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# Combination of the fetal urinary metabolome and peptidome for the prediction of postnatal renal outcome in fetuses with PUV

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ABSTRACT

Most of biomarker panels, extracted from single omics traits, still need improvement since they display a gray zone where prediction is uncertain. Here we verified whether a combination of omics traits, fetal urinary metabolites and peptides analyzed in the same sample, improved prediction of postnatal renal function in fetuses with posterior urethral valves (PUV) compared to individual omics traits.

Using CE-MS, we explored the urinary metabolome of 13 PUV fetuses with end stage renal disease (ESRD) and 12 PUV fetuses without postnatal ESRD at 2 years postnatally. This allowed the selection of 24 differentially abundant metabolite features which were modelled into predictive classifiers, alone or in combination with 12 peptides previously identified as predictive of ESRD. Validation in 35 new fetuses showed that the combination of peptides and metabolites significantly outperformed the 24 metabolite features with increased AUC (0.987 *vs* 0.905), net reclassification improvement (36%) and better sensitivity accuracy (86% *vs* 60%). In addition, the two trait combination tended to improve, but without reaching statistical significance, the already high performances of the 12 peptide biomarkers (AUC 0.967, accuracy 80%).

In conclusion, this study demonstrates the potential of cumulating different omics traits in biomarker research where single omics traits fall short.

*Significance:* Although increasingly proposed in disease-diagnosis and -prognosis because of their improved efficacy over single markers, panels of body fluid biomarkers based on single omics analysis still fail to display perfect accuracy, probably due to biological variability.

Here, we hypothesized that combination of different omics traits allowed to better capture this biological variability. As proof of concept, we studied the added value of fetal urine metabolites and peptides using CE-MS, starting from the same urine sample, to predict postnatal renal outcome in fetuses with posterior urethral valves. We observed that the prognostic power of combined metabolite and peptide markers was clearly higher than that of metabolites alone and slightly, but non-significantly, improved compared to the peptides alone. To our knowledge, this report is the first to demonstrate that combining multiomics traits extracted from (fetal) urine samples displays clear promise for kidney disease stratification.

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## 1. Introduction

Biomarker panels identified by urinary omics-based strategies display clear promise for clinical use in kidney disease because of their improved efficacy over single markers [1, 2]. Using approaches based on capillary electrophoresis coupled to mass spectrometry (CE-MS), we and others have shown the usefulness of urinary peptidome analysis for the potential management of kidney disease in adults and children as well as in fetal medicine [1–5]. In contrast, a limited number of studies explored the urinary metabolome for the identification of clinically relevant metabolites. This is surprising since metabolites are considered to be final proxies of physiological homeostasis that reflect more closely the cell/organ/organism activity than the other omics traits [6, 7]. Recently, we described an optimized CE-MS setup and analysis pipeline to reproducibly explore the urinary metabolome [8]. We demonstrated the feasibility to use such tool in a clinical context for the diagnosis of ureteropelvic junction obstruction in children [8].

Despite these promising results in urinary omics analysis, advances still need to be made since most approaches, based on panels using single omics traits, display a so-called gray zone defined by the uncertainty of the prediction [9–12]. We hypothesized that cumulating urinary peptidome and metabolome analysis would improve the assessment of kidney disease. As proof of concept, we tested this hypothesis for the prediction of postnatal renal outcome in fetuses with posterior urethral valves (PUV).

PUV are congenital anomalies associated with a wide spectrum of outcomes ranging from extremely severe phenotypes with prenatal death to normal phenotypes with a nearly normal renal function. The management of disease is hampered by the lack of tools for adequate antenatal screening of PUV fetuses at high risk for developing early end-stage renal disease (ESRD) [4, 13–17]. This leads to a number terminations of pregnancy (TOP) sometimes contradicted by fetopathology or continuations of pregnancies resulting in early ESRD [13–17]. Therefore, although a rare disease (1/5000–1/8000 children [18]), PUV are a common cause of ESRD [19, 20].

Recently, we identified a set of 12 fetal urinary peptides which predicted with high accuracy the postnatal renal function in fetuses with PUV [4]. In the current study we explored the fetal urinary metabolome of the same patients and we evaluated the potential of metabolites, alone or in association with the 12 previously published fetal urinary peptides, to prognosticate postnatal renal outcome in PUV fetuses. The predictive efficacy of the multiomics trait combination was compared to the performance of the single omics traits.

## 2. Material and methods

### 2.1. Patients, samples and ethics statement

This study is a retrospective analysis of a previously described prospective study focusing on fetal urine peptides [4]. It included patients consecutively enrolled in 26 authorized and accredited multidisciplinary French centers [4]. Eligible patients were mothers carrying fetus with suspected PUV according to the following inclusion criteria: i) male singleton fetus with megabladder associated with urinary tract anomalies detected in a first ultrasound and confirmed in a second ultrasound; ii) collection of fetal urine taken during the routine management of the disease for evaluation of fetal urinary biochemistry. Samples taken from cysts or urinoma were excluded. The decision to recommend fetal urine sampling was made by the clinician in charge of the patient. Among 76 fetuses with PUV assessed for eligibility, 66 were allocated to urinary peptidome analysis [4] but only 60 fetuses were allocated to urinary metabolome analysis because samples of 6 patients were not anymore available (Fig. 1). The mean age of the 60 fetuses was 27.8 weeks of amenorrhea (wa) [95%CI: 26.3–29.3] at the date of urinary sampling and the mean age of mothers was 28.6 years [95%CI: 27.0–30.2].

The judgment criterion was the development - or not - of ESRD at 2 years postnatally. The ESRD group included PUV patients (29/60) that died in the neonatal period due to ESRD or that were subjected to TOP due to the severity of renal lesions (Fig. 1). Indeed, in accordance with the French law, TOP was possible at parental request in case of poor renal prognosis indicated by disappearance of normal renal parenchyma and/or severe oligohydramnios, both detected by ultrasound, or abnormal biochemical results. When TOP was decided, a detailed histological examination of the kidneys was performed at autopsy. In all cases, histological kidney lesions incompatible with normal life were observed. In contrast, the no-ESRD group was composed of PUV patients (31/60) that led to liveborn infants having glomerular filtration rate (GFR) (estimated using Schwartz formula) $\geq$15 ml/min at 2 years of life (Fig. 1). The date at sampling was slightly earlier for fetuses with early ESRD (25.9 wa [95%CI: 23.6–28.2] *vs.* 29.5 wa [95%CI: 27.9–31.1], $p = 0.024$) probably due to the severity of renal lesions which are visible at an earlier stage and lead to earlier requests for fetal urine biochemistry analysis.

The cohort of 60 PUV fetuses was divided in independent discovery and validation sets (Supplementary Tables 1–2) for generation and then testing the omics-based prognosis classifiers, respectively. We used the same distribution of patients over the discovery and validation cohorts as previously studied for the fetal urinary peptides, except for 6 no-ESRD fetuses (4 in the discovery cohort, 2 in the validation cohort) for whom urinary samples were not anymore available [4]. The discovery set was balanced in terms of gestational age at sampling thereby avoiding selection of biomarkers associated to normal fetal tubular maturation (28.1 wa [95%CI: 25.0–31.2] for ESRD *vs.* 27.9 wa ([95%CI: 24.8–31.0]) for no-ESRD fetuses. The study design is presented in Fig. 2.

The work was performed according to the ethical principles expressed in the Declaration of Helsinki. Precisely, appropriate written informed consent for fetal urine sampling and for laboratory testing was obtained from all parents of fetuses participating in the study. According to current French law, this observational study did not need to be declared or submitted to the opinion of a research ethics board i) since it did not change the routine management of patients (urine assessment for biochemistry analysis was part of the current diagnostic work-up) and ii) because of its retrospective nature.

### 2.2. Metabolome analysis

The fetal urine metabolite content was analyzed by CE-MS as previously described [8]. Briefly, a 170 µl aliquot of fetal urine was diluted with the same volume of a denaturing solution composed of 2 M urea, 0.0125% $NH_4OH$, 100 mM NaCl and 0.01% SDS. To remove higher molecular mass proteins, the samples were ultrafiltered using a Centrisart 20 kDa cut-off centrifugal filter device (Satorius, Göttingen, Germany) at 2000 × *g* for 45 min at 4 °C. In order to remove urea, electrolytes and SDS, 200 µl of filtrate was applied onto a NAP5 gel filtration column (GE Healthcare Bio Sciences, Uppsala, Sweden), washed and then eluted with 700 µl of 0.01% $NH_4OH$. Finally, all samples were lyophilized in a Savant speedvac SVC100H connected to a Virtis 3 L Sentry freeze dryer (Fischer Scientific, Illkirch, France) and then stored at 4 °C. Shortly before CE-MS analysis, lyophilisates were resuspended in HPLC grade water, so that the protein concentration was at 1 µg/µl (BCA assay, Pierce Biotechnology, Rockford, USA).

CE-MS analyses were performed using a Beckman Coulter Proteome Lab PA800 capillary electrophoresis system (Beckman Coulter, Fullerton, USA) on-line coupled to a micrOTOF II MS (Bruker Daltonic, Bremen, Germany). The electro-ionization sprayer (ESI, Agilent Technologies, Palo Alto, CA, USA) was grounded, and the ion spray interface potential was set between −4 and − 4.5 kV. The CE separation buffer contained 20% (v/v) acetonitrile and 250 mM formic acid (Sigma-Aldrich) in HPLC-grade water. Data and MS acquisition methods were automatically controlled by the CE *via* contact-close-