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Urinary Kidney Injury Biomarkers Are Associated with Ischemia-Reperfusion Injury Severity in Kidney Allograft Recipients

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BACKGROUND: We explored the potential of emerging and conventional urinary kidney injury biomarkers in recipients of living donor (LD) or donation after circulatory death (DCD) kidney transplantation, patients with chronic kidney disease (CKD), and individuals from the general population.

METHODS: Urine samples from kidney allograft recipients with mild (LD; n = 199) or severe (DCD; n = 71) ischemia-reperfusion injury (IRI) were analyzed for neutrophil gelatinase-associated lipocalin (NGAL), insulin-like growth factor-binding protein 7 (IGFBP7), tissue inhibitor of metalloproteinases 2 (TIMP2), kidney injury molecule-1 (KIM-1), chemokine C-X-C motif (CXCL9), solute carrier family 22 member 2 (SLC22A2), nephrin, and uromodulin (UMOD) by quantitative multiplex LC-MS/MS analysis. The fold-change in biomarker levels was determined in mild and severe IRI and in patients with CKD stage 1–2 (n = 127) or stage ≥3 (n = 132) in comparison to the general population (n = 1438). Relationships between the biomarkers and total protein, β2-microglobulin (B2M), creatinine, and osmolality were assessed.

RESULTS: NGAL, IGFBP7, TIMP2, KIM-1, CXCL9, and UMOD were quantifiable, whereas nephrin and SLC22A2 were below the limit of detection. Kidney injury biomarkers were increased up to 6.2-fold in allograft recipients with mild IRI and 8.3-fold in recipients with severe IRI, compared to the reference population, with the strongest response observed for NGAL and B2M. In CKD stage 1–2, B2M, NGAL, IGFBP7, TIMP2, KIM-1, UMOD, and CXCL9 were not altered, but in individuals with CKD stage ≥3, B2M, NGAL, and KIM-1 were increased up to 1.3-fold. IGFBP7, TIMP2, NGAL, and CXCL9 were strongly correlated (all $r \geq 0.8$); correlations with B2M and TP were smaller (all $r \leq 0.6$).

CONCLUSIONS: IRI, but not stable CKD, was associated with increased urinary levels of kidney injury biomarkers determined by LC-MS/MS. Absolute and multiplexed protein quantitation by LC-MS/MS is an effective strategy for biomarker panel evaluation for translation toward the clinical laboratory.

Introduction

Acute kidney injury (AKI) significantly contributes to overall in-hospital patient morbidity and mortality, but its prevalence and impact on long-term health is generally underestimated (1). Pre-existing chronic kidney disease (CKD) is a major risk factor for AKI development, and vascular procedure-related ischemia-reperfusion injury (IRI), cardiothoracic surgery, or kidney allograft transplantation can provoke an AKI event (1). IRI is an inevitable consequence of the kidney transplant (KT) procedure and affects short-term allograft function and survival (2). In particular, donation after circulatory death (DCD) kidney transplantation is associated with longer cold ischemia times and more severe IRI, resulting in higher risk for early graft loss and delayed graft function in comparison to donation after brain death or living donation (LD) (3, 4). IRI is characterized by increased tissue damage upon reperfusion of ischemic tissue and mainly affects the tubular system, in particular peritubular capillaries and interstitium

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(2, 3). Tubular injury is poorly recognized by current diagnostic laboratory tests, such as estimated glomerular filtration rate (eGFR) for estimation of the filtration capacity of the kidneys or total urinary protein (TP) to determine glomerular permeability (5, 6). In addition, kidney biopsy is considered to be the gold standard for classification of kidney pathology but is not suitable for early kidney injury recognition or regular kidney allograft monitoring due to the invasive nature of the procedure. To this end, urinary biomarker proteins have been proposed as an additional noninvasive diagnostic tool for early-stage kidney injury screening and allograft surveillance (7, 8).

Notwithstanding the unmet clinical needs for kidney injury detection, the search for single kidney injury biomarkers has not led to breakthroughs comparable to the cardiac troponins for early detection of myocardial injury (9, 10).

To target the unmet clinical need for early detection of kidney injury, we designed a multiplex protein panel that combines kidney injury biomarkers neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM-1), tissue inhibitor of metalloproteinases 2 (TIMP2), insulin-like growth factor-binding protein 7 (IGFBP7), the inflammatory chemokine C-X-C motif (CXCL9) and kidney-enriched proteins uromodulin (UMOD), solute carrier family 22 member 2 (SLC22A2), and nephrin (11). NGAL, KIM-1, TIMP2, IGFBP7, and CXCL9 have been studied to predict AKI in critically ill patients after major surgery or to enable noninvasive kidney allograft monitoring (7, 8, 12–16), whereas UMOD has been proposed for AKI risk stratification prior to elective surgery (15) and SLC22A2 and nephrin as biomarkers for detecting tubular and glomerular kidney-tissue damage, respectively (11). To explore the response of the kidney injury panel in conditions of kidney injury, an in-house mass spectrometry (MS)-based quantitative method was developed (17). Direct MS-based measurement of proteins through their specific proteotypic peptides was proposed as an alternative technology in the translational phase because it allows for biomarker comparison independent of the manufacturer and reagents used, which is not the case when using multiple uniplex immunoassays that may come from different manufacturers (18).

Since May 2022, the EU In-Vitro Diagnostic Regulation 2017/746 has been applied in the EU, which means that in-house tests should meet clinical evidence and general safety and performance requirements (19). For running an in-house developed test within the clinical laboratory, an assessment of scientific validity and clinical and analytical performance is required. The term *scientific validity* here refers to the specific association of a biomarker with the clinical condition or physiological state to be detected (19). Although some of the kidney injury biomarkers in our panel—NGAL, TIMP2, IGFBP7, KIM-1, and UMOD—have already been studied in defined clinical population groups (15, 20–22), knowledge about their concentrations in healthy and diverse patient populations and their relation to conventional laboratory parameters is currently lacking. In this study we explore the association of the urinary biomarker levels in a general population sample compared to urinary levels in LD and DCD allograft recipients, reflecting, respectively, mild and severe IRI, and in patients with different CKD stages. Furthermore, we evaluated the relation between emerging kidney injury biomarkers and conventional laboratory parameters.

Materials and Methods

RETROSPECTIVE SUBJECT AND COHORT SELECTION

In this cross-sectional analysis, urinary samples were collected from patient cohort studies, clinical trials, and a population-based cohort study (study profile in Supplemental Fig. 1). To include patient populations with mild and severe IRI, spot urine samples were obtained from patients in 2 KT studies. The mild IRI patients were Dutch and British KT recipients included in the REnal Protection Against Ischemia-Reperfusion in transplantation (REPAIR) trial, conducted between 2010 and 2013 (23, 24). In this trial, 406 adult LD KT donor-recipient pairs were enrolled and in a substudy 199 urinary samples were collected from allograft recipients 1 day after kidney transplantation for biomarker analysis. Patients with severe IRI were obtained from the Prospective Trial on Erythropoietin in Clinical Transplantation (PROTECT) trial, conducted at the Leiden University Medical Center (25). Urinary samples (n = 71) from recipients of donation circulatory death (DCD) kidney transplantation were collected 1 day after transplantation.

To study biomarker levels in CKD, baseline spot urine samples were obtained from 4 previous patient studies on autosomal dominant polycystic kidney disease that have been described in detail elsewhere (26). To obtain 2 populations representing mild and moderate CKD, we stratified all autosomal dominant polycystic kidney disease patients with an available urine sample based on the boundaries from the KDIGO CKD classification criteria, to create a population with CKD stage 1–2 (eGFR ≥60 mL/min/1.73 m^2, n = 127) and a population with CKD stage ≥3 (eGFR < 60 mL/min/1.73 m^2, n = 132) (27).

The population-based Netherlands Epidemiology of Obesity Study cohort included middle-aged men and women aged 45 to 65 years from one municipality (Leiderdorp, the Netherlands) (28). Urine samples collected between 2008 and 2012 from this control population (n = 1443) were previously analyzed for kidney

injury biomarkers using the LC-MS/MS analytical platform to establish reference intervals (29). In the current study, 5 individuals with self-reported history of CKD were excluded from the reference population (n = 1438) (29).

Ethical approval for the REPAIR trial in the United Kingdom was given by the Joint University College London/University College London Hospital Committees on the Ethics of Human Research. For the studies including Dutch participants, the Medical Ethical Committee of the Leiden University Medical Center approved the design of the population-based Netherlands Epidemiology of Obesity Study and the aforementioned clinical trials and cohort studies, which included the anonymized use of collected urinary samples for biomarker evaluation studies.

URINE SPECIMEN PROCESSING

Spot urine samples from CKD patients were collected and centrifuged at 1000 *g* for 10 min. Spot urine samples from LD-KT recipients were collected 1 day after transplantation and centrifuged at 400 *g* for 10 min. Twenty-four-hour urine samples from DCD-KT recipients were collected 1 day after transplantation and centrifuged at 1000 *g* for 10 min. From all urine samples, the supernatant after centrifugation was aliquoted and stored at −80°C until analysis. The urine samples underwent multiple freeze-thaw cycles until biomarker analysis (2–4 times).

LABORATORY ANALYSIS CONVENTIONAL LABORATORY MARKERS

Urine osmolality was determined by freezing point depression using an Osmo-Station (Auto & Stat model OM-6060, Arkray Inc.). TP concentration in urine was determined by turbidimetry, urinary β2-microglobulin (B2M) was determined by immunoturbidimetry, and urinary creatinine by an enzymatic method, all using a Cobas C8000 c702 analyzer and Roche Reagents (Roche Diagnostics).

MULTIPLEX LC-MS/MS ANALYSIS OF EMERGING BIOMARKERS

Urinary NGAL, IGFBP7, KIM-1, TIMP2, CXCL9, UMOD, SLC22A2, and nephrin were quantified in 36 batches between January 2021 and November 2021 using an in-house developed multiplex LC-MS/MS test. The preanalytical and analytical phases of this LC-MS/MS test were carried out according to the standard operating procedure described elsewhere (17). To ensure acceptable performance of the LC-MS/MS instrument, a system suitability test was carried out prior to each analysis batch of 81 study samples. In addition, 2 urine-based internal quality control samples were prepared and analyzed together with the study samples. The test performance was considered stable over 1 year based on internal quality control monitoring (Supplemental Table 1; Supplemental Fig. 2).

DATA ANALYSIS

The mean and SD were calculated to describe population characteristics and the median and interquartile range for urinary biomarker concentrations. Biomarker data were ^{10}log transformed and the analytical lower limit of detection of 1 pmol/L was imputed when biomarkers were not detected, to avoid the loss of observations. Linear discriminant analysis (LDA) was conducted to examine the suitability of the multiparameter biomarker data for population discrimination. The coefficients in the first 2 linear discriminants were obtained to study the contribution of the different proteins in separating reference, CKD, LD, and DCD populations. Group means and 95% confidence intervals (CI) of the ^{10}log transformed biomarker concentrations in the 4 patient populations were compared to the reference population and expressed as fold change. To investigate the effect of urine specimen dilution, analyses were performed with and without creatinine normalization. Relations between age and biomarkers were determined by regression analysis accompanied by the Spearman correlation. To assess the relation between biomarkers and kidney function, we stratified CKD patients further based on eGFR: (*a*) < 30 mL/min/1.73 m^2 (n = 29), (*b*) 30 to 60 mL/min/1.73 m^2 (n = 107), (*c*) 60 to 90 mL/min/1.73 m^2 (n = 86), and (*d*) > 90 mL/min/1.73 m^2 (n = 53). We then tested whether the biomarker concentrations are similar among eGFR groups by Kruskal-Wallis rank testing. Finally, the percentage of individuals within the different patient groups with one or multiple biomarker(s) outside the predefined reference lower or upper limit in the general population were determined using the predefined limits provided in Supplemental Table 2.

SOFTWARE

Mass Hunter Workstation software (version 10.0, Agilent Technologies) was used for LC-MS/MS peak integration. Data visualization and statistical analysis were conducted in R (version 4.0.2, R Core Team [2020]). The R package "MASS" was used for LDA.

Results

STUDY COHORT CHARACTERISTICS

In total, 127 samples from patients with CKD stage 1–2, 132 samples from those with CKD stage ≥3, 199 samples from LD-KT recipients, and 71 DCD-KT recipients were included in this study (Table 1). The LD-KT (71% men) and DCD-KT (72% men) populations consisted of more men as compared to the general population and the 2 CKD populations. Compared to

Table 1. Population characteristics.

Characteristics		Reference	Population A CKD stage 1–2 eGFR ≥ 60 mL/min/1.73 m^2	Population B CKD stage ≥3 eGFR < 60 mL/min/1.73 m^2	Population C Mild IRI	Population D Severe IRI
Description	—	General	Outpatient CKD	Outpatient CKD	LD KT recipient	DCD KT recipient
n	M&F	1438	127	132	199	71
Sex (% male)	—	624 (43)	56 (44)	69 (52)	142 (71)	51 (72)
Age (mean ± SD)	M&F	56 ± 6	41 ± 11	51 ± 9	44 ± 15[a]	53 ± 13[a]
Serum creatinine (μmol/L)	M	98 ± 13	97 ± 15	181 ± 58	236 ± 91	903 ± 324
	F	79 ± 12	79 ± 13	143 ± 48	207 ± 109	761 ± 239
CKD-EPI eGFR (mL/min/1.73 m^2)	M	86.0 ± 11.6	86.0 ± 17.7	40.2 ± 11.7	28.7 ± 18.5	6.2 ± 3.4
	F	85.4 ± 12.5	82.6 ± 19.0	40.8 ± 12.6	39.4 ± 27.3	5.9 ± 3.8
Urine sample characteristics						
Osmolality, (mOsmol/kg)	M&F	**441 (339–603)**	**422 (248–560)**	**357 (284–461)**	**395 (323–490)**	**315 (297–356)**
Creatinine, (mmol/L)	M&F	**6.2 (4.4–9.0)**	**6.0 (3.0–9.9)**	**5.7 (4.1–8.3)**	**6.0 (4.0–9.7)**	**5.2 (3.4–8.8)**
B2M, (mg/L)	M&F	**0.2 (0.2–0.2)**	**0.2 (0.2–0.2)**	**0.2 (0.2–0.6)**	**17.4 (5.2–38.2)**	**30.6 (18.1–52.3)**
Total protein, (g/L)	M&F	**0.04 (0.03–0.05)**	**0.07 (0.04–0.11)**	**0.07 (0.04–0.15)**	**0.32 (0.16–0.65)**	**1.90 (0.84–2.49)**

[a]Allograft recipient age. The variables eGFR and serum creatinine normalized biomarker concentrations are stratified by sex (M = male and F = female). Mean ± SD shown or median (interquartile range). Individuals who received a kidney transplant from a LD or a DCD organ donor.

CKD stage 1–2 patients and LD-KT recipients, the reference individuals, the CKD stage ≥3 patients and DCD-KT patients were older (one way ANOVA, $P < 0.001$). The mean (SD) eGFR of individuals with CKD stage 1–2 was 86.0 (17.7) mL/min/1.73 m^2 for men and 82.6 (19.0) mL/min/1.73 m^2 for women and resembled kidney function in the 15-year-older aged reference population. On the first postoperative day, the mean (SD) eGFR of the LD-KT recipients was 29 (19) and 39 (27) mL/min/1.73 m^2 for men and women, respectively. The mean eGFR in DCD-KT recipients was 6 (3) mL/min/1.73 m^2 for men and 6 (4) mL/min/1.73 m^2 for women, and 97% of all DCD-KT recipients had an eGFR ≤15 mL/min/1.73 m^2.

URINARY BIOMARKER CONCENTRATIONS NORMALIZED BY CREATININE AND STRATIFIED FOR SEX

The biomarkers NGAL, IGFBP7, TIMP2, KIM-1, CXCL9, and UMOD were detectable in urine by LC-MS/MS analysis (Table 2). Of note, KIM-1 was detected in only 63% of the samples (59% in the reference population, 44% in CKD, 65% in LD, and 73% in DCD) and CXCL9 was exclusively detected in conditions of IRI (29% of the LD-KT and 63% of the DCD-KT population). In addition, nephrin and SLC22A2 were detected in <1% of all individuals, and therefore excluded from further analyses. The mean urine creatinine concentration was similar in CKD and KT patients compared to the reference population [0.9-fold (95% CI, 0.9–1.0) difference], and biomarker normalization by creatinine did not affect the overall biomarker response, as detailed for each biomarker in Supplemental Table 3. Creatinine normalization, however, increased the within-group variance as a consequence of sex-dependent creatinine concentrations. (Fig. 1 and Supplemental Fig. 3).

To address the age differences between the studied populations, regression analysis was performed between age and biomarker concentrations grouped by population. Weak correlations were observed ($r < 0.2$) between B2M, IGFBP7, KIM-1, and age in specific population groups but were not confirmed in the other independent populations (Supplemental Fig. 4).

KIDNEY INJURY BIOMARKERS ARE INCREASED IN KIDNEY ALLOGRAFT RECIPIENTS AND ASSOCIATE WITH IRI SEVERITY

The biplot plot of the LDA indicated the ability of the urinary kidney injury biomarker panel to separate the

Table 2. Urinary kidney injury biomarker concentrations with and without creatinine-normalization and stratification for sex.

Characteristics		Sex	Reference	CKD stage 1–2 eGFR ≥ 60 mL/min/1.73m^2		CKD stage ≥3 eGFR < 60 mL/min/1.73m^2		Mild IRI LD KT recipients		Severe IRI DCD KT recipients	
					RL		RL		RL		RL
			Median (IQR[a])	Median (IQR[a])	(%)	Median (IQR[a])	(%)	Median (IQR[a])	(%)	Median (IQR[a])	(%)
NGAL	pmol/L	M&F	165 (75–302)	126 (40–415)	9	227 (96–659)	13	1710 (586–4519)	56	10830 (6576–22036)	82
	pmol/mmol creatinine	M	12 (8–23)	11 (7–27)	4	24 (13–55)	11	284 (127–789)	62	2307 (873–11337)	58
		F	43 (22–73)	31 (12–87)	6	79 (24–196)	11	186 (106–613)	11	2432 (899–4561)	25
IGFBP7	pmol/L	M&F	382 (249–577)	395 (132–835)	11	230 (126–457)	4	225 (125–365)	6	614 (382–908)	10
	pmol/mmol creatinine	M	56 (42–73)	48 (33–86)	4	41 (29–57)	2	33 (26–47)	5	96 (71–232)	25
		F	65 (48–83)	72 (38–109)	6	41 (24–75)	3	45 (31–59)	2	85 (53–333)	11
TIMP2	pmol/L	M&F	151 (95–233)	167 (62–316)	9	150 (81–227)	4	220 (129–381)	17	1198 (556–3375)	53
	pmol/mmol creatinine	M	21 (16–27)	19 (13–25)	2	19 (14–17)	5	34 (22–54)	21	211 (81–1162)	51
		F	26 (19–35)	29 (20–45)	7	32 (20–43)	5	40 (26–68)	8	257 (82–647)	23
KIM-1	pmol/L	M&F	12 (1–23)	17 (1–54)	17	25 (12–57)	16	27 (1–71)	24	29 (1–55)	18
	pmol/mmol creatinine	M	2 (1–3)	3 (1–7)	7	5 (3–7)	10	3 (1–9)	19	5 (1–13)	24
		F	2 (1–4)	4 (1–7)	7	5 (1–7)	5	7 (1 -14)	11	9 (6–15)	8
CXCL9	pmol/L	M&F	< 1[b]	< 1[b]	0	< 1[b]	1	< 1[b]	16	17 (1–38)	63
	pmol/mmol creatinine	M	—	—	0	—	1	—	11	2 (1–7)	45
		F	—	—	0	—	0	—	5	2 (1–12)	18
UMOD	mg/L	M&F	2.7 (1.9–3.8)	2.6 (1.6–3.5)	5	2.3 (1.6–3.2)	11	0.7 (0.2–2.3)	44	0.1 (0.0–0.6)	66
	µg/mmol creatinine	M	319 (182–546)	416 (257–693)	2	333 (245–524)	5	79 (25–246)	36	14 (3–77)	44
		F	576 (367–896)	497 (288–804)	7	487 (335–692)	2	248 (66–416)	8	15 (4–105)	24
[TIMP2]x[IGFBP7] (ng/mL)^2/1000)		M&F	0.04 (0.01–0.09)	0.05 (0.01–0.20)	5	0.02 (0.01–0.07)	2	0.04 (0.1–0.10)	6	0.47 (0.14–1.36)	31
	/µmol creatinine	M	7 (4–12)	5 (1–17)	1	4 (2–8)	1	6 (3–12)	6	113 (23–312)	31
		F	6 (3–11)	10 (3–31)	9	6 (1–12)	2	6 (4–11)	5	79 (21–657)	60

Continued

Table 2. (continued)

Characteristics	Sex	Reference	CKD stage 1–2 eGFR ≥ 60 mL/min/1.73m^2	RL	CKD stage ≥3 eGFR < 60 mL/min/1.73m^2	RL	Mild IRI LD KT recipients	RL	Severe IRI DCD KT recipients	RL
[NGAL]×[TIMP2] (ng/mL)^2/1000)	M&F	0.01 (0.00–0.03)	0.01 (0.00–0.09)	0	0.02 (0.01–0.07)	1	0.19 (0.05–0.83)	6	7.78 (2.51–31.48)	49
/µmol creatinine	M	1 (0.6–3)	1 (0.3–3)	0	1.8 (0.8–5.4)	0	32 (11–133)	5	1347 (321–11662)	31
	F	3 (1–6)	4 (1–18)	3	9 (2–22)	3	32 (10–107)	10	1568 (336–9050)	75

[a]Interquartile range.
[b]Concentrations below the analytical limit of detection (1 pmol/L). Median and IQR provided. The creatinine-normalized biomarker concentrations are stratified by sex (M = male and F = female). The reporting of the product [TIMP2]×[IGFBP7] and [NGAL]×[TIMP2] in mass units are based on the stripped and canonical amino acid sequence reported in the UniProt database. The percentage of individuals in the population with a kidney injury biomarker exceeding the reference limit (%RL) is provided. The upper limit (97.5th percentile in the reference population) was set as threshold for TP, B2M, NGAL, IGFBP7, TIMP2, KIM-1, and CXCL9, and the lower limit (2.5th percentile in the reference population) was used for uromodulin (see Supplemental Table 1 for concentration cut-offs).

general population, all CKD patients and LD- and DCD-KT recipient populations (Supplemental Fig. 5). CXCL9 and NGAL contributed the most in population separation in the first linear discriminant dimension, accounting for 94% of information in the data. When adding conventional laboratory markers to the biomarker panel for LDA, it enabled discrimination of CKD patients from the reference population.

In conditions of IRI, B2M levels were most discriminating of all biomarkers with a mean fold-change of 6.2 (95% CI, 5.6–6.8) in LD-KT and 8.3 (95% CI, 7.3–9.1) in DCD-KT recipients, compared to the reference population (Fig. 2, Supplemental Table 3). In addition, TP levels were 2.4-fold (95% CI, 2.3–2.5) higher in LD-KT recipients and 4.8-fold (95% CI, 4.4–5.2) higher in DCD-KT recipients. Of the emerging biomarkers, the highest response was observed in NGAL with a 2.8-fold (95% CI, 2.6–3.1) concentration increase in mild IRI and a 6.7-fold (95% CI, 5.9–7.5) increase in severe IRI. In addition, NGAL most frequently exceeded the upper reference limit with 73% in the LD-KT and 83% in the DCD-KT population (Table 2). TIMP2 and CXCL9 were associated with IRI severity with respectively fold increases of 1.3 (95% CI, 1.2–1.4) and 1.4 (95% CI, 1.3–1.6) in LD-KT recipients and 2.7 (95% CI, 2.3–3.0) and 2.7 (95% CI, 2.2–3.1) in DCD-KT recipients (Fig. 2, Supplemental Table 3). To demonstrate the effect of combined biomarker reporting, we provided the product [TIMP2]×[IGFBP7] and [TIMP2]×[NGAL]—the best responders analyzed by the same method—among the different study populations (Table 2). After biomarker multiplication, the response tended to be attenuated compared to TIMP2 alone and enlarged compared to IGFBP7, although the test sensitivity and specificity of this approach remains to be evaluated in specific clinical settings.

MINIMAL KIDNEY INJURY BIOMARKER RESPONSE IN CKD

In the CKD populations, urinary creatinine and osmolality were similar [0.9-fold (95% C, :0.9–1.0)] compared to the reference population, whereas TP was 1.2-fold (95% CI, 1.2–1.3) higher in CKD stage 1–2 and 1.3-fold (95% CI, 1.3–1.4) higher in CKD stage ≥3 (Fig. 1 and Supplemental Table 3). The other conventional marker, B2M, was not increased in CKD stage 1–2 (1.1; 95% CI, 1.0–1.1) but 1.3-fold (95% CI, 1.3–1.6) increased in CKD stage ≥3 as compared to the reference population. The urinary biomarkers NGAL, TIMP2, KIM-1, and CXCL9 were not altered in CKD stage 1–2, while in CKD stage ≥3, NGAL and KIM-1 both increased 1.3-fold (95% CI, 1.1–1.4) compared to the reference population. IGFBP7 and UMOD tended to be lower in patients with CKD stage ≥3 compared to the general population with fold-changes of 0.8 (95% CI, 0.7–0.9)

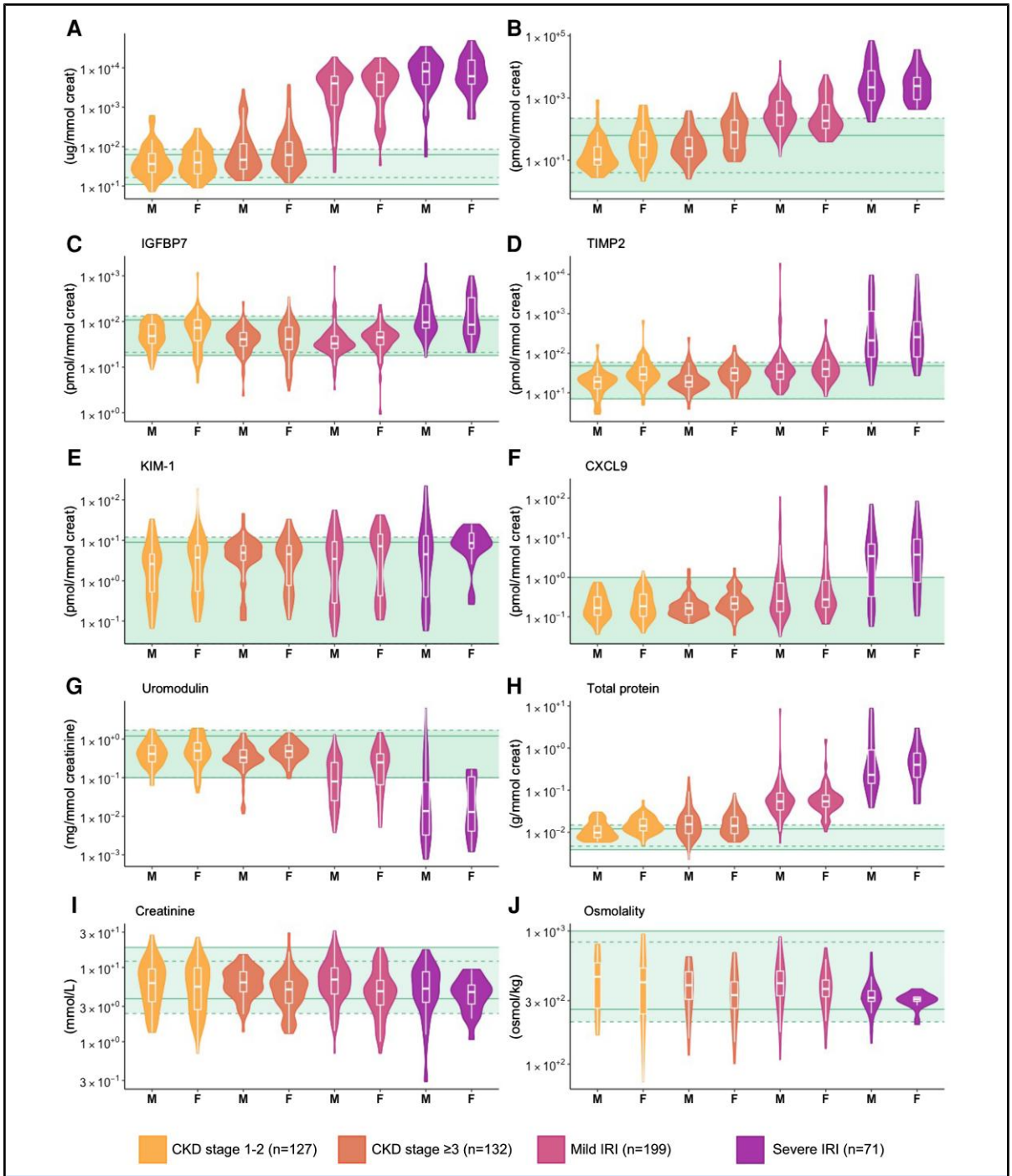

Fig. 1. Biomarkers in CKD patients and kidney allograft recipients stratified by sex. Violin plots of kidney injury biomarkers in CKD patients and kidney transplant recipients with mild or severe IRI. Biomarkers are normalized by urinary creatinine and grouped by sex (M = males, F = females). The rectangles represent the reference intervals (solid = males, dashed = females) and the boxplot the median with interquartile range.

and 0.9 (95% CI, 0.8–0.9), respectively. In 34% of the CKD stage 1–2 patients, at least one emerging kidney injury biomarker exceeded its reference limit. None of the CKD patients had all 6 injury biomarkers exceeding the reference limit, whereas all emerging biomarkers were concurrently increased in 41% of the DCD-KT patients (Supplemental Fig. 6). Of all biomarkers, only NGAL was inversely related to eGFR ($P < 0.001$), whereas IGFBP7 concentrations tended to be different among eGFR groups ($P < 0.015$) (Supplemental Fig. 7).

PROTEIN-BASED BIOMARKERS CLUSTER IN THEIR RESPONSE TO IRI IN KIDNEY ALLOGRAFT RECIPIENTS

To study the association between conventional and emerging biomarkers in health, CKD, and conditions of IRI, correlations were calculated between the biomarkers in the different patient populations. The relation between the concentration of biomarkers in CKD was similar to the correlation pattern found in the general population (Fig. 3, A and B). Herein, TP, B2M, and UMOD were positively correlated ($r \geq 0.29$) and negatively correlated with osmolality ($r \geq -0.35$) as an indicator of dilution. In addition, TIMP2 was correlated to IGFBP7 ($r = 0.51$), NGAL ($r = 0.52$), and KIM-1 ($r = 0.46$).

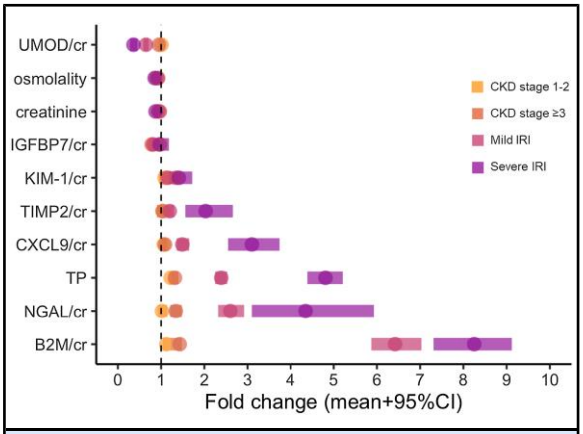

Fig. 2. Kidney injury biomarker concentration fold change in CKD patients and in kidney allograft recipients with IRI compared to reference population. The mean concentration fold change with 95% CI is shown of the kidney injury biomarker concentration in patients with CKD stage 1–2 (n = 127) or CKD stage ≥3 (n = 132) and kidney transplantation recipients with mild (n = 199) or severe (n = 71) IRI compared to the mean concentration in the reference population is shown (dashed line). All mean concentrations were calculated from the ^{10}log transformed data and back calculated to provide in the original scale.

In the LD-KT patients the correlation for the protein cluster, consisting of TP, B2M, and UMOD, was smaller than in the reference population ($r = 0.14$–0.30), whereas the correlations between TIMP2 and IGFBP7 became stronger ($r = 0.61$) (Fig. 3, C). Furthermore, NGAL was correlated to B2M ($r = 0.60$) and TIMP2 to TP ($r = 0.40$). In the DCD-KT population, NGAL, TIMP2, IGFBP7, and CXCL9 correlate in their response ($r = 0.80$–0.91) (Fig. 3, D). These proteins tend to correlate to B2M (all $r > 0.39$), whereas KIM-1 did not ($r = 0.01$). Moreover, UMOD was inversely correlated ($r = -0.24$ to -0.61) to B2M, TP, and all injury biomarkers. The biomarker clusters were identified for both crude and creatinine-normalized biomarker concentrations (Supplemental Fig. 8).

Discussion

This study was intended to explore the scientific validity (i.e., biomarker panel association with disease) of a multiplex urinary kidney injury protein panel in patients with IRI in the context of a living donor or donation after circulatory death KT procedure. Results were compared to those obtained in relevant control populations including patients with different stages of CKD and individuals from the general population. Urinary NGAL, IGFBP7, KIM-1, TIMP2, UMOD, and CXCL9 were quantifiable using multiplex quantitative protein LC-MS/MS, whereas nephrin and SLC22A2 were below the detection limit. The biomarkers B2M, TP, NGAL, IGFBP7, TIMP2, and CXCL9 were marginally responsive in CKD but increased up to 8.3-fold in conditions of IRI and were associated with the severity of IRI. Surprisingly, the amplification of the response of TIMP2 and IGFBP7 and TIMP2 and NGAL did not improve the individual biomarker response of TIMP2 or NGAL, although the test sensitivity and specificity for AKI prediction prior to serum creatinine increase could not be evaluated in this cross-sectional study.

Nowadays, serum creatinine or cystatin-C based GFR estimations and urinary protein or albumin excretion are conventional laboratory tests for kidney allograft monitoring, whereas tubular injury markers are less frequently used. B2M (12 kDa) has regained interest as tubular dysfunction marker, independent of eGFR and proteinuria, because urinary concentrations of this low molecular weight plasma protein rapidly increase in AKI due to impaired reabsorption by tubular epithelial cells (15, 30). In this study, B2M was the most responsive marker for kidney IRI, which highlights the potential for extending its intended clinical use for detecting AKI.

Urinary TIMP2 and IGFBP7 were not associated with CKD or its stages, which is in agreement with observations in previous studies determining urinary

TIMP2, IGFBP7, and NGAL with immunoassays (26, 31, 32). In our hands, urinary NGAL concentrations did gradually increase with the decline in kidney function (Kruskal-Wallis, $P < 0.001$), The median concentration difference between the eGFR group >90 mL/min/1.73m^2 and eGFR < 30 mL/min/1.73 m^2 of 0.4 nmol/L was considered small compared to the median NGAL concentration increase of 11.0 nmol/L observed in conditions of IRI. The biomarker responsiveness to transplantation procedure-related IRI tended to be similar to the concentration increase in IRI-induced AKI after cardiothoracic surgery. For instance, after cardiothoracic surgery, TIMP2 and IGFBP7 levels increased about 1.5- and 2-fold relative to presurgery levels, respectively (33). Moreover, in IRI after cardiopulmonary bypass surgery, urinary

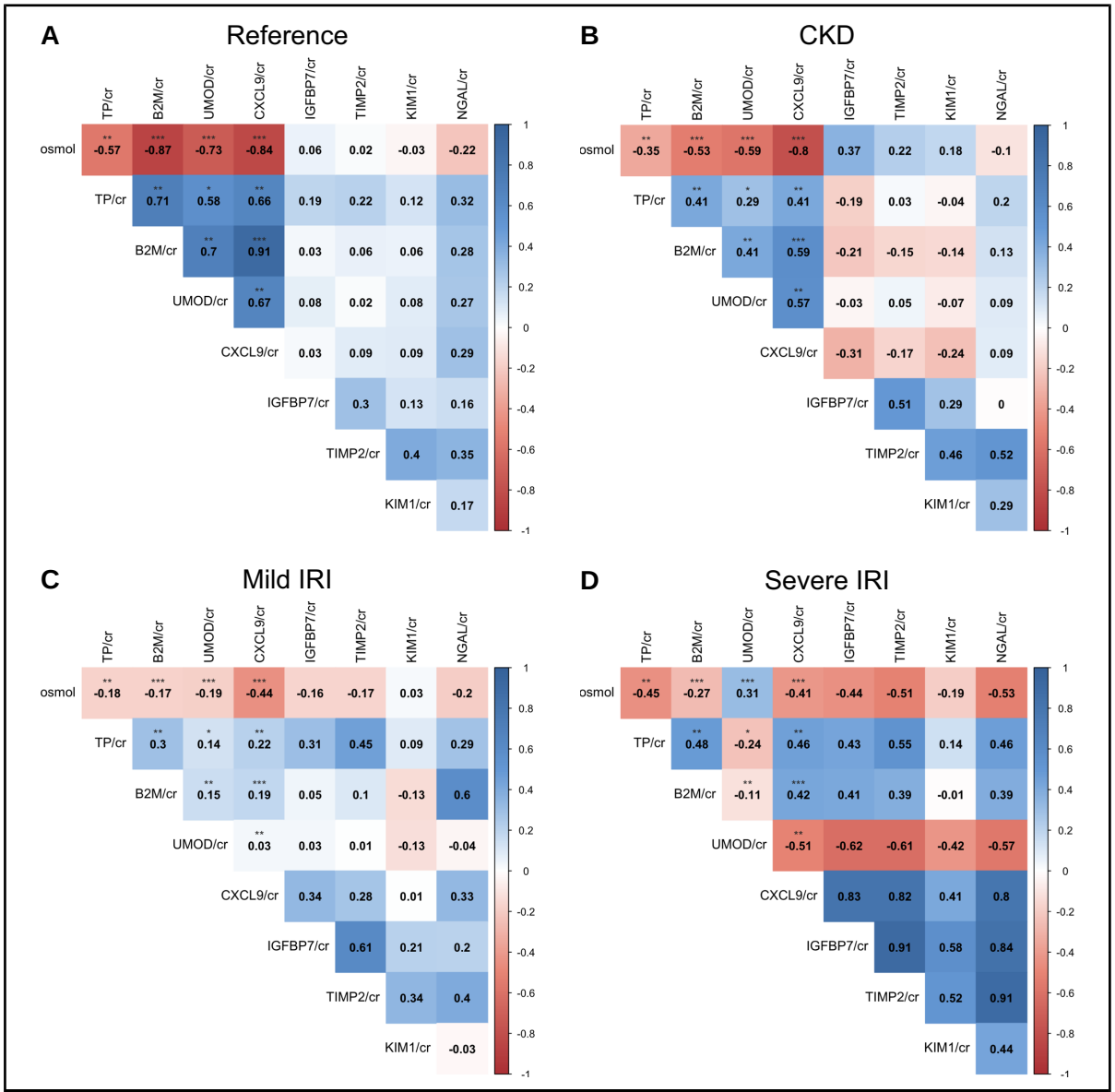

Fig. 3. Biomarker correlation matrices grouped by population. Correlation matrices of conventional and emerging urinary markers. The Spearman rank correlation coefficient is provided in the different populations. (A), Reference population (n = 1438); (B), all CKD patients (n = 259); (C), LD allograft recipients (n = 199) with mild IRI; and (D), DCD allograft recipients (n = 71) with severe IRI. Significance level: * = 0.5; ** = 0.01; ***0.001.

NGAL concentrations were increased 15-fold at 2 h and 25-fold at 4 to 6 h after surgery, which highlights the influence of timing of sample collection on the biomarker response (12). To this end, follow-up studies are now planned to examine the release kinetics of the kidney injury biomarkers and determine the optimal time window for their analysis.

The correlation analysis in this study supports similar principal mechanisms for biomarker excretion in urine. The kidney injury biomarkers were partially correlated to TP, a marker for increased glomerular permeability due to glomerular tissue damage and/or increased hydraulic pressure. In conditions of IRI, the low molecular weight plasma proteins CXCL9 (14 kDa), TIMP2 (24 kDa), IGFBP7 (29 kDa), and NGAL (23 kDa) were related to the levels of B2M (12 kDa) (34, 35). Indeed, this mechanism of increased protein excretion by impaired reabsorption has been described for glomerular filtrated TIMP2, IGFBP7, NGAL, and Cystatin C (13 kDa) (36–38). Interestingly, KIM-1 (39 kDa) was only partially correlated to the cluster of low molecular weight proteins. This may indicate an additional mechanism of excretion in IRI, such as increased expression on tubular kidney tissue (7, 39).

A strength of using the MS-based biomarker analysis is that it was embedded in a clinical chemistry laboratory with strict quality management systems. System suitability testing and internal quality control monitoring were implemented and enabled batch-wise biomarker analysis in study samples. In addition, LC-MS/MS results were assessed for validity by (*a*) peptide comparison to study tryptic digestion effects, (*b*) internal standard signal intensity monitoring to monitor ion suppression, and (*c*) ion ratio monitoring for measurement accuracy assessment, as detailed elsewhere (18). This LC-MS/MS test should be considered as a second-tier high-complexity LDT that was developed to facilitate translational biomarker research and remains to be evaluated for its clinical performance and effectiveness prior to potential implementation for patient diagnostics. As the current study is limited to a cross-sectional analysis, the design does not allow clarification of biomarker kinetics or suitability for early detection of AKI in the individual or injury progression monitoring after a surgical or medical intervention. Longitudinal studies on biomarker kinetics are needed before clinical performance and clinical utility of kidney injury biomarkers can be appreciated. Carter et al. illustrated how reference change values of urinary NGAL, KIM-1, and TIMP2 can facilitate personalized interpretation of these biomarkers in subsequent samples (40).

There are limitations to this study that should be acknowledged. First, some of the urinary samples had been stored for long periods prior to biomarker analysis, and the results in this study rely on available long-term stability data. Van de Vrie et al. showed 6-month stability of urinary NGAL and KIM-1, whereas Schuh et al. reported the stability of urinary NGAL and KIM-1 over 5 years of storage at −80°C (41, 42). Since the stability data does not cover the age span and all biomarkers included in this study, absolute biomarker concentrations should be interpreted with caution. Second, the biomarker analyses were performed in 24-h (reference and DCD population) and spot urine (LD population), which may impact interpretation of results. Future efforts are needed to evaluate whether biomarkers normalized for creatine concentration in spot urine can be used as the preferred specimen. In addition, the biomarker findings obtained in our cross-sectional studies reflect the association of the biomarkers 24 h after transplantation. The study designs do not allow for the evaluation of test performance for the purpose of AKI prediction, as the clinical test performance is highly dependent on the timing of specimen collection, normalization strategy, analytical methodology definition, and time to clinical outcome of interest (43). Studies evaluating the biomarker concentration in fresh urine specimens by both conventional high-throughput laboratory tests authorized by regulatory agencies and the LC-MS/MS strategy are needed to clarify and explore the additional value of protein biomarker panels reported in SI units and the role of biomarker evaluation by LC-MS/MS. Lastly, urine samples were not available for all patients enrolled in the clinical studies. The missing samples (n = 23, 24%) due to no or limited urine production after DCD-KT surgery could have introduced selection bias.

In conclusion, we demonstrated the scientific validity of a urinary kidney injury panel in patients with healthy and diseased kidneys using quantitative and multiplex LC-MS/MS. A 6- to 8-fold increased level of urinary NGAL and B2M was found in recipients 1 day after either LD or DCD kidney transplantation with mild or severe IRI, respectively. To examine the clinical utility of the kidney injury biomarkers in the future, their clinical performance needs to be evaluated for the intended uses that address an unmet clinical need in kidney injury diagnosis.

Supplemental Material

Supplemental material is available at *Clinical Chemistry* online.

Nonstandard Abbreviations: AKI, acute kidney injury; CKD, chronic kidney disease; IRI, ischemia-reperfusion injury; DCD, donation after circulatory death; eGFR, estimated glomerular filtration rate; TP, total protein; NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule 1; TIMP2, tissue inhibitor of metalloproteinases 2; IGFBP7, insulin-like growth factor-binding protein 7;

CXCL9, C-X-C motif chemokine 9; UMOD, uromodulin; SLC22A2, solute carrier family 22 member 2; MS, mass spectrometry; KT, kidney transplant; B2M, β2-microglobulin; LDA, linear discriminant analysis; CI, confidence interval.

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