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Review

Title: Urinary biomarkers for renal tract malformations

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Abstract

Introduction: Renal tract malformations (RTM) are congenital anomalies of the kidneys and urinary tract, which are the major cause of end stage renal disease in children. Using immunoassay-based approaches (ELISA, western blot), individual urinary proteins including transforming growth factor β, tumor necrosis factor and monocyte attractant proteins 1 were found to be associated to RTM. However, only mass spectrometry (MS) based methods leading to the identification of panels of protein-based markers composed of fragments of the extracellular matrix allowed the prediction of progression of RTM and its complications.
Areas covered: In this review, we summarized relevant studies identified in “Pubmed” using the keywords “urinary biomarkers” and “proteomics” and “renal tract malformations” or “hydronephrosis” or “ureteropelvic junction obstruction” “posterior urethral valves or “vesicoureteral reflux”. These publications represent studies on potential protein-based biomarkers, either individually or combined in panels, of RTM in human and animal models.
Expert commentary: Successful use in the clinic of these protein-based biomarkers will need to involve larger scale studies to reach sufficient power. Improved performance will potentially come from combining immunoassay- and MS-based markers.

Keywords: proteomics; children; urine; biomarkers; renal tract malformation

1. Introduction

Renal tract malformations (RTM) are congenital anomalies of the kidneys and/or lower urinary tract. Severe RTM is life-threatening and only a limited number of RTM can be successfully (surgically) corrected, e.g. ureteropelvic junction obstruction (UPJO) and to a lesser extent posterior urethral valves (PUV). Severe, not surgically correctable, RTM will

lead in many cases to end stage renal disease (ESRD) (1;2). Hence, key issues in the clinical management of RTM, which is often detected *in utero*, are the prediction of disease progression and management of its complications.

Hippocrates linked health problems to diagnostic changes in the urine two millennia ago. Presently, measurement of urinary components such as proteins to test for the presence of kidney disease, using dipsticks and standard laboratory tests, is routine in diagnostics. However, these tests lack sensitivity and consequently detect kidney disease often at a late stage since there has to be significant kidney damage before frank proteinuria occurs. Therefore, identification of earlier and prognostic markers using, for example proteomics techniques, would be desirable. Indeed the use of proteome analysis for the discovery of clinically relevant proteins, known as “clinical proteomics”, is perceived to be the most relevant discipline to foster the translation of basic discoveries into clinical applications for the benefit of the patient (3). Clinical proteomics needs to adhere to strict guidelines to generate clinically useful biomarkers of disease. Therefore, Mischak *et al.* asserted that successful clinical proteome studies should in general use stringent statistical approaches for biomarker definition and that results should always be confirmed in independent test sets (4). In addition, they described a brief set of practical and feasible recommendations for investigators to properly identify and qualify proteomic (or any other) biomarkers, which could also be used as reporting requirements. Such recommendations should help put proteomic biomarker discovery on solid ground.

2. Urine as a source of biomarkers of RTM

Easily accessible biological samples, such as plasma, serum or urine are valuable sources of biomarkers. Particularly urine which can be collected in large quantities and in non-invasive fashion and is less complex than for example plasma (5;6). Under physiological conditions, 70% of urinary proteins are produced by kidney and urinary tract and hence urine is a potential source of biomarkers for RTM. However, urine also allows to obtain information related to various other organs because of the glomerular filtration of blood (7). The urinary

proteome is relatively stable, because it is stored for hours in the bladder at 37°C and any proteolysis has been completed at time of voiding (8). Therefore, the urinary proteome does not change significantly and urine can be stored for 6 h at room temperature or 3 days at 4ºC as well as for several years at -20°C (9). In addition to full length proteins, urine also contains many naturally occurring low molecular weight proteins (peptides), which can be directly analysed by mass spectrometry, without additional sample processing, such as tryptic digestion and the depletion of abundant blood-derived proteins (10;11). Variability in the protein/peptides concentration is the main disadvantage of urine. These variations are caused by the differences of the protein quantity during the day, circadian rhythms, exercise, diet and metabolic or catabolic processes (10;12). However, this can be corrected by using normalization methods (13).

3. Proteome analysis-based approaches for the identification and validation of biomarkers

The main goal of clinical proteomics is the identification of biomarkers, providing information to improve and personalize medicine (14). Since, by definition, the proteome is complex, its analysis needs in general two steps: firstly, fractionation of the proteins into smaller less complex subsets followed by, secondly, analysis of the protein abundance and protein identity by mass spectrometry (MS) of those fractions. Fractionation is/has been often performed by chromatographic techniques including two-dimensional gel electrophoresis (2-DE), liquid chromatography (LC) and capillary electrophoresis (CE). The protein fractions are then analysed off- or on-line by MS. 2-DE, cumbersome and low-throughput, has now mostly been abandoned. The advantages and disadvantages of these proteomics techniques have been extensively described in several reviews (10;15;16) and will not be discussed here. Enzyme-linked immunosorbent assays (ELISA) are frequently cited for validation and clinical application of protein-based markers of disease. However, antibodies have the limitation that they only recognize a particular epitope and therefore one antibody cannot differentiate between isoforms (17;18) or highly similar peptides (19-21) that are often disease specific.

Another drawback of the use of antibodies is antibody cross-reactivity and protein/protein interactions that may modify the quantification of the results (22;23). Furthermore, although ELISAs supports multiplexing this is limited to a maximum number of proteins which often does not cover the several 10-100 of proteins of which biomarker panels are currently composed of (24-26). Finally, although MS-based techniques need an initial large investment for the MS-equipment, ELISAs are relatively expensive in use and in the long run will cost more than MS-based techniques (27;28).

Selected reaction monitoring (SRM) and multiple reaction monitoring (MRM) are selective/targeted MS-based techniques for protein quantification and identification of small proteins/peptides. High specificity, sensitivity, fast analysis, and the quantification of targeted proteins/peptides are some advantages of these proteome analysis based methods that can be employed in biomarker discovery, verification and validation (29-31). These techniques might become the references for clinical analysis of sets of proteome-based markers although large scale studies proving the feasibility still need to be undertaken. To our knowledge MRM has not yet been used in the context of RTMs. Finally, in recent years several studies have also demonstrated the importance of CE-MS in validation of biomarkers, due to low cost and high-throughput, as well as high reproducibility, allowing this proteomic approach to be essential for clinical application.

4. Renal tract malformations

RTM are considered as the primary cause of chronic renal failure in children (30-50%). RTM can be unilateral or bilateral involving the upper or low urinary tract or only the kidney and combinations thereof, as depicted in **figure 1** (30;31). For example ureteropelvic junction obstruction (UPJO) is often unilateral and represents an obstruction at the intersection of the kidney pelvis and the ureter, blocking normal urine flow and inducing in severe cases a hydronephrotic kidney (32). Posterior urethral valves (PUV) normally disappear during

development, but in PUV remain and block urine outflow from the bladder leading to bilateral obstruction of urinary pathways with associated (often severe) lesions in the kidney (33;34). Vesicoureteral reflux (VUR) is characterized by a backward flow of the urine from the bladder towards the kidneys often due to urinary tract infection or urinary tract abnormalities. Severe VUR can lead to renal scarring, arterial hypertension and impaired renal function (35). RTM display a wide spectrum of pre- and postnatal outcomes ranging from death *in utero* to normal postnatal function. Most of the diagnostic methods of RTM are based on pre- and post-natal imaging, either ultrasound or radiological tests. However, imaging even if in some cases combined with fetal urine/amniotic fluid biochemistry, does often not help to provide the prediction of RTM progression and or its complications (31). Hence a number of studies have looked at the value of proteome analysis in RTM. **Table 1** summarizes potential human urinary proteome analysis-based biomarkers of RTM that will be described in detail in the next sections.

4.1. *UPJO*

UPJO is the most common cause of upper tract obstruction and can be detected by ultrasound before birth (20;32). Currently UPJO is treated surgically in the easily detectable severe cases. However for the other patients with mild forms of obstruction close follow-up during the first years of life is required which is necessitating imaging and frequent hospital visits to determine the progression of the disease. ELISA and MS-based urinary proteome studies have been performed with the aim to identify urinary biomarkers of UPJO. Several groups used sandwich ELISA kits to discover and identify potential biomarkers of UPJO based on predefined knowledge obtained in animal models of obstruction or processes repeatedly observed in kidney disease progression (e.g. fibrosis, inflammation).

Taha *et al*. were the first to study urinary concentrations of transforming growth factor β1 (TGF-β1) in UPJO. Using a quantitative sandwich ELISA kit and a cohort of 35 children with UPJO and 30 healthy children, they observed that the expression of TGF-β1 was significantly increased in urine of children with UPJO (36). Interestingly, urinary TGF-β1 decreased after

pyeloplasty (corrective surgery to remove the UPJO). These results were confirmed by Gawłowska-Marciniak *et al.*, who used ELISA for their investigation of urine from the bladder and renal pelvis of 45 patients undergoing pyeloplasty and 25 controls (37) and by Sager *et al.* who used bladder urine from 19 UPJO patients and 19 matched controls (38). In another, more recent study, based on a cohort of 25 healthy controls and 25 children with UPJO, the authors also detected increased TGF- β1 levels in urine of patients with UPJO and a significant correlation of TGF-β1 with hydronephrosis grade (p=0.0001) (39).

Studies of association between urinary EGF concentrations and UPJO were less successful because several studies described contradictory results. In 2000, ELISA was used in a study examining 24 patients with UPJO and 15 healthy children. Grandaliano *et al*. (40) established that EGF levels were significantly lower in UPJO patients when compared with healthy controls. These results, employing ELISA, were confirmed by Bartoli *et al.* (41) using a cohort of 76 UPJO patients and 30 healthy children. Li *et al*. (42) analyzed the urinary EGF values in 33 healthy subjects and 12 patients with UPJO (surgical group) and they also observed, using ELISA, a significant decrease in abundance in patients group, during the first six months of life. The data presented also supported the hypothesis that urinary EGF changes over time are inversely correlated with Society of Fetal Urology (SFU) hydronephrosis grade. However, Taha *et al*. did not find significant differences in urinary EGF concentrations between controls and surgical groups as well as preoperative and postoperative values one year after surgery (36). Furthermore, using a cohort of 28 UPJO children and 13 controls and a bead-based multiplex sandwich immunoassay, EGF levels according to Madsen *et al.* (43) were significantly increased in UPJO patients (submitted to pyeloplasty) compared to controls. Furthermore, a 3 months and 1 year follow-up showed that these values normalized with similar values between patients and controls. Based on these contradictory results, it seems that EGF is not a valid biomarker for UPJO, because EGF levels were decreased in some studies (40-42), increased in another (43) or without changes (36).

The same studies also investigated specific urinary cytokines, such as monocyte chemotactic peptide 1 (MCP-1) for its use as a biomarker for UPJO (40;41;43). Comparing

patients subjected to pyelopasty (preoperative and postoperative) and healthy controls, Grandaliano *et al.* (40) demonstrated that preoperative values of MCP-1were significantly increased. These findings were verified by Bartoli *et al*. (41) and Madsen et al. (43). They reported increased concentrations of this cytokine in urine of preoperative UPJO patients and a decrease in postoperative UPJO patients (similar to healthy).

Several, more anecdotal, studies focusing on the association of urinary angiotensinogen (AGT), kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) to UPJO were recently published: Taranta-Janusz *et al*. compared 31 children with severe hydronephrosis (who needed surgery); a non-surgical group, 20 patients with mild hydronephrosis, and 19 healthy children to study the urinary AGT concentration with the use of a commercially available ELISA (44). They observed that increased levels of AGT were directly correlated with UPJO children. AGT was also found in a later study (45), but the researchers turned no special attention to this protein.

A study involving 20 children with severe hydronephrosis, two groups of healthy individuals, 20 patients with mild non-obstructive hydronephrosis and 25 healthy children, demonstrated using immunoenzymatic ELISA commercial kits an increase in urinary NGAL and KIM-1 and established a correlation with worsening obstruction (46). The results for NGAL were confirmed in another study, presenting an increase in urine from patients with obstructed kidneys at the time of surgery. This increase was followed by a decrease and stabilization to the same level as that of the controls (47).

Although these individual proteins were associated, with different degrees of confidence, to the severity and grade of UPJO, none of the studies evaluated their predictive power which would be the main use of urinary markers in UPJO.

MS-based proteomics approaches have been performed to predict the progression of UPJO, and the results were validated in several studies. The urinary proteome of 13 healthy newborns, 19 UPJO patients with mild obstruction (non-operated individuals (no-OP)) and 19 UPJO patients with severe obstruction (operated individuals (OP)) was analysed using CE-

MS by Decramer *et al.* (51). They established a classifier based on 51 UPJO-specific urinary peptide biomarkers to differentiate resolution of UPJO or the requirement of pyeloplasty. Validation of these 51 biomarkers combined in a support vector machine (SVM) panel in an independent group of OP and no-OP patients (n=16) resulted in 94% sensitivity and 80% specificity. In a blinded cohort of 36 UPJO patients with 9 months of follow-up this panel predicted with 95% accuracy whether an infant with UPJO needed surgery. This biomarker panel was further validated in 19 children where the classifier displayed 83% sensitivity and 92% specificity. However in UPJO patients >1 year of age, this classifier lost sensitivity (66%) and specificity (20%) (52). This is probably due to the change of the urinary proteome after the age of 1-2 years (53). It also points to the fact that the context of use of disease classifiers will often not be beyond the population in which it was defined.

Bandin *et al.* used CE-MS-based urinary proteome analysis to determine whether early surgically corrected UPJO would normalize the urinary proteome after long-term follow-up compared to conservatively followed UPJO patients (54). Studying 42 patients with UPJO 5 year after either pyelopasty or spontaneous resolution of the obstruction, they observed that the urinary proteome was very similar in patients with early surgical correction of UPJO and age matched controls. However the urinary proteomes of UPJO patients leading to spontaneous resolution or late surgical intervention did not normalize and displayed a significantly different pattern (54). This suggests ongoing renal or ureteral remodeling in the conservatively followed patients that is not visible clinically.

LC-MS/MS coupled to bioinformatics analysis was used in a number of studies to evaluate the urinary proteome of infants with UPJO. According to these studies, the most prominent proteins found were related to pathways involving inflammation, oxidative stress, fibrosis and renal disease (48;55-57). Mesrobian *et al*. (56;57) have identified the proteome differences between normal infants and infants with UPJO. In the first study, using 21 healthy infants and 25 infants with grade IV unilateral ureteropelvic junction obstruction, they identified 31 proteins changing at different time-points (56). In the second study, differences in individual urine samples were assessed in 21 healthy infants and 25 infants with grade IV unilateral

UPJO followed for 5 years. The urinary proteome from patients with UPJO was different from the age-matched controls, based on the activation of processes of inflammation, apoptosis, tubular injury and fibrosis, and oxidative stress (57).

A proteomic study of urine samples (n=5/group) from newborns with UPJO at different stages and controls was performed. Urine protein profiles of these patients were obtained by label free quantitative nanoLC-MS/MS and 970 urinary proteins were identified (52). In a recent case-control study (8 controls and 8 subjects with unilateral obstruction), Froehlich *et al*. (45) reported correlation between identified and quantified proteins associated with this type of RTM. They identified 1113 proteins, but only 76 were significantly different between the two groups. In particular biological processes such as inflammation and renal disease pathways showed significant variations, targeted oxidative stress proteins were also presented over expressed.

Based on these studies, potential regulated proteins in RTM such as arginase 1, glutathione S-transferase Mu 1 (GSTM1) and heat shock 70 kDa protein 1A (HSPA1A) were further studied and validated. Using Western Blot and MRM analysis, a decrease in arginase 1 in UPJO was validated in independent samples. In addition, in a mouse model of obstructive nephropathy, arginase 2 and total arginase activities were found to be increased (52). GSTM1 and HSPA1A were also validated by Western Blot and confirmed that these proteins were elevated in urine of UPJO patients, supporting the idea that alterations in the processing of reactive oxygen species (ROS) are related to UPJO.

These urinary proteome analysis studies were also an opportunity to re-evaluate the unclear association of urinary EGF to UPJO obstruction observed using ELISA. However, again contradicting results were obtained. Lacroix *et al*. (52) observed that urinary EGF decreased in UPJO using a combination of targeted (MRM) and non-targeted MS/MS analyses (using an independent cohort, n=10/group), and also demonstrated that EGF was among the top differentially excreted proteins. In contrast, Froehlich *et al.* (45) did not report EGF among the differentially expressed urinary proteins in their study. Hence EGF appears, again, not to be a reliable marker of UPJO. This might be due to the fact that urinary EGF is also modified in

other renal diseases including acute kidney injury, diabetic nephropathy, chronic kidney disease, IgA nephropathy, Lupus nephritis etc. (55) or even in different types of cancer (56), which demonstrates that EGF is not exclusively specific to UPJO.

4.2. *PUV*

Posterior urethral valves (PUV), the prototypic bilateral RTM (37), is an abnormal congenital obstructing membrane that is located within the posterior male urethra; this valve is the most common cause of bladder outlet obstruction in male children (36). The valve is believed to result from abnormal embryologic development of the fetal posterior urethra. The classic categorization of posterior urethral valves into types I, II, and III was developed by Young *et al.* in 1919 (60) and has undergone modification over time based on clinical observation and a better understanding of the embryologic events that lead to normal urethral development. This anomaly is not related with genetic disorders; however it was reported with familial inheritance (61). In PUV the major aim is the timing of surgical intervention (pre- or post-natal) and the prediction of the progression stage of chronic kidney disease (CKD) (34). CE-MS has been applied in a cohort of 28 patients with PUV with the aim to identify fetal urinary biomarkers that allow prediction of post-natal renal function (62). In that study Klein *et al.* identified 26 specific fetal urinary peptides biomarkers that characterized early ESRD. Twelve of these 26 peptides were combined in a classifier called the “12PUV” classifier, which displayed a sensitivity of 86% and a specificity of 95% for the prediction of post-natal renal function in an independent validation cohort of 38 patients with PUV. The main markers associated to early ESRD in PUV were collagen fragments. One particularity of this study was the abundance of these collagens in disease. Collagens were increased in fetal urine of patients with PUV displaying early ESRD. This is the opposite to what is observed in postnatal urine in patients with CKD (62) suggesting that instead of fibrosis in CKD, patients with PUV display increased extracellular matrix turnover due to disruption of nephrogenesis (visible as dysplasia or hypoplasia in fetopathology). One fetal urinary biomarker of early ESRD was identified as a fragment of the XLαs variant of the G-protein-α subunit (GNAS).

GNAS and its variants represent imprinted genes (specific maternally or paternally transmitted active copies of genes due to methylation patterns) and are described to have major effects on growth in utero and after birth.

Trnka *et al.* studied the potential correlation between post-natal urinary protein levels and kidney function in patients with PUV (63). In total, 47 subjects were studied: 27 patients with PUV and 20 controls, performing immunoblotting technique as well as determination of glomerular filtration rate (GFR). They were able to demonstrate significant differences in the excretion of some proteins between the two groups. Aquaporin-2 was significantly decreased. On the other hand, the urinary protein-to-creatinine, whole-urine TGF-β and L1 cell adhesion molecule (L1CAM) were considered the best potential biomarkers, because they were significantly increased in urine of PUV patients and presented better correlation with GFR.

Using post-natal urine of 30 patients with PUV, Mandelia *et al.* investigated a number of urinary proteins and also the effects of angiotensin-converting enzyme inhibitors (ACE-I) on renal recovery (64). The pre- and postoperative protein levels of TGF-β1, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and microalbuminuria were measured over a period of 1 year. TGF-β1, TNF-α, and microalbuminuria were increased in patients with PUV. These proteins can be potential biomarkers because they reflect potential activation of the renin–angiotensin system, as well as they can provide an early recognition of children with ongoing renal damage. The treatment with ACE-I indicated an improvement of the kidney physiology and decrease of urinary TGF-β1 and microalbuminuria, allowing the retarding of renal injury in PUV patients and preserve the renal function in PUV patients (64).

4.3. *VUR*

Vesicoureteral reflux (VUR) can be detected and diagnosed by voiding cystourethrography which is an invasive and uncomfortable method for the children (38). VUR is frequently genetically heterogeneous, and emerges from interruption of complex signaling pathways as well as cell differentiation, which may be influenced by environmental factors (65). Urine from

73 children was collected and analyzed by CE-MS. To identify potential biomarkers, 18 patients with primary VUR (grade IV or V) and 19 patients without VUR were used. Nine urinary peptides were found, which were differentially regulated and a VUR-classifier was generated based on these peptides. A subsequent blinded evaluation has been performed on 17 patients with VUR grade IV or V and 19 patients without VUR with 88% sensitivity and 79% specificity. Five of the nine urinary biomarkers were successfully sequenced. They included 3 collagen alpha-1 (I) chain fragments, a sodium/ potassium-transporting ATPase and a CD99 antigen fragment.

5. Urinary RTM biomarkers from animal models

The UPJO animal model is the most frequently used RTM animal model. This model was used to study potential protein-based urinary markers of the disease including interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin 10 (IL-10)), TNF-α, and interferon-γ (IFN-γ), etc. that are known to act as intercellular mediators in the cellular and molecular events of in UPJO.

Madsen *et al.* performed a study using two different rat UPJO models (the basic rat model is depicted in **figure 2**) to analyze the urinary abundance of these different potential biomarkers with the use of a bead-based multiplex sandwich immunoassay (66). One complete acute obstruction model (complete unilateral ureteral obstruction (CUUO) for 48 hours) and one partial chronic obstruction model (partial unilateral ureteral obstruction (PUUO) for 10 weeks) was employed. In the CUUO model IL-1β, IL-6, TNF-α, and IL-10 showed significant differences in comparison with controls: IL-1β and IL-10 were detected at significantly decreased levels, with IL-6 and TNF-α found at significantly higher concentrations in urine from the left obstructed kidney. In the PUUO model only IL-6 showed a significant increase in urinary abundance. Another study compared the protein profiles in a PUUO model in neonatal rats (obstruction at 1 day of age, randomized into 4 groups: 1, 2 and 5 days of obstruction and sham surgery) using 2-D gel electrophoresis coupled to MS (2DE-MS) (67). Forty-three proteins with differential urinary abundance were identified, which were reported

to be involved in the regulation of the cytoskeleton and actin, glucose metabolism, cell apoptosis, mitochondrial energy metabolism, oxidative stress, and endoplasmic reticulum stress. Electron transfer flavoprotein subunit beta (ETFB) identified as downregulated in urine by 2DE-MS was validated by immunoblot analysis. ETFB mRNA levels was also decreased in renal tissue from PUUO rats.

Another 2DE-MS study using an adult CUUO (three time-points (12 h, 24 h and 72 h)) rat model was performed by Zhao *et al*. They identified 39 proteins with different urinary abundance between the sham operated group and the CUUO group (68). Cell apoptosis, energy metabolism, injuries of mitochondrion and oxidative stress were some of the biological processes associated with these proteins. In addition, based on immunoblot analysis and immunofluorescence staining and assessment of the mRNA levels in renal tissue, they confirmed changes of 3 proteins: peroxiredoxin-1 (PRDX-1) was increased, glutathione peroxidase 1 (GPX1) and glutathione S-transferase P1 (GSTP1) were decreased. This study allowed a better understanding between oxidative stress and obstructive nephropathy.

To study the acute urinary proteomic alterations induced by bilateral ureteral obstruction (BUO), MS-based proteomics was applied in rats that were subjected for three different time-points (2, 6, 24 h) to BUO (69). In this study 109 proteins associated with different biological processes involved in cytoskeleton and cytoskeletal regulation were identified. Western blots confirmed the selected results, demonstrating acute downregulation of proteins belonging to all three cytoskeletal components: the microfilament protein β-actin and the intermediate filament proteins pankeratin and vimentin, as well as β-tubulin, an important microtubular protein, were all downregulated. Furthermore, there was a significant upregulation of cofilin, an actin-binding protein. **Table 2** summarizes potential urinary biomarkers of RTM identified in animal models.

6. Expert commentary

In recent years a substantial number of studies have performed urinary proteome analysis in order to identify potential biomarkers of RTM. Only a few urinary biomarkers have been found to be unequivocally associated to UPJO. This is the case for urinary TGF-β1 that in all studies increased in UPJO, and decreased after relief of the obstruction. Studies on urinary EGF, although initially promising, turned out to be less clear and with time it appeared that EGF can be a urinary marker for many diseases thereby losing its specificity for UPJO. This also points to the important fact that to be marked as a "biomarker" validation should be performed in independent studies.

TGF-β1 and EGF were investigated as "isolated" markers of UPJO obstruction using ELISA and might perform better in a panel of markers. It has now been repeatedly observed that complex disease such as RTM cannot be faithfully described with single markers and panels of markers are more likely to "catch" the perturbed system, similarly to everyday medicine where a diagnosis is rarely based on a single observation. MS-based proteomics serves this goal. In this context it was observed, as described in detail above (sections 4.1-4.3), that panels of urinary peptides could predict the outcome in the different RTM, such as 51 UPJO-specific biomarkers (51), 12 related to PUV (62) and 9 concerning VUR (38).

7. Five-year view

In the coming years these initial successes obtained with single or multiple biomarkers still need to be confirmed to be applicable in the clinic. This involves larger scale studies (several hundreds of individuals/trial) the reach sufficient power. This might involve, in addition to the peptide panels discovered with MS-based tools, combination of markers (e.g. ELISA panels including TGF-β1, EGF, cytokines observed in animal models, etc.).

Finally, studies including other omics traits in urine (non-coding RNAs, metabolites, etc.) might allow adding additional markers to the panels and i) increase their specificity for the stratification of patients with RTM and ii) improve the understanding of the pathophysiology to allow optimised management of this frequent disease in children.

8. Key issues

- Urine is an important source of potential biomarkers, presenting several advantages such as the collection in large quantities, as a non-invasive method and as its stability.
- Biomarker validation in an independent cohort is a crucial step for clinical proteomics, and it can be performed through mass spectrometry techniques (e.g. LC-MS, CE-MS) or immunoassay techniques (e.g. ELISA, WB).
- RTM are congenital anomalies of the kidneys and urinary tract, and are more common in infants, but can be also presented in childhood and adulthood.
- Development of non-invasive methods, such as urinary proteomics, can be useful to understand the progression of the different types of RTM, as well as to allow an early diagnosis, prognosis and a better clinical decision-making.
- Several studies in clinical proteomics have been performed, and a panel of biomarkers is more efficient than the use of a single biomarker.

Studies with high numbers of samples coupled to statistical analysis and an independent validation should be conducted to generate a classifier for RTM.

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Declaration of Interest

H. Mischak is cofounder and a shareholder of mosaiques diagnostics GmbH. P. Magalhães and P. Zürbig are employees of mosaiques diagnostics GmbH. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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Reference annotations

** Of interest*

*** Of considerable interest*

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Figure 1: Different types of urinary tract malformations in humans.

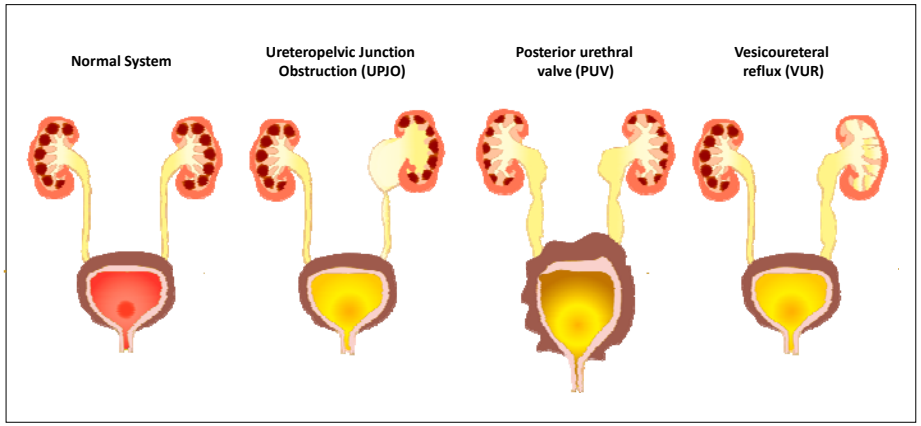

Figure 2: Example for an induced ureteral obstruction in rats. Reproduced with permission from the Danish Medical Journal. Madsen MG. Urinary biomarkers in hydronephrosis. Dan Med J 2013 Feb;60(2):B4582 (66).

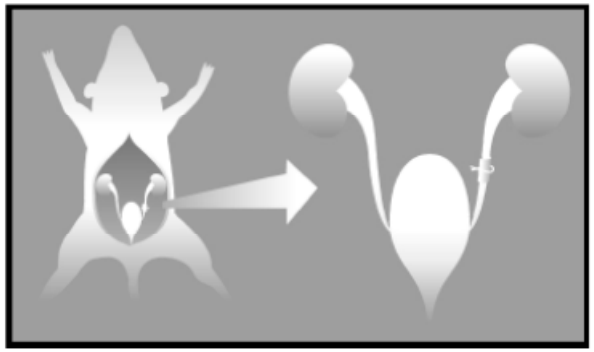

Table 1: Summary of potential urinary biomarkers for RTM in studies concerning human samples.

Disease	Biomarker	Method	Species	Performance	Pathways/ Biological Process	Validity	Reference
UPJO	TGF-β1	ELISA	human	Diagnosis	Fetal development, cell differentiation (leading to renal fibrosis)	significant (AUC=0.75)	(36-39)
	EGF	ELISA	human	Diagnosis	Modulation of tubular cell growth	questionable (AUC=0.79)	(36;40-43;52;54)
	MCP-1	ELISA	human	Diagnosis	Specific chemotactic and activating factor for monocytes	significant (AUC=0.78)	(40;41;43)
	ATG	ELISA	human	Diagnosis	Growth factor activity	significant (AUC=0.84)	(44;45)
	KIM-1	ELISA	human	Diagnosis	Kidney injury, tubulointerstitial inflammation and fibrosis	significant (AUC=0.80)	(46)
	NGAL	ELISA	human	Diagnosis	Activation of specific iron-dependent	significant (AUC=0.92)	(46;47)
	51 peptides (including different collagen fragments)	CE-MS	human	Diagnosis and Prognosis	Collagen turnover	significant (AUC=0.92)	(48;49;51)
	Different proteins	LC-MS	human	Diagnosis	Cell differentiation, signalling pathway	questionable	(53;54)

	Arginase 1 (beside other proteins)	LC-MS, WB, MRM	human	Diagnosis	Urea Cycle (development of renal fibrosis)	significant	(52)
	GSTM1, HSPA1A (beside other 74 proteins)	LC-MS, WB	human	Diagnosis	Stress response, transferase	significant	(45)
PUV	12 peptides (including different collagen and GNAS fragments)	CE-MS	human	Diagnosis and Prognosis	Collagen turnover, Signal transduction	significant (AUC=0.92)	(58)
	AQP2, TGFβ, L1CAM	2D-MS, WB	human	Diagnosis	Cell differentiation, transport	significant ($AUC_{TGF\beta}$=0.79) (AUC_{L1CAM}=0.80)	(59)
	TGF-β1, TNF-α	ELISA	human	Diagnosis	Cell differentiation	significant	(60)
VUR	9 peptides (including different collagen fragments, sodium/ potassium-transporting ATPase fragment, CD99 antigen fragment)	CE-MS	human	Diagnosis	Collagen turnover	significant	(35)

Table 2: Summary of potential urinary biomarkers for RTM identified on animal models.

Disease	Biomarker	Method	Species	Performance	Pathways/ Biological Process	Validity	Reference
UPJO	Arginase 1 (beside other proteins)	LC-MS, WB, MRM	mice	Diagnosis	Urea Cycle (Development of renal fibrosis)	significant	(55)
CUUO	IL-1β, IL-6, TNF-α, IL-10	WB	rat	Diagnosis	Cytokine activity, Signaling and regulatory/growth factors	significant	(66)
	PRDX1, GSTP1, GPX1	2D-MS, WB	rat	Diagnosis	Antioxidation related to oxidative stress	significant	(68)
PUUO	IL-6	WB	rat	Diagnosis	Cytokine activity, Growth Factor	significant	(66)
	ETFB	2D-MS, WB	rat	Diagnosis	Specific electron acceptor (associated to fibrotic processes)	significant	(67)
BUO	β-actin, pankeratin, vimentin, β-tubulin, cofilin	LC-MS, ELISA	rat	Diagnosis	Cytoskeletal regulation	significant	(69)