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Avaliação de biomarcadores em pacientes pediátricos com Refluxo Vesicoureteral

UNIVERSIDADE FEDERAL DE MINAS GERAIS
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Dissertação apresentada ao Curso de Pós-Graduação da Faculdade de Medicina da Universidade Federal de Minas Gerais, como requisito para a obtenção do grau de Mestre.

Área de concentração: Saúde da Criança e do Adolescente.

Orientadora: Dra. Ana Cristina Simões e Silva

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FOLHA DE APROVAÇÃO

AVALIAÇÃO DE BIOMARCADORES EM PACIENTES PEDIÁTRICOS COM REFLUXO VESICOURETERAL.

FLÁVIA CORDEIRO VALÉRIO

Dissertação submetida à Banca Examinadora designada pelo Colegiado do Programa de Pós-Graduação em Ciências da Saúde - Saúde da Criança e do Adolescente, como requisito para obtenção do grau de Mestre em Ciências da Saúde - Saúde da Criança e do Adolescente, área de concentração em Ciências da Saúde..

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Dedico este trabalho aos meus pais e aos meus irmãos.

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“Devemos julgar um homem mais pelas suas perguntas que pelas suas respostas”.

Voltaire

RESUMO

O refluxo vesicoureteral (RVU) é a quarta causa mais comum de doença renal crônica (DRC) na infância. O manejo dos pacientes com RVU inclui o uso de antibioticoprofilaxia contínua, tratamento da disfunção do trato urinário inferior e, em casos selecionados, intervenção cirúrgica, de forma a evitar ou ao menos adiar a progressão para DRC. O grande desafio na condução dos pacientes com RVU é discernir quais pacientes irão evoluir para DRC em estágio final. Estudos sobre a fisiopatologia da nefropatia do refluxo são escassos e biomarcadores clinicamente significativos ainda não foram determinados. Existem inúmeros achados clínicos e experimentais sobre o papel da inflamação nas doenças renais. Avaliar os efeitos de citocinas, quimiocinas, fatores de crescimento e moléculas de adesão celular sobre o início e progressão da lesão renal é, portanto, primordial para o desenvolvimento de novos marcadores prognósticos e de novos alvos terapêuticos.

Os dados existentes na literatura até o momento evidenciam que a citocina fibrogênica TGF-β, a citocina anti-inflamatória IL-10 e as citocinas pró-inflamatórias IL-6, IL-8 e TNF devem ser melhor estudadas como potenciais biomarcadores para cicatriz renal e para o desenvolvimento de DRC na nefropatia do refluxo. IL-1β também deve ser melhor investigada em pacientes com RVU, uma vez que essa citocina parece exercer um importante papel na fase aguda da pielonefrite, ao mesmo tempo em que pode prevenir a formação de cicatrizes renais. CCL2/MCP-1 pode se tornar um biomarcador útil em fases iniciais da progressão da lesão renal, além de predizer o risco de lesão renal a longo prazo. Fatores de crescimento e moléculas de adesão celular também estão envolvidos na fisiopatologia da nefropatia do refluxo. A respeito dessas moléculas conclui-se que a molécula de adesão celular ICAM-1 e os fatores de crescimento, FGF e VEGF, são potenciais biomarcadores para nefropatia do refluxo. Por outro lado, a molécula VCAM parece estar associada à gravidade do refluxo, independentemente de sua progressão para lesão renal.

O objetivo desse estudo foi identificar biomarcadores não invasivos clinicamente relevantes em pacientes pediátricos com RVU primário. Fatores inflamatórios foram dosados na urina de pacientes com RVU em comparação a controles saudáveis. As dosagens de todos os biomarcadores foram obtidas simultaneamente através de citometria de fluxo. Foi possível demonstrar que os biomarcadores MCP-1, IL-12p70, TNF, VEGF e IL-10 encontram-se significativamente elevados na urina de pacientes com RVU. Além disso, naqueles pacientes com DRC estágio ≥ 3 observou-se também níveis urinários elevados de CXCL9/MIG, IL-8, FGF e VCAM-1.

Novos estudos são necessários para identificar dentro desse grupo de proteínas e polipeptídeos aquelas moléculas com potencial de se transformar em biomarcadores capazes de auxiliar em decisões clínicas. Além disso, acreditamos que um painel de biomarcadores, composto por uma combinação de fatores pró-inflamatórios e anti-inflamatórios, poderia apresentar maior acurácia em predizer o risco de progressão para DRC em comparação a avaliação de um único biomarcador.

Palavras-chave: biomarcadores; citocinas; fatores de crescimento celular; inflamação; moléculas de adesão celular; nefropatia de refluxo; quimiocinas; refluxo vesicoureteral.

ABSTRACT

Vesicoureteral reflux (VUR) is the fourth most common cause of chronic kidney disease (CKD) in children. The management of VUR patients involves the use of continuous antibiotic prophylaxis, treatment of lower urinary tract dysfunction and, in selected cases, surgical approach, to avoid or at least delay progression to CKD. The great challenge in the management of VUR patients is to identify those at potential risk of end-stage renal disease. Studies on the pathophysiology of reflux nephropathy are scarce and clinically significant biomarkers have not yet been determined. There are several clinical and experimental findings on the role of inflammation in kidney disease.

Assessing the effects of cytokines, chemokines, growth factors, and cell adhesion molecules on the onset and progression of kidney injury is therefore essential for the development of new prognostic markers and new therapeutic targets.

An extensive review of the literature showed that the fibrogenic cytokine, TGF-β, the anti-inflammatory cytokine, IL-10, and the proinflammatory cytokines, IL-6, IL-8, and TNF, should be more intensively evaluated as potential biomarkers for renal scarring and for progression to CKD in reflux nephropathy. IL-1β should also be further investigated in VUR patients, since this cytokine seems to play an important role in acute phase of pyelonephritis and might prevent the formation of renal scarring. CCL2/MCP-1 might be an early biomarker of progressive renal damage and might have a potential role in predicting the long-term renal outcome. Growth factors and cellular adhesion molecules are also involved in the physiopathology of reflux nephropathy. In this regard, the cellular adhesion molecules ICAM-1 and the growth factors, FGF and VEGF, should be assessed as potential biomarkers for reflux nephropathy. Whereas VCAM appears to be associated with reflux severity, independent of progression to renal scarring.

The objective of this study was to identify clinically relevant non-invasive biomarkers in pediatric patients with primary VUR. Inflammatory factors were investigated in the urine of VUR patients compared to healthy controls. All biomarkers were assessed simultaneously by flow cytometry method. It was showed that the inflammatory molecules MCP-1, IL-12p70, TNF, VEGF, and the anti-inflammatory IL-10 were highly increased in urine samples of VUR patients. In those VUR patients with CKD ≥ 3, we also observed elevated urinary levels of CXCL9/MIG, IL-8, FGF, and VCAM-1.

Further studies are needed to identify within this group of proteins and polypeptides those molecules with the potential to become biomarkers capable of assisting in clinical decisions. Moreover, perhaps a panel of biomarkers composed of a combination of pro-inflammatory and anti-inflammatory factors would have greater accuracy in predicting the risk of progression to CKD than the measurement of a single marker.

Keywords: adhesion molecule; biomarkers; chemokines; cytokines; growth factors; inflammation; reflux nephropathy; vesicoureteral reflux.

LISTA DE ABREVIATURAS E SIGLAS

ACEi Angiotensin-converting enzyme inhibitors

ANH Antenatal hydronephrosis

APD Anteroposterior diameter

APN Acute pyelonephritis

APRPD Anteroposterior renal pelvis dilatation

ARB Angiotensin receptor antagonists

Basic FGF Basic Fibroblast Growth Factor

BBD Bladder and bowel dysfunction

CAKUT Congenital anomalies of kidney and urinary tract

CAP Continuous antibiotic prophylaxis

CCL2/MCP-1 Monocyte chemoattractant protein-1

CCL5/RANTES Regulated on activation, normal T cell expressed and secreted

CD54(ICAM-1) Intercellular adhesion molecule 1

CD106/VCAM-1 Vascular cell adhesion molecule 1

CKD Chronic kidney disease

CXCL9/MIG Monokine induced by interferon-γ

CXCL10/IP-10 Interferon-γ-induced protein-10

DAP Diâmetro ântero-posterior

DMSA 99m-Tc ácido dimercaptosuccínico / 99m- Tc dimercaptosuccinicacid

DRC Doença renal crônica

DTPA 99m-Tc ácido dietilenotriaminopentacético / 99m- Tc dimercaptosuccinicacid

eGFR Estimated glomerular filtration rate

ESRD End stage renal disease

FGF Fibroblast growth factor

HAS Hipertensão arterial sistêmica

IL-1β Interleukin-1β

IL-6 Interleukin-6

IL-8 Interleukin-8

IL-10 Interleukin-10

IL-12p70 Interleukin-12p70

ITU Infecção do trato urinário

LUTD Lower urinary tract dysfunction

MAG3 99m-Tc ácido mercaptoacetiltriglicinico /99m-Tc mercaptoacetyltriglycineacid

OJUP Obstrução da junção ureteropélvica

PNH Prenatal hydronephrosis

PUV Posterior urethral valve

RBUS Renal and bladder ultrasonography

RMD Rim multicístico displásico

RN Reflux nephropathy

RPD Renal pelvis dilatation

RS Renal scarring

RUS Renal ultrasonography

RVC Radionuclide voiding cystourethrogram

RVU Refluxo vesicoureteral

SFU Society of fetal urology

TGF-β Transforming growth factor-beta

TNF Tumor necrosis factor

UCM Uretrocistografia miccional

UE Urografia excretora

UPJO Uretero pelvic obstruction

US Ultrasonography / ultrassonografia

UTI Urinary tract infection

VCUG Voiding cystourethrogram

VEGF Vascular endothelial growth factor

VUP Válvula de uretra posterior

VUR Vesicoureteral reflux

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SECÇÃO RESULTADOS E DISCUSSÃO

Artigo original: Evaluation of molecules related to inflammation and fibrosis in pediatric patients with primary vesicoureteral reflux.

SUMÁRIO

1. INTRODUÇÃO

Refluxo vesicoureteral (RVU) é definido como o retorno do fluxo urinário da bexiga em direção ao ureter e pelve renal. Estima-se sua ocorrência em 0,4-1,8% da população pediátrica (1). RVU está presente em 31,1% das crianças com infecção do trato urinário (ITU) (2) e, em aproximadamente, 16,2% (variando de 7-35%) das crianças com diagnóstico de hidronefrose fetal (3). De acordo com dados do *North American Pediatric Renal Trials and Collaborative Studies* (NAPRTCS), a nefropatia do refluxo foi responsável por 5,1% dos transplantes renais (4) e foi a quarta causa mais comum de doença renal crônica (DRC) em pediatria (8,4%) (5).

Apesar dos evidentes avanços no conhecimento sobre a evolução clínica do RVU (6-9), ainda permanece como grande desafio no manejo desses pacientes identificar aqueles em risco de progressão para DRC. Estudos de imagem contribuem na identificação dos pacientes com maior potencial de evolução desfavorável. No entanto, alguns desses exames são invasivos, impõem exposição a radiação e são dispendiosos. Além disso, em diversos momentos as técnicas de imagem não são suficientes para definir o prognóstico e auxiliar na indicação de tratamento clínico ou cirúrgico. Dessa forma, o estudo de biomarcadores poderia auxiliar na compreensão da complexa fisiopatologia da nefropatia do refluxo, em sua definição prognóstica e, até mesmo, na identificação de novos alvos terapêuticos.

Os achados histopatológicos da nefropatia do refluxo incluem infiltrado intersticial com células inflamatórias crônicas, fibrose periglomerular e atrofia de células tubulares (10). É interessante ressaltar que a lesão renal pode progredir na ausência de infecção do trato urinário (ITU) e, inclusive, após a resolução do RVU (11, 12). As células inflamatórias infiltradas no interstício produzem quimiocinas que amplificam a resposta inflamatória renal. Além disso, diversos fatores produzidos pelas próprias células renais, como fatores de crescimento, moléculas de adesão celular e citocinas/quimiocinas, levam a progressão da fibrose intersticial (13, 14). Vários estudos já foram realizados com o intuito de encontrar biomarcadores na urina ou no sangue capazes de predizer a evolução dos pacientes com RVU (15). No entanto, até o presente momento, nenhum biomarcador clinicamente expressivo foi encontrado. Conforme descrito anteriormente, o processo inflamatório crônico possui um importante papel na etiopatogenia da nefropatia de refluxo, justificando-se, portanto, a investigação de fatores inflamatórios, como citocinas,

quimiocinas, fatores de crescimento e moléculas de adesão celular como biomarcadores do RVU.

A Unidade de Nefrologia Pediátrica do Hospital das Clínicas da UFMG tem desenvolvido várias pesquisas clínicas com o objetivo de melhor compreender o heterogêneo grupo de pacientes com uropatias. O presente estudo pertence a uma abrangente linha de pesquisa que pretende investigar aspectos clínicos, laboratoriais e biomarcadores das anomalias congênitas do trato urinário. Modelos de predição de desfechos clínicos em CAKUT (C*ongenital Anomalies of the kidney and Urinary Tract*) têm sido desenvolvidos em nosso grupo, com o objetivo de identificar precocemente pacientes com risco de evolução para DRC (16). Com relação à pesquisa de biomarcadores em nefropatias destacam-se três trabalhos publicados por nosso grupo. No primeiro estudo, foi encontrada associação entre redução da captação de DMSA com elevados níveis urinários de TGF-β, indicando um papel dessa citocina no processo fibrogênico das nefrouropatias congênitas (17). Um segundo estudo comparou pacientes com DRC a controles saudáveis e encontrou-se níveis urinários e séricos significativamente elevados de MCP-1/CCL-2. Ressalta-se, no entanto, que nesse trabalho foram avaliados pacientes com DRC secundária a nefrouropatias e também a glomerulopatias. Nesse mesmo estudo, níveis urinários elevados de IL-8/CXCL8 correlacionaram-se com redução de ritmo de filtração glomerular em pacientes com DRC secundária a CAKUT, sugerindo que essa quimiocina pode estar associada à formação de cicatrizes renais e à progressão da DRC (18). Recentemente, um intenso perfil inflamatório foi identificado em amostras urinárias de pacientes com VUP, com níveis significativamente elevados de IL-2, IL-4, IL-6, TNF, sTNFRI, sTNFRII, IFN-γ, MCP-1/CCL2, Eotaxin/CCL11 e IL-8/CXCL8. Níveis significativamente elevados da citocina antiinflamatória, IL-10, e de TGF-β também foram encontrados. Nesse estudo, evidenciou-se que moléculas inflamatórias estão elevadas desde o período fetal na VUP, sugerindo que o processo inflamatório possui um importante papel fisiopatológico nessa nefropatia desde o momento da embriogênese (19).

Essa dissertação de mestrado tem, portanto, o objetivo de identificar biomarcadores não invasivos clinicamente significativos no RVU. Conforme resolução previamente aprovada no Programa de Pós-Graduação em Ciências da Saúde – Saúde da Criança e do Adolescente, essa dissertação será apresentada em formato de artigos científicos que serão posteriormente submetidos à publicação. Serão apresentados dois artigos de revisão: 1. *Vesicoureteral Reflux: Clinical approaches;* 2. *Biomarkers in*

vesicoureteral reflux: an overview e um artigo original: *Evaluation of molecules related to inflammation and fibrosis in pediatric patients with primary vesicoureteral reflux*. As referências bibliográficas foram citadas no texto seguindo as Normas de Vancouver - (*Uniform requirements for manuscripts submitted to biomedical journals: writing and editing for biomedical publication*- www.ICMJE.org).

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2. REVISÃO

2.1 Review article

Title: Vesicoureteral Reflux: Clinical approach

1. Introduction

Vesicoureteral reflux (VUR) is defined as the anomalous backward flow of urine from the urinary bladder into the ureter and renal pelvis. It is a complex disease that reflects a dysfunction of the lower urinary tract and an anatomical anomaly at the ureterovesical junction. According to the dilatation of the renal collecting system and the extent of retrograde filling on a voiding cystourethrography (VCUG), VUR is classified into five grades by the International Reflux Grading system (1).

VUR can occur as an isolated anomaly or associated with other congenital anomalies of the kidney and urinary tract (CAKUT). It is also classified as primary or secondary. Primary reflux (PR) is the most prevalent type and occurs because of an anatomic insufficiency of the ureterovesical junction. Secondary VUR is the consequence of clinical conditions that lead to abnormally high intravesical pressure, including neurogenic bladder or posterior urethral valves.

2. Incidence and Prevalence

VUR affects 0.4-1.8% of otherwise healthy children (2). It is also present in 31.1% of children with urinary tract infection (UTI) (3), and in approximately 16.2% (range: 7–35%) of patients with prenatal hydronephrosis (4). VUR is more common in white versus black children (5), and is twice as prevalent in girls as in boys (5). Nevertheless, there is a male predominance in VUR diagnosed during follow-up of prenatal hydronephrosis (6-9).

Familial VUR implies that primary VUR can be a genetic disorder (10, 11). VUR is found in 35.7% (range: 16.4–61%) of the offspring of patients with primary VUR, and 27.4% (range: 2.9–51.9%) of siblings of children with VUR (4). Nowadays, VUR is considered a genetically heterogeneous disease and cumulative effect of rare mutations in distinct genes justifies the high frequency of VUR (12). However, the currently available data is conflicting as to whether which genes are involved in the formation of a refluxing ureterovesical junction (12-15).

3. VUR and Reflux Nephropathy

Reflux nephropathy (RN) consists of VUR associated with renal scarring (RS) and is classified as congenital or acquired. High grade primary reflux associated with perinatal hydronephrosis is more common in male infants and is generally more likely to progress to spontaneous resolution. Renal pathophysiological changes in these males are due to congenital kidney dysplasia and hypoplasia. In contrast, renal scars acquired after recurrent episodes of urinary tract infection (UTI) in the presence of VUR are more common in females (9, 16-18).

In patients with VUR, UTI is considered one of the main factors involved in the pathophysiology of renal scars. The immune response against this infection directly damages tubular cells, causes renal ischemia, and leads to reperfusion injury. The inflammatory process that is established after UTI with the aim of tissue regeneration, leads to fibrosis and, consequently, to the formation of renal scars (19).

Intrarenal reflux is also an important causative factor in the development of RS. However, intrarenal reflux of uninfected urine produces renal scars only in the context of abnormally high intravesical pressure (20). Additionally, RS has been observed in children without VUR (21) and even in those whose VUR has already been resolved. Factors responsible for progression of parenchymal renal injury even after VUR resolution include release of autologous renal antigens, hyperfiltration of undamaged nephrons, increased superoxide production, and persistent glomerular hypertension (19).

4. Clinical evolution

VUR may produce end-stage renal disease (ESRD), but it can also be a benign condition with few long-term clinical consequences. Effects of reflux nephropathy include proteinuria, hypertension, growth impairment and ESRD.

Hypertension was detected in up to 30% of children with RS, being RN an important cause of severe hypertension in children and young adults (16, 22, 23). The exact mechanism of hypertension needs to be better understood. It was suggested that the renin-angiotensin system could be activated by segmental ischemia present in RN (24, 25). However, in the past, studies were not conclusive on the role of the renin-angiotensin system in this pathology (26). Other vasoactive compounds and cytokines appear to influence the hemodynamic and structural changes that occur in RN, such as nitric oxide, eicosanoides, tumor necrosis factor, tumor growth factor, epithelial growth factor, platelet-derived growth factor, and beta-fibroblast growth factor (27).

Microalbuminuria has been reported in 51% of children with RS and an inverse correlation was found between urine albumin excretion and GFR (28). Although proteinuria is not common in childhood, it has been described in 31% of adult patients with RN (29). The presence of proteinuria is a marker of progressive glomerulosclerosis and worse renal prognosis. Hyperfiltration in the remaining nephrons leads to a change in permselectivity of macromolecules (including albumin) and to the progression of reflux nephropathy (30).

RN is an important cause of progressive renal failure and ESRD. Most renal scars develop in early childhood but worsening in renal function persists over the next few years. The incidence of ESRD among children with VUR is difficult to assess. According to the 2014 North American Pediatric Renal Trials and Collaborative Studies (NAPRTCS) report, RN is present in 5.1% of transplanted patients. In 2008, NAPRTCS reported that RN was the fourth most common cause of CKD in children (8.4%). Data from the Italkid project concluded that the estimated risk of children with primary VUR progressing to ESRD by the age of 20 years was 56%. Patients with a creatinine clearance rate (ClCr) of less than 40 mL/min/1.73 m^2 at baseline had a fourfold higher risk to progress to ESRD than those with a greater ClCr (22).

The possibility of developing renal scars depends on a set of factors. In a systematic meta-analysis, the American Urological Association (AUA) has identified increasing frequency of UTI, increasing grade of VUR and presence of bladder and bowel dysfunction (BBD) as risk factors for RS (31).

As mentioned, VUR may have few long-term sequelae and can evolve to spontaneous resolution. Negative predict factors for reflux resolution are the presence of renal cortical abnormalities, bladder dysfunction, high grade reflux at diagnosis, and breakthrough febrile UTI (32-34). Persistence of VUR is more common in patients with VUR grades IV–V, bilateral VUR and, when the age of diagnosis is over one year old (33, 35-37).

Bladder and bowel dysfunction was associated with an increased risk of febrile UTI in children with VUR on continuous antibiotic prophylaxis (CAP) and with an increased rate of UTI in postoperative treatment, as well as being linked with a decreased rate of reflux resolution 24 months after diagnosis and with a decreased rate of cure following endoscopic therapy (31). A questionnaire with dysfunctional voiding scoring system can be used for the diagnosis of BBD (38). VUR surgical treatment should preferably be indicated only after BBD treatment. Possible treatment options for BBD are

behavioral therapy, biofeedback, and medications (anticholinergics or alpha blockers). Constipation should also be treated (31).

5. Diagnosis

Primary reflux is usually diagnosed after urinary tract infection or during prenatal hydronephrosis follow-up. Another situation that leads to VUR investigation is the screening of a sibling of a VUR patient.

VCUG is the gold standard imaging test for the diagnosis of VUR. It provides accurate anatomic details, including the uretra, and allows grading of VUR. Radionuclide voiding cystourethrogram (RVC) is a highly sensitive diagnostic test but presents poor anatomic detail definition and is unable to assess the male urethra. VCUG is the most appropriate starting image and RVC is used for follow-up. RVC is also useful for postoperative evaluation, and for screening siblings of patients.

Ultrasonography is very useful to obtain information about renal growth and scarring. However, it is unable to provide a quantitative assessment of relative renal function, may not diagnose all RS and is operator dependent. DMSA (99m-Tcdimercaptosuccinic acid) is the best test to assess renal function and to detect renal scars. It can also confirm acute pyelonephritis.

The first exam for assessment of children with prenatally diagnosed hydronephrosis (PNH) is ultrasonography of the kidney and bladder. A normal postnatal ultrasonography does not exclude VUR; however, it is useful to exclude the presence of significant obstruction. VCUG is indicated for infants with high-grade hydronephrosis (SFU Grades 3-4), ureteral dilation or abnormal bladders on ultrasound, or for those who manifested UTI (4). The European Association of Urology (EAU) Guidelines on Vesicoureteral Reflux in Children recommend the use of VCUG in PNH patients with ultrasonographic findings of bilateral high-grade hydronephrosis, both kidneys with hydronephrosis, ureterocele, ureteral dilatation, and abnormal bladders (39).

For children under the age of 2 years, VCUG is recommended after the first febrile UTI by the American and European Urological Associations. However, the American Academy Pediatrics (AAP) recommend that VCUG should be performed at this population only in cases of recurrent or atypical febrile UTI, or when renal ultrasound reveals abnormalities (scarring, hydronephrosis, indirect signs of severe VUR or obstructive uropathy). DMSA scan is the next evaluation if reflux is diagnosed (39-41).

A top-down approach is also an alternative option. In this approach, a VCUG is done if a DMSA scan reveals renal involvement close to the time of a febrile UTI. A normal DMSA scan, without follow-up VCUG, leads to non-detection of 5–34% of VUR cases. However, unnecessary VCUG would be avoided in 35-49% of individuals screened if DMSA is normal (42-45).

Sibling reflux is usually of low severity, asymptomatic and resolves spontaneously. In siblings of children with VUR and in offspring of patients, a VCUG or RVC is recommended if the ultrasound shows renal cortical abnormalities, renal size asymmetry or findings that suggest high-grade VUR or if there is a history of UTI (4).

6. Management of VUR

Conservative approach includes strict clinical monitoring, intermittent or CAP, and treatment of bladder and bowel dysfunction. Follow-up with no intervention has been proposed as another option for low-grade VUR.

6.1 Follow-up

Clinical evaluation and imaging studies are indicated to evaluate spontaneous resolution and kidney status. The AUA (31) recommends that on initial diagnosis of VUR and annually, the child should undergo general medical evaluation including measurement of height, weight and blood pressure. Urinalysis to detect proteinuria and bacteriuria is also recommended. At initial presentation serum creatinine should be measured if bilateral renal cortical abnormalities are present.

Regarding regular imaging follow-up, the AUA (31) recommends renal ultrasonography at baseline and every 12 months to assess renal growth and the development of parenchymal scars. The EAU Guidelines recommend biannual ultrasonography (39). DMSA scintigraphy is recommended when there are abnormalities in renal ultrasound, in VUR grade III-IV, after episodes of UTI with the possibility of generating new scars, or when there is worsening of renal function (31). Voiding cystography is recommended every 1 to 2 years. In those patients with the possibility of low spontaneous resolution rates, the intervals between studies should be longer (31). Resolution of VUR can be confirmed by a single normal voiding cystogram.

6.2 Antibiotic prophylaxis

Prolonged use of antibiotics is intended to avoid pyelonephritis and RS by preventing the ascending flow of bacteria from the bladder into the upper urinary tract. However, the effectiveness of CAP is still controversial with current data.

Recent meta-analysis and systematic reviews concluded that CAP significantly reduced the risk of febrile and symptomatic UTI in children with VUR. However, CAP did not prevent the occurrence of new RS (46-48). These conclusions should be viewed with caution because of the variable subpopulations and methodologies of these RCTs. Meta-analysis by Hewitt *et al.* pointed out that, with the exception of the Swedish trial, all the other trials analyzed were unable to demonstrate any reduction in new RS with CAP (48). In this trial a beneficial effect of prophylaxis was observed only in girls (49). Another interesting fact is that when RIVUR data were not included in meta-analyses by Bessa Jr *et al*., only children with high-grade VUR would benefit from CAP (46).

The RIVUR Trial (50) concluded that antimicrobial prophylaxis reduced the risk of recurrence of UTI but did not avoid the occurrence of new RS. Patients in which antibiotic prophylaxis was most effective in terms of reducing UTI recurrence were children whose first episode of UTI was febrile and those with BBD. As expected, resistant pathogens were more common in the recurrences of children who received antibiotic prophylaxis (50).

Currently, the AUA (31) recommend CAP for patients under one year of age with VUR with a history of febrile UTI, regardless of the degree of reflux. In the context with no history of UTI, CAP is indicated for patients under one year of age with VUR grade III-V, and for patients with VUR and BBD (31). The EAU Guidelines consider that all VUR patients diagnosed within the first year of life should receive CAP (39). In children over than one year of age, CAP should be maintained in the presence of BBD, renal cortical abnormalities, and recurrent febrile UTI (31). For CAP discontinuation, it is interesting that children have already been toilet trained and they should not have symptoms of BBD (39).

The antibiotics of choice are trimethoprim–sulfamethoxazole and nitrofurantoin. These drugs are effective against the most common urinary pathogens, have minimal impact on intestinal flora and reach high urine concentration.

6.3 **Surgical Theraphy**

Traditional indications for surgical correction include presence of ureterovesical junction pathologies (e.g., ureterocele or diverticuli), grade V VUR, breakthrough febrile

UTIs in the correct use of CAP, deterioration of renal function, and development of new renal scars or decreased renal growth during clinical management.

The International Reflux Study in Children evidenced that either surgical or medical management of patients with grade III–IV reflux can be safe (51). Ten years of follow-up data were available for 252 children randomly assigned to either antimicrobial prophylaxis or ureteral reimplantation. Renal growth, UTI recurrence, renal function, radionuclide imaging and somatic growth were similar in both groups. However febrile infections were less frequent in surgical managed patients.

Surgical therapeutic options include open and endoscopic methods. Although the endoscopic injection method can be effective in low-grade reflux, open surgery has superior results for higher grades (39). A renal ultrasound should be obtained to rule out obstruction after both, open or endoscopic procedures (31).

The success rate for endoscopic methods was evaluated in a meta-analysis including 8101 renal units. Reflux resolution rates following the procedure were progressively lower as the grades of reflux got higher. The rates were better after more injections. Factors associated with worse response to endoscopic treatment were duplicated systems and neuropathic bladders (52). The success rate for open surgical correction is > 95% (53, 54).

7. Follow up after resolution of VUR

If there is abnormality in kidney by ultrasound or DMSA scanning, a general evaluation, including monitoring of blood pressure, height and weight, and urinalysis for proteinuria and UTI is recommended by the AUA annually through adolescence.

8. Conclusion

Although VUR may be a benign condition with few long-term clinical consequences, it can also progress to end-stage renal disease (ESRD). Nowadays, the management of VUR has been focused on CAP or surgical intervention in selected cases to avoid or at least delay CKD progression. VUR pathophysiology is complex and involves a wide variety of interacting factors such as genetic, environmental, fibrogenic, and inflammatory molecules. Identify VUR patients at higher risk for renal scarring and progression to CKD is imperative to avoid unnecessary complementary exams and therapeutics in those patients at low risk for ESRD.

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2.2. Review Article

Title: Biomarkers in Vesicoureteral Reflux: an overview

Introduction

Vesicoureteral reflux (VUR) is defined as the anomalous backward flow of urine from the urinary bladder into the ureter and renal pelvis. VUR can occur as an isolate anomaly or associated with other congenital anomalies of the kidney and urinary tract (CAKUT), including multicystic kidney, hypodysplastic kidneys, renal agenesia, and renal or ureteral ectopia. VUR is classified as primary or secondary. Primary reflux occurs because of an anatomic insufficiency of the ureterovesical junction, while secondary VUR is the consequence of clinical conditions that lead to abnormally high intravesical pressure.

VUR affects 0.4–1.8% of otherwise healthy children (1). It is also present in 31.1% of children with urinary tract infection (UTI) (2) and in approximately 16.2% (range: 7–35%) of patients with prenatal hydronephrosis (3). Effects of reflux nephropathy include proteinuria, hypertension, growth impairment and end-stage renal disease (ESRD). According to the North American Pediatric Renal Trials and Collaborative Studies, reflux nephropathy (RN) was present in 5.1% of transplanted patients (4) and was the fourth most common cause of chronic kidney disease in children (8.4%) (5).

The great challenge in the management of VUR patients is to identify patients with potential risk of ESRD. Imaging techniques contribute to patient risk stratification. However, they are often not sufficient for a precise prognostic definition, as well as for determining between a surgical or clinical approach. Furthermore, imaging techniques can be invasive, require radiation exposure, and are expensive. In this way, the study of biomarkers may help understanding the complex pathogenesis of VUR and may become alternative therapeutic targets.

Despite the investigation of several serum and urinary biochemical markers, there are no reliable markers that predict renal function in patients with VUR. The objective of this paper is to review the role of cytokines and chemokines including interleukin-6 (IL-6), interleukin-8 (CXCL8/IL-8), interleukin-1 beta (IL-1β), tumor necrosis factor (TNF), interleukin-10 (IL-10), transforming growth factor-beta (TGF-β) and monocyte chemotactic protein-1 (CCL2/MCP-1); growth factors such as vascular endothelial grown factor (VEGF) and fibroblast grown factor (FGF) and cellular adhesion molecules

comprising intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM), as potential biomarkers for VUR and RN. For this purpose, we have searched for articles at PubMed and Scopus by using the combination of words: “VUR” or “RN” and each one of these biomarkers.

Pathophysiology of vesicoureteral reflux

Renal scarring linked to VUR is called reflux nephropathy (RN) and is categorized as “congenital” or “acquired”. The exact mechanism by which reflux produces renal scars lacks clarification. Histological alterations related to RN are sclerosis and enlargement of glomeruli, separation of glomeruli from proximal tubules, tubular dilatation and tubular cells atrophy, interstitial infiltration of inflammatory cells and tissue fibrosis. Glomerular changes are secondary and probably occur as a consequence of periglomerular fibrosis contraction leading to ischemia. These histopathological findings of renal scarring are characteristic of “acquired” RN and do not contain dysplastic features (6, 7). Differently, congenital RN shows dysplasia and its main histological findings are the presence of primitive ducts associated with increased mesenchyme, reflecting intrauterine anomaly of kidney development as an important element in its pathogenesis (8).

Renal injury in children with RN may progress even after reflux has resolved and even when episodes of UTI have not occurred. Infiltrating leukocytes produce chemokines that may amplify inflammatory response in the kidney. In addition, the hydronephrotic kidney produces a variety of intrarenal factors that contribute to progressive interstitial fibrosis, including growth factors, cell adhesion molecules and cytokines/chemokines (9, 10). Altered renal expression of growth factors and cytokines also modulate this chronic inflammatory process. In contrast, several anti-fibrotic mechanisms were triggered in response to RN, supporting the potential role for the stimulation of renal endogenous counter-regulatory molecules against progressive fibrosis (9, 11). Continuous renal parenchyma damage may occur even after the resolution of VUR. Factors responsible for this process include release of autologous renal antigens and of Tamm–Horsfall protein, hyperfiltration of undamaged nephrons, increased superoxide production, and persistent glomerular hypertension (12). Glomerular hyperfiltration and hypertension result in proteinuria, glomerulosclerosis, excessive stimulation of renin angiotensin system, and progressive chronic renal disease (6, 13).

Biomarkers in Vesicoureteral Reflux

Several molecules measured in serum or urine has been investigated as possible biomarkers of VUR and/or RN. This article aims to review the role of cytokines/chemokines (IL-1β, IL-12p70, TNF, IL-6, IL-10, TGF-β, MCP-1, IL-8), growth factors (FGF, VEGF), and cellular adhesion molecules (ICAM-1, VCAM). Besides that, associations between VUR and gene polymorphisms of TNF-α, TGF-β, IL-10, IL-12 and VEGF were also discussed (14-18).

Cytokines and Chemokines in Vesicoureteral Reflux:

1. CCL2/MCP-1

CCL2/MCP-1 is an important mononuclear cell chemoattractant produced by some mesenchymal cells, including glomerular cells (19). This chemokine induces the release of fibrogenic factors and consequently, leads to an increase in the extracellular matrix. MCP-1 may also induce injury via release of oxygen radicals and lysosomal enzyme, resulting in tubular atrophy and necrosis (20, 21). Many studies have associated CCL2/MCP-1 levels with glomerulopathies (19, 22) and with renal transplant (10, 23). This chemokine was also evaluated in uropathies and its expression was strictly correlated with tubular damage and extent of monocytes infiltration (20, 21, 24-27).

Increased MCP-1 expression and reduction of epidermal growth factor (EGF) expression at tubulointerstitial sites were correlated with severity of apoptosis in RN (21). The downregulation of EGF and enhanced production of CCL2/MCP-1 may lead to increased release of proinflammatory and profibrotic cytokines. This process may stimulate apoptosis, which, in turn, contributed to tubular atrophy, kidney tissue damage and deterioration of renal function in RN (21). EGF is mainly produced by the ascending portion of Henle's loop and the distal convoluted tubule of the kidney and is a potent modulator of tubular cell proliferation during development and maturation of the kidney (20, 24).

Regarding ureteropelvic junction obstruction (UPJO), it has been reported that CCL2/MCP-1 urinary concentrations and gene expression at tubulointerstitial sites were increased, while the EGF urinary levels and gene expression were notably reduced (24, 28). Surgical treatment of UPJO produced a significant elevation of urinary concentration of EGF associated with a decrease of CCL2/MCP1 levels (24). However, the measurement of urinary levels of CCL2/MCP-1 failed to identify those patients with UPJO who would require surgical correction (29).

2. CXCL8/IL-8

CXCL8/IL-8 is a chemokine produced by mesangial and epithelial cells of the kidney in response to inflammatory stimuli. This chemokine attracts neutrophils and lymphocytes to the inflammation site. Levels of IL-8/CXCL8 were found elevated in the urine of patients with acute pyelonephritis (30, 31), VUR (32, 33), and renal scars (30, 33, 34).

Elevated urinary levels of CXCL8/IL-8 in children with VUR may contribute to RN, even in the absence of episodes of UTI (32, 33, 35). According to this, CXCL8/IL-8 was proposed as a biomarker for the diagnosis of VUR in infants without a recent UTI episode (32). Our research group has reported a correlation between high urinary levels of CXCL8/IL-8 and reduced glomerular filtration rate (GFR) in congenital anomalies of the kidney and urinary tract (CAKUT) patients, suggesting that this chemokine might be associated with renal scarring and CKD (22). These findings suggest that urinary measurements of CXCL8/IL-8 may be useful in detecting VUR patients with more advanced kidney damage and those who need close follow-up.

Contrary to the previously mentioned studies, some authors did not find an association between urinary or serum concentrations of CXCL8/IL-8 and RN (36-39). These contradictory results regarding the association of CXCL8/IL-8 and VUR indicate that further studies are needed. However, this interleukin appears to play an important role in chronic interstitial inflammation in RN and may be a promising biomarker for renal damage in these patients.

3. IL-6

Interleukin-6 (IL-6) is a proinflammatory cytokine that induces the activation and differentiation of B and T cells, being one of the main mediators of inflammation in response to bacterial infection. Several cell lineages including fibroblasts, activated macrophages, T and B cells, endothelial, mesangial, and renal tubular cells can produce IL-6. It was shown by an immunohistochemical study that the main source of this protein was tubular cells within and adjacent to fibrotic areas, corroborating the hypothesis that tubular IL-6 may be involved in the pathogenesis of tubulointerstitial injury in RN (40, 41).

Although some studies have shown an increase in serum and urinary levels of IL-6 in the acute phase of pyelonephritis, no associations were found between this biomarker and the presence of VUR (31, 33, 38) and of RN (33). On the other hand, other authors

have associated high levels of IL-6 with the presence of VUR (34) or RN (38, 39). Sheu *et al* have detected that high levels of IL-6 in serum and in urine of young children at first time febrile acute pyelonephritis were associated to an enhanced risk of future renal scarring (42). In addition, there was found to be a significant relationship between the grade of renal scars and urinary levels of IL-6 (40, 43).

Some factors may justify the discrepancies between these results. First, a recent acute inflammatory process (for instance, a febrile UTI) can probably elevate serum and urinary levels of IL-6, leading to a misinterpretation on the association between this cytokine and VUR or RN. Second, prophylactic antibiotics given to patients with VUR could also affect IL-6 production. Additionally, surgical correction of VUR prior to study entry could influence the outcome. Another consideration is that, despite similar methods being used for the measurement of urinary levels of IL-6 in most studies, there were differences in the detection limits, sensitivity, and specificity of the assays.

In conclusion, measurements of the urinary concentration of IL-6 seem to reflect renal production and the severity of tubulointerstitial damage. Confounding factors including acute or recent UTI and the chronic use of chemoprophylaxis should be taken into account to interpret the results. However, the role of IL-6 in the progression of RN should be further investigated.

4. IL-1β

IL-1 has two distinct forms: IL-1α and IL-1β. IL-1α is usually associated to a membrane and/or a cell, while IL-1β is found free in the serum, urine and synovial fluid. One of the most important biological activity of IL-1 is the activation of T lymphocytes and stimulation of B-cell proliferation, thereby enhancing immunoglobulin synthesis (41).

It has been shown that urine IL-1β is elevated in acute pyelonephritis (44) and acts in both inflammatory and anti-inflammatory responses. Urinary levels of IL-1β were also positively correlated with other inflammatory biomarkers. After antibiotic treatment, urinary levels of IL-1β returned to normal range and there was no difference in urine measurements of IL-1β levels in children with or without VUR (44). On the other hand, urinary levels of IL-1β and IL-1α were reduced in patients with renal scarring, suggesting that persistent elevation of these cytokines might control kidney tissue inflammation and fibrosis (44, 45). In a study of experimentally induced acute *E. coli* pyelonephritis in IL-1β deficient mice, Hertting *et al* found that this cytokine has an essential anti-

inflammatory function in renal pathogenesis (46). These findings show that changes in kidney tissue release of IL-1β may exert a crucial role in acute pyelonephritis and in subsequent development of renal scarring in children.

5. TNF

Data evaluating TNF in VUR is still very limited. However, considering the proinflammatory properties of TNF, it seems reasonable to investigate its role on tubulointerstitial damage in RN and on the progression to CKD. TNF is cytotoxic to tubular and epithelial cells of the renal tissue, leading to direct renal injury, necrotic cell death, and apoptosis. TNF can also affect intraglomerular blood flow and decrease of GFR as a result of disequilibrium between vasoconstrictor and vasodilator molecules (17).

TNF is produced by the renal cortical tubular cells in response to ureteral obstruction and seems to initiate tubulointerstitial injury by upregulating monocyte chemoattractants (such as CCL2/MCP-1) at early stages of obstruction (9, 47). Studies have shown elevated TNF levels in animal models and patients with ureteral obstruction, which return to normal after surgical correction of the obstruction (47, 48).

In regard to VUR, significant elevations of IL-6 and TNF levels were detected in patients with RN (39). Schwentner *et al* evaluated the extracellular microenvironment and cytokine profile of the ureterovesical junction in children with VUR and found that while profibrotic cytokines and extracellular matrix proteins are increased, smooth muscle cell related growth factors are downregulated in the refluxing ureteral wall. In this study, insulin-like growth factor-1, nerve growth factor and VEGF were more expressed in healthy controls, whereas TNF and TGF-β1 were significantly higher in VUR patients (49).

It has been shown that specific polymorphism at position -308 in the TNF gene promoter is associated with an increase in TNF gene transcription *in vitro* (50), which can influence the occurrence of inflammatory diseases. Although some studies have attempted to find an association between TNF AA genotype and the susceptibility to renal scarring in patients with VUR, the results were contradictory (14-16).

6. TGF-β

TGF-β is a cytokine intimately related to tubulointerstitial fibrosis in renal tissue. This cytokine stimulates the synthesis of matrix proteins, the deposition of collagen in

renal tissue, and apoptosis of kidney tubules (9). TGF-β is produced by renal tubular cells, but interstitial fibroblast cells appear to be the main source of TGF-β (51). TGF-β induces the production of proinflammatory cytokines in the proximal tubule cells, including IL-8, MCP-1, MCP-4 and RANTES (52). Therefore, it is believed that elevation of urinary concentration of TGF-β may reflect renal tissue fibrosis. On the other hand, TGF-β can also exert regulatory or even anti-inflammatory effects by inhibiting the proliferation of many different cell types (41).

Different animal models of CAKUT support the role of TGF-β as a potential biomarker for urinary tract obstruction and interstitial fibrosis (53-57). Preliminary investigations in humans have also suggested that the urinary concentrations of TGF-β seem to be helpful for the identification of patients with obstructive uropathies and clinically relevant CAKUT (58-63).

Alterations of TGF-β expression during kidney development might increase the likelihood of developing renal dysplasia (64). TGF-β regulates the growth and branching of the ureteric bud contributing to the formation of the collecting system and ureters. Furthermore, exposure of cultured metanephron to high concentrations of TGF-β inhibits ureteric duct growth and reduces the number of nephrons (65).

Concerning VUR and RN, Sabasiñska *et al*. showed that urinary TGF-beta levels increased proportionally with reflux severity, being even higher in cases of bilateral VUR (66). Since renal fibrosis is the major histological change in RN, urinary and serum levels of TGF-β may be associated with the presence of renal parenchyma injury or renal scarring in VUR. Another interesting study demonstrated that mechanical stress mimicking VUR stimulated renal tubular epithelial cells to secrete TGF-β in response to intermittent-pressure-loading (67). This study supports the hypothesis that mechanical stress induces renal cells to release cytokines that will trigger subsequent cellular events.

An increasing amount of evidence suggests a significant role of the genetically determined host response to UTI and subsequent scar formation. In this regard, genetic polymorphism of the gene encoding TGF-β was correlated with a genetic predisposition for renal scarring (18, 68, 69). However, studies are still contradictory regarding the association of TGF-β gene polymorphisms and the occurrence of VUR (69, 70).

TFG-β has also been investigated as a therapeutic target for nephropathies. Monoclonal antibody use for all three isoforms of TGF-β in rats with unilateral ureteral obstruction led to a decreased interstitial volume, collagen concentration, tubular apoptosis, and tubular atrophy (71). Isaka *et al.* found a significantly smaller extent of

fibrotic area after the use of TGF-β antisense oligodeoxynucleotides in unilateral ureteral obstruction (51). In theory, angiotensin receptor blockers (ARBs) and angiotensin converting enzyme (ACE) inhibitors may exert a protective effect against RN. Accordingly, the administration of an ARB, losartan, reduced the expression of TGF-β in a unilateral ureteral obstruction model (72). Unfortunately, long-term use of these drugs (enalapril and losartan) can be harmful for neonates or young babies. These medications may impair nephrogenesis and normal renal growth (73).

7. IL-10

IL-10 is an anti-inflammatory cytokine that exerts a counter-regulatory role in immune response by opposing the actions and by inhibiting the synthesis of TNF, IL-1, IL-6, IL-12, and IFN-γ. This cytokine is produced by T lymphocytes, monocytes, and B cells. IL-10 plays an important role in controlling and interrupting the inflammatory response. Moreover, this cytokine regulates the proliferation and differentiation of various immune cells, including T cells, B cells, natural killer cells, antigen presenting cells, mast cells, and granulocytes (41, 74).

Concerning nephrouropathies, polymorphisms of the gene encoding IL-10 were associated with increased risk of VUR (17). An experimental study revealed significantly lower levels of IL-10 in renal parenchyma and urine of acute unilateral obstructed animals, while renal levels of IL-1β, IL-6, and TNF were increased (75).

8. Other cytokines

Other cytokines were also associated to CAKUT and VUR. For instance, an increased incidence of congenital hydronephrosis was detected in transgenic animals that overexpressed IL-9. This anomaly was also dependent to the presence of the cytokines IL-4 and IL-13 (76). In children with RN, Konda *et al* reported that serum levels of soluble IL-2 receptor increased proportionally to the progression of renal injury (77). Serum soluble IL-2 receptor is a sensitive and quantitative marker of lymphocyte activation. KordiTamandani *et al* showed a significant association between IL-12 (AA) gene polymorphism and increased risk of VUR (17). IL-12 stimulates the differentiation of naive T cells into Th1 cells, resulting in the increased production of IFN-γ.

Growth Factors and cellular adhesion molecules in VUR

In addition to cytokines and chemokines, altered renal expression of growth factors can induce apoptosis or phenotypic transformation of glomerular, tubular, and vascular cells. Furthermore, cellular adhesion molecules have an important role in the immune response by modulating cell–cell interactions during inflammatory process. Thus, it seems reasonable to investigate the role of these molecules in VUR and RN.

1. VEGF and FGF

VEGF is expressed in glomerular podocytes and tubular cells. VEGF is a key mediator of physiological and pathological angiogenesis and an important regulator of vascular permeability. It also exerts chemoattraction of monocytes and macrophages and by so doing VEGF amplifies the inflammatory response (78). In glomerulopathies and in chronic tubulo-interstitial disorders, the expression of VEGF is enhanced (18, 79).

Concerning VUR, studies have not yet been able to independently associate VEGF polymorphisms with the presence of VUR, VUR severity and evolution to renal scarring (18, 80, 81). In CKD, the VEGF −460 C allele has been found strongly associated with progressive renal failure (82).

Interestingly, Konda *et al*. did not find an association between plasma and urinary levels of VEGF in patients with RN. The authors interpreted that urinary levels of VEGF seem to reflect the local production of this factor in renal tissue. Since urinary VEGF levels raised before the increase in serum ß2-microglobulin and/or urinary α1-microglobulin, urine measurements of this factor might indicate early stages of RN (83).

FGF is a growth factor that was also evaluated in patients with VUR. FGF is an important mitogenic and chemotactic factor for fibroblasts. In summary, macrophages, T lymphocytes, as well as glomerular and tubular epithelial cells secrete profibrogenic cytokines that stimulate fibroblasts. The release of these profibrogenic cytokines, particularly TGF-β and platelet-derived growth factor (PDGF), lead to fibroblast activation and, consequently, cell proliferation, extracellular matrix synthesis and autocrine stimulation, mediated by FGF (84). Kobayashi *et al*. found that VUR patients with renal scarring have elevated serum levels of FGF than those VUR patients without renal scarring, indicating that this molecule could be associated with RN (85).

2. VCAM/CD106 and ICAM/CD54

VCAM-1 and ICAM-1 are members of the immunoglobulin supergene family of adhesions molecules that is expressed in several cell types, including resting endothelial

cells and leukocytes. Proinflammatory cytokines and lipopolysacharides activate VCAM-1 and ICAM-1 expression on cell surface. VCAM-1 plays an important role in the firm adhesion and transendothelial migration of lymphocytes by binding to the very late antigen-4, while ICAM-1 plays a critical role in mediating lymphocyte adhesion to tubular cells (86).

Overexpression of VCAM-1 was detected in renal tubules with intense infiltration of inflammatory cells, suggesting a role for this adhesion molecule in leukocyte-tubular epithelial interactions related to tubulointerstitial inflammation. This inflammatory process was detected in both primary tubulointerstitial disorders as well in secondary inflammatory alterations produced by glomerulopathies. Kaneyama *et al* showed that serum VCAM-1 levels were significantly higher in patients with VUR if compared with healthy controls. Moreover, serum VCAM-1 levels were significantly higher in patients with high grade VUR (IV or V) than in those with grade III, independently of the presence of renal scarring (87).

Previous studies have suggested that ICAM-1 plays an important role in renal immune injury. An increased expression of ICAM-1 has been reported in allograft rejection, glomerulonephritis, and tubulointerstitial inflammation (86). Regarding VUR, serum levels of ICAM-1 were significantly higher in young children (age <2 years) with VUR and renal scarring than VUR patients without renal scarring. On the other hand, in children older than 2 years of age, there was no difference in serum ICAM-1 levels among VUR patients with or without renal scarring (88). Therefore, ICAM-1 could represent an interesting biomarker of renal tissue damage in younger children with VUR. In line with these findings, renal tissue of patients with RN exhibited increased mRNA expression and immunoreactivity for ICAM-1 in the glomerulus, interstitium and proximal tubulus (89). These findings suggest that ICAM-1 may play an important role in the pathogenesis of renal damage in RN.

Future Perspectives

VUR is the fourth most common cause of CKD in children (5). The management of VUR has been focused on continuous antibiotic prophylaxis or surgical intervention in selected cases to avoid or at least delay CKD progression. VUR pathophysioloy is complex and involves a wide variety of interacting molecules. Moreover, renal injury is well known to progress even after reflux resolves and even in the absence of episodes of UTI. Genetic, environmental, epigenetic, inflammatory, and fibrogenic factors

responsible for renal scarring need to be better understood and investigated. New diagnostic approaches to VUR and predictive factors for RN development are needed. In this context, research on novel biomarkers of VUR and RN has become essential. Clinical and experimental studies demonstrate the role of inflammation in renal diseases. Understanding the effects of cytokines, chemokines, growth factors, and cellular adhesion molecules on renal injury onset and progression is therefore of great importance.

Studies about VUR and RN pathogenic process are still scarce, precluding conclusive remarks. Based on this review, we consider that the fibrogenic cytokine, TGF-β, the anti-inflammatory cytokine, IL-10, and the proinflammatory cytokines, IL-6, IL-8 and TNF, should be more intensively evaluated as potential biomarkers for renal scarring and for progression to CKD in RN. IL-1β seems to play an important role in acute phase of pyelonephritis and might prevent the formation of renal scarring. Gene polymorphism of IL-12 was associated with increased risk of VUR, but further studies are necessary to confirm this finding. In addition, urinary levels of CCL2/MCP-1 might be an early biomarker of progressive renal damage and might have a potential role in predicting the long-term renal outcome. Growth factors and cellular adhesion molecules are also involved in the physiopathology of RN. In this regard, the cellular adhesion molecules ICAM-1 and the growth factors, basic FGF and VEGF, should be assessed as potential biomarkers for RN. Whereas VCAM appears to be associated with reflux severity, independent of progression to renal scarring.

Further studies are needed to identify the measurement of which among these molecules can really help clinical decisions. Moreover, perhaps a panel of biomarkers or a combined measurement of several markers may identify VUR patients at higher risk for renal scarring and progression to CKD with more accuracy than the measurement of a single marker.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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3. OBJETIVOS

Objetivo geral

Identificar biomarcadores não invasivos clinicamente relevantes em pacientes pediátricos com refluxo vesicoureteral primário.

Objetivos específicos

1. Verificar a associação das concentrações urinárias de quimiocinas e citocinas (CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, CXCL10/IP-10, IL-8, IL-1β, IL-6, IL-10, TNF, IL-12p70), fatores de crescimento (*basic* FGF, VEGF) e moléculas de adesão (CD54/ICAM-1, CD106/VCAM-1) com a presença de RVU e a progressão para doença renal crônica secundária à nefropatia do refluxo.

2. Avaliar os níveis urinários desses biomarcadores em pacientes com progressão para ritmo de filtração glomerular ≤ 60 mL/min/1.73m² em comparação com pacientes com doença renal crônica estágios 1-2 e crianças saudáveis.

4. PACIENTES E MÉTODOS

4.1 Delineamento

Estudo transversal, com amostra de conveniência de pacientes pediátricos portadores de refluxo vesicoureteral (RVU) primário, para avaliação de biomarcadores urinários.

4.2. População, local e período do estudo

4.2.1 Critério de inclusão

O critério de inclusão foi a confirmação do diagnóstico de RVU primário, por meio da uretrocistografia miccional (UCM), em pacientes pediátricos encaminhados à Unidade de Nefrourologia Pediátrica do Hospital das Clínicas da UFMG entre 1990 e 2010. Os dados clínicos dos pacientes foram coletados até dezembro de 2016.

4.2.2 Critérios de exclusão

Pacientes com RVU secundário ou associado a outras malformações urológicas e renais, como válvula de uretra posterior (VUP), ureterocele e bexiga neurogênica foram excluídos do presente estudo. Além disso, pacientes com cromossomopatias ou múltiplas malformações maiores também foram excluídos. Ressalta-se que a coleta de urina não foi realizada em vigência de quadros clínicos agudos (infecções, instabilidade clínica) ou durante o uso de imunossupressores. Pacientes acima de 18 anos não foram incluídos no estudo.

4.2.3 Controles

Os controles compreendiam crianças e adolescentes saudáveis do Centro de Atendimento Primário de Pediatria de nossa instituição. Todos os controles eram normotensos e possuíam dosagens de creatinina sérica normais à época da coleta de amostra de urina. A revisão da história clínica dessas crianças, associada ao depoimento de seus pais, foi utilizada para descartar a presença de doenças agudas ou crônicas.

4.3 Aspectos éticos

O estudo foi aprovado pelo Comitê de Ética em Pesquisa (COEP) da UFMG sob o parecer ETIC 109/07, anexo B.

Assinatura pelos responsáveis legais do Termo de Consentimento Livre e Esclarecido foi obtida à época de admissão no estudo.

4.4 Protocolo do estudo

Refluxo vesicoureteral primário foi diagnosticado em crianças e adolescentes investigados pelo quadro de infecção urinária, hidronefrose fetal, disfunção miccional e, menos frequentemente, em rastreio familiar. À admissão os pacientes foram submetidos a exame físico completo (destacando-se mensuração de dados antropométricos e da pressão arterial sistêmica, palpação abdominal e exame da genitália), avaliação laboratorial e propedêutica de imagens. Os pacientes foram acompanhados no Ambulatório da Unidade de Nefrourologia Pediátrica do Hospital das Clínicas da UFMG, trimestralmente nos dois primeiros anos após o diagnóstico do refluxo e, então, semestralmente até a alta da Unidade.

Às consultas médicas de seguimento eram avaliados, além da evolução clínica, crescimento ponderoestatural, pressão arterial sistêmica, adesão ao uso do antibiótico profilático e presença ou não de infecção do trato urinário (ITU) (urina rotina e urocultura). As dosagens séricas de ureia e creatinina foram obtidas semestralmente no primeiro ano de vida e, após esse período, anualmente. Nos pacientes com ClCr < 60 mL/min/1.73m² as dosagens séricas de ureia e creatinina eram repetidas a cada três meses. Ajustes no intervalo de coletas foram realizados conforme demanda clínica(1-3).

Antibioticoprofilaxia nos pacientes com hidronefrose fetal foi iniciada no primeiro dia de vida e mantida conforme diagnóstico nefrourológico pós-natal (1, 4). Nos pacientes com história de ITU com idade inferior a 2 anos também foi prescrita antibioticoprofilaxia e essa foi mantida conforme graduação do refluxo e evolução clínica do paciente. Quimioprofilaxia para ITU foi realizada utilizando-se cefalosporina de primeira geração (50 mg/dia) nos dois primeiros meses de vida para os casos de diagnóstico fetal. A partir dessa faixa etária, foi instituída a profilaxia com sulfametoxazol+trimetoprim (2mg/kg/dia de trimetoprim) ou nitrofurantoína (2mg/kg/dia) em dose única diária. A quimioprofilaxia foi suspensa após a resolução do RVU ou, nos casos sem ITU de repetição e sem outros fatores de risco, aos cinco anos de idade aproximadamente.

Doença renal crônica (DRC) foi classificada de acordo com os critérios do *National Kidney Foundation practice guidelines* (5). DRC estágio 5 foi definida como

um ritmo de filtração glomerular estimado < 15 mL/min/1.73m² em três medidas consecutivas e/ou pela necessidade de terapia renal substitutiva.

Pacientes com *clearance* de creatinina < 60mL/min/1.73m² foram acompanhados conforme nosso protocolo institucional de pacientes com DRC ≥ 3. Esses pacientes eram avaliados clínica e laboratorialmente pelo menos a cada três meses (3, 6).

Acompanhamento nutricional foi realizado em conformidade com as referências dietéticas preconizadas pela literatura (7). Suplementos de eletrólitos, vitamina D e eritropoetina foram prescritos conforme orientado pelos *guidelines* de referência (8, 9). Hipertensão foi definida como valores acima do percentil 95 para idade, sexo e estatura em três consultas consecutivas conforme recomendado pelo *Fourth Task Force Guidelines* (10). Aferição acima de 140/90 mmHg foi considerada para o diagnóstico de hipertensão em pacientes com idade superior a 17 anos. Medicamentos anti-hipertensivos foram prescritos para pacientes com níveis pressóricos persistentemente acima do percentil 95. Proteinúria foi definida como relação proteína/creatinina > 0,2 em amostra única de urina ou >150 mg/dia em urina de 24 horas, em pelo menos duas medidas consecutivas.

Definiu-se disfunção miccional como presença de incontinência diurna, sintomas de urgência e/ou alterações na frequência miccional, associados a parâmetros ultrassonográficos que incluíam presença de resíduo pós-miccional (maior que 20% da capacidade vesical) e/ou capacidade vesical aumentada (acima de duas vezes o valor normal para a idade). Em caso de disfunção miccional diagnosticada, tratamentos específicos eram instituídos como o uso de anticolinérgicos, terapêuticas comportamentais, *biofeedback* e tratamento adequado para constipação.

4.4.1 Investigação por método de imagem

A propedêutica por metodologia de imagem foi realizada de forma sequencial conforme algoritmo da figura 2. Na primeira década do estudo, os pacientes com diagnóstico pré-natal de CAKUT (*Congenital anomalies of kidney and urinary tract*) foram investigados conforme protocolo descrito em estudos anteriores (11). Resumindo, todos os pacientes com dilatação anteroposterior da pelve renal maior ou igual a 5 mm iniciaram antibioticoprofilaxia ao nascimento e foram submetidos a extensa propedêutica de imagem, incluindo ultrassonografia (US) de rins e vias urinárias e UCM (12). A partir de 2009, UCM passou a ser indicada somente para pacientes com dilatação do diâmetro anteroposterior (DAP) da pelve > 10 mm ou com dilatação de ureter. Cintilografia renal

(Tc-99m DMSA e Tc-99m-DTPA) foi realizada nos pacientes com DAP da pelve ≥ 10 mm.

4.4.1.1 Ultrassonografias sequenciais

O primeiro estudo ultrassonográfico foi realizado à admissão. US de rins e vias urinárias subsequentes foram realizados anualmente nos pacientes com hidronefrose fetal e a cada 2 anos nos pacientes com DRC, ou a intervalos menores caso a evolução clínica justificasse.

As dimensões renais obtidas de forma sequencial permitiram acompanhar longitudinalmente o crescimento renal, comparativamente aos padrões de referência descritos por Han e Babcock (13). O surgimento de novas cicatrizes renais também foi acompanhado ao US.

4.4.1.2 Uretrocistografia miccional

Uretrocistografia miccional foi realizada no Serviço de Radiologia do Hospital das Clínicas da UFMG, segundo técnicas e normas padronizadas. Esse exame era executado após o término do tratamento de ITU ou, nos casos de hidronefrose fetal, no primeiro mês de vida. A graduação do refluxo foi realizada conforme proposto pelo *International Reflux Study Comittee* (14). Para análise estatística, RVU foi classificado como leve (graus I-II) e moderado/grave (graus III-V). Foi considerado o refluxo de maior grau para a classificação daqueles com refluxo bilateral.

UCM ou cistografia radioisotópica direta foi repetida a cada 2 anos nos primeiros dois anos de acompanhamento e a cada três anos a partir de então. O critério utilizado para resolução do RVU foi baseado em um único exame negativo de UCM ou cistografia radioisotópica direta.

4.4.1.3 Cintilografia renal

A morfologia do parênquima renal foi avaliada, além da ultrassonografia, pela cintilografia renal estática (99mTc-DMSA). Para realização dos estudos, aguardava-se um intervalo de dois meses após o episódio de ITU ou, em caso de hidronefrose fetal, completar um mês de vida. Os estudos cintilográficos foram realizados nos setores de Medicina Nuclear do Hospital Felício Rocho, da Santa Casa de Misericórdia de Belo Horizonte e do Hospital das Clínicas da UFMG conforme técnicas e normas padronizadas. A avaliação da lesão renal foi feita por examinadores que desconheciam o

contexto clínico do paciente. As lesões renais foram qualitativamente classificadas como nenhuma, unilateral ou bilateral.

4.4.2 Exames laboratoriais

4.4.2.1 Dosagem de ureia e creatinina séricas

Os exames laboratoriais analisados já estavam presentes nos protocolos de manejo clínico dos pacientes com hidronefrose fetal e DRC de nosso serviço. A creatinina plasmática foi obtida à admissão e sequencialmente a cada 6 meses. Nos pacientes com DRC ≥ 3 as dosagens de ureia e creatinina eram repetidas a cada três meses (3, 6).

A dosagem de creatinina era realizada pelo método de *Jaffe* até novembro de 2011 em nosso serviço. Portanto, a taxa de filtração glomerular foi estimada através da fórmula de Schwartz convencional (15) para os dados obtidos até esse período. Após novembro de 2011, a dosagem de creatinina passou a ser realizada por diluição isotópica por espectrometria de massa (*IDMS – isotope dilution mass spectrometry*). A partir de então, a fórmula de Schwartz modificada (16) começou a ser usada para estimar o ritmo de filtração glomerular.

4.4.2.2 Urina rotina e urocultura

Amostras de urina de todos os pacientes foram obtidas para realização de urina rotina e urocultura à admissão e, posteriormente, a cada consulta médica ou quando havia probabilidade clínica de ITU. Os exames foram realizados no Laboratório Central do Hospital das Clínicas da UFMG, de acordo com técnicas padronizadas.

ITU foi definida como o crescimento de pelo menos 10^5 UFC/mL de uma única bactéria em urina coletada com bolsa coletora ou amostra de jato médio, associado a febre (temperatura axilar ≥ 38º) e/ou sintomas urinários.

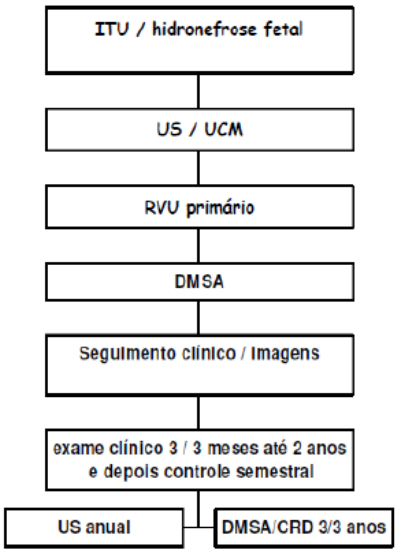

Figura 1 – Algoritmo para avaliação de seguimento do paciente com refluxo vesicoureteral.
US: ultrassonografia; UCM: uretrocistografia miccional; DMSA: cintilografia renal estática; CRD: cistografia radioisotópica direta.

4.4.2.3 Amostras de urina para dosagem de biomarcadores

Uma única amostra de urina foi coletada de cada paciente entre 07:30-09:30 a.m. no mesmo dia da coleta dos exames de rotina. As amostras foram coletadas entre 2008 e 2011. As amostras foram coletadas em frascos estéreis e imediatamente transportadas em gelo para nosso laboratório de pesquisa. O processo de centrifugação e armazenamento da urina ocorreram quase que imediatamente após sua coleta. Após serem homogeneizadas, as amostras de urina foram centrifugadas a 4ºC durante 20 minutos a 1300 x g. As amostras de urina livres de células foram então aliquotadas em tubos de 0.5mL e armazenadas a -80ºC até a realização das medidas dos biomarcadores.

4.4.2.4 Dosagens de biomarcadores

Os seguintes biomarcadores foram dosados em amostras urinárias: citocinas e quimiocinas (IL-8, IL-1β, IL-6, IL-10, TNF, IL-12p70, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, CXCL10/IP-10), fatores de crescimento (basic FGF e VEGF) e moléculas de adesão celular (CD54 - ICAM-1 e CD106 -VCAM-1). As amostras urinárias foram descongeladas e foram obtidas as dosagens de todos os biomarcadores simultaneamente através de citometria de fluxo utilizando *Human Flex Set kits for Cytometric Bead Array* (CBD, BD Bioscience, San Jose, CA, USA). A leitura dos testes foi realizada em um citômetro de fluxo FACSCanto II (BD Biosciences, San Jose, CA, USA). Resultados

quantitativos foram gerados a partir do software *FCAP array* versão 1.0.1. (Soft Flow Inc., Pecs, Hungary).

4.5 Análise estatística

As análises estatísticas foram realizadas utilizando os softwares SPSS versão 22.0 (SPSS Inc., Chicago, IL, USA) e GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, California, USA). Todas as variáveis foram submetidas ao teste de Shapiro-Wilk para avaliação de normalidade. Os resultados foram expressos como mediana e intervalo interquartil (percentis 25-75) ou média ± desvio padrão, conforme o resultado do teste de normalidade. Para variáveis sem distribuição Gaussiana, foi utilizado o teste de Mann-Whitney para a comparação das medianas dos dois grupos (pacientes x controles). Teste t-Student foi utilizado para comparar as médias dos pacientes com as médias dos controles naquelas variáveis de distribuição normal. Coeficiente de correlação de postos de Spearman foi realizado para avaliar se existia correlação entre idade dos controles e seus níveis urinários de biomarcadores. Assumiu-se um nível de significância (α) de 0,05 para todos os testes realizados.

4.6 Revisão da literatura

A base de consulta para a realização da revisão de literatura pertinente a este estudo foi a MEDLINE, consultada através da internet, via *U.S. National Library of Medicine*, no seguinte endereço: http://www.ncbi.nlm.nin.gov/PubMed/.

Foram utilizadas as seguintes palavras-chave: *vesicoureteral reflux, reflux nephropathy, biomarkers, cytokines, chemokines, growth factors, cellular adhesion molecules* e cada um dos biomarcadores testados.

As referências bibliográficas foram citadas no texto seguindo as Normas de Vancouver - (*Uniform requirements for manuscripts submitted to biomedical journals: writing and editing for biomedical publication*- www.ICMJE.org).

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5. RESULTADOS E DISCUSSÃO

5.1 ARTIGO ORIGINAL

Original Article

Title: Evaluation of molecules related to inflammation and fibrosis in pediatric patients with primary vesicoureteral reflux

Introduction

Vesicoureteral reflux (VUR) is defined as the anomalous backward flow of urine from the urinary bladder into the ureter and renal pelvis. It can occur as an isolate anomaly or associated with other congenital anomalies of the kidney and urinary tract (CAKUT).

VUR affects 0.4-0.8% of otherwise healthy children (1). It is also present in 31.1% of children with urinary tract infection (UTI) (2) and in approximately 16.2% (range: 7–35%) of patients with prenatal hydronephrosis (3). According to the North American Pediatric Renal Trials and Collaborative Studies, reflux nephropathy (RN) was present in 5.1 % of transplanted patients (4) and was the fourth most common cause of chronic Kidney disease (CKD) in children (8.4%) (5).

Renal scarring linked to VUR is called RN. Histological alterations related to RN are sclerosis and enlargement of glomeruli, separation of glomeruli from proximal tubules, tubular dilatation and tubular cells atrophy, interstitial infiltration of inflammatory cells and tissue fibrosis. These histopathological findings of renal scarring are characteristic of acquired RN (6, 7). Differently, congenital RN shows dysplasia and its main histological findings are the presence of primitive ducts associated with increased mesenchyme (8). Infiltrating leukocytes produce chemokines that may amplify inflammatory response in the kidney. In addition, the hydronephrotic kidney produces a variety of intrarenal factors that contribute to progressive interstitial fibrosis, including growth factors, cell adhesion molecules and cytokines/chemokines (9, 10). In contrast, several anti-fibrotic mechanisms were triggered in response to RN, supporting the potential role for the stimulation of renal endogenous counter-regulatory molecules against progressive fibrosis (9, 11).

Continuous renal parenchyma damage may occur even after the resolution of VUR and even in the absence of a history of UTI. Factors responsible for this process include release of autologous renal antigens, hyperfiltration of undamaged nephrons, increased superoxide production, and persistent glomerular hypertension (12). Glomerular hyperfiltration and hypertension result in proteinuria, glomerulosclerosis,

excessive stimulation of renin angiotensin system, and progressive chronic renal disease (6).

Identifying VUR patients at higher risk of progression to ESRD is essential to rationalize the indication of therapeutic and complementary tests. In this way, measurement of urinary, plasma, and renal tissue levels of biomarkers could be useful. Cytokines and chemokines as IL-1β, IL-6, IL-8, IL-10, IL-12, TNF-α, TGF-β and CCL2/MCP-1 have been evaluated in VUR. Despite some progress, there is still a need for reliable markers that predict renal function in VUR patients. These markers might help in understanding the complex pathogenesis of VUR, in defining prognosis and may become alternative therapeutic targets. Therefore, in this study, we investigated cytokines/chemokines, growth factors and cellular adhesion molecules in urinary samples of VUR patients with CKD ≥3 compared to healthy controls and to VUR patients with CKD ≤ 2.

Patients and Methods

Ethical aspects

This study was approved by our institutional Ethics Committee. Informed consent was obtained from the parents of patients and controls. The research protocol did not interfere with any medical recommendations or prescriptions.

Patients and Controls Subjects

The present cross-sectional study included 18 patients with primary *VUR* admitted at the Pediatric Nephrology Unit of our institution (University Hospital-UFMG, Belo Horizonte, Brazil) between 1990 and 2010. The study group comprised 12 patients with antenatal diagnosis of hydronephrosis due to VUR and 6 children and adolescents with CKD at stages 2–4 secondary to VUR.

Patients with acute illness (e.g., infections, clinical instabilities) were not scheduled for urine collection during the period of the disease activity. We also excluded patients receiving immunosuppressive medication at the time of sample collections and patients with chromosome disorders or with multiple major malformations. The age limit was less than 18 years at baseline. Healthy controls were patients followed-up at the Pediatric Primary Care Center of our institution. All controls were normotensive and had serum creatinine levels within the normal range at the time of urine sampling. The healthy

status was determined through the medical history and either a parental report to rule out the presence of chronic or acute diseases.

The records of the 18 patients were retrospectively reviewed. Of the 18 patients, 10 continued to be followed-up at our institution until 2016, 2 were discharged, 2 underwent hemodialysis, and 1 was transplanted. Three patients missed follow-up.

Follow-up protocol

The clinical protocol for the management of infants with perinatal diagnosis of CAKUT has undergone some adjustments during the period of this study. Until 1999, infants were investigated according to a systematic protocol described elsewhere (13). Regarding imaging workup, a renal ultrasonography (RUS) was performed after the first week of postnatal life, and, until 2009, all infants underwent a voiding cystourethrogram (VCUG) (14). Since 2009, VCUG has been indicated for patients with fetal or postnatal anteroposterior renal pelvis dilatation (APRPD) > 10 mm or ureter dilatation. In those patients with APRPD equal or greater than 10 mm, renal scintigraphy (Tc-99m DMSA and Tc-99m DTPA) was indicated after the first month of life. Antibiotic prophylaxis was started on the first day of life and its maintenance was performed according to the definitive diagnosis and clinical evolution. Chemoprophylaxis was discontinued after VUR resolution or, in cases without recurrent UTI and without other risk factors, at 5 years of age. After initial clinical and propaedeutic evaluation, clinical visits, RUS scan, and laboratory reviews (including serum creatinine and urine culture) were scheduled at 6-month intervals (15). Renal damage was investigated by renal scintigraphy with 99mTc-DMSA in patients with VUR.

CKD patients were followed-up according to a clinical approach described in detail elsewhere (16). The visits were scheduled at 3-months intervals and a complete physical evaluation and laboratory tests were repeated on each occasion. Associated conditions were managed according to our unit protocol (16-20).

For both groups (antenatal hydronephrosis and CKD patients) urine cultures were obtained at each follow-up visit and it was recommended that urine samples should be collected in the presence of UTI symptoms or during any unexplained febrile episode. Urine specimens for culture were carefully collected by bag or mid-stream sample at our hospital outpatient laboratory. Plasma creatinine concentration was determined at baseline and six months intervals or more frequently, whether clinically needed. Glomerular filtration rate (eGFR) was estimated by the conventional Schwartz formula

(21) or the modified Schwartz formula (22) according to the method of creatinine measurement. CKD was classified according to the stages proposed by the National Kidney Foundation practice guidelines (23). CKD stage 5 was defined as eGFR < 15 ml/min/1.73m^2 in three consecutive tests and/or the need for renal replacement therapy.

Follow-up imaging consisted of conventional VCUG or a direct isotope cystogram every 2 years for the first 48 months after diagnosis and at 3-year intervals thereafter. A DMSA scan and RUS were performed at approximately the same intervals.

Clinical characteristics, imaging and laboratory test results were evaluated during the clinic visit or by reviewing medical records. The following variables were analyzed: age at diagnosis, clinical presentation (fetal hydronephrosis/UTI), unilateral/bilateral reflux, VUR grade, renal damage (absence/presence), renal function, constipation, and dysfunctional voiding.

Definitions:

Reflux grade was classified according to the system proposed by the International Reflux Study Committee (24). For statistical analysis, VUR was categorized as mild (grades I-II) and moderate/severe (grades III-V). Patients with bilateral reflux were classified by the highest grade of reflux. Renal damage classification was qualitative according to the findings obtained in imaging studies and defined as none, unilateral and bilateral. Resolution of reflux was confirmed by a single negative VCUG or direct isotope cystogram.

UTI was defined by the growth of at least 100,000 cfu/ml in a urine collected by bag or from a midstream sample, in patients with fever (38.0°C or more) and/or urinary symptoms.

Hypertension was defined as values constantly above the 95th percentile for age, gender, and height on three consecutive visits. Normal blood pressure values were based on The Fourth Report on High Blood Pressure in Children and Adolescents (20). Antihypertensive drugs were used for patients with BP persistently above the 95th percentile. The presence of proteinuria was considered when urinary protein:creatinine ratio was above 0.2 or 24-hour protein excretion was higher than 150 mg/day in at least two consecutive evaluations.

Urine samples

A single urine sample was collected from 7:30 AM to 9:00 AM during collection of routine exams. The samples were collected from 2008 to 2011. The spot urine samples

were collected in sterile recipients, which were immediately placed on ice and transported to our research laboratory. After homogenization, urine samples were centrifuged at 4°C for 20 min at 1,300 × g. Cell-free urine was stored at -80°C until the measurements.

Biomarkers measurements

Multiple cytokines/chemokines (IL-8, IL-1β, IL-6, IL-10, TNF, IL-12p70, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, CXCL10/IP-10), human growth factors (basic FGF, VEGF) and cellular adhesion molecules (ICAM-1, VCAM-1), were assessed simultaneously using Human Flex Set kits for Cytometric Bead Array (CBA, BD Bioscience, San Jose, CA, USA) following manufacture's instruction. Acquisition was performed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The instrument was checked for sensitivity and performance with Cytometer Setup and Tracking beads (BD Biosciences) prior to data acquisition. FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary) was used to generate quantitative results. All samples were analyzed in duplicate and in a single assay to avoid interassay variation. Our intra-assay variation was lower than 3%.

Statistical Analysis

The software SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism5.0 (GraphPad Software, Inc., La Jolla, California, USA) were used for statistical analysis. All variables were tested for Gaussian distribution by the Shapiro–Wilk test. According to the results of normality test, data were expressed as median and interquartile range (25th percentile and 75thpercentile) or mean±standard error of mean. For variables without Gaussian distribution, Mann–Whitney test was used to compare the medians of two groups (patients *vs.* controls). Unpaired student t test was used to compare means between patients and controls for variables with normal distribution. Spearman's correlation analyses were performed to examine the relationship between age and urinary levels of biomarkers. All statistical tests were two-tailed and were performed using a significance level of α=0.05.

Results

Patient's characteristics (Demographic, clinical and laboratory findings)

The mean age of patients with eGFR $\leq$ 60mL/min/1.73m^2 was 152.8±18.35 (83.56- 221.4) months and the mean age of controls was 168±6.718 (108-216) months

(p=0.3502). As expected, patients with eGFR > 60mL/min/1.73m^2 were significantly younger, with a mean age of 65.16 ± 17.17 (3.73-206.9) months. Of the total number of patients, 61.1% (14) were male and 38.9% (7) were female. Comparative groups did not differ in terms of sex distribution.

The mean eGFR at the time of sampling was 96.50±53.53 (12.87-166.41) mL/min/1.73m^2 and it was lower than 60mL/min/1.73m^2 in 38.9% (7) of patients. Renal cortical scarring was detected in 44.4% (8) patients (11.1% unilateral and 33.3% bilateral). VUR was bilateral in 72.22% of patients and 77.8% were classified as moderate/severe. At least one episode of urinary tract infection was diagnosed in 66.7% (12) of patients. Chemoprophylaxis for urinary tract infection was prescribed for 94.4% of patients and the mean age of its suspension was 82.48±57.21 (10.80-190.73) months. Dysfunctional voiding was present in 50% of patients and 16.7% had intestinal constipation. Proteinuria was diagnosed in 38.9% and 61.1% of the patients were classified as hypertensive. Angiotensin-converting enzyme inhibitors (ACEi) or angiotensin receptor antagonists were used for 38.9% of patients, 16.7% for the management of proteinuria and 22.2% for antihypertensive and antiproteinuric therapy. The mean age of diagnostic of arterial hypertension and proteinuria was 143.70±63.03 and 133.35±45.15 months, respectively. Surgical treatment was performed in 22.2% of patients at a mean age of 45.57±36.61 (0.26-89.23) months.

Urinary levels of biomarkers

As shown in Table 1, urinary levels of proinflammatory cytokines and chemokines were not homogenous. While urinary levels of IL-8 ($p<0.0001$) were significantly higher in VUR patients with eGFR ≤ 60 ml/min/1.73m^2 compared to healthy controls indicating an inflammatory profile, urinary levels of IP-10 (p=0.0007) and RANTES (p=0.0002) were significantly lower in those patients. Besides that, there was no significant difference in urinary levels of MCP-1 (p=0.0564), MIG (p= 0.2727), IL-12p70 (p=0.1336), TNF (p=0.1247), IL-6 (p=0.2769), IL-1β (p=0.0824) and the anti-inflammatory cytokine IL-10 (p=0.6314) when urine samples of VUR patients with CKD ≥3 were compared to healthy controls.

It is interesting to note that when we compare all the 18 patients to healthy controls, urinary levels of MCP-1 (p=0.0030), IL-12p70 (p=0.0003), TNF (p=0.0016) and IL-10 (p=0.0128) were significantly higher in VUR patients. The comparison between VUR patients with CKD ≥ 3 and healthy controls did not show this difference probably

due to the small sample size. However, when patients with eGFR > 60 ml/min/1.73m^2 were included in the analysis, there was a significant difference in age between patients and healthy controls. To exclude the influence of age factor, Spearmann's test was performed on samples from healthy controls and there was no correlation between age and urinary level of any of the biomarkers evaluated. In addition, there was no difference in urinary biomarkers levels comparing healthy controls younger than 12 years with those older than 12 years. Another curious finding was that urine levels of MIG (p=0.0260) were significantly higher in VUR patients with eGFR ≤ 60 ml/min/1.73m^2 when compared to patients with eGFR > 60 ml/min/1.73m^2.

Regarding the expression of growth factors, urinary levels of VEGF (p<0.0001) and FGF (p=0.0385) were significantly higher in urine samples of VUR patients with eGFR ≤ 60 ml/min/1.73m^2 compared to healthy controls. Urinary levels of cellular adhesion molecules were also interesting. Although urinary levels of CD54(ICAM-1) (p=0.0003) were lower in urine samples from VUR patients with CKD ≥3, CD106 (VCAM-1) (p=0.0197) levels were significantly higher in VUR patients with eGFR ≤ 60 ml/min/1.73m^2 than in healthy controls.

Urinary levels of all measured molecules [median (interquartile range: 25th percentile -75th percentile)] and statistics are provided in Table 1.

Discussion

In this study, we showed that the pro-inflammatory cytokines and chemokines MCP-1, IL-12p70, TNF and the anti-inflammatory cytokine IL-10 were highly increased in urine samples of VUR patients (Figure 1). IL-8 was increased in CKD≥3 patients compared to healthy controls. CXCL9/MIG was significantly higher in patients with eGFR ≤ 60 ml/min/1.73m^2 than in patients with CKD ≤2 (Figure 2). Regarding growth factors we found that VEGF were significantly higher in VUR patients and FGF were higher in urine samples of those with CKD≥3 (Figure 3). Urinary levels of the cellular adhesion moleculeVCAM-1 were significantly higher in CKD≥3 patients than in healthy controls (Figure 4).

CCL2/MCP-1 induces the release of fibrogenic factors and, consequently, leads to an increase in the extracellular matrix. It may also induce injury via release of oxygen radicals and lysossome enzyme, resulting in tubular atrophy and necrosis (25, 26). This chemokine was evaluated in uropathies, and its expression was strictly correlated with tubular damage and extent of monocytes infiltration (25-30). In our study, CCL2/MCP-1

was significantly higher in urine samples of VUR patients compared to healthy controls (p=0.0030). Urinary levels and gene expression of MCP-1/CCL2 were also increased in animal obstructive nephropathy models and in UPJO patients (26-33) and these markers reduced after surgical treatment (27, 31, 32). Regarding VUR, Chertin *et al* have showed that increased MCP-1 expression and reduction of epidermal growth factor (EGF) expression at tubulointerstitial site were correlated with severity of apoptosis in RN (26).

CXCL9 is also known as MIG (monokine induced by gamma interferon) and interacts with the chemokine receptor CXCR3, exerting its function as a T-cell chemoattractant. Although urinary CXCL9/MIG levels were significantly lower in VUR patients compared to healthy controls (p=0.0052), patients with CKD ≥3 had higher urinary levels of this chemokine than patients with eGFR> 60 ml/min/1.73m^2 (p=0.026) which leads us to believe that their levels increase with the advancement of RN. To our knowledge, no previous study has reported any association between CXCL9/MIG and VUR.

Urinary levels of IP-10 **(**Interferon gamma-induced protein 10) were lower in VUR patients compared to controls (p<0.0001). A study of animal models of unilateral ureteral obstruction revealed an increased level of CXCL10/IP-10 in obstructed kidneys (30). As far as we know, no previous study reported any association between this proinflammatory chemokine and VUR.

In the present study, CCL5/RANTES was significantly higher in urine samples from healthy controls than from VUR patients (p<0.0001). Contrary to our results, Vielhauer *et al* found an increase in CCL5/RANTES expression at sites of progressive tubulointerstitial injury in murine obstructive nephropathy models. CCR5-positive monocytes and CCR5-positive lymphocytes are recruited by the release of CCL5/RANTES, leading to chronic interstitial inflammation (28).To our knowledge, there are no studies evaluating the association between RANTES and VUR.

There was no significant difference in urinary levels of IL-8 (p=0.3288) in VUR patients compared to controls. However, the stratified analysis showed that the urinary levels of IL-8 were significantly higher in CKD≥3 than in healthy controls (p<0.0001). Urinary IL-8 levels were also elevated in patients with CKD≥3 compared to patients with eGFR>60 ml/min/1.73m^2, but this difference was not significant, probably due to the small sample size (p=0.1430). Interleukin-8 (IL-8/CXCL8) is a pro-inflammatory chemokine that was previously associated to renal diseases (10). In patients with VUR, high urinary levels of IL-8/CXCL8 were found to be associated with RS and renal

function deterioration, even in the absence of UTI (34, 35). Our research group has recently reported a correlation between high urinary levels of CXCL8/IL-8 and reduced eGFR in CAKUT patients (36). Contradictory results were observed regarding the association between the presence of VUR and serum or urinary IL-8 concentration (37-41). Kuroda *et al* also found no association between IL-8 gene polymorphism and familial VUR with or without RS (42). More studies are needed to clarify the role of IL-8 in VUR.

Although some studies have shown an increase in serum and urinary levels of IL-6 in the acute phase of pyelonephritis, no associations were found between this biomarker and the presence of VUR (43, 44). We also did not find a significant difference in urinary levels of IL-6 ($p= 0.2370$) when urine samples from VUR patients were compared to healthy controls. However, high levels of IL-6 were found to be associated with RS and renal function deterioration in patients with VUR (41, 45, 46).

In our study, there was no significant difference in urinary levels of IL-1β ($p= 0.0746$) when urine samples of VUR patients were compared to controls. IL-1 induces the expression of adhesion molecules and chemokines, leading to the migration of leukocytes to the damaged tissue (47). Some studies suggest that the initial urine IL-1β levels during acute pyelonephritis could predict the risk for later development of RS. However, urine IL-1β is not useful in discriminating patients with or without VUR. Urine levels of IL-1β (48) and IL-1α (49) were lower in patients with RS suggesting that persistent levels of these cytokines may have an anti-inflammatory function in renal pathogenesis. Besides that, in a study of experimentally induced acute *E. coli* pyelonephritis in IL-1β deficient mice, Hertting *et al* found that this cytokine has an essential anti-inflammatory function in renal pathogenesis (50).

IL-12 stimulates the differentiation of naive T cells into Th1 cells, resulting in the increased production of IFN-γ (51). We found that urinary levels of IL-12p70 ($p=0.0003$) were significantly higher in VUR patients compared to healthy controls, which leads us to believe that this cytokine may be involved in the physiopathology of VUR. In agreement with these findings, Kordi Tamandani *et al* showed a significant association between IL-12(AA) gene polymorphism and increased risk of VUR (52).

Considering the proinflammatory properties of TNF, it seems reasonable to investigate its role on tubulointerstitial damage in RN. TNF is cytotoxic to tubular and epithelial cells of the renal tissue, leading to direct renal injury. TNF can also affect intraglomerular blood flow and decrease GFR as a result of disequilibrium between vasoconstrictor and vasodilator molecules (52). Our results showed that urinary levels of

TNF (p=0.0016) were significantly higher in urine samples of VUR patients compared to healthy controls. Schwentner *et al* evaluated the extracellular microenvironment and cytokine profile of the ureterovesical junction in children with VUR. In this study, TNF and TGF-β1 were significantly more abundant in VUR samples, whereas insulin-like growth factor-1, nerve growth factor and VEGF were less prevalent in VUR samples compared to healthy controls (53). Although some studies have attempted to find an association between TNF-α gene polymorphisms and the susceptibility to RS, the results were contradictory (54-56). Further studies are needed to determine the role of TNF-α in VUR and the development of RS.

IL-10 is an anti-inflammatory cytokine that exerts a counter-regulatory role in immune response by opposing the actions and by inhibiting the synthesis of some chemokines such as TNF, IL-1, IL-6, IL-12, and IFN-γ (57). Concerning nephrouropathies, polymorphisms of the gene encoding IL-10 were associated with increased risk of VUR (52). Our urinary levels of IL-10 (p=0.0128) were significantly higher in urine samples of VUR patients compared to healthy controls. Subgroup analysis evidenced that urinary IL-10 levels were higher in patients with eGFR>60ml/min/1.73m^2 than in patients with CKD ≥ 3 (p=0.1067). The small size of our sample did not allow this study to have statistical power to detect difference between these two groups with a significance level of 0.05. We believe that levels of IL-10 may increase as a counter-regulation of the inflammatory process in patients with RN. Casio *et al* evaluated serum levels of IL-10 in children with RN and found no significant difference compared to controls (58). An experimental study revealed significantly lower levels of IL-10 in renal parenchyma and urine of acute unilateral obstructed animals, while renal levels of IL-1β, IL-6, and TNF-α were increased (59).

It is important to emphasize that some factors may justify the discrepancies in the results of these cytokines and chemokines studies. First, a recent acute inflammatory process can probably elevate levels of these proinflammatory biomarkers, leading to a misinterpretation on the association between these biomarkers and VUR or RN. Moreover, prophylactic antibiotics given to patients with VUR and surgical correction of VUR prior to study entry could influence the outcome. Another consideration is that, despite similar methods being used for the measurement of biomarkers, there were differences in the detection limits, sensitivity, and specificity of the assays. It should be emphasized that, we are not aware of other studies that used CBA in the urinary dosage of biomarkers in VUR.

In addition to cytokines and chemokines, altered renal expression of growth factors may modulate the inflammatory process leading to progression of renal disease (11). Furthermore, cellular adhesion molecules are responsible for modulating cell-cell interactions after antigen presentation, playing an important role in the immune response. Based on this fact we investigated the urinary dosage of two growth factors (VEGF and FGF) and two cellular adhesion molecules (VCAM and ICAM) in patients with VUR.

Urinary levels of VEGF ($p<0.0001$) were significantly higher in urine samples of VUR patients compared to healthy controls. VEGF, expressed by glomerular podocytes and tubular cells, is a key mediator of physiological and pathological angiogenesis and an important regulator of vascular permeability. It also exerts chemoattraction of monocytes and by doing so VEGF amplifies the inflammatory response (60). Some studies have attempted to find an association between VEGF gene polymorphisms and VUR or susceptibility to RS, however, the results are still contradictory (61-63). Konda *et al*. did not find an association between plasma and urinary levels of VEGF in patients with RN. The authors interpreted that urinary levels of VEGF seem to reflect the local production of this factor in renal tissue. In this study, urinary VEGF showed an increase directly proportional to the severity of renal scarring (64). Schwentner *et al.* demonstrated that patients without VUR have higher expression of growth promoting factors like insulin growth factor-1 (IGF-1), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) than those with VUR (53).

There was no significant difference in urinary levels of Basic FGF ($p= 0.2208$) when urine samples of VUR patients were compared to controls. However, patients with CKD ≥3 had significantly higher urinary levels of FGF than patients with eGFR> 60 ml/min/1.73m^2 ($p=0.0385$, table 4). The only study we found correlating FGF to VUR found that VUR patients with RS have elevated serum levels of basic FGF compared to those without RS (65).

VCAM-1 and ICAM-1 are members of the immunoglobulin supergene family of adhesions molecules that is expressed in several cell types, including resting endothelial cells and leukocytes. Proinflammatory cytokines and lipopolysacharides activate VCAM-1 and ICAM-1 expression on cell surface. VCAM-1 plays an important role in the firm adhesion and transendothelial migration of lymphocytes by binding to the very late antigen-4, while ICAM-1 plays a critical role in mediating lymphocyte adhesion to tubular cells (66).

Previous studies have suggested that ICAM-1 plays an important role in renal immune injury. An increased expression of ICAM-1 has been reported in allograft rejection, glomerulonephritis, and tubulointerstitial inflammation (66). Furthermore, serum levels of ICAM-1 were significantly higher in young children (age <2 years) with VUR and RS than in patients without RS (67). In line with these findings, renal tissue of patients with RN exhibited increased mRNA expression and immunoreactivity for ICAM-1 in the glomerulus, interstitium and proximal tubulus (68). These findings suggest that ICAM-1 probably participates in the pathogenesis of renal damage in RN. We are not able to explain why urinary levels of CD54(ICAM-1) ($p<0.0001$) were significantly lower in urine samples from our VUR patients.

Overexpression of VCAM-1 was detected in renal tubules with intense infiltration of inflammatory cells, suggesting a role for this adhesion molecule in leukocyte-tubular epithelial interactions related to tubulointerstitial inflammation. Kaneyama *et al* showed that serum VCAM-1 levels were significantly higher in patients with VUR if compared with healthy controls. Moreover, serum VCAM-1 levels were significantly higher in patients with high grade VUR (IV or V) than in those with grade III, independently of the presence of renal scarring (69). We found no significant difference in urinary levels of CD106 (VCAM-1) ($p= 0.0690$) when urine samples of VUR patients were compared to healthy controls. However, the stratification of the sample according to the severity of CKD showed interesting results of this biomarker. Urinary levels of VCAM-1 were higher in CKD≥3 patients than in healthy controls (0.0197). The small size of our sample did not make it possible to demonstrate a significant difference between urinary VCAM-1 levels of patients with CKD ≥ 3 of those patients with eGFR greater than> 60ml/min/1.73m^2 ($p=0.1031$).

Summarizing, we found that the inflammatory molecules MCP-1, IL-12p70, TNF, VEGF and the anti-inflammatory IL-10 were highly increased in urine samples of VUR patients. In those VUR patients with CKD ≥ 3, we also observed elevated urinary levels of CXCL9/MIG, IL-8, FGF and VCAM-1.

We are aware of the limitations of our study. The main weakness is the cross-sectional design. Another limitation is the small number of subjects. Furthermore, our study did not allow us to understand the mechanisms by which the biomarkers studied contribute to ESRD in VUR patients. Nevertheless, some aspects may increase the strength of our findings, including strict inclusion criteria for both groups and well-

established protocols for cytokine measurements and renal function parameters evaluation.

Conclusion

In this study, we showed that the inflammatory molecules MCP-1, IL-12p70, TNF, VEGF and the anti-inflammatory IL-10 were highly increased in urine samples of VUR patients. In those VUR patients with CKD ≥ 3, we also observed elevated urinary levels of CXCL9/MIG, IL-8, FGF and VCAM-1.

In conclusion, clinical and experimental studies demonstrate the role of inflammation in renal diseases. Understanding the effects of cytokines, chemokines, growth factors, and cellular adhesion molecules on renal injury onset and progression is therefore of great importance.

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Table 1: *Urinary biomarkers of VUR patients* **with eGFR** ≤ *60 mL/min/1.73m²* ***compared to VUR patients with*** **eGFR** > *60 mL/min/1.73m² and healthy controls*

	Controls	*VUR patients with eGFR > 60 mL/min/1.73m²*	*VUR patients with eGFR ≤ 60 mL/min/1.73m²*
IP-10 (pg/mL)	39.90 (20.73-65.95)	2.215 (1.093-5.195)	3.700 (2.600-4.480) *
MCP-1 (pg/mL)	7.76 (3.16-25.38)	39.31 (12.56-86.31)	28.91 (18.56-54.97)
MIG (pg/mL)	4.390 (1.610-11.220)	1.025 (0.6150-1.320)	2.370 (1.208-4.733) †
RANTES (pg/mL)	250.5 (136.6-815)	4.370 (3.950-4.580)	4.210 (4.060-4.513) *
IL-8 (pg/mL)	1.670 (1.470-1.930)	1.530 (1.270-10.380)	7.160 (1.540-17.160) *
IL-12p70 (pg/mL)	1.065 (0.860-1.250)	1.780 (1.428-2.253)	1.650 (0.900-2.280)
TNF (pg/mL)	1.260 (1.060-1.510)	1.780 (1.633-2.048)	1.920 (1.220-2.390)
IL-10 (pg/mL)	1.210 (0.930-1.330)	1.460 (1.383-1.795)	1.150 (0.880-1.760)
IL-6 (pg/mL)	1.790 (1.480-2.220)	1.885 (1.773-2.123)	2.260 (1.440-3.440)
IL-1β (pg/mL)	1.610 (1.410-1.900)	1.810 (1.573-1.858)	2.010 (1.320-2.290)
VEGF (pg/mL)	15.84 (11.10-19.43)	46.65 (36.90-55.37)	36.45 (27.34-47.45) *
FGF (pg/mL)	58.51 (54.06-65.27)	61.57 (49.49-71.62)	67.18 (60.60-78.95) *
VCAM-1 (pg/mL)	7873 (6809-8745)	818.3 (327.2-4264)	13540 (968.8-19100) *
ICAM (pg/mL)	18350 (11520-40550)	484.4 (256.8-795.6)	780.5 (313.6-973.5) *

Values are given as median (interquartile range: 25th percentile - 75th percentile).
Abbreviations: IP-10 = Interferon-γ-inducible protein-10; MCP-1= Monocyte chemotatic protein-1; MIG = Monokine induced by gamma interferon; RANTES = regulated on activation normal T cell expressed and secreted); IL=Interleukin; TNF = Tumor necrosis factor; VEGF = Vascular endothelial growth factor; FGF = Fibroblastic growth factor; VCAM-1 = Vascular cell adhesion protein 1; ICAM-1 = Intercellular adhesion molecule 1.
* p value <0.05, comparing VUR patients with eGFR ≤ 60 mL/min/1.73m^2 and healthy controls
† p value <0.05, comparing VUR patients with eGFR ≤ 60 mL/min/1.73m^2 and VUR patients with eGFR > 60 mL/min/1.73m^2

Figure 1 – Cytokines and chemokines levels in urine samples from vesicoureteral reflux (VUR) patients and controls. Significant differences were considered when p< 0.05 (Mann-Whitney U test).

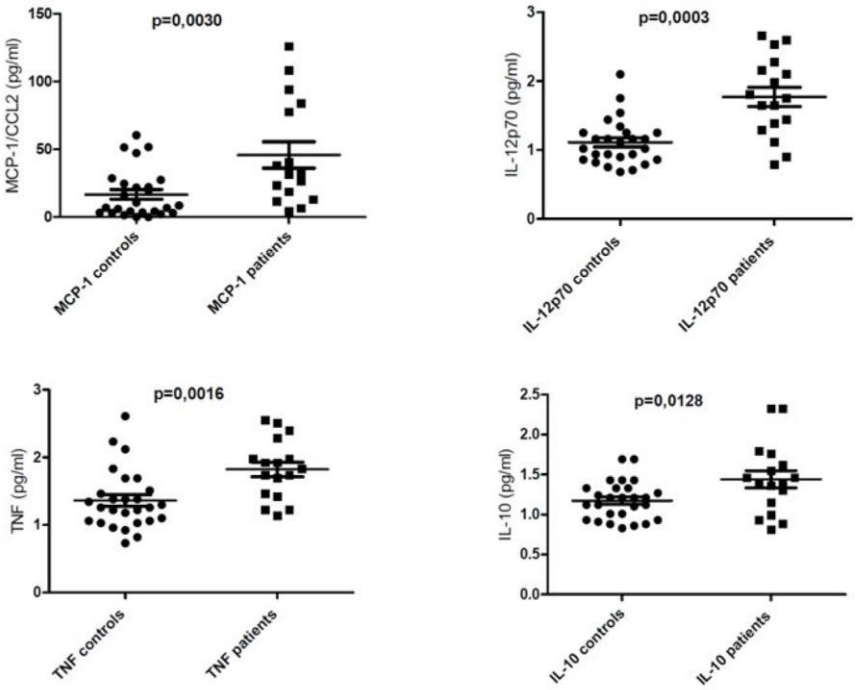


Figure 2 – Cytokines and chemokines levels in urine samples from vesicoureteral reflux (VUR) patients with eGFR ≤ 60 ml/min/1.73m^2 compared to healthy controls and VUR patients with eGFR > 60 ml/min/1.73m^2. Significant differences were considered when p< 0.05 (Mann-Whitney U test).

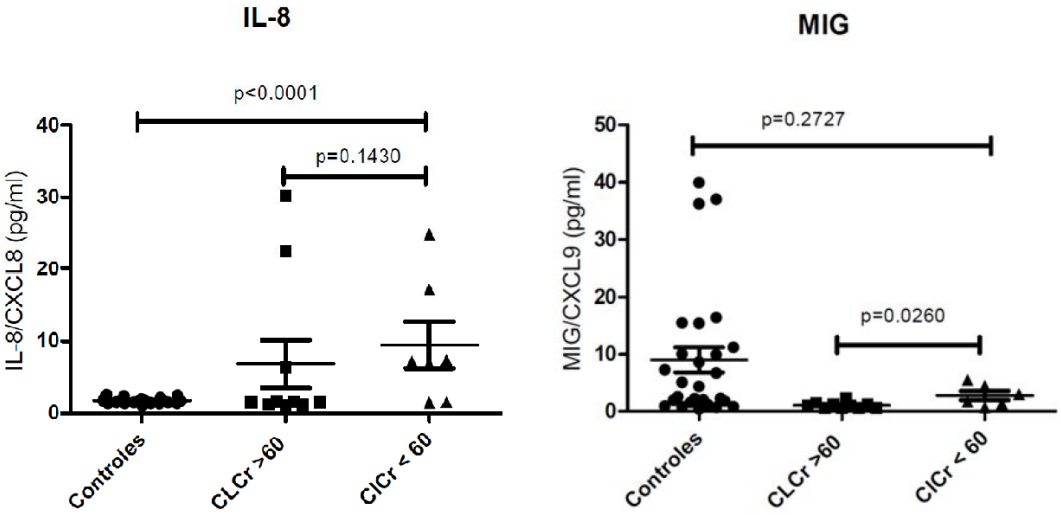


Figure 3 – Growth factors levels in urine samples from vesicoureteral reflux (VUR) patients and controls. Significant differences were considered when p< 0.05 (Mann-Whitney U test).

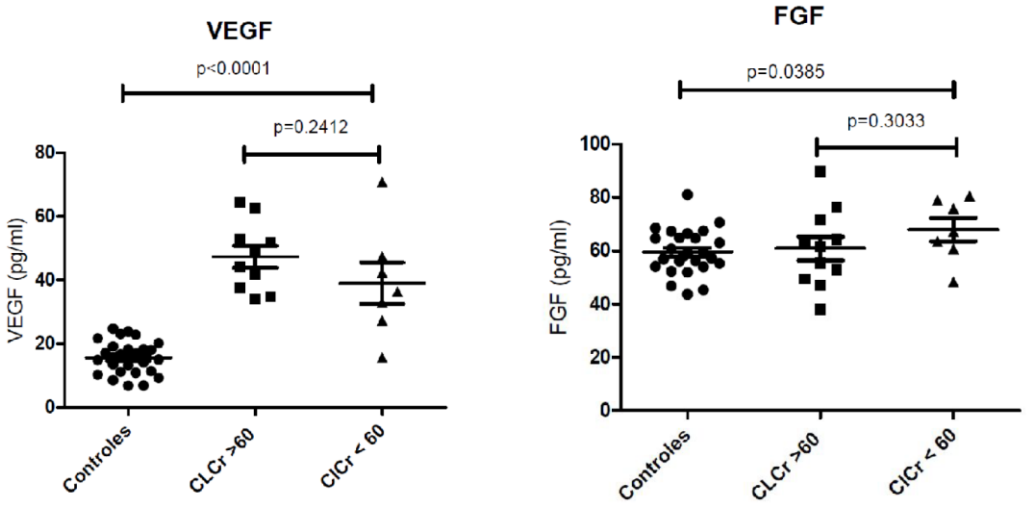
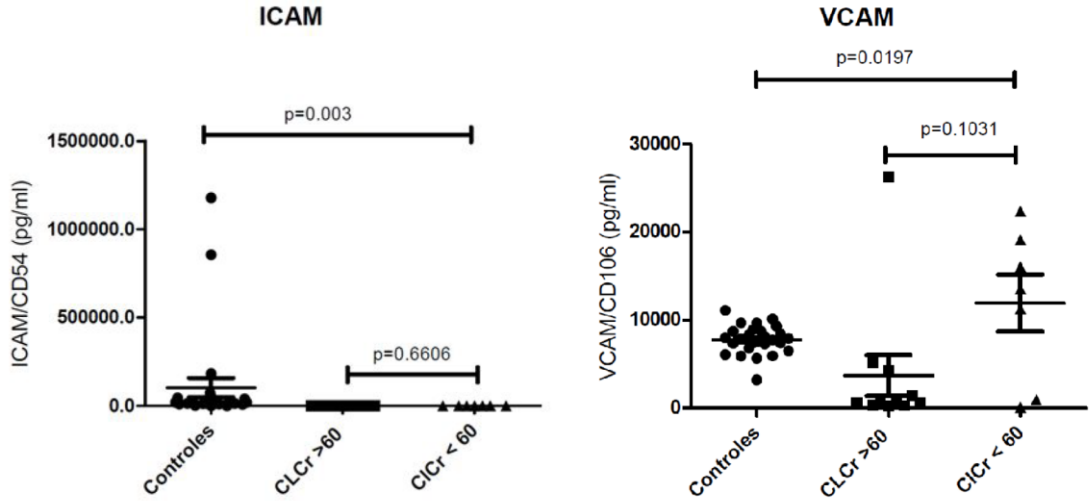


Figure 4 - Cellular adhesion molecules in urine samples from vesicoureteral reflux (VUR) patients and controls. Significant differences were considered when p< 0.05 (Mann-Whitney U test).



6. CONSIDERAÇÕES FINAIS

Refluxo vesicoureteral (RVU) é a quarta causa mais comum de DRC na infância (1). A fisiopatologia da nefropatia do refluxo é complexa e envolve uma grande variedade de moléculas que interagem entre si. Além disso, sabe-se que a lesão renal no RVU pode progredir na ausência de ITU e até mesmo após a resolução do refluxo. O manejo dos pacientes com RVU envolve o uso de antibioticoprofilaxia contínua, tratamento da disfunção do trato urinário inferior e, em casos selecionados, intervenção cirúrgica, de forma a evitar ou ao menos atrasar a progressão para DRC (2, 3). O grande desafio na condução dos pacientes com RVU é discernir quais pacientes irão evoluir com doença renal estágio final. Os exames de imagem auxiliam na identificação desses pacientes com maior potencial de evolução desfavorável. No entanto, esses exames muitas vezes são invasivos, expõem o paciente a radiação e apresentam um custo financeiro elevado. Além disso, tais exames não são capazes de predizer em todos os casos sua real potencialidade para evolução para DRC.

Novas abordagens diagnósticas para o RVU e novos fatores preditivos para o desenvolvimento de nefropatia do refluxo são necessários. Nesse contexto, a pesquisa de biomarcadores apresenta grande importância. Existem inúmeras evidências clínicas e experimentais sobre o papel da inflamação nas doenças renais. No entanto, fatores genéticos, inflamatórios, fibrogênicos, ambientais e epigenéticos envolvidos na fisiopatologia da nefropatia do refluxo precisam ser melhor compreendidos. Avaliar os efeitos de citocinas, quimiocinas, fatores de crescimento e moléculas de adesão celular sobre o início e progressão da lesão renal é, portanto, primordial para o desenvolvimento de novos marcadores prognósticos e de novos alvos terapêuticos.

Estudos sobre a fisiopatologia da nefropatia do refluxo são escassos e biomarcadores clinicamente significativos ainda não foram determinados. Após extensa revisão da literatura, consideramos que a citocina envolvida na fibrogênese TGF-β (4-7), a citocina anti-inflamatória IL-10 (8) e as citocinas pró-inflamatórias IL-6 (9-13), IL-8 (14-17) e TNF (13, 18) devem ser melhor estudadas como potenciais biomarcadores para cicatriz renal e para o desenvolvimento de DRC na nefropatia do refluxo. IL-1β (19) também deve ser melhor investigada em pacientes com RVU, uma vez que essa citocina parece exercer um importante papel na fase aguda da pielonefrite ao mesmo tempo em que previne a formação de cicatrizes renais. MCP-1 (20) pode se tornar um biomarcador útil em fases iniciais da progressão da lesão renal, além de predizer o risco de lesão renal a longo prazo. Fatores de crescimento e moléculas de adesão celular também estão

envolvidos na fisiopatologia da nefropatia do refluxo. A respeito dessas moléculas conclui-se que a molécula de adesão celular ICAM-1 (21, 22) e os fatores de crescimento *basic* FGF (23) e VEGF (7, 24) são potenciais biomarcadores para nefropatia do refluxo. Por outro lado, VCAM (25) parece estar associada a gravidade do refluxo, independentemente de sua progressão para lesão renal.

Nessa dissertação de mestrado, fatores inflamatórios foram investigados em pacientes com RVU e foi possível demonstrar que os biomarcadores MCP-1, IL-12p70, TNF, VEGF e IL-10 encontram-se significativamente elevados na urina desses pacientes. Além disso, naqueles pacientes com DRC estagio ≥ 3 observou-se também níveis urinários elevados de CXCL9/MIG, IL-8, FGF e VCAM-1.

Novos estudos são necessários para identificar dentro desse grupo de proteínas e polipeptídeos aquelas moléculas com potencial de se transformar em biomarcadores capazes de auxiliar em decisões clínicas. Além disso, acredita-se que um painel de biomarcadores, composto pela combinação de marcadores que se elevam e/ou diminuem de acordo com a gravidade da nefropatia do refluxo, será provavelmente mais eficaz no auxílio às decisões clinicas do que um único biomarcador isoladamente. Nesse sentido, a pesquisa de biomarcadores em amostra de urina pela técnica de citometria de fluxo é extremante interessante, pois permite a investigação simultânea de múltiplos biomarcadores em uma amostra biológica coletada de forma não invasiva e de fácil manuseio e conservação.

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ANEXOS

ANEXO A: Resumo do artigo de revisão publicado em julho de 2020. Biomarkers in vesicoureteral reflux: an overview. Biomark Med. 2020 Jun;14(8):683-696. doi: 10.2217/bmm-2019-0378. Epub 2020 Jul 9.

Review

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Biomarkers in Medicine

Biomarkers in vesicoureteral reflux: an overview

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Aim: This article aimed to review the role of cytokines, chemokines, growth factors and cellular adhesion molecules as biomarkers for vesicoureteral reflux (VUR) and reflux nephropathy (RN). **Methods:** We reviewed articles from 1979 onward by searching PubMed and Scopus utilizing the combination of words: 'VUR' or 'RN' and each one of the biomarkers. **Results:** Genetic, inflammatory, fibrogenic, environmental and epigenetic factors responsible for renal scarring need to be better understood. TGF-β, IL-10, IL-6, IL-8 and TNF seem to exert a role in VUR particularly in RN based on the current literature. Serum levels of procalcitonin have been also associated with high-grade VUR and RN. These molecules should be more intensively evaluated as potential biomarkers for renal scarring in VUR. **Conclusion:** Further studies are necessary to define which molecules will really be of utility in clinical decisions and as therapeutic targets for VUR and RN.

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Keywords: adhesion molecules procalcitonin, biomarkers • chemokines • cytokines • growth factors • inflammation • reflux nephropathy • vesicoureteral reflux

Future Medicine

ANEXO B: Parecer do Comitê de Ética em Pesquisa

Parecer nº. ETIC 109/07

Interessado(a): Prof. Eduardo Araújo Oliveira
Depto. Pediatria
Fac. Medicina -UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 15 de maio de 2007,após atendidas as solicitações de diligência, o projeto de pesquisa intitulado **"Anomalias congênitas do trato urinário: do diagnóstico pré-natal à prevenção da doença renal crônica"** bem como o Termo de Consentimento Livre e Esclarecido.

O relatório final ou parcial deverá ser encaminhado ao COEP um ano após o início do projeto.

Profa. Dra. Mariza Santos Castro
Vice -Presidente do COEP-UFMG
Presidente em Exercício