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Biological variation of plasma and urinary markers of acute kidney injury in patients with chronic kidney disease

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List of abbreviations: AKI, acute kidney injury; RCV, reference change value; NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule-1; TIMP-2, tissue inhibitor of metalloproteinases-2; NAG, N-acetyl-β-D-glucosaminidase; IL-18, interleukin-18; CV_I, within-subject biological variability; RIFLE, Risk, Injury, Failure, Loss, End-stage; AKIN, Acute Kidney Injury Network; CKD, chronic kidney disease; II, index of individuality; GFR, glomerular filtration rate; ANOVA, analysis of variance; CV_T, total variability; CV_A, analytical variability; CV_G, between-subject variability;

Brief summary: Several novel biomarkers of acute kidney injury (AKI) have recently been described but little is known of their biological variability, a prerequisite to their use in clinical practice. We characterised the biological variability of whole blood, plasma and

urinary neutrophil gelatinase-associated lipocalin (NGAL), urinary kidney injury molecule-1 (KIM-1), tissue inhibitor of metalloproteinases-2 (TIMP-2) and interleukin-18 (IL-18), in addition to more traditional markers of kidney damage (plasma cystatin C and creatinine, urinary N-acetyl-β-D-glucosaminidase, albumin and α_1-microglobulin). Biological variability of the novel markers is high, but compared against the scale of change described in disease situations would not preclude their use as sensitive markers of AKI.

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Abstract:

Background:

Identification of acute kidney injury (AKI) is predominantly based on changes in plasma creatinine concentration, an insensitive marker. Alternative biomarkers have been proposed. The reference change value (RCV), the point at which biomarker change can be inferred to have occurred with statistical certainty, provides an objective assessment of change in serial tests results in an individual.

Methods:

In 80 patients with chronic kidney disease weekly measurements of blood and urinary biomarker concentrations were undertaken over 6 weeks. Variability was determined and compared before and after adjustment for urinary creatinine and across subgroups stratified by level of kidney function, proteinuria and presence/absence of diabetes.

Results:

RCVs of whole blood, plasma and urinary neutrophil gelatinase-associated lipocalin (NGAL, 111%, 59%, 693% respectively), plasma cystatin C (14%) and creatinine (17%), urinary kidney injury molecule-1 (KIM-1, 497%), tissue inhibitor of metalloproteinases-2 (TIMP-2, 454%), N-acetyl-β-D-glucosaminidase (NAG, 361%), interleukin-18 (IL-18, 819%), albumin (430%) and α_1-microglobulin (216%) were determined. Blood biomarkers exhibited lower variability than urinary biomarkers. Generally, adjusting urinary biomarker concentrations for creatinine reduced ($p < 0.05$) within-subject biological variability (CV_I). For some markers, variation differed ($p < 0.05$) between subgroups.

Conclusions

These data can form a basis for application of these tests in clinical practice and research studies and are applicable across different levels of kidney function, proteinuria and in the presence/absence of diabetes. Most of the studied biomarkers have relatively high CV_I (noise) but also have reported large concentration changes in response to renal insult (signal); thus evolutional change should be detectable (high signal: noise ratio) when baseline data are available.

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Introduction

Acute kidney injury (AKI) is common, harmful, and potentially preventable.([1, 2])
Definitions of AKI, including the RIFLE (Risk, Injury, Failure, Loss, End-stage) and
AKIN (Acute Kidney Injury Network) criteria, are based upon recognition of changes in
plasma creatinine concentration.([3]) However, creatinine is a poor biomarker for AKI.([4])
Earlier identification with more sensitive biomarkers has potential to improve outcomes
including mortality, hospital length of stay and progression to chronic kidney disease
(CKD).([5]) Widely studied markers include plasma and urinary neutrophil gelatinase-
associated lipocalin (NGAL)([6, 7]) and kidney injury molecule-1 (KIM-1).([8]) Interleukin-
18 (IL-18)([9]) and, more recently, tissue inhibitor of metalloproteinases-2 (TIMP-2) have
also been proposed as early markers of AKI.([10]) Historically, a range of tubular enzymes
and filtered proteins have been measured in urine as indicators of tubular functional
integrity including N-acetyl-β-D-glucosaminidase (NAG)([11]) and α_1-microglobulin.([12,
13]) Many of these have recently been re-evaluated as markers of AKI.([14, 15])

It is appreciated that there is a need for a better understanding of these potential AKI
markers, including their biological variability, amongst people with CKD.([16]) CKD is
prevalent in the general population, increases susceptibility to AKI,([17]) and is potentially
amenable to surveillance for AKI-avoidance measures. Biological variation may differ in
chronic disease states compared to health:([18]) there is little data on variability of
biomarkers in CKD patients. An understanding of biological variation of markers is
essential to interpretation of changes in response to disease events. Data on biological
variation should be generated early in the evolution of new diagnostic and disease
monitoring tests.([19]) Critical evaluation of the significance of changes in results obtained
on analysis of serial specimens can be performed only by consideration of biological and
analytical variation.([20]) Such data enables the derivation of the reference change value
(RCV), the point at which a true change in a biomarker in an individual can be inferred to
have occurred. Knowledge of biological variability is imperative to objectively compare
available tests; to set analytical performance goals; and to determine the utility of reference
intervals through derivation of the index of individuality (II).([20, 21])

The primary aim of this study was to measure biological variation of biomarkers of AKI
amongst patients with CKD. We focussed on within-subject variation (CV_I) as the

parameter of most relevance when detecting change within an individual. Subsidiary research questions were (i) should urinary biomarker results be expressed as a concentration or as a ratio to urinary creatinine and (ii) does variability differ amongst different groups of CKD patients?

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Subjects and Methods

Patients with CKD (n=80), who did not have kidney failure (glomerular filtration rate [GFR] >15 mL/min/1.73 m^2), were recruited at the Kent Kidney Care Centre, UK between January 2012 and March 2014. The sample was a convenience sample targeted to include representative numbers of patients with diabetes and/or proteinuria. Exclusion criteria included patients aged <18 years, renal transplant recipients, terminal illness or significant cognitive impairment. Patients provided written informed consent. The study had ethical approval (South-East Coast Research Ethics Committee reference 11/LO/1304). The study conforms to the internationally agreed checklist for the reporting of studies of biological variation.([22](#22))

Demographic details, blood pressure, height, weight and relevant medical and drug history and cause of CKD were recorded (http://www.era-edta-reg.org/prd.jsp , last accessed 28th July 2015). Samples were either taken at the Kent Kidney Care Centre or in patient's homes by registered nursing staff. Patients provided a random urine sample and a blood sample weekly for 6 consecutive weeks. To standardise preanalytical variables, samples were collected on the same day of the week for each patient and consistently either in the morning (n=62) or afternoon (n=18). Blood samples were collected using standard venepuncture procedures, including the use of a tourniquet, into ethylenediaminetetraacetic acid- and lithium heparin-containing Vacuette^{TM} tubes (Greiner Bio-One International) following manufacturer's recommended order of draw. Patients provided urine samples in a plain sterilin pot. Plasma/serum was separated by centrifugation within 4 h of venepuncture and sample aliquots were stored at -80°C pending analysis. All analyses were undertaken within 15 months and the majority within 6 months of venepuncture.

For each patient, at each of the six time points in the study, blood and plasma NGAL, plasma cystatin C and creatinine, and urinary biomarkers, NGAL, KIM-1, NAG, albumin, IL-18, TIMP-2, α1-microglobulin and creatinine were measured. Blood NGAL was measured using a point of care testing device (Triage Biosite, Inverness Medical Innovations) and plasma NGAL using a turbidimetric immunoassay (The NGAL Test^{TM}, BIOPOPRTO Diagnostics, Denmark) on an Abbott Architect analyser (Abbott Laboratories, Abbott Park, Illinois, USA). Urinary NGAL was measured using a chemiluminescent microparticle immunoassay (Abbott Architect).([23](#23)) KIM-1 (Quantikine

DKM100, R&D Systems, www.RnDSystems.com), IL-18 (MBL International Corporation, Woiburn, MA, www.mblintl.com) and TIMP-2 (Quantikine DTM200, R&D Systems) were measured using enzyme-linked immunosorbent assays. Urinary NAG was measured using a colorimetric assay (PPR Diagnostics Ltd). Urinary α1-microglobulin (Beckman Immage),(12) plasma cystatin C and urinary albumin (both Abbott Architect) were measured using turbidimetric immunoassays. Plasma and urinary creatinine were measured using an enzymatic assay (Abbott Architect) traceable to a reference isotope dilution-mass spectrometry method. GFR was estimated using the Modification of Diet in Renal Disease (MDRD) Study equation.(24) Prior to analysis samples were thawed at room temperature and mixed by inversion. All samples from each individual subject were measured in duplicate in random order in a single assay. Each of the biomarker analyses was undertaken by a single operator using a single instrument. Quality control data for the respective assays are given in Supplementary Table 1.

Data analysis

Normality tests were performed using the Shapiro-Wilk test (Analyse-It, Leeds, UK). For estimation of variance components data were log-transformed using natural log. Outliers between duplicate measurements and of within-subject variance were excluded using Cochran's test and outliers amongst mean values of subjects were excluded using Reed's test, in the entire cohort and in the subgroups (Supplementary Table 2).(20) Log transformation improved the normality of the data as assessed by determining the percentage deviation from one of the mean over the median,(25) an increase in Shapiro-Wilk W statistic, minimisation of excess kurtosis and skewness scores, and by visual examination of the distributions (Supplementary Table 3). Terminology used was as proposed by Simundic et al.(26) Total (CV_T), analytical (CV_A), CV_I and between-subject (CV_G) components of variation were calculated using standard approaches(20) with general linear model ANOVA (Minitab; Coventry, UK) followed by back-transformation of the data. Confidence intervals for CV_I were estimated using the method of Roraas et al.(27) CV_A was considered acceptable if minimal performance standards were met ($CV_A \leq 75\% CV_I$).(21) The RCV (p<0.05) for change in analyte concentration between two results was calculated using a log-normal approach.(25) The number of specimens (n) required to produce a precise estimate of the homeostatic set-point with 95% confidence within $\pm 10\%$ was calculated as:

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$$n = [1.96 \cdot (CV_I^2 + CV_A^2)^{1/2}/10]^2$$

For each biomarker the index of individuality (II) was calculated as:

$$II = (CV_I^2 + CV_A^2)^{1/2}/CV_G$$

The value of population-based reference ranges was assessed based on the II, following the conventional approach that when the II is low (<0.6)(i.e. individual results stay within a narrow range compared with the population reference interval) then a population-based reference interval will be of limited sensitivity in detecting changes in an individual. The converse applies to a high II (>1.4).[20]

Urinary data was reanalysed after adjustment of biomarker concentrations for urinary creatinine, to establish whether such adjustment reduced CV_I. A comparison of CV_I between males and females was also undertaken. Differences were tested using the F-test (Minitab).

In addition to studying variability in the whole cohort, analyses were also undertaken across 4 groups (groups A-D) divided on the basis of glomerular filtration rate (GFR, <60 or $\geq$60 mL/min/1.73 m^2), albuminuria (albumin to creatinine ratio <30 or $\geq$30 mg/mmol),[28] and in the presence and absence of diabetes mellitus (Table 1). For native data, the Kruskal-Wallis test (non-parametric analysis of variance (ANOVA)) was used to assess the significance of differences in continuous variables between the groups and chi-squared test was used for categorical variables (Analyse-It). Variation was compared across the groups using multilevel regression methods. Three level models were used to divide the total variation into that between patients, between weeks (within patient), and between repeat measurements (within patient/week). The variation between weeks was allowed to vary between the patient groups (A-D), and the significant of this difference was assessed. Due to the skewed distribution of all markers the analysis was performed on the log scale. The analyses were performed using MLwiN version 2.25 (Centre for Multilevel Modelling, University of Bristol, Bristol BS8 1TX, UK).

Results

Characteristics of the study subjects are shown in Table 1. Causes of CKD were autosomal dominant polycystic kidney disease (3), chronic hypertensive nephropathy – no histology (9), diabetic nephropathy (6), IgA nephropathy – histologically proven (5), membranous nephropathy – idiopathic (8), mesangial proliferative glomerulonephritis (3), mesangiocapillary glomerulonephritis type 1 (3), other (17), systemic vasculitis (3), tubulo-interstitial nephritis – no histology (2) and aetiology unknown (21). No patients developed AKI(3) during the study.

Variance components for all biomarkers are given in Table 2. Blood biomarkers exhibited lower CV_I than urinary biomarkers. Plasma creatinine and cystatin C exhibited the lowest, and plasma NGAL the highest, CV_I values of the blood markers. The concentration of serial plasma NGAL measurements would need to increase by 59% between any two measurements before it can be considered a significant change: serial changes in all urinary markers would need to be greater than this (Table 2). With the exception of α1-microglobulin, adjusting urinary biomarker concentrations for urinary creatinine siginificantly reduced their biological variability ($p<0.05$). Significant ($p<0.05$) differences for CV_I were observed between males and females for urinary NGAL, KIM-1, NAG, IL-18, and TIMP2 both with and without adjustment for urinary creatinine concentration in all cases (Supplementary Table 4). Most biomarkers satisfied minimal analytical performance standards except for whole blood NGAL (CV_A 17.8%, CV_I 20.8%).

For all biomarkers, concentrations differed across the sub-groups (Table 1, $p<0.001$ in all cases). Within-subject variation differed significantly between the four patient subgroups for some of the markers examined (Table 3). For plasma NGAL variation was largest in group A (estimated GFR >60 mL/min/1.73 m^2 with or without proteinuria). For the majority of the urine markers where a difference was observed, the least variation was observed for Group C (proteinuric, non-diabetic patients with estimated GFR 15-59 mL/min/1.73 m^2). Supplementary Tables 5-8 provide full variance component data for the four subgroups in addition to causes of CKD.

Discussion

We report the biological variability characteristics of a range of biomarkers of AKI amongst patients with CKD. Generally the within-subject variability of these markers is relatively high and the index of individuality low, suggesting that use of conventional reference ranges for disease detection will be unsuitable.[20] This discussion places the biological variability of the markers we have studied in the context of changes in concentration that have been observed in AKI, and also compares our data in CKD patients with that previously reported in healthy individuals, where such is available (Supplementary Table 9).

We studied variability of NGAL in urine and plasma and also in whole blood using a point of care testing device. The latter did not meet minimum analytical performance criteria as defined by biological variability, such that >25% of the variability observed may be attributable to analytical imprecision.[21] Establishment of biological variability data for whole blood NGAL will require use of a device with improved analytical performance. We have not identified previous studies of the biological variability of plasma NGAL. Plasma NGAL demonstrated lower variability than urinary NGAL. In some carefully controlled situations plasma NGAL has been shown to increase rapidly (within 2 h) and markedly (290% increase) in response to kidney injury:[6, 29] concentrations then remain increased and relatively stable for some time (e.g. 48 h[17]) after insult. Such large changes in biomarker concentration could be detected against background biological variability. However, in some studies pre-AKI plasma NGAL concentrations have not been available: monitoring rate of change is not an option and analysis is based on presenting plasma NGAL concentrations amongst patients who have AKI compared to those that do not. Studies have been undertaken in an attempt to define a cut-off value above which AKI can be diagnosed. Whilst differences in plasma NGAL concentrations between patients with, and without, AKI are marked, in the presence of pre- and coexisting CKD such discrimination is less clear-cut. For example, in the study of Soto et al plasma NGAL concentrations amongst a non-AKI sub-group of their cohort with stable CKD almost completely overlapped values observed in the AKI group.[17] One third of patients with AKI in that study had pre-existing CKD, illustrating the significance of this issue.[17]

The CV_I of urinary NGAL was considerable and was slightly reduced by adjusting the concentration for urinary creatinine. Our data are in broad agreement with other reports (Supplementary Table 9). Bennett et al reported 20-fold increases in urinary NGAL concentrations amongst paediatric cardiac surgery patients who developed AKI, well in excess of the RCV.(23) However, as for plasma NGAL, the increase in urinary NGAL concentration occurs early (within 2 h) after insult(29) and the concentration remains increased for at least 24 h.(30) Consequently, in more general situations AKI patients presenting to emergency departments will most likely already have had an increase in their urinary NGAL concentration and recognition of AKI will require use of a diagnostic cut-off for NGAL. Whilst such a cut-off may allow identification of patients with more severe AKI (e.g. RIFLE stage F), discrimination of lesser degrees of injury compared to non-AKI may be more difficult.(30)

The observed CV_I of urinary KIM-1 reported here is lower than that for urinary NGAL, especially after adjustment for creatinine concentration, and agrees reasonably with a previous report amongst healthy volunteers (Supplementary Table 9). In contrast to NGAL, urinary KIM-1 concentration shows a later increase after insult in patients with AKI, with for example more than two-fold increases being observed after cardiopulmonary bypass surgery after 12 h, with further increases up to 24 h.(30) The delayed rise and marked increase in urinary concentration of KIM-1 may allow serial monitoring of this marker to be a useful tool in the detection of AKI. IL-18 also exhibited a high CV_I in the present and a previous study (Supplementary Table 9), but changes in IL-18 concentration after renal insult are also likely to exceed the RCV.(30)

TIMP-2 is a relatively novel marker of AKI.(10) We found no other studies of urinary TIMP-2 biological variation. Biological variability was of a similar order of magnitude to other urinary markers and was reduced by adjustment for creatinine concentration. Yamashita et al report five-fold increases in urinary TIMP-2 concentration in patients developing AKI.(14)

We also studied the biological variability of some more established markers of kidney damage: plasma creatinine and cystatin C and urinary albumin, NAG and α1-microglobulin. Criteria for diagnosis of stage 1 AKI are currently met when there is a ≥26.4 umol/L (≥0.3 mg/dL) or a 50% increase in plasma creatinine concentration above

baseline within 48 h.(3) This definition has primarily been based on increased risk associated with such a change rather than considerations of what is detectable analytically against background variation.(31) The overall CV_I we obtained for plasma creatinine amongst stable CKD patients (5.7%) is similar to that reported in healthy individuals (Supplementary Table 9) and would facilitate AKI detection at baseline physiological concentrations (e.g. 1 mg/dL).

We included cystatin C in our study as it is increasingly being proposed as an alternative marker of kidney damage to creatinine, including in the AKI setting.(32) The biological variability we observed amongst CKD patients is in keeping with data of others, being similar to, but slightly lower than, the CV_I of creatinine (Supplementary Table 9). It should be noted that plasma creatinine and cystatin C were the only two markers we studied where measurement of a single sample would enable a useful assessment of the true concentration (homeostatic set point) in an individual.

Albuminuria is associated with AKI(15) and predicts progression of AKI after surgery in individuals with AKI.(33) The CV_I of urinary albumin we observed is broadly similar to that previously reported (Supplementary Table 9); was reduced by adjustment for creatinine concentration; and was highest amongst those with the lowest levels of albuminuria, as also noted by others.(34) Urinary NAG showed similar within-subject biological variability to KIM-1, including a reduction after adjustment for creatinine and differences in CV_I across the patient groups which disappeared after creatinine adjustment. Our data compares reasonably well with earlier estimates of CV_I derived in first morning urines of healthy individuals (Supplementary Table 9). The CV_I of urinary α1-microglobulin was lower than that for other urinary markers but was not reduced by adjustment for creatinine concentration, as noted by others (Supplementary Table 9). α1-microglobulin in its free form is relatively freely filtered at the glomerulus, following which it undergoes proximal tubular reabsorption.(13) Possibly variability in urinary α1-microglobulin loss is determined by variability in tubular reabsorption rates, irrespective of urinary flow.

For most biomarkers included in the present study adjustment for urinary creatinine concentration reduces within-subject variability, since it effectively adjusts for changes in urinary flow rate. Generally this is considered an advantage when monitoring markers over

time within an individual or comparing marker concentrations between individuals. However, creatinine adjustment itself can also be misleading as it may distort differences between individuals with differing muscle mass or changes within individuals at times of change in creatinine excretion (e.g. AKI).(35) Although achieving statistical significance for some markers, variances across different groups of CKD patients were relatively consistent, with the possible exception of non-diabetic proteinuric patients with CKD who appeared to have lower variance for some markers (e.g. urinary NGAL, Table 3). Given their higher levels of proteinuria, we postulate that this could be related to saturation of tubular reabsorptive mechanisms in such individuals.(36) We also observed higher within-subject variability for some urinary, but not plasma, markers in females compared to males. This difference persisted after adjustment for urinary creatinine. As noted above, these analyses do not take into account other potential influences on variability (e.g. age, level of proteinuria and GFR) and would require further study to confirm.

In most cases the variability we have observed in CKD patients is similar to that observed in earlier studies amongst healthy individuals (Supplementary Table 9). A limitation of our study is that we have not directly established the magnitude of marker changes in patients with AKI, but have compared our data to that reported elsewhere. However, it is generally accepted that estimates of biological variability can be generalised across studies. In most cases, reported AKI-related changes in marker concentrations are likely to exceed biological variability, suggesting that evolutional change in markers will allow AKI detection when patients can be followed from onset of insult (e.g. in elective surgical situations). However, in the general acute medical setting, knowledge of baseline biomarker concentrations is unlikely to be the norm. The high individuality of the markers (low II), and higher concentrations in CKD patients compared to healthy individuals, suggest that interpretation against reference intervals or simple decision thresholds may result in reduced sensitivity for AKI detection amongst patients with pre-existing CKD. A single biomarker may be unable to diagnose all aspects of a complex multifactorial process such as AKI, and a panel of biomarkers may therefore be necessary.(37) Biological variability is clearly not the only criterion of importance in biomarker selection e.g. the temporal delay in increase in creatinine compared to NGAL following an episode of AKI will remain a major limitation of creatinine. Practical considerations also impinge on choice of biomarker employed e.g. experience suggests that blood sampling is more commonly undertaken than urine sampling in patients presenting with AKI.(2) The high

reagent cost of some of the newer markers (e.g. NGAL, KIM-1) should also be tested in health economic analysis against the more traditional, and less expensive, markers (e.g. NAG).

In conclusion, we have established baseline data on biological variation of AKI markers in a carefully designed study in a clinically relevant population, namely patients with CKD. The data reported herein can form a basis for the application of these tests in clinical practice and research studies. Most of these biomarkers have relatively high within-subject variability (noise) but also have reported large concentration changes in response to renal insult (signal): thus evolutional change should be detectable (high signal to noise ratio) when baseline data is available. Broadly similar variability should be anticipated in the presence of proteinuria and diabetes. Generally variability will be reduced by adjustment of urinary markers for creatinine concentration: this may facilitate disease detection.

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design; data collection, analysis, and interpretation; writing the report; or the decision to submit the report for publication.

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Table 1. Characteristics of study subjects in whole cohort and in four sub-groups.

Continuous data shown as median (range) unless stated otherwise. Biomarker data represents the median (range) of data from all six sampling points.
Group A: estimated glomerular filtration rate [GFR] $\geq$60 mL/min/1.73 m^2 with or without proteinuria (albumin:creatinine ratio $\geq$30 mg/mmol)
Group B: estimated GFR 15-59 mL/min/1.73 m^2 non-proteinuric (albumin to creatinine ratio <30 mg/mmol) non-diabetic patients
Group C: estimated GFR 15-59 mL/min/1.73 m^2 proteinuric (albumin to creatinine ratio $\geq$30 mg/mmol) non-diabetic patients
Group D: estimated GFR 15-59 mL/min/1.73 m^2 proteinuric (albumin to creatinine ratio $\geq$30 mg/mmol) diabetic patients

*One patient initially assigned to group B was subsequently excluded from the subgroup analysis after developing proteinuria across the course of the study. This patient's data is included in the 'All' column but not in the 'B' column.

	All*	Group A	Group B*	Group C	Group D	p
n	80	18	25	20	16	-
Age, years	67.9 (33.8, 89.1)	61.5 (33.8, 74.9)	68.5 (53.7, 81.7)	73.5 (38.2, 88.1)	67.4 (47.5, 89.1)	0.013
M:F	47:33	9:9	12:13	14:6	11:5	0.328
Caucasian (n)	78	18	24	19	16	0.660
Height, m	1.71 (1.50, 1.95)	1.70 (1.50, 1.95)	1.67 (1.55, 1.95)	1.71 (1.57, 1.88)	1.73 (1.57, 1.83)	0.660
Weight, kg	80.1 (38.1, 150.0)	77.8 (48.3, 128.0)	75.7 (38.1, 114.0)	85.0 (46.7, 150.0)	90.3 (57.6, 142.0)	0.165
Body mass index	27.2 (15.9, 60.9)	27.0 (18.9, 42.8)	25.1 (15.9, 33.5)	26.3 (18.9, 60.9)	32.5 (23.0, 47.4)	0.061
Systolic blood pressure (mm Hg)	136 (98, 206)	134 (106, 176)	132 (110, 182)	134 (98, 206)	147 (101, 169)	0.398
Diastolic blood pressure (mm Hg)	77 (56, 106)	83 (66, 106)	75 (58, 102)	76 (57, 93)	77 (56, 105)	0.180
Diabetes (n)	20	4	0	0	16	-
Estimated GFR (mL/min/1.73 m^2)	41 (15, 90)	67 (60, 90)	40 (17, 56)	34 (15, 57)	31 (19, 51)	-
Plasma creatinine (µmol/L)	138 (53, 354)	84 (53, 116)	146 (76, 265)	154 (83, 354)	158 (113, 348)	<0.0001
Plasma cystatin C (mg/L)	1.76 (0.59, 3.93)	1.02 (0.59, 2.13)	1.75 (1.12, 3.93)	1.93 (1.06, 3.51)	2.23 (1.33, 3.86)	<0.0001
Plasma NGAL (µg/L)	148 (31, 10387)	78 (31, 368)	123 (60, 300)	181 (51, 549)	243 (108, 10387)	<0.0001
Whole blood NGAL (µg/L)	197 (46, 1245)	125 (46, 737)	175 (84, 1195)	270 (64, 1245)	363 (133, 798)	<0.0001
Urine NGAL (µg/L)	32 (1, 1501)	19 (2, 310)	20 (1, 501)	33 (3, 1501)	44 (6, 1456)	<0.0001
Urine KIM-1 (µg/L)	1.04 (0.01, 11.75)	0.90 (0.06, 3.07)	0.84 (0.01, 11.75)	1.19 (0.13, 5.85)	1.28 (0.09, 8.04)	<0.001
Urine NAG (µmol/L)	360 (23, 5678)	350 (23, 1632)	323 (44, 2456)	505 (79, 5678)	521 (58, 1520)	<0.0001
Urine albumin (mg/L)	160.4 (5.0, 10824.0)	112.1 (5.0, 10824.0)	18.7 (5.4, 369.7)	379.3 (25.3, 2591.4)	450.3 (9.6, 2508.4)	<0.0001
Urine IL-18 (ng/L)	39.4 (2.1, 1242.7)	38.6 (2.9, 569.6)	29.2 (2.3, 575.7)	45.0 (2.1, 1242.7)	45.58 (3.86,658.3)	<0.0001
Urine TIMP-2 (µg/L)	2.9 (0.1, 31.3)	3.5 (0.4, 19.5)	2.2 (0.1, 24.0)	2.9 (0.3, 31.3)	3.2 (0.2, 10.7)	<0.0001
Urine α1-microglobulin (mg/L)	22 (4, 306)	16 (4, 137)	18 (4, 146)	23 (5, 164)	33 (4, 306)	<0.0001
Urine creatinine (mmol/L)	7.1 (1.7, 22.8)	7.1 (1.3, 27.0)	7.8 (5.4, 22.8)	6.1 (1.7, 18.7)	6.4 (1.1, 20.0)	<0.0001

Abbreviations: CKD, chronic kidney disease; IL-18, interleukin-18; KIM-1, kidney injury molecule-1; NAG, N-acetyl-β-D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin; TIMP-2, tissue inhibitor of metalloproteinase-2

To convert plasma creatinine concentration from umol/L to mg/dL, divide by 88.4.

Table 2. Variance components for CKD patients in whole cohort (n = 80)

Variance components were used to calculate the critical difference for change in serial results (positive and negative reference change value, RCV) to be considered as significant (p<0.05), the number of specimens required to estimate the homeostatic set point of an individual (within $\pm10\%$), and the index of individuality (II).

Biomarker	CV_I, % (CI)	CV_G, %	CV_A, %	Positive RCV, %	Negative RCV, %	No. of samples for set-point estimation	II
Plasma creatinine	5.7 (5.0, 6.3)	51.3	0.6	17	-15	1	0.1
Plasma cystatin C	4.8 (4.3, 5.3)	49.9	0.9	14	-13	1	0.1
Plasma NGAL	16.1 (14.4, 17.7)	127.5	5.1	59	-37	11	0.1
Whole blood NGAL	20.8 (17.8, 23.8)	91.9	17.8	111	-53	29	0.3
Urine NGAL	86.3 (79.7, 92.9)	196.2	5.2	693	-87	287	0.4
Urine NGAL/creatinine	70.7 (65.1, 76.4)*	219.0	5.3	486	-83	193	0.3
Urine KIM-1	71.6 (65.9, 77.3)	124.6	4.8	497	-83	198	0.6
Urine KIM-1/creatinine	29.8 (27.1, 32.5)*	103.2	5.1	127	-56	35	0.3
Urine NAG	59.5 (54.6, 64.4)	86.5	4.0	361	-78	137	0.7
Urine NAG/creatinine	33.4 (30.4, 36.3)*	83.1	4.3	148	-60	43	0.4
Urine albumin	66.0 (60.7, 71.4)	462.9	2.9	430	-81	168	0.1
Urine albumin/creatinine	44.9 (41.1, 48.8)*	509.2	2.9	229	-70	78	0.1
Urine IL-18	94.6 (87.5, 101.8)	130.5	4.5	819	-89	345	0.7
Urine IL-18/creatinine	65.0 (59.8 70.3)*	128.9	5.0	420	-81	164	0.5
Urine TIMP-2	67.9 (62.4, 73.3)	119.7	6.0	454	-82	178	0.6
Urine TIMP-2/creatinine	29.6 (26.6, 32.5)*	104.2	8.4	130	-57	36	0.3
Urine α1-microglobulin	43.3 (39.6, 47.1)	140.6	1.3	216	-68	72	0.3
Urine α1-microglobulin /creatinine	41.1 (37.5, 44.6)	147.0	1.9	199	-67	65	0.3

Abbreviations: CI, confidence interval; CV_A, analytical variation; CV_G, between-subject variation; CV_I, within-subject variation; II, index of individuality; IL-18, interleukin-18; KIM-1, kidney injury molecule-1; NAG, N-acetyl-β-D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin; RCV, reference change value; TIMP-2, tissue inhibitor of metalloproteinase-2

*Significantly different than the CV_I in patients with the equivalent urinary biomarkers not corrected for creatinine concentration (p<0.05).

Table 3. Within-subject variation [CV_I (confidence interval)] in the four different subgroups of CKD patients. Multilevel regression methods were used to test for differences in CV_I across the four groups.

Group A: estimated glomerular filtration rate [GFR] $\geq$60 mL/min/1.73 m^2 with or without proteinuria (albumin:creatinine ratio $\geq$30 mg/mmol)
Group B: estimated GFR 15-59 mL/min/1.73 m^2 non-proteinuric (albumin to creatinine ratio <30 mg/mmol) non-diabetic patients
Group C: estimated GFR 15-59 mL/min/1.73 m^2 proteinuric (albumin to creatinine ratio $\geq$30 mg/mmol) non-diabetic patients
Group D: estimated GFR 15-59 mL/min/1.73 m^2 proteinuric (albumin to creatinine ratio $\geq$30 mg/mmol) diabetic patients

Biomarker	CV_I, % (confidence interval)				p
	Group A	Group B	Group C	Group D	
Plasma creatinine	5.1 (4.3, 6.0)	4.5 (3.7, 5.3)	6.7 (5.7, 7.7)	6.1 (5.1, 7.1)	<0.001
Plasma cystatin C	4.6 (3.8, 5.4)	4.4 (3.6, 5.2)	5.9 (5.0, 6.7)	3.9 (3.2, 4.5)	<0.001
Plasma NGAL	24.6 (20.4, 28.8)	13.1 (11.2, 15.1)	14.8 (12.7, 16.9)	10.9 (9.1, 12.6)	<0.001
Whole blood NGAL	18.8 (14.1, 23.4)	24.5 (15.2, 33.8)	22.9 (18.9, 26.9)	14.8 (8.8, 20.8)	0.20
Urine NGAL	93.9 (81.2, 106.5)	99.8 (86.9, 112.7)	48.6 (43.0, 54.2)	87.6 (75.7, 99.5)	<0.001
Urine NGAL/creatinine	82.2 (70.9, 93.5)	79.3 (68.3, 90.4)	41.3 (36.1, 46.4)	74.5 (64.1, 85.0)	<0.001
Urine KIM-1	74.5 (64.1, 85.0)	72.2 (63.0, 81.2)	60.3 (53.1, 67.5)	82.9 (71.5, 94.3)	<0.001
Urine KIM-1/creatinine	32.7 (27.7, 37.8)	29.6 (24.9, 34.2)	26.1 (22.6, 29.7)	27.9 (23.5, 32.3)	0.48
Urine NAG	64.7 (55.5, 74.0)	57.0 (50.0, 64.0)	47.4 (41.5, 53.3)	60.6 (51.9, 69.4)	<0.001
Urine NAG/creatinine	30.7 (26.0, 35.5)	33.6 (25.8, 41.4)	29.4 (25.5, 33.3)	40.1 (34.0, 46.1)	0.12
Urine albumin	68.9 (59.1, 78.6)	70.2 (63.2, 83.8)	46.7 (35.8, 57.5)	47.3 (40.2, 54.3)	<0.001
Urine albumin/creatinine	42.6 (36.2, 49.0)	49.8 (41.6, 57.9)	33.6 (29.2, 38.1)	33.4 (28.2, 38.5)	<0.001
Urine IL-18	94.6 (81.9, 107.4)	107.0 (92.9, 120.9)	74.5 (64.1, 85.0)	96.8 (70.9, 122.7)	0.14
Urine IL-18/creatinine	68.2 (58.5, 77.9)	70.9 (60.9, 80.9)	51.3 (43.7, 58.8)	61.6 (43.9, 79.3)	0.09
Urine TIMP-2	68.7 (59.0, 78.4)	71.9 (61.8, 82.1)	52.2 (44.5, 59.8)	79.0 (57.1, 100.8)	0.03
Urine TIMP-2/creatinine	23.0 (19.0, 27.0)	26.5 (21.9, 31.0)	28.5 (24.1, 33.0)	35.1 (23.3, 46.9)	0.004
Urine α1-microglobulin	39.2 (33.3, 45.2)	40.6 (34.5, 46.8)	39.1 (33.2, 45.0)	46.5 (32.7, 60.4)	0.06
Urine α1-microglobulin /creatinine	32.5 (27.4, 37.5)	38.1 (32.3, 43.9)	37.0 (31.4, 42.7)	43.2 (30.2, 56.2)	0.27

Abbreviations: IL-18, interleukin-18; KIM-1, kidney injury molecule-1; NAG, N-acetyl-β-D-glucosaminidase; NGAL, neutrophil gelatinase-associated lipocalin; TIMP-2, tissue inhibitor of metalloproteinase-2