluation of performance to predict AKI of the clinical model versus the clinical model + biomarker

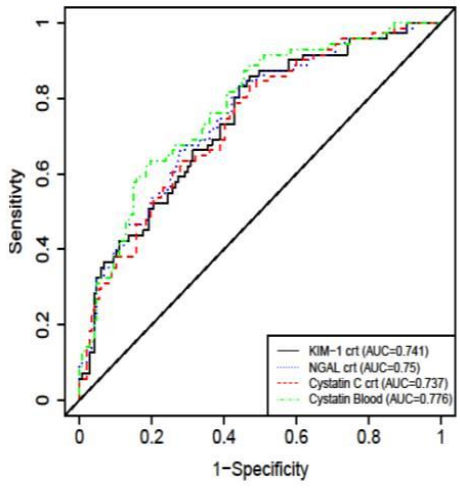
Model	T= 3h (n=218;71 AKI)		T=6h (n=233;75 AKI)		T=12h (n=230;72 AKI)	
	G^2	AUC	G^2	AUC	G^2	AUC
Clinical Model	238.88	0.737	244.07	0.767	245.98	0.745
+ KIM-1_CRT	237.84	0.741	244.02	0.766	245.8	0.743
+ NGAL_CRT	234.52*	0.753	244.07	0.767	239.38*	0.759
+ Cyst-C_CRT	238.49	0.737	240.4	0.781	240.69*	0.775*
+ s-Cyst-C	227.03*	0.776*	221.16	0.823*	228.39*	0.798*

Goodness-of-fit (G^2) and area under the curve (AUC) values for the clinical model (a combination of the pre-op estimated glomerular filtration rate, duration of cardiopulmonary bypass and body mass index) and for the clinical model in combination with every biomarker separately (i.e. urinary KIM-1, urinary NGAL, urinary Cystatin C and serum cystatin C). The urinary biomarkers are normalized to urinary creatinine to account for urinary dilution. * $p<0.05$ compared with the clinical model. Abbreviations: T: time point; AKI: acute kidney injury, G^2: goodness-of-fit; AUC: area under the curve; KIM-1_CRT: urinary kidney injury molecule 1 normalized for urinary creatinine; NGAL_CRT: urinary neutrophil gelatinase-associated lipocalin normalized for urinary creatinine; cyst-C_CRT: urinary cystatin C normalized for urinary creatinine; s-cyst-C: serum cystatin C.

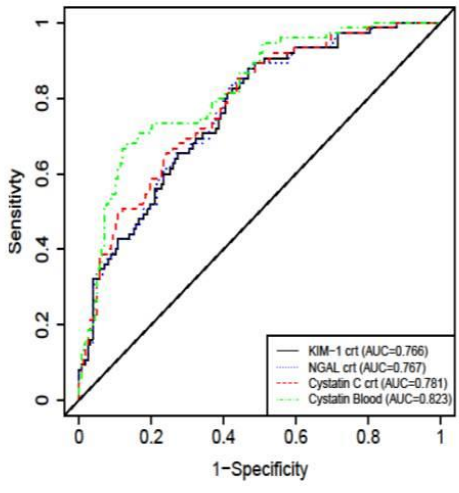
A. pre-op



B. 3h post-op



C. 6h post op



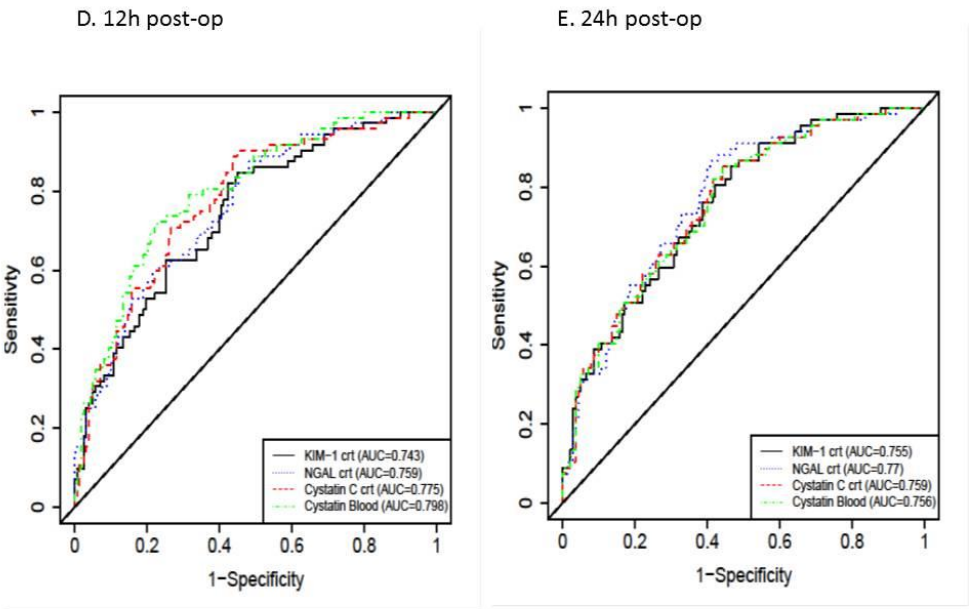


Figure 6.3 ROC curves with corresponding Area Under the Curve (AUC)scores for the prediction of AKI for the clinical model (duration of CPB, BMI and pre-operative eGFR) combined with KIM-1 crt (in black), NGAL crt (in blue), urinary cystatin C normalized for urinary creatinine (cystatin C crt in red) and serum cystatin C (cystatin blood in green) at time point pre-op (A), 3h post-op (B), 6h post-op (C), 12h post-op (D) and 24h post-op (E). ROC curves are generated through simple logistic regression. Abbreviations: KIM-1 crt: urinary kidney injury molecule 1 normalized for urinary creatinine, NGAL crt: urinary neutrophil gelatinase-associated lipocalin normalized for creatinine; cystatin C crt: urinary cystatin C.

6.5 Discussion

AKI is a complication occurring frequently after cardiac surgery involving CPB, with incidence rates reported between 3 and 30% depending on the definition of AKI (Huen and Parikh 2012). The detection of AKI in a timely fashion is a challenge because the definitions and categorization of AKI are based on increases in s-CRT and/or decreases in UO (Han et al. 2009). s-CRT is only increased after a delay of several hours and is not sufficiently sensitive for the early detection of AKI, as it does not discriminate between the time and type of renal insult or the site and extent of glomerular or tubular injury (Bellomo et al.

2004). Recently some promising results were published on novel biomarkers, which could detect AKI after cardiac surgery faster, resulting in opportunities for earlier therapeutic interventions. Most of these promising results were however obtained in research in pediatric settings with known timing and amount of renal impact and without comorbidities such as diabetes, CKD and vascular disease (Vanmassenhove et al. 2013). As these confounding factors are more prevalent in adult patients, the results obtained in the pediatric setting may not be generalizable to adults undergoing CPB (Krawchzeski et al. 2011).

In the present prospective, multicenter study we investigated the discriminative power of a panel of novel biomarkers for the early detection of AKI after cardiac surgery in an adult patients group. The results are less conclusive compared to previous studies with pediatric patients (Krawchzeski et al. 2011, Kwiatkowski et al. 2012). From the biomarkers that were measured only s-Cystatin C was significantly different between the AKI and the non-AKI group for all the different time-points. For u-KIM-1, a significant increase was observed only at 3h post-op, while for u-NGAL and u-Cystatin C, the mean concentrations were significantly different at time-points 3h post-op, 6h post-op and 12h post-op (u-NGAL) and 6h post-op, 12h post-op and 24h post-op (u-Cystatin C).

The AUC scores, representing the discriminative power of the individual biomarkers, are relatively low and were statistically significant only for u-NGAL and s-Cystatin C (at time point 3h post-op) and for u-NGAL, u-Cystatin C and s-Cystatin C (at time points 6h post-op and 12h post-op). The AUC score of the combined biomarkers ranged from 0.612 to 0.787, with only s-Cystatin C being a significant predictor for AKI at all the time points, except for 24h post-op.

When adding the different biomarkers to a clinical model (consisting of pre-op eGFR, BMI and duration of CPB), only s-Cystatin C (at time points 3h post-op, 6h post-op and 12h post-op) and u-Cystatin C (at time point 12h post-op), improved the discriminative power of the clinical model significantly.

These findings are similar to the results obtained by Han et al. in 2009, investigating the predictive power of u-KIM-1, u-NGAL and u-NAG in an adult cardiac setting (Han et al. 2009). The AUC scores for u-NGAL to detect AKI after cardiac surgery are also comparable to previous findings in similar settings (Wagener et al. 2008; Koyner 2010, 2012a). Unfortunately none of these studies investigated the additional power of the biomarkers when added to more

customary clinical models. Although the positive discriminative power of the single biomarkers or a panel of novel biomarkers is promising, the clinical utility is limited if it is no better than the classically used clinical models (Vanmassenhove et al. 2013). The added predictive ability of biomarkers above a clinical model was investigated in a pediatric setting, with positive results for almost all investigated biomarkers: u-NGAL, interleukin 18 (IL-18), liver type fatty acid binding protein (L-FABP) and u-KIM-1. However, the clinical model only consisted of age and duration of CPB, without taking into account the eGFR values (Krawczeski et al. 2011).

The most promising results in the present study are found to be for s-Cystatin C. These findings are in accordance with previous studies, investigating the added predictive value of s-Cystatin C above the RIFLE and AKIN classification methods for AKI after cardiac surgery in adults (Magro and Vattimo Mde 2013; Shilipak 2011, Zhang et al. 2012, Zapitelli et al. 2011). However, Bagshaw and Bellomo concluded in their review on s-Cystatin C that the results are inconsistent in adult cardiac patients and more research is needed (Bagshaw and Bellomo 2010).

Based on the literature and previous in-house research, it was rather surprising that u-KIM-1 did not exhibit any discriminative power in the detection of AKI and only a small significant difference is seen between the AKI and non-AKI group at only one time point, i.e. 3h post-op. AUC curves in the different statistical models used were at no time point significant for u-KIM-1, in contrast to previous studies conducted in adult cardiac patients, reporting significant AUC scores between 0.60 and 0.90 (Han et al. 2009, Liangos et al. 2009, Liang et al. 2010, Koyner et al. 2010). Possible explanations for this discrepancy are the rather small patients groups used in the previous studies (ranging around 100 patients), the different definitions used to distinguish between AKI and non-AKI and differences in underlying comorbidities in the patient groups. In a recent study of Parikh et al. (Parikh et al. 2013), an association between u-Kim-1 and AKI was seen, with a peak elevation at 2 days post-op. However, the association did not remain significant after adjusting for other biomarkers and only moderate discrimination for AKI was observed for the panel of biomarkers. In our study the latest time point at which the different biomarkers were tested was 24h post-op, making it impossible to investigate the discriminative power of

the biomarkers at later time points, comparable with the study of Parikh et al. (2013).

Our study has several strengths. First of all, in this multi-center prospective study we employed a rigorous protocol for collection of samples and of demographic and clinical data, followed by a blinded measurement of the biomarkers and other clinical parameters. Secondly, as the samples for the biomarker measurements were collected at similar time points as the collection of samples for the routine analyses (such as s-CRT and UO, which are used in the definition of AKI) a precise determination of the temporal rise in biomarkers in comparison with the changes in s-CRT and UO was possible. Thirdly, a rather robust patient group of over 250 patients was enrolled. Fourthly, patients were selected in a routine clinical setting and were not selected based on morbidity, which makes our findings representative for everyday clinical practice.

On the other hand, this study does have important shortcomings. First of all, some important data on comorbidities (such as diabetes mellitus incidence) are lacking, making it impossible to adjust biomarkers' performance for these parameters. Secondly, the definition of AKI was based on the elevation of s-CRT. This could possibly have as a consequence that patients, suffering from sub-clinical AKI, are not categorized in the AKI group, although these patients may exhibit elevations in early biomarker concentrations. As a consequence, the association between the clinical parametes (such as BMI, CPB duration and eGFR) with AKI might be biased. Thirdly, the incidence of RRT and mortality in the patient group was rather low, making it difficult to adequately assess the performance of the biomarker and the models in the prediction of these clinical outcomes. The statistical models which are used (AUC) are sensitive to sample size and incidence of predictive outcome.

Moreover, up till date, we did not examine if there was a difference between data gathered in the two hospitals. This difference could be an additional random effect, which could influence the estimation of the variances. Further statistical analysis is needed to elucidate the effect of the multicenter design on the results of this study.

Future research is needed to validate the discriminative power of in particular s-Cystatin C, in a large cohort (with higher incidence of RRT and mortality), taking into account more underlying comorbidities such as diabetes mellitus.

Our study is one of the first to investigate a panel of biomarkers (u-KIM-1, u-NGAL, u-Cystatin C and s-Cystatin C) in a multicenter prospective setting of adult patients undergoing cardiac surgery requiring CPB. A rather low association of u-KIM-1 and u-Cystatin C with AKI was observed, while both NGAL and s-Cystatin C demonstrated higher associations with AKI. The discriminative power for AKI of the different biomarkers was moderate, with only s-Cystatin C showing significant added value when combining with the clinical model (consisting of CPB duration, BMI and eGFR).

7 Summary and general discussion

The aim of this thesis was to contribute to the next step in the implementation of these new biomarkers for AKI. In **chapter 2** and **chapter 3**, we focused on the determination of the most ideal collection and storage conditions prior to the biomarker measurements. In **chapter 4** the goal was to establish reference values for these novel biomarkers for healthy subjects with special attention for age and gender. In the following chapters we investigated the utility of these biomarkers in different clinical settings. **Chapter 5** was attributed to the research we conducted on the association between KIM-1 and urinary cadmium concentrations. Finally, in **chapter 6** we investigated a panel of novel biomarkers in an attempt to detect AKI after cardiac surgery requiring a cardiopulmonary bypass.

The AKI Network defined AKI as a functional and structural disorder or signs of renal damage including any defect, detected through blood and urine test or tissue imaging, that is present for less than 3 months (Bellomo et al. 2004). The structural damage can take place in the different parts of the nephron (i.e. the glomerulus, the proximal and the distal tubule) (de Geus et al. 2012). AKI is associated with worse patient outcomes characterized by higher mortality, prolonged duration of hospitalization, need for RRT and increased risk of developing CKD (Bagshaw et al. 2005, Ponte et al. 2008).
Although modern medicine has advanced enormously over the last few decades, the outcomes from AKI have remained relatively unchanged (Cruz and Ronco 2007). This stagnation can partly be attributed to the diagnostic tools that are applied in the detection of both AKI and CKD. Both conditions are diagnosed based on formulas estimating the glomerular filtration rate (eGFR) and do not take into account actual renal tubular damage. Therefore they are not appropriate diagnostical tools. Instead, they only indirectly reflect kidney damage (McCullough et al. 2013) without providing any information on the etiology of the AKI (Koyner et al. 2012a). The AKI classification systems (AKIN and RIFLE) are based on the eGFR (estimated by the s-CRT levels) and UO while the classification of CKD uses s-CRT in combination with albuminuria (Bellomoet al. 2004, Mehta et al. 2008, KDIGO 2013). Although these classification systems are until now the gold standard in the clinic and are associated with length of hospital stay and mortality (Koyner et al. 2012a), they exhibit some major

shortcomings. First of all the functional markers that are used in the classification systems are subject to extrarenal influences. Muscle mass, volume status and use of medication influence s-CRT (Slocum et al. 2012), while UO can be altered through the use of diuretics and dehydratation (McCullough et al. 2013). Secondly the classification systems require baseline values of the different biomarkers, which is often impossible in ICU settings.

The major problem however lies in the fact that a rise in the functional biomarkers, used in the classification system, will occur only after a 50% loss of kidney function has already occurred (Martensson et al. 2012). When put in a time frame, this delay in the rise of functional biomarkers means there is a gap of 24h to 48h between the actual initiation of renal damage and the detection of AKI (Koyner et al. 2012b), impairing early therapeutic intervention (**Figure 7.1**).

During this gap, which is referred to as the subclinical phase of AKI, cell damage will occur before any clinical dysfunction is observed (Honoré et al. 2012). This structural damage will cause many genes to be up-regulated in the kidney cells, resulting in an increase in protein products in the urine and/or plasma. As these structural alterations initiate in a much shorter time period after the renal injury, these proteins are potential biomarkers for earlier diagnosis of AKI (de Geus et al. 2012.)

As the specific place of up-regulation or secretion of these potent biomarkers is known, such markers would allow the discrimination between different etiologies of AKI in a clinical environment, which would in turn result in a more appropriate or adjusted treatment (Devarajan et al. 2003). These understandings, together with the possibilities of innovative techniques such as genomics and proteomics, have led to the discovery and characterization of 'earlier' biomarkers for AKI, such as NGAL, KIM-1, NAG and Cystatin C. A detailed description of the different biomarkers studied in this thesis is given in the general introduction.

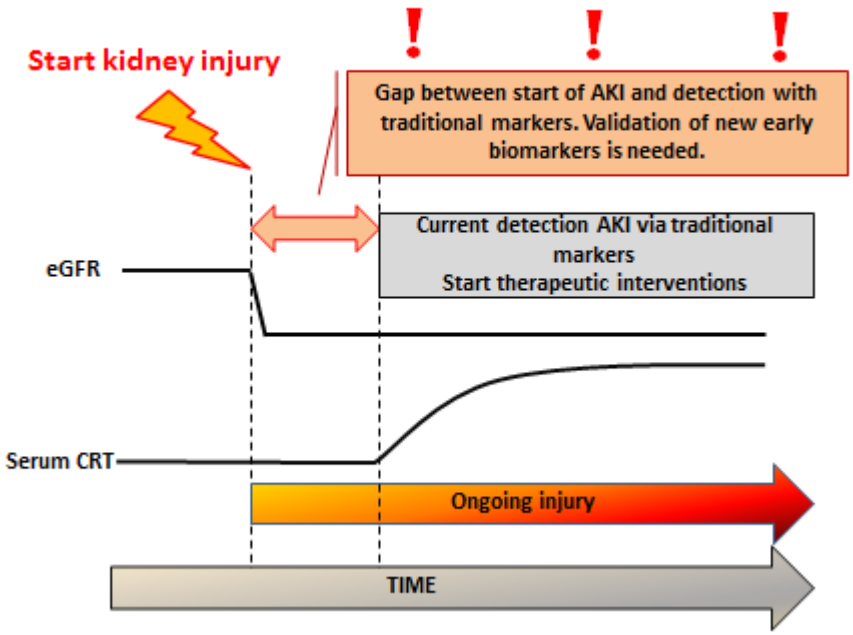


Figure 7.1 Time frame: at the start of kidney injury novel biomarkers are required allowing early detection of AKI. The figure represents the timing and changes in eGFR and the time-delayed rise in serum creatinine (CRT) levels after severe renal injury. Several novel biomarkers are upregulated prior to the changes in the traditional markers (creatinine and urine output). This early diagnosis could provide a temporal advantage for the initiation of therapeutic interventions and possibly improve outcomes in the setting of AKI. Abbreviations: eGFR = estimated glomerular filtration rate; CRT = creatinine; AKI = acute kidney injury

In **Figure 7.2** the anatomical site of action is illustrated. Functional biomarkers (representing injury-induced functional changes to the nephron) are shown in orange panels, structural biomarkers (representing early injury-induced structural changes in the nephron, even before functional changes occur) in blue panels.

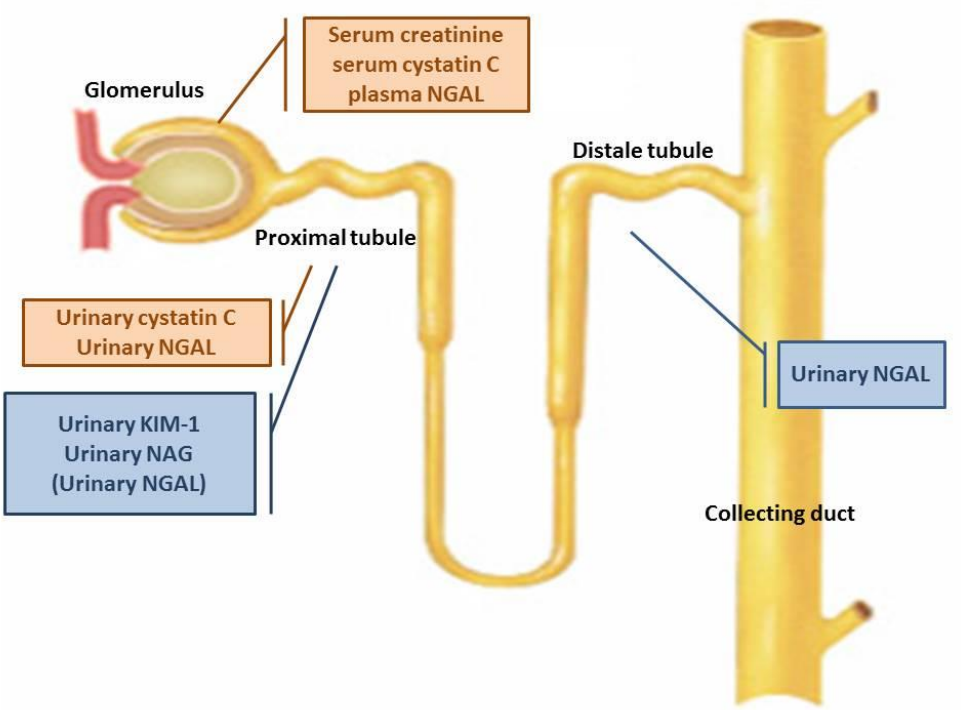


Figure 7.2 Biomarkers of acute kidney injury: anatomic site of action. The different biomarkers discussed in this thesis are stratified according to their anatomic site of action. Biomarkers can either be upregulated in the serum or urine due to functional changes (i.e. functional biomarkers; in orange panels) or due to structural changes (i.e. structural biomarkers; in blue panels).

Collection and storage conditions prior to biomarker research

In order to validate novel biomarkers for kidney damage, it is important to standardize the collection and storage conditions of the urine and plasma samples prior to measurement. This will optimize the comparability of the results from different labs, different patients and/or of patients as a function of time. It will facilitate the ultimate evaluation of the clinical utility of a potent biomarker.

In **chapter 2** we evaluated the possible effect of urinary pH on the measurement of u-KIM-1 values. **Figure 2.1** shows that KIM-1 itself is not pH-dependent, i.e. the urinary concentration remains the same when the samples are adapted to different pH-values. However, the measurement technique itself is pH dependent. On the one hand, when samples were adapted to different pH values during the storage period, but readapted to a neutral pH value just before measurement, KIM-1 recovery was close to 100%. However, when samples were not readapted to a neutral pH before measurement, recovery was significantly less. The pH-dependency of the ELISA measurement can be attributed either to the pH-dependency of the activity of HRP (Straus 1964), or to the fixation itself (Hundgen 1977). This effect of pH on the avidity of antibody-epitope interactions was described before by using QSAR (quantitative structure−activity relationship) and molecular modeling approaches (Dejaeghere et al. 2005). According to these findings, we recommend urine samples to be adapted to pH values near neutral pH, i.e. between pH 6 and 8, the range at which the KIM-1 measurements are not affected by the urinary pH, prior to KIM-1 measurement. For the subsequent chapters, we adapted the pH of urine samples that were used for u-KIM-1 measurements in the different study designs according to these recommendations. Moreover, since analogous ELISA kits (purchased at the same firm; i.e. R&D systems) are used to measure u-NGAL and u-Cystatin C, we also adapted the pH of these urine samples, prior to measurement.

In **chapter 3** we further investigated different collection and storage conditions prior to u-KIM-1 measurement. Although the addition of protease inhibitors and centrifugation of the urine samples did not affect the measurement of u-KIM-1, in **Figure 3.1** and **Figure 3.2** it is shown that both the time before freezing and the pre-measurement thawing period affect the KIM-1 measurement. In **Table**

3.1 it is shown that the preferred storage temperature is -80°C, but more importantly, freeze-thaw cycles should be avoided prior to measurement. Similar effects of freezing methods were seen in the measurement of other urinary biomarkers, such as microalbuminuria (Schultz et al. 2000), urinary transferrin and urinary immunoglobulin G (Vittinghus 1990). Storage up to 1,5 years at -80°C did not significantly influence the KIM-1 concentrations. According to these research findings the optimal storage temperature for urine samples, collected for KIM-1 measurements, is -80°C (even for long periods), with a limited number of freeze thaw cycles. Both the time between collection and freezing and the time between thawing and measurement should be kept as short as possible, preferable under 3h.

Reference values for novel biomarkers

The next step in the validation process of the novel biomarkers is the establishment of reference values for a 'healthy' population. In **chapter 4**, we established such reference values for u-KIM-1, u-NAG, u-NGAL and u-Cystatin C. We investigated the effect of age and gender on the normal physiological biomarker concentrations. If necessary age- and/or gender specific reference values were provided. This was done not only for the absolute urinary concentrations, but also for the values that were normalized to u-CRT and to USG (as shown in **Table 4.1**). Except for u-Cystatin C, all the biomarker concentrations increased with increasing age. A constant rate of u-cystatin C levels was observed in other studies as well (Uchida and Gotoh 2002). The other biomarkers (u-KIM-1, u-NAG and u-NGAL) increasing over age can be attributed to the normal physiological aging of the kidney, which is associated with structural and physiological changes in the different parts of the nephrons (Karam and Tuazon 2013, Weinstein and Anderson 2010). Although these reference values will provide a framework for further research in the role these biomarkers may play in the detection of early AKI, it has to be kept in mind that the biomarker concentrations were measured with manual laboratory assays. As there are technical differences across different measurement assays, the use of these reference values is limited to measurements conducted with the same manual assays. Future availability of commercial laboratory platforms for biomarker measurement is needed to obtain analytical harmonization.

Establishment of reference values for biomarkers measured with these standardized laboratory platforms will be the next step to facilitate the use of these novel biomarkers in the diagnosis and prognosis of AKI, in the same way this was done for serum s-CRT values for estimation of GFR (Levey et al. 2006).

Association between urinary kidney injury molecule 1 and long term Cd exposure in humans

In **chapter 5** we investigated the association between u-KIM-1 and u-Cd excretion, in a population that is environmentally exposed for a long time to a low-dose of Cd. For this purpose, we selected a population living near a non-ferrous heavy metal industrial zone in Genk (Belgium), with known elevated Cd levels ((VMM: Mira-T indicator rapport 2008). To ensure the subjects selected for this study had indeed been subject to a long term Cd exposure, we chose to only include patients over 65 years of age. The average age of the participants was 71 years and the average time they lived at their current address was 36 years (SD= 15,4 years). Average U-Cd concentration was 0.80 µg/l (CI= 0.73 – 0.88 µg/l) (absolute urinary concentrations) and 0.76 µg/g CRT (CI= 0.70 – 0.84 µg/g CRT) (corrected for u-CRT). These values are similar to another Belgian studies (Chaumont et al. 2013) in environmentally exposed subjects. According to Prozialeck and Edwards, urinary levels of Cd in non-exposed populations are normally less than 0.5 µg/g CRT and values higher than 1 to 2 µg/g are indicative of exposure or elevated body burden (Prozialeck and Edwards 2012). At these levels of low Cd burden, we found a positive correlation between u-Cd and u-KIM-1 after correction for confounding factors (sex, age, past smoking, BMI and SES), while other classical biomarkers for Cd (albuminuria, proteinuria and BUN) did not correlate with u-Cd. These findings could be explained by the fact that the mean u-Cd levels in the studied group were below the critical threshold associated with renal damage of 2-10 µg/g CRT (Thévenod and Lee 2013). At this threshold, which corresponds with kidney Cd levels of 150-200 µg/g wet tissue, Cd-induced damage is observed in the proximal tubule cells of the kidney. This will lead to a decreased reabsorption function of the proximal tubule cells, which is characterized by an increase in u-Cd and the onset of polyuria and proteinuria (Thévenod and Lee 2013). Indeed, to our knowledge, this is one of the strengths of KIM-1 as a biomarker for early

Cd-induced renal damage. As KIM-1 is a structural biomarker (in contrast to the classical biomarkers, which are functional biomarkers) which is upregulated before either necrosis or apoptosis is observed in the proximal tubule cells (Prozialeck et al. 2009a), it can be detected at Cd-concentrations below the critical threshold, at which the classical biomarkers are upregulated.

In a recent publication, Chaumont et al. state that the urinary excretion of Cd is based on two mechanisms. The first mechanism, the release or secretion of Cd that was accumulated in the kidney tissue, is probably responsible for the u-Cd excretion in industrial workers and experimental animals, who were exposed to high doses of Cd and therefore suffered from high Cd body burden. In patients with low Cd body burden, the second mechanism is more dominant. This mechanism is the glomerular filtration of Cd-Mt, which is followed by the excretion of Cd-Mt, that is not reabsorbed by the proximal tubule. In this mechanism, the urinary output of Cd is determined both by the amount of circulating Cd (which reflects the acute exposure), and by the capacity of the kidneys to filter and reabsorb low molecular weight proteins. They therefore suggest that the association of urinary cadmium with age and urinary proteins is due to physiological variations unrelated to metal accumulation and toxicity (Chaumont et al. 2013). However, KIM-1, the renal biomarker we investigated in our study, is not influenced by possible co-excretion. This co-excretions is indeed observed in classical biomarkers (such as low molecular weight proteins and albumin), as they follow the same glomerular filtration – tubular reabsorption pathways as Cd (Chaumont et al. 2011).

In the analysis of the association between Cd exposure and the concentrations of renal biomarkers for Cd-induced nephropathy, the possible effect of diabetes mellitus is not taking into account. With mean fasting glucose levels of 99,95 mg/dl, it can however be assumed that a certain amount of persons are suffering from pre-diabetes (fasting glucose levels between 100 mg/dl and 120 mg/dl) or diabetes (fasting glucose levels >120 mg/dl). As described in a review of Edwards and Prozialeck (2009), both epidemiologic studies as well as animal studies show an association between Cd exposure, increased blood glucose levels, the development of diabetes and diabetes-related nephropathy. Although the precise mechanisms are not yet fully elucidated, cadmium and blood glucose appear to have a synergetic relation in the development in nephropathy.

Unfortunately, we did not collect details on the prevalence of diabetes mellitus among our population. Moreover, it is a shortcoming of the study that the fasting glucose levels were not taken into account as a possible confounding factor in the analysis of the association between the concentrations of u-Cd and the renal biomarkers.

Although the findings in **chapter 5** show considerable promise for Kim-1 as an early biomarker, this pilot study in humans, in combination with some pioneer work in rats (Prozialeck et al. 2007a, 2007b and 2009a), is only a very first step. Further research is definitely needed to resolve several important questions. First of all, it remains unclear how KIM-1 expression changes in higher levels of cadmium exposure. Secondly, since KIM-1 is detected prior to functional changes in the PTC, it has to be unraveled which mechanisms are responsible for the upregulation of KIM-1 in case of increased Cd burden. Lastly, the possible role of KIM-1 as an early biomarker for Cd-induced renal damage needs to be investigated in large cohorts, after different exposure routes of Cd and in different age classes.

Biomarkers of AKI after cardiac surgery requiring CPB

The clinical setting of cardiac surgery involving CPB is an example in which the early detection of AKI could enhance rapid intervention (Gude and Jha 2012). This hypothetical temporal gain (as shown in **Figure 7.1**) could lead to improved prognosis. It is therefore that, in **Chapter 6,** we assessed the possible role that novel biomarkers, i.e. u-KIM-1, u-NGAL, u-Cystatin C and s-Cystatin C, might play in the early detection of AKI after cardiac surgery involving CPB in a cohort of 259 adult patients. With a high incidence rate (up to 30%) of AKI, this clinical setting is ideal to study the predictive value of novel biomarkers as the timing and amount of renal damage is known and biomarkers, together with other clinical parameters can be obtained before and after the renal insult (Vanmassenhove et al. 2013). Previous studies in pediatric cardiac patients have already demonstrated good predictive values for the biomarkers we studied (Krawchzeski et al. 2011, Kwiatkowski et al. 2012, Peco-Antic et al. 2013, Zheng et al. 2013). The results obtained in our study were however less impressive as the ones obtained in pediatric patients. For u-KIM-1 we only found a significant difference between AKI and non-AKI patients at 3h post op. u-NGAL and u-

Cystatin C only correlated significantly with AKI at 3h post-op, 6h post-op and 12h post-op and at 6h post-op, 12h post-op and 24h post-op, respectively. The best associations were found between s-cystatin C and AKI, with significant associations at all pre- and post-op measurements. When investigating the predictive value for AKI of the biomarkers only moderate AUC scores were found for the different biomarkers. When adding the different biomarkers to a clinical model, consisting of BMI, eGFR and duration of CPB, only u-Cystatin C (at 3h post-op) and s-Cystatin C (at 3h, 6h and 12h post-op) added predictive value to the clinical model in predicting AKI. Although there are several possible explanations for the deviating result compared to the clinical studies published, such as different panels of biomarkers, different definitions for AKI and different clinical panels used, the most plausible explanation is the difference in age. In a patient group of children the amount of comorbidities and confounding factors on renal damage such as older age, diabetes mellitus, CKD, … are kept at a minimum allowing a less complicated association between the current renal insult and the temporal up-regulation of renal biomarkers. This is also confirmed by the fact that more results similar to ours are found in other studies on biomarkers for AKI in adult cardiac patients (Wagener et al. 2008; Han et al. 2009; Koyner 2010; 2012a; Vanmassenhove 2013) .

In our study the most promising novel biomarker was s-Cystatin C, being the only biomarker adding discriminative power to the clinical model. s-Cystatin C is a functional biomarker that indicates the presence of glomerular damage (as shown in **Figure 7.2**). In previous studies, conflicting results were obtained concerning this biomarker in adult cardiac patients (Bagshaw and Bellomo 2010), which can again be attributed to differences in patient groups, in the definition of AKI, in the timing of sampling and in the different clinical models used. It is clear that more research is needed to elucidate the exact predictive power of novel biomarkers in the setting of cardiac patients and until then, the detection of AKI can only be based on changes in s-CRT and UO, although our findings corroborate the added value of using cystatine C as an alternative marker for s-CRT.

Future perspectives

As described by a recent article of Horvath at al. (2013) the validation of novel biomarkers is a complex cyclic process, consisting of several phases, which are interrelated. Although we contributed to the evaluation of the analytical and clinical performance, further study is required. To improve the cost effectiveness of the use of the novel biomarkers, commercial laboratory platforms need to be developed. Once commercial laboratory platforms are available for the different biomarkers, it is necessary to re-establish reference values for the different age groups, comparable to the reference values we established using the manual measurement techniques (**chapter 4**). Preferably, manufacturers should try to first standardize their assays to a standard of higher order, conform the CE Guideline 98/79/EG on in-vitro diagnostics.

Subsequently, the clinical performance and clinical effectiveness of the novel biomarkers need to be tested in different pathologies and for various patient groups. As previous results are rather conflicting with both positive and negative results for the individual biomarkers as well as for panels of biomarkers, further research is needed to investigate which biomarker (or panel of biomarkers) can add predictive value to the definitions and clinical models that are used nowadays in the clinic to detect AKI. In the setting of cardiac surgery involving CPB, we need to further elucidate the predictive value of s-Cystatin C, alone and in combination with other biomarkers, in the prediction of post-surgical AKI. A large cohort is needed, with higher incidence rates for post-surgical RRT need and mortality, in order to test the predictive value of the biomarker(s) in these alternative endpoints. Another requirement is to collect more patient details on comorbidities such as diabetes mellitus, to investigate the possible confounding effect on the predictive value of the biomarkers in the early detection of AKI.

To further ameliorate the prediction of AKI after cardiac surgery involving CPB, the panel of biomarkers has to be broadened with other novel biomarkers for renal damage. The addition of L-FABP and IL-18, which have been investigated recently as predictive renal biomarkers in this clinical setting (Mao et al. 2013; Vanmassenhove 2013), should be added to the predictive clinical model.

After the positive association that was found between U-Cd and U-KIM-1 (**chapter 5**), future research is needed to investigate whether there is a causal

relationship between Cd exposure and the up-regulation of KIM-1 in the kidney. Although animal studies are the ideal setting to investigate this relationship, future epidemiological studies in humans are recommended as well, taking into account possible confounding factors and comorbidities that can influence the relationship between Cd and Kim-1.

8 Nederlandse samenvatting

Momenteel is het vroegtijdig opsporen van acuut en chronisch nierfalen gebaseerd op de stijging van de creatinine concentratie in het serum in combinatie met een daling in urine volume. Deze detectiemethode heeft het nadeel dat de vermelde veranderingen pas enkele uren tot dagen na het eigenlijk ontstaan van het nierprobleem waargenomen kunnen worden. Er is dan ook nood aan nieuwe, snellere detectiemethoden of biomerkers. Via proteomische onderzoeksmethoden werden een aantal eiwitten in het bloed en de urine ontdekt, die mogelijkerwijze als biomerker voor nierschade kunnen dienen. Deze eiwitten blijken in experimenten bij dieren en mensen immers verhoogd tot expressie te komen in geval van nierschade. Deze nieuwe biomerkers dienen echter gevalideerd te worden: er moet nagegaan worden op welke wijze ze het best gemeten worden, wat normale waarden zijn bij gezonde mensen en bij welke pathologieën en in welke patiëntengroepen ze verhoogd zijn. In deze doctoraatsthesis hebben we ons gericht op het validatieproces van enkele nieuwe biomerkers voor nierschade, nl. KIM-1, NGAL, NAG en Cystatine C. We hebben vooreerst aangetoond dat KIM-1, gemeten in de urine, niet afhankelijk is van de pH van de urine, maar de commerciële kit die gebruikt wordt voor de meting wel. Het is dan ook van belang om urinestalen voor de meting van KIM-1 op een pH rond 7 te brengen (**hoofdstuk 2**). Enkele andere verzamel- en opslagspecificaties voor de urinestalen waarin de biomerkers gemeten worden (zoals het toevoegen van protease inhibitoren, centrifugatie, maximale duur voor invriezen en de invriestemperatuur en –periode) zijn vervolgens onder de loep genomen, en richtlijnen hieromtrent zijn uitgewerkt (**hoofdstuk 3**). In het volgende deel van ons onderzoeksproject, hebben we urinestalen van verondersteld gezonde proefpersonen van alle leeftijden verzameld om zo referentiewaarden voor KIM-1, NGAL, Cystatine C en NAG op te stellen (**hoofdstuk 4**). Uit dierproeven van andere onderzoeksgroepen werd een positieve associatie afgeleid tussen de blootstelling aan het zware metaal cadmium, waarvan geweten is dat het schadelijke effecten heeft op de nier enerzijds en verhoogde KIM-1 concentraties in de urine anderzijds. We onderzochten een proefgroep van oudere personen die wonen in de omgeving van een industrieterrein met gekende verhoogde cadmiumwaarden in de bodem en de lucht en dus gedurende een lange periode blootgesteld zijn aan cadmium. Eenzelfde associatie werd aangetoond tussen cadmium en KIM-1 in de urine van

deze proefpersonen (**hoofdstuk 5**). In het laatste deel van dit doctoraat werd onderzocht of de nieuwe biomerkers een rol kunnen spelen in de vroege detectie van acuut nierfalen, na het ondergaan van cardiale chirurgie waarbij extra-corporele circulatie noodzakelijk is. Hierbij werden de concentraties van de urinaire biomerkers voor en op verschillende tijdstippen na de operatie vergeleken tussen volwassen patiënten die acuut nierfalen hadden ontwikkeld of niet. Voor elke gemeten biomerker (urinair KIM-1, NGAL, cystatine C en serum cystatine C) kon een associatie met acuut nierfalen gevonden worden op tenminste één tijdstip. Alleen serum Cystatine C bleek echter een voorspellende meerwaarde te hebben bovenop een klinisch model, dat werd samengesteld uit klinische parameters die momenteel reeds gebruikt worden in de detectie van AKI in het ziekenhuis (**hoofdstuk 6**).

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Curriculum vitae

Curriculum Vitae

Valérie Pennemans werd geboren op 20 maart 1982 te Lommel. In 2000 behaalde ze haar diploma Algemeen Secundair Onderwijs (ASO) in de studierichting Latijn-Wiskunde aan het Sint-Jozefscollege van Lommel. Datzelfde jaar ving ze haar studies aan aan de Katholieke Universiteit Leuven (KUL) waar ze in 2004 afstudeerde met onderscheiding als licentiaat Lichamelijke Opvoeding. In de periode 2004 – 2005 was ze werkzaam als medisch vertegenwoordiger voor chirurgische instrumenten. Tussen 2005 en 2006 doceerde ze Lichamelijke opvoeding, wiskunde, fysica, chemie, biologie, wetenschappelijk werk aan leerlingen van de 2^{e} en 3^{e} graad van volgende middelbare scholen: Salus Nostra Instituut in Mol en het Heilig Hart Instituut Maria Opdracht in Maasmechelen. In september 2006 startte ze aan de Katholieke Universiteit als praktijkassistent in de Onderzoeksgroep Cardiovasculaire en Respiratoire Revalidatie van het departement Revalidatiewetenschappen. Tijdens de periode 2008 – 2013 was ze werkzaam aan de Uhasselt en bereidde haar doctoraatsthesis binnen de onderzoeksgroep Fysiologie van de faculteit Geneeskunde en levenswetenschappen. Tijdens deze periode was ze lid van het onderwijsteam voor diverse blokken, zoals Nier en Homeostase, Circulatie, Zieke organen, fysische modellen,… . Ze volgde tevens Verschillende cursussen zoals proefdierkunde (module 1 en module 2), blackboard en Transport en communicatie tussen cellen en weefsels. De resultaten die werden bekomen tijdens dit doctoraat werden grotendeels gepubliceerd in internationale tijdschriften en werden gepresenteerd op verschillende (inter)nationale meetings.

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Oral presentations

- Kidney Injury Molecule 1 (KIM-1): an early biomarker for kidney damage in chronic cadmium exposure in humans.
  Cadmium Symposium 2012. Sassari (It.) 8 june 2012.
- New markers in kidney function testing.
  Annual KBVKC-SRBCC symposium -State of the art in diagnostic markers. Groot-Bijgaarden (B) 18 April 2013.

Selected poster presentations

- Effect of pH on the stability of Kidney Injury Molecule 1 (KIM-1) and on the accuracy of its measurement in human urine.
  Pennemans, V., De Winter, L.M., Faes, C., VanKerkhove, E., Reynders, C., Rigo, JM., Swennen, Q., Penders, J**.**
  European Biomarker summit, Florence (It.) 9 – 10 november 2010.
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- Novel urinary biomarkers for proximal tubular damage: is age an issue? **Pennemans, V.,** Swennen, Q., Rigo, JM., Faes, C. Penders, J. 5th ISN Update Course in Nephrology, Dubai (UAE) 9-12 December 2011.
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