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Machine Learning-Based Urine Peptidome Analysis to Predict and Understand Mechanisms of Progression to Kidney Failure

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Introduction: The identification of patients with chronic kidney disease (CKD) at risk of progressing to kidney failure (KF) is important for clinical decision-making. In this study we assesed whether urinary peptidome (UP) analysis may help classify patients with CKD and improve KF risk prediction.

Methods: The UP was analyzed using capillary electrophoresis coupled to mass spectrometry in a case-cohort sample of 1000 patients with CKD stage G3 to G5 from the French CKD-Renal Epidemiology and Information Network (REIN) cohort. We used unsupervised and supervised machine learning to classify patients into homogenous UP clusters and to predict 3-year KF risk with UP, respectively. The predictive performance of UP was compared with the KF risk equation (KFRE), and evaluated in an external cohort of 326 patients.

Results: More than 1000 peptides classified patients into 3 clusters with different CKD severities and etiologies at baseline. Peptides with the highest discriminative power for clustering were fragments of proteins involved in inflammation and fibrosis, highlighting those derived from α-1-antitrypsin, a major acute phase protein with anti-inflammatory and antiapoptotic properties, as the most significant. We then identified a set of 90 urinary peptides that predicted KF with a c-index of 0.83 (95% confidence interval [CI]: 0.81−0.85) in the case-cohort and 0.89 (0.83−0.94) in the external cohort, which were close to that estimated with the KFRE (0.85 [0.83−0.87]). Combination of UP with KFRE variables did not further improve prediction.

Conclusion: This study shows the potential of UP analysis to uncover new pathophysiological CKD progression pathways and to predict KF risk with a performance equal to that of the KFRE.

Kidney Int Rep (2023) **8,** 544–555; https://doi.org/10.1016/j.ekir.2022.11.023
KEYWORDS: α-1-antitryspin; case-cohort design; KFRE; kidney failure; proteomics

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Received 4 November 2022; accepted 28 November 2022; published online 12 December 2022

CKD, defined as the presence of irreversible structural or functional kidney damage, increases the risk of poor kidney and cardiovascular outcomes.[1] The identification of patients at risk of progression to KF is important for clinical decision-making and trial enrolment. Risk equations have been developed to predict KF, including the widely used 4-variable KFRE from Tangri *et al.*,[2] on the basis of age, sex, estimated glomerular filtration rate (eGFR), and urinary

albumin-to-creatinine ratio (UACR); and that from the German CKD cohort using 6 routine laboratory tests.[3] However, UACR used as marker of kidney damage in both equations is not present in all kidney diseases, and within-person UACR variability is high among patients with albuminuria. A number of other urinary proteins, including uromodulin, tubular epithelium derived glycoprotein Dickkopf-3, neutrophil gelatinase-associated lipocalin/lipocalin 2, or kidney injury molecule 1 have been assessed as potential biomarkers of CKD progression,[4,5] with inconsistent or still limited findings.[6–8] Single biomarkers may nevertheless not fully describe the heterogeneous origin of CKD, and the implication of a wide array of risk factors that contribute to CKD progression.

Omics-based strategies, which assess multiple molecular features simultaneously, seem promising to better capture such heterogeneity and complexity of CKD. In this context, analysis of proteomic urine peptide content (urinary peptidomics) has emerged as one of the most attractive areas in the identification and quantification of biomarkers to characterize CKD.[9,10] A urinary signature composed of 273 peptides, called the CKD273 classifier, was developed to predict the occurrence of CKD[11]; however, it was also shown to be associated with kidney fibrosis.[12] Although CKD273 was able to detect CKD at a very early stage as recently shown in the CKD273 guided intervention trial PRIORITY,[13] this classifier loses its performance in predictingf eGFR decline at late-stage CKD.[14] Whether the analysis of the urine peptidome can predict and/or improve the prediction of CKD progression to KF has never been explored so far.

The main objectives of this study were therefore to describe the urine peptidome diversity in patients with CKD, and to assess its potential to better characterize patients' profile and predict their progression to KF. To achieve these objectives, we selected a case-cohort sample of 1000 patients with CKD stages G3 to G5 from the CKD-REIN cohort,[15] and used machine learning to first classify patients according to their UP, then identify the best performing peptide signature to predict KF. We also assessed the prediction performance of this signature as compared to that of the KFRE in the case-cohort study, and in an independent validation cohort of 326 patients with CKD stages G3 to G5 from Ghent, Belgium.[16]

METHODS

Study Design and Participants

The CKD-REIN study is a prospective cohort carried out in 40 nephrology clinics in France, nationally representative with respect to geography and facility legal status, public or private. From 2013 through 2016, we included 3033 adult patients with CKD stage G3 to G5 (eGFR <60 ml/min per 1.73 m^2) and any type of primary kidney disease, not on dialysis or transplanted. At baseline, fasting blood and second morning urine samples were collected together with extensive information about patient and disease characteristics. Patients were then followed-up annually over 5 years. Study protocol and cohort profile have been published in detail elsewhere.[15,17] The study was approved by the institutional review board of the French National Institute of Health and Medical Research (IRB00003888) and is registered at ClinicalTrials.gov (NCT03381950). For the peptidome analysis, a case-cohort sample of 1054 participants was selected, including all incident cases of KF and cardiovascular events over the first 3-year follow-up, and a random sample of enrolled patients, all of them with urinary sample stored at baseline or within the next 3 months (Figure 1). The validation cohort included 326 patients with CKD stage G3 to G5 and UP data from the Ghent cohort study, a single-center study that included 526 patients with CKD Stages G1 to G5 not on dialysis, from Ghent University Hospital outpatient nephrology clinic (Belgium), between 2011 and 2014.[18,19] This study was approved by the local ethical committee at the Ghent University Hospital (2010/033; B67020107926). Written informed consent was obtained from all participants.

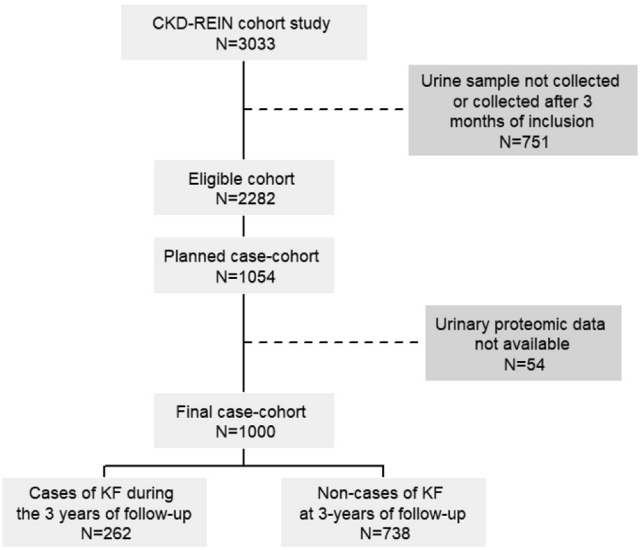

Figure 1. Study flow chart. KF, kidney failure.

Outcome of Interest

KF was defined as initiation of dialysis or pre-emptive transplantation. In the CKD-REIN cohort study, KF and deaths before KF (concurrent event) were identified from patient medical records or their family, or by

record linkage to the national REIN and the death registries until December 2020. In the Ghent cohort study, all outcomes were recorded prospectively by a single nephrologist (FV) until June 2017.

Demographic, Clinical and Laboratory Data

In the CKD-REIN study, patients' characteristics (age, sex, body mass index, blood pressure, and primary kidney disease), and comorbidities (cardiovascular disease, hypertension, and diabetes) were collected at baseline from medical records. GFR was estimated using the CKD-epidemiology collaboration equation[20] on the basis of cystatin C and serum creatinine (isotope dilution mass spectrometry traceable enzymatic method) measured in a single laboratory from stored blood samples. UACR was also measured centrally based on stored urine samples. For missing data (8 for eGFR, 13 for UACR), we used the closest routine laboratory value to the inclusion date. In the Ghent cohort study, GFR was estimated using the CKD- epidemiology collaboration equation with serum creatinine.

Sample Preparation and Capillary Electrophoresis–Mass Spectrometry Analysis

Detailed sample preparation and capillary electrophoresis–mass spectrometry analysis can be found in the Supplementary Methods. Spot urine samples (second morning urine) were collected during morning consultation at the hospital in urine collection vials (Vacuette) without the use of protease inhibitors. Samples were immediately stored at 4 °C and aliquoted within 6 hours without additional processing (no centrifugation or pH modification). All CKD-REIN urine samples were stored frozen at −80 °C at the Biobanque de Picardie (BRIF number: BB-0033-00017) and shipped in the frozen form to Toulouse, France, for analysis. Both teams were blind for outcome and patients characteristics. Urine samples from Ghent, Belgium, were immediately centrifuged after collection, aliquoted, and stored at −80 °C. Sample preparation was performed using established standard protocols, which have been applied on >85,000 samples and which have been described and evaluated in detail, also with respect to reproducibility.[21–23] Capillary electrophoresis–mass spectrometry analyses were performed on a PrinCE Next 840 capillary electrophoresis system (Prince Technologies) on-line coupled to a micrOTOF II mass spectrometer (Bruker Daltonic) as previously described.[24] Of the 1054 patients selected in the case-cohort study, 1000 had urinary pepidome data available for this analysis (Figure 1). Urine samples from Ghent, Belgium, were analyzed in Hannover, Germany on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter) coupled on-line coupled to a micrOTOF II mass spectrometer (Bruker Daltonic).

Data Processing

Mass spectral ion peaks representing identical molecules at different charge states were deconvoluted into single masses with MosaiquesVisu software.[25] The software automatically examined all mass spectra from a capillary electrophoresis–mass spectrometry analysis for signals with a signal-to-noise ratio of at least 4 present in 3 consecutive spectra. Capillary electrophoresis migration time was calibrated by local regression with more than 1700 reference signals. Normalization of urine peptidome data require correction for both analytical variances during profiling (e.g., signal suppression in the mass spectrometer) and for variability caused by biological issues (e.g., dilution of urine by different hydration states of the urine donors). With that aim, mass spectrometry signal intensities were normalized relative to 29 "housekeeping" peptides.[26] These highly abundant endogenous collagen fragments are generally present in at least 75% of all urine samples with small relative SDs. For calibration, linear regression was performed and a sample-specific dilution factor was calculated and subsequently used for normalization of urinary peptides. The resulting peak list characterized each peptide by its molecular mass (Da) and normalized capillary electrophoresis migration time (min). Normalized signal intensity (i.e., peptide abundance) was used as a measure of relative abundance of the peptides. All detected mass signals of peptides were deposited, matched, and annotated in Microsoft SQL Server.

Peptide Sequencing

For sequencing of peptides, the urine samples were analyzed on a Dionex Ultimate 3000 RSLC nano flow system (Dionex, Camberly, UK) coupled to an Orbitrap Velos MS instrument (Thermo Fisher Scientific), using data-dependent higher-energy collision dissociation MS/MS sequencing of a maximum of the top 20 ions, as described in Klein *et al*.[27] Data files were analyzed using Proteome Discoverer 1.2 (Thermo Fisher Scientific) and were searched against the Uniprot database. No fixed modifications were selected; oxidation of methionine, lysine and proline were selected as variable modifications. The peptide data were extracted using high confidence peptides, which are best ranking peptides with an Xcorr ≥1.9, a delta mass between experimental and theoretical mass ± 5 ppm, absence of cysteines in the sequence (because cysteines without reduction and alkylation form disulphide bonds), absence of oxidized proline in protein precursors other than collagens or elastin.

Enzyme-Linked Immunosorbent Assay Measurement of α-1-Antitrypsin

Urine α-1-antitrypsin concentration was determined in a blinded manner with a commercially available enzyme-linked immunosorbent assay kit for human (Bio-techne, # DY1268), according to the manufacturer's protocol.

Statistical Analysis

Patients' baseline characteristics were reported as percentage for qualitative variables and as mean ± SD or median (lower quartile–upper quartile) for quantitative variables. In all analyses, peptides abundances and continous variables were used as linear predictors. In the CKD-REIN case-cohort, a clustering algorithm with selection of relevant variables and number of groups was first carried out with the sequenced urinary peptides to classify patients at baseline. Peptides present in less than 20 patients or with a near zero variance were excluded. The algorithm used the integrated complete-data likelihood (VarSelLCM package in R Statistical software, R Core Team).[28] Patients' baseline characteristics were described according to identified clusters. Several 3-year KF risk prediction models were then developed in the CKD-REIN case-cohort with different sets of predictors as follows: (i) sequenced peptides; and (ii) sequenced peptides and risk factors including age, sex, eGFR, and UACR (log transformation); two supplementary models were created to explore the prediction potential of nonsequenced peptides as follows: (iii) all peptides, (iv) all peptides and risk factors. Only peptides that had more than 20 patients with a nonzero value were predictor candidates. We used a Cox's proportional hazards model regularized by elastic net penalty to develop each model (glmnet package in R Statistical software, R Core Team).[29] All predictors were standardized before training. Two-fold stratified cross-validation repeated 50 times was performed to choose the optimal hyperparameters with the "one standard error" rule[30] and the Harrell c-index. Mean c-index was estimated and its 95% CI obtained with the percentile method through resampling. Finally, each optimal model was fitted in the entire data set.

In accordance with clinical proteomics guidelines,[31,32] we then assessed the performance of the identified peptide KF signature in Ghent, an external validation cohort, using the c-index and its 95% bootstrap CI (percentile method). The overall calibration was verified for the absolute risk of KF at 3 years. Mean of estimated probability of KF at 3-years was calculated. Because of the competing risk of death before KF, the cumulative incidence function of Kalbfleisch and Prentice was used to estimate the observed probability. In the CKD-REIN case-cohort, the performance of the 4-variables KFRE was also estimated using the c-index [95% CI]. All statistical analyses were performed with R software (v4.0.2; R Core Team 2020).

Table 1. Patient characteristics at inclusion in the CKD-REIN case-cohort and the Ghent cohort subsample

Characteristics	CKD-REIN case-cohort (*N* = 1000)		Ghent cohort subsample (*N* = 326)	
	% or mean ± SD or median (Q1–Q3)	$N_{missing}$	% or mean ± SD or median (Q1–Q3)	$N_{missing}$
Men	69%		60%	
Age, yr	69 (61–77)		71 (61–79)	
CKD stages				
2–3	36%		67%	
4	54%		28%	
5	10%		5%	
Estimated GFR (CKD-EPI), ml/min/1.73 m^2	28 ± 11		36 ± 13	
Urine albumin-to-creatinine ratio, mg/g	228 (39–1201)	1	NA	
Urine albumin-to-creatinine ratio categories		1	NA	
Normal or minimal increase (A1)	21%			
Moderate increase (A2)	32%			
Severe increase (A3)	46%			
Urine protein-to-creatinine ratio, g/g creatinine	NA		0.5 (0.2–1.5)	167
Primary kidney disease		106		-
Diabetic nephropathy	22%		20%	
Glomerular nephropathy	19%		9%	
Hypertensive or vascular nephropathy	31%		24%	
Tubulointerstitial nephropathy	12%		7%	
Polycystic kidney disease	6%		6%	
Other or unknown	10%		34%	
Kidney biopsy	24%	35		
Diabetes	43%	2	38%	
History of acute kidney injury	23%	76	NA	
Hypertension	92%		NA	
Cardiovascular disease	57%	6	45%	

CKD-EPI, chronic kidney injury epidemiology collaboration; GFR, glomerular filtration rate; N, number; NA, not available; Q1, lower quartile; Q3, upper quartile; REIN, Renal Epidemiology and Information Network.
Cardiovascular disease includes the presence or history of coronary artery disease, myocardial infarction, coronary artery bypass surgery, percutaneous coronary intervention, atrial fibrillation, other cardiac rhythm disorder, implanted pacemaker or defibrillator, heart failure, pericarditis, valvular disease, prosthetic heart valve, stroke, transient ischemic attack, cerebral hemorrhage, peripheral vascular disease, intermittent claudication, heart valve prosthesis, arterial bypass grafting/percutaneous intervention.

RESULTS

Characteristics of the CKD-REIN Case-Cohort Subsample

Patients were mostly men, had a median age of 69 years at baseline, any type of primary kidney disease, a median eGFR of 25 ml/min per 1.73 m^2, and a median UACR of 228 mg/g (Table 1). Over 3-year follow-up, 262 patients progressed to KF (227 started dialysis; 35 had preemptive kidney transplantation; incidence rate,

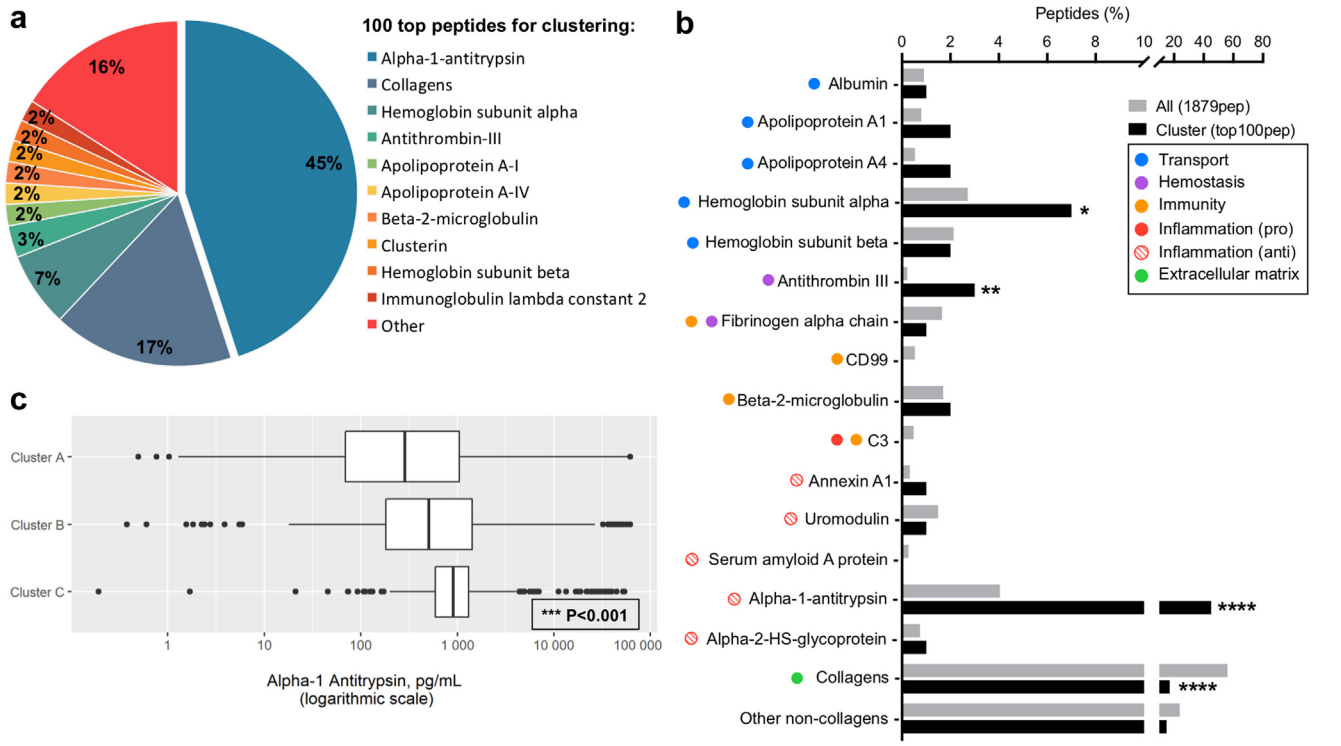

Figure 2. Representation of the peptides with highest discriminative power for clustering at baseline. (a) Identities of 100 peptides with highest discriminative power for clustering at baseline. (b) Percentage of peptides originating from collagenic or noncollagenic proteins among all identified urinary peptides ($N = 1879$) and among the top peptides for clustering at baseline ($N = 100$). (c) Enzyme-linked immunosorbent assay analysis of urinary α-1-antitrypsin in the CKD-REIN case-cohort.***$P < 0.001$ using Kruskal-Wallis rank sum test.

10.5 per 100 person-years [95% CI: 9.2−11.8]), and 108 died before KF.

Urinary Peptidome-Based Classification of Patients at Baseline

A total of 5616 different peptides were detected in urine of the 1000 case-cohort patients. Sequence information was available for 2157 peptides, of which 1879 were detected in more than 20 patients. Clustering algorithm classified patients at baseline into 3 clusters with large between-cluster differences in both abundance and types of fragments. The 100 peptides with the highest discriminative power for clustering were mainly fragments of α-1-antitrypsin (45%). Other peptides included various collagen fragments (17%), and fragments of hemoglobin subunit alpha and beta (7% and 2%, respectively), antithrombin-III (3%), apoliprotein A-1 (2%) and A-4 (2%), beta-2-microglobulin (2%), clusterin (2%), and immunoglobulin lambda (2%) (Figure 2a). The 3 groups of patients substantially differed according to diabetes status, primary kidney disease, and CKD severity, with decreasing eGFR and increasing UACR levels from cluster A to cluster C (Table 2). The incidence rate of KF was 4.2 per 100 person-years (95% CI: 3.0–5.5) for cluster A, 10.4 (95% CI: 8.3–12.4) for cluster B and 23 (95% CI: 18.9–27.1) for cluster C. Protein fragments of α-1-antitrypsin were strongly enriched compared with total peptidome (Figure 2b) and more abundant in the urine of cluster C, including patients with more severe CKD than in cluster A and B patients (Supplementary Figure 1). Other protein fragments showing enrichment in these top 100 peptides included hemoglobin subunit alpha and antithrombin-III, whereas fragments of collagens were significantly less represented compared with total peptidome (Figure 2b). The link between α-1-antitrypsin and patient classification was confirmed using a commercial enzyme-linked immunosorbent assay as increasing levels of urinary α-1-antitrypsin were observed from cluster A to cluster C (Figure 2c).

Development And Internal Validation Of A Urinary Peptidome Signature To Predict 3-Year Risk Of Kf

Among the 1879 sequenced peptides, the regularized Cox model selected 90 peptides that best predicted the 3-year risk of KF with a c-index of 0.83 (95%CI: 0.81–0.85) (Figure 3). The majority of the 90 peptides were fragments of various collagens (59%, Figure 4a and Supplementary Table S1). Other peptides included fragments of apolipoprotein A-1 (4%), α-1-antitrypsin (4%), serum amyloid A protein (2%), albumin (2%), fibrinogen alpha chain (2%), complement C3 (2%), and annexin A1 (2%) (Figure 4a and Supplementary

Table 2. Patients characteristics at baseline according to the 3 urinary peptidomic clusters in the CKD-REIN case-cohort ($N = 1000$)

Characteristics	Cluster A ($n = 384$)	Cluster B ($n = 374$)	Cluster C ($n = 242$)	*P*-value[a]	$N_{missing}$
Baseline characteristics					
Male	67%	69%	74%	0.134	
Age, yr	70 (63–77)	68 (60–77)	67 (59–75)	0.007	
CKD stages				<0.001	
2–3	53%	32%	17%		
4	41%	59%	66%		
5	5%	9%	17%		
Estimated GFR (CKD-EPI), ml/min/1.73 m^2	31.8 ± 12.2	26.4 ± 10	22.5 ± 8.4	0.004	
Urine albumin-to-creatinine ratio categories				<0.001	1
Normal to mildly increased	43%	12%	2%		
Moderatly increased	35%	42%	14%		
Severly increased	22%	46%	84%		
Primary kidney disease				<0.001	106
Diabetic nephropathy	15%	23%	30%		
Glomerular nephropathy	14%	19%	26%		
Hypertensive or vascular nephropathy	39%	29%	22%		
TubuloInterstitial nephropathy	15%	11%	8%		
Polycystic kidney disease	7%	7%	6%		
Other	10%	11%	8%		
Diabetes	35%	45%	53%	<0.001	2
History of acute kidney injury	19%	26%	22%	0.077	76
History of cardiovascular disease	58%	57%	58%	0.946	6

CI, confidence interval; CKD-EPI, chronic kidney injury epidemiology collaboration; GFR, glomerular filtration rate; N, number; Q1, lower quartile; Q3, upper quartile; REIN, Renal Epidemiology and Information Network.
[a]*P*-value of analysis variance test, Kruskal-Wallis test, Fisher exact test, or the χ^2test as appropriate.
Data of baseline characteristics are presented as %, mean ± SD or median (Q1–Q3);

Table S1). Peptides that contributed most to the predictive power of the signature were fragments of fibrinogen alpha chain, albumin, apolipoprotein A-1 and apolipoprotein A-4 (positive association, Supplementary Figure S3), and fragments of CD99 (MIC2 or single-chain type-1 glycoprotein; negative association, Supplementary Figure S3). Fragments of collagens contributed both positively and negatively to the prediction (Supplementary Figure S3). At the peptide level, only 5 fragments (3 α-1-antitrypsin, 1 annexin A1, and 1 uromodulin fragment) of the 90 predictive peptides were also present in the top 100 discriminative peptides at baseline. However, most parent proteins were commonly represented in both signatures (Figure 4b), except for protein fragments of antithrombin-III and α-2-HS-glycoprotein that were only found in the top 100 clustering peptides at baseline; and fragments of CD99, complement C3, and serum amyloid A protein that were only found in the KF signature (Figure 4b). Comparing the 90 KF predictors with the total peptidome showed a significant enrichment in fragments of apoliprotein A-1 that were more abundant in the urine of KF cases compared with that of noncases (Figure 4c and Supplementary Figure S2). Other protein fragments showing enrichment were peptides from anti-inflammatory proteins annexin A1 and serum amyloid protein (Figure 4c).

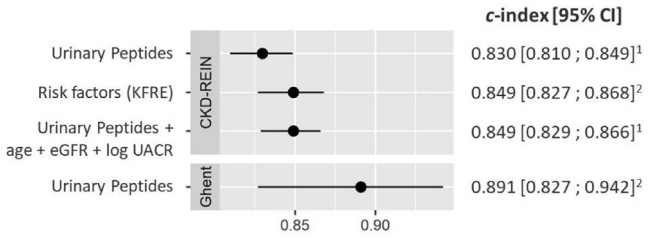

Figure 3. Performance metrics of the prediction models. Performance of the prediction models with the different sets of predictors was estimated in the CKD-REIN case-cohort ($N = 1000$, E = 262) and in the Ghent cohort ($N = 326$, E=28). *N*: number of patients; E: number of kidney failure events at 3 years; Urinary peptides: sequenced and present among more than 20 CKD-REIN patients; 4-variable Kidney Failure Risk Equation[2] including age, sex, log UACR, and eGFR. [1]Estimated by 2 folds cross-validation repeated 50 times; [2]Estimated in the entire data set. CI, confidence interval; eGFR, estimated glomerular filtration rate; KFRE, kidney failure risk equation; UACR, urinary albumin-to-creatinine ratio.

When analyzed according to the different underlying CKD etiologies, the 90-peptide KF signature showed similar score distribution among the various etiologies (Supplementary Figure S4). Finally, based on all 5616 differentially abundant peptides, not restricted to the sequenced peptides, the Cox model selected 169 peptides that similarly predicted the 3-year risk of KF with a c-index of 0.83 (95% CI: 0.82–0.85) (Supplementary Table S2).

Prediction Performance of the Peptidomic Signature as Compared to the KFRE

The performance of the 4-variable KFRE[2] was close to that of the 90-peptide signature to predict the 3-year

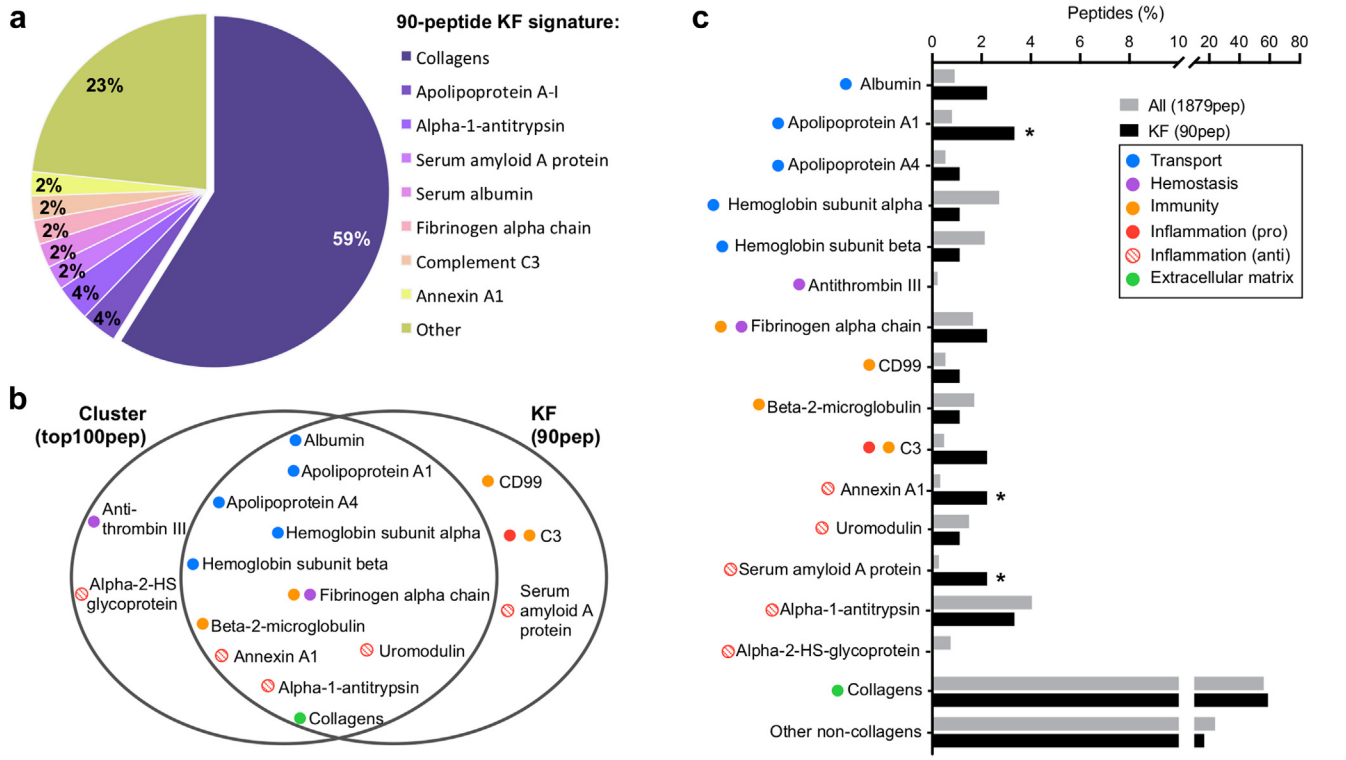

Figure 4. Representation of the peptides predictive of kidney failure. (a) Identities of 90 peptides included in the 3-year KF model. (b) Comparison of the parent proteins represented in the 100 peptides with highest discriminative power for clustering at baseline and the 90 peptides included in the 3-year KF model. (c) Percentage of peptides originating from collagenic or noncollagenic proteins among all identified urinary peptides ($N = 1879$) and among the peptides identified as KF predictors ($N = 90$). KF, kidney failure.

risk of KF, with c-index of 0.85 (95% CI: 0.83–0.87) versus 0.83 (95% CI: 0.81–0.85), respectively (Figure 3). When the 1879 sequenced peptides were combined with the 4 KFRE variables, the optimal model selected 24 peptides and 3 of these 4 variables (age, eGFR, and log [UACR]) with a c-index of 0.85 (95%CI: 0.83–0.87), similar to that of the above models (Figure 3). All of those 24 peptides were also present in the initial 90-peptide signature. Because the loss of 66 of the 90-peptides in this combined model did not modify the predictive performance, this suggests that the information contained in the peptides is partially redundant with these 3 variables. This is supported by the strong correlation between the peptide-based and the KFRE-based scores (Rho = 0.83, Figure 5). Similar results were obtained when all peptides, sequenced or not were considered. Twenty-five peptides combined with age, eGFR and log (UACR) predicted KF with a c-index of 0.85 (95% CI: 0.83–0.87) (Supplementary Table S2).

External Validation of the Peptidomic Signature to Predict KF

Compared to the CKD-REIN case-cohort, the Ghent cohort used for external validation included patients at an earlier CKD stage; 28 of 326 patients progressed to KF over the 3-year follow-up and 61 died before KF (Table 1). The incidence rate of KF was 3.3 per 100 person-years (95% CI: 2.0–4.5). The performance of the 90-peptide signature was high with a c-index of 0.89 (95%CI: 0.83–0.94) for the prediction of the 3-year risk of KF (Figure 3). Overall calibration diagnostic showed an overestimation of the 3-year predicted risk of KF by the 90-peptide signature compared to the observed risk, 0.16 (95% CI: 0.14–0.17) versus 0.09 (95% CI: 0.06–0.12), respectively. Similar results were obtained with the 169-peptide signature based on all peptides with a c-index of 0.90 (95% CI: 0.84–0.96) (Supplementary Table S2).

DISCUSSION

In this case-cohort study nested in a large cohort of well-phenotyped patients with CKD, 5000 urinary peptides were detected and quantified using peptidome analysis. Unsupervised machine learning classified the patients into 3 groups with homogeneous peptidome profiles at baseline. These groups differed significantly in terms of CKD severity. The 100 peptides with the highest discriminative power for clustering were mainly fragments of inflammation and fibrosis proteins, highlighting those derived from α-1-antitrypsin as the most significant. Using supervised machine learning, we further identified a set of 90 peptides which

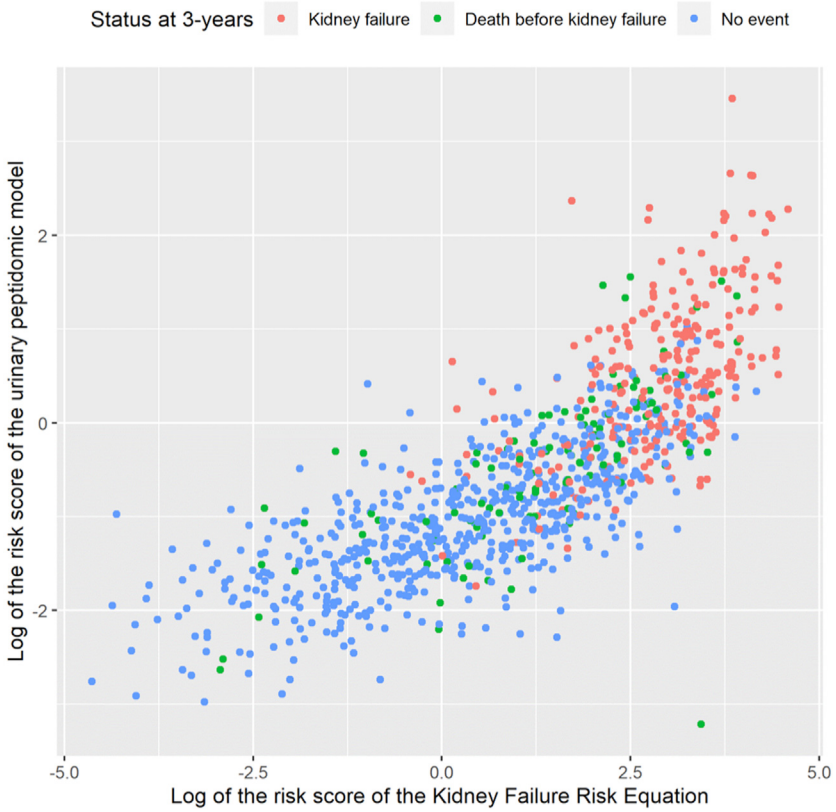

Figure 5. Correlation between the peptide-based and KFRE-based scores. Logarithm of the urinary peptidomic model risk score according to the logarithm of the KFRE risk score and patient outcome at 3-years in the CKD-REIN case-cohort ($N = 1000$).

achieved excellent discrimination in predicting the risk of progression to KF, in both the development and validation cohorts with c-index largely above 0.80. However, this 90-peptide signature or the combination of these peptides with age, eGFR, and UACR did not improve the prediction provided by the KFRE alone. These findings have both clinical and research implications.

One objective of this study was to better understand the pathophysiological mechanisms associated with CKD severity and progression (e.g., glomerular and tubular injury, inflammation, extracellular matrix remodeling, etc) using untargeted peptidome analysis. Among the peptides identified in the present study, the anti-inflammatory α-1-antitrypsin urinary fragments emerged as the most abundant peptides in the cluster of patients with most severe CKD, and this was confirmed at the protein level by enzyme-linked immunosorbent assay. Three of the α-1-antitrypsin fragments were also present in the signature predicting progression to KF.

Alpha-1-antitrypsin is a member of the serpin family, a major acute phase protein, and a physiological inhibitor of serine proteases such as neutrophil elastase, resulting in a plethora of anti-inflammatory and anti-apoptotic effects.[33] The lack of such an important circulating proteinase inhibitor predisposes homozygous individuals with severe α-1-antitrypsin deficiency to early-onset emphysema ("loss of function").[33] Although lung and liver diseases are the most prominent associated disorders, several other conditions, such as CKD and diabetes are overrepresented in persons with α-1-antitrypsin deficiency.[33]

In patients with diabetes, plasma levels and activity of α-1-antitrypsin have been shown to be significantly decreased,[34] whereas increased levels of urinary α-1-antitrypsin peptides were observed in an animal model of diabetes.[35,36] Our present data extend the findings that increased levels of urinary α-1-antitrypsin peptides and protein are also associated with CKD severity and progression in patients with diabetic and nondiabetic kidney disease. A possible mechanism to explain the increased levels of urinary α-1-antitrypsin peptides and protein is an increased excretion because of local synthesis by various kidney cell types combating inflammatory processes in the kidney. In keeping with this hypothesis is the observation that transgenic mice which were expressing the normal human α-1-antitrypsin gene in tubular epithelial cells exhibited α-1-antitrypsin protein in the cisternae of the rough endoplasmic reticulum.[37] Another alternative consequence of enhanced renal loss could be decreased plasma α-1-antitrypsin levels, an activity that might be of clinical significance. Elastin is the main component of elastic fibers, and the

destruction of elastin fibers by elastase causes loss of elasticity of the arterial wall.[38] Therefore, a possible consequence of plasma α-1-antitrypsin deficiency could be increased elastase activity leading to a greater arterial stiffness, that is, arteriosclerosis as a major contributor to nonatheromatous vascular disease. In this context, it is noteworthy that arteriosclerosis has been shown to predict rapid GFR decline among patients with high cardiovascular risk.[39] Whether therapy with α-1 antitrypsin, which is available on the market but indicated only for the treatment of the severe forms of α-1 antitrypsin deficiency, will be helpful to protect the risk of progression from CKD to KF remains to be demonstrated by clinical studies.

Many blood-derived protein fragments (e.g., albumin, apolipoproteins, hemoglobin, fibrinogen, and β-2-microglobulin) were present in both the top 100 clustering peptides and the 90-peptide KF signature. This could be interpreted as functional change because of the loss of function of the glomerular filtration barrier[40] and confirms observations of Pontillo *et al.*,[14] who observed more blood-derived urinary peptides in patients with advanced CKD and in those that showed CKD progression. Our data also confirm the modification of urinary collagen peptide abundance in CKD[10,11] and the suggested link to in-situ fibrosis previously observed.[12] Other fragments such as β-2-microglobulin and uromodulin[4] present in both selection of peptides, however, may reflect tubular damage.

The other objective of this study was to evaluate the potential of the urine peptidome to predict CKD progression to KF, and its added value compared to other predictors. In CKD stages G3 to G5, it has been reported that the urinary peptide signature CKD273, developed to identify CKD, does not have an added value in predicting CKD progression (defined as eGFR decline >5 ml/min per 1.73 m^2 per year) compared to urinary albumin excretion.[14] In our population of patients with CKD stages G3 to G5, the urinary peptide signature displayed similar predictive performance as the 4-variable KFRE, and its combination with KFRE variables did not further improve this performance, thereby confirming these observations. This was not surprising given the high c-index of 0.85 (0.83–0.87) observed for the equation in this cohort, as in many others worldwide.[2] Interestingly, the 6-variable risk equation based on routine laboratory parameters and developed using machine learning by the German CKD cohort was shown to provide a slight improvement in KF prediction of 0.010 in the CKD-REIN cohort compared to the KFRE.[3] Altoghether, this suggests that in moderate to advanced CKD, the information contained in the urine peptidome, analyzed with an untargeted holistic approach, is redundant with those captured by age, sex, eGFR, and UACR alone. Analysis of different biological levels (e.g., metabolome) or other biological fluids (e.g., plasma) could help to better grasp the multifactorial disease character.[41,42] Although urinary peptide signatures do not seem to have added value for KF risk prediction in advanced CKD compared to the routinely used KFRE, they emerge as good predictors for patients at risk of CKD, in individuals with type 2 diabetes and normoalbuminuria.[13] Our findings that the 90-peptide KF signature showed similar score distribution across the various types of primary kidney disease may prompt further reseach to assess its predictive performance in patients at early stages of various nonalbuminuric CKD phenotypes.

This study has major strengths with respect to standards requested for proteomic analyses.[31,43,44] It is based on a large cohort of well-phenotyped CKD patients with adequate demographic and clinical data (type of nephropathy and comorbidities), clearly defined clinical questions, patient selection, and outcomes, and detailed information about the sampling methodology because urinary samples of the CKD-REIN study have been collected and stored following strict standard operating procedures. The case-cohort design of the study was cost-effective and allowed us to use Cox proportional hazards models and Harell c-index to evaluate the 90-peptide signature performance as for the entire cohort. Moreover, we validated our findings in an external independent cohort with less severe CKD. Interestingly, the samples were collected from another site (Ghent University Hospital) than the CKD-REIN cohort, and peptidome analysis was also performed in another laboratory confirming the high inter-laboratory precision, stability, and reproducibility of our biomarkers regardless of the analytical platform.[21,45] Our study also has some limitations. The small number of events in the validation cohort leads to a low precision of c-statistics and does not allow to evaluate calibration by level of risk. Finally, we did not explore early CKD stages, which limits the clinical implication of the present results to CKD stage 3 to 5 patients, and lacks sufficient study power to assess the performance of the signature in patients with low albuminuria level.

In conclusion, UP appears to represent a useful tool for the detection of new pathophysiological pathways involved in CKD severity and progression, and KF risk prediction with a performance equal to that of the KFRE. The combination of urinary peptides with the KFRE variables does not improve the prediction, indicating that the KFRE and peptidome probably reflect similar mechanisms of progression to KF.

APPENDIX

Members of the CKD-REIN Study Group Collaborators

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DISCLOSURE

ZAM reports having received grants for CKD-REIN and other research projects from Amgen, Baxter, Fresenius Medical Care, GlaxoSmithKline, Merck Sharp and Dohme-Chibret, Sanofi-Genzyme, Lilly, Otsuka, Astra Zeneca, Vifor, and the French government, and fees and grants to charities from Astra Zeneca, Boehringer Ingelheim, and GlaxoSmithKline. HM is the cofounder and co-owner of Mosaiques Diagnostics. JS is employed by Mosaiques Diagnostics. BS coordinates the CKD-REIN cohort study, funded by the *Agence Nationale de la Recherche* through the 2010 *Cohortes-Investissements d'Avenir program* and by the 2010 national *Program Hospitalier de Recherche Clinique*, and supported through a public-private partnership currently involving Fresenius Medical Care, GlaxoSmithKline, Vifor Fresenius and Boehringer Ingelheim France. CKD-REIN is supported by a public-private partnership with funding from 10 pharmaceutical companies as listed in the Funding section.

ACKNOWLEDGMENTS

We acknowledge the CKD-REIN study coordination staff for their efforts in setting up the CKD-REIN cohort (Marie Metzger, Elodie Speyer, Céline Lange, and all the clinical research associates) as well as the CKD-REIN clinical sites and investigators: Alsace: Prs. T. Hannedouche et B. Moulin (CHU, Strasbourg), Dr. A. Klein (CH Colmar); Aquitaine: Pr. C. Combe (CHU, Bordeaux), Dr. J.P. Bourdenx (Clinique St Augustin, Bordeaux), Dr. A. Keller, Dr. C. Delclaux (CH, Libourne), Dr. B. Vendrely (Clinique St Martin, Pessac), Dr. B. Deroure (Clinique Delay, Bayonne), Dr. A. Lacraz (CH, Bayonne); Basse Normandie: Dr. T. Lobbedez (CHU, Caen), Dr. I. Landru (CH, Lisieux); Ile de France: Pr Z. Massy (CHU, Boulogne – Billancourt), Pr. P. Lang (CHU, Créteil), Dr. X. Belenfant (CH, Montreuil), Pr. E. Thervet (CHU, Paris), Dr. P. Urena (Clinique du Landy, St Ouen), Dr. M. Delahousse (Hôpital Foch, Suresnes); Languedoc – Roussillon: Dr. C. Vela (CH, Perpignan); Limousin: Pr. M. Essig, Dr. D. Clément (CHU, Limoges); Lorraine: Dr. H. Sekhri, Dr. M. Smati (CH, Epinal), Dr. M. Jamali, Dr. B. Hacq (Clinique Louis Pasteur, Essey-les-Nancy), Dr. V. Panescu, Dr. M. Bellou (Polyclinique de Gentilly, Nancy), Pr. Luc Frimat (CHU, Vandœuvre-les-Nancy); Midi-Pyrénées: Pr N Kamar (CHU, Toulouse); Nord-Pas-de-Calais: Prs. C. Noël et F. Glowacki (CHU, Lille), Dr. N. Maisonneuve (CH, Valenciennes), Dr. R. Azar (CH, Dunkerque), Dr. M. Hoffmann (Hôpital privé La Louvière, Lille); Pays-de-la Loire: Pr. M. Hourmant (CHU, Nantes), Dr. A. Testa (Centre de dialyse, Rezé), Dr. D. Besnier (CH, St Nazaire); Picardie: Pr G. Choukroun (CHU, Amiens), Dr. G. Lambrey (CH, Beauvais) Provence-Alpes - Côte d'Azur: Pr. S. Burtey (CHU, Marseille), Dr. G. Lebrun (CH, Aix-en-Provence), Dr. E. Magnant (Polyclinique du Parc Rambot, Aix-en-Provence); Rhône-Alpes: Pr M. Laville, Pr. D. Fouque (CHU, Lyon-Sud) et L. Juillard (CHU Edouard Herriot, Lyon), Dr. C. Chazot (Centre de rein artificiel Tassin Charcot, Ste Foy-les-Lyon), Pr. P. Zaoui (CHU, Grenoble), Dr. F. Kuentz (Centre de santé rénale, Grenoble).

The authors are grateful to all biological resource centers for their participation in the project: the Biobanque de Picardie (BB-0033-00017), NeuroBioTec (BB-0033-00046), Centre de ressources biologiques (CRB)-Centre Hospitalier Universitaire de Nantes Hôtel Dieu (BB-0033-00040), CRB-Centre Hospitalier Universitaire Grenoble Alpes (BB-0033-00069), CRB-Centre Hospitalier Régional Universitaire de Nancy (BB-0033-00035), Service de Néphrologie, Centre Hospitalier de Perpignan, the Plateforme de Ressources Biologiques-Hôpital Henri Mondor (BB-0033-00021), the Centre d'Investigation Clinique Plurithématique CIC-1435, Plateforme de Ressources Biologiques-Hôpital européen Georges-Pompidou (BB-0033-00063), L'Etablissement Français du sang (EFS) Hauts de France–Normandie (Site de Bois-Guillaume, Site de Loos-Eurasanté), EFS Nouvelle Aquitaine (site Pellegrin), EFS Ile de France (Site Avicenne), EFS Occitanie (Site de Toulouse), EFS Grand-Est (Site de Colmar, Site de Metz), EFS PACA-Corse (Site de Marseille).

Funding

CKD-REIN is funded by the Agence Nationale de la Recherche through the 2010 «Cohortes-Investissements d'Avenir » program (ANR-IA-COH-2012/3731) and by the 2010 national Program Hospitalier de Recherche Clinique. CKD-REIN is also supported through a public-private partnership with Fresenius Medical Care and GlaxoSmithKline (GSK) since 2012 and Vifor France since 2018, Sanofi Genzyme (2012 to 2015), Baxter and Merck Sharp & Dohme-Chibret (MSD France) from 2012 to 2017, Amgen (2012 to 2020), Lilly France (2013 to 2018), Otsuka Pharmaceutical (2015 to 2020), AstraZeneca (2018 to 2021) and Boehringer Ingelheim France since 2022. Inserm Transfert set up and has managed this partnership since 2011. The work was also supported by a grant from the Agence Nationale de la Recherche (Proteomark_CKD,

ANR-17-CE17-0006-01) and by the "Fondation pour la Recherche Médicale" (DEQ20170336759).

Authorizations

All legal autorizations were obtained including those from the Comité consultatif sur le traitement de l'information en matière de recherche dans le domaine de la santé (CCTIRS N°12.360), the Commission nationale de l'informatique et des libertés (CNIL N°DR-2012-469), and from the Kremlin-Bicêtre Comité de protection des personnes (CPP N°IDRCB 2012-A00902-41). CKD-REIN biological collection is registered in the management application of the COnservation D'Eléments du COrps Humain (CODECOCH N°-2012-1624). The Institut national de la santé et de la recherche médical (Inserm) Institutional Review Board approved the study protocol (IRB00003888). ClinicalTrials.gov Identifier: NCT03381950.

Data Availability

All data, including the risk equation with the predictors list, are available on reasonable request from the authors.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods.

Figure S1. Urinary peptide abundance in the CKD-REIN case-cohort according to the peptidome clusters. Peptides are grouped by proteins. Only the 100 peptides with the highest discriminating power are represented.

Figure S2. Urinary peptide abundance in the CKD-REIN case-cohort according to the status case/noncase at 3 years. Peptides are grouped by proteins. Only the 90 peptides present in the KF signature are represented.

Figure S3. Coefficients of standardized abundance of the 90 peptides present in the urinary peptidome-based KF prediction model. Positive coefficients increase the risk score, negative coefficients decrease the risk score.

Figure S4. Risk score of the urinary peptidome model according to the primary kidney disease and patient outcome at 3 years in the CKD-REIN case-cohort.

Table S1. Sequence of the 90 urinary peptides predicting KF.

Table S2. Performance metrics of regularized Cox's models with all urinary peptides.

TRIPOD Checklist.

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