Published in final edited form as:

Pediatr Nephrol. 2015 June ; 30(6): 881–887. doi:10.1007/s00467-014-2880-x.

Metabolomics in pediatric nephrology: Emerging concepts

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Abstract

Metabolomics, the latest of the “omics” sciences, refers to the systematic study of metabolites and their changes in biological samples due to physiological stimuli and/or genetic modification. Because metabolites represent the downstream expression of genome, transcriptome and proteome, they can closely reflect the phenotype of an organism at a specific time. As an emerging field in analytical biochemistry; metabolomics has the potential to play a major role for monitoring real-time kidney function and detecting adverse renal events. Additionally, small molecule metabolites can provide mechanistic insights for novel biomarkers of kidney diseases, given the limitations of the current traditional markers.

The clinical utility of metabolomics in the field of pediatric nephrology includes biomarker discovery, defining as yet unrecognized biologic therapeutic targets, linking of metabolites to relevant standard indices and clinical outcomes, and providing a window of opportunity to investigate the intricacies of environment/genetic interplay in specific disease states.

Keywords

Metabolomics; Biomarkers; Pediatric Nephrology

Introduction

Metabolomics, the latest of the “omics” sciences, refers to the systematic study of metabolites and their changes in biological samples due to physiological stimuli and/or genetic modification (1). Because metabolites represent the downstream expression of genome, transcriptome and proteome, they can closely reflect the phenotype of an organism at a specific time (2). As an emerging field in analytical biochemistry; metabolomics has the potential to play a major role for monitoring real-time kidney function and detecting adverse renal events. Additionally, small molecule metabolites can provide mechanistic insights for novel biomarkers of kidney diseases, given the limitations of the current traditional markers.

The clinical utility of metabolomics in the field of pediatric nephrology includes biomarker discovery, defining as yet unrecognized biologic therapeutic targets, linking of metabolites

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Disclosure: All the authors declared no conflicts of interest.

to relevant standard indices and clinical outcomes, and providing a window of opportunity to investigate the intricacies of environment/genetic interplay in specific disease states. In this review, the recent advancements in the field of metabolomics and their application in pediatric nephrology are described, based on published studies that applied metabolomics to a variety of kidney diseases. We searched MEDLINE/PubMed for English-language articles using the term “metabolomics” along with various specific kidney diseases.

Metabolomics Technologies

The two major analytical techniques applied in metabolomics are Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS). These techniques can handle complex biological samples with a high sensitivity, selectivity and throughput (3). NMR spectroscopy is a quantitative nondestructive, noninvasive, perturbing technique that provides detailed information on solution-state molecular structures, based on atom-centered nuclear interactions. It gives detailed simultaneous information on both the structure and molecular mobility of metabolites without the need for the preselection of analytical parameters or sample derivatization procedures. As it is a nondestructive technology, it can be used for the analysis of tissue biopsies and thus can directly be compared to histopathological findings using the same tissue samples. Sensitivity is a limiting factor and often metabolite concentrations in the range of 1 to 10 $\mu\text{mol/L}$ are required for detection and quantification by NMR (4).

Mass spectrometry platforms tend to have much higher analytical sensitivity, enabling broader surveys of the metabolome. Prior to analysis, the samples need to be separated using chromatography, commonly either gas or liquid chromatography (GC or LC) followed by ionization in a fluid or matrix; and subsequently, metabolites are identified using a mass spectrometer on the basis of their mass-to-charge ratio (m/z) and their representation in a spectrum. The combination of chromatographic separations with mass spectrometry increases the biological information obtained, and enhances the sensitivity and the ability to identify metabolites in complex biological systems. An advantage of MS-based techniques is the ability to identify metabolites either through the measurement of molecular mass (indicative of the molecular formula) to a high mass accuracy (typically parts per million, ppm) or by collection of fragmentation mass spectra (indicative of molecular structure). This allows the identification of novel metabolites not currently described in metabolomic databases as well as previously characterized metabolites across large sample sets. Ion suppression in complex biological samples may be caused by the interaction of multiple analytes that are present in the ionization source at the same time, thus limiting the ability of MS to quantify metabolites (5, 6). The differences between NMR and MS are summarized in table 1.

Both NMR and MS can be used to characterize metabolite data either in a targeted manner, or in a non-targeted (pattern-recognition) manner (7). The target analysis involves identification and quantification of specific analytes in a given biofluid or tissue extract by comparing the spectrum of interest to a library of reference spectra of pure compounds. In order to establish a knowledge base that can serve as reference, metabolomics databases have been actively developed, such as the Human Metabolite Database (HMDB: <http://>

www.hmdb.ca/), METLIN (<http://metlin.scripps.edu/>), and MetaCyc (<http://www.metacyc.org/>). While these publically available databases are accessible, proprietary databases do exist and may be accessed through contractual/research agreements with the entities that have developed them. Alternatively, the non-targeted (global) analysis serves as a hypothesis-generating tool whose results often require follow-up with more targeted approaches. The global pattern-recognition method can also screen for a multitude of key compounds in specific metabolic pathways such as carbohydrates, amino acids, fatty acids, phospholipids and NO-synthesis pathway, which provides valuable information for metabolic fingerprinting. Such pathway analysis can provide insights into real time disease processes, developmental processes and potentially reveal biological therapeutic targets.

After data collection a statistical analysis method is chosen to suit the study objective. Univariate analyses, such as t test, analysis of variance, Mann-Whitney U test, Wilcoxon signed-rank test, and logistic regression, are applied to identify metabolites that are capable of differentiating between groups. Multivariate analysis methods are used to develop statistical pattern recognition models. By extracting and displaying the systematic variation in the data based on projection methods, multivariate analysis reduces the variability of the data and combines complex interactions. Unsupervised principal component analysis (PCA) is applied by summarizing the data into much fewer variables called scores which are weighted averages of the original variables. Each Principle Component (PC) is a linear combination of the original data parameters and each successive PC explains the maximum amount of variance possible, not accounted for by the previous PCs. In contrast, supervised partial least-squares discriminant analysis (PLS-DA) enables the identification and characterization of metabolic perturbations signatures through a linear regression model by projecting the predicted variables and the observed variables to a new space. Therefore PLS-DA is the main tool used in chemo-metrics for classification and discrimination purposes (8). Orthogonal partial least squares (OPLS) regression is an extension of PLS, where only the variation in the set of predictor variables that correlates with the response variable is retained. Discriminant Analysis of Principal Components (DAPC) has recently been proposed as a way to combine the flexibility and efficiency of PCA and the discriminating power of DA. The use of dimension reduction techniques in a regression context requires the specification of the number of latent variables to be retained in the model. This usually relies on cross-validation procedures aiming at the identification of the number of components that optimizes both interpretability and prediction error (9).

Metabolomic data sets are intrinsically multidimensional with the number of measured metabolites typically ranging from a few dozen to hundreds. A key step in placing statistically significant findings from chemo-metric analyses into a meaningful biological context is to identify significantly altered pathways represented by certain spectral peaks. Therefore, the identified metabolites are integrated into metabolic correlation networks in order to better understand the complex relationships among various metabolites. The analysis and interpretation of these data generated networks reflect the structure of the underlying biochemical pathways. Furthermore, pathways mapping represents a snapshot of the physiological state at a given point in time which is a useful tool in metabolic fingerprinting. Figure 1 illustrates the flow of the processes of metabolomics.

Metabolomics as biomarkers

A biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes or pharmacologic responses to therapeutic intervention” (10). Each cell type and biologic fluid has a genetically predetermined characteristic set of metabolites that reflects the organism under particular environmental conditions and that fluctuates in response to physiologic and environmental stimuli. Thus, the interest in metabolomics biomarkers has been growing exponentially over the last several years and recent metabolomic studies have not only advanced biomarker discovery, but also allowed the elucidation of mechanisms underlying different diseases such as diabetes mellitus, chronic kidney disease and obesity. In a review about the application of metabolomics for specific kidney diseases, Weiss et al outlined the phases of a metabolomics-based biomarker discovery strategy including phase 1: discovery, phase 2: pre-validation and quantification and phase 3: validation and application (11).

Fukui et al. applied ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) based metabolomic approach to identify a candidate metabolite associated with interstitial cystitis (IC) (12). Urine samples from the following were collected and analyzed: 10 IC patients, 10 bacterial cystitis (BC) patients, and 10 healthy volunteers. The urinary levels of phenylacetylglutamine (PAGN) relative to creatinine (Cr) were significantly high in patients with IC (mean 0.47 mg/mg Cr) compared with BC patients (mean 0.25mg/mg Cr) and with healthy volunteers (mean 0.11 mg/mg Cr). These results established the urinary PAGN/Cr ratio as a potential new urinary biomarker of IC. Additionally it may indicate an underlying pathological condition in early IC patients, for example, abnormal amino acid metabolism.

Gronwald et al was able to differentiate subjects with autosomal dominant polycystic kidney disease (ADPKD) from other kidney diseases and from subjects with normal kidney function, using NMR metabolic fingerprinting of the urine (13). They grouped the subjects into 5 cohorts: 54 patients with ADPKD and slightly reduced estimated glomerular filtration rates, 10 ADPKD patients on hemodialysis with residual renal function, 16 kidney transplant patients, 52 type 2 diabetic patients with chronic kidney disease, and 46 healthy volunteers with normal renal function. ADPKD patients showed increased excretion of proteins and methanol compared to the control and other patient groups suggesting the existence of a set of urinary compounds specific to early stages of ADPKD. However, this study was designed as a cross-sectional proof-of-concept study and, therefore, does not allow firm conclusions on changes of urinary fingerprints with disease progression. In a case control longitudinal study, Nevedomskay et al used a metabolomics approach to profile UTI patients, using as study groups not only healthy subjects and affected patients, but also patients who had recovered (14). They were able to identify molecular discriminators that characterize different patients groups. Among those discriminators (e.g. acetate, trimethylamine) showed association with the degree of bacterial contamination of urine, whereas others (e.g., para-aminohippuric acid (PAH), scyllo-inositol) were identified as more likely to be markers of morbidity. The discriminative model was able to classify most of the independent samples correctly according to their diagnosis; however its real clinical utility needs careful examination.

Many benefits have been shown from the use of metabolomics to identify biomarkers of CKD. Sato et al developed an UPLC–MS approach to analyze the plasma samples of 10 patients with end stage renal disease (ESRD) who were being treated with hemodialysis, as well as in 16 healthy subjects (15). 1-Methylinosine was found to be an effective candidate biomarker to estimate an adequate dose of hemodialysis. Several experimental studies have utilized metabolomics to study AKI induced by toxins and antibiotics in animal models (16–18). Using a combination of gas chromatography/mass spectrometry (GC/MS) and liquid chromatography/mass spectrometry (LC/MS), Boudonck et al performed a global, non-targeted metabolomics analysis on Sprague-Dawley rats treated with gentamicin, cisplatin, or tobramycin (16). A significant increase in polyamines and amino acids was observed in urine from drug-treated rats after a single dose and prior to observable histological kidney damage and conventional clinical chemistry indications of nephrotoxicity.

Membranous nephropathy (MN) is an important glomerular disease characterized by podocyte injury and proteinuria. In order to comprehensively profile systematic metabolic variations associated with MN, Gao et al divided MN patients clinically into two groups: one with low levels of urinary proteins < 3.5 g/24 h (Low Urinary Protein MN group—LUPM) and the other group with high levels of urinary proteins > 3.5 g/24 h (High Urinary Protein MN group—HUPM) (19). Citric acid and 4 amino acids were markedly increased only in the serum samples of HUPM patients, implying more impaired filtration function of kidneys of HUPM patients than LUPM patients. The dicarboxylic acids, phenolic acids, and cholesterol were significantly elevated only in urines of HUPM patients, suggesting more severe oxidative stress. Thus parallel metabolomics of urine and serum revealed the systematic metabolic variations associated with LUPM and HUPM patients. This study exhibited a promising application of parallel metabolomics in renal diseases and provided novel insights for oxidative stress during the pathogenesis and progression of HUPM patients. The small number of patients enrolled in all the aforementioned studies is a major limitation. Table 2 presents a summary of these studies applying metabolomics technologies for the detection of potential biomarkers.

A particular strength of metabolomics compared with the other ‘omic’ technologies is that it provides an instantaneous snapshot of the cellular physiology at a functional level. Therefore, it can be used as a direct read out of the physiologic state of a cell or tissue at any given point in time as well as identifying biomarkers of diseases. Although, the correlation analysis provided by the biostatistical methods that are commonly used in metabolomics does not establish a causal relationship, the application of metabolomics as an extension of functional genomics provides a useful link between genotypes and phenotypes. Additionally, changes in specific metabolite patterns may reflect changes in real-time pathways and processes. Ideally, metabolomics should be integrated with other omics such as genomics and proteomics in order to connect the experimentally observed correlations to the underlying biochemical system. The recent advances in biotechnological platforms to analyze such complex data provide a powerful tool to identify biomarkers.

Metabolomics in pediatric nephrology

Although metabolomics has enormous potential in the field of pediatric nephrology, it has been used only in a very limited number of clinical applications so far. In our experimental study, gentamicin-induced acute kidney injury in newborn rats resulted in a distinct urinary metabolic profile characterized by glucosuria, phosphaturia, and aminoaciduria that preceded changes in serum creatinine (20).

Beger et al. studied 40 children undergoing cardiac surgery, prospectively collecting urine at 4 and 12 hours after surgery (21). Twenty-one of these children developed acute kidney injury (AKI) defined as an increase of serum creatinine concentrations 50% or greater from baseline after 48–72 hours. They identified a metabolite of dopamine (homovanillic-acid-sulfate) as a marker indicating AKI with 90% sensitivity and 95% specificity using a cutoff value of 24 ng/ml at 12 hours after surgery. Despite the limitation of the small number, this finding suggests that urinary homovanillic-acid-sulfate is a novel promising sensitive and specific biomarker of AKI following pediatric cardiac surgery.

Applying a proton nuclear magnetic resonance (^1H NMR)-based metabolomic analysis, Atzori et al. were able to differentiate children with nephrouropathies (renal dysplasia, vesicoureteral reflux, urinary tract infection, and acute kidney injury) from healthy children through urine metabolic profiling. In particular, cortical renal pathology has been associated with alterations of purine, pyridine, and urea cycle (22). The same group showed a correlation between urinary metabolic profiles and neutrophil gelatinase-associated lipocalin (NGAL) concentration in a cohort of young adults born extremely low-birth weight (ELBW), using partial least-squares discriminates analysis (23). The combined approach applied in this study illustrated the relevance of using the metabolomics technique in conjunction with a novel promising biomarker of renal injury.

Immunoglobulin A nephropathy (IgAN) is a leading cause of chronic kidney disease, frequently associated with hypertension and renal inflammation. Zivkovic et al. applied a targeted metabolomic approach using liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) to analyze oxylipin profiles of IgAN patients before and after supplementation with fish oil or corn oil (placebo) (24). In this cohort of adolescents and young adults, plasma total oxylipins, hydroxyoctadecadienoic acids, hydroxyeicosatetraenoic acids, and leukotriene B₄ metabolites were significantly lower in patients whose proteinuria improved, compared to patients whose proteinuria either did not improve or worsened. These data support the involvement of oxylipins in the inflammatory component of IgAN as well as the potential use of oxylipin profiles as biomarkers to monitor the response to ω -3 fatty acid supplementation in IgAN patients.

Classical xanthinuria is a rare inherited metabolic disorder caused by either isolated xanthine dehydrogenase (XDH) deficiency (type I) or combined XDH and aldehyde oxidase (AO) deficiency (type II). Peretz et al demonstrated that urinary metabolic profiling allows differentiating type I from type II xanthinuria (25). Urine metabolomic analysis by liquid chromatography-mass spectrometry was performed on children and adult xanthinuric patients. Novel endogenous products of AO, hydantoin propionic acid, N1-methyl-8-

oxoguanine and N-(3-acetamidopropyl)pyrrolidin-2-one were identified as being lower in type II xanthinuria. The identified biomarkers have the potential to replace the allopurinol-loading test used in the past to type xanthinuria, thus facilitating appropriate pharmacogenetic counseling and gene directed search for causative mutations. Additionally, it is expected that this patient-friendly test will improve patient compliance and facilitate patient-tailored pharmacogenetic counseling. Table 3 presents a summary of these studies.

Conclusion

The application of metabolomics in the pediatric nephrology field, while still in its infancy, is holding yet another promise of how metabolomics may offer an innovative approach for early diagnosis and treatment of renal diseases. Metabolomic studies, combined with modern multivariate data analysis methods, allow researchers to perform multifactorial biomarker discovery in a highly efficient manner (26). Careful study design, consistent sample collection methods and the application of an appropriate statistical analysis are important key steps in the design and execution of future metabolomic studies. Metabolomic studies often require the analysis of many samples prepared simultaneously to reduce variability and improve workflow efficiency. Utilization of preservative techniques, such as the addition of protease inhibitors to bio-specimens should be considered in terms of study design and generalizability. While the addition of such preservatives may allow identification of a larger range of metabolites, which may inform detailed mechanistic insights, their use does not necessarily reflect “real world” bedside scenarios in the era of bedside point-of care testing. Therefore the specific goals and long-term objectives of the study must be considered carefully. In general, bio-fluid samples should be frozen at -20°C , then transferred to lower storage temperatures within one week, sample preparation and the choice of a specific technology depend on the research question(s). Finally, validation of the prediction model is necessary in order to generate meaningful results.

In the era of personalized medicine, the incorporation of metabolomic biomarkers into clinical research and practice will allow the identification and stratification of patients into subpopulations who will derive more or less benefit from an intervention. The sensitivity of metabolomics relies on the ability to identify small differences in pathological conditions. Therefore, the initial step in metabolomics research is to identify the metabolic signature associated with various diseases by mapping early biochemical changes in biological systems. After establishing renal metabolomic profiles in the pediatric and neonatal population, the next step is metabolic fingerprinting. In such investigations, the intention is not to identify each observed compound but to compare patterns or fingerprints of metabolites that change in response to disease or drug exposure. Furthermore, experimental work in model systems and integration with other omics approaches will provide insight into the pathophysiologic interactions between select biomarkers and disease pathogenesis. Finally, large epidemiological cohort studies are needed to assess whether metabolomic biomarkers improve upon existing disease markers and to determine the validity of their application in different clinical settings.

Acknowledgments

This research was supported by a pilot grant from the University of Iowa Institute for Clinical and Translational Science UL1RR024979 (ICTS- CTSA).

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Figure 1.
Metabolomic workflow

Table 1

The difference between nuclear magnetic resonance (NMR) and mass spectrometry (MS)

	NMR	MS
Sample preparation	Minimal, no extraction or derivatization	Samples have to be extracted into a suitable solvent
Quantitation	Quantitative because the signal intensity is only determined by the molar concentration	Ion suppression limits quantitation
Sensitivity	Low, therefore less suited for analysis of trace compounds	High
Analytical reproducibility	High	Poor
Technique	Nondestructive	Destructive as metabolite identification is based on fragmentation patterns

Table 2

Summary of studies applying metabolomics as biomarkers

First author (year)	Biological sample	Clinical setting	Method	Metabolite(s)
Fukui (2009)	Urine	Interstitial Cystitis	UPLC-MS	phenylacetylglutamine (PAGN)
Gronwald (2011)	Urine	ADPKD	NMR	Methanol, alanine, proteins
Nevedomskay (2012)	Urine	UTI	NMR	acetate, trimethylamine, para-aminohippuric acid (PAH), scyllo-inositol
Sato (2011)	Plasma	Patients with ESRD undergoing hemodialysis	UPLC-MS	1-Methylinosine
Gao (2012)	Urine and Serum	Membranous Nephropathy	GC-MS	Citric acid, L-serine, L-asparagine, L-threonine, dicarboxylic acids, phenolic acids, cholesterol

UPLC-MS, ultra-performance liquid chromatography-mass spectrometry; NMR, nuclear magnetic resonance; GC, gas chromatography; ADPKD, autosomal dominant polycystic kidney disease; UTI, urinary tract infection; ESRD, end stage renal disease

Table 3

Summary of studies applying metabolomics in pediatric nephrology

First author (year)	Biological sample	Clinical setting	Method	Metabolites
Beger (2008)	Urine	AKI following cardiac surgery	LC-MS	Homovanillic-acid sulfate (HVA-SO ₄)
Atzori (2010)	Urine	Nephrouropathies	NMR	Purine, pyridine
Atzori (2011)	Urine	Young adults born extremely low-birth weight	NMR	N-Methylhydantoin, glycine, valine, glutamine
Zivkovic (2012)	Serum	Immunoglobulin A nephropathy	LC-MS/MS	Total oxylipins, hydroxyoctadecadienoic acids, hydroxyeicosatetraenoic acids, leukotriene B ₄
Peretz (2011)	Urine	Children and adults from 11 families affected by classical xanthinuria	LC-MS	hydantoin propionic acid, N1-methyl-8-oxoguanine, N-(3-acetamidopropyl)pyrrolidin-2-one

LC-MS, liquid chromatography-mass spectrometry; NMR, nuclear magnetic resonance;