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Identification of a urine metabolomic signature in patients with advanced-stage chronic kidney disease

Maria Posada-Ayala[1], Irene Zubiri[1], Marta Martin-Lorenzo[1], Aroa Sanz-Maroto[1], Dolores Molero[2], Laura Gonzalez-Calero[1], Beatriz Fernandez-Fernandez[3], Fernando de la Cuesta[4], Carlos M. Laborde[4], Maria G. Barderas[4], Alberto Ortiz[3], Fernando Vivanco[1,5] and Gloria Alvarez-Llamas[1]

[1]Department of Immunology, IIS-Fundacion Jimenez Diaz, Madrid, Spain; [2]CAI-RMN, Universidad Complutense de Madrid, Madrid, Spain; [3]Department of Nephrology, IIS-Fundacion Jimenez Diaz-UAM/IRSIN, Madrid, Spain; [4]Department of Vascular Physiopathology, Hospital Nacional de Paraplejicos, SESCAM, Toledo, Spain and [5]Department of Biochemistry and Molecular Biology I, Universidad Complutense de Madrid, Madrid, Spain

The prevalence of chronic kidney disease (CKD) is increasing and frequently progresses to end-stage renal disease. There is an urgent demand to discover novel markers of disease that allow monitoring disease progression and, eventually, response to treatment. To identify such markers, and as a proof of principle, we determined if a metabolite signature corresponding to CKD can be found in urine. In the discovery stage, we analyzed the urine metabolome by NMR of 15 patients with CKD and compared that with the metabolome of 15 healthy individuals and found a classification pattern clearly indicative of CKD. A validation cohort of urine samples from an additional 16 patients with CKD and 15 controls was then analyzed by (Selected Reaction Monitoring) liquid chromatography-triple quadrupole mass spectrometry and indicated that a group of seven urinary metabolites differed between CKD and non-CKD urine samples. This profile consisted of 5-oxoproline, glutamate, guanidoacetate, α-phenylacetylglutamine, taurine, citrate, and trimethylamine N-oxide. Thus, we identified a panel of urine metabolites differentially present in urine that may help identify and monitor patients with CKD.

Kidney International (2013) **85,** 103–111; doi:10.1038/ki.2013.328; published online 18 September 2013

KEYWORDS: chronic kidney disease; mass spectrometry; metabolomics; nuclear magnetic resonance; selected reaction monitoring; urine

Correspondence: *Gloria Alvarez-Llamas, Department of Immunology, IIS-Fundacion Jimenez Diaz. Avda. Reyes Catolicos, 2 28040 Madrid, Spain. E-mail: galvarez@fjd.es*

Received 25 July 2012; revised 17 May 2013; accepted 29 May 2013; published online 18 September 2013

The prevalence of chronic kidney disease (CKD) is rising, becoming a major public health problem worldwide, with a great impact on national healthcare budgets because of the cost of renal replacement therapy and a shortened life expectancy. CKD is categorized into five stages of increasing severity (CKD1-5) based on an estimation of the glomerular filtration rate (eGFR), and, more recently, additional categorization based on a combination of eGFR and proteinuria has been proposed to more closely reflect the potential risk for progression. In general terms, clinicians are alerted to the presence of CKD on the basis of serum creatinine data and calculation of eGFR from serum creatinine. However, serum creatinine is a late marker and it is affected by muscle mass, age, and race, and assessment of renal function based on eGFR is less reliable when this value is higher than 60 ml/min. Addition of cystatin C to the combination of creatinine and albuminuria or proteinuria has been recently proposed to improve the predictive accuracy for all-cause mortality and end-stage renal disease,[1] pointing to the continuous demand for candidate markers associated with CKD. Recent clinical trials have highlighted the inadequacy of albuminuria or proteinuria as a surrogate therapeutic target for therapy aimed at preventing CKD progression, as a therapy-induced reduction of proteinuria was dissociated from kidney and survival outcomes.[2] Although not ready for clinical practice, novel markers such as NGAL and cystatin C have been related to CKD progression.[3]

The metabolome represents the downstream changes in the genome, transcriptome, and proteome as a reflection of real-time processes occurring in living organisms. Compared with more than 10 million proteins in the proteome, a few thousand metabolites present in an organism imply a considerable reduction in complexity. The main advantage of 'omics' technologies is that there is no preselection of candidate molecules (metabolites) to be investigated for a potential influence on the disorder under study. In particular, nuclear magnetic resonance (NMR)–based metabolomics is a robust technique that does not require sample treatment,

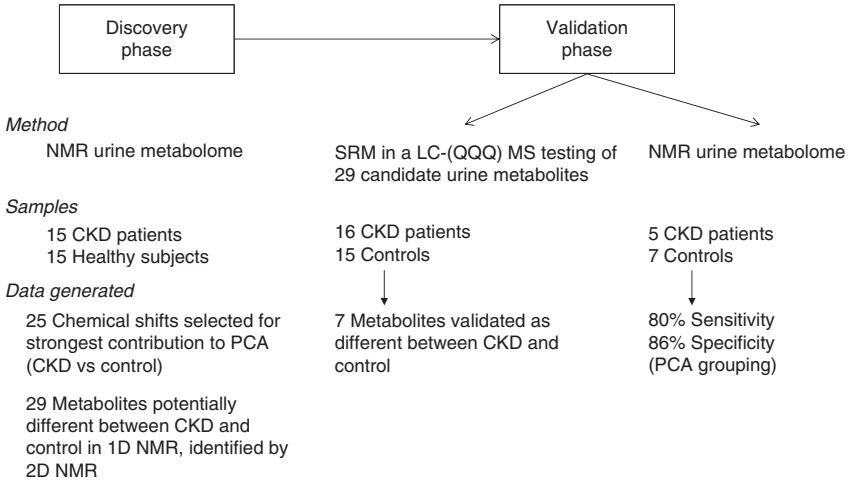

Figure 1 | Overview of study design and flow of results. Urine samples in each phase and in each of the two validation studies were obtained from independent patient and control cohorts. The discovery phase was approached by nuclear magnetic resonance (NMR); the validation phase was carried out by Selected Reaction Monitoring (SRM) liquid chromatography-mass spectrometry (LC-MS) and the urine metabolome from 12 new individuals was analyzed by NMR, and grouping in the principal component analysis (PCA) graph was inspected. QQQ, triple quadrupole.

chromatography, or analyte ionization and allows unambiguous identification of analytes. NMR was used here to approach the nonbiased, nontargeted study of a potential association of CKD with a panel of urinary metabolites. The role of proteomics[4] and metabolomics[5] in the study of CKD has been recently reviewed. However, few studies have investigated the whole subset of low–molecular weight compounds present in a biological fluid or tissue and their relationship with the presence of CKD. In particular, plasma metabolomics profiling was investigated recently at different stages of CKD by GC–MS and LC-MS, finding major differences with increasing stage.[6] In a prospective study, a set of urine metabolites was found to predict progression from normoalbuminuria to macroalbuminuria or diabetic kidney disease,[7] and specific urine metabolites were found to respond to early kidney damage in patients with acute heart failure at the time of presentation.[8] Clearly, novel markers are required that allow identification of CKD patients and may eventually be used to monitor disease progression and even to identify early CKD stages. As a proof of principle, we have applied here NMR-based metabolomics to urine for the nonbiased, nontargeted study of CKD. A subset of metabolite candidates with potential significant variation in CKD were validated by Selected Reaction Monitoring (SRM) in a triple quadrupole (QQQ) mass spectrometer. As a result, we show here a metabolomic urine fingerprint that responds to CKD.

RESULTS

An overview of the study design and flow of the results is shown in Figure 1. Urine samples in each phase were obtained from independent patient and control cohorts. The discovery phase was approached by NMR, followed by a validation phase carried out by SRM (liquid chromatography–mass spectrometry). Urine metabolome from 12 new individuals were additionally analyzed by NMR, and grouping in the principal components analysis (PCA) graph was inspected (blinded study). Further details are explained in the following sections.

Discovery phase

Thirty urine samples from CKD patients (Table 1) and healthy subjects (Table 2) were analyzed by ^{1}H NMR. For comparison, spectra were divided into buckets (spectral regions of 0.04 p.p.m. width), and a bucket table was created for every sample. The nonsupervised PCA showed a good clustering for cases and controls and so did the PLS analysis (Figure 2), indicating that main variability was not attributed to differences related to age, medication, sex, or urine collection date (samples collected over 1 year), but mainly to CKD. The particular contribution of each chemical shift (spectral region, p.p.m.) to the principal component space is represented in the loading plot graph (see Supplementary Material online; Figure 1). A total of 25 chemical shifts (p.p.m. value ± 0.02 p.p.m., bucket size 0.04 p.p.m.), with the strongest contribution to the PCA according to the loading plots graph, were selected as potentially significant (see Supplementary Material online; Table 1). Two of the most significant shifts are the two singlets of creatinine (3.06 and 4.06 p.p.m.). These could be unequivocally identified in the spectrum of the first dimension based on literature data, but other metabolites mainly contributing to disease classification cannot be rigorously identified based only on ^{1}H NMR owing to overlapping of some signals in one-dimensional spectra. Two-dimensional NMR experiments (homonuclear ^{1}H–^{1}H correlation spectroscopy, total correlation total correlation spectroscopy and ^{1}H–^{13}C heteronuclear single-quantum correlation spectroscopy) (see Supplementary material online; Figure 2) allowed unequivocal identification of those metabolites whose signals (chemical shifts) in 1D

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