GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de biomedische wetenschappen: milieu en gezondheid

Masterproef
Validation of early biomarkers in urine: studies in healthy subjects and cardiac patients

Promotor :
Prof. dr. Jean-Michel RIGO
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Masterproef voorgedragen tot het bekomen van de graad van master in de biomedische wetenschappen , afstudeerrichting milieu en gezondheid

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Maastricht University

universiteit hasselt

KNOWLEDGE IN ACTION

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ABSTRACT

Background   The novel urinary biomarkers for the early detection of kidney injury after ischemic or toxic insults have recently been well studied, including neutrophil gelatinase associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), cystatin C (Cys-C) and N-acetyl-β Dglucosaminidase (NAG). The lack of reference values have greatly impeded progress in the diagnosis and treatment of patients with AKI and have a detrimental effect on the possibly outcomes of clinical trials of cardiac surgery.

Objective   The first objective of this study was to estimate the normal value of urinary NGAL, KIM-1, Cys-C and NAG in a healthy population.
The second objective was to evaluate the diagnostic performance of urinary biomarkers in a prospective pilot study including adults after cardiopulmonary bypass (CPB) surgery.

Methods   The age study population consist of 337 healthy non-smoking subjects (191 female, 146 male ranging from 4 months to 90 years). Urine samples were obtained at routine visit to the ZOL hospital. ELISA was used to measure urinary NGAL, KIM-1 and Cys-C. BIOQuant N-acetyl-B-D-Glucosaminidase (NAG) assay was used to measure NAG.

The prospective pilot study consists of 25 adults (7 females, 18 males ranging from 51 to 84 years) who required CPB surgery. Urine and blood samples were obtained before and 3, 6, 12 and 24 hours after CPB surgery. Patients were divided into the AKI and non-AKI groups according to developed AKI after surgery, AKI was defined as a ≥ 0.3 mg/dl increase in sCr from the base line within 48h after the surgery. Urinary KIM-1 and NGAL were measured using ELISA assay.

Results   Age study: significant age related differences were found for KIM-1, NGAL, Cys-C and NAG. In addition, significant differences between gender were found for KIM-1, NGAL and NAG. Cardiac study: 25 patients were enrolled, 20% developed AKI. Urinary KIM-1/Cr level was significantly increased 24h post surgery and was significantly associated with AKI within 24h ,while urinary NGAL level did not show such a performance.

Conclusions   Based on our results, further study in larger population with biomarker panel is needed for the evaluation of normal values with variance factors as well as to establish the prediction value to diagnose AKI for long and short outcomes after cardiac surgery.

INTRODUCTION

1.1 Acute Kidney Injury

Acute Kidney injury (AKI) is a common clinical syndrome, which refers to sudden decline in kidney function that causes disturbances in fluid, electrolyte, and acid-base homeostasis. This is due to loss in clearance of small solutes and a decreased glomerular filtration rate (GFR). Severe AKI may even result in oliguria or even anuria [1].

A recent multinational, multicenter and epidemiological study of AKI in critically ill adult patients reported an overall hospital mortality rate of 60% of those who survived to hospital discharge, 13% of survived patient remained dialysis-dependent. Also, they found that the major reason for intensive care unit (ICU) admission of patients with AKI was medical in 58.9% of patients and surgical in the remaining 41.1%. Furthermore, they reported that cardiovascular surgery was the most common diagnostic grouping with AKI, followed by medical respiratory surgery, gastrointestinal tract surgery and sepsis [2].

AKI can result from a toxic or obstructive insult to the renal tubule, decreased renal or intra-renal perfusion, tubule-interstitial inflammation and edema, or primary reduction in the filtering capacity of the glomerulus. Ischemia and toxins, usually in the setting of sepsis, account for the largest number of cases of AKI [3].

Several pathophysiological mechanisms can lead to AKI after toxic and ischemic insult. These include (*a*) alterations in renal perfusion resulting from loss of auto-regulation and increased renal vasoconstriction (*b*) tubular dysfunction and cell death by apoptosis and necrosis (*c*) desquamation of viable and dead cells contributing to intra-tubular obstruction (*d*) metabolic alterations resulting in transport abnormalities that can lead to abnormalities of tubule-glomerular balance and (*e*) local production of inflammatory mediators resulting in interstitial inflammation and vascular congestion [4].

Especially on a cellular level, injury results in rapid loss of cytoskeletal integrity and cell polarity, with mislocalization of adhesion molecules and other membrane proteins such as the Na^+K^+-ATPase and β-integrins, shedding of the proximal tubule brush border, as well as apoptosis and necrosis [5] (Figure 1). This injury to the epithelium results in the generation of inflammatory and vasoactive mediators, which can act on the vasculature to worsen the vasoconstriction and inflammation. Hence, inflammation contributes in a significant way to the pathophysiology of AKI [6].

Conversely to the heart or brain, the kidneys completely recover cells that were lost because of an ischemic or toxic insult that result in cell death, although it is becoming increasingly recognized that there are longer term detrimental effects of even brief periods of AKI [7].

When the kidney recovers from acute injury it relies on a sequence of events, including epithelial cell spreading and migration to cover the exposed areas of the basement membrane, cell de-differentiation and proliferation to recover cell number, followed by differentiation, which results in restoration of the functional integrity of the nephron (Figure 1-B). The contribution of non-tubular progenitor cells to this repair of the tubules is likely to be minimal if any at all. Numerous studies suggest that there is a very delicate and dynamic relationship between tissue repair and progression or regression of renal injury [8].

AKI as classified by the RIFLE criteria (acronym for Risk, Injury, Failure, Loss and End stage) has been reported to occur in approximately 30% of critically ill patient and is common after major

surgery such as open heart surgery. Although the AKIN (Acute Kidney Injury Network) criteria, based on the RIFLE classification system are being used increasingly in hospitals by clinicians and are presumed to improve sensitivity in diagnosing AKI (Table 1), they have not been shown to improve the ability to predict outcomes [10, 11].

To date, the conventional biomarkers (creatinine, urea, GFR and urine output) have serious limitations as early detectors of AKI. However, the current classification is still entirely based on an increase in serum creatinine (sCr) or decrease in urine volume.

Serum Creatinine is a suboptimal marker following injury, the levels are often not reflective of GFR owing to a number of renal and non-renal influences on creatinine levels. In the setting of AKI, the delay between changes in sCr and changes in GFR inhibits the ability to accurately estimate timing of injury and severity of dysfunction following injury [10, 12]. There is a need for early and specific renal biomarkers that would enable the early diagnosis of AKI and timely intervention and treatment.

1.2 Cardiopulmonary bypass

Cardiopulmonary bypass (CPB) is the most frequent major surgical procedure performed in hospitals worldwide since 1950's, with over a million operations undertaken each year. CPB has allowed surgeons to empty the heart of blood, stop it from beating as necessary, open any desired chamber, and safely carry out reparative procedures. CPB surgery has improved the patients quality of life, although serious postoperative risk factors for complications still exist, such as infection, respiratory failure, shock states and multiple organ failure [15, 16-17].

AKI is a common and serious complication in critically ill patient after CPB surgery [2]. It has significant implications on both short- and long-term outcomes, encountered in 30–40% of adults and children after CPB surgery [12, 17-18] with high persistently mortality in patient who require dialysis of up to 60-70% [19]. Furthermore, Loef et al. [20] confirmed that patients with postoperative AKI not only showed an increase in hospital mortality, but also had higher mortality rates five years later.

Several studies have explored the risk factors associated with the development of post-operative AKI-CPB [14, 18-19]. In addition, knowledge of risk factors may help to identify the patient with serious risk for AKI after CPB surgery, including factors related to patients and procedures, such as advance age, sex and CPB duration (Table 2).

The pathogenesis of AKI associated with CPB can be divided into pre-operative, intra-operative and post-operative events [14, 19-21].

Pre-operative events: Patients who had recent myocardial infarctions or sever valvular disease, a pre-existing renal problem, often present the major pro-operative events (Table 3). The pre-existing renal problem may worsen pre-operative events by the use of medication such as diuretics, angiotensin receptor blockers and non-steroidal anti-inflammatory drugs, which can affect the auto-regulation of renal blood flow.

The intra-operative events: this period presents the most critical events when the patient undergoes CPB surgery that would lead to hemodynamic effects, and activate immune responses [19, 21]. Pro-inflammatory events during CPB include operative trauma, contact of the blood components with the artificial surface of the CPB circuit, ischemia-reperfusion injury and endotoxemia. Hence, inflammation plays a central role in the development of ischemic kidney injury and it is thought that

the systemic inflammatory response caused by CPB has similar damaging effects. Furthermore, CPB may further predispose patients to ischemic kidney injury through the generation of free hemoglobin and iron from hemolysis that occurs during CPB [19, 22-23]. Finally, a recent study has provided evidence that supports the association between prolonged CPB duration and morbidity and mortality with AKI after surgery [24].

The post-operative events such as vasoactive agents, hemodynamic instability, sepsis, volume depletion and exposure to nephrotoxic medication all are critical factors that can affect kidney and lead to AKI [14]. Perhaps the most critical factor is post-operative cardiac performance and need for mechanical support of left ventricular (LV) function. Thus, in patients at high risk of AKI after presence of post-operative LV dysfunction, the vulnerable kidney is subjected to marginal perfusion pressures and extension of ischemic injury. Moreover, any additional complications such as infections may lead to tubular injury [19].

1.3 Biomarkers

AKI is diagnosed by increase in sCr measured over time in the clinical status. sCr is widely known to be a suboptimal biomarker after kidney injury; it often does not reflect GFR or the degree of tubular injury. Also sCr concentration is greatly influenced by numerous non-renal factors such as: body weight, race, age, total body volume, drugs, muscle metabolism and protein intake [25]. Additionally, patients with cardiac disease often have decreased muscle mass, and that will reflect on the diagnosis [26].

Under physiological conditions, 70% of urinary proteins originated from the kidney, whereas 30% of urinary proteins are plasma proteins. Furthermore, the kidneys are subject to aging as it shows as decrease in function with age. Therefore urinary proteins generally reflect normal kidney physiology [27, 28].

Neutrophil Gelatinase-Associated Lipocalin (NGAL) is a 25 kDa protein and a member of the lipocalin family. Lipocalins include a class of proteins that can be identified by eight β-strands that form a β-barrel defining a calyx, which binds and transports low molecular weight molecules. NGAL binds siderophores with high affinity; and siderophores trap iron with high affinity as well [29, 30-31]. Naturally, NGAL is expressed in very low levels by neutrophils and various epithelial cells including renal proximal tubule. It is expressed in several human organ including lung, stomach, and colon [27]. Goetz et al. [32] discovered NGAL for the first time, while subsequent studies provided illustration of massive up-regulation and release of NGAL by injured distal nephron; and thus it can be measured in plasma and urine [30, 33].

Evidently, NGAL in AKI appears in plasma derived from distal tubular back leakage into the blood and from extra-renal sources as a result of organ cross-talk of the injured kidney. On the other hand, NGAL is excreted into the urine after kidney injury via collecting ducts and ascending loop of Henle or by excessively filtered plasma NGAL [34, 35]. Hence, NGAL in urine has been investigated as early biomarker for AKI in preclinical and clinical setting including ICU and CPB patients [12, 27-29]. In recent studies, in patients who developed AKI after CPB surgery, urinary NGAL levels were significantly elevated within 1 h to 3 h after CPB surgery [36]. These finding in adults were widely confirmed in paediatric patients within 2 h after CPB surgery [12, 26]. Finally, in a meta-analysis study urinary and plasma NGAL were considered as a vigorous early biomarker for AKI [37].

Interleukin-18 (IL-18) is a proinflammatory cytokine produced as inactive form of 23 kDa by monocytes, macrophages, and proximal tubule epithelial cells [18, 38-39]. The intracellular protease caspase-1 cleaves the inactive form IL-18 into the mature active IL-18 form with high specificity after inflammatory states as well as proximal tubular injury. Melnikov et al. [40] has demonstrated IL-18 associated with AKI. A subsequent study established urinary IL-18 as early diagnostic biomarker for AKI 24 h before the sCr level increased. Moreover they found that urinary IL-18 level was an independent predictor of death [41]. Finally, recent studies stressed that urinary IL-18 dramatically increased in paediatric patients 6 h after CPB [26, 42].

Cystatin-C (Cys-C) is a low molecular weight (13 kDa) protein that is produced by all nucleated cells in the body. It is a member of lysosomal protease cystein inhibitors [10, 11-43]. Cys-C is involved in the extra-cellular proteolysis, antiviral and antibacterial activity, and immune system [44]. Obviously Cys-C is filtered via the basal membrane of the glomeruli, then Cys-C is reabsorbed immediately and degraded in the proximal tubules, and thus it will not be secreted or reabsorbed into plasma. Therefore it is normally not be found in urine and can be considered as an endogenous biomarker for GFR [44, 45-46]. In addition Cys-C levels in blood are not affected by gender, age, race, or muscle mass, hence it would be a more accurate biomarker of GFR than sCr [11, 27].

Urinary Cys-C is a useful early biomarker in the early diagnosis of AKI after cardiac surgery for adult patients according to earlier study [45, 46]. The authors found that Cys-C levels were increased after 6 h following the surgery. Moreover, Wald et al. [43] have found that elevated levels of plasma Cys-C within 2 h after CPB surgery. Given this, it is widely accepted that Cys-C is one of the superior novel biomarker for the early diagnosis of AKI following CPB.

Liver Fatty Acid Binding Protein (L-FABP) is a member of a family of small cytoplasmic proteins that facilitate β-oxidation via binding and transportation of fatty acid chain. These proteins are widely expressed in tissues with fatty acid metabolism activity [47, 48-49]. The FABP family consists of nine tissue specific type, including heart, intestine, muscle, adipocyte, epidermis, ileum, myelin, testes and liver. L-FABP (14 kDa) is expressed in the epithelial cells of proximal tubules of the kidney. L-FABP binds fatty acids then transports them to the mitochondria or peroxisomes, where they can metabolised by β-oxidation [50].

The glomeruli filter L-FABP and due to its small size, it will be reabsorbed in the proximal tubules cells. Urinary L-FABP has been identified in preclinical and clinical studies and it becomes apparent that L-FABP expression is up-regulated and urinary excretion levels are elevated after insult in ischemia and toxic-induced AKI [10, 47].

Negishi et al. [51] have demonstrated that urinary L-FABP levels were increased in transgenic mice at one hour, even after only 5 min of exposure of ischemia. In a clinical study, the levels of urinary L-FABP were elevated one day after cardiac surgery [47]. In addition, a recent study reported that urinary L-FABP levels correlated with ischemia occurring after transplantation, and reflecting with clinical outcome in hospital stay [52]. Furthermore, Matsui et al. [53] found increases in urinary L-FABP levels within 3 h after cardiac surgery. Interestingly, another study demonstrated that L-FABP level significantly increased within 6 h after CPB with paediatric patients [26].

Consequently, urinary L-FABP shows promise as an early biomarker of AKI.

Kidney Injury Molecule-1 (KIM-1) is a type 1 transmembrane glycoprotein found on renal proximal tubule epithelial cells. It consists of an extracellular portion with a unique 6-cysteine immunoglobulin-like domain and a mucin-domain. An intracellular highly conserved tyrosine kinase phosphorylation motif is a strong indicator that KIM-1 is a cell signalling molecule [54, 55]. KIM-1 expression is virtually undetectable in normal kidneys, whereas with AKI it is significantly up-regulated [55, 56].

Han et al. [57] found that the ectodomain of KIM-1 (90 kDa) is shed and can be detected in the urine of patients with ischemic AKI. A subsequent cohort study with AKI patients showed that urinary KIM-1 has better prognostic utility than routine biomarkers, such as the urine output and sCr. Also they demonstrated that urinary levels are associated with disease severity degree, composite outcome of dialysis or hospital death as well [58]. Furthermore, Liangos et al. [59] found that levels of normalized KIM-1 were increased rapidly by 2 h after CPB surgery in adult patients with AKI. In contrast, in a study with pediatric patients undergoing CPB surgery, urinary levels of KIM-1 were elevated markedly within 12 h in patients who developed AKI [26].

N-Acetyl-β-glucosaminidase (NAG) is a lysosomal enzyme (130 kDa) found in the proximal tubular epithelial cells. The glomeruli preclude filtration of NAG due to its large molecular weight. Hence, elevated urinary NAG levels can give evidence for kidney glomeruli [58, 11]. Urinary NAG was considered as predicting and a useful biomarker for AKI in ICU patients as articulated by Westhuyzen et al. [60]. Subsequently, another study reported markedly increased urinary NAG levels in elderly patients within the fist operative day after CPB surgery, and values were higher compared to younger patients until the end of the study [61]. Furthermore, a recent study confirmed that urinary NAG levels have utility of AKI prognosis, and also are associated with degree of AKI severity and dialysis requirement [58]. Lastly, urinary NAG levels were increased before sCr in AKI adult patient after CPB [62].

1.4 Objective

The first objective of this study was to estimate the normal value of urinary NGAL, KIM-1, Cys-C and NAG in a healthy population.

Prior studies demonstrated the performance of these biomarkers to be an early diagnostic indicator of kidney injury, irrespectively whether the kidney injury was induced by ischemia or toxicant [33, 39, 40, 45, 47, 56-58]. Should be noted, however that biomarkers have performed well for early diagnosis/prognosis of AKI in the clinical studies, it will need to assess in a broad spectrum for healthy populations [25].

The second objective was to evaluate the diagnostic performance of urinary biomarkers in a prospective pilot study including adults after cardiopulmonary bypass (CPB) surgery.
Several studies in which patients developed AKI after CPB have demonstrated the use of those biomarkers in the early diagnosis of AKI. Notes that, there were an obvious variation in the values [10, 12, 20, 26, 29, 38-55], our interest is to determine the relevance performance of biomarkers in the absence of the increases of sCr.

MATERIAL AND METHODS

2.1 Age study

2.1.1 Selection of participants

Local ethics committee approval was obtained for analysis of routinely collected clinical urine samples, and all the participants were healthy non-smoking population from the Ziekenhuis Oost-Limburg (ZOL), Belgium. The participants were divided in 9 groups according to their age (Table 4) and selected for the study.

2.1.2 Urine samples collection and storage

Urine samples were collected from healthy participants at ZOL hospital lab. The pH of urine samples was adjusted with a pH meter (Ankersmit 420A, Orion, Boston, MA, USA) to range 6.0 - 8.0 by adding a droplet of 1 mol/ml hydrochloric acid or 1 mol/ml sodium chloride. The adjusted urine samples were aliquoted into 2 ml eppendorf tubes (n = 6) and frozen within 2 hours of collection at -80 ºC.

2.1.3 Measurements of urinary biomarkers

Urinary routine analyses of the samples were obtained from the clinical laboratory of the regional hospital ZOL in Genk. Serum creatinine was measured by automated analyzer (Modular® P800-ISE900 System, Roche Diagnostics; Mannheim, Germany) according to the kinetic Jaffe methods [63].

Urinary KIM-1 measurement was performed using sandwich enzyme-linked immunosorbent assay (ELISA) (Human TIM-1/KIM-1/HAVCR Duoset, R&D System; Abingdon, U.K), which was validated for KIM-1 by Chaturvedi et al.[64]. Briefly, the 96-well microplate was coated with 100 µl per well of Capture Antibodies (diluted with PBS), the plate was sealed and incubated overnight at room temperature. The next day, urine samples were thawed for measurement, the plates was aspirated and washed three times with 300 µl per well of wash buffer, then 300 µl per well of reagent diluent was added and incubated at room temperature for 90 min. This was followed by aspirating and washing steps three times as before, samples and standard solution were added (100 µl per well) for two hours incubation. Aspirating and three washing steps were repeated, and 100 µl per well of detection antibodies were added and incubated at room temperature (2 h). Streptavidine-HRP was added (100 µl) for each well after washing steps, and incubated 20 min at room temperature (covered by aluminium foil to protect from light). After washing steps, 100 µl per well of substrate solution was added for 20 min incubation at room temperature, protected from light. Finally, 50 µl per well of stop solution was added to stop the reaction and measurements were performed. The absorbance was measured using BMG LABTECH FLUOstar OPTIMA plate reader (Isogen life sciences, De Meern, The Netherlands) at 450 nm with a correction wavelength of 540 nm. Urine samples were analysed in duplicate and concentration of the urinary KIM-1 was calculated based on four parametric logarithmic standard curves at pg/mL.

The assay to measure urinary NGAL was ELISA (human Lipocalin-2/NGAL, Duoset, R&D System; Abingdon, U.K) and followed the same procedure as of KIM-1. The urine samples were diluted with reagent diluents if necessary, urinary NGAL concentration was calculated based on four parametric logarithmic standard curves at pg/mL.

Urinary Cys-C was measured using ELISA (Biovendor, R&D, Brno, Czech Republic) [65].

Urinary NAG was measured using BIOQuant N-acetyl-B-D-Glucosaminidase (NAG) assay [66].

2.2 Cardiac surgery study

2.2.1 Study design and patient selection

Adult patients aged between 51 to 84 years undergoing cardiac surgery with cardiopulmonary bypass (CPB) system in the department of cardiovascular surgery, Ziekenhuis Oost-Limburg (ZOL, Belgium) were eligible for enrolment in the study from April to August 2012. Exclusion criteria were age under 18 years and patients with acute kidney injury within the prior year. Medical records were reviewed to retrieve hospitalization data, including: age, gender, height, weight, preoperative clinical and laboratory data, intra-operative variables including surgery type, surgery time, CPB time, postoperative data including vasopressin and inotrope medication, clinical laboratory data including sCr measurement within 48 hours.

The study was approved by the ethics committee of the Ziekenhuis Oost-Limburg, Belgium, and all patients gave written informed consent.

2.2.2 Urine and blood samples collection and storage

The urine samples were obtained at the following time points from each patient: preoperatively,3, 6, 12 and 24 hours postoperatively. Blood samples were obtained at the same time point as urine samples, and centrifuged (SORVALL® RT 6000D) at 3000 rpm for 10 min at room temperature. The serum was aliquoted into 2 ml eppendorf tube and stored at -80°C. The pH of urine samples was measured with a pH meter (Ankersmit 420A, Orion, Boston, MA, USA) and adjusted to range 6.0 - 8.0 by adding a droplet of 1 mol/ml hydrochloric acid or 1 mol/ml sodium chloride. Specific gravity for urine samples was measured by refractometry (ATAGO, Japan). The adjusted urine samples were aliquoted into 2 ml eppendorf tubes (n = 6) and stored at -80 ℃ until analysis.

2.2.3 Clinical assay

Measurements of routine biomarkers were performed in the clinical laboratory in ZOL hospital, Genk. An automated analyzer (Modular® P800- ISE900 System, Roche Diagnostics; Mannheim, Germany) was used. The following urinary analyses were performed, according to manufacturer's instructions: creatinine according to the kinetic Jaffe method (compensated, rate blanked), total protein by a colorimetric biuret test and α1Microglobuline based on immunological agglutination. Red and white blood cells (RBC, WBC) were measured in urine samples by means of flow cytometry (UF-100™, Sysmex; Hoeilaart, Belgium). Microalbumin was determined by nephelometer (Immage® Immunochemistry system, Beckman Coulter; Suarlée, Belgium). Hemoglobin, pH and specific gravity were obtained by dipstick (Roche).
In blood samples serum creatinine, urea and total protein were determined as the same assay as urinary analysis.

Estimated Glomerular filtration rates (eGFR) for each patient was calculated using chronic kidney disease epidemiology collaboration (CKD-EPI) formula, as defined GFR for male = 141^{α} x (serum creatinine/0.9)$^{-0.411}$ x (0.993)^{age}, and GRF for female = 144^{α} x (serum creatinine/0.7)$^{-0.329}$ x (0.993)^{age} [72].

2.2.4 Measurement of urinary biomarkers

The level of urinary KIM-1 was measured by sandwich ELISA Human TIM-1/KIM-1/HAVCR Duoset (R&D System; Abingdon, U.K). The level of urinary neutrophil gelatinase-associated lipocalin (NGAL) was measured using ELISA human Lipocalin-2/NGAL, Duoset (R&D System; Abingdon, U.K). It was previously described in detail for the age study (2.1.3).

2.2.5 Outcome measurement

Patients were divided into the AKI and non-AKI groups according to weather they develop AKI within 48 h after cardiac surgery.

The outcome of AKI was measured according to AKIN criteria by increase in serum creatinine $\geq$ 0.3 mg/dl from the baseline within the first 48 h after cardiac surgery [14]. Also we determined GFR by decrease > 25% from the baseline according to RIFLE criteria [13].

2.2.6 Statistical analysis

To illustrate differences over time of the biomarkers concentration, mean ± standard deviation and standard error were determine. To determine statistically significant differences between urinary biomarkers at various times after surgery two way ANOVA test was used. To examine the correlation of biomarkers with kidney injure at all time followed surgery post-hoc Bonferroni was used. Differences at each time point among the same group were analyzed using non-parametric one-way ANOVA analysis (Kruskal-Wallis test). Pearson's correlation coefficient analysis was calculated to evaluate the association between the concentrations of urinary biomarkers at each time point and also the different variables clinical data (age, sex, height, weight, time of surgery, CPB time, vasopression and inotropic agent). Correlation and differences were considered significant at $P < 0.05$.

Statistics were performed by using GraphPad Prism (version 5.0); while Microsoft Excel 2007 was used for figures and tables.

RESULTS

3.1 Age study

In our study using urine samples from 337 healthy subjects aged between 4 months to 90 years old, changes in the concentrations of Cr, KIM-1, Cys-C, NAG and NGAL were investigated as shown in Table 5.

The urinary Cr concentration increased rapidly with age between <10 and group B (Figure 2) peaking at group B (Table 4) ranging on average from 24.3-362.6 mg/dl. Subsequently the concentrations decreased gradually till >80 group ranging on average from 1.72-163 mg/dl.

There were significant age-related differences for KIM-1 with elevated level for groups F, G, and H (Figure 2), even when it was normalized to creatinine (Figure 3). Also we found significant differences between gender at group G and group >80 (Figure 4). NGAL showed significant elevated level for group B, D and >80 (Figure 2), significant differences between gender at the group A, B, C, D, F and >80 (Figure 4).

In the same way, urinary Cys-C showed significant elevated levels for group B and C, and no significant gender effect. The urinary NAG showed significant age-related differences between group F, G and >80, and we found significant correlation with gender effect.

3.2 Cardiac surgery study

3.2.1 Patient characteristics

A total of 40 patients were enrolled of whom 15 patients were excluded, because their data not completed. The characteristics of the AKI and non-AKI groups are given in Table 6. Five patients (20%) developed AKI according to AKIN and RIFLE criteria within the first 48h after cardiac surgery and were categorized in the AKI group. The remaining 20 patients were categorized in the non-AKI group. Renal replacement therapy was required in only one case in AKI group.

3.2.2 Detectability of AKI biomarkers preoperatively

Changes in various parameters preoperatively in the AKI and non-AKI groups are shown in Table 7 (A) and Figure 5. Urinary Cr levels were higher in AKI group, but it was not statistically significant (121.6 mg/dL vs. 106.4 mg/dL non-AKI, P > 0.05). Moreover, serum albumin level was significantly higher in non-AKI group (36 g/L vs. 31.6 g/L AKI, P = 0.0015), while no significant effects of kidney injury were found preoperatively for the rest of the biomarkers.

 The urinary KIM-1 level in AKI group was higher than non-AKI group (601.8 pg/ml vs 387.3 pg/ml, respectively). Similarly, the urinary KIM-1/Cr and KIM-1/SG levels in the AKI group were higher than non-AKI group. Although there were differences in the KIM-1, KIM/1-Cr and SG levels between the two groups preoperatively, these differences were not statistically significant (Table 7 (B), Figure 6). In non-AKI and AKI groups, there were no significant differences in urinary NGAL, NGAL/Cr and NGAL/SG levels preoperatively.

To evaluate the correlation between biomarkers pre- and postoperatively, Pearson's correlation coefficient analysis was performed. The results indicated that biomarkers concentrations were mostly poorly correlated with one another in non-AKI and AKI groups preoperatively. A positive correlation was found for KIM-1with creatinine, total protein and α1-microglobulin in non-AKI group ($r = 0.71$; $r = 0.70$; $r = 0.68$ respectively; $P < 0.05$). In addition, KIM-1/Cr ratio in non-AKI was significantly positive correlated with microalbumin ($r = 0.73$, $P < 0.05$). Furthermore, total protein positively correlated with creatinine and α 1-microglobulin ($r = 0.75$; $r = 0.63$, $P < 0.05$, respectively) in non-AKI group (Table 8 A). At last, no significant correlation between biomarkers and clinical characteristic (data not shown).

3.2.3 Changing temporal of biomarkers level postoperatively

The urine and blood samples were obtained at 3, 6, 12, 24 hours postoperatively for clinical analysis. The urinary Cr level was decreased at 3 h after surgery in both groups compared with the respective level preoperatively. At later time points, Cr values were increased postoperatively again. Also we found that α1-microglobulin level increased at 3h postoperatively in the two groups and then remained at stable level. Similarly, the α1-microglobulin/Cr ratio level was increased at 3 h up to 6 h postoperatively and slightly decreased at 12h, then stayed stable level at 24h in two groups. Likewise, urinary total protein level was elevated in both groups at 3h, 6 h and 12h postoperatively. On the other hand, serum total protein was decreased after the surgery in non-AKI and AKI group compared to preoperative values. Furthermore, albumin level in two groups was significantly decreased after CPB compared with the preoperative level (Table 7 (A), Figure 5).

Urinary KIM-1and KIM-1/Cr levels remained stable in non-AKI and AKI groups at 3, 6 and 12 hours postoperatively. Then the KIM-1and KIM-1/Cr levels were elevated significantly at 24 h in AKI group (2076 pg/ml, $P = 0.001$; 32.8 pg/mg, $P < 0.0001$) compared with non-AKI group. In addition, KIM-1/SG level was decreased in the two groups at 3, 6hafter CPB surgery (Table 7 (B), Figure 6). Afterwards at 12h KIM-1/SG level were elevated. Urinary KIM-1/SG level at 24h was significantly higher in AKI group postoperatively (1995, $P < 0.0001$).

In contrast, there were no differences found in urinary NGAL, NGAL/Cr and NGAL/SG levels between AKI and non-AKI groups. Furthermore, there were no effect of time and kidney in NGAL, NGAL/Cr and NGAL/SG levels at each time point after surgery.

Furthermore, KIM-1 level at 24h postoperatively was significantly different from all time points in non-AKI and AKI groups. A significant difference was also found in KIM-1/Cr at 24h in non-AKI group (Table 7 B).

Pearson's correlation coefficient analysis found positive correlations between NGAL and α1-microglobulin ($r = 0.60$, $P < 0.05$) and negatively correlated with GFR ($r = -0.65$, $P < 0.05$) in non-AKI group at 3 h after CPB surgery. Similarly, a positive correlation was found between Cr and total protein in non-AKI ($r = 0.79$, $P < 0.05$) and AKI groups ($r = 0.99$, $P < 0.05$), and strong positive correlation with microalbumin ($r = 0.91$, $P < 0.05$) in AKI group. Also, in AKI group a negative correlation between total protein and GFR ($r = -0.95$, $P < 0.05$) at 3 h was found (Table 8 B).

At 6 h after surgery, a negative correlation in KIM-1, creatinine and NGAL with GFR in non-AKI (r = -0.65 KIM-1; r = -0.72 Cr and r = -0.59 NGAL, P < 0.05) was found. In the AKI group, there was strong positive correlation between NGAL and KIM-1/Cr (r = 0.93, P < 0.05), while KIM-1/Cr and total protein levels were positively correlated with microalbumin (r = 0.97 and r = 0.99, respectively; P < 0.05) Table 8 (C).

At 12 h postoperatively as shown in Table 8 (D), there was negative correlation found between KIM-1 with α 1-microglobulin (r = -0.58, P < 0.05) in non-AKI group, and with serum albumin (r = -0.92, P < 0.05) in AKI group. Moreover, in AKI group was found positive correlation between NGAL and microalbumin (r = 0.97, P < 0.05).

Furthermore, there was a moderately positive correlation between KIM-1 and microalbumin (r = 0.36, P < 0.05) in AKI group at 24 h postoperatively. In non-AKI group, positive correlation between NGAL and albumin (r = 0.64, P < 0.05) was found. A negative correlation was also found in KIM/Cr with GFR, but it was not statistically significant (r = -0.98, P>0.05). No significant correlation between other biomarkers was found. (Table 8 E).

Finally, we did not find any statistically significant correlation with biomarkers and clinical characteristics in two groups (data not shown).

DISCUSSION

4.1 Age study

Several promising novel biomarkers of AKI, including: NGAL, KIM-1, Cys-C and NAG, have been studied clinically in a variety of renal patho-physiologic states, such as intensive care unit patients, cardiac surgery, renal damage due toxic and drug exposure and renal transplantation. However, for the use of these biomarkers in patients, an understanding of their normal reference values and their relationships with age is essential. The present study was aimed at measuring the reference range of the most promising biomarkers for AKI, urinary KIM-1, Cys-C, NAG and NGAL in healthy population. Availability of such reference values would assist the physicians in the management of AKI patients.

In this study, we represent data from healthy population spanning all age groups (inclusive of infants and those over 80 years), and we found a significant correlation between biomarkers and age (Figure 2), in addition to a significant sex difference (Figure 4).

Our findings are compatible with other studies that confirm the association of age with urinary biomarkers as a marker for kidney function, it is evident that a significant different interaction between aging and gender with urinary poly-peptides [28].

In this study, we found that a significant correlation between KIM-1 concentration and age. Absolute KIM-1 levels increased with age, and the increase is more apparent in males than in females. Many studies have established that the KIM-1 ectodomain produced by the proximal tubule and can be measured in urine after ischemic or toxin-induced AKI, suggesting it is sensitive and predictive biomarker [55, 57]. To our knowledge, there are no published data on reference KIM-1 values. KIM-1 levels were significantly lower in infants and children (group A) than all other age groups. The renal immaturity of healthy newborn and suckling infant is not risky under normal circumstances [67], but may explain the lower KIM-1 level.

The second urinary biomarker analyzed in this study was Cys-C. The results showed that urinary Cys-C levels increase gradually with age, but no significant gender differences was found. In this study the higher values were (86.03 ng/ml) noticed among young participants (group B). Cystatin C is a protease inhibitor that is released into the blood, filtered through the glomerulus, and completely reabsorbed in the proximal tubule. Literature state that Cys-C was a better biomarker for measured GFR compared with creatinine [44, 45].
Oden et al [68] observed a strong association of age with Cys-C levels in healthy population and they provide some illustration suggesting that aging could considerably change physiological function in kidney even among healthy population.
Moreover, a previous study [69] supported that finding and did not observe gender differences, but they indicate a positive association between Cys-C level and BMI in healthy population.

NAG is a lysosomal enzyme found in proximal tubular cells that has been shown to be a sensitive biomarker for proximal tubular injury in the setting of a wide variety of drugs, environmental toxicants, contrast-induced toxicity, and ischemic acute tubular injury [62].

In our study, the NAG levels were nearly stable. Equally important, there were significant gender differences: females had higher values than males.

By contrast, previous study [70] found a significant age difference with NAG level. In the neonates and infants groups, the urinary NAG level was higher than the level in children and teenager groups.

Certainly, the specific differences in age groups and methods could contribute to the differences with our results. Besides that, taking into account circadian variations in the urinary NAG enzyme activity and time of collecting samples can be recommended.

Furthermore, we presented the correlation between NGAL concentration and age. The absolute NGAL level were higher in B, D and >80 age groups than all other age groups. In addition, in the same age group the urinary NGAL level in females was significantly higher than males. Notably, Cullen et al [71] reported that there was considerable difference in urinary NGAL level within age and gender. On the other hand, they reported the optimum cut-off value for total reference adults (ages ranging from < 40 to 88 years) of 107 µg/L, which is higher than our result. Our data suggests that urinary NGAL concentration is related to the age and sex, and so comparatively it is suggested that at different age both of the different sex should have a different cut-off value.

NGAL is an epithelial protein freely filtered by glomerulus and reabsorption at proximal tubule; it's expressed in very low levels by neutrophils and various epithelial cells including renal proximal tubule [29, 31]. NGAL values in infants and children (group A) reveal that the concentration (714.1 pg/ml) is lower than all other age groups in the present study. Although the mechanism of the dependency of NGAL on age and sex is unknown, many immunological and physiological features in young children, including immature immune system in infants may explain the lower NGAL concentration at this age group.

In all biomarkers, we found that the urinary KIM-1, Cys-C, NAG and NGAL concentration at older age groups were elevated, even when it was normalized to Cr (Figure 3). The low urinary Cr concentration in the <10, A, G and >80 age groups show higher urinary biomarkers/Cr ratio with the extrapolated biomarkers average concentration, predictably determined the fact that unites were different (Cr mg/dL; Cys-C ng/ml; NAG U/ml; KIM-1 pg/ml and NGAL ng/ml). Conversely, in the age groups with high urinary Cr concentration this led to lower biomarkers/Cr ratio compared with the biomarker average concentration. Worthwhile to consider when normalizing a biomarker with Cr, it behaves exactly like Cr, then the normalized level will not be affected differences in Cr excretion. Given this, normalization may be unnecessary and misleading in some clinical setting [72]. In this study, we measured the absolute biomarker value and that presented in linear scale, while the biomarker-Cr ratio presented as quadratic scale. This may present another possible explanation for this observation. As previously explained (1.1 introduction), Cr level was strongly affected by age, gender and muscle mass. In our study, Cr level was significantly elevated with younger age, then urinary Cr level dramatically decreased in older age groups.

4.2 Cardiac surgery study

Herein, we presented a prospective pilot study of adults undergoing cardiac surgery; we compared the predictive value of urinary KIM-1 and NGAL levels pre and post time elapsed following cardiac surgery.

Urinary KIM-1/Cr level displayed the best diagnostic performance. In the AKI group, urinary KIM-1/Cr level was significantly higher than the non-AKI group at 24h after the operation. Moreover, the urinary KIM-1/Cr level was significantly associated with AKI within 24h ($P < 0.0001$). While urinary KIM-1 performed less well than KIM-1/Cr, its level was also significantly higher at 24h in the AKI group compared with non-AKI group ($P < 0.001$).

Recent literature [26, 59-73] has stressed promising results for urinary KIM-1 as a diagnostic biomarker for AKI. In contrast to our result, Han et al. [62] and Liangos et al. [59] showed that urinary KIM-1/Cr level was induced at 2 to 3h among adults undergoing cardiac surgery. It must be taking into account that the numbers of patients in the AKI group, which was relatively small (n = 5), could contribute to the differences with our result.

We noted a significant positive correlation between urinary KIM-1/Cr and microalbumin at 6h in AKI group, this positive correlation was also found at 3 and 12h postoperatively but not statistically significant. A marked correlation in non-AKI group in urinary KIM-1/Cr with microalbumin at preoperative and 3h after surgery was observed. Moreover, similar positive significant correlation was found between KIM-1 and microalbumin in AKI group at 24h postoperatively, which suggests an interference of microalbumin with the renal excretion of KIM-1. Comparable to our result, recent literature [55] reported a strong positive association in diabetic patients between KIM-1 and microalbumin. Considering the high rate of diabetic patients among AKI (3 of 5) and non-AKI (12 of 20) groups in our study, this may corroborated our findings.

Furthermore, urinary KIM-1/Cr correlated positively with NGAL at 6h in AKI group. According to the increased urinary NGAL level at 6h in AKI group, this may suggests that the positive correlation between urinary KIM/Cr and NGAL could be combined for tubular damage detection at this time point.

Moreover, decreasing albumin has generally been attributed to tubular injury as well as in patients with diabetes [55, 77], an inverse correlation in AKI group were found in KIM-1 level with albumin at 12h postoperatively.

Urinary NGAL was stated to be another early biomarker for detection AKI after cardiac surgery [25, 36, 37-75]. Previous literature reported that urinary NGAL level were significantly elevated within 1 h to 3 h after CPB surgery [36]. In contrast, in the present study, urinary NGAL did not perform markedly well in the two groups, with even higher levels in the non-AKI group at every time point compared with the AKI group.

In the study of Thraikill et al. [76] in diabetic patients, the urinary concentration of NGAL increased comparing with non-diabetic patient. Therefore, it is possible that the higher urinary NGAL level observed in non-AKI patients may explained due to diabetic patients in non-AKI group.

Similarly, urinary NGAL correlated positively with microalbumin at 12h in AKI group postoperatively. At first sight, this correlation could also indicate the association of NGAL in diabetic patients.

Interestingly, a negative correlation in GFR with NGAL and KIM-1 were found at 6h in non-AKI group as well as with KIM-1/Cr at 24h in AKI group (r = -0.98, P > 0.05). Of interest, Boliganano et al. [78] confirmed significant an inverse correlation between GFR and NGAL. Moreover, a recent study [79] hypothesized that decreased GFR level in AKI as a result of loss of nephron function, while the increase in NGAL level occurs due to inflammation in the tubular cells. These evidence suggests that the inverse correlation of GFR with KIM-1 and NGAL under the same conditions may combined to assess residual renal function at time elapsed after cardiac surgery.

Lastly, however there were significant differences in the routine biomarkers levels: urinary creatinine, α 1 microglobulin, and α 1 microglobulin/Cr and total protein levels increased at 24h after operation compared with preoperative in the two group were found, while serum albumin and serum total protein levels decreased at 24h postoperative compared with preoperative time after surgery in the two groups, their levels were not higher than the reference range.

CONCLUSION

Acute kidney injury is a common and complex condition associated with significant morbidity and mortality. Recently, several protein biomarkers have been evaluated as an early detection for AKI.

The objective of the first part in the present study was to explore the normal value of urinary NGAL, KIM1, NAG and Cys-C with increasing age. Obviously, a significant correlation between urinary biomarkers and age and gender, respectively in healthy population were found. This is the first study to look at age effect on urinary biomarkers in large scale healthy populations. Thus, a panel of carefully selected biomarkers may prove to be convenient in the diagnosis and prognosis of AKI.

Prospective studies should also examine the influence of physical activity, BMI, alcohol consumption and ethnicity in healthy population to clarify the effect on normal urinary biomarkers levels. Moreover, daily serial measurements with comparison for sCr can reveal the optimal timing of biomarkers measurement. To that end, biomarkers will need accurate assessment to provide accurate prognostic information of kidney function.

In the prospective pilot study the objective was comparing the diagnostic performance of urinary biomarker (KIM-1 and NGAL) for early detection of AKI among adults undergoing CPB surgery. We reported that KIM-1/Cr increased at 24h after CPB surgery in AKI group and performed significantly in differentiating between patients with and without AKI. Unfortunately, we did not find that a combination of urinary biomarkers that enhances diagnostic performance of AKI in our prospective pilot study, probably due to the small population size.

Importantly, the results have a numbers of important diagnostic implications were observed, such as the correlation between KIM-1/Cr and GFR, which have to be further validated in large population undergoing cardiac surgery in a multi-center study as well as a panel of selected biomarkers, may explain the biomarkers performance after elapse time following the surgery.

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Table 1

Classification system for Acute Kidney Injury (RIFLE and AKIN)

	RIFLE			AKIN	
Stage	**Creatinine or GFR**	**Urine output**	**Stage**	**Creatinine**	**Urine output**
Risk	Increase sCr of ≥ 1.5 times or GFR decrease > 25%	UO < 0.5 ml /Kg/h for 6h	1	0.5 -2 times (baseline) or increase of > 0.3 mg/dl	UO < 0.5 ml/kg/h for > 6h
Injury	Increase sCr to > 2 times or GFR decrease > 50%	UO < 0.5 ml/Kg/h for 12h	2	2-3 times (baseline)	UO < 0.5 ml/kg/h for > 12h
Failure	Increase sCr to > 3 times or GFR decreased > 75%	UO < 0.3 ml/Kg/h for 24 h or Anuria† for 12h	3	> 3 times (baseline) or level of 4.0 mg/dl with an acute increase of 0.5 mg/dl or RRT	<0.3 ml/kg/h for 24 h or Anuria for 12 h
Loss	Persistent ARF= complete loss of kidney function > 4 weeks				
ESKD	End-Stage Kidney Disease > 3 months				

RIFLE: Risk-Injury-Failure-Loss-End-stage kidney depending on the renal replacement therapy; RIFLE classification uses a 7 days window for assessment of renal function. **AKIN**: Acute Kidney Injury Network; AKIN classification uses a 48 h widow. **sCr**: Serum Creatinine; **UO**: Urine Output; **ARF**: Acute renal failure; **RRT**: Renal replacement therapies. † Anuria means non-passage of urine (adapted from Srisawat et al.[13]; Kumar et al.[14]).

Table 2

Risk factors associated AKI after CPB

Patients	Procedure
Age	Haemolysis
Gender	Haemodilution on CPB
Diabetes mellitus	CPB duration
Peripheral vascular disease	off-pump vs. on-pump
Renal insufficiency	
Cardiac shock	
Sepsis	
Emergent surgery	

Table modified from Kumar et al. [14] to current study concept.

Table 3

Patho-physiological factors in AKI and CPB

Pre-operative	Intra-operative	Post-operative
Lack of renal reserve	Decrease renal perfusion	Systemic inflammation
Reno-vascular disease	Embolism generated by CPB	Reduced left ventricular function
Nephro-toxins	Free hemoglobin	Hemodynamic instability
Inflammation		Mechanical circulatory support devices

Table modified from Kumar et al. [14]; Rosner et al. [19] to current study concept.

Table 4

Participants anthropometrics

Group	No (337)	Age range (years)	Gender (Male/Female)
<10	33	0 - 9	21/12
A	40	10 - 20	15/25
B	33	21 - 30	9/24
C	35	31 - 40	15/20
D	40	41 - 50	10/30
E	40	51 - 60	21/19
F	41	61 - 70	20/21
G	40	71 - 80	22/18
>80	35	more than 80 years	13/22

Table 5
The concentration and ratios of urinary biomarkers versus creatinine for age groups

*Age	Cr	ST e	†KIM-1	ST e	KIM-1/Cr	ST e	Cys-C	ST e	Cys-C/Cr	ST e	NAG	ST e	NAG/Cr	ST e	NGAL	ST e	NGAL/Cr	ST e
<10	39.3	5.67	397.9	67.1	1.68	0.47	49.11	8	1.34	0.38	34.89	3.87	1.87	0.44	714.13	224.24	50.77	25.89
A	102.4	13.53	419.6	75.6	0.48	0.05	78.41	13.56	1.53	0.85	35.10	3.38	0.57	0.10	1069.71	245.63	44.17	27.24
B	165.9	13.88	561.6	84.8	0.34	0.04	86.03	5	0.51	0.02	36.78	3.86	0.36	0.09	2045.69	394.32	25.66	11.91
C	131.6	12.63	738	92.4	0.67	0.11	83.70	8.13	0.74	0.11	33.06	4.25	0.39	0.09	1099.98	144.28	11.09	1.77
D	107.7	14.18	655.1	119.4	0.80	0.14	70.012	9	0.69	0.10	34.76	3.48	0.66	0.21	1795.16	449.19	34.27	14.21
E	90.8	11.05	592	87.2	0.78	0.12	58.83	6.47	0.71	0.05	36.17	3.55	0.61	0.10	1192.74	311.57	19.90	6.46
F	81.4	8.13	1161.2	169.5	1.63	0.25	70.32	11	1.01	0.20	44.21	3.65	0.87	0.17	1284.14	386.09	18.50	4.53
G	76.5	8.87	966.2	130	1.54	0.27	76.31	12.43	0.92	0.07	46.09	3.98	0.89	0.19	2197.03	595.33	43.85	13.49
> 80	60.6	6.81	1045.1	115	1.92	0.18	73.54	13	1.51	0.27	54.71	3.14	1.15	0.12	2038.3	384.86	43.34	11.53

*Age range: <10 (0-9 years); A (10-20 years); B (21-30 years); C (31-40 years); D (41-50 years); E (51-60 years); F (61-70 years); G (71-80 years); > 80 (more than 80 years).

† (Cr) Creatinine mg/dl ;(KIM-1) Kidney injury molecule-1 pg/ml; (NAG) N-acetyl-β-glugosaminidase U/ml; (Cys-C) Cystatine-C ng/ml; (NGAL) Neutrophil gelatinase-associated lipocalin pg/ml.

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Table 6

Patient characteristics and clinical outcome

Characteristic	Non-AKI (n = 20)	AKI (n = 5)
Age, y	69.9 (10)	73 (8.6)
Female gender	4	3
Height, cm	170. (8.8)	166
Weight, kg	79 (12)	56
Comorbid disease		
Diabetes	12	3
Operative procedure		
CABG	20	4
CABG + AVR	1	1
Duration of surgery time, h	5.3 (1.3)	6 (2.8)
Extra corporation circulation time, min	133.7 (44)	181 (94)
RRT		1

Data of clinical parameters expressed as mean (standard deviation) or absolute number. **Non-AKI**, non acute kidney injury group; **AKI**, acute kidney injury group; **CABG**, coronary artery bypass graft; **AVR**, aortic valve replacement; **RRT**, renal replacement therapy.

Table 7

(A) Biomarkers concentration at various postoperative time points

	Non-AKI					AKI					P value†
	Pre-op	3h	6h	12h	24h	Pre-op	3h	6h	12h	24h	
Urine											
Creatinine	106.47 (77.3)	39.6 (29.4)	57.2 (32.7)	121 (51.5)	134.1 (53.7)	121.65 (30.5)	54.1 (50)	20.2 (6.3)	83.3 (36)	122.7 (77.7)	<0.0001
α1-M	16.5 (14.2)	44.5 (34.5)	38.2 (32.5)	47.5 (12.8)	49.4 (26.5)	7.27 (7)	40.1 (39.4)	21.2 (15.7)	40 (10.6)	37.2 (27.6)	0.0069
Microalbumin	37.3 (41)	23 (22.6)	19 (23.5)	34.6 (35.2)	40.7 (30)	12 (5.7)	20.2 (11)	10.5 (12.6)	51.2 (50)	33.7 (19.3)	NS
α1-M/Cr	18.3 (15.5)	139.4 (113)	82 (83)	48.4 (32)	41.6 (24.3)	7 (7)	99.2 (77.7)	111.5 (70.7)	60 (40)	37.2 (20.3)	0.0003
Total protein	12.9 (8.4)	30.2 (26)	133 (140)	269.4 (226)	207 (177)	10.75 (3.6)	25 (20.3)	62 (74)	448 (248)	159 (150)	<0.0001
Albumin/Cr	49.4 (82.4)	74.3 (80)	44.5 (65.4)	32.6 (32)	36.4 (31)	10 (5.4)	61.2 (44.6)	45.2 (43.8)	61.7 (46.7)	81.5 (127)	NS
SG	1.017 (0.005)	1 (0.01)	1.043 (0.01)	1.05 (0.01)	1.04 (0.01)	1.02 (0.003)	1.03 (0.01)	1.02 (0.008)	1.05 (0.01)	1.03 (0.01)	<0.0001
pH	6.3 (0.7)	5.8 (0.8)	5.6 (0.8)	5.8 (0.8)	5.7 (0.8)	6.3 (1.2)	5.7 (0.1)	5 (0)	5.3 (0.7)	5.5 (1)	0.034
RBC	16.5 (20.5)	705.4 (1724)	88.6 (110)	433 (1011)	595.3 (1037)	9 (4)	18.2 (15)	147.5 (215)	84.7 (69)	230.7 (365)	NS
WBC	4 (3)	20.3 (34)	12.4 (24)	39.4 (50)	51 (47)	4.5 (1.3)	6 (4)	6.5 (7)	44.7 (59)	46.5 (26)	0.003
Blood											
Creatinine	1 (0.2)	0.99 (0.2)	1 (0.2)	1.1 (0.3)	0.98 (0.3)	0.8 (0.2)	0.85 (0.2)	0.98 (0.2)	1.2 (0.3)	1.3 (0.7)	NS
Urea	44.5 (17.2)	42.8 (14.6)	41.7 (14.3)	43.5 (14.5)	43 (14.8)	49.7 (15)	46.7 (14.7)	47 (14)	50.5 (14.8)	60.3 (14.8)	NS
Albumin**	36 (3)	22.4 (3.5)	22 (3.2)	23.6 (3.5)	26 (3)	31.6 (6.3)	16.3 (2.3)	20.1 (2.3)	24 (2)	24.1 (1.6)	<0.0001
eGFR	70.5 (18)	71.6 (17.5)	69.6 (17.6)	67 (18.8)	71.6 (19)	78.75 (13.3)	78.7 (12.8)	68.5 (17)	54.2 (12.5)	53.3 (23.7)	NS
Total protein	6 (0.5)	3.9 (0.4)	4.1 (0.3)	4.3 (0.3)	4.6 (0.4)	6.6 (1.7)	3.5 (0.8)	4.2 (0.5)	4.5 (0.6)	4.7 (0.4)	<0.0001

B) Urinary KIM-1 and NGAL concentration and ratio with Cr and SG at various time points after CPB surgery

	Non-AKI					AKI					P value†
	Pre-op	3h	6h	12h	24h	Pre-op	3h	6h	12h	24h	
KIM-1*	387.3 (263) BC	221.8 (296) C	173.1 (115) C	564.3 (354) AB	1267.6 (1112) A	601.8 (246) AB	393 (320) B	319 (261) B	810.2 (524) AB	2076 (924) A	< 0.0001
KIM-1/Cr***	4.05 (2.8) B	7.2 (6.6) AB	4 (3) B	9.3 (7) AB	9.3 (7.1) A	5.06 (2.6)	10.8 (11)	10.8 (7.3)	10.5 (2.8)	32.8 (34)	< 0.0001
KIM-1/SG*	380.1 (257)	213.6 (282)	165.6 (110)	537 (338)	1214.3 (1060)	589.2 (240)	380.2 (308)	309.1 (253)	767.4 (497)	1995 (873)	< 0.0001
NGAL	9951 (19979)	16046 (16583)	21872 (29976)	57201 (168347)	66111 (142318)	6277.2 (8018)	8766 (8449)	12446 (11966)	30424 (39490)	27697 (23394)	NS
NGAL/Cr	116 (232)	906 (1246)	405.5 (679)	700.4 (2311)	669.2 (1814)	51.4 (49)	232.7 (158)	595.9 (592)	522.3 (513)	389 (433)	NS
NGAL/SG	9785.4 (19665)	15522 (15971)	20919 (28677)	54800 (162211)	63645 (137543)	6146.3 (7852)	8430 (8015)	12047 (11530)	28683 (37020)	26616 (22346)	NS

Data of clinical parameters expressed as mean (standard deviation). **Urinary biomarkers and reference range (ZOL, Genk): Creatinine** 39-259 mg/dL; α1-M, α1- microglobulin, < 12 mg/L; **Microalbumin** <20 mg/L; **α1-M-Cr**, α1- microglobulin- creatinine ratio < 14 mg/g; **Total protein** <15 mg/dL; **Albumin-Cr**, Albumin- creatinine ratio 0-30 mg/g; **SG**, specific gravity 1.002-1.035; **RBC**, red blood cells < 25 RBC/µL; **WBC**, White blood cells < 25 WBC/ µL; **pH** 4.8-7.4; **KIM-1**, kidney injury molecule; **NGAL** neutrophil gelatinase-associated lipocalin; **KIM-1-Cr**, KIM-1–creatinine ratio(pg/mg); **NGAL-Cr**, NGAL-creatinine ratio (pg/mg); **KIM-1-SG**, KIM-1-specific gravity ratio; **NGAL-SG**, NGAL-specific gravity ratio. **Blood biomarkers and reference range (ZOL, Genk): Creatinine** 0.70-1.20 mg/dL; **Urea** 18-55 mg/dL; **Albumin** 35-52 g/L; **eGFR** estimated glomerular filtration rate 90-120 ml/min; **Total protein** 6.40-8.30 g/dL. NS, not significant.

Different letters in the same row indicate significant differences between subsequent time points after cardiac surgery.

Dagger sign (†) indicates a significant effect of postoperative time on the biomarkers.

Asterisks indicates a significant effect of kidney injury on the biomarkers: *P value = 0.001; **P value = 0.0015; ***P value < 0.0001.

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Table 8

Pearson's correlation coefficients between biomarkers in non-AKI and AKI groups after CPB surgery at subsequent time points

(A) Preoperative

	Non-AKI									AKI								
	KIM-1	NGAL	Cr	α1-M	TP	KIM-1/Cr	MA	ALB	GFR	KIM-1	NGAL	Cr	α1-M	TP	KIM-1/Cr	MA	ALB	GFR
KIM-1		0.36	0.71*	0.68*	0.70*	-0.29	0.32	-0.24	0.26		0.86	0.66	0.77	0.61	0.85	0.69	0.6	0.22
NGAL			0.17	0.30	0.11	-0.16	-0.19	-0.33	0.18			0.47	0.06	0.16	0.36	0.08	0.13	0.48
Cr				0.48	0.75*	-0.20	0.32	0.19	0.31				0.41	0.50	0.74	0.85	0.97	0.43
α1-M					0.63*	0.19	0.04	-0.02	0.13					0.025	0.37	0.18	0.23	0.21
KIM-1/Cr						-0.03	0.73*	0.16	0.18						0.33	0.25	0.28	0.12
TP							-0.16	-0.01	-0.14							0.13	0.08*	0.66
MA								0.06	-0.05								0.007*	0.65
ALB									0.32									0.69
GFR																		

(B) 3h Postoperative

	Non-AKI I									AKI								
	KIM-1	NGAL	Cr	α1-M	TP	KIM-1/Cr	MA	ALB	GFR	KIM-1	NGAL	Cr	α1-M	TP	KIM-1/Cr	MA	ALB	GFR
KIM-1		-0.20	-0.001	-0.17	0.13	0.74*	-0.22	-0.16	-0.02		-0.46	-0.51	-0.69	-0.57	0.68	-0.13	-0.68	-0.80
NGAL			0.47	0.60*	0.13	-0.30	0.43	-0.32	-0.65*			-0.49	-0.11	-0.41	-0.20	-0.79	-0.03	0.48
Cr				0.84	0.79*	-0.10	0.79	-0.14	-0.39				0.86	0.99*	-0.32	0.91*	0.80	0.20
α1-M					0.78*	-0.25	0.79	-0.32	-0.28					0.91	-0.15	0.67	0.99*	0.19
KIM-1/Cr					-0.32		0.63*	-0.02	-0.07					-0.30		0.87	0.86	0.21
TP							-0.29	0.32	0.40							-0.07	-0.09	-0.95*
MA								-0.18	-0.47*								0.60	-0.12
ALB									0.43*									0.15
GFR																		

(C) 6h Postoperative

	Non-AKI									AKI								
	KIM-1	NGAL	Cr	α1-M	TP	KIM-1/Cr	MA	ALB	GFR	KIM-1	NGAL	Cr	α1-M	TP	KIM-1/Cr	MA	ALB	GFR
KIM-1		0.35	0.50*	0.33	0.09	0.20	0.20	0.12	-0.65*		-0.14	0.044	-0.30	-0.11	-0.29	-0.32	-0.01	-0.42
NGAL			0.21	0.03	-0.25	-0.08	-0.18	0.28	-0.59*			0.55	-0.76	0.92	0.93*	0.88	0.14	-0.69
Cr				0.03	-0.16	-0.57*	0.14	0.16	-0.72*				0.042	0.8	0.67	0.74	-0.74	-0.88
α1-M					0.20	-0.02	0.85	-0.18	-0.30					-0.458	-0.50	-0.39	-0.67	0.31
KIM-1/Cr					0.35		0.13	-0.26	0.007					0.97		0.97*	-0.22	-0.80
TP							-0.098	-0.35	0.31							0.99*	-0.06	-0.63
MA								-0.20	-0.16								-0.18	-0.66
ALB									-0.006									0.48
GFR																		

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(D) 12h Postoperative

Non-AKI

	KIM-1	NGAL	Cr	α1-M	TP	KIM-1/Cr	MA	ALB	GFR
KIM-1		-0.007	0.16	-0.58*	-0.101	0.73*	-0.01	-0.13	-0.27
NGAL			-0.19	-0.08	-0.19	0.12	-0.06	-0.33	-0.29
Cr				0.35	0.05	-0.49*	0.38	-0.27	-0.38
α1-M					-0.05	-0.74	0.14	-0.24	-0.03
KIM-1/Cr					-0.16		-0.25	-0.07	0.35
TP							-0.30	0.15	-0.09
MA								0.20	-0.25
ALB									0.14
GFR									

AKI

	KIM-1	NGAL	Cr	α1-M	TP	KIM-1/Cr	MA	ALB	GFR
KIM-1		0.23	-0.14	0.53	0.52	-0.94*	-0.05	-0.92*	0.48
NGAL			0.26	-0.92	0.60	0.03	0.97*	0.51	-0.88
Cr				-0.05	-0.24	0.31	0.26	-0.03	-0.62
α1-M					-0.41	-0.27	-0.83	-0.78	0.81
KIM-1/Cr					-0.75		0.72	-0.18	-0.20
TP							-0.16	0.75	-0.38
MA								0.32	-0.81
ALB									-0.57
GFR									

24h postoperative

(E)

	Non-AKI									AKI								
	KIM-1	NGAL	Cr	α1-M	TP	KIM-1/Cr	MA	ALB	GFR	KIM-1	NGAL	Cr	α1-M	TP	KIM-1/Cr	MA	ALB	GFR
KIM-1		-0.02	0.41	-0.03	0.22	0.18	0.37	-0.35	0.30		-0.47	-0.86	-0.78	-0.35	0.97	0.36*	0.98	-0.79
NGAL			-0.17	-0.20	-0.03	0.16	0.29	0.64*	-0.25			0.25	0.77	-0.26	-0.54	-0.37	-0.99	0.58
Cr				0.50*	0.16	0.28	-0.04	-0.30	0.27				0.65	0.45	-0.94	-0.01*	0.56	0.23
α1-M					0.31	0.09	-0.26	0.006	0.41					-0.28	-0.78	0.14	-0.73	0.99
KIM-1/Cr					0.37		0.30	0.03	0.13					-0.36		-0.59	0.80	-0.98
TP							-0.17	0.04	0.31							0.21	0.27	-0.89
MA								0.004	-0.41								0.11	0.66
ALB									-0.36									-0.67
GFR																		

KIM-1, kidney injury molecule; **NGAL** neutrophil gelatinase-associated lipocalin; **Cr,** creatinine; **α1-M,** α1- microglobulin; **KIM-1-Cr,** KIM-1-creatinine ratio; **MA,** microalbumin; TP, total protein ; **ALB,** albumin; **GFR,** glomerular filtration rate.

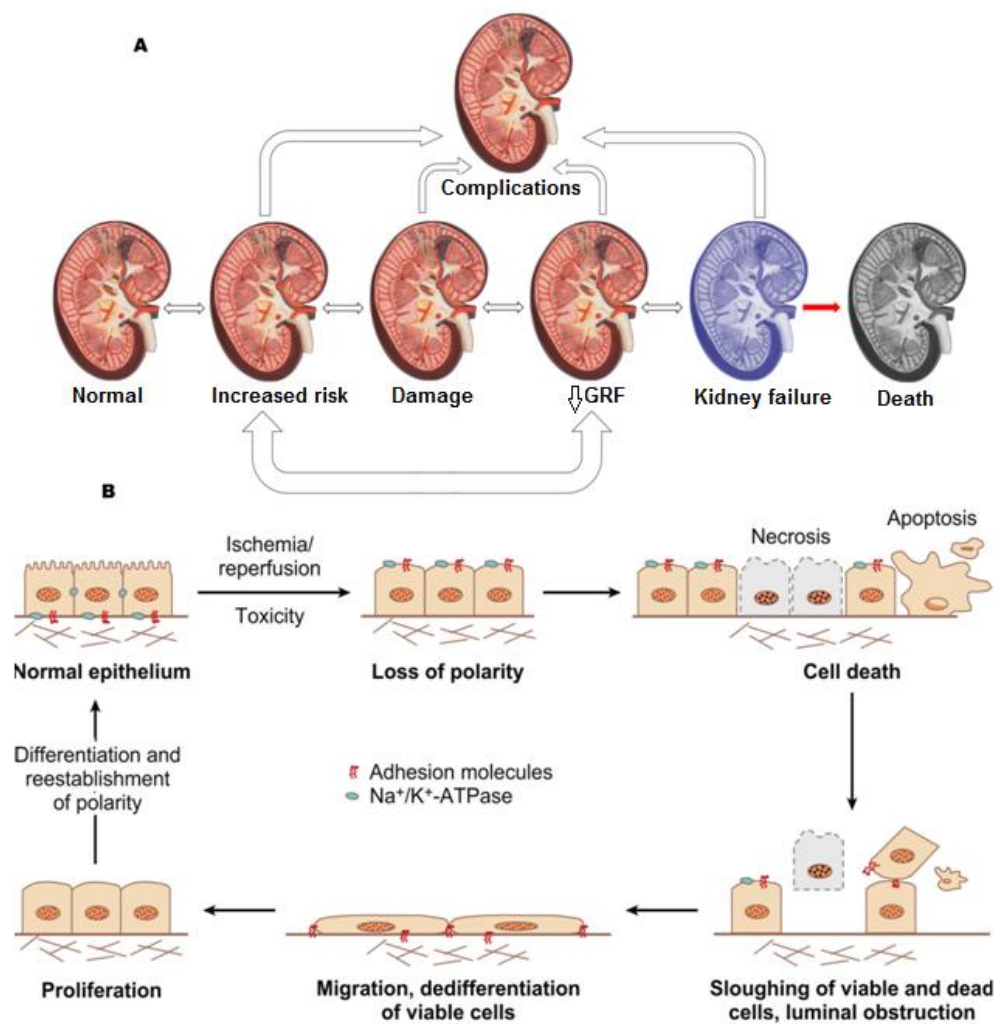Asterisks indicate a significant correlation: * P value < 0.05.

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Figure 1: Acute Kidney injury (**A**) The process of acute kidney injury can be divided into various reversible stages depending on the severity of insult, starting from increased risk to damage followed by decrease in glomerular filtration rate (GFR) further progressing to kidney failure and death. (**B**) **Schematic illustration of epithelial cell injury and repair after AKI**: The Na+/K+-ATPase localizes to basolateral plasma membranes. Some of the injured epithelial cells undergo necrosis and/or apoptosis detaching from the underlying basement membrane into the tubular space where they contribute to tubular occlusion. Cells that remain attached, dedifferentiate, spread, and migrate to repopulate the denuded basement membrane. With cell proliferation, when cell-cell and cell-matrix contacts are restored and the epithelium redifferentiates, forming a functional, normal epithelium. (Modification of figure from Bonventre et al.[6] and Vaidya et al. [9]).
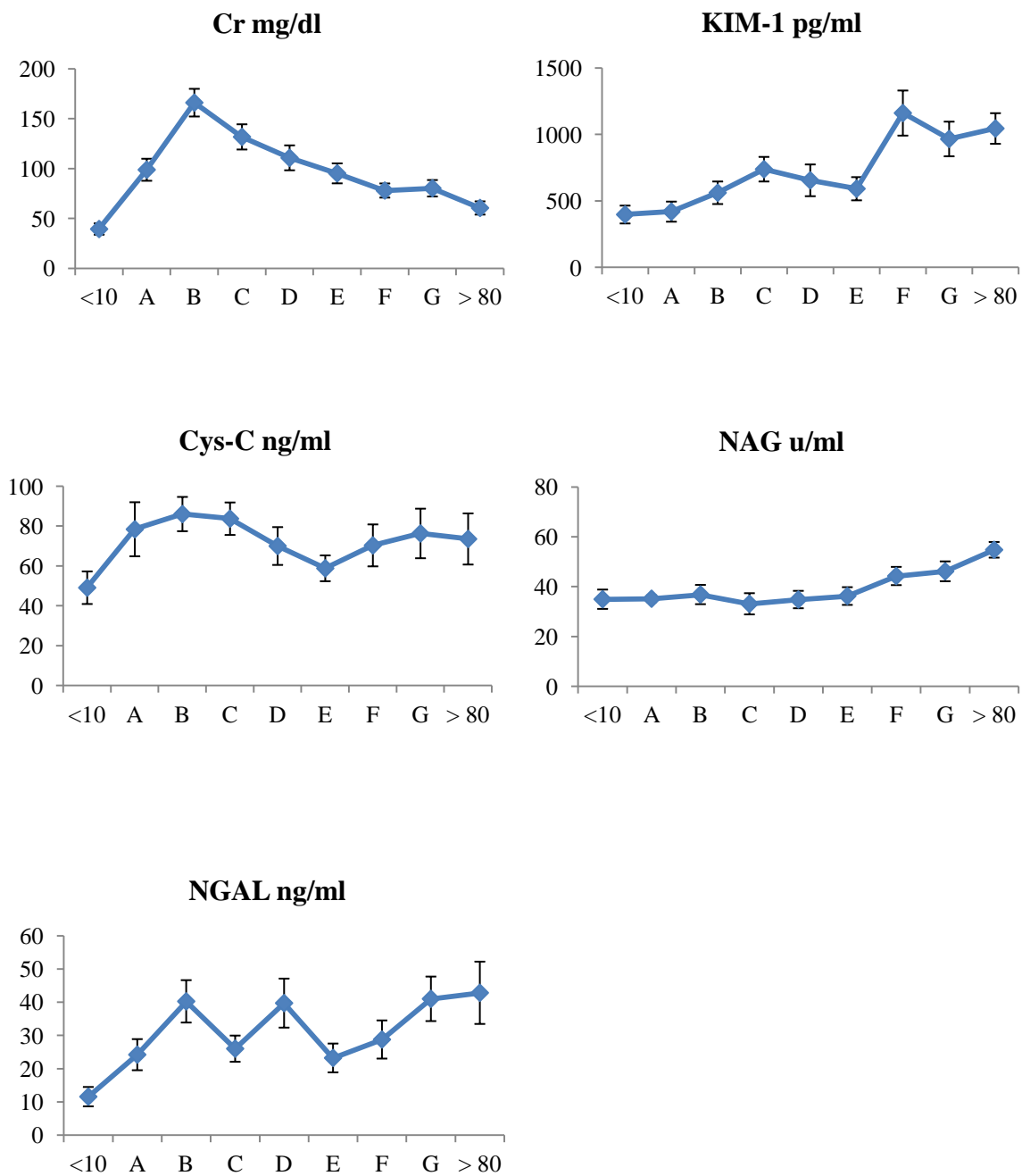
Figure 2: Analysis of urinary biomarkers: Changes in mean urinary biomarkers parameter relating to age groups**: <10** (0-9 years); **A** (10-20 years); **B** (21-30 years); **C** (31-40 years); **D** (41-50 years); **E** (51-60 years); **F** (61-70 years); **G** (71-80 years); **>80** (more than 80 years). **Cr** Creatinine; **KIM**-1,Kidney Injury Molecule-1; **NGAL** Neutrophil Gelatinase-Associated Lipocalin; **NAG** N-acetyl-β-glugosaminidase and **Cys-C** Cystatin-C.
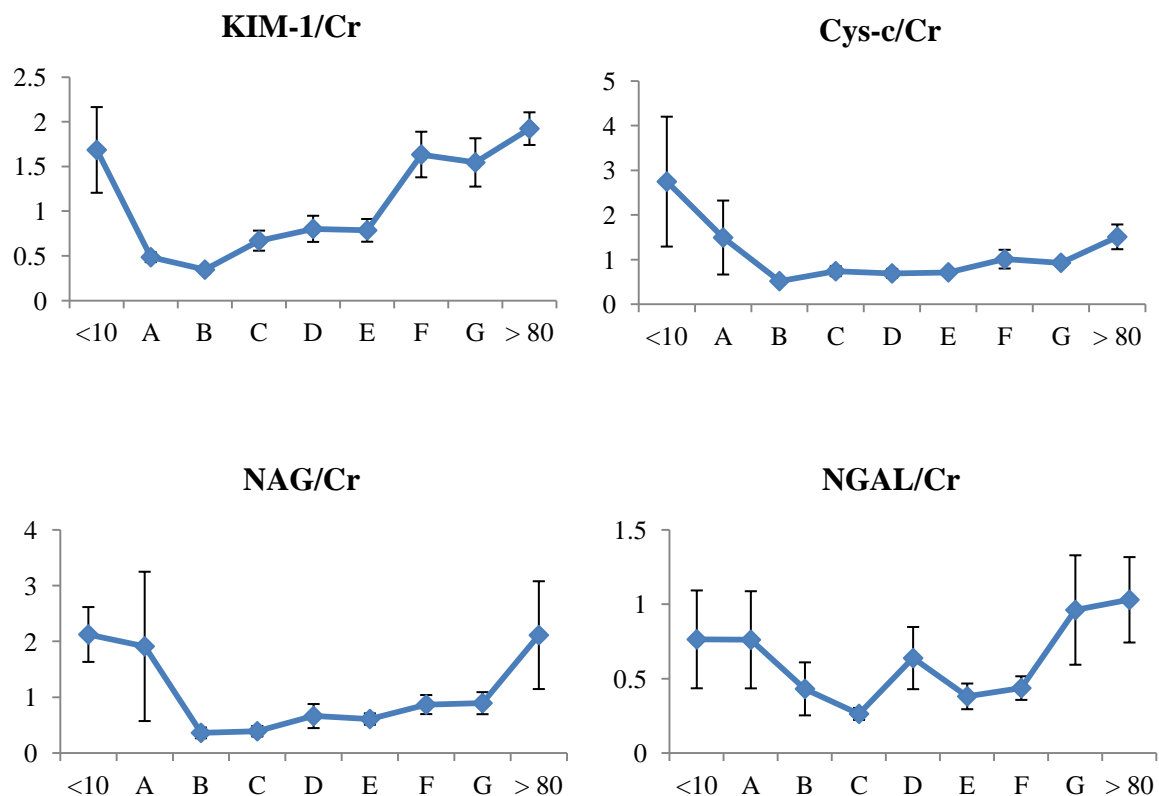
Figure 3: Ratio between the concentrations of urinary biomarkers and urinary creatinine level : represent the ratio between the concentration of KIM-1/Cr; the ratio the concentration of Cys-C/Cr; the ratio between the concentration of NAG/Cr and ratio between the concentration of NGAL/Cr. **<10** (0-9 years); **A** (10-20 years); **B** (21-30 years); **C** (31-40 years); **D** (41-50 years); **E** (51-60 years); **F** (61-70 years); **G** (71-80 years); **>80** (more than 80 years).
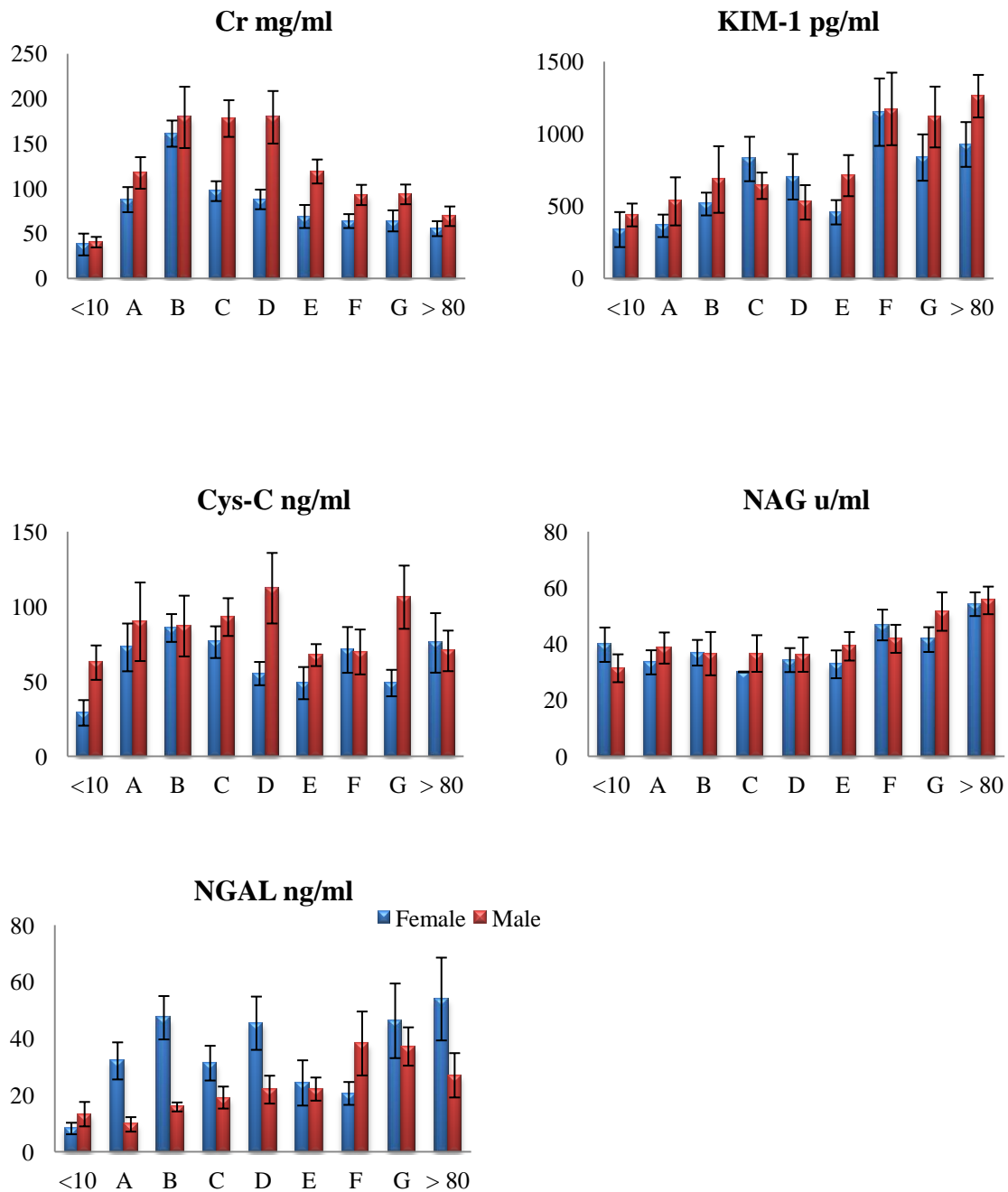
Figure 4: Gender related differences in urinary biomarkers for age groups : <10 (0-9 years); **A** (10-20 years); **B** (21-30 years); **C** (31-40 years); **D** (41-50 years); **E** (51-60 years); **F** (61-70 years); **G** (71-80 years); **>80** (more than 80 years). **Cr** Creatinine; **KIM**-1 Kidney Injury Molecule-1; **NGAL** Neutrophil Gelatinase-Associated Lipocalin; **NAG** N-acetyl-β-glugosaminidase and **Cys-C** Cystatin-C.
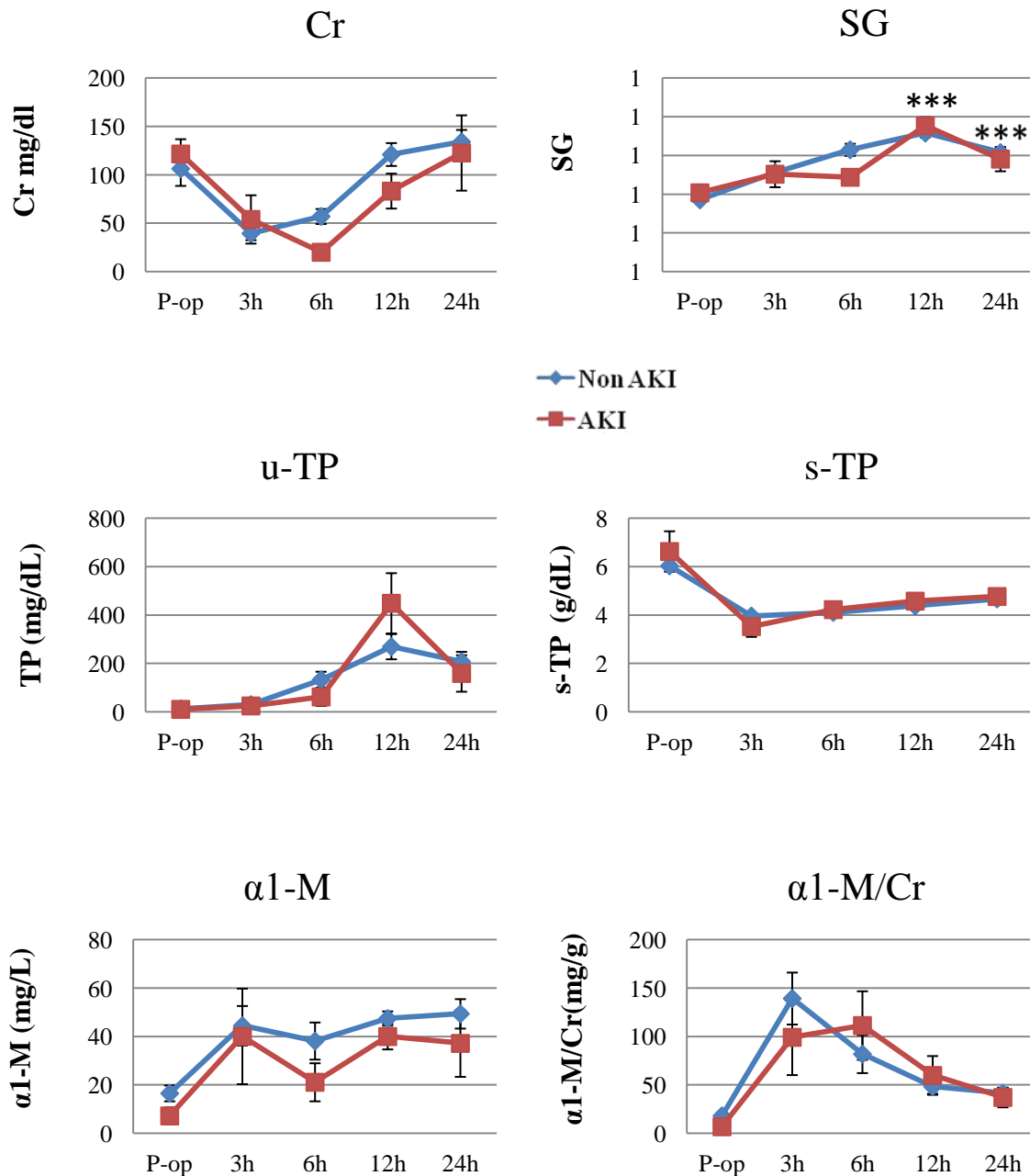
Figure 5: Changes in various biomarkers in the acute kidney (AKI) and non-acute kidney patients groups (non-AKI) after CPB surgery.

Cr, Creatinine mg/dl; **SG**, specific gravity; **u-TP**, urinary total protein (mg/dL); **s-TP**, serum total protein (g/dL); **α1-M**, α1- microglobulin (mg/L); **α1-M/Cr**, α1- microglobulin/creatinine ratio (mg/g); **P-op**, preoperative value; **3h**, postoperative 3 hours; **6h**, postoperative 6 hours; **12h**, postoperative 12 hours; **24h**, postoperative 24 hours.

Asterisks indicates a statistically significant differences for AKI group compared with non-AKI group at the same time point after CPB surgery: * * * P <0.001.
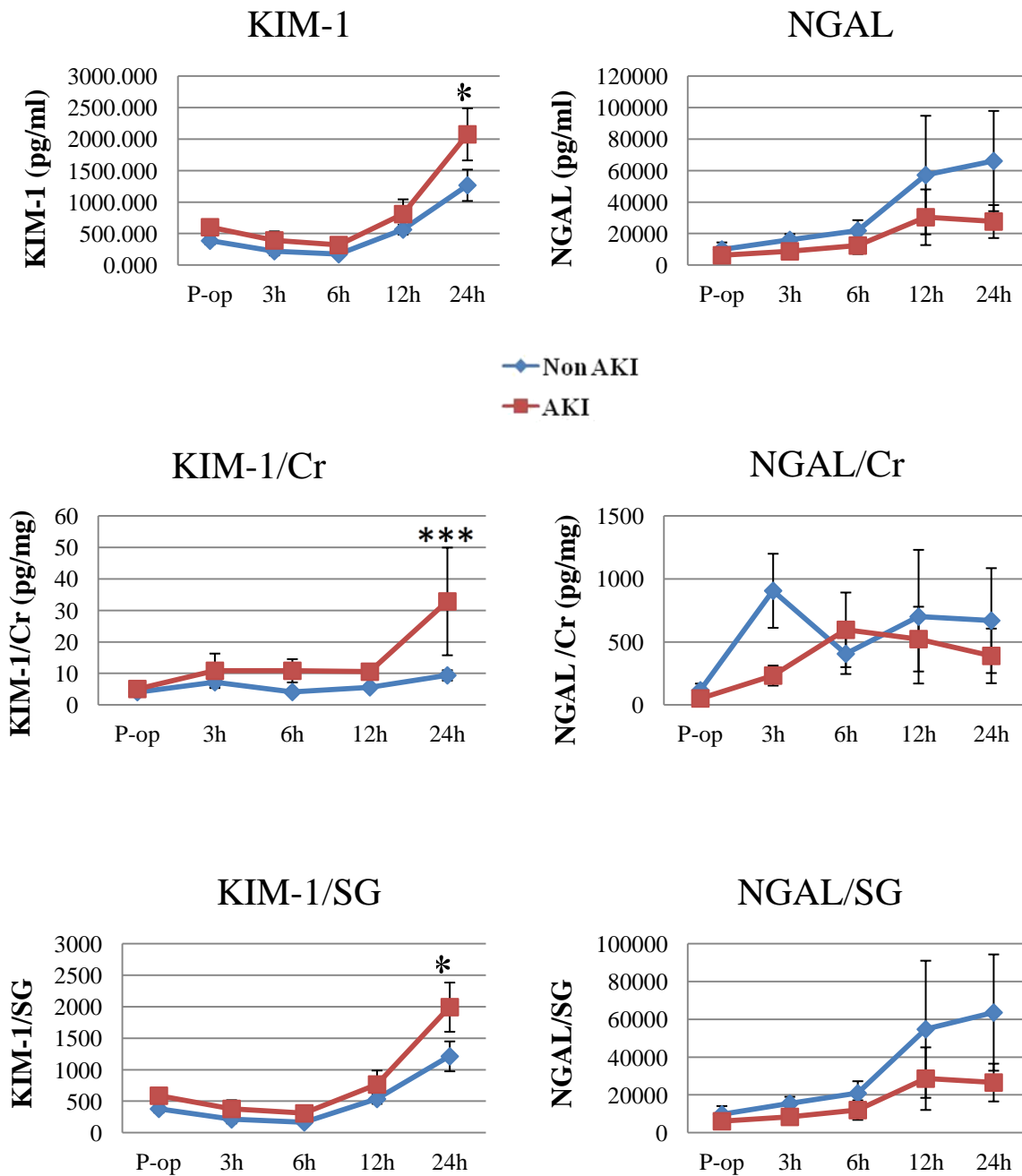
Figure 6: Changes in urinary KIM-1 and NGAL in acute kidney (AKI) and non-acute kidney patients groups (non-AKI) after CPB surgery.

P-op, preoperative value; **3h**, postoperative 3 hours; **6h**, postoperative 6 hours; **12h**, postoperative 12 hours; **24h**, postoperative 24 hours. **KIM-1**, kidney injury molecule; **NGAL**, neutrophil gelatinase-associated lipocalin; **KIM-1/Cr** (pg/mg), KIM-1/creatinine ratio; **KIM-1/SG**, KIM-1/specific gravity ratio; **NGAL/Cr** (pg/mg), NGAL/creatinine ratio; **NGAL/SG**, NGAL/specific gravity ratio.

Asterisks indicate a statistically significant differences for AKI group compared with non-AKI group at the same time point after CPB surgery: * $P < 0.05$; * * * $P < 0.001$.

Informed consent

Deel I: Informatiebrief patiënt

Opdrachtgever: Uhasselt, in samenwerking met Ziekenhuis Oost-Limburg en Jessa Ziekenhuizen.

Geachte,

We willen u hierbij graag uitnodigen om deel te nemen aan het onderzoek 'validatie van nieuwe biomerkers voor nierschade na cardiale chirurgie met cardiopulmonaire bypass'. Deelname aan deze studie is volledig op vrijwillige basis. Dit document beschrijft het doel, de onderzoeken, de voordelen, risico's en eventuele ongemakken gepaard gaande met de studie. Ook het recht om op elk moment de studie te verlaten, zijn hieronder beschreven. U mag op elk moment vragen stellen over deze studie aan de medewerkers. Als u beslist om deel te nemen aan deze studie, dient u het toestemmingsformulier aan het eind van dit document te ondertekenen.

Doel van de studie

Cardiale chirurgie leidt soms tot postoperatieve nierproblemen. Aangezien nierproblemen in veel gevallen geassocieerd zijn met een verlengd verblijf op de eenheid intensieve zorgen, is het van belang om deze nierproblemen zo snel en precies mogelijk op te sporen. Met de huidige detectiemethoden (zoals serumcreatinine) worden de eventuele nierproblemen pas zichtbaar in een gevorderd stadium. Indien men over snellere biomerkers voor nierproblemen zou beschikken, zou het mogelijk zijn om eventuele therapeutische interventies in een vroeger stadium te starten wat het ziekteverloop?? vooruitzicht ten goede zou komen.

De bedoeling van het onderzoek is om bij 250 patiënten die cardiale chirurgie ondergaan, na te gaan of enkele recent ontdekte urinaire biomerkers voor nierschade de detectie van nierproblemen kunnen versnellen of verbeteren.

Onderzoek op lichaamsmateriaal kan meer inzicht leveren in het ontstaan van ziektes en hoe ze kunnen voorkomen worden. Het is mogelijk dat uw lichaamsmateriaal wordt gebruikt voor genetisch onderzoek. Daarnaast kan dit onderzoek resulteren in betere methoden voor het stellen van een diagnose of prognose en leidt dit tot betere behandelingswijzen. Aldus kan in de toekomst een beter antwoord gevonden worden op vele vragen rond ziektes zoals kanker, diabetes of hartziekten.

Uw materiaal zal enkel voor huidige en toekomstige onderzoeksdoeleinden in verband met nierschade of andere ziekten worden gebruikt en zal niet worden verkocht. Het uigevoerde onderzoek kan bijdragen tot de ontwikkeling van nieuwe producten in de toekomst.

Procedures en studieprotocol

Indien u toestemt om aan de studie deel te nemen en indien u voldoet aan alle voorwaarden voor deelname aan de studie, dan zullen de volgende tests en onderzoeken worden uitgevoerd:

Er zullen bloed- en urinestalen van u worden afgenomen. Dit zal zowel gebeuren vlak voor uw chirurgische ingreep als tijdens uw verblijf op intensieve zorgen na de operatie, nl. 2 u, 6 u, 12 u en 24 u na de ingreep. Op ieder tijdstip zal 5 ml bloed en 20 ml urine afgenomen worden. Bloedname zal gebeuren via venipunctie, urinestalen worden afgenomen via de blaassonde die naar aanleiding van uw ingreep wordt aangebracht. Aangezien de staalnames voor de studie gecombineerd worden met de bloed- en urineverzameling die standaard worden uitgevoerd in functie van uw ingreep, zal u geen bijkomende hinder ondervinden van de staalnames voor deze studie.

Het ongebruikt/bijkomend lichaamsmateriaal zal worden bewaard in de Limburgse Biobank. Dit materiaal kan dan worden gebruikt voor verder onderzoek naar biomerkers voor nierschade, maar kan ook worden gebruikt voor onderzoek naar andere ziektes door verschillende onderzoekers.

Duur van de studie

Het onderzoek zal niet langer duren dan uw opname in het ziekenhuis naar aanleiding van uw geplande cardiale ingreep.

Mogelijke neveneffecten en risico's

Deelname aan deze studie omvat geen extra lichamelijk risico's. De materiaalverzameling gebeurt via procedures die nodig zijn voor de ingreep met de daarbij geassocieerde standaardrisico's van de ingreep en het aanprikken van de bloedbaan voor het plaatsen van een katheter.

De opdrachtgever heeft een verzekering afgesloten die de risico's en de schade dekken die eventueel door deelname aan de studie werden opgelopen

Het risico gepaard met bewaring van lichaamsmateriaal in een biobank bestaat uit het ongeoorloofd verspreiden van uw persoonlijke informatie. De biobank neemt alle voorzorgsmaatregelen in acht om te verzekeren dat uw gegevens confidentieel blijven. Zo krijgen de onderzoekers nooit toegang tot de gegevens die u kunnen identificeren. Daarnaast worden het materiaal en de gekoppelde klinische gegevens altijd gecodeerd vooraleer deze worden doorgegeven. De kans dat uw gegevens verspreid worden is daarom zeer klein. Bovendien is door de Limburgse Biobank een verzekeringspolis afgesloten die de eventuele risico's en/of schade gekoppeld aan bewaring van lichaamsmateriaal en gegevens in een biobank dekt.

Deel II: toestemmingsverklaring

Lees zorgvuldig onderstaande tekst en denk goed na over u keuze. Vink vervolgens uw antwoord aan. Voor verdere vragen kan u steeds terecht bij de arts, de verpleging of bij de biobank (zie onderaan). Uw medische zorgverlening zal niet beïnvloed worden door uw keuze. U ontvangt een kopie van dit document.

Ik ontving een kopie van alle pagina's van dit document. Ik heb het document gelezen of het is mij voorgelezen. Ik begrijp de gegeven informatie. Ik kreeg voldoende kans om mijn vragen te stellen en de vragen zijn tot mijn voldoening beantwoord. Ik stem vrijwillig toe om deel te nemen aan het beschreven onderzoek.

Ik geef toelating tot afname en bewaring van mijn lichaamsmateriaal voor biomedische onderzoeksdoeleinde volgens de aangeduide wijze en doelstellingen.

Naam van de deelnemer   Datum   Handtekening

Ik bevestig dat ik het informatiedocument en het bijhorende procedures accuraat aan de deelnemer heb uitgelegd. Ik bevestig dat de deelnemer de kans kreeg om vragen te stellen, en alle gestelde vragen werden correct en naar mijn beste vermogen beantwoord. Ik bevestig dat de deelnemer niet tot toestemming werd gedwongen en dat de toestemming vrij en vrijwillig werd gegeven.

Een kopie van dit toestemmingsformulier werd aan de deelnemer overhandigd

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Voor akkoord,

AL Hamdani, Wasen

Datum: **3/09/2012**