Urinary biomarkers in hydronephrosis

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THE FOUR ORIGINAL PAPERS ARE

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INTRODUCTION

Hydronephrosis is the most common genitourinary tract anomaly detected on prenatal ultrasound studies, reported in approximately 1–5% of all pregnancies [1]. The prevalence depends on the diagnostic criteria for significant dilatation. A Danish review from 2006 showed that the prevalence of prenatal urological anomalies was 1–2%, with prenatal hydronephrosis being the most common (anterior-posterior (AP) diameter > 5 mm at the 20th gestational week). The prevalence in newborns was 0.5% (AP diameter > 10 mm), boys were more commonly affected, and 80% of cases were unilateral [2].

Hydronephrosis is an anatomical entity that is defined as an enlargement of the capacity of the collecting system of the kidney, calices, and pelvis [3], and represents a wide spectrum of urological conditions. The most common causes of hydronephrosis in neonates in order of frequency are ureteropelvic junction obstruction (UPJO) (35 %), ureterovesical junction anomalies, vesicoureteral reflux, multicystic kidney, and posterior urethral valves. Other causes include obstructive and non-obstructive megaureter, ureterocele, neurogenic bladder, prune-belly syndrome, and urethral atresia [4].

Conversely, obstruction can be defined clinically as a condition of impaired urinary drainage that, uncorrected, will limit the ultimate functional potential, including the renal functional reserve capacity and response to stress [5]. Therefore, it is clear that hydronephrosis is not synonymous with obstruction, and differentiation between a dilated obstructed and a dilated nonobstructed kidney is often a problem [6].

UPJO shows different degrees of severity, with several possible causes. The most common causes of UPJO are an adynamic ureteral segment at the junction between the ureter and the renal pelvis, or an extrinsic compression of the proximal ureter by the presence of accessory lower pole renal vessels, and, more rarely, it may be the result of an abnormal departure of the ureter from the pelvis or intrinsic anomalies [7].

Symptoms of obstructive hydronephrosis show a wide spectrum from asymptomatic patients to patients with flank pain, urinary tract infection (UTI), a palpable abdominal mass, or symptoms of impaired kidney function. The obstruction may change temporally, i.e., diminish over time, become progressive, or occur intermittently [8]. Previously, patients with hydronephrosis presented with one or more of these symptoms, which were the compelling indications for surgical intervention. Occasionally, patients with a severe obstruction were diagnosed and/or treated too late, resulting in impaired renal function and, in a few of the worst cases, renal insufficiency.

The impact of prenatal ultrasonography on diagnosing hydronephrosis has increased over the last few decades, creating a new group of patients that are diagnosed early and, thereby, a population of asymptomatic infants with varying degrees of unilateral hydronephrosis. Some patients benefit from this early diagnosis, resulting in timely intervention and preserved kidney function, but the majority ends up with prenatal findings of uncertain long-term clinical significance, and possibly unnecessary interventions.

The clinical management of these patients remains a controversial topic. The aim is to preserve renal function by selecting the 15–20% of children who require early surgical intervention from those for whom watchful waiting may be appropriate because of spontaneous resolving/stabilization without a significant loss of renal function. Today this requires repetitive ultrasonographies, diuretic renographies and, in selected cases, determinations of the glomerular filtration rate (GFR). These examinations can be time-consuming and distressing to the child and, still, are not sensitive or specific enough to identify those kidneys that require treatment in all cases.

Today the most used indications for surgery are (1) declining function of the hydronephrotic kidney by more than 5% and to less than 40% of the total renal function estimated by renography, (2) ipsilateral flank pain, (3) frequent pyelonephritis, (4) massive hydronephrosis, and (5) social indications [2]. However, these indications do not guarantee that the "right" patients are operated on, since there may be a tendency of redundant operations in a number of individuals, or observing for too long so that the reduced kidney function, especially in later life, could cause health problems [9].

Consequently, there is a great need for the development of new methods to monitor patients, and the urinary biomarker research field is a promising approach for this purpose. Urinary proteins provide a snapshot of the physiological situation and have the potential to be used as prognostic tools for early disease detection and the choice of the optimal treatment and monitoring [10].

BACKGROUND
Pathophysiology of ureteral obstruction
The renal response to a urinary tract obstruction in the developing kidney is complex and only partly elucidated. The pathological changes include interstitial inflammation, tubular apoptosis, and interstitial fibrosis [11,12], and the cellular and molecular events are dependent on interstitial cells and a variety of locally and systematically produced molecular products. These signaling molecules include an endless list of cytokines that act as intercellular mediators of paracrine communication [13]. Cytokines are small proteins or peptides that embrace several subgroups such as chemokines, interleukins, growth factors, interferons, lymphokines etc. [14]. They play a significant role in cell growth, death, and differentiation, and the function of the developing and mature kidney, but they also appear to be involved in the pathogenesis of obstructive nephropathy [11,15].

The initial reaction to an acute obstruction of the ureter with the subsequent increase in pressure is a prompt renal hemodynamic response, mediated by increased activity of the renin-angiotensin system, which leads to an increase in the renal vascular resistance of the obstructed kidney [16]. Various vasoactive mediators such as angiotensin [17], thromboxane [18], and endothelin [19] contribute to this complex and not completely understood response [14]. As mentioned previously, this is followed by an interstitial inflammatory response that is initially characterized by macrophage infiltration, tubular dilatation, and renal tubular apoptosis, leading to tubular atrophy and interstitial fibrosis with nephron loss [16].

Interstitial inflammation. The activation of the intrarenal renin-angiotensin system [20] with increased levels of angiotensin II activates, among others, nuclear factor-kappa B (NF-κB) and rho-associated coiled-coil forming protein kinase (ROCK), which initiate interstitial macrophage infiltration and activation [16,21]. In addition, the macrophages are stimulated by, among other factors, selectins, intercellular-adhesion molecule 1 (ICAM1), interleukin 1 (IL-1), monocyte chemoattractant peptide 1 (MCP-1), colony-stimulating factor 1 (CSF-1), and osteopontin (OPN). In contrast, the macrophages are inhibited by endogenous anti-inflammatory compounds, e.g., retinoids and inducible nitric oxide synthase (iNOS) [16]. The macrophages participate in the inflammatory response through the release of cytokines and growth factors, e.g., tumor-necrosis factor-α (TNF-α) [22], transforming growth factor-β1 (TGF-β1) [23], and platelet-derived growth factor (PDGF) [16].

Tubular apoptosis. The mechanical stretch of epithelial cells stimulates TGF-β1- and TNF-α-induced apoptosis in dilated tubules [24], and the infiltration of macrophages also induces apoptosis via the release of proinflammatory cytokines [22]. This tubular apoptosis is inhibited by several molecules, e.g., iNOS, epidermal growth factor (EGF), and insulin-like growth factor 1 (IGF-1). Several of these compounds have conflicting effects on the interstitial and tubular compartments [16].

Interstitial fibrosis. Deposition of the extracellular matrix is a consequence of the increased synthesis and reduced degradation. An epithelial-mesenchymal transformation, which is a transformation of renal tubular cells to fibroblasts, is the major mechanism contributing to interstitial fibrosis [25]. This reorganization is followed by the migration of cells into the interstitial space, and there is a further phenotypic transformation of the fibroblasts to myofibroblasts [12]. TGF-β1 has a central role in this, and is produced by tubular epithelial cells and interstitial fibroblasts, and has fibrogenic actions, which are partly mediated by the stimulation of the α-smooth muscle actin produced by interstitial fibroblasts [26]. Furthermore, angiotensin II stimulates the expression of TGF-β1 and α-smooth muscle actin, and angiotensin-dependent pathways account for 50% of interstitial fibrosis resulting from unilateral ureteral obstruction (UUO) [16]. Additional mechanisms for the progression of fibrosis include the release of compounds by infiltrating macrophages [26].

Urinary biomarkers
Definitions of biomarkers
A biomarker has been defined as *a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention* [27].

Most biological markers are not simply present or absent but have wide ranges of values that overlap in patients and healthy individuals, which is one of the challenging tasks in the identification of an ideal biomarker, since the biomarker has to be highly sensitive, specific, and have a high predictive value. Finally, a test for the biomarker should be available, and its low cost and ease of performance are also desirable elements.

Potential biomarkers in ureteropelvic junction obstruction – well-known and novel
Given the multifactorial nature of UPJO, it is unlikely that a single urinary biomarker will be identified that meets all of the criteria described above. The optimal urinary biomarker in UPJO should measure renal injury and the impairment of renal function. If the marker correlates with renal function, it will be a good marker of disease progression and may have the potential to predict which patients will require surgery and in which patients the UPJO will resolve. The potential exists to identify a panel of biomarkers that generates the required specificity and sensitivity and that is reproducible with a high predictive value [28].

To date, the most well-known candidate biomarkers of UPJO include TGF-β1, EGF, endothelin-1, MCP-1, and selected tubular enzymes. These biomarkers show different degrees of compliance to the above mentioned "ideal criteria." TGF-β1 has been examined in several clinical studies [29,30,31,32,33,34], and Taha et al. suggested that the urinary levels of TGF-β1 could be used as a non-invasive tool in the long-term follow-up of children with UPJO after pyeloplasty [29]. Further, Almodhen et al. showed that the urinary levels of TGF-β1 in the first 3 months of life were 82% sensitive and 86% specific in predicting the need for surgery in

newborns with prenatal hydronephrosis (grade 4 or 5) [30]. Taha et al. also examined a panel of 3 tubular enzymes (i.e., NAG, ALP, and GGT) and showed their significantly increased urinary concentration in children with UPJO, leading them to suggest a combination of these markers as non-invasive markers for the long-term follow-up of children with UPJO and also to differentiate children with UPJO needing surgery from those with a dilated non-obstructed kidney [35]. Their results confirm the study of Carr et al. [36]. Likewise, MCP-1 and endothelin-1 have been examined in clinical studies and showed increased urinary concentrations in children with UPJO [37,38,39]. Several studies have shown that children with UPJO have a marked reduction of renal EGF gene expression [32,38,40] but studies examining the urinary levels of EGF have shown contradictory results. Grandaliano et al. demonstrated decreased urinary concentrations of EGF in children with UPJO [38], whereas Taha et al. showed no significant differences between children with UPJO and controls [29]. Carbohydrate antigen 19-9, which has been applied as a clinically valuable tumor marker for pancreatic and gastrointestinal carcinoma, was examined in a study that included children with UPJO, showing significantly increased urinary levels compared to controls [41]. Recently, a study by Wasilewska et al. demonstrated significantly increased urinary concentrations of neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule 1 (KIM-1) in children with UPJO, and that these correlated negatively with differential renal function (DRF) [42].

Potential novel urinary biomarkers include a long list of cytokines, micro-proteins, etc. that act as intercellular mediators in the cellular and molecular events of obstructive nephropathy. One approach in the search for biomarkers is to investigate the cytokines that are already known to be up- or downregulated in either UPJO or other types of nephropathy [14].

Study I examined 6 cytokines: interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin 10 (IL-10), TNF-α, and interferon-γ (IFN-γ). They are reviewed in Table 1.

Study II focused on 9 potential urinary biomarkers: the above-mentioned growth factor EGF, 4 chemokines, i.e., MCP-1, interferon-γ-inducible protein 10 (IP-10), regulated on activation normal T-cell expressed and secreted (RANTES), and macrophage inflammatory protein-1α (MIP-1α), and 4 well-known biomarkers of kidney damage, i.e., beta-2-microglobulin (β2-M), cystatin C (CyC), NGAL, and OPN. They are all reviewed in Tables 2 + 3.

Table 1. Review of the 6 examined cytokines in Study I

Cytokine	Experimental studies	Clinical studies	References
Interleukin-1β (IL-1β)	Regulation of TGFβ-1. Activation of endothelin-1.	Induction of an increased expression of the NGAL receptor in mesangial cell in patients with glomerulonephritis.	[43,44,45,46]
Interleukin-2 (IL-2)	Stimulation of lymphoid cell proliferation.	Induction of apoptosis of renal tubular epithelial cells in patients with kidney transplant rejection.	[47,48,49,50]
Interleukin-6 (IL-6)	Activation and proliferation of lymphocytes. Recruitment of leukocytes.	Increased urinary levels in children with vesicoureteral reflux, in children with pyelonephritis, and in patients with IgA nephropathy.	[51,52,53,54]
Interleukin-10 (IL-10)	Downregulation of proinflammatory cytokines. Reduced production of chemotactic factors.	A high production have a long-term protective effect on kidney transplant outcome.	[51,55,56,57,58,59]
Tumor Necrosis Factor-α (TNF-α)	Induction of renal tubular cell apoptosis, renal fibrosis and dysfunction.	Increased levels correlate with severity of renal disease in patients with type 2 diabetes. Increased urinary excretion in membranous glomerulonephritis.	[51,60,61,62,63,64,65]
Interferon-γ (IFN-γ)	Stimulation of macrophages with induction of direct antimicrobial and antitumor mechanism.	Treatment with IFN-γ was associated with focal segmental glomerulosclerosis.	[66,67,68]

Experimental and clinical studies of the cytokine with respect to ureteral obstruction are mentioned. If there are no studies of ureteral obstruction, studies of the cytokine in other kidney diseases are mentioned.

Table 2. Review of the 5 examined cytokines in Study IIa

Cytokine	Experimental studies	Clinical studies	References
Epidermal growth factor (EGF)	Rats with chronic UUO had decreased renal EGF production. Exogenous EGF inhibited tubular apoptosis in rats with UUO.	Children with UPJO had a marked reduction of renal EGF expression. Urinary EGF levels in UPJO are still not clarified since contradictory results have been presented.	[29,32,38,40,69,70]
Interferon-γ-inducible protein 10 (IP-10)	Inhibitory effect upon renal tubular cell proliferation after ischemia-reperfusion injury. Increased levels within the tubular compartment in mice at 24 h after UUO.	Correlation between elevated IP-10 urinary levels after kidney transplantation and short- and long-term graft function.	[71,72,73]
Monocyte chemotactic peptide-1 (MCP-1)	Increased levels of MCP-1 within the tubular epithelium in rats with UUO. Clear correlation between the urinary levels of MCP-1 with the grade of obstruction in rats with PUUO.	Increased renal gene expression in children with UPJO. Increased urinary MCP-1 levels in children with UPJO.	[38,39,74,75,76]
Macrophage inflammatory protein-1α (MIP-1α)	Recruits macrophages to the kidney in a mouse model of hemolytic-uremic syndrome.	Strong correlation of MIP-1α with infiltrating macrophages within glomeruli in glomerulonephritis.	[77,78,79]
Regulated on activation normal T-cell expressed and secreted (RANTES)	Increased levels of RANTES within the tubular compartment in mice at 24 h and 7 days after UUO.	Increased urinary levels in children with UPJO (pilot study).	[71,80]

Abbreviations: UUO, unilateral ureteral obstruction; UPJO, ureteropelvic junction obstruction; PUUO, partial unilateral ureteral obstruction. Experimental and clinical studies of the cytokine with respect to ureteral obstruction are mentioned. If there were no studies of ureteral obstruction, studies of the cytokine in other kidney diseases are mentioned.

Table 3. Review of the 4 examined urinary proteins in Study IIb

Cytokine	Experimental studies	Clinical Studies	References
β2-microglobulin (β2-M)	Increased urinary levels in rats with UUO and PUUO.	Decreased urinary levels in infants after relief of UPJO. Increased urinary levels in children with UPJO.	[39,81,82, 83,84]
Cystatin C (CyC)	Increased urinary CyC levels in rats with glomerular injury.	Increased serum concentration of CyC in children with UPJO.	[85,86,87]
Neutrophil gelatinase-associated lipocalin (NGAL)	Increased pelvic urinary levels in mice with UUO.	Increased urinary levels in children with severe UPJO. Early and sensitive marker of kidney injury in several kinds of nephropathy.	[42,88,89, 90]
Osteopontin (OPN)	Increased levels in adult and neonatal mice with UUO. Mediates early interstitial macrophage influx and fibrosis in UUO/PUUO. Suppresses apoptosis.	Associated with inflammation in IgA nephropathy.	[91,92,93, 94,95]

Abbreviations: UUO, unilateral ureteral obstruction; UPJO, ureteropelvic junction obstruction; PUUO, partial unilateral ureteral obstruction. Experimental and clinical studies of the cytokine with respect to ureteral obstruction are mentioned. If there are no studies of ureteral obstruction, studies of the cytokine in other kidney diseases are mentioned.

PURPOSE

The aim of this study was to further elucidate the pathophysiology of UPJO. This was done by investigating the variations in the secretion of urinary cytokines after the release of UUO, and the examination of whether a potential change in the concentration of these compounds in the urine reliably reflects changes in the renal parenchyma. This was tested using 2 experimental animal models: an acute obstruction model and a chronic obstruction model.

An additional aim of this study was to search for potential candidate biomarkers that may have a predictive and/or diagnostic value in the management of UPJO. For this, a prospective study was conducted with the inclusion of children referred for pyeloplasty due to hydronephrosis caused by UPJO. With the collection of pre-, peri- and postoperative urine samples, the focus was on the dynamics of the urinary excretion pattern of selected potential biomarkers after the relief of UPJO and also a comparison to the urinary levels in healthy controls.

Hypotheses
Study I (Paper I)
i.   A potential change in the concentration of selected urinary cytokines after the release of experimental UUO reliably reflects changes of their levels in the renal parenchyma.
ii.   The excretion pattern of selected urinary cytokines after the release of experimental acute obstruction can be reproduced in an experimental model of chronic obstruction.

Study IIa (Paper II)
i.   Selected urinary cytokines are increased in children with severe UPJO compared to healthy controls.
ii.   Selected urinary cytokines are increased in urine from the obstructed kidney compared to urine from the contralateral kidney.
iii. The cytokines normalize to control values after the relief of UPJO.
iv.   The mRNA expression of the selected cytokines in renal

pelvic tissue from children with UPJO is up-regulated compared to renal pelvic tissue from healthy controls.

Study IIb (Paper III)
i.   Well-known kidney injury biomarkers are increased in urine from children with UPJO compared to healthy controls.
ii.   They are increased in urine from the obstructed kidney compared to urine from the contralateral kidney.
iii. They normalize to control values after the relief of UPJO.

MATERIALS AND METHODS
Study I: Experimental animal study
Experimental animals

Male Wistar rats (Møllegaard Breeding Center, Eiby, Denmark) were used and they were maintained on a standard rodent diet with free access to water. During the entire experiment, 2 rats were kept in each cage, with a 12:12 h artificial light-dark cycle, a temperature of 21°C ± 2°C, and humidity of 55% ± 2%.

Experimental unilateral ureteral obstruction (UUO) and partial unilateral ureteral obstruction (PUUO)

Anesthesia: The adult rats were anaesthetized with 0.5% Isoflurane (Abbott Scandinavia AB, Sweden). During surgery, they were placed on a heating board under an operating microscope. After each operation 0.2 mL of buprenorphine (Temgesic 0.3 mg/mL; Schering-Plough, Denmark) were injected subcutaneously. The newborn rats were placed on crushed ice for 8 min, which was sufficient to maintain anesthesia for 30 min. Additional ice was placed around the neck/head during the operation.

UUO operation: Through a midline abdominal incision, the left ureter was exposed and ligated with a 5-0 silk ligature (Ethicon, Denmark) (Fig. 1).

PUUO operation: Through a midline abdominal incision, the left ureter was exposed. The underlying psoas muscle was split to form a groove into which the upper two-thirds of the left ureter were embedded.

SHAM operation: Through a midline abdominal incision, the left ureter was exposed and left untouched. The abdominal muscle layers and skin were closed.
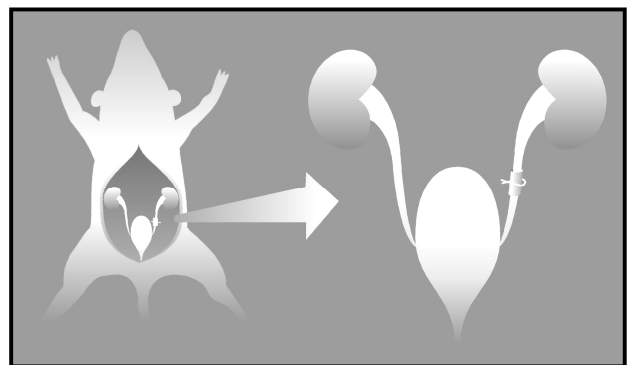

Figure 1. The unilateral ureteral obstruction

Urine collection: A polyethylene tube (Portex; Smiths Industries, England) was inserted into each ureter to allow separate collection of urine from the kidneys. The urine was collected into CryoPure Tubes (Sarstedt, Germany), and Complete Mini Protease Inhibitor Cocktail Tablets (Roche, Denmark) were added. An intravenous infusion (through a tail vein) of a 25 mM glucose solution (40 µL/min) was provided simultaneously to maintain an adequate minimum urine flow rate for biochemical analysis of the collected urine. Urine was collected for 3 h.

Handling of the kidneys: The kidneys were dissected into the inner medulla and cortex. The tissue was homogenized in 500 µL of Tissue Extraction Reagent (Invitrogen, CA, USA). The total protein concentration of the homogenate was measured using a Pierce BCA protein assay kit (Roche, Denmark).

Experimental protocols

UUO protocol: UUO was performed on 12 rats (weighing 220 g). After 48 h of obstruction, 6 rats were used for urine collection, and 6 rats had their kidneys removed. All rats were then sacrificed. Age- and time-matched sham-operated controls were prepared in parallel (*n* = 12).

PUUO protocol: One-day-old newborn Wistar male rats (5–7 g) were used. They were operated upon and separated from their mother on day 21. PUUO was performed on 12 rats. After 10 weeks of partial obstruction, 6 rats were used for urine collection and 6 rats had their kidneys removed. All rats were then sacrificed. Age- and time-matched sham-operated controls were prepared in parallel (*n* = 12).

Study II: Clinical prospective study
Inclusion of patients

Children (3–15 years old) diagnosed with unilateral UPJO were included in a prospective study from 2007–2011. Their inclusion took place at referral for a scheduled Anderson-Hynes pyeloplasty at the Department of Urology, Pediatric Section, Aarhus University Hospital, Skejby, Denmark.

Inclusion criteria: surgical treatment of UPJO indicated by either ipsilateral flank pain or declining function of the hydronephrotic kidney that was more than 5% and to less than 40% of the total renal function.

Exclusion criteria: bilateral hydronephrosis; previous surgery of the urinary system except surgery for phimosis and other deformations of the external genital organs; malformations in the lower ureter, bladder, and urethra; urinary stones; reflux; UTI; neurogenic bladder dysfunction; GFR < 40 % standardized to age; and non-compliance.

The day before the planned surgery, the patients underwent renal ultrasonography by an experienced pediatric radiologist to assess the AP diameter. The degree of hydronephrosis was graded using the Society of Fetal Urology (SFU) system: grade 1 is a visualization of the renal pelvis; grade 2 is a dilated renal pelvis and a few visualized calyces; grade 3 is a dilated renal pelvis with many identified calyces; and grade 4 is a similar appearance as grade 3, but the involved kidney has parenchymal thinning when compared with the normal kidney [96]. The patients also underwent a diuretic technetium-99m mercaptoacetyltriglycine (MAG3) renography. The labelled substance was injected (50 Ci/kg 99mTc MAG3), and after a 20-30 min observation, furosemide stimulation (0.5 mg/kg IV bolus) was given to the patients who did not eliminate at least 50% of the substance in the pelvis (T½). Elimination was monitored for another 20 min, and patients who did not achieve T½ by the end of the test were considered to have obstructive hydronephrosis. DRF of the obstructed kidney < 40% was considered abnormal.

All patients were operated on by a pediatric urologist with a robot-assisted retroperitoneoscopic Anderson-Hynes pyeloplasty with the insertion of a thin stent (Salle Pyeloplasty Stent 4.7 Cook Urological, Spencer, IN, USA) which was inserted to reduce the load on the anastomosis between the pelvis and ureter (Fig. 2). After the retroperitoneal approach, the ureter and pelvis were exposed. The ureter was spatulated at 1–2 cm past the stenotic area. After the stent was inserted, the anastomosis was sutured,

and the stent was guided through the skin and carefully attached with a bandage. The stent was closed on the first postoperative day and removed at the outpatient clinic after 3 weeks without anesthesia. It was possible to collect urine samples (e.g., in case of fever), and to rinse the stent in case of blockage due to blood-clots.
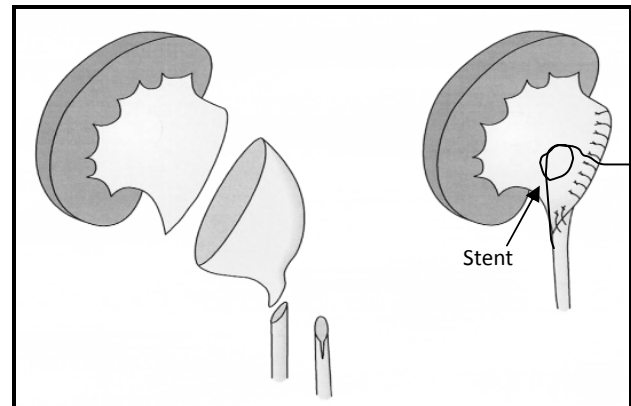

Figure 2. Anderson-Hynes pyeloplasty.

All patients followed an evaluation program (Table 4). The preoperative and postoperative (3 weeks, 3 months and 1 year) bladder urine samples were collected as voided midstream urine samples. The perioperative and postoperative (1 day) bladder urine samples were collected from a bladder catheter, and the urine was primarily from the non-obstructed kidney because urine from the obstructed kidney drained through the stent. Conversely, the postoperative (3 weeks) bladder urine was a mixture of urine from both kidneys since the stent was closed prior to sample collection.

A small piece of renal pelvic tissue was collected during the surgical procedure.

Table 4. Evaluation program

	Preoperative (1 day prior to operation)	Operation	Postoperative (1 day and 3 weeks)	Postoperative (3 months and 1 year)
Contacts	1	2	3+4	5+6
Evaluation	Renal ultra-sound and MAG3[1] renography			Renal ultra-sound and MAG3 renography
Urine sample from bladder	X	X	X	X
Urine sample from stent		X	X	

[1] MAG3, Technetium-99m mercaptoacetyltriglycine (performed < 3 months before the operation).

Inclusion of controls
Urine:

Healthy sex- and age-matched children were included. A voided midstream urine sample was collected. Morning urine was avoided to reduce the degradation of cytokines in the bladder/urinary tract.

Pelvic tissue:

From children: Pelvic tissue specimens were collected from children who had a nephrectomy due to congenital Finnish nephrotic syndrome. This is a rare autosomal recessively inherited disease caused by mutations in the gene encoding nephrin, which is a podocyte protein located at the glomerular slit diaphragm of the kidney [97]. The infants present with massive proteinuria and

a progression of glomerular and tubulointerstitial scarring [98]. In this study, we assumed that the pelvic tissue from the infants was normal. The children were included from the Helsinki Children's Hospital, Finland.

From adults: Pelvic tissue specimens were collected from adults who had a nephrectomy due to renal cell carcinoma. All patients underwent a renography, which showed a non-obstructed elimination phase. We assumed that they had healthy pelvic tissue that was comparable to pelvic tissue from children. They were included from the Department of Urology, Odense University Hospital, Denmark.

The exclusion criteria for all controls were the same as for the patient group and, in addition, they were excluded if they had contralateral hydronephrosis.

Storage and preservation of the collected material: Urine samples and pelvic tissue specimens were rapidly frozen and stored at –80°C until assayed. All analyses were performed in 2011 in order to limit the inter-assay variation by using assays with the same batch number. Multiple freeze/thaw cycles were avoided.

The urinary concentrations of creatinine (cr.) were measured by an enzymatic method (Vitros 950; Johnson & Johnson, Denmark).

Ethics statement

All animals were treated in accordance with the Danish National Guidelines for the care and handling of animals. The animal protocols were approved by The Institute of Clinical Medicine, Aarhus University, Denmark, according to the licenses for the use of experimental animals issued by the Danish Ministry of Justice. The clinical study was approved by the Local Ethics Committee (M-20070141) and was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki. The parents gave their informed consent prior to the inclusion of their child in the study. The inclusion of adult patients (pelvic tissue) was approved as a supplement to the clinical study (M-20070141, appendix 4). The inclusion of children (pelvic tissue) from Helsinki was approved by the Ethics Committee of the Children's Hospital, University of Helsinki, Finland.

Luminex

Cytokine profiles were measured by a bead-based multiplex sandwich immunoassay that utilizes the xMAP® detection technology developed by Luminex. This system allows for the sensitive and precise quantification of multiple analytes within a single sample. The Luminex 100 IS Total System (Luminex Corporation, Austin, TX, USA) equipped with StarStation version 2.3 software (Applied Cytometry, Sheffield, UK) was used. The assay principle is essentially an enzyme-linked immunosorbent assay (ELISA) on a bead. The assay includes analyte-specific antibody-conjugated differentially-dyed capture beads that allow for the capture and detection of analytes from various samples. Biotinylated detector antibodies are added to and incubated with a mixture of the sample and beads, and the detector antibodies bind to the appropriate immobilized analytes and form a bead-immuno-sandwich complex. Streptavidin R-Phycoerythrin is added and it binds to the detector antibodies during the incubation step. This allows for the quantification and detection of bound analytes. A mean fluorescence intensity (MFI) is generated by the Luminex instrument when measuring the assay reporter molecule on the multi-fluorescent coded microspheres. The limit of detection (LOD) is the lowest analyte concentration that can be detected in a sample, and the lower limit of quantification (LLOQ) is the low-

est analyte concentration that can be quantified with acceptable precision and accuracy. To measure the LOD of the assay, the standard deviation was multiplied by 2 and added to the MFI value of the blank assay. The LOQ range is dependent on the algorithm used to create the standard curves. The actual MFI versus the known concentration of standards was plotted and a 4- or 5-parameter logarithmic algorithm was used to generate a standard curve. This curve-fitting equation was used to calculate the concentration of unknown samples using the MFI as the input value. The standard recovery was calculated by taking the ratio of the calculated concentration value divided by the expected amount of standard and expressing that as a percentage. An acceptable range of recovery was between 70–130%. Accuracy is the closeness of the test results obtained by the analytical method to the true value, and is reported as the percent recovery of known added amounts of analyte in the sample matrix. An acceptable range of accuracy was within 70–130% recovery. Precision is the degree of agreement among individual test results when the analytical method is repeated for multiple measurements of a sample. The inter-assay precision should be less than 20% CV (CV represents the variation in concentrations).

Study I: IL1-β, IL-2, IL-6, IL-10, TNF-α, and IFN-γ were measured simultaneously in urine and tissue homogenates of kidney inner medulla and cortex using a Rat Cytokine 6-plex assay (Invitrogen, CA, USA). The assay was set up for duplicate measurements of standard analyte mixtures and samples (urine or tissue homogenate) according to the manufacturer's instructions. The measured concentrations were normalized to either the total protein concentration (tissue homogenates) or urinary creatinine concentration (urine).

Study IIa: EGF, IP-10, MCP-1, MIP-1α, and RANTES were measured simultaneously in urine using a Procarta Cytokine Assay Kit (Affymetrix, Ramcon, Denmark). The assay was set up for duplicate measurements of standard analyte mixtures and triplicate measurements of urine samples according to the manufacturer's instructions.

Study IIb: CyC, β-2 M, NGAL, and OPN were measured simultaneously in urine using a BeadPlex Human Kidney Toxicity/Injury Panel 2 (Widescreen; EMD Chemicals Inc., Merck, Germany). The assay was set up for duplicate measurements of standard analyte mixtures and urine samples according to the manufacturer's instructions.

To avoid dilution effects and to standardize samples, urinary levels were expressed as the ratio of cytokine to urinary creatinine.

Quantitative real-time polymerase chain reaction (QPCR)

To evaluate the quantitative mRNA levels of the 5 cytokines in study IIa, QPCR was performed on control pelvic tissue and on pelvic tissue from 9 patients with a DRF < 40% of the obstructed kidney.

Total RNA was isolated from the pelvic samples using a Machery-Nagel's NucleoSpin® RNA II kit and DNAse digestion was routinely performed. RNA was quantified using spectrophotometry and stored at –80oC. cDNA synthesis was performed on 1 μg RNA using a RevertAidTM First Strand cDNA Synthesis Kit (MBI Fermentas, Ontario, Canada) according to the manufacturer's instructions.

For QPCR, 100 ng of cDNA in duplicate were used as the template and mixed with the respective primers (see Paper II, Table 6) for PCR amplification. We used a Maxima® SYBR Green QPCR Master Mix according to the manufacturer's instruction (Stratagene, AH Diagnostics, Denmark). The mixture was dena-

tured for 3 min at 95°C and 40 cycles were run on a MX3000P QPCR machine (Stratagene, AH Diagnostics, Denmark) as follows: denaturation for 30 s at 95°C and annealing and extension for 60 s at 60°C. Emitted fluorescence was detected during the annealing/extension step in each cycle. The standard curve was constructed by plotting threshold cycle (Ct-values) against serial dilutions of the purified PCR product. Negative controls with no cDNA were run in each plate. The specificity of the PCR product was confirmed post-run for each plate setup by melting curve analysis. Random samples from each plate were loaded on agarose gels to confirm a single amplification product of the expected size.

Statistical analysis

The STATA/IC 11.0 (StataCorp LP, College Station, TX, USA) software package was used for statistical analyses.

Study I: Statistical comparisons between groups were made by a two-sample t-test or a two-sample Wilcoxon rank-sum test, if the data did not follow a normal distribution and/or had equal variance. Statistical comparisons between the obstructed and the non-obstructed kidneys in the UUO and PUUO groups were made by a paired t-test or a Wilcoxon signed rank test. A p-value < 0.05 was considered significant.

Studies IIa+b: Due to the small sample size, non-parametric statistical analyses were used. A Mann Whitney U test was used to compare the patients and controls. Kruskal-Wallis and Wilcoxon signed rank tests were used for comparisons within the patient group. Correlations were calculated using Spearman's test. A receiver operating characteristic (ROC) curve analysis was used to determine the cut-off values of urinary NGAL (uNGAL) and uβ2-M that yielded the best sensitivity and specificity. A p-value < 0.05 was considered significant. The data from the measurements of the urinary cytokines were not normally distributed; therefore, the values are presented as medians with ranges in parentheses. The mRNA data from the pelvic tissue were normally distributed; therefore, the values are presented as means with standard error.

RESULTS
Study I: Experimental animal study

Obstruction was associated with significant differences in the concentrations of IL1-β, IL-6, IL-10, and TNF-α between the groups. IFN-γ and IL-2 were measurable in kidney tissue homogenates and in urine, but there was no significant difference between either of the groups.

The weight of the left obstructed kidney was significantly higher, and the creatinine concentration in the urine from the obstructed kidney was significantly lower in the UUO group than in the SHAM group. These differences were not found between the PUUO group and the SHAM group.

IL1-β: In the UUO group, there were significantly lower concentrations in the inner medulla and urine from the right non-obstructed kidney. There was no significant change in the PUUO group.

IL-6: In the UUO group, there were significantly higher concentrations in the inner medulla and in urine from the left obstructed kidney. In the PUUO group there were significantly higher concentrations in urine from the left obstructed kidney.

IL-10: In the UUO group, there were significantly lower concentrations in the cortex and urine from the left obstructed kidney. IL-10 was not detectable in urine samples from the PUUO group.

TNF-α: In the UUO group, there were significantly higher concentrations in urine from the left obstructed kidney and, corresponding to this, a tendency toward a higher concentration in the left obstructed inner medulla. There was no significant change in the PUUO group.

Study II: Characterization of the study group

Twenty-eight children operated on for unilateral UPJO were included in the study. Pre-, peri-, and postoperative urine samples were collected (1 day, 3 weeks, 3 months, and 1 year), and the dynamics of the measured urinary proteins were analyzed and compared to the measured concentrations in urine samples from 13 healthy sex- and age-matched controls.

The clinical data from the study group are presented in Table 5. Six patients were excluded after the first postoperative day; 2 patients had a nephrostomy catheter inserted due to pain, 2 patients had a UTI, and 2 patients had the stent removed before the scheduled time at 3 weeks due to pain. One patient did not attend the 3 month control evaluation, and 3 patients did not attend the 1-year control evaluation. Eighteen patients attended the 1-year follow-up exam. Nine patients had a DRF < 40% of the total kidney function, indicating that the hydronephrotic kidney was impaired.

The control group was comparable to the patient group with respect to gender (8 males and 5 females) and age (median age 8.3 [3.5–14.5] years). The median age of the patients was 8.1 (3.5–14.5) years.

Control pelvic tissue was collected from 4 infants (median age 8 [6–8] months) and 6 adults (median age 66 [52.5–82] years).

Table 5. Clinical characteristics of the patient group

Category	Subcategory	Results
Gender	Female	12
	Male	16
Age (years)	At time of diagnosis	6.5 (0–14)
	At time of surgery	8.1 (3.5–15.0)
Laterality	Left	15
	Right	13
Operative findings	Stenotic segment	10
	Accessory vessels	18
SFU[1] grade	1	0
	2	3
	3	19
	4	6
Differential renal function (%)	Preoperative < 40%	9
	Preoperative (n = 28)	44 (20–54)
	3 months postoperative (n = 21)	49 (20–53)
	1 year postoperative (n = 18)	49 (33–53)
Anterior-posterior diameter (mm)	Preoperative (n = 28)	35.5 (13–65)
	3 months postoperative (n = 21)	16.0 (5–40)
	1 year postoperative (n = 18)	20.0 (7–30)

The values are presented as medians with ranges given in parentheses.
[1]SFU, Society for Fetal Urology grading, see text.

Study IIa

The concentrations of EGF, IP-10, MCP-1, MIP-1α, and RANTES were measured in urine samples from all 28 patients and 13 controls. Figure 3 illustrates the dynamics of the urinary excretion of the 5 cytokines after relief of the obstruction, and the levels in patients are compared with controls. The perioperative and postoperative (1 day) concentrations of all 5 cytokines were significantly different from the controls. In addition, the preoperative concentrations of EGF, MCP-1, and MIP-1α were significantly different from the controls. All 5 cytokines showed the

same excretion pattern with significantly increased concentrations on the day of surgery and the first postoperative day, after which their concentrations declined and stabilized to a lower level after 3 months.
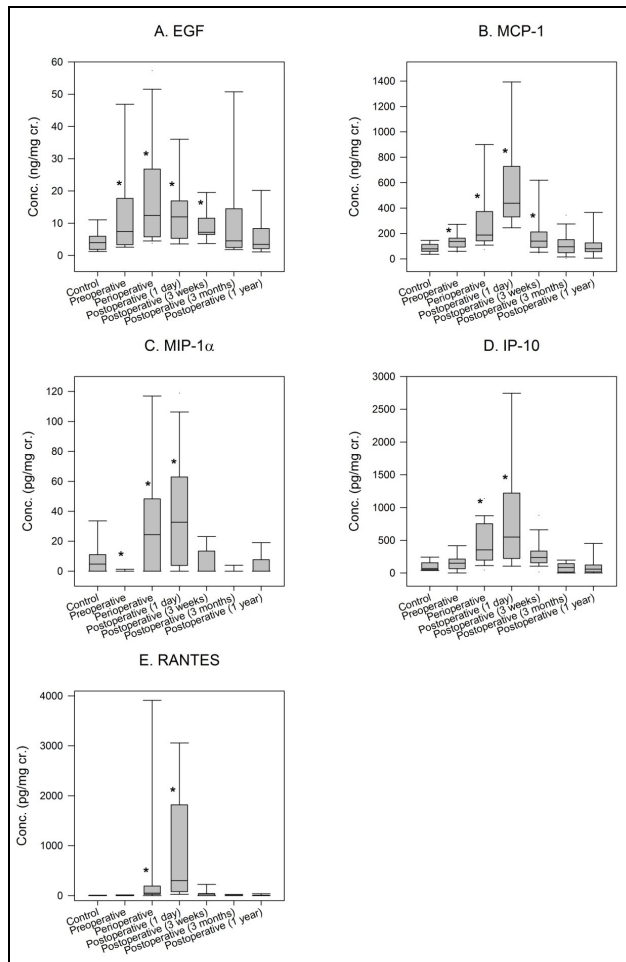

Figure 3. Urinary concentrations of EGF, MCP-1, MIP-1α, IP-10, and RANTES in patients and controls
The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 90th and 10th percentiles, respectively. In order to make the medians comparable, the perioperative and postoperative (1 day) concentrations were calculated as an average of the concentrations in urine from the obstructed kidney and the non-obstructed kidney, respectively. The postoperative (3 weeks) concentrations are from bladder urine.
*$p < 0.05$, patients compared to controls.

The concentrations in urine from the obstructed kidney were significantly higher than in urine from the non-obstructed kidney in the perioperative samples (MCP-1, MIP-1α, IP-10, and RANTES) and in the samples from the first postoperative day (EGF, MCP-1, IP-10, and RANTES). Figure 4 illustrates the individual values of EGF and MCP-1 in each patient ($n = 16$) in the preoperative and postoperative (1 year) samples. The urinary concentrations of EGF and MCP-1 were decreased in 73% and 81% of the patients, respectively.

ROC curve analysis was used to determine the diagnostic profiles of the preoperative urinary concentrations of EGF and

MCP-1. The area under the curve (AUC) was EGF: 0.75 (95% confidence interval [CI], 0.60–0.90) with the best cut-off value of 4.71 ng/mg cr. (sensitivity, 70.4%; specificity, 69.2%), and MCP-1: 0.78 (95% CI, 0.63–0.92) with the best cut-off value of 93.19 pg/mg cr. (sensitivity, 77.8%; specificity, 69.2%).
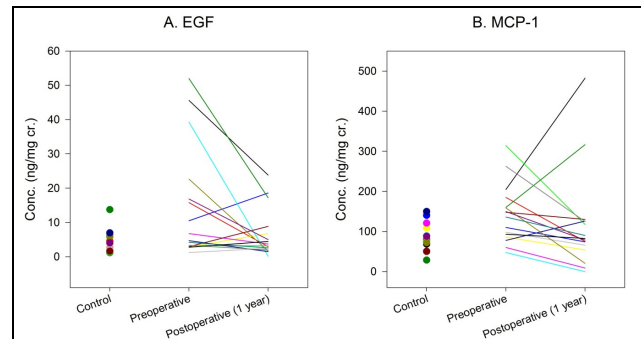

Figure 4. Urinary concentrations of EGF and MCP-1 in controls and patients
Patients who attended the 1-year follow-up ($n = 16$) are presented together with controls ($n = 13$).
A. Eleven patients showed decreased uEGF in the follow-up period, whereas 5 patients showed an increase; therefore, the difference between the pre- and postoperative concentrations was non-significant ($p = 0.079$). The preoperative concentration of uEGF was significantly higher compared to the controls ($p = 0.012$), whereas the postoperative concentration did not differ from the controls ($p = 0.693$).
B. Thirteen patients showed decreased uMCP-1 in the follow-up period, whereas 3 patients showed an increase; therefore, the difference between the pre- and postoperative concentrations was non-significant ($p = 0.121$). The preoperative concentration of uMCP-1 was significantly higher compared to the controls ($p = 0.005$), whereas the postoperative concentration did not differ from the controls ($p = 0.861$).

The preoperative urinary concentrations of EGF and IP-10 were negatively correlated with the AP diameter (EGF: $r = -0.461$, $p = 0.016$; IP-10: $r = -0.469$, $p = 0.014$). The IP-10 and MCP-1 concentrations in urine from the obstructed kidney (perioperative) were negatively correlated with DRF (IP-10: $r = -0.572$, $p = 0.002$; MCP-1: $r = -0.415$, $p = 0.031$), and the same tendency was seen for EGF, MIP-1α, and RANTES although it was not significant ($p = 0.359$, $p = 0.064$, and $p = 0.066$, respectively). There was no significant correlation between the measured urinary cytokines and the gender, age, or operative findings.

QPCR was performed on control pelvic tissue and on pelvic tissue from 9 patients with a DRF < 40% of the obstructed kidney. In the control group, a comparison between the infants and the adults showed no significant difference in the mRNA expression of the cytokines and the reference gene, TATA box-binding protein (TBP), respectively, and therefore the data were pooled. The results are presented in Figure 5. The mRNA levels of MIP-1α were significantly down-regulated in the pelvic tissue from patients compared to controls ($p = 0.027$). There was no significant difference in the regulation of EGF, IP-10, MCP-1, and RANTES, although there was a tendency toward an up-regulation of MCP-1 mRNA in the pelvic tissue from patients compared to controls.
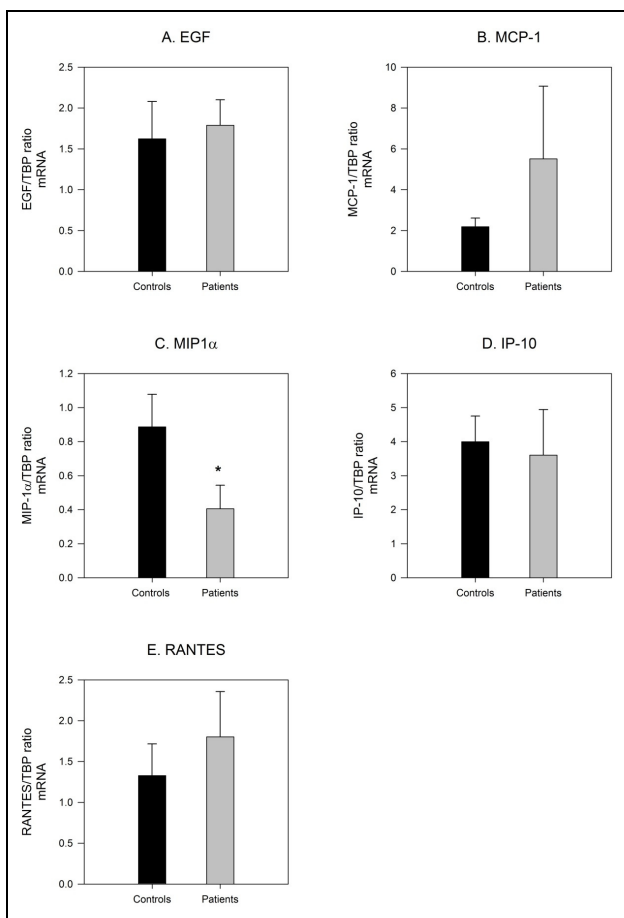
Figure 5. EGF, IP-10, MCP-1, MIP-1α, and RANTES mRNA expression in pelvic tissue
The values are expressed as means with standard errors. TBP, TATA box-binding protein.
*$p < 0.05$, patients compared with controls (rank-sum test).

Study IIb

The concentrations of NGAL, CyC, β2-M, and OPN were measured in urine samples from 24 patients and 13 controls. Four patients from the group of 28 patients were excluded due to an insufficient amount of urine sample.

Figure 6 illustrates the dynamics of the urinary excretion of the 4 proteins after relief of the obstruction, and the levels in patients are compared with controls. The perioperative and first day postoperative concentrations of NGAL and β2-M were significantly different from the controls. This was followed by a decline in the follow-up period to levels that were not significantly different from the controls after 3 months and 1 year. CyC showed significantly changes in its urinary excretion pattern in the patient group with an increase in the perioperative and postoperative (1 day) samples. However, there was no significant difference compared to the controls. OPN did not show a significant difference in its excretion pattern in the patients group within the 6 time points.

All 4 proteins showed significantly increased concentrations in urine from the obstructed kidney compared to urine from the non-obstructed kidney (postoperative [1 day] samples) and to urine from the controls. This was also the case for NGAL and CyC in the perioperative samples.
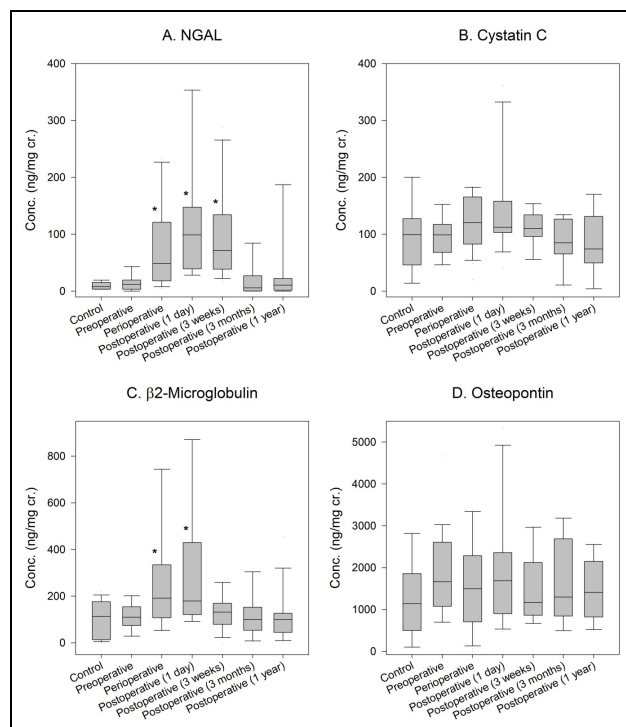

Figure 6. Urinary concentrations of NGAL, CyC, β2-M and OPN in patients and controls
The boundary of the box closest to zero indicates the 25th percentile, a line within the box marks the median, and the boundary of the box farthest from zero indicates the 75th percentile. The error bars above and below the box indicate the 90th and 10th percentiles, respectively. In order to make the medians comparable, the perioperative and postoperative (1 day) concentrations were calculated as an average of the concentrations in urine from the obstructed kidney and the non-obstructed kidney, respectively. The postoperative (3 weeks) concentrations are from bladder urine.
*$p < 0.05$, patients compared to controls.

Figure 7 illustrates the individual values of NGAL and β2-M in each patient ($n = 18$) in the perioperative (i.e., urine from the obstructed kidney) and postoperative (1 year) samples. The urinary concentrations of NGAL and β2-M decreased in 75% and 81% of the patients, respectively.
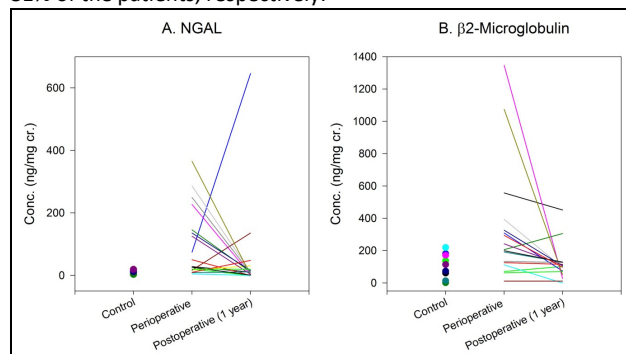

Figure 7. Urinary concentrations of NGAL and β2-M
Only patients who attended the 1-year follow-up evaluation are presented ($n = 18$) together with controls ($n = 13$). The decrease of uNGAL and uβ2-M were significant ($p = 0.013$, $p = 0.007$, respectively), and the decrease was observed in 75% and 81% of the patients, respectively. The preoperative concentrations of uNGAL and uβ2-M were significantly higher compared to the controls ($p = 0.001$, $p = 0.002$, respectively), whereas the postoperative concentrations did not differ from the controls ($p = 0.96$, $p = 0.87$, respectively).

ROC curve analysis was used to determine the diagnostic profiles of the perioperative urinary concentrations of NGAL and β2-M. The NGAL AUC was 0.92 (95% CI, 0.84–1.00) with the best cut-off value of 20.57 ng/mg cr. (sensitivity, 82%; specificity, 100%). The β2-M AUC was 0.81 (95% CI, 0.61–0.95) with the best cut-off value of 191.8 ng/mg cr. (sensitivity, 68%; specificity, 95%).

There was no correlation between the measured urinary proteins and the DRF, AP diameter, gender, or operative findings. Conversely, a negative correlation was shown between age and preoperative uβ2-M ($r = -0.430$, $p = 0.04$), uCyC ($r = -0.689$, $p = 0.0003$), and uOPN ($r = -0.686$, $p = 0.0003$).

DISCUSSION
Methodological aspects
Choice of animal model

The use of animal models is often essential in the study of pathophysiologic processes. For more than 50 years, different animal models of ureteral obstruction have been developed to further elucidate the pathogenesis of obstructive nephropathy. The early studies were mainly performed in rabbit and dog models, whereas the majority of the current studies are based on rat and mouse models [99], but also sheep, guinea pig, opossum, and especially pig models have also contributed to the understanding of this condition [100]. Spontaneous genetic models with congenital UUO have been identified, but the infertility or low reproduction rate of these strains limits their use [100,101]. The development of several genetic models of obstruction (mainly gene knockout technology) in the past 2 decades has contributed to the current understanding of the development of interstitial fibrosis in obstructive nephropathy [102]. Especially important information about the beneficial and deleterious roles of specific gene products can be investigated with the use of genetically engineered animals [102].

Contrary to the genetic models, the surgical models of obstruction have the advantage of controlling the onset, duration, and severity of the obstruction and the opportunity to study recovery following relief of the obstruction [99,102]. Complete UUO is easy to perform and is a well-established model of renal fibrosis. The rat is a manageable animal to work with due to its size, which makes the surgical set-up simpler compared to the pig, for example. It is also more affordable, especially taking the long stabling period of the operated pups into account. The manageable and affordable characteristics of the rat facilitates surgical training, which is required, since the development of reproducible animal models of UUO/PUUO requires an experienced animal surgeon [99].

Taken into account that the response of the developing kidney differs from that of the mature kidney as well as the fact that most cases of clinical congenital obstructive nephropathy are partial rather than complete obstruction justifies the use of a PUUO model in neonatal rats. Nephrogenesis in rats continues into the first postnatal week and a ureteral obstruction performed within the first 24 h mimics the midtrimester human fetus in which nephrogenesis is complete by the 34th week of gestation [103]. The PUUO model allows us to study the effects of obstruction during the period of most rapid nephrogenesis [99]. Besides the learning curve of performing PUUO in neonatal rats (5–7 g), the challenge is to ensure that the pups are accepted by their mother and nursed successfully following surgery. In addition, the PUUO model is also challenged by the variability in the degree of partial obstruction [104].

Comments on the design of the clinical study

Two inclusion criteria were defined (i.e., declining function of the hydronephrotic kidney or ipsilateral flank pain) to ensure that a sufficient number of patients were included in the study. Both these groups present with hydronephrosis, but the hydronephrotic kidney is affected in different ways and the patients present with different degrees of severity. This is taken into account by analyzing the results independently for each group, and since there were no differences in the results, the data were pooled and analyzed together.

The patients underwent a surgery, which was not the case for the controls. It is well-known that surgery leads to specific endocrine, immunologic, and metabolic changes and affects the functions of organs [105]. In comparison with open surgery, a laparoscopic procedure reduces surgical stress, but instead the establishment of pneumoperitoneum alters certain physiologic functions [106]. The increase in abdominal pressure affects the kidneys by decreasing in renal blood flow, glomerular filtration, and urinary output [106]. Gomez et al. confirmed this in a clinical study with children, and they also showed that urine output recovered within 6 h after desufflation of the abdomen; there was no change in serum creatinine or CyC, indicating that the perioperative oliguria has little or no postoperative implications [107]. Surgical trauma initiates immunologic and inflammatory responses, and acute-phase proteins are produced in response to tissue injury. The levels of these proteins increase at 4–12 h after surgery, peak at 24–72 h, and remain elevated for up to 2 weeks [108]. The inflammatory response is mediated by cytokines and, especially, IL-6 levels have been shown to increase proportionally to the amount of tissue trauma. Studies have shown a significantly lower release of pro-inflammatory cytokines within the CO_2 environment of laparoscopy compared to open surgery [106]. The physiological effects of the laparoscopic procedure are important to consider when the results from study II are interpreted. The observed increase in the urinary concentrations of the proteins might simply be a response to the surgical stress.

A drawback of this study was the control groups. With regard to the collection of the urine samples from the controls, the parents were interviewed about the health status of their child, but none of the children underwent renal ultrasonography to rule out hydronephrosis. Furthermore, the controls were not exposed to a surgical trauma, which, in any case, would not be ethically justifiable. The optimal control group would have been children with healthy kidneys and no infections who underwent a laparoscopic procedure.

In addition, an inclusion of control groups consisting of children with non-obstructed UPJO and children with different degrees of UPJO would have contributed to address the predictive value of the examined potential biomarkers.

With regard to the collection of pelvic tissue from the controls, we included 4 infants who were nephrectomized due to congenital Finnish nephrotic syndrome. This is a rare autosomal recessively inherited disease caused by mutations in the gene encoding nephrin [97]. The infants present with massive proteinuria and a progression of glomerular and tubulointerstitial scarring [98]. We assumed that the pelvic tissue from these infants was normal. In addition, we included 5 adults who were nephrectomized due to renal cell carcinoma, and we also assumed that they had healthy pelvic tissue that was comparable to pelvic tissue from children. These patients all underwent a renography, which demonstrated a non-obstructed elimination phase. The ideal control group for the collection of pelvic tissue would have been sex- and age-matched children who were nephrectomised;

however this is a very rare occurrence since children with a renal tumor could not be included due to preoperative chemotherapy.

Significance of urine collection and storage

All urine samples were collected aseptically using sterile tubes. Protease inhibitors were added to the urine samples from rats since they were collected over 3 h, which increased the risk of cytokine degradation. The human urine samples were collected in 4 tubes of which 2 of them contained protease inhibitors. Prior to the analyses in studies IIa + b, a pilot study was performed to test the significance of the protease inhibitors. It was decided to use samples without protease inhibitors as the pilot study showed no differences in cytokine concentrations with respect to the addition of protease inhibitors.

After a literature review of urine sample storage [109,110,111], it was decided to freeze all samples immediately on crushed ice followed by storage at –80°C within 10 min. In addition, morning urine was avoided to reduce the degradation of cytokines, freeze-thaw cycles were avoided, and the samples were defrosted to 5°C prior to analysis and kept on ice during the procedures.

All analyses were performed in 2011 to limit inter-assay variation by using assays with the same batch number. By doing so, the samples were kept at –80°C for 0.1–3.5 years. A comparison between the oldest samples (from 2007–2009) and the newest samples (from 2009–2011) showed no significant differences in the concentrations between the different time points.

Advantages and limitations of Luminex

The advantage of fluorescent bead-based technologies is their multiplexing capability, allowing the measurement of multiples analytes in a single 2–50 µL sample. The assays are a time-saving and cost-effective choice, since distinct bead species can be mixed individually to target the desirable analytes in a single sample, analyze them simultaneously, and thus save reagent costs and reduce sample volume.

It is an attractive method to use as a screening tool for the selection of promising biomarkers even though it is important to take the limitations of the assays into account when planning experiments. A number of studies have validated Luminex systems, and stated that they should be used with caution [112]. When using the kits, it is important to include replicates of samples as well as negative and positive controls with known concentrations of the examined cytokines. A study concluded that the cytokine concentrations, as measured by a bead-based multiplex assay and ELISA, respectively, showed similar trends, although the absolute concentrations measured were different [113]. Another study in which 3 different commercial bead-based Luminex cytokine assays were evaluated concluded that the most appropriate use for these tests is as a screening tool for the selection of promising markers that can be validated using a method with higher accuracy and proven reliability, such as ELISA [112].

The choice of analysis methods in this Ph.D. study was limited by the small amounts of urine available. It was only possible to collect 1–2 mL of urine in the experimental animal studies, and the urine samples from the affected pelvis and the stent in the clinical study were also a small volume. In addition, the aim was to screen for candidate markers; therefore Luminex was the obvious choice.

Three different bead-based assays were used: 2 by request, mix-to-order customer defined assay panels (Rat Cytokine 6-plex assay [Invitrogen, CA, USA] and Procarta Cytokine Assay Kit [Affymetrix, Ramcon, Denmark]) and 1 fixed, pre-formatted assay panel (BeadPlex Human Kidney Toxicity/Injury Panel 2 [Widescreen; EMD Chemicals Inc., Merck, Germany]).

The measured concentrations were analyzed with regard to the standard recovery, accuracy and inter-assay precision of each assay (see page 6).

Determination of mRNA abundance

QPCR was used to quantify mRNA levels in study IIa. Several other methods are available, e.g., Northern blotting and RNAse detection assays. In Northern blotting, purified RNA is separated by agarose gel electrophoresis, transferred to a solid matrix, and probed with a specific probe. This technique provides only qualitative or semi-quantitative information on mRNA levels and, like the RNAse detection assays, it requires relatively large amounts of RNA and is time-consuming. In study IIa, only small amounts of pelvic tissue were collected; thus, QPCR was the ideal method for analysis, since it only needs a small amount of template (50–100 ng) and it only takes 3–4 h to perform one set of QPCR.

QPCR uses fluorescent labeling as a detection system (e.g., the non-specific SYBR Green detection system or specific fluorescent reporter probes). The use of specific fluorescent reporter probes requires the design and synthesis of one or more custom-made fluorescent probes for each PCR assay, which significantly increases specificity. The non-specific SYBR Green detection system was used in study IIa, which has the advantages that it can be incorporated into optimized and long-established protocols, and is significantly cheaper, as there is no probe-associated cost. However, the non-specific detection system can potentially interfere with, or prevent, the accurate quantification of the intended target sequence. The specificity of the detection system was confirmed by testing for interactions between the primers using melting curve analysis and agarose gel separation.

The values generated by QPCR do not have absolute units associated with them. To ensure the accuracy of the quantification, it is necessary to normalize mRNA expression to a stably expressed reference gene that corrects for possible differences in RNA quantity or quality across experimental samples, and in addition, it ensures that the same amount of cDNA is loaded. The selection of the reference gene has to fulfill the criteria of being constitutively expressed and minimally regulated under the experimental conditions. In study IIa, 2 reference genes (β-actin and GAPDH) were tested and rejected as they were both regulated, whereas TBP was found to be stably expressed. TBP is a transcription factor, and it has previously been used as a reference gene under the same experimental conditions [114].

Choice of cytokines to test

Many approaches are possible in the search for urinary biomarkers. The animal model of UUO has been used extensively to elucidate the role of the regulation and function of cytokines in obstructive nephropathy and renal fibrosis. This basic research might identify potential biomarkers that, subsequently, can be tested in clinical studies. Another approach is to examine cytokines that are already known to be up- or down-regulated in other types of nephropathy or to perform a large-scale search using urinary proteome analysis. With proteome analysis, the entire set of proteins expressed in urine at a given time under certain conditions is defined and followed by a validation of potential candidate biomarkers in urine samples from large patient cohorts with UPJO.

In study I, a review of the literature concerning obstruction was performed prior to the selection of the cytokines to be examined. Of the available cytokine assays for Luminex, we chose to

use the Rat Cytokine 10-plex, which includes GM-CSF, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IFN-γ, and TNF-α. This 10-plex was tested in a pilot study after which a 6-plex was designed, which includes IL-1β, IL-2, IL-6, IL-10, IFN-γ, and TNF-α, since they were all detectable in urine and kidney parenchyma. This 6-plex was used in the acute and chronic rat models of obstruction.

In study II, a similar procedure was performed with a review of the literature, investigation of the available assays, and, additionally, a pilot study that included the 4 cytokines (IL-1β, IL-6, IL-10, and TNF-α) identified as significantly regulated in the animal experiments. Furthermore, eotaxin, IL-8, and macrophage migration inhibitory factor (MIF) were examined in a pilot study together with the 9 selected cytokines presented in studies IIa + b.

Urine and kidney cytokine profiles in acute and chronic experimental hydronephrosis

The complex pathophysiology of obstructive nephropathy remains poorly understood. The interstitial inflammatory response is characterized by macrophage infiltration, tubular dilatation, apoptosis, and, finally interstitial fibrosis with nephron loss [16]. These processes are mediated by the release of cytokines, chemokines, growth factors, and other signaling molecules acting as intercellular mediators of paracrine communication. Study I sought to identify potential new urinary biomarkers by testing the selected cytokines among the molecules involved in these processes.

The selected cytokines were detectable in renal parenchyma and urine from rats with experimental UUO. In the acute obstruction model, significantly increased renal levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6, and, consistent with this, significantly increased urinary concentrations were detected. The changes concerning IL-6 and the urinary concentration of IL-1β were reproduced in the chronic model of obstruction, whereas there was no evidence for the regulation of IL-10 and TNF-α. Notably, significantly increased concentrations of IL-10 were detected in the cortex and urine from the non-obstructed kidney in the acute model. This was consistent with the immunomodulatory and anti-inflammatory qualities that characterize IL-10, which down-regulates proinflammatory cytokines and reduces the production of chemotactic factors [51]. IL-10 was not detected in any of the urine samples in the chronic model.

IL-1β is produced by macrophages in response to inflammatory stimuli and has been shown to be an important regulator of several other inflammatory mediators, e.g., IL-6, TGF-β1, and ET-1 [44,45]. Interestingly, TGF-β1 and ET-1 have been measured in urine from children with UPJO and both were found at higher concentrations than in urine from healthy children [33,37,115,116].

Besides from being regulated by IL-1β, IL-6 is also stimulated by TNF-α and is produced by numerous immune cells in response to physiologic stimuli, e.g., bacterial endotoxins, physical exercise, and oxidative stress [51]. IL-6 is a complex cytokine since it functions by a combination of distant and local effects. It is present in the circulation and can be detected in the 1 pg/mL range in healthy individuals [51]. Ruiz-Deya et al. reported an up-regulation of IL-6 in the ureteropelvic junction segments from patients with UPJO following a failed pyeloplasty [117]. As expected, this study confirmed the increased concentrations of IL-1β and IL-6 in urine and renal parenchyma in the obstruction models.

TNF-α is released mainly from activated macrophages infiltrating the obstructed kidney as little as 4 h after the onset of obstruction, and it induces renal tubular cell apoptosis, renal

fibrosis, and dysfunction [62]. Since the urinary levels of TNF-α are reportedly increased in children with UPJO [118], we expected to observe a higher renal and urinary concentration of TNF-α in the chronic model of obstruction as this model was intended to model children with UPJO.

In the chronic obstruction model, PUUO was created by embedding two-thirds of the ureter into a slit in the underlying psoas muscle, which, according to previous studies, produces a severe partial obstruction [119]. There was great biological variation between the animals, especially in the partial obstruction model as the grade of hydronephrosis was unknown. The degree of obstruction was only evaluated visually, which was an obvious weakness of this study. Previously, it was shown that a severe partial obstruction caused compensatory growth in the contralateral non-obstructed kidney after 14 weeks [119]. We investigated the kidneys after 10 weeks and, as expected, we did not find a significant change in the weights of the kidneys. It seemed reasonable to question the grade of hydronephrosis, but due to the significant increase in the concentration of IL-6, a partial obstruction resulting in hydronephrosis was believed to be present. Still, the grade of hydronephrosis might have been too low to induce renal changes with altered levels of TNF-α and IL-10.

A translational link between the animal experimental studies and the clinical study?

A selected panel of cytokines was examined in the animal model of acute obstruction followed by an examination of the reproducibility of the data in the animal model of chronic obstruction. Originally, we intended to test the same panel in all urine samples from the clinical study, but due to a limited amount of sample volume, it was initially tested in a pilot study. This study showed disappointing results since the cytokines were not detectable in several of the samples. Accordingly, we chose to test other cytokine panels in the clinical study.

Significantly increased concentrations of EGF and MCP-1 in urine samples collected before the surgical procedure

In the clinical study, 2 of the 9 examined cytokines showed significantly increased concentrations in the preoperative urine samples from children with UPJO compared to healthy children. This is very interesting, since an ideal biomarker should be measurable in bladder urine, and sufficiently sensitive and specific to diagnose obstruction and suffering kidneys in children with hydronephrosis.

Markedly increased preoperative urinary concentrations of EGF were detected, followed by a further perioperative increase, and then a decrease to the same level as the controls after 3 months (Fig. 3).

Studies of renal EGF expression revealed EGF as a key-player in the modulation of epithelial cell growth and metabolism and thereby a mediator of tubular regeneration after renal injury. Experimental models of hydronephrosis have shown decreased renal EGF expression [120], which has been restated in several clinical studies including children with UPJO [32,38,40,69]. Importantly, opposing renal responses to the exogenous supply of EGF have been shown in rats and mice. Exogenous EGF acts as a survival factor in neonatal rats, whereas it acts as a death factor in mice [121]. It has been suggested that the response to EGF in humans is more similar to that of rats than of mice [12].

The urinary levels of EGF in children with UPJO have also been examined in several studies [29,38,39], but the results have been unclear and partly contradictory. Grandaliano et al. reported a study with 24 patients operated due to UPJO [38]. They were

diagnosed with renal ultrasonography and diuretic MAG3 renography, but data about the hydronephrosis grade and the indication for surgical intervention were missing, and the results are therefore difficult to interpret. In addition, the collection of urine samples was not standardized as they used both pelvic urine and bladder urine. They demonstrated decreased urinary concentrations of EGF in children with unilateral UPJO compared to controls. Conversely, a study by Taha et al. showed no significant difference between the urinary levels of EGF before surgery in patients and controls, and no significant difference in the urinary levels of EGF before and after surgery over the 1-year follow-up [29]. The study cohort included children with unilateral UPJO, SFU grade 3 or higher, and bladder urine was used. Recently, a study by Bartoli et al. reported the urinary concentrations of EGF, MCP-1, and β2-M in children with UPJO and controls [39]. The UPJO patients were divided into obstructive (no response to diuretic stimulation on a MAG3 scan), functional (response to diuretic stimulation on a MAG3 scan), and operated UPJO. The indications for surgical intervention were DRF < 45%; persistent obstructive curve at 6-month follow-up renal scan; recurrent abdominal pain; or UTIs. They demonstrated significantly decreased urinary concentrations of EGF in the obstructed UPJO group compared to the controls and the functional UPJO group, while the operated UPJO group did not show any significant changes. A review of the literature with contradictory reports on urinary EGF levels emphasizes the necessity for further investigations of urinary EGF concentrations.

MCP-1 followed the same urinary excretion pattern as EGF with a high preoperative concentration, followed by a further perioperative increase, and a decrease to the same level as the controls after 3 months (Fig. 3), which confirmed the results from previous studies demonstrating significantly higher urinary concentrations of MCP-1 in patients with UPJO compared to controls [38,39]. Experimental animal studies have demonstrated increased renal MCP-1 expression in rats subjected to UUO [75,76], which have been confirmed in clinical studies [38,39].

Six of the remaining 9 cytokines did not show any differences in their preoperative urinary concentrations between the patients and controls, whereas MIP-1α showed significantly lower urinary concentrations in the patients.

Significantly increased concentrations of selected cytokines in urine samples collected during the surgical procedure and the following day

All cytokines, except OPN, showed significantly increased concentrations in the perioperative and postoperative (1 day) urine samples compared to the preoperative samples and controls, respectively. This increase is probably, partly, a response to the surgical stress as discussed on page 10. The comparisons between the cytokine concentrations in urine from the obstructed and non-obstructed kidneys, respectively, showed the higher excretion of NGAL, CyC, MCP-1, MIP-1α, IP-10, and RANTES from the obstructed kidney in the perioperative samples. In addition, all cytokines, except MIP-1α, showed higher excretion from the obstructed kidney on the first postoperative day.

It seems obvious to conclude that the surgical trauma inflicted on the obstructed kidney gave rise to this increase, but this may not be the sole explanation. The perioperative urine samples were collected from the affected pelvis at an early stage of the surgical procedure, and since the urine was collected before the dilated pelvis and kidney were "touched," it is believed to be a reliable indication of their physiological excretion from the obstructed kidney. Conversely, the postoperative (1 day) increase of

the concentrations in urine from the obstructed kidney could well be caused by the surgical trauma to the obstructed kidney as well as the presence of the stent. The preoperative urine samples consisted of bladder urine, which means that urine from the obstructed kidney was diluted with urine from the contralateral kidney, and thereby, the concentrations were, most likely, decreased. Accordingly, the perioperative increase of NGAL, CyC, MCP-1, MIP-1α, IP-10, and RANTES in urine from the obstructed kidney compared to the contralateral kidney indicates that the majority of these 6 cytokines were excreted from the obstructed kidney. An explanation for the lower concentrations in the preoperative samples could be that these cytokines were too sensitive to be measured in bladder urine, and, perhaps, the cytokines were degraded in the bladder.

Significantly decreased concentrations of selected cytokines in the postoperative urine samples

Three months after the surgical procedure, all of the cytokines, except OPN, MIP-1α, and RANTES, were at the same urine level as observed in the controls.

The urinary excretion pattern of OPN in the patients was different from the other 8 examined cytokines. Notably, the analysis of the dynamics at the 6 time points was non-significant (Fig. 6). According to previous animal experiments showing high levels of renal OPN expression following ureteral obstruction [92,122], a high urinary concentration of OPN was expected. In these 2 previous animal studies, they suggested that OPN facilitates macrophage recruitment to sites of inflammation during ureteral obstruction, and, furthermore, that OPN suppresses apoptosis and may function as a survival factor for renal tubulointerstitial cells [92,122]. The lack of a postoperative decrease in the urinary concentration of OPN may be explained by the dual actions of OPN raised in the previous animal experiments, e.g., the kidney might still be regenerating after 1 year. The fact that OPN shows dual actions complicates the potential use of OPN as a urinary biomarker in hydronephrosis.

MIP-1α and RANTES showed the same urinary level as the controls after 1 year, and, thereby, 8 of the cytokines showed decreased concentrations after 1 year compared to their perioperative concentrations. This postoperative decrease is particularly interesting when the results of EGF and MCP-1 are interpreted. With the significantly increased preoperative urinary concentrations, the fact that their concentrations normalize in the postoperative follow-up period further emphasizes the promising potential of these cytokines as biomarkers for UPJO.

The postoperative decrease of the other 6 cytokines is also worthy of consideration as it illustrates the dynamics in the excretion pattern of cytokines, and these cytokines may be potential markers of convalescence, indicating the success of the surgical treatment.

Correlations between the functional parameters and the urinary cytokine concentrations

Statistical analyses were performed to study the significance of the clinical characteristics of the patients. The results were not affected by gender, and neither the SFU grade nor the operative findings had an impact on the urinary excretion of the cytokines.

Interestingly, the preoperative concentrations of uβ2-M, uCyC, and uOPN were negatively correlated with age. This negative correlation between age and urinary cytokines has been discussed previously in several studies, and it has been suggested that the lower urinary levels in older children with UPJO might reflect a lower and more steady-state production of inflammatory

cytokines with long-standing obstruction [34]. Several studies examining urinary biomarkers in UPJO have reported different thresholds for infants and children [29,37], which further emphasize the importance evaluating potential urinary biomarkers in different age groups to determine age-adjusted cutoff values.

Study IIa showed a negative correlation between DRF and the perioperative uIP-10 and uMCP-1 levels, respectively. Furthermore, there was a tendency (non-significant) toward a negative correlation between DRF and the preoperative uEGF, uMIP-1α, and uRANTES levels. A correlation with DRF is very valuable, since the power of the biomarker to detect disease progression improves, when there is a correlation with the renal function. This negative correlation between DRF and MCP-1 confirmed the results of a previous study by Grandaliano et al. [38], in which the concentration of MCP-1 in urine from the obstructed kidney was inversely correlated with the DRF. There was no significant linear correlation of NGAL with DRF, which was shown in a study by Wasilewska et al. [42]. Their study was a case-control prospective study that included one study group (20 children with severe UPJO who underwent surgery) and 2 control groups (20 children with mild non-obstructive hydronephrosis and 25 healthy children). They observed a negative correlation between the preoperative uNGAL levels and DRF, but no significant correlation with serum creatinine or GFR. Study IIb did not reproduce their findings with respect to the negative correlation, which may be explained by the low number (n = 6) of patients with DRF < 40%.

In addition, study IIa showed a negative correlation between the AP diameter and the preoperative levels of uEGF and uIP-10. The AP diameter is not a reliable parameter for renal function or the severity of the hydronephrosis [1]; therefore, a correlation between urinary cytokine concentrations and the AP diameter is not important.

Notably, there were no correlations between the urinary cytokine concentrations and the SFU grade of the hydronephrosis. This might be explained by the fact that most of the included patients were grade 3 (68%).

Significance of the calculated cut-off values

ROC curve analyses were performed to define the diagnostic profile of the preoperative urinary EGF and MCP-1 concentrations and the perioperative urinary NGAL and β2-M concentrations for the identification of children with UPJO among healthy children.

The diagnostic profiles of the preoperative uEGF and uMCP-1 levels described AUCs of 0.75 (95% CI, 0.60–0.90) and 0.78 (0.63–0.92), respectively. The diagnostic profiles improved when the analyses were performed on the perioperative uEGF and uMCP-1 levels from the affected pelvis, describing AUCs of 0.85 (95% CI, 0.72–0.97) and 0.89 (95% CI, 0.79–0.99), respectively. When the analyses were performed on the perioperative samples, the cut-off values of EGF and MCP-1 were clearly higher with improved sensitivity and specificity.

The diagnostic profiles of perioperative uNGAL and uβ2-M levels were good as they described AUCs of 0.92 (95% CI, 0.84–1.00) and 0.81 (95% CI, 0.61–0.95), respectively. ROC curve analyses were not performed on the preoperative concentrations since they were not found to be significantly different from that of the controls.

The usefulness of cut-off values in the urine from the affected pelvis is limited in the clinical setting. The collection of urine from the pelvis is quite invasive compared to the collection of voided urine samples. The cut-off values presented in this study are not transferable to the clinic. Nevertheless, it is believed to be important to present them in this exploratory study to clarify the diag-

nostic power of these urinary proteins. Before implementation in the clinic, it is necessary to perform larger studies, in which children with severe UPJO can be compared with children with milder degrees of UPJO, and thereby more useful cut-off values can be determined, since the clinical challenge is to distinguish between these 2 groups of patients.

EGF, MCP-1, MIP-1α, IP-10 and RANTES mRNA expression in the pelvic tissue

The mRNA expression of EGF, MCP-1, MIP-1α, IP-10, and RANTES was evaluated in pelvic tissue from children with UPJO in order to investigate the molecular pathologic changes. Significantly lower MIP-1α mRNA expression was demonstrated in patients compared to controls, whereas there was no differential regulation of EGF, MCP-1, IP-10, and RANTES expression.

The etiology and mechanism of obstructive lesions involving the ureteropelvic junction remain unclear. Although different types of UPJO are recognized, e.g., intrinsic stenosis/valves, insertional anomaly, fibrous bands, and crossing vessels, there are still unanswered questions concerning the causative factors as well as speculation about the initial obstructive event [8]. For example, an insertional anomaly may develop as a result of the initial obstructive event and thereby cause a further progression of the obstruction, and similarly, fibrous bands/adhesions may be secondary to an existing obstruction. Studies on the histopathologic changes within the stenotic segment have shown muscle hypertrophy, collagen deposition and proliferation of fibrous tissue, which all contribute to a hypoplastic adynamic ureteral segment [123,124]. Only a few studies have addressed the molecular events associated with these histopathologic changes. A study by Yang et al. evaluated the mRNA expression of EGF and TGF-β1 in stenotic tissue from children with UPJO [124]. They included 25 children with UPJO who underwent surgery indicated by reduced (< 40%) DFR, and control ureters from patients who underwent nephrectomy for nephroblastoma. Increased TGF-β1 and decreased EGF mRNA expressions was demonstrated in children with UPJO compared to controls. However, when interpreting their results, it is important to note that they used a semi-quantitative RT-PCR technique in order to measure the mRNA levels as an alternative to QPCR, and immunohistochemistry did not verify their EGF mRNA results [124]. The TGF-β1 mRNA results confirmed the results from the study by Seremetis et al. that demonstrated increased TGF-β1 mRNA expression in the renal pelvis following clinical and experimental UPJO [125]. A study by Knerr et al. evaluated the mRNA expression of endothelin-1 and adrenomedulin in stenotic tissue from 20 children with UPJO and 21 controls [126]. Increased endothelin-1 and decreased adrenomedullin mRNA expressions was demonstrated in children with UPJO compared to controls, and the authors concluded that these changes in the local production of endothelin-1 and adrenomedullin may contribute to the micro-anatomical changes in the obstructed segments [126].

Study IIa demonstrated significantly lower MIP-1α mRNA levels in the stenotic tissue from patients compared to that of controls, which was reflected in the low preoperative MIP-1α concentrations in the urine samples. Surprisingly, there was a remarkable increase in the concentrations of uMIP-1α in the samples from the operation and the first postoperative day; after 1 year, the concentrations declined to the same levels as in the controls. MIP-1α is a chemokine that attracts inflammatory cells but, contrary to our expectations, we did not demonstrate high preoperative levels in the patients. In our study, the levels of MIP-

1α in the renal parenchyma were unknown, even though they could be speculated to be high as seen in other inflammatory kidney diseases [77,78,79], but must be kept inside the cells instead of being secreted in the urine.

There was no significant difference in EGF, MCP-1, IP-10, and RANTES mRNA expression between the patients and controls; thus, the results from the study of Yang et al. showing decreased EGF mRNA expression in stenotic tissue were not confirmed.

In this study, we assumed that the pelvic tissue from children was comparable to the pelvic tissue from adults. This issue has not been investigated previously in the literature. Within the control group, the cytokine mRNA expression patterns of tissue specimens from the 4 infants and the 5 adults showed a similar pattern, and therefore age-related effects were excluded. The median age of the control group differed from that of the patient group for reasons of availability, but the similar excretion pattern within the control group in combination with the stable expression of the reference gene (TBP) supported the view that the observed change in MIP-1α mRNA expression might be disease-specific rather than age-related. Another limitation of this study was the lack of histological confirmation that tissue specimens from the controls were unaffected, and additionally, there was no histologic assessment of the obstructed segment due to the limited amount of sample.

SUMMARY AND CONCLUSIONS
Study I
i.   Significant changes were demonstrated in the urinary concentrations of IL-1β, IL-6, TNF-α, and IL-10 after the release of obstruction in an experimental model of acute UUO, which were also reflected in the renal parenchyma. No significant change of IL-2 and IFN-γ concentrations was observed.
ii. The changes of the urinary concentration of IL-6 after the release of acute UUO were reproduced in an experimental model of chronic UUO, whereas the results from the acute study regarding IL1-β, TNF-α, and IL-10 were not reproduced.
iii. In conclusion, study I demonstrated significantly higher renal levels of IL-1β, IL-6, and TNF-α in the acute obstruction model compared to those of controls, and these were associated with increased urinary concentrations. In addition, significantly lower levels of IL-10 in renal parenchyma and urine were demonstrated. These results confirmed the hypothesis that the urinary excretion of these cytokines is reflected in the renal parenchyma. The results from the acute obstruction model were not reproduced in the chronic obstruction model in which only IL-6 was found to be up-regulated. The fact that most clinical cases of UPJO are partial rather than complete, supports the use of the chronic obstruction model, and the studied cytokines are therefore of limited value as future urinary biomarkers for UPJO in hydronephrosis.

Study IIa
i.   The urinary concentrations of EGF and MCP-1 were significantly increased in preoperative samples from children operated on for UPJO compared to healthy children.
ii. The concentrations of MCP-1, MIP-1α, IP-10, and RANTES were increased in urine from the obstructed kidney compared to urine from the non-obstructed kidney in the perioperative samples.
i.   The 1-year postoperative urinary concentrations of EGF, MCP-1, MIP-1α, IP-10, and RANTES were decreased compared to the preoperative (EGF and MCP-1) and/or perioperative (all) samples, and their concentrations were comparable to those of the controls.
ii. The mRNA levels of MIP-1α were significantly down-regulated in the pelvic tissue from patients compared to controls. There was no significant regulation of EGF, MCP-1, IP-10, and RANTES.

In conclusion, study IIa demonstrated significantly increased urinary concentrations of EGF and MCP-1 in children with UPJO. This indicates that EGF and MCP-1 are regulated as a response to the obstruction, suggesting that they may have a potential as urinary biomarkers for UPJO in hydronephrosis. The ROC curve analyses yielded relative good diagnostic profiles for EGF and MCP-1 to distinguish children with UPJO from healthy children. Furthermore, MCP-1 in the perioperative samples was negatively correlated with DRF, which enhances the candidacy of MCP-1 as a future urinary biomarker for UPJO in hydronephrosis.

The results for urinary EGF should be interpreted with caution due to the contradictory results in previous studies.

Study IIb
i.   The urinary concentrations of NGAL, β2-M, CyC, and OPN were not increased in the preoperative urine samples from children with UPJO compared to controls. The urinary concentrations of NGAL and β2-M were increased in the perioperative urine samples from the obstructed kidney compared to the controls.
ii. The concentrations of NGAL and CyC were increased in urine from the obstructed kidney compared to urine from the non-obstructed kidney in the perioperative samples.
iii. The 1-year postoperative urinary concentrations of NGAL, β2-M, and CyC were decreased compared to the perioperative samples, and their concentrations were comparable to the levels observed in the controls.

In conclusion, study IIb confirmed the increased concentrations of NGAL and β2-M in urine from obstructed kidneys when compared to the contralateral kidneys and controls. The ROC curve analyses yielded good diagnostic profiles of NGAL and β2-M to distinguish children with UPJO from healthy children. The implementation of NGAL and β2-M as urinary biomarkers for UPJO in hydronephrosis is limited by the need to collect pelvic urine. Nevertheless, this study showed a tendency toward higher concentrations of NGAL and β2-M in preoperative bladder urine samples; therefore, they should be tested in larger studies that should have more power to detect a significant difference. With the significant decline in the concentrations of uNGAL, uCyC, and uβ2-M after the first postoperative day, these proteins may be potential markers for convalescence, indicating the success of the surgical treatment.

Furthermore, the urinary concentrations of β2-M, CyC, and OPN in the preoperative samples were negatively correlated with age, which is important to consider in the design of future studies.

FUTURE PERSPECTIVES
Further investigations are required to test the ability of urinary proteins to identify kidney obstructions and reveal disease progression and, thereby, be useful diagnostic and prognostic tools in the clinic. It is evident that EGF and MCP-1 merit a more thorough analysis, but also NGAL, β2-M, IP-10 and RANTES are interesting since they were increased in urine from the obstructed kidney in the perioperative samples. Conversely, CyC,

OPN, and MIP-1α are not tempting for further investigation due to their aberrant excretion patterns.

Several potential new urinary biomarkers for obstruction in hydronephrosis have been introduced during the past decade, but none have yet been implemented in daily clinical practice due to their limitations and because of the need for further validation in clinical studies. A significant issue is the consistent non-standardization of the various studies, e.g., different definition criteria for UPJO, different inclusion criteria, different procedures for sample collection, etc. [14]. The most recognized definition for obstruction is *a condition of impaired urinary drainage that, uncorrected, will limit the ultimate functional potential, including renal functional reserve capacity and response to stress* [5]. As Craig A. Peters pointed out in an editorial comment on a biomarker study, *the definition of "obstructed" itself remains controversial...in that the fact of undergoing pyeloplasty is not a proof of obstruction* [127]. The criteria for surgery vary widely, which make the study groups in reported studies difficult to compare. In addition, some studies also include bilateral UPJO, patients with UTIs, and there is a wide age-range.

Future studies should be designed with strict definitions of the patient groups, and the inclusion of different patient groups, e.g., children with different degrees of UPJO, children with non-obstructive hydronephrosis, and healthy children, is necessary in order to investigate the ability of biomarkers to distinguish between these groups. The reference values are also an essential challenge since there is a high variation in the measured values depending on the methods of analysis and the biological variation between individuals and probably also for the age of the child. Larger clinical studies with a longer follow-up period are required to determine the reference ranges that might need to be individualized.

SUMMARY

Hydronephrosis is diagnosed in 0.5–1% of all newborns, and ureteropelvic junction obstruction (UPJO) accounts for 35% of those cases. A urinary tract obstruction that occurs during early kidney development affects renal morphogenesis, maturation, and growth, and in the most severe cases, this will ultimately lead to progressive renal tubular atrophy and interstitial fibrosis with the loss of nephrons.

The clinical management of these patients remains a controversial topic. The aim is to preserve renal function by identifying the 15–20% of children who require early surgical intervention from those for whom watchful waiting may be appropriate because of spontaneous resolving/stabilization without significant loss of renal function. Although the patients attend regular follow-ups, including repetitive blood tests, ultrasonographies, and the more invasive diuretic renograms, the surgeon still miss reliably biomarkers that could be used as predictors for renal parenchymal damage and decreased renal function, and thereby provide more clear indications for surgical intervention.

The aim of this Ph.D. thesis was to further elucidate the pathophysiology of obstructive nephropathy (study I) and to search for potential candidate biomarkers that may have a predictive and/or diagnostic value in the management of hydronephrosis (study II).

Study I: Urine and kidney cytokine profiles in experimental unilateral acute and chronic hydronephrosis.

Aim: To study the dynamics of the urinary secretion of cytokines after the release of unilateral ureteral obstruction, and to study whether the urinary concentrations of these compounds reliably reflects changes in the renal parenchyma.

This was tested in 2 experimental rat models: an acute obstruction model and a chronic obstruction model.

Results: The acute obstruction model demonstrated significant differences in the renal levels of IL-1β, IL-6, TNF-α, and IL-10 in comparison with controls, and these differences were associated with similar differences in their urinary excretion. Such results were not obtained in the chronic obstruction model in which significant differences were only demonstrated in the urinary concentrations of IL-6.

Study II: Candidate urinary biomarkers in hydronephrosis – a clinical study.

Aim: To study the dynamics of the urinary excretion of selected potential biomarkers in children after the relief of UPJO, and to compare their findings with healthy controls.

Results: Twenty-eight children with UPJO were included in the study from 2007–2011 together with 13 healthy children. Pre-, peri- and postoperatively (1 year) urine samples were collected. The median age of the patients was 8.1 (3.5–14.5) years. Five proteins (EGF, IP-10, MCP-1, RANTES, and MIP-1α) were examined in study IIa, and 4 proteins (NGAL, CyC, β2-M, and OPN) were examined in study IIb. In brief, significantly increased urinary concentrations of EGF and MCP-1 were demonstrated in children with UPJO compared to controls, which was followed by a decline in the postoperative period to levels similar to the controls. This indicates that the urinary concentrations of EGF and MCP-1 are regulated as a response to the obstruction, suggesting that they may have a potential as urinary biomarkers in hydronephrosis. In general, urine from the obstructed kidney exhibited higher concentrations of the proteins compared to urine from the non-obstructed kidney. Furthermore, CyC, β-2M, and OPN were negatively correlated with age, and IP-10 and MCP-1 were negatively correlated with DRF.

In conclusion, this Ph.D. study confirmed increased concentrations of selected proteins in urine from kidneys suffering from obstruction. Interesting, it was observed that some urinary proteins had an age-dependent excretion. Further investigations are required to test the ability of the examined proteins to identify an obstruction and reveal disease progression and, thereby, be useful clinical tools.

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