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► **To cite this version:**

Frank Bienaimé, Mordi R. Muorah, Melanie Broeuilh, Pascal Houiller, Martin Flamant, et al.. Combining robust urine biomarkers to assess chronic kidney disease progression. *EBioMedicine*, 2023, 93, 10.1016/j.ebiom.2023.104635 . hal-04191676

**HAL Id: hal-04191676**

**<https://hal.science/hal-04191676v1>**

Submitted on 20 Sep 2023

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# Combining robust urine biomarkers to assess chronic kidney disease progression



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## Summary

**Background** Urinary biomarkers may improve the prediction of chronic kidney disease (CKD) progression. Yet, data reporting the applicability of most commercial biomarker assays to the detection of their target analyte in urine together with an evaluation of their predictive performance are scarce.

**Methods** 30 commercial assays (ELISA) were tested for their ability to quantify the target analyte in urine using strict (FDA-approved) validation criteria. In an exploratory analysis, LASSO (Least Absolute Shrinkage and Selection Operator) logistic regression analysis was used to identify potentially complementary biomarkers predicting fast CKD progression, determined as the <sup>51</sup>CrEDTA clearance-based measured glomerular filtration rate (mGFR) decline (>10% per year) in a subsample of 229 CKD patients (mean age, 61 years; 66% men; baseline mGFR, 38 mL/min) from the NephroTest prospective cohort.

**Findings** Among the 30 assays, directed against 24 candidate biomarkers, encompassing different pathophysiological mechanisms of CKD progression, 16 assays fulfilled the FDA-approved criteria. LASSO logistic regressions identified a combination of five biomarkers including CCL2, EGF, KIM1, NGAL, and TGF- $\alpha$  that improved the prediction of fast mGFR decline compared to the kidney failure risk equation variables alone: age, gender, mGFR, and albuminuria. Mean area under the curves (AUC) estimated from 100 re-samples was higher in the model with than without these biomarkers, 0.722 (95% confidence interval 0.652–0.795) vs. 0.682 (0.614–0.748), respectively. Fully-adjusted odds-ratios (95% confidence interval) for fast progression were 1.87 (1.22, 2.98), 1.86 (1.23, 2.89), 0.43 (0.25, 0.70), 1.10 (0.71, 1.83), 0.55 (0.33, 0.89), and 2.99 (1.89, 5.01) for albumin, CCL2, EGF, KIM1, NGAL, and TGF- $\alpha$ , respectively.

**Interpretation** This study provides a rigorous validation of multiple assays for relevant urinary biomarkers of CKD progression which combination may improve the prediction of CKD progression.

**Funding** This work was supported by Institut National de la Santé et de la Recherche Médicale, Université de Paris, Assistance Publique Hôpitaux de Paris, Agence Nationale de la Recherche, MSD Avenir, Pharma Research and Early Development Roche Laboratories (Basel, Switzerland), and Institut Roche de Recherche et Médecine Translacionnelle (Paris, France).

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**Keywords:** Chronic kidney disease progression; Urinary biomarkers; EGF; TGF- $\alpha$ ; CCL2; NGAL

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eBioMedicine

2023;93: 104635

Published Online 6 June 2023

<https://doi.org/10.1016/j.ebiom.2023.104635>

1016/j.ebiom.2023.104635

### Research in context

#### Evidence before this study

Patients with chronic kidney disease usually experience a progressive reduction in kidney function, but the rate of the decline varies widely from one individual to another one. Well established risk factors for fast kidney function decline include older age, male gender, a reduced baseline glomerular filtration rate and a high urine albumin to creatinine ratio. However, the prediction of kidney function decline provided by these elements remains imprecise. Several studies investigated if the urinary excretion rate of additional biomolecules could improve risk stratification among patients suffering from chronic kidney disease. The quantification of these biomolecules in urine usually relies on Enzyme Linked Immunosorbent Assays that were not tested for their ability to robustly detect their analyte in urine, a very peculiar matrix. In addition, the majority of reported studies focused on the detection of one or a few additional biomolecules precluding the evaluation of redundancy or complementarity among potential urinary biomarkers of kidney function decline.

#### Added value of this study

Among 30 commercial assays for the detection of potential urinary biomarkers that we tested, only 16 full-filled FDA-approved criteria for the detection of their analyte into urine. Exploratory analyses in a deeply phenotype cohort of 229 patients suffering from chronic kidney disease identified a non-redundant combination of 5 biomarkers, which improved the prediction of fast kidney decline in this cohort.

#### Implications of all the available evidence

The discovery of biomarkers for the prediction of kidney function decline requires a robust validation of the assays' performance in the urine matrix. The combination of distinct biomarkers reflecting different aspects of the pathophysiology of chronic kidney disease may not only improve patients risk stratification but also pinpoint underlying druggable pathophysiological processes.

## Introduction

Chronic Kidney Disease (CKD) affects about 10% of the Western population and is associated with increased risk of cardiovascular death as well as all-cause mortality.<sup>1-3</sup> CKD is characterized by the progressive decline of kidney function that occurs, regardless of the initial cause, once a critical number of functional nephrons has been lost. While almost all kidney diseases can lead to nephron loss, the degradation of the remnant nephrons occurs through common pathophysiological pathways, allowing common therapeutic interventions for CKD patients. Epidemiological studies have shown that the rate of CKD progression varies widely among individuals.<sup>4</sup> In fact, if some patients will progress rapidly to end stage kidney disease (ESKD), others may remain stable or even improve over the time.<sup>5,6</sup> Accordingly, there is a need for new strategies to discover biomarkers able to identify patients at highest risk for CKD progression, who might maximally benefit from increased surveillance, early prevention and targeted therapies. In addition, such biomarkers might also serve as important surrogate markers for treatment response in clinical trials for which we tremendously lack valuable end points. Decreased glomerular filtration rate and increased albumin urinary excretion are established markers of CKD progression.<sup>7,8</sup> However, these markers may appear relatively late in the course of CKD and reflect more the functional changes than the early structural alterations in the kidney. Moreover, albuminuria can regress in spite of on-going CKD and patients can progress without albuminuria.<sup>9</sup> Thus, increasing prediction accuracy by adding biomarkers has become an important concern for the community.

In the last decade, a number of studies have focused their interest on the discovery of CKD biomarkers.<sup>10-22</sup> Although urinary biomarkers have emerged, many of them maintain an undefined pathophysiological significance and limited practical clinical application.<sup>22-24</sup> Importantly, few assays have been validated for the measurement of those biomarkers in urine, a very specific matrix with unusual biochemical characteristics.<sup>25</sup> Validating the applicability of biochemical assays to urine matrices should rely on rigorous homogenous criteria, which are often lacking or not mentioned in a significant number of publications.<sup>26</sup> In addition, the vast majority of the studies performed in CKD have focused on one or a few biomarkers.<sup>27,28</sup> Considering the complexity of CKD progression, it is, however, unlikely that a unique molecule can alone reflect the evolution of the disease. Recent studies using proteomics, metabolomics or specific bioassays for urinary biomarkers detection have shown that combining biomarkers may ameliorate the prediction of CKD progression and/or outcomes.<sup>29-31</sup> In this context, defining valid and potentially complementary assays for urinary biomarker in CKD is of major importance.

To provide insights into the validity and the complementarity of multiple bioassays for candidate biomarkers of CKD progression, we used a pipeline incorporating robust technical validation and multivariable assessment of the ability of the selected biomarkers to improve the prediction of CKD progression in a well-phenotyped cohort of patients with serially measured glomerular filtration rate (mGFR). We selected an a-priori panel of 24 molecules based on their implication in known pathophysiological common processes involved in CKD

progression, broadly covering fibrosis (CTGF, Fibronectin, MMP9, Procollagen III N-terminal peptide, Periostin, Osteopontin, TIMP1, and TGF- $\beta$ ),<sup>10–12,32–35</sup> inflammation (CCL2, GDF15, IL6, IL18, and LIF),<sup>36–42</sup> cell growth and proliferation (EGF and TGF- $\alpha$ ),<sup>15,27,43</sup> angiogenesis (VEGFA and VEGFC),<sup>44–46</sup> oxidative stress (Carbonyl protein) and tubular damage (Cystatin C, FABP1, KIM1, N-acetyl- $\beta$ -D-glucosaminidase, NGAL, Uromodullin).<sup>16–21,47,48</sup> To measure these biomarkers in urine, we tested a total of 30 commercial kits according to the strict criteria defined by the Food and Drug Administration (FDA) guidelines for immunoassay validation.<sup>49</sup> We then studied the associations of each individual biomarker with fast mGFR decline in a subsample of the NephroTest prospective cohort study,<sup>50</sup> and used Lasso (least absolute shrinkage and selection operator) regression to select the best combination of those to improve the prediction of CKD progression as compared to the 4-variables Kidney Failure Risk Equation (KFRE).<sup>51</sup>

## Methods

### Analytical method validation

The methods used to validate the assays according to the FDA approved criteria for industry are detailed in [Supplementary Methods](#).

### Patient cohorts

#### Pilot cohort

For the technical validation of the assays, we consecutively collected urines (under anti-proteases) from CKD patients admitted at the Nephrology Department of Necker hospital from March to September 2011 or healthy subjects recruited in our Institute. We estimated GFR (eGFR) using four-variable modification of diet in renal disease (MDRD) equation ( $eGFR = 175 \times \text{standardized serum creatinine in mg/dL}^{-1.154} \times \text{age in years}^{-0.203} \times 1.212$  [if black]  $\times 0.742$  [if female]).<sup>52</sup> For this convenience sampling, we planned to collect urine from 5 patients for each CKD stage and for each of the three main aetiologies followed in our clinical unit: CKD of all origins (except genetics origin), Autosomal Dominant Polycystic Kidney Disease (ADPKD) and transplanted patients. We first enrolled 13 healthy individuals and 19 CKD patients with different level of GFR (pilot study 1; [Supplementary Table S1](#)), to validate each tested assay according to the FDA-approved criteria. To confirm the ability of assays to detect the endogenous concentration of their analytes in urines from CKD patients, we systematically applied the assays fulfilling FDA criteria to the urine of sixty patients known to have CKD of any cause and of 15 subjects with no known renal impairment or albuminuria, recruited in the same manner (pilot study 2). The characteristics of these patients are presented in [Supplementary Table S2](#). The sample size was determined a priori according to the good laboratory practice for the validation

of ELISA for urinary biomarkers. We did not perform a power analysis.

Of note the urines were collected, processed, handled and stored exactly as in the NephroTest cohort.

#### NephroTest cohort

NephroTest is a prospective cohort study which included 1825 adult patients with all-cause and any stage of CKD from three large nephrology centers in Paris: European Georges Pompidou hospital, Bichat hospital and Tenon hospital.<sup>50</sup> Exclusion criteria were patients on any form of kidney replacement therapy and pregnant women. The urine collection biobank in the protocol started in 2009. Among the 1825 patients, we selected 229 patients who had stored urine collections under anti-proteases and 2 or more mGFRs overtime (127 patients with 2 mGFR, 92 with 3, 10 with 4) at the time of the study in order to assess disease progression. The median follow-up time for these patients in the cohort from the time of the first urine collection was 21.6 (IQR, 13.6–24.7) months. First urine collection from these 229 patients was analyzed. The clinical and biological data were prospectively collected from patients as previously described.<sup>50</sup>

### Measurement and definition of CKD progression

As reported elsewhere,<sup>53</sup> all patients in NephroTest cohort had GFRs measured by chromium-51 labelled ethylenediaminetetraacetic (EDTA) clearance. Individual slopes in mL/min/year were estimated using ordinary least-squares (OLS) linear regression. We then calculated relative mGFR slopes in % per year and classified patients into two groups of slow and fast progressors, defined by a relative mGFR decline  $\leq 10\%$  per year vs.  $>10\%$  per year, respectively.<sup>4</sup>

### Urine collection and storage

Mid-stream urine was collected into a sterile pot and stored at  $-80^\circ\text{C}$  after a maximal 4-h period at  $4^\circ\text{C}$ . We stored the urine supernatant after initial centrifugation at 1000g for 10 min (to removed particulate matter) in a 15 mL polypropylene centrifuge tube containing  $\frac{1}{4}$  protease inhibitor cocktail tablet (cOmplete; Roche) and subsequently aliquoting in smaller volumes (to avoid protein degradation due to freeze-thaw cycle). We simultaneously collected urine in a similar fashion but omitting the either protease inhibitor, the centrifugation step or both.

### Analytical method validation

#### Matrix-based standard curve preparation

In order to determine the capacity of each kit to adequately measure their target analyte in urine matrix, we created a standard curve in urine and calculated the recovery of the amount of analyte added to the matrix compared to the actual amount added. For each assay, we spiked urine samples with 6–8 increasing levels of recombinant protein, generating a matrix-based standard curve. We

calculated the mean percent recovery of the assay in urine as  $[(\text{final concentration} - \text{initial endogenous concentration}) / \text{added concentration}] \times 100$ . Matrix effect was excluded when the percent recovery stand within a range of 70%–130%. We also determined the lower and upper limits of quantification (LLOQ and ULOQ) defined as the lowest and highest concentrations of the calibration curve in the matrix, which can be determined with an inter-assay precision of <20% CV (coefficient of variation) and an accuracy of within 20% of the actual value.<sup>49</sup>

#### Quality controls selection

To obtain an overview of analytes endogenous concentrations in human samples a convenience sample from healthy patients (n = 13) and CKD patients (n = 19) were analyzed (see “pilot cohort” section for details). Among these samples, we selected three quality controls (QCs) that covered the range of quantification of the analyte in this population (low, medium, and high).

#### Selectivity

To assess the selectivity of each assay (ability to solely measure the biomarkers of interest irrespective of the presence of other molecules), the three QCs were measured by ELISA after depletion of the analyte of interest by overnight immunoabsorption at 4 °C. Measured analyte concentrations of depleted QCs were compared to those of QC incubated overnight in absence of depleting antibody.

#### Assay linearity

To assess the dilution linearity of each assay, we selected six random urine samples (3 from the healthy donors and 3 from the patients) with measured high analyte levels and undertook two-fold serial dilutions (from the starting dilution recommended by the manufacturer) using assay diluent. When there were no samples with high endogenous levels, we diluted the three QCs.

For the molecules for which urinary concentration appeared below the assay detection threshold, we evaluate the effect of concentration on the detection of the analyte. We concentrated ten-fold 3 samples spiked with low quantities of recombinant protein using Amicon® Ultra-0.5 centrifugal with the appropriate cut-off for the molecule.

#### Stability

To evaluate analyte stability, the three QC were treated with 1 or 2 thaw-freeze cycle (s) (15 min at room temperature and then 2 h at –80 °C). In addition, the effect of overnight 4 °C and room temperature storage were also evaluated.

#### Intra- and inter-assay precision

Intra-assay precision (within a plate) was evaluated by 8 repeated measures of the 3 QCs and 3 determinations per each six to eight concentrations of the standard in

the biological matrix. Likewise, we measured the inter-assay precision (between plates), using the same controls as above, but measured on two different plates. The mean CV for intra and inter-assay precisions is shown in [Supplementary Table S3](#) and did not exceed 10% and 19% respectively for all the kits.

#### Biomarker measurements

ELISA used to measure biomarkers in urine are presented in [Table 1](#). All ELISA were performed according manufacturer’s instructions and all samples assayed in duplicates. For each immunoassay plate, we generated a calibration curve, as per the manufacturers’ instruction, consisting in a zero-sample and six to eight non-zero standards covering the expected range. We used a sigmoid function, four-parameter logistic regression model for curve fitting, with a coefficient of regression closest to 1 ( $r^2 > 0.99$ ). All biomarker values were normalized for urinary creatinine to correct for differences in concentrations related uniquely to the hydration status or urinary volume of the subject. Urine creatinine was measured in the hospital laboratory using the enzymatic technique with standardization to isotope dilution mass spectrometry.

#### Ethics

All the patients signed an informed consent. The NephroTest study was approved by an ethics committee (*Direction Generale pour la Recherche et l’Innovation & Comité Consultatif sur le Traitement de l’Information en matière de Recherche dans le domaine de la Santé*: MG/CP09.503, July 9, 2009). Nephrotest cohort has been approved by the national commission on informatics and liberty (CNIL; DR 2010-149). The biobank and the database from the Department of Physiology of Necker Hospital have been declared to the French ministry of research (DC-2020-3940) and to the national commission on informatics and liberty (1764193).

#### Statistics

Clinical and laboratory data were expressed as percentages, means ( $\pm$  standard deviation, SD) or median (interquartile range, IQR), as appropriate. All urinary biomarkers had skewed distributions and were subsequently log-transformed. NGAL and cystatin C required two sequential log-transformations to approach a normal distribution, which was assessed using QQ-plots before and after transformation. To account for gender-related differences in biomarker distribution, log-transformed values were then standardized to a mean of 0 and SD of 1, using gender-specific means and SDs [i.e.,  $(\text{measured value} - \text{mean}) / \text{SD}$ ].

We compared baseline clinical and laboratory data and CKD risk factors between slow and fast progressors as defined above. Continuous variables were compared with the Wilcoxon rank-sum test and categorical variables with the chi-squared or Fisher’s exact test. Gender

Analyte	Bibliography	Company	Reference	Matrix effect	Detection range in urine	Linearity	Precision		Specificity		
				Recovery in urine (%)	LLOQ-ULOQ	Recovery after 4 fold dilution (%)	Coefficient of variation (%)		Depletion by specific antibody (%)		
							Intra-assay	Inter-assay	QC Low	QC Medium	QC high
Carbonyl protein		Cell Biolab	STA-310	1	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
Connective tissue growth factor	11,54	Usnc	E90010Hu	46	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
		Peprotech	900-K317	227	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
Cystatin C	55-57	R&D	CTC0	101	3.12-100 ng/mL	108	4.7	4.4	86	65	66
Chemokine ligand 2	58,59	R&D	CP00	103	62.5-2000 pg/mL	108	4.9	11.9	67	77	82
Epidermal growth factor	27	R&D	EG00	81	3.9-250 pg/mL	100	4.7	6.8	98	95	96
Fatty acid binding protein 1	60-62	R&D	Z-001	104	6.25-400 ng/mL	92	8.3	4.1	66	67	53
Fibronectin	63	ThermoFisher Scientific	BMS2028	110	0.62-20 ng/mL	119	6.4	4.8	71	81	83
Growth and differentiation factor 15	42,64	R&D	GD150	101	23.4-1500 pg/mL	100	4.7	4.1	95	92	97
Interleukin 6	65	R&D	D6050	93	3.12-300 pg/mL	79	4.6	5.5	96	97	98
Interleukin 18	58,66,67	Cusabio	CSB-E07450h	46	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
		eBioscience	BMS267/2	163	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
Kidney injury molecule 1	28,58	R&D	KM100	100	0.31-10 ng/mL	80	6.8	16.9	60	78	83
Leukaemia inhibitory factor	68,69	R&D	LF00	55	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
		eBioscience	BSM242TEN	88	3.13-200 pg/mL	97	4.4	11.9	100	96	99
Matrix metalloprotease 9	70	R&D	MP9	81	1.25-20 ng/mL	119	5.1	2.7	100	96	100
N-Acetyl-β-D-glucosaminidase	71,72	Roche	10 875 406 001	65 to 407	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
		Diazyme	DZ062A-K	11 to 226	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
Neutrophil gelatinase associated lipocalin	15,17,66	BioPorto	036RUO	97	25-1000 pg/mL	108	9.9	14.4	100	100	100
Osteopontin	73,74	R&D	OST00	112	2.5-20 ng/mL	95	5.3	5.7	78	78	79
Periostin	75	R&D	Y3548	47	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
		Aviscera Bioscience	SK00072-06	34	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
Pro-collagen III N-terminal peptide	76	Usnc	E90573Hu	43	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
Tissue inhibitor of metalloprotease 1	77	R&D	TM100	77	0.15-10 ng/mL	87	4.2	19.3	95	94	91
Transforming growth factor-α	43	CellScience	CKH188	32	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
		R&D	TGA00	91	15.6-1000 pg/mL <sup>a,b</sup>	81	5.7	5.8	100	100	100
Transforming growth factor-β	78	R&D	DB100B	97	31.2-2000 pg/mL <sup>a</sup>	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
		eBioscience	BMS249/3	85	31-2000 pg/mL <sup>a</sup>	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
Uromodulin	31	MBD bioscience	M036020	91	4.68-150 ng/mL	112	8.7	7.9	61	64	77
Vascular endothelial growth factor A	79	R&D	VE00	93	15.6-1000 pg/mL	144	5.5	15.2	30	67	85
Vascular endothelial growth factor C	80	R&D	VEC00	111	219-7000 pg/mL <sup>a</sup>	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded
		eBioscience	BMS297/2	91	230-15000 pg/mL <sup>a</sup>	Discarded	Discarded	Discarded	Discarded	Discarded	Discarded

QC: quality control, LLOQ: lower limit of quantification. ULOQ: upper limit of quantification. Bibliography is articles containing pathophysiological evidences in favor of the selected biomarker. <sup>a</sup>Indicates assays with a detection range above the naturally occurring concentration of the analyte in urine. <sup>b</sup>Indicates assays with a detection range overlapping the naturally occurring concentration of the analyte in urine after urine concentration.

**Table 1: Validation of the candidate biomarker assays.**

differences regarding biomarkers and mGFR slopes were similarly tested. We also assessed Pearson's correlations between log-transformed biomarkers. Logistic regression was used to estimate crude and adjusted odds ratios (OR) of fast progression for each biomarker considered individually. ORs were sequentially adjusted

for baseline covariates: age, gender, ethnicity, mGFR, BMI, mean blood pressure, diabetes mellitus, history of cardiovascular disease, smoking, renin angiotensin system (RAS) blockade such as angiotensin converting enzyme inhibitor (ACEI) or angiotensin receptor blocker (ARB) treatment, and finally for albuminuria. We used

Holm and Bonferroni's method to provide p-values corrected for multiple testing. In order to select the best combination of biomarkers to predict CKD progression and to quantify the potential gain in discrimination ability as compared to traditional risk factors, we used logistic regression regularized by LASSO (Least Absolute Shrinkage and Selection Operator) penalty with the complete set of biomarkers and/or risk factors from the 4-variables KFRE (age, sex, mGFR, albumin-to-creatinine ratio).<sup>51</sup> To provide an unbiased estimation of the model performances, we used a resampling approach. Two-fold stratified cross-validation repeated 50 times were performed both to choose the penalty parameter of the LASSO maximizing the area under the ROC curve (AUC), and to re-estimate the mean AUC and 95% confidence interval (obtained with the percentile method) through the 100 resamples. Finally, a logistic model was fitted including biomarkers and risk factors selected by the LASSO regression with the highest mean AUC.

For the pilot cohort studies, levels of biomarkers between different groups (e.g.: control vs. CKD patients or CKD stage I-II vs. stage III-V) were compared using Mann Whitney t-test. Statistical analyses were performed with SAS 9.2 (SAS Institute Inc., Cary, NC USA), R 2.3 (R Foundation for Statistical Computing, Vienna, Austria, 2014).

### Role of funders

The funders of this study were Institut National de la Santé et de la Recherche Médicale, Université de Paris Cité, Assistance Publique Hôpitaux de Paris, Agence Nationale de la Recherche, Pharma Research and Early Development Roche Laboratories (Basel, Switzerland), Institut Roche de Recherche et Médecine Translationnelle (Paris, France). They were not involved in study design, data collection, data analyses, interpretation, or writing of report.

## Results

### Biomarker selection

In order to identify robust biomarkers that would improve the prediction of CKD progression, we first selected a panel of biomarkers that full-fill at least two of the following criteria: (i) reflect tissue damage or kidney functional mass; (ii) participate to the mechanisms leading to kidney damage; (iii) availability of a commercial assay is for its measurement. To refine our analysis, we selected molecules whose function does not overlap and tried to cover all the cellular events known to be involved in CKD progression, as cell proliferation, matrix balance and inflammation. This pipeline led us to define a panel of 24 distinct biomarkers (Table 1).

### Assay validation

We then determined the validity of the assays allowing the detection of these molecules in urine according to

the FDA guidelines for immunoassay validation.<sup>49</sup> In this aim, we first evaluated if urine interferes with the capacity of each assay to adequately measure the analyte. Notably, when an assay showed a matrix effect, we eventually tested an additional commercially available assay for that analyte. Among the 30 tested assays, 10 displayed significant matrix interference [i.e., the percentage of recovery of the analyte in the urine overstepped the accepted range (70%–130%) for more than 4 out of 6 standard concentrations; Table 1 and Supplementary Fig. S1]. In addition, we were unable to validate the spectrophotometric assays for N-acetyl- $\beta$ -D-glucosaminidase measurement, due to matrix interference and a high variability between sample replicates and experiments (Table 1). These matrix interferences led us to discard assays for Carbonyl Protein, CTGF, IL18, Periostin and Pro-collagen III N-terminal peptide.

We then assessed if the detection range of the assays was compatible with the usual endogenous level of the molecule in the urine from healthy individuals ( $n = 13$ ) and CKD patients ( $n = 19$ ). Results showed that the majority of the ELISA kits displayed good linearity with a deviation percentage from undiluted sample not exceeding the 20% limit (Table 1). Only TGF- $\alpha$ , TGF- $\beta$  and VEGFC assays had lower limits of detection (LLOD, defined by manufacturers), above the naturally occurring endogenous levels of the analyte in the urine. After concentration by ultrafiltration, TGF- $\beta$  and VEGFC remained below LLOD, suggesting protein degradation or very small urinary levels. In contrast, TGF- $\alpha$  reached quantifiable levels. In addition, urine concentration was associated with acceptable recovery (83.8%) when recombinant TGF- $\alpha$  was added to heat inactivated urines before concentration.

We next evaluate the selectivity of each kit (i.e., the ability to solely measure the biomarker of interest irrespective of the presence of other molecules) through depletion experiments. For all the assays tested, except for the low-quality control in VEGFA, we achieved analyte depletion above 50%, which was deemed sufficient for appropriate selectivity (Table 1).

One of the problems of studying a large panel of biomarkers is the time required for sample processing particularly when applied to a large cohort of patients, since this may compromise correct quantification if the stability of the analyte is reduced over the time. Hence, we analyzed the values of analyte concentration in samples that underwent thaw/freezing cycle and overnight storage at 4 °C or room temperature compared to freshly thawed samples. We observed that many of the tested analytes were stable in these conditions (i.e., less than 20% protein degradation; Supplementary Tables S3 and S4). However, cystatin C, FABP1, MMP9 and TIMP1 were unstable after 1 or 2 thaw-freeze cycles (Supplementary Table S3). In addition, cystatin C, IL6, KIM1, MMP9, TIMP1 and uromodulin

were unstable after overnight storage at 4 °C or room temperature (Supplementary Table S4).

Finally, we analyzed the intra-assay precision of each assay within a plate and between two different plates by repeating 8 measures of the 3 quality controls and 3 measures of the standard in urine. As shown in Table 1, the mean coefficient of variation (CV) for intra and inter-assay precisions did not exceed 10% and 19%, respectively.

### Biomarker concentrations in CKD patients and controls

This rigorous evaluation pipeline allowed us to identify 16 assays that fulfilled all the technical requirements for the detection of a urinary molecule relevant to CKD progression. Then, we wondered, which of the biomarkers was associated with CKD and its severity. In this aim, we measured the levels of the 16 biomarkers in 15 healthy controls and 60 CKD patients (Pilot cohort). All biomarker values were normalized for urinary creatinine to correct for differences in concentrations related exclusively to the hydration status or urinary volume output. The mean biomarker levels were significantly increased in CKD patients when compared with healthy controls for CCL2, cystatin C, FABP1, fibronectin, KIM1, LIF, MMP9, NGAL, and TIMP1. In contrast, the concentration of EGF and osteopontin was significantly decreased (Supplementary Fig. S2). In an exploratory analysis, we compared patients with early, mild stages of CKD (stages I and II) to those with more severe stages (stages III to V), we observed higher levels of cystatin C, FABP1, fibronectin, GDF15 and KIM1 and TIMP1 in patients with more severe CKD (Supplementary Fig. S3). Conversely, EGF and uromodulin excretion were lower in advanced stages of CKD (Supplementary Fig. S3).

### Characteristics of the NephroTest cohort patients

We then evaluated the association of each individual biomarker with CKD progression in a subsample of 229 patients with CKD stage 2–5 from the NephroTest cohort with both urine collection and at least two subsequent <sup>51</sup>CrEDTA clearance-based mGFR measurements. Participants were mostly men (66%) with a mean age of 61 ± 13 years and a median mGFR of 38.3 (IQR, 26.4–49.6) mL/min at the time of urine collection; a majority (89%) were prescribed renin-angiotensin system (RAS) blockers (Table 2).

Over a median follow-up time of 21.6 months, the median absolute change in mGFR was –1.4 (IQR, –4.2 to 1.1) mL/min/year and the relative change was –3.9 (IQR, –12 to 2.7) % per year from baseline (negative values represent a loss). Sixty-eight (30%) patients were ‘fast progressors’ as defined by a mGFR decline >10% per year. Fast progressors had significantly higher albuminuria, and more often diabetes than slow progressors; they were also more often prescribed RAS blockers (all

$P < 0.05$ ). They did not significantly differ, however, according to age, gender, ethnicity, as well as follow-up duration (median 18 vs. 23 months,  $P = 0.07$ ) or number of mGFR measurements over the study period (>2 visits for 47% vs. 38%, respectively,  $\chi^2$  test:  $P = 0.2$ ).

### Distribution of urinary biomarkers, overall and by gender

The baseline distribution of the 16 studied biomarkers is shown in Supplementary Table S5. For MMP9, IL6, and LIF, more than 20% of the patients had values below the LLOD. These biomarkers were not included in subsequent analyses. The distributions varied by gender for seven of the biomarkers. Levels of LIF, MMP9, NGAL, TGF- $\alpha$ , and uromodulin were significantly higher and those of TIMP1 and VEGFA, lower, in women than in men. There was no difference in baseline mGFR normalized for body surface area (BSA) between men and women (36.8 vs. 34.6 mL/min/1.73 m<sup>2</sup>, t-test:  $P = 0.3$ ). Correlations between biomarkers were shown in Supplementary Table S6.

	All	Slow progressors	Fast Progressors	P-value
N	229	161	68	
Age, years	61 ± 13	60 ± 13	62 ± 13	0.4
Men	66% (152)	66% (106)	68% (46)	0.8
African origin	10% (23)	12% (19)	6% (4)	0.2
Diabetes	25% (57)	21% (34)	34% (23)	0.04
mGFR (mL/min)	38 [26–50]	40 [30–50]	35 [24, 44]	0.06
Mean blood pressure (mm Hg)	92 ± 13	92 ± 12	92 ± 14	0.9
Elevated blood pressure (>140/90 mm Hg)	26% (57)	24% (38)	28% (19)	0.5
ACEI or ARB	89% (202)	86% (137)	96% (65)	0.04
Body mass index, kg/m <sup>2</sup>	27.2 ± 5.9	27.0 ± 5.8	27.7 ± 6.1	0.3
History of cardiovascular disease	17% (38)	16% (25)	19% (13)	0.5
Smoking				0.4
Past	38% (87)	35% (57)	44% (30)	
Current	10% (22)	11% (17)	7% (5)	
Kidney disease				0.2
PKD	7% (16)	4% (7)	13% (9)	
Diabetic nephropathy	8% (19)	7% (12)	10% (7)	
Glomerular disease	16% (37)	16% (26)	16% (11)	
Vascular disease	25% (57)	26% (42)	22% (15)	
Interstitial nephritis	17% (39)	19% (30)	13% (9)	
Unknown	27% (61)	27% (44)	25% (17)	
Proteinuria (mg/mmol)	24.6 [0.0620, 848]	17.7 [0.0620, 544]	59.2 [1.7, 848]	<0.001
Albuminuria (mg/mmol)	12.4 [0.03, 547]	7.9 [0.03, 401]	31.7 [0.05, 547]	<0.001

mGFR: measured glomerular filtration rate; ACEI: Angiotensin converting enzyme inhibitor; ARB: angiotensin receptor blocker; PKD: polycystic kidney disease. Data are expressed as mean ± standard deviation (SD), median [interquartile range (IQR)] or percent % (number of patients (n) as appropriate).

Table 2: Baseline characteristics of NephroTest cohort patients.



**Crude and adjusted associations of individual urinary biomarkers with CKD progression**

As expected, odds-ratio of “fast progression”, estimated per one gender-specific standard deviation (SD) increase of log-transformed biomarkers, was significantly higher in patients with higher albuminuria and proteinuria, before and after adjusting for common progression risk factors (Table 3). Crude and adjusted odds-ratios were also significantly higher with higher levels of cystatin C, CCL2, FABP1, fibronectin, GDF15, KIM1, NGAL, TIMP1, TGF- $\alpha$  and VEGFA, and lower for higher EGF level, but only those associated with CCL2, TIMP1, and TGF- $\alpha$  remained statistically significant after further adjusting for albuminuria and multiple testing. There was no significant association with osteopontin or uromodulin, either before or after adjustment (Table 3). Interestingly, we observed a stronger odds-ratio of fast progression for TGF- $\alpha$  than for albumin. Further adjustment for center did not change any of these results (data not shown). Of note, we also tested the associations between each biomarker and mGFR, as a continuous variable, and found consistent results between the logistic and the linear regression models (data not shown).

**Combining urinary biomarkers to predict CKD progression**

We then performed exploratory analysis to investigate if a combination of several biomarkers was susceptible to classify patients into slow vs. rapid progressors better than the 4-variables KFRE based on age, gender,

mGFR, and albuminuria.<sup>51</sup> Some biomarkers were highly correlated ( $r > |0.5|$ ) as shown in Supplementary Table S6. Therefore, we used LASSO (Least Absolute Shrinkage and Selection Operator) to obtain the most parsimonious model for patients classification.<sup>81</sup> We first performed three LASSO regressions to test three sets of covariates including: (1) the 4 KFRE variables, (2) the 13 urinary biomarkers selected at the first step of the study, and (3) both the 13 biomarkers and the 4 KFRE variables. Models 2 selected 5 biomarkers, including CCL2, EGF, NGAL, TGF- $\alpha$ , and KIM1, which were also selected in Model 3 (Supplementary Table S7). The discriminatory power as measured with mean area under the curves (AUC) estimated from 100 re-samples (two-fold stratified cross-validation repeated 50 times), slightly improved from model 1 to model 2 and 3, with AUC of 0.673 [95% confidence interval, 0.564–0.751], 0.703 [0.625–0.783], and 0.715 [0.632–0.786], respectively. Model 3, combining the 4 KFRE variables with CCL2, EGF, NGAL, TGF- $\alpha$ , and KIM1 was the best predictive model regarding mean AUC, but the empirical confidence intervals overlapped between the 3 models. In 81% of the re-samples, the performance of the model combining the 4 KFRE variables with BMs was better to that of the model which only uses the 4 KFRE variables, with a median of the paired differences in AUC of 0.0466 (Q1 = 0.0139-Q3 = 0.072, max = 0.220). Adding biomarkers to a model including all covariates (age, sex, mGFR, albuminuria, ethnicity, BMI, mean blood pressure, diabetes mellitus, history of

	Model 1		Model 2		Model 3	
	OR (95%CI) <sup>a</sup>	P <sup>b</sup>	OR (95%CI) <sup>a</sup>	P <sup>b</sup>	OR (95%CI) <sup>a</sup>	P <sup>b</sup>
Protein	2.15 (1.52–3.03)	<0.001	2.24 (1.52–3.31)	<0.001		
Albumin	2.08 (1.48–2.91)	<0.001	2.12 (1.44–3.12)	0.002		
Cystatin C	1.92 (1.40–2.65)	<0.001	2.13 (1.45–3.14)	0.001	1.69 (1.10–2.60)	0.15
CCL2	2.10 (1.51–2.93)	<0.001	2.10 (1.49–2.97)	<0.001	1.87 (1.31–2.66)	0.006
EGF	0.62 (0.46–0.84)	0.015	0.55 (0.35–0.86)	0.038	0.63 (0.40–1.01)	0.37
FABP	1.87 (1.37–2.54)	<0.001	1.88 (1.32–2.69)	0.004	1.49 (1.00–2.22)	0.37
Fibronectin	1.59 (1.18–2.15)	0.015	1.62 (1.16–2.26)	0.034	1.43 (1.01–2.03)	0.37
GDF15	1.60 (1.17–2.19)	0.017	1.62 (1.16–2.27)	0.034	1.41 (1.00–2.00)	0.37
KIM1	1.70 (1.16–2.51)	0.023	1.68 (1.10–2.57)	0.05	1.38 (0.90–2.13)	0.43
NGAL	1.50 (1.12–2.00)	0.023	1.58 (1.14–2.18)	0.034	1.34 (0.95–1.89)	0.39
Osteopontin	1.09 (0.80–1.49)	0.59	1.11 (0.78–1.60)	0.56	1.03 (0.75–1.42)	0.86
TIMP1	2.02 (1.45–2.83)	<0.001	2.08 (1.44–3.02)	0.001	1.75 (1.19–2.57)	0.049
TGF- $\alpha$	2.08 (1.48–2.92)	<0.001	2.39 (1.64–3.48)	<0.001	2.33 (1.57–3.44)	<0.001
Uromodulin	1.26 (0.94–1.69)	0.24	1.27 (0.94–1.73)	0.25	1.24 (0.91–1.70)	0.43
VEGFA	1.82 (1.31–2.53)	0.003	1.95 (1.33–2.87)	0.005	1.67 (1.12–2.48)	0.12

Model 1: crude model. Model 2: adjusted for mGFR, age, gender, ethnicity, body mass index, mean blood pressure, diabetes, history of cardiovascular disease, smoking, angiotensin converting enzyme inhibitor or angiotensin receptor blocker. Model 3: model 2 + albumin-to-creatinine ratio, mGFR, measured glomerular filtration rate; CCL2, C-C motif chemokine ligand 2; EGF, Epidermal Growth Factor; FABP1, Fatty Acid Binding Protein; GDF15, Growth and Differentiation Factor 15; KIM1, Kidney Injury Molecule 1; NGAL, Neutrophil Gelatinase Associated Lipocalin; TIMP1, Tissue Inhibitor of Metalloprotease 1, TGF- $\alpha$ , Transforming Growth Factor alpha, VEGFA, Vascular Endothelial Growth Factor A. <sup>a</sup>Odds-ratios (95% confidence intervals) estimated per one gender-specific standard deviation unit increase (after log-transformation). All biomarker values were normalized for urinary creatinine. <sup>b</sup>Adjusted *p*-value for multiple-tests (Holm-Bonferroni).

**Table 3: Crude and adjusted odds-ratios of fast CKD progression (mGFR decline >10% per year) associated with individual urinary biomarker.**

cardiovascular disease, smoking, and RASi) also increased model's AUC (AUC without biomarkers: 0.666 [0.5000–0.742]; with biomarkers: 0.708 [0.615–0.787]). We then estimated adjusted odds ratios of fast progression for all biomarkers and covariates selected by the LASSO (Fig. 1). Odds-ratios were significantly higher for albumin, CCL2, and TGF- $\alpha$ , and lower for EGF and NGAL. This model fitted the data significantly better than a model based on age, mGFR, and albuminuria alone (likelihood-ratio test,  $p$ -value < 0.001).

### Impact of urine collection on the detection of the selected biomarkers

In the NephroTest study, we used a very rigorous, but costly and cumbersome method to collect urine. It is possible that this might limit the clinical applicability of our molecular signature. Therefore, we tested whether urine centrifugation and protease inhibitors were required for the proper detection of the four most promising biomarkers. Neither the absence of protease inhibitors nor that of centrifugation appeared to impact the detection of the four analytes (Supplementary Table S8), indicating that these biomarkers are amenable to clinical practice.

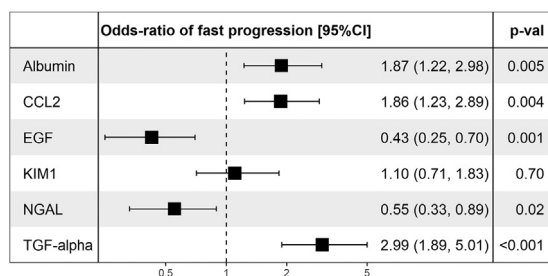
### Discussion

Identifying urinary biomarkers predicting GFR decline has long been an area of intense investigation in the field of nephrology.<sup>82,83</sup> Most candidates proposed to date originate from pathophysiologic models<sup>70,84</sup> or transcriptional screens performed on human or rodent kidney tissues.<sup>15,27,85</sup> Such pipelines are essentially based upon protein biomarkers, which detection in urine relies mainly, to date, on ELISA. While the number of candidate biomarkers has impressively increased over the past two decades, the data regarding

the applicability of the commercially available ELISA targeting these biomarkers to their quantification in urine are surprisingly scarce.<sup>25</sup> In addition, whether distinct biomarkers play a complementary or a redundant role in refining the prediction of CKD progression remains unclear.

The first aim of this study was to determine if a consequent panel of 30 commercial ELISA for 24 promising urinary biomarkers of CKD progression meet FDA criteria for analyte quantification in urine. This unprecedented effort in the field of CKD progression led to identify important pitfalls. First, we found unacceptable matrix interference for a third of the tested assays. Of note, we obtained reasonable performance with assays that were not previously validated in urine matrices (IL6, LIF, TGF- $\alpha$ , and VEGFA). In contrast, some of the kits that were supposed to allow analyte quantification in urine had significant matrix effect (e.g., CTGF and NAG). In addition, we observed that LLOQ below the naturally occurring concentration of the analyte in patient urine represented another important limitation for additional assays. Although urine concentration was able to solve this issue in the case of TGF- $\alpha$ , this approach complexify the pre-analytic pipeline, which may compromise the use of this otherwise promising assay in routine screening. Our results further revealed the poor stability of cystatin C and uromodulin in urine, which limits their use as routine urinary biomarkers. An appreciable positive finding of this study is that neither the use of antiprotease or urine centrifugation is required to achieve accurate quantification of the most promising biomarkers tested in urine, precluding the future use of costly and/or cumbersome urine collection protocol for these molecules.

Having identified robust assays for the detection of 16 candidate biomarkers of CKD progression in urine, our second goal was to explore the ability of these markers to predict fast GFR decline either individually or in combination in a subsample of 229 CKD patients from the NephroTest prospective cohort. Considering the biomarkers individually, we observed a significant and independent association with the risk of fast CKD progression for cystatin C, CCL2, fibronectin, GDF15, TGF- $\alpha$ , TIMP1, and VEGFA, and on the borderline of significance for EGF. CCL2 is an important chemoattractant for monocytes derived macrophage<sup>86</sup> and plays an instrumental role in the progression of distinct rodent CKD models.<sup>87–90</sup> CCL2 urinary excretion rate has been previously associated with the risk of GFR decline in diabetic nephropathy<sup>91</sup> and autosomal dominant polycystic kidney disease.<sup>19,92</sup> Thus, our findings generalize the concept that CCL2 represents a promising biomarker for the progression of CKD. In contrast to CCL2, the data regarding the use of urinary TIMP1,<sup>93,94</sup> fibronectin<sup>63</sup> or TGF- $\alpha$  for the prediction of CKD progression are to date scarce or inexistent. Nonetheless, concordant evidence suggests that these molecules are



**Fig. 1: Adjusted odds-ratios of fast CKD progression (mGFR decline > 10% per year) associated with a combination of urinary biomarkers.** Odds-ratios were adjusted for all variables selected by the LASSO including age, mGFR, albuminuria and five biomarkers. Chemokine (C-C motif) Ligand 2 (CCL2), Epidermal Growth Factor (EGF), Kidney Injury Molecule 1 (KIM 1), Neutrophil Gelatinase Associated Lipocalin (NGAL), Transforming Growth Factor- $\alpha$  (TGF- $\alpha$ ) and albuminuria in the model.

linked to CKD progression. This is particularly notable for the epidermal growth factor receptor (EGFR) ligand TGF- $\alpha$ , which has been shown to fuel renal parenchyma deterioration through EGFR activation in distinct rodent models of CKD.<sup>43,95,96</sup> While fibronectin and TIMP1 are clearly associated with renal fibrosis, their instrumental role in CKD progression remains debated.<sup>97–101</sup> Thus, this study sheds light on the potential use of these overlooked potential biomarkers as predictors of CKD progression.

We further investigate if a combination of different biomarkers could improve the prediction of fast GFR decline compared to the 4 KFRE variables alone. Using LASSO logistic regression, we observed, that a model incorporating CCL2, EGF, TGF- $\alpha$ , NGAL, and KIM1 offered the strongest improvement in patient classification. However, the small size of the cohort did not allow us to demonstrate significant improvement of the prediction, when comparing AUC between models including age, gender, mGFR and albuminuria with and without the 5 selected biomarkers. Additional investigation involving independent cohorts are required to confirm or infirm the potential of these biomarkers' combination. In addition, a more sensitive assay for urinary TGF- $\alpha$  that will not require urine concentration step to detect its target analyte is also mandatory.

Nonetheless, our study enhances the notion that the combined use of rigorously validated biomarkers increases the predictive accuracy for GFR decline. The nature of the molecules involved and their specific association with the outcome in our exploratory model further raise interesting questions. First, it is worth noting that our prediction model incorporates two EGFR ligands (i.e., EGF and TGF- $\alpha$ ), with opposite associations with the outcome. While EGF and TGF- $\alpha$  activate EGFR, they differentially impacts on its activation kinetic and trafficking, leading to the non-parallel recruitment of downstream effectors with divergent cellular effects.<sup>102,103</sup> Intriguingly, while TGF- $\alpha$  expression is associated with deleterious EGFR activation in different CKD models,<sup>15,43,95</sup> EGF perfusion has been shown to promote recovery from acute kidney injury,<sup>104–106</sup> raising the hypothesis that EGF and TGF- $\alpha$  ligand may differentially orient the fate of the renal parenchyma toward repair or deterioration. A reduction in EGF urinary excretion has been associated with deleterious outcome in CKD cohorts.<sup>27,107</sup> Unexpectedly, while univariate analysis pinpointed NGAL as a weak predictor of fast GFR decline, we observed that high NGAL excretion was associated with a reduction of the risk of CKD progression, when included in our model incorporating EGF, TGF- $\alpha$ , albuminuria, KIM1 and CCL2. NGAL is a well-characterized marker of kidney injury. Previous studies of this biomarker alone or in association with other biomarkers showed a

modest significant association between NGAL urinary excretion and CKD outcome.<sup>17,108</sup> However, NGAL measurement did not improved risk stratification compared to traditional risk factors.<sup>22,24</sup> NGAL is a secreted molecule with pleiotropic, context specific effects. Exogenous NGAL administration mitigated renal damage in different models of acute kidney injury.<sup>109–112</sup> On the contrary, NGAL has been show to relay the deleterious effect of EGFR activation or albuminuria in rodents models of CKD.<sup>15,113</sup> The difference that we observed regarding the association of NGAL excretion with CKD progression depending on the incorporation of additional biomarkers in the model may therefore reflects the versatile functions of NGAL in kidney diseases.

Our study has notable limitations. First, we did not include all the available ELISAs for each targeted biomarker, nor extensively tested multiple lots for each assay. Second, the selected population of the NephroTest cohort precludes generalization of our findings to the overall CKD patient population, but is representative of the nephrology patients. Third, as we used a convenient sample reflecting our intuition recruitment for assays validation, we cannot exclude that some assays may not perform as well in patients suffering from kidney disease that were not included in this initial convenient sample. Fourth, we lacked an independent external cohort to validate our results. Nevertheless, we did perform an internal validation of our models by cross validation, as recommended by current TRIPOD guidelines,<sup>114</sup> a method which proved to be a most effective to minimize overfitting when applied to the entire sequence of modeling steps as we did.<sup>115</sup> Finally, the small size of the study precluded stratified analyses per kidney disease etiology. Nevertheless, our hypotheses relied on the assumption that mechanisms of CKD progression and its prediction were independent of disease.<sup>116</sup>

Our study has also notable strengths including: the use of a robust validation pipeline for multiple assays to detect a broad panel of biomarkers in the urine, the use of measured GFR to define CKD progression and the simultaneous assessment of multiple biomarkers accounting for multiple testing, which allowed us to questioned their redundancy and/or complementarity for the prediction of fast GFR decline.

In conclusion, this study provides a robust validation of several assays carefully selected for the detection in urine of relevant biomarkers reflecting various pathophysiological mechanisms of CKD progression. It also suggests that the combination of some of these biomarkers may improve the prediction of CKD progression. Further studies are needed to refine the optimal combination of biomarkers predicting GFR decline in individuals with CKD and allowing personalized therapeutic intervention.

### Contributors

F.B. and M. Mu. analyzed the data and wrote the paper. M.Me. analyzed the data, performed the statistical analyses, and wrote the paper. M.B. designed and performed the experiments and contributed writing the manuscript. P.H., M.F., and J.P.H. measured GFR and collected the clinical data, J.V., J.M. and G.F. discussed the results. B.S. designed the study, verified the analyzes and discussed the results. F.T. provided the conceptual framework and designed the study, supervised the project and wrote the paper. B.S. and M.Me. verified the underlying data.

All the contributors read and approved the final version of the manuscript.

### Data sharing statement

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

### Declaration of interests

J.V. is CEO of ENYO Pharma, Board member of Step Pharma and Inathersys, and owns shares of Hoffmann-La-Roche.

### Acknowledgments

This work was supported by Institut National de la Santé et de la Recherche Médicale, Université de Paris, Assistance Publique Hôpitaux de Paris, Agence Nationale de la Recherche, Pharma Research and Early Development Roche Laboratories (Basel, Switzerland) and Institut Roche de Recherche et Médecine Translationnelle (Paris, France).

### Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.ebiom.2023.104635>.

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