UNIVERSIDADE FEDERAL DE MINAS GERAIS

FACULDADE DE MEDICINA

PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

ROBERTA DA SILVA FILHA

AVALIAÇÃO DE POTENCIAIS BIOMARCADORES NA SÍNDROME NEFRÓTICA PRIMÁRIA EM PACIENTES PEDIÁTRICOS

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AVALIAÇÃO DE POTENCIAIS BIOMARCADORES NA SÍNDROME NEFRÓTICA PRIMÁRIA EM PACIENTES PEDIÁTRICOS

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ROBERTA DA SILVA FILHA

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Às crianças e adolescentes acompanhados no ambulatório do HC-UFMG.

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RESUMO

Introdução: A síndrome nefrótica primária (SNP) é uma doença glomerular comum na faixa etária pediátrica, relacionada a alterações intrínsecas dos glomérulos sem causa estabelecida, que leva à proteinúria, edema, hipoalbuminemia e dislipidemia. Inúmeros fatores têm sido relacionados aos mecanismos de desenvolvimento e evolução da doença como alterações dos componentes da barreira de filtração glomerular, modificações no sistema imune e atuação do sistema renina angiotensina (SRA). No entanto, a fisiopatologia da doença ainda não está elucidada e persistem diversas questões referentes ao tratamento mais apropriado. **Objetivos:** Avaliar a atuação do SRA, das moléculas inflamatórias, adipocinas, hormônios do metabolismo e marcadores de função endotelial em pacientes pediátricos com SN primária. **Métodos:** Foi realizado um estudo transversal que incluiu 33 crianças e adolescentes com SNP em remissão parcial e completa. As mensurações dos biomarcadores foram comparadas entre os pacientes e indivíduos saudáveis (grupo controle), pareados por idade e sexo. Os biomarcadores foram relacionados às variáveis clínicas e laboratoriais. **Resultados:** Em nosso primeiro estudo original, verificamos que as concentrações urinárias de Ang II, Ang-(1-7), ECA e MCP-1 encontravam-se aumentadas nos pacientes com SNP em comparação ao grupo controle. Por outro lado, os níveis urinários de ECA2 eram significativamente menores nos pacientes com SNP e correlacionavam negativamente com a proteinúria. No segundo estudo, os níveis urinários de leptina e as concentrações plasmáticas de resistina e grelina estavam significativamente aumentadas em relação ao grupo controle. Em contrapartida as concentrações urinárias de adiponectina e PAI-1 foram menores nos pacientes SN quando comparados aos controles. **Conclusão:** As moléculas do SRA, as quimiocinas, as adipocinas, os hormônios do metabolismo e marcadores de função endotelial foram encontrados em diferentes concentrações em pacientes com SNP comparados ao grupo controle, sugerindo que essas moléculas participam da fisiopatologia e da progressão da lesão renal.

Palavras-chave: Síndrome Nefrótica, SRA, moléculas inflamatórias, adipocinas, marcadores metabólicos, marcadores de função endotelial.

ABSTRACT

Introduction: Primary nephrotic syndrome (PNS) is a common glomerular disease in the pediatric age group, related to intrinsic changes in the glomeruli without an established cause, which leads to proteinuria, edema, hypoalbuminemia and dyslipidemia. Numerous factors have been related to the mechanisms of development and evolution of the disease, including changes in the components of the glomerular filtration barrier, alterations in the immune system and the effects of the renin angiotensin system (RAS). However, the pathophysiology of the disease remains to be elucidated and several questions regarding the most appropriate treatment persist. **Objectives:** To evaluate the role of RAS, inflammatory molecules, adipokines, metabolism hormones and endothelial function markers in pediatric patients with primary NS. **Methods:** This cross-sectional study included 33 children and adolescents with SNP in partial and complete remission. Biomarker measurements were compared between patients and healthy individuals (control group), matched for age and sex. Biomarkers were related to clinical and laboratory variables. **Results:** In our first original study, we found that urinary concentrations of Ang II, Ang-(1-7), ACE and MCP-1 were increased in patients with PNS compared to the control group. On the other hand, urinary ACE2 levels were significantly lower in patients with PNS and negatively correlated with proteinuria. In the second study, urinary leptin levels and plasma concentrations of resistin and ghrelin were significantly increased compared to the control group. In contrast, urinary adiponectin and PAI-1 concentrations were lower in PNS patients when compared to controls. **Conclusion:** RAS molecules, chemokines, adipokines, metabolism hormones and endothelial function markers were found in different concentrations in patients with PNS compared to the control group, suggesting that these molecules participate in the pathophysiology and progression of renal damage.

Keywords: Nephrotic Syndrome, RAS, inflammatory molecules, adipokines, metabolic markers, endothelial function marker.

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ARTIGO ORIGINAL 1

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LISTA DE ABREVIATURAS E SIGLAS

Ang-(1-7)	Angiotensina-(1-7)
Ang II	Angiotensina II
BRA	Bloqueadores de Receptores Angiotensinérgicos do tipo 1
CBA	Cytometric Bead Array
CCL/CXCL	Quimiocinas
COEP	Comitê de Ética em Pesquisa
DRC	Doença Renal Crônica
ECA	Enzima Conversora de Angiotensina
ECA2	Enzima Conversora de Angiotensina 2
ELISA	Enzyme-Linked Immunosorbent Assay
GESF	Glomeroesclerose Focal e Segmentar
GIP	Polipeptídeo Inibitório Gástrico
GLP-1	Peptídeo Semelhante ao Glucagon
iECA	Inibidor da Enzima Conversora de Angiotensina
IL	Interleucina
IMC	Índice de Massa Corpórea
IP-10	Proteína Induzida por Interferon Gama
IRA	Injúria Renal Aguda
MCP-1	Proteína Quimioatraente de Monócitos 1
MIG	Monocina Induzida pelo Interferon Gama
PAI-1	Inibidor do Ativador de Plasminogênio-1
RANTES	Regulado na Ativação, Normal T Expresso e Secreto
RFG	Ritmo de Filtração Glomerular

sVCAM	Molécula de Adesão Celular Solúvel
SN	Síndrome Nefrótica
SNCD	Síndrome Nefrótica Corticodependente
SNCR	Síndrome Nefrótica Corticorresistente
SNCS	Síndrome Nefrótica Corticossensível
SNLM	Síndrome Nefrótica por Lesões Mínimas
SRA	Sistema Renina Angiotensina
TCLE	Termo de Consentimento Livre e Esclarecido
TNF	Fator de Necrose Tumoral

SUMÁRIO

1 INTRODUÇÃO

A síndrome nefrótica (SN) é uma doença prevalente na faixa etária pediátrica associada à elevada morbidade, apesar de recentes avanços em seu tratamento[1]. Definida como a glomerulopatia mais comum em crianças e adolescentes,[1,2,3] decorre de alterações na permeabilidade/seletividade da barreira de filtração glomerular, levando a edema, proteinúria maciça e hipoalbuminemia[4]. Outros achados clínicos da SN são dislipidemia, lipidúria, fenômenos tromboembólicos e infecções de repetição, além de outras complicações como a injúria renal aguda (IRA) e evolução para doença renal crônica (DRC)[5].

Etiologicamente, a SN se classifica em primária ou idiopática e secundária a doenças sistêmicas. A forma primária é responsável por cerca de 90% dos casos de SN na faixa etária pediátrica. Quanto à histopatologia, a SN primária se apresenta mais comumente como síndrome nefrótica por lesões mínimas (SNLM), seguida da glomeruloesclerose segmentar e focal (GESF) e da nefropatia membranosa[6]. Embora a patogênese permaneça desconhecida, alguns mecanismos são identificados como possíveis causadores da doença, tais como defeitos genéticos na expressão de proteínas específicas dos podócitos ou, mais comumente, resposta anormal do sistema imune a estímulos exógenos[2,7]. Existem hipóteses de que fatores circulantes afetariam a permeabilidade glomerular e, em consequência, ocasionariam a perda de proteínas na urina[8,9]. Em contrapartida, a forma secundária da SN é predominante em adultos, ocorrendo durante o curso clínico de doenças sistêmicas que afetam os rins tais como síndromes genéticas, doenças metabólicas, doenças infecciosas e auto-imunes[10]. Consta-se que, menos de 10% e mais de 50% dos casos acometem crianças e adultos, respectivamente.

A SN pode também ser classificada com base na resposta ao tratamento com corticoides e nos achados histopatológicos da biopsia renal[2,11]. Os pacientes que não respondem ao uso de corticosteroides são denominados corticorresistentes (SNCR), enquanto aqueles que apresentam remissão clínica e laboratorial após o seu uso são chamados de corticossensíveis (SNCS). Os pacientes que dependem da manutenção do uso de corticoides para permanecerem em remissão ou que apresentam recidiva até duas semanas após a suspensão do tratamento são considerados corticodependentes (SNCD). As crianças responsivas ao tratamento apresentam melhor prognóstico, com curso clínico favorável da doença.

Por outro lado, pacientes com SNCR geralmente são diagnosticados com GESF à biopsia renal e apresentam pior prognóstico. Pelo menos 20% das crianças apresentam recidivas frequentes e/ou dependência a corticoides durante ou após terapia imunossupressora (SNCD) e

cerca de 1-3% são resistentes à esteroides (SNCR)[12] com alto risco de progressão para os estágios finais da DRC em 5 anos, não alcançando remissão completa ou parcial[11,13].

Dentre as complicações frequentes da SN, as alterações do metabolismo lipídico podem contribuir substancialmente para o desenvolvimento e progressão da doença. A hipercolesterolemia, um achado onipresente na SN, vem acompanhada não só por anormalidades nos níveis de lipídios, mas também por modificações na função das lipoproteínas[1,14]. A gravidade da dislipidemia, no entanto, é diretamente correlacionada à intensidade da proteinúria, uma vez que, para repor as perdas de proteínas essenciais, como a albumina, ocorre elevação na produção dos lipídios[14]. Os podócitos, assim como os glomérulos e túbulos renais, sofrem lesões decorrentes do acúmulo de lipoproteínas e colesterol com consequente produção de agentes citotóxicos, citocinas[1], liberação de possíveis marcadores de função endotelial[15,16], adipocinas[17,18] e hormônios peptídicos[19-21].

Além disso, alguns estudos têm abordado o papel do sistema renina-angiotensina (SRA) na evolução da lesão renal em pacientes com SN primária[22-24]. A ação de componentes do sistema, sobretudo da angiotensina II (Ang II), desencadeia uma cascata de eventos lesivos que incluem o recrutamento de células inflamatórias e estimulação da expressão e liberação de citocinas e quimiocinas[25] que contribuirão para proteinúria, inflamação e fibrose renais[26]. Neste contexto, o uso de inibidores da enzima conversora de angiotensina (iECA) e bloqueadores de receptores angiotensinérgicos do tipo 1 (BRA) tem-se mostrado efetivo no controle da proteinúria e na redução de inflamação e fibrose renais[22]. As ações renoprotetoras desses medicamentos independem da etiologia da glomerulopatia[27-29]. Além disso, estudos experimentais sugerem efeitos benéficos nas doenças renais de ativadores da enzima conversora de angiotensina 2 (ECA2) e do heptapeptídeo angiotensina-(1-7)[26,29].

Em síntese, os desafios atuais em relação à SN primária consistem sobretudo no conhecimento dos mecanismos de progressão da doença, além de investigação de novas abordagens terapêuticas baseadas nos prováveis fatores implicados na doença. Dentro deste contexto, o presente estudo teve como objetivo investigar o papel do SRA, das moléculas inflamatórias, adipocinas, e dos marcadores do metabolismo energético e de função endotelial na SN primária, comparando com essas mesmas moléculas dosadas em indivíduos saudáveis, pareados por idade e sexo. Foram também avaliadas associações e/ou correlações das moléculas avaliadas com dados clínicos e laboratoriais dos pacientes.

2 REFERÊNCIAS BIBLIOGRÁFICAS

1 Agrawal S, Zaritsky JJ, Fornoni A, Swoyer WE (2018) Dyslipidaemia in nephrotic syndrome: mechanisms and treatment. Nephrol 14:57-70. doi: https://doi.org/10.1038/nrneph.2017.155

2 Bierzynska A, Saleem M (2017) Recent advances in understanding and treating nephrotic syndrome. F1000 Research 6:121. doi: 10.12688/f1000research.10165.1

3 Hang H, Wang Z, Dong LQ, Guo YN (2016) Children with steroid-resistant nephrotic syndrome: long-term outcomes of sequential steroid therapy. Biomed Environ Sci 29(9):650-655. doi: 10.3967/bes2016.087

4 Wenderfer SE, Gaut JP (2017) Advances in chronic kidney disease. Adv Chronic Kidney Dis 24(6):364-371. doi: 10.1053/j.ackd.2017.09.005

5 Park SJ, Shin JI (2011) Complications of nephrotic syndrome. Korean J Pediatr 54(8):322-328. doi: 10.3345/kjp.2011.54.8.322

6 Eddy AE, Symons JM (2003) Nephrotic syndrome in childhood. The Lancet 362(9384):629-639. doi: https://doi.org/10.1016/S0140-6736(03)14184-0

7 Dorval G, Gribouval O, Martinez-Barquero V, Machuca E, Tête M-J, Baudouin V, Benoit S, et al (2018) Clinical and genetic heterogeneity in familial steroid-sensitive nephrotic syndrome. Pediatr Nephrol 33:473-483. doi: 10.1007/s00467-017-3819-9

8 Colucci M, Corpetti G, Emma F, Vivarelli M (2018) Immunology of idiopathic nephrotic syndrome. Pediatr Nephrol 33:573-584. doi: 10.1007/s00467-017-3677-5

9 Maas RJ, Deegens JK, Wetzels JF (2014) Permeability factors in idiopathic nephrotic syndrome: historical perspectives and lessons for the future. Nephrol Dial Transplant 29:2207-2216. doi: 10.1093/ndt/gfu355

10 Souto MFO, Teixeira MM, Penido MGMG, Simões e Silva AC (2008) Fisiopatologia da síndrome nefrótica em crianças e adolescentes. Arch Latin Nefr Ped 8(1).

11 Noone DG, Iijima K, Parekh R (2018) Idiopathic nephrotic syndrome in children. Lancet 392(10141):61-74. doi: https://doi.org/10.1016/S0140-6736(18)30536-1

12 Kazumoto I, Mayumi S, Kandai N (2017) Rituximab for nephrotic syndrome in children. Clin Exp Nephrol 21(2):193-202. doi: 10.1007/s10157-016-1313-5

13 Zagury A, Oliveira AL, Montalvão JA, Novaes RHL, de Sá VM, Moraes CAP, Tavares MS (2013). Steroid-resistant idiopathic nephrotic syndrome in children: long-term follow-up and risk factors for end-stage renal disease. J Bras Nefrol 35(3):191-9. doi: 10.5935/0101-2800.20130031

14 Hari P, Khandelwal P, Smoyer WE (2020) Dyslipidemia and cardiovascular health in childhood nephrotic syndrome. Pediatr Nephrol 35(9):1601-1619. doi: https://doi.org/10.1007/s00467-019-04301-y

15 Zhang J, Gu C, Lawrence DA, Cheung AK, Huang Y (2014) A PAI-1 mutant retards diabetic nephropathy in db/db mice through protecting podocytes. Exp Physiol 99 (5):802-815. doi: 10.1113/expphysiol.2013.077610

16 Ross R (1998) Atherosclerosis - an inflammatory disease. N Engl J Med 340:115-26. doi: https://doi.org/10.1056/NEJM199901143400207

17 Sharma B, Saha A, Dubey NK, Kapoor K, Anubhuti, Batra VV, Upadhayay AD (2014) Endothelial dysfuntion in children with idiopathic nephrotic syndrome. Atherosclerosis 233 (2):704–706. doi: https://doi.org/10.1016/j.atherosclerose.2014.01.055

18 Wasilewska A, Tomaszewska B, Zoch-Zwierz W, Biernacka A, Klewinowska K, Koput A (2005) Serum and urine leptin concentration in children with nephrotic syndrome. Pediatr Nephrol 20(5):597-602. doi: https://doi.org/10.1007/s00467-004-1772-x

19 Gubina NV, Kupnovytska IH, Mishchuk VH, Markiv HD (2020). Ghrelin levels and decreased kidney function in patients with early stages of chronic kidney disease against the background of obesity. Journal of Medicine and Life 13(4):530–535. doi: doi.org/10.25122/jml-2020-0152

20 Ozkorucu D, Cetin N, Sav NM, Yildiz B (2016). Urine and serum ghrelin, sCD80 and sCTLA-4 levels in doxorubicin-induced experimental nephrotic syndrome. Int Urol Nephrol 48(7):1187-1196. doi: 10.1007/s11255-016-1249-4.

21 Katsurada K, Nandi SS, Zheng H, Liu X, Sharma NM, Patel KP (2020) GLP-1 mediated diuresis and natriuresis are blunted in heart failure and restored by selective afferent renal denervation. Cardiovascular Diabetology 19(1):57. doi: https://doi.org/10.1186/s12933-020-01029-0

22 Yi Z, Li Z, Wu X-C, He Q-N, Dang X-Q, He X-J. Effect of fosinopril in children with steroid-resistant idiopathic nephrotic syndrome. Pediatr Nephrol 2006; 21:967-972.22

23 Gipson DS, Chin H, Presler TP, et al. Differential risk of remission and ESRD in childhood FSGS. Pediatr Nephrol 2006; 21(3):344-349.

24 Filha RDS, Pinheiro SVB, Macedo E Cordeiro T, Feracin V, Vieira ÉLM, Miranda AS, Simões E Silva AC (2019) Evidence for a role of angiotensin converting enzyme 2 in proteinuria of idiopathic nephrotic syndrome. Biosci Rep 39(1):BSR20181361. doi: oi: https://doi.org/10.1042/BSR2018136116

25 Capettini LS, Montecucco F, Mach F, Stergiopulos N, Santos RA, da Silva RF (2012) Role of renin-angiotensin system in inflammation, immunity and aging. Curr Pharm Des 18:963-970. doi: 10.2174/138161212799436593

26 Simões e Silva AC, Silveira KD, Ferreira AJ, Teixeira MM (2013) ACE2, angiotensin-(1-7) and Mas receptor axis in inflammation and fibrosis. British J Pharmacol 169:477-492. doi: 10.1111/bph.12159

27 Ourbakhsh N, Mak RH (2017) Steroid-resistant nephrotic syndrome: past and current perspectives. Pediatric Health, Medicine and Therapeutics 8:29-37. doi: 10.2147/PHMT.S100803

28 McCaffrey J, Lennon R, Webb NJA (2016) The non-immunosuppressive management of childhood nephrotic syndrome. Ped Nephrol 31:1383-1402. doi: 10.1007/s00467-015-3241-0

29 Simões e Silva AC, Teixeira MM (2016) ACE inhibition, ACE2 and angiotensin-(1-7) axis in kidney and cardiac inflammation and fibrosis. Pharmacological Research 107:154-162. doi: 10.1016/j.phrs.2016.03.018

3 MANUSCRITO DE REVISÃO (submetido ao periódico Current Pediatric Reviews)

Idiopathic Nephrotic Syndrome in Pediatrics: an up-to-date

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ABSTRACT

Background: Idiopathic or Primary or Idiopathic Nephrotic Syndrome (INS) is a common glomerulopathy in pediatric population, basically characterized by proteinuria, edema and hypoalbuminemia with diverse findings in renal histopathology.

Objective: The present review aimed to update knowledge on the etiopathogenesis diagnoses, current protocols of treatment and potential therapeutic advances in INS.

Methods: This integrative review searched for articles on histopathology, physiopathology, genetic causes, diagnosis and treatment of INS in pediatric patients. The databases evaluated were PubMed and Scopus.

Results: The disease is related to a primary alteration in the permeability of the glomerular filtration barrier due to unknown etiology. There are several gaps in the etiopathogenesis, response to treatment and evolution of the syndrome that justify further investigation. Novel advances include the greater understanding about the role of podocytes in INS and the identification of genes associated with the occurrence of the disease. The role of immune system cells and molecules have also been investigated. The diagnosis relies on clinical findings, laboratory exams and renal histology for selected cases. The treatment is primarily based on steroids administration. In case of failure, other medications should be tried. Recent studies have also searched for novel biomarkers for diagnose and alternative therapeutic approaches.

Conclusion: The therapeutic response to corticosteroids still remains the main predictive factor for the prognosis of the disease. Genetic and pharmacogenomics tools may allow the identification of cases not responsive to immunosuppressive medications.

Key words: idiopathic nephrotic syndrome, children, proteinuria, podocyte, steroid therapy, focal and segmental glomerulosclesrosis

INTRODUCTION

Nephrotic syndrome (NS) is clinically characterized by marked proteinuria, edema and hypoalbuminemia. Lipiduria and hyperlipidemia may also be present. The protein loss is due to changes in permeability of the glomerular filtration barrier [1].

According to the etiology, NS is classified into primary or idiopathic glomerular disease (INS) and in secondary NS. INS is more common in children and resulting from intrinsic changes in the glomeruli without determinant causes. Secondary NS is due to systemic diseases, including diabetes, systemic lupus erythematosus (SLE), viral hepatitis, HIV infection, among others [2,3]. Secondary forms of the disease affect mainly adolescents and adults. In the pediatric population, minimal-change nephrotic syndrome (MCNS) is the most common histopathological form of INS, but a recent increase in the incidence of focal and segmental glomerulosclerosis (FSGS) has been observed [2,3,4].

INS may have a positive family history. There is a predominance of male patients during childhood [5]. The population distribution is very diverse, as the incidence varies according to the geographical area, race and age, presenting, approximately, 2 to 7 cases per year for every 100,000 children under the age of 16. The prevalence is about 16 cases/100,000 in the same population group, being children aged 2 to 3 years most frequently affected [6].

According to epidemiological data, viral infections had already been associated with the development of INS. There is an association between the typical age of the disease onset and the time of highest incidence of viral infections [7]. Some cases of INS are also triggered by bacterial, fungal and parasitic infections, and antigens from infectious agents have been identified in the glomeruli of children with the disease [7-9].

INS can also be classified according to the therapeutic response, relapse pattern, histopathology and presence of genetic mutations [10]. Depending on the response to treatment, about 70 to 85% of pediatric patients respond to treatment with corticosteroids, being called steroid-sensitive (SSNS). On the other hand, some patients present recurrence during the corticosteroids reduction or suffer from frequent relapses after discontinuation of the medication, being called steroid-dependent (SDNS). In addition, there are patients who are

resistant to corticosteroid therapy (SRNS), comprising 10 to 20% of cases with a tendency to progress to renal fibrosis and chronic kidney disease (CKD) [10]. Table 1 shows the terms related to the therapeutic response and the clinical stages of the disease. Two thirds of the NS cases that manifest during the first year of life can be explained by genetic mutations (NPHS1, NPHS2, WT1 or LAMB2, among other genes) [11,12] and represent congenital NS [3].

Regarding the primary form of the disease, the precise mechanisms for its development have not been elucidated yet, but it seems to be associated with changes in the immune system, specifically in the T lymphocytes activity, with a consequent change in the podocytes structure [6], aside from changes in the expression of cytokines and immune inflammatory cells [13-18]. The therapeutic action of immunosuppressive drugs in INS and the presence of polymorphism in the human histocompatibility complex genes corroborate the hypothesis that changes in the immune system play a key role in the pathogenesis of the disease [14]. However, there are still many issues regarding INS. In this context, this review article aims to describe the most recent knowledge about pathophysiology, histopathology, clinical manifestations, diagnosis, as well as to summarize the current treatment protocols and new therapeutic approaches related to INS.

METHODOLOGY

To perform this review, we conducted researches in the PubMed and Scopus databases, including English-language articles, published in the last 15 years and using the following term combinations: “nephrotic syndrome”, “epidemiology”, “treatment”, “pediatric”, “Childhood”, “focal and segmental glomerulosclerosis”, “minimal-change nephrotic syndrome”, “diagnosis”, “genetic basis”.

HISTOPATHOLOGY

INS has distinct histological variants, with emphasis on MCNS, FSGS and membranous glomerulopathy (MGN) [15]. In children, MCNS is detected in about 84.5% of patients with INS, while 9.5% have FSGS and 3.5% have MGN or other diseases that present massive proteinuria [16-18].

In MCNS, there are few glomerular changes, with evidence of foot processes fusion and formation of microvilli in the epithelial cells, without any inflammatory injury or immune complex deposition [2,4,15,17]. Immunofluorescence is negative or exhibits only mild positivity for C3 and IgM [19].

Table 1 - Classification of Idiopathic Nephrotic Syndrome in association to disease stages and response to corticosteroids

Disease stage and response to steroids	*Clinical and laboratory criteria*
Complete remission	Diuresis, edema and hypoalbuminemia improvement with normalization of proteinuria
Partial remission	Different from complete remission because of persistency of elevated proteinuria
Relapse	Resurgence of nephrotic proteinuria ($\geq$ 40 mg/m^2/h or protein-creatinine ratio $\geq$ 2 in urine sample)
Steroid sensitive	Complete remission (up to 4 weeks of treatment)
Steroid resistant	Absence of complete remission (after 4 weeks of treatment)
Steroid dependent	Relapses during medication withdrawal or up to 2 weeks after withdrawal

It is believed that 70% of patients diagnosed as MCNS are under the age of 5 [6]. The incidence of MCNS decreases as age increases. In general, MCNS is considered relatively benign and the risk of progressing to terminal CKD is extremely low [19].

As a result of changes in the histological pattern reported in the last years, an increase in the incidence of FSGS, the most severe form of INS, has been observed [4,6]. FSGS is responsible for about 20% of cases in children and 40% in adults [20]. The racial and geographic differences make it impossible for FSGS incidence and prevalence data to be accurate. FSGS is characterized by glomerular lesions with focal distribution of sclerosis, affecting few glomeruli, mainly at the corticomedullary junction, and with a segmental pattern that occurs only in part of the glomerular tuft. It happens capillary lumen obliteration, hypercellularity and hyalinosis, which are revealed by optical microscopy [5,15], in addition to immune complex and complement proteins, mainly C3 deposits, detected by immunofluorescence [2,5].

Unlike MCNS and FSGS, MGN is the main cause of NS in adults (20% of cases) [21], being less representative in the children population [15,16,21]. It is associated with the immune

complex deposition on the glomerular basal membrane with projections of the epithelial cells cytoplasm [6,15,21], without changes in the mesangial matrix and cellularity or in the glomerular capillaries. Immunofluorescence analysis identifies a granular pattern of IgG deposits (IgG4 and IgG1 subclasses) and C3 complement on the walls of glomerular capillaries [15,21].

PHYSIOPATHOLOGY

Despite several efforts made to elucidate the pathophysiological mechanisms of INS, these have not been fully understood yet. However, changes in the glomerular filtration barrier, activation of the immune system, deregulation of T cells and the production of permeability factors have been implicated in the pathogenesis of this disease. A schematic view of the physiopathology of INS is shown in Figure 1.

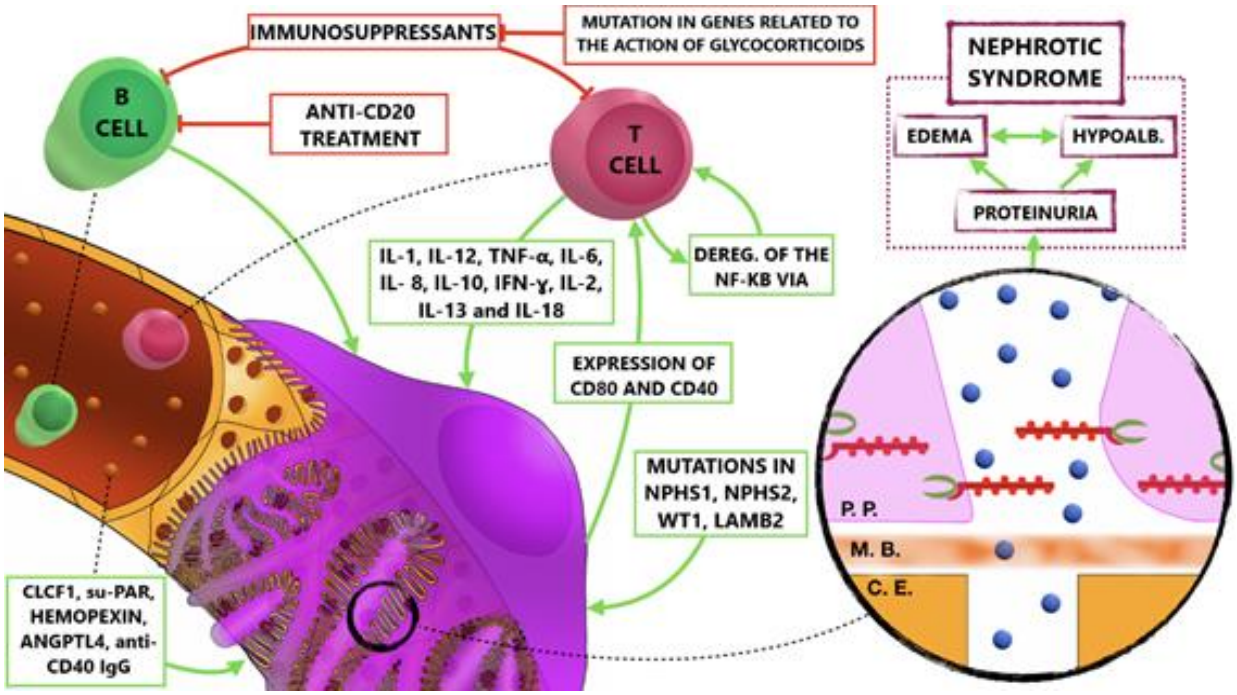
Podocytes play a crucial role in INS [17, 22-26] since structural changes and lesions in these cells directly interfere with the selectivity of the glomerular filtration barrier [10]. More recent studies emphasize that the podocytes themselves, under inflammatory stimuli, may be responsible for the activation of cellular pathways that cause the proteins loss in the urine [27]. Kidney injuries can cause foot process effacement (FPE), which may induce podocytes de-differentiation and result in massive proteinuria. The functional imbalance of the cytoskeleton (Rho family of GTPases) also may provoke FPE [26].

CXCL12 is a cytokine normally produced by healthy podocytes and responsible for providing feedback to progenitor cells to keep them quiescent and suppress the intrinsic capacity of producing new podocytes. The production of this cytokine is reduced in podocytopathies. This phenomenon promotes the stimulation of the NOTCH gene, leading to podocyte dedifferentiation as an attempt to protect cells, but leads to functional defects, worsening proteinuria [26].

There is also interaction between podocytes and the immune system. There are reports of increased expression of co-stimulatory molecules, such as CD80 (B7-1) and CD40, capable of inducing T cells activation [28] and supposedly causing proteinuria and genetic or immunological changes [17,18] in kidney diseases. The expression of CD80 in podocytes can be induced by cytokines or by dysfunction of regulatory T cells (Treg) [29-32], with damage in the self-regulatory function of podocytes [25]. Cytotoxic T lymphocytes have been associated with the presence of persistent proteinuria [18].

The role of B-lymphocytes has also been the subject of recent research. Some clinical trials have shown the efficacy of anti-CD20 antibodies [15] and antibodies against cells that express

CD20 [17] in maintaining prolonged remission in INS patients. B cells can act as antigen-presenting cells, modifying T lymphocytes function. This mechanism is present in SDNS patients [26]. Moreover, SRNS patients have lower B lymphocyte counts [18,31]. Natural killer cells (NK) have also been studied and there are reports of reduced activity in SRNS patients [31].



IL = interleukin; TNF-α = tumor necrosis factor alpha; IFN-γ = interferon gamma; NF-kB = nuclear factor kappa B; CLCF1 = cardiotrophin-like cytokine 1; su-PAR = urokinase type plasminogen activator receptor; AGNPTL4 = angiopoietin-related protein 4; IgG = immunoglobulin G; DEREG.= deregulation; HYPOALB.= hypoalbuminemia; P.P.= process of podocytes; M.B.= basal membrane; C.E = endothelial cell;

Figure 1 - Etiopathogenic factors of Idiopathic Nephrotic Syndrome

In addition, changes in the inflammatory profile and expression of cytokines, as well as the therapeutic response of corticosteroids and immunosuppressants, also highlight the role of the immune system in the INS [2,6,13,17,18,22,23,26,32-34]. Studies show deregulation of signaling by the transcription factor NF-kB, responsible, among other functions, for the lymphocytes T response [2,22] and an association of some cytokines and chemokines (IL-1, IL-12, TNF, IL-6, IL- 8, IL-10, IFN-ɣ, IL-2, IL-13 and IL-18) [2,6,17,18,26] with the presence of proteinuria. As the interaction between cytokines and the cellular component of the immune system is complex, it is suggested that INS is the result of numerous changes, successive or simultaneous, in different cell types, as well as in the expression profile of cytokines [23].

Studies show a tendency of changing from a T helper 1 (less inflammatory) profile to a T helper 2 (more inflammatory) profile in INS [18,26]. Figure 1 summarizes changes in the immune system that have been associated with the pathophysiology of INS.

In addition, the hypothesis that there is a circulating factor capable of increasing glomerular permeability continues to be a target of investigation in INS. Circulating factors such as cardiotrophin-like cytokine 1 (CLCF1), hemopexin, soluble urokinase type plasminogen activator receptor (su-PAR), angiopoietin-related protein 4 (Angptl4) and anti-CD40 IgG [2,17,26,35] have been associated with the occurrence of proteinuria [10] by affecting the permeability of the glomerular barrier.

Furthermore, molecules from the renin angiotensin system (RAS) have also been evaluated in INS (Figure2). Recently, our research group showed an increase in angiotensin-converting enzyme (ACE), angiotensin II (Ang II) and angiotensin-(1-7) [Ang-(1-7)] in the urine of nephrotic patients and a significant reduction in angiotensin-converting enzyme 2 (ACE2). It is believed that the elevation of Ang-(1-7) is a compensatory mechanism to the increase in Ang II or may be due to a dysfunction of the Mas receptor in the kidney. This last hypothesis may explain the fact that despite the increase in Ang-(1-7), the heptapetide may not exert its nephroprotective effects [36,37]. Also, ACE2 deficiency is associated with elevated pro-inflammatory cytokines and chemokines (TNF, IL-1B and MCP1/CCL2) in the urine. This relationship was proven to be stronger in patients with SRNS than in those with SSNS, and greater in the last one than in the control group [37]. Treatments with RAS blockers, ACE inhibitors or type 1 angiotensin receptor antagonists have been shown to be effective in reducing proteinuria and have a nephroprotective effect on glomerulopathies [36].

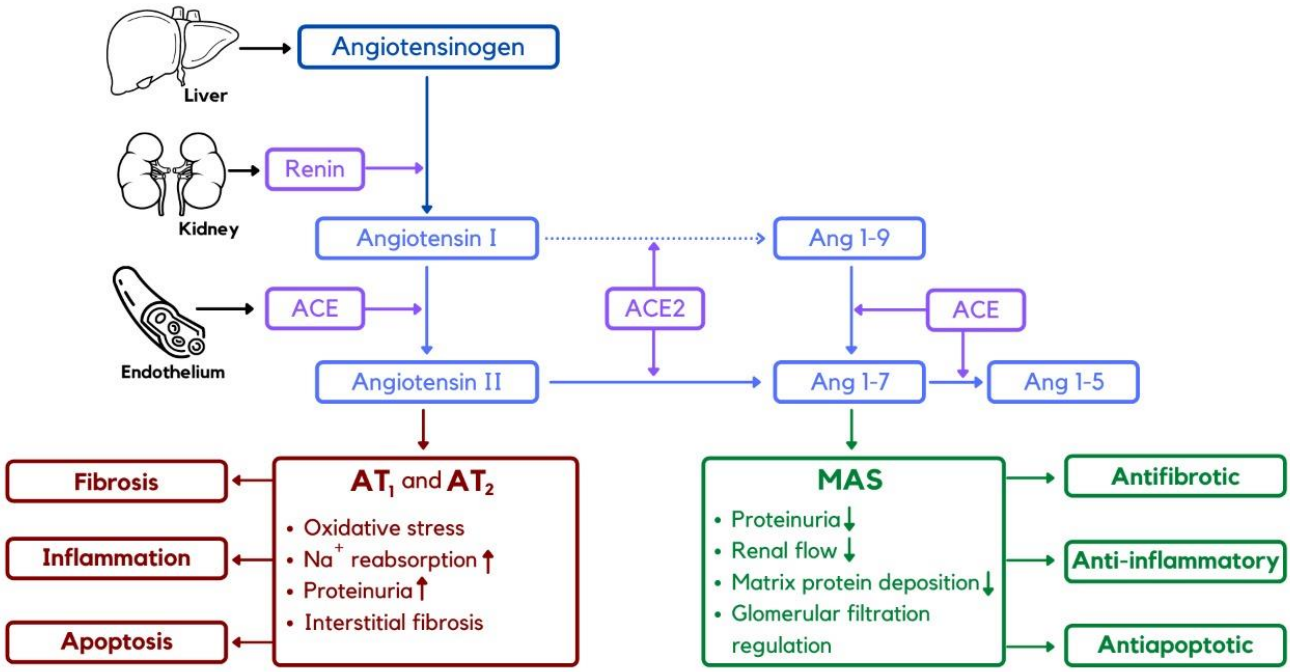


Figure 2 - Proposed mechanisms for the role of the RAS axis at renal level

GENETIC FACTORS

Genetic factors have been related to the development of NS, especially congenital forms. In congenital NS, resistance to treatment with corticosteroids is very common, being observed in 70% of the cases that manifest the disease until the third month of life and in 50% of those that manifest the disease between 4 months and one year of life [38]. In addition, two thirds of NS cases that occur during the first year of life can be explained by mutations in the NPHS1, NPHS2, WT1 and LAMB2 genes [12,26]. Such mutations represent the most common genetic causes of SRNS, affecting approximately 69 to 85% of patients who manifest the disease in the first 3 months of life and 50 to 66% of cases in which the disease appears until the age of 12 months. The genetic forms of FSGS occur until the first year of life and are frequently associated with mutations in genes that encode proteins present in podocytes [11,12,38-40], producing structural changes in the signaling pathways and in the components of the slit diaphragm [12,26,41]. For these reasons, congenital or genetic etiology of NS presents early onset [13] and resistance to treatment with corticosteroids. Depending on the age that resistance to treatment with corticosteroids begins, the chance of being a genetic NS decreases substantially [37]. However, in these cases, there are less cases of recurrence after kidney transplantation [12].

Some genes are related to the presence of NS associated with syndromic conditions. Mutations in the WT1 gene, for example, are associated with urogenital abnormalities, ambiguous genitalia and nephroblastoma. Mutations in LAMB2 and LMX1B typically cause Pierson's syndrome (ocular abnormalities) and Nail-Patella's syndrome (musculoskeletal abnormalities and inguinal changes), respectively. Moreover, SRNS has been observed in association with myopathy, encephalopathy, lactic acidosis, diabetes, and convulsive-like episodes, in the presence of mitochondrial genes mutations (most commonly MTTL1) [12].

On the other hand, genetic factors can also play a role in the pathophysiology of INS. For example, changes in genes that encode molecules related to the action mechanisms of glucocorticoids, such as glucocorticoid receptors (GRα), P glycoproteins and cytochromes P450 and polymorphisms in genes that encode cytokines (IL-4 and IL-6) may contribute to resistance to treatment with corticosteroids development in INS [42].

Another mechanism that has been associated, although more rarely, is the deficiency or dysfunction of coenzyme Q10 (CoQ10), due to mutations in the genes COQ2, COQ6, PDSS2 and ADCK4, part of mitochondrial DNA, which manifests as FSGS alone or associated with extrarenal manifestations (encephalopathy, deafness, hypotonia, cerebellar ataxia, developmental delay). These patients benefit from oral CoQ10 supplementation [12,26].

In addition, many genome-wide association studies have shown participation of the loci HLA-DR and HLA-DQ in SSNS [26]. These data confirm the importance of performing genetic tests [11,38,39] in children who manifest NS before the first year of life or in the presence of SRNS [38]. The identification of genetic causes and/or genetic factors related to the evolution of NS can contribute to early diagnosis, avoid the unnecessary use of immunosuppressive therapies and allow a better understanding of the disease pathophysiology.

CLINICAL MANIFESTATIONS

Symptoms of INS can start in the first year of life, but usually begin between 2-7 years. The main complaint is bilateral periorbital morning edema, which tends to progress to anasarca. Pleural effusion, ascites and genital edema can also happen [3].

Edema is the result of pathophysiological mechanisms due to primary (overfilling) or secondary (underfilling) retention of water and sodium. In the case of primary sodium and water retention by the renal tubules, the patient presents with hypervolemia, cardiopulmonary congestion and arterial hypertension. On the other hand, in cases where sodium and water retention is secondary as a compensatory mechanism, the abrupt and sharp drop in oncotic pressure due to hypoalbuminemia initially produces hypovolemia with decreased blood pressure (BP). As a

compensatory response, activation of the RAS occurs, which stimulates the tubular reabsorption of sodium and water. Thus, a patient with NS can present hypovolemia with normal BP or even hypotension (more common in MCNS) or hypervolemia with hypertension (more common in FSGS) [1].

Clinical and laboratorial changes also include hypoalbuminemia, dyslipidemia, oliguria, foamy urine, apathy, cellulitis, hypercoagulability, hydroelectrolytic imbalance and appetite loss [2,3,17]. The disease evolution and pharmacological treatment itself may also increase the propensity for systemic infections, pulmonary embolism and other clinical complications [2,3,26].

Following infections and thromboembolism, acute kidney injury is considered to be the third most common complication of INS in children, whose main risk factors are SRNS, presence of infection and treatment with nephrotoxic drugs [38].

MCNS is characterized by massive and selective proteinuria, mainly albuminuria. On the other hand, in FSGS, there is a loss of selectivity in the urinary excretion of proteins, causing urinary excretion of immunoglobulins, hormone-transporting proteins, factors related to coagulation and fibrinolysis, among others [16]. It should also be mentioned that it is yet not known if MCNS and FSGS are isolated clinical entities or spectral variations of the same disease [15].

The clinical course of INS is quite variable, ranging from patients with infrequent relapses, whose disease goes into permanent remission in adolescence, to others who rapidly progress to end stage renal disease [15].

DIAGNOSIS

The first approach of a child with suspected NS should include confirmation of the syndrome, investigation of secondary causes, assessment of renal function and BP, as well as of possible acute complications [1,43], including bacterial infections, metabolic and blood volume disorders, coagulation disorders and adverse effects to treatment.

To confirm NS diagnosis, there must be 24-hour proteinuria above 50 mg/kg/day (or $\geq$ 40 mg/m^2/hour) or protein/creatinine ratio in a single urine sample greater than 2.0 associated with hypoalbuminemia (serum albumin $\leq$ 2.5 g/dL) and edema. There are also recommended exams to assess possible acute complications associated with the disease and to detect secondary causes of NS as listed in Table 2. The most common laboratory changes in INS are shown in Table 3.

Renal biopsy is indicated in case of secondary cause suspicion, in congenital NS, in SRNS, and in cases of dependency to doses of corticosteroids. There are not validated biomarkers capable

of predicting disease progression and/or early detecting cases that may evolve with an inadequate response to treatment with corticosteroids. The determination of the excreted fraction of magnesium (EFMg) in pediatric patients with INS was linked to the presence of resistance to corticosteroids [44]. The authors found significantly higher levels of magnesium excretion in 20 children with SRNS compared with 20 SSNS patients. The explanation was that an increased EFMg could indicate an initial tubulointerstitial lesion, which would be associated with resistance to corticosteroids [44]. However, subsequent studies have not confirmed this conclusion. In a retrospective cohort study that included 294 pediatric patients with INS, it was found that the later age of disease onset, the presence of hematuria in spot urine collected during the first episode of edema and resistance to corticosteroids are independent risk factors for deterioration of renal function [45].

Table 2 - Laboratory tests for first approach of pediatric patients with Nephrotic Syndrome

Purpose	*Laboratory test requested*
To confirm Nephrotic Syndrome	Urinalysis
	24-hour proteinuria and protein/creatinine ratio in a single urine sample
	Serum albumin measurement
To evaluate associated complications	Serum biochemistry (urea, creatinine, uric acid, total cholesterol, LDL, HDL, triglycerides, Na, K, Cl, ionic Ca, Mg and P, D vitamin)
	Fraction of sodium excretion in a single urine sample
	FBC and coagulogram
To search the etiology	Fasting glycemia, glycated hemoglobin
	Complementenemia (C3 and C4)
	ANF, Anti-DNA double helix
	Serologies (Hepatitis B and C, HIV, CMV, EBV, syphilis and toxoplasmosis)
	Parasitological examination of feces (schistosomiasis, strongyloidiasis)

LDL = low density lipoprotein; HDL = high density lipoprotein; Na = sodium; K = potassium; Cl = chlorine; Ca = calcium; Mg = magnesium; P = phosphor; FBC = full blood count; ANF = antinuclear factor; HIV = human immunodeficiency virus; CMV = cytomegalovirus; EBV = Epstein Barr virus.

Table 3 - Clinical and laboratory changes present in Nephrotic Syndrome in pediatrics.

Parameters	*Nephrotic Syndrome*
Clinical condition	Edema associated with hypoalbuminemia (serum albumin ≤ 2.5 g/dL) and 24-hour proteinuria (≥ 50 mg/kg body weight or > 40 $mg/m^2/h$[42]) or protein/creatinine ratio in a single urine sample ≥ 2.0
Urine analysis	Proteinuria, lipiduria, oval fatty bodies, fatty cylinders and some cases of hematuria
Serum biochemistry	Total proteins < 5.0 mg/dL Dyslipidemia (total cholesterol > 170 mg/dL, LDL > 110 mg/dL, TG ≥ 75 mg/dL in children up to 9 years old and ≥ 90 in adolescents) Fasting blood glucose < 100 mg/dL and/or HbA1C < 7%
Renal function	Normal (MCNS) and GFR< 70 mL/min/1.73 m^2 (FSGS) Reduction associated with hypovolemia in the edematous phase
Blood count and serum complement	Normal Increased hematocrit in hypovolemic patients

LDL = low density lipoprotein; TG = triglycerides; HbA1C = glycated hemoglobin; MCNS = minimal change nephrotic syndrome, GFR = glomerular filtration rate; FSGS = focal segmental glomerulosclerosis.

TREATMENT

NS is a chronic disease that presents periods of remissions and relapses. In the case of MCNS, patients often respond to corticosteroid therapy and have a good prognosis. On the other hand, NS due to more complex glomerular lesions, such as FSGS and MGN, resistance to corticotherapy often occurs, making treatment challenging [3,5,46].

Regardless of the evolution and response to treatment, it is very important to clarify to the patient and their family members the peculiarities of the syndrome, such as the possibilities of recurrence, the warning signs of complications and the necessary general care [1,3].

The vaccination with live vaccines is contraindicated while on immunosuppressive or cytotoxic agents and should be postponed for up to 3 months after stopping treatment with immunosuppressive agents and/or until prednisone dose is <20 mg/dl, except for the influenza vaccine. The children are at increased risk of invasive pneumococcal infection and should receive pneumococcal vaccination with the heptavalent conjugate vaccine and the 23-valent polysaccharide vaccine. As well, exposure to varicella can cause serious damage, being the Zostavax® live attenuated vaccine contraindicated in immunosuppressed and immunodeficient patients and although immunosuppression reduces its effectiveness, the recombinant Shingrix vaccine is safer [1,3,26,46].

Nutritional monitoring aims to decrease proteinuria, to prevent protein malnutrition and to delay the onset of CKD. A balanced ingestion of micro and macronutrients is recommended. Diet rich in proteins and lipids is contraindicated. Fiber ingestion is also indicated, and statin use may be necessary in cases of chronic dyslipidemia. Salt ingestion should be reduced to approximately 2 g per day in the presence of severe edema [1,3,26].

Children with NS have an increased risk to develop hypovitaminosis D. Supplementation of vitamin D with 600 to 1000 IU per day for children aged 1 year may be necessary to maintain 25 (OH)-vitamin D above the ideal level of 20-25 ng/ml [1,3]. In this sense, the determination of the serum concentration of 25 (OH)-vitamin D can be useful to determine the need or not for the supplementation of vitamin D. In addition, it is important to ensure adequate calcium ingestion in the diet (700 to 1,300 mg of calcium element/day), in order to prevent bone complications, some of them due to chronic treatment with corticosteroids. Thus, the ingestion of milk and dairy products should be encouraged. Dairy products are considered the main foods that contribute to adequate daily calcium ingestion [1,3].

During the edema phase, the skin deserves special attention due to the major possibility of infections and tissue rupture in regions very stretched by the edema. Some patients complain of itching in some parts of the body, needing to moisturize the skin. Subcutaneous medications are banned. Venous punctures should be restricted to situations of recognized need. Treatment with loop diuretics may be necessary. However, patients should be monitored closely because of the risk of hypovolemia [1,3,26].

The child's normal activities should always be stimulated, and their restriction is the responsibility of the patient. No benefit has been observed with rest, even in the edematous phase of the disease [1,3]. On the contrary, inactivity can increase the risk of thromboembolic events. However, in patients with symptomatic arterial hypertension, rest should be indicated until blood pressure levels improve. There is no impediment for children to attend school,

except in those cases in which there is a risk of exposure to infectious diseases, especially chickenpox [1,3].

The treatment of the disease is usually in the outpatient facilities. Hospitalizations are rarely necessary and due to the risk of infections, should be avoided and restricted to cases of severe infections and accentuated edema. These complications may be difficult to manage on an outpatient basis, increasing the risk of hemodynamic instability, worsening renal function and significant hydroelectrolytic disorders.

The introduction of corticosteroids has considerably reduced the mortality and morbidity of children with INS [1,3]. The mortality rate of children with NS has dropped to about 3% [47]. Corticosteroid therapy has as main objectives to induce total or partial remission of proteinuria and preserve renal function (Figure 3). About 80% of children show complete remission of symptoms after the introduction of treatment, while the remaining 20% may progress to SRNS. These cases may respond well to other immunosuppressors or may progress to CKD, requiring dialysis or transplantation [38]. In addition, approximately 80% of children who respond to corticosteroid therapy have recurrences. Half of these children have relapses frequently, which increases the risk of adverse effects from the long-term or repeated use of corticosteroids [48]. Those patients who initially present a good response to corticosteroids, but with frequent relapses or dependence, may progress to drug resistance [49]. In these cases, renal biopsy is indicated to guide the therapeutic strategy [1,46].

There is no indication for the use of corticosteroids for more than 2-3 months at the initial episode of INS, considering that there is a low risk of relapse if the duration of treatment is shorter than this period [50]. On the other hand, patients with corticosteroid addiction should consider the use of a minimum amount of prednisone, which maintains remission and minimizes side effects for a prolonged period (6 months to 1 year). Corticosteroid therapy is associated with a wide range of adverse effects, which predominate in children who use high doses or for a long period of time [51]. The administration of other immunosuppressive medications is recommended If the use of a high dose of prednisone is necessary or if the side effects of the medication are significant, as detailed below. Calcineurin inhibitors have been shown to be effective in patients with SSNS, as they significantly increase the chance of partial or total remission [52]. In contrast, patients who have genetic forms of corticosteroid resistance have worse results with this treatment than those who do not have these mutations [53].

As a measure of renoprotection, RAS blockers have been widely recommended [1,6,53,54]. Treatment with angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin type 1 receptor blockers (ARBs) slow down the progression of glomerular injury, reduce inflammatory and fibrogenic processes in renal tissue and control proteinuria [54]. The effectiveness of ACEIs and ARBs has been reported in different studies that show improvement in renal survival [55-58]. In children with INS, the use of ACEIs and/or ARBs can promote a 50-80% decline in proteinuria, determining partial and/or total remission of the disease [58].

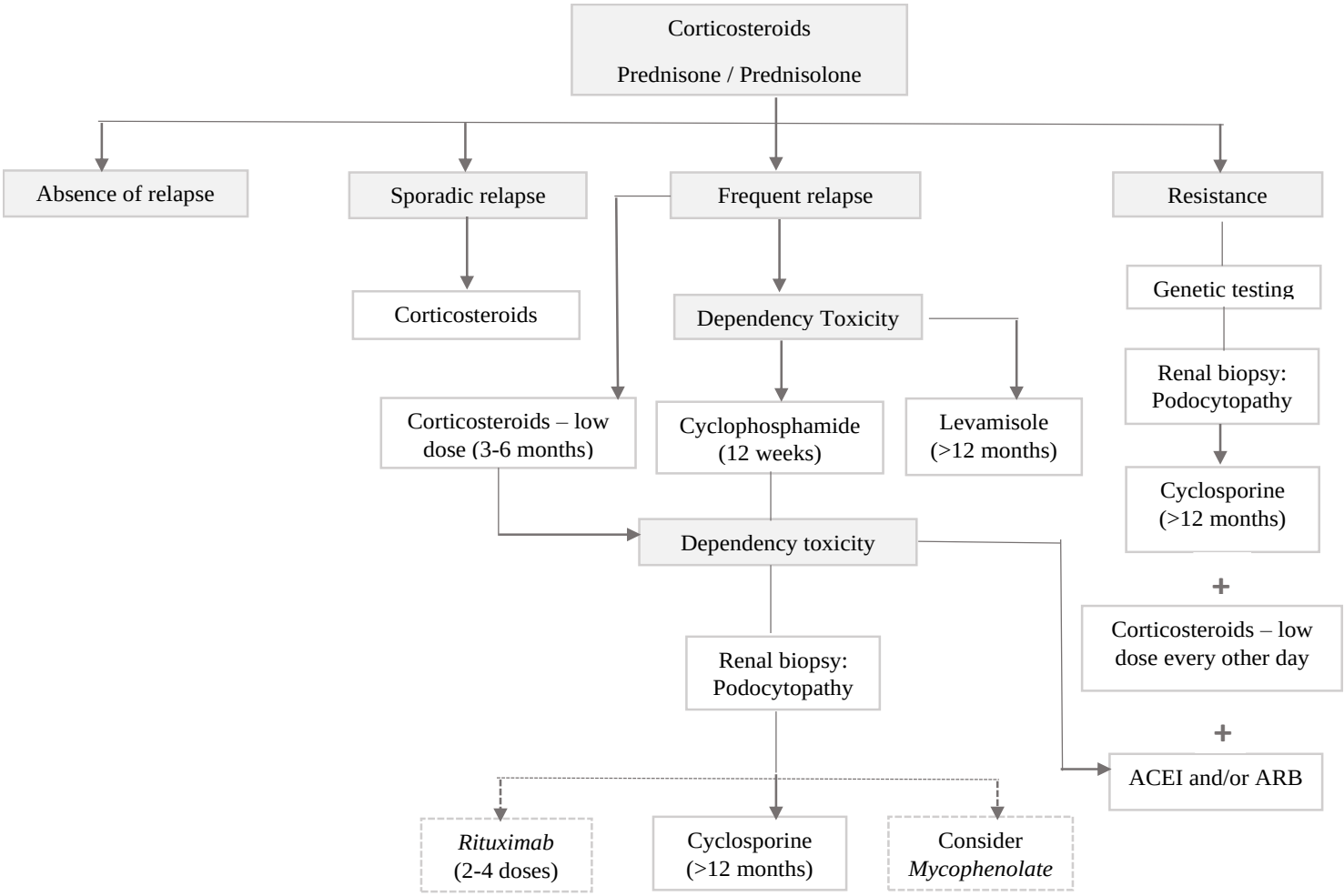


Figure 3 - Therapeutic approach to Pediatric Idiopathic Nephrotic Syndrome

There are several schemes of corticosteroid administration for INS. The immunosuppression scheme of the international group KDIGO (Kidney Disease Improving Global Outcomes, 2020) [46] recommends:

- *First episode:* the first episode should be treated with prednisone/prednisolone at a dose of 2 mg/kg/day or 60 mg/m²/day (maximum 60 mg/day), in a single dose in the morning, for 4-6 weeks. Some studies have shown that there is no significant difference in the number of relapses, over a 6-month period, using dose according to weight or to surface area [46]. The single daily dose was shown to be as effective as multiple administrations in maintaining remission. The occurrence of serious side effects was reduced in single-dose treatment compared to patients who received divided doses [51]. Some patients will have a slow response to corticosteroids and, in this case, the treatment time can be extended to 8 weeks in an attempt to achieve remission. After this period, in that patient who showed complete remission, the dose is reduced to 1.5 mg/kg or 40 mg/m² (maximum 40 mg), every other day, for another four to six weeks, making at least 12 weeks of treatment. Some protocols no longer use the slow and progressive withdrawal of corticosteroids, suspending treatment immediately after the alternate day schedule, without taking into account the risk of acute adrenal insufficiency. However, in our service, we use progressive corticosteroid reduction as detailed below.
- *Relapses:* Relapses should be treated with prednisone/prednisolone at a dose of 2 mg/kg/day or 60 mg/m²/day (maximum 60 mg/day) in a morning single dose, until the child completes 3 days of complete remission, which usually occurs in 4 weeks. Then, the dose is reduced to 1.5 mg/kg or 40 mg/m² (maximum 40 mg) on alternate days for another 4 weeks. In children with relapsing SSNS, some studies have not identified differences in the time of remission between patients using prednisone doses of 1 mg/kg compared with patients using the conventional dose of 2 mg/kg. However, it is still necessary to carry out a large study to determine whether the lower doses of prednisone are sufficient to treat relapses [46].
- *Gradual reduction of prednisone/prednisolone*: the gradual reduction of prednisone/prednisolone is performed after the treatment of the first episode or recurrences, due to the risk of acute adrenal insufficiency. Individual factors may contribute to the development of acute adrenal insufficiency. The risk is greater when the dose is close to physiological levels of cortisol. After the initial immunosuppressive treatment or in relapses, the gradual withdrawal of prednisone/prednisolone should be made, considering the particularities of each patient. A 25% reduction of the initial dose every 7-15 days, until reaching the dose 2.5 mg/m2 every other day is recommended. The dose of 2.5 mg/m2 every other day should be maintained for 1-2 weeks and then suspended. If the patient presents an infectious condition during corticosteroid

withdrawal, the medication should be used daily, at the same dose that was being administered, on alternate days, at the time of the patient infection. A recent study showed that therapy with low doses of prednisone, administered daily during an infectious process, reduces the risk of relapse compared to using the drug every other day [46].

At the beginning of treatment with corticosteroids, sodium and water retention occurs with worsening of edema. Then, the anti-inflammatory effects of the medication surpass the mineralocorticoid action, stimulating diuresis and the beginning of remission in steroid-sensitive patients [51, 59].

In SDNS, a dose of prednisone should be administered sufficient to maintain remission, but as low as possible, to minimize side effects [46]. It is used for a minimum period of 6 months, on alternate days. Most children need a dose of 0.5 to 1.0 mg/kg every other day. The use of other immunosuppressors is indicated for children who need high doses of prednisone and/or in those with more intense adverse reactions to corticosteroids [46, 60].

SRNS is considered when complete remission of proteinuria does not occur after 8 weeks of corticosteroids [26]. Some patients show clinical improvement, with the disappearance of edema, however, remaining with significant proteinuria. In addition, some children who are initially steroid-sensitive or steroid-dependent may later develop resistance to steroid treatment [46]. Only about 5% of MCNS cases develop resistance to corticosteroids [61]. The majority of children with SRNS have another type of histopathological lesion, predominantly FSGS [26]. For this reason, all SRNS patients should be submitted to renal biopsy for assessment of the prognosis and to decide the therapeutic approach [53].

Other immunosuppressors

The subsequent treatment of INS varies according to the initial response to corticosteroids (Figure 3). It is essential to replace corticosteroids when there is dependence on high doses of corticosteroid due to risk of complications or toxicity, including Cushing's syndrome, obesity, metabolic changes, bone reabsorption, cardiovascular alterations, increase of intraocular pressure, psychological or aesthetic changes [38,60]. In addition, the treatment with other immunosuppressors fundamentally aims complete remission and should be preferentially prescribed by pediatric nephrologists.

- *Cyclophosphamide (CPA)* is an alkylating agent whose immunosuppressive action involves direct cytotoxicity in inflammatory cells [62]. CPA has been shown to be

effective in providing prolonged remissions in patients with SDNS and/or SSNS with frequent relapses (Figure 3). The duration of remission is significantly shorter in more severe patients [62]. However, the use of CPA reduced the number of children who relapsed over a period of up to 24 months, compared to prednisone therapy [48]. Preferably after complete remission, CPA is used at a dose of 2 to 3 mg/kg/day, for 8 to 12 weeks with abrupt suspension. The maximum recommended cumulative dose is 168 mg/kg [53]. It is not advisable to administer a second cycle of the drug. The results are best when administered during a discontinued corticosteroid regimen, after remission of proteinuria [46]. After treatment, some children may become steroid-sensitive. The drug should be given in the morning, stimulating water intake, to decrease the risk of hemorrhagic cystitis. Other adverse drug events are leukopenia, alopecia, bone marrow suppression, gastrointestinal toxicity, disorders of the central nervous system, increased susceptibility to infections (which can happen up to three months after discontinuation of the medication) and gonadal toxicity, which is more common in males and in adolescence, but rarely seen at a cumulative dosage below 150 mg/kg [53]. Azoospermia and infertility are dose dependent, but rarely occur at the doses usually prescribed. However, for safety, its use is recommended between the age of 6 to 12 years [62]. The leukocyte count should be performed every 7 to 15 days for leukopenia monitoring. Dose reduction is in case of leukocytes below 4.500/mm^3 and the drug suspension if leukocytes below 3.000/mm^3 [38].

- *Levamisole* is an anthelmintic with immunomodulatory properties, but its mechanism of action has not yet been fully understood. Levamisole increases the Th1 response and reduces the Th2 response, in addition to activating $CD4^+$ T cells more than $CD8^+$ T cells. Although its action predominates on T cells, studies have shown that it can also increase B cells and the humoral immune response. Finally, Levamisole can also act on podocytes, by increasing the expression and activity of the glucocorticoid receptor [61]. Levamisole has been shown to be effective in promoting remission in patients with SDNS and/or SSNS with frequent relapses (Figure 3). In addition, it is a good option in SDNS children and in the presence of important side effects related to prednisone. The risk of NS recurrence has reduced by half with the use of levamisole for four to 12 months [62,63]. The recommended dose is 2.0-2.5 mg/kg every other day, for 12-24 months, initially in combination with prednisone/prednisolone. It is a safe medication, generally well tolerated, with few side effects that include leukopenia, reversible with

the suspension of the drug, skin rash, and gastrointestinal disorders [46,62,63]. After the suspension of levamisole, recurrences of NS are common.

- *Cyclosporine* is a calcineurin inhibitor that blocks the production and release of IL-2 by T lymphocytes, thus preventing the induction and proliferation of effector T lymphocytes [62]. It is indicated for SRNS and for SDNS at high doses of prednisone or with corticosteroid toxicity. Its use should also be reserved for patients with a glomerular filtration rate greater than or equal to 60 ml/min/1.73m² and without pre-existent tubulointerstitial fibrosis in histopathological examination [46]. The dose is 3-6 mg/kg/day divided into two doses (12/12 hours), which can be increased up to 10 mg/kg/day. Although its efficacy is dose-dependent, the dose increase must be slow and guided by the serum level, which must be closely monitored and maintained between 80-125 ng/ml (collected immediately before administration of the medication administration, level C0). Some recent studies indicate that the use of cyclosporine in combination with ACEI/ARB seems to be more effective in keeping the patient in remission [46]. In addition, studies have shown that treatment with cyclosporine increased the number of SRNS patients who achieved complete remission of the disease [63]. Side effects include arterial hypertension, hypertrichosis, gingival hypertrophy, tremors, gastrointestinal symptoms, hepatotoxicity and nephrotoxicity. For this reason, when using cyclosporine, kidney function and drug level should be monitored carefully [65]. It is still uncertain whether its use reduces the number of patients who progress to end-stage renal disease [64]. Renal biopsy should be performed in all patients under cyclosporine treatment, periodically every 3 to 5 years. Significant tubulointerstitial changes may occur during treatment. *Tacrolimus* is another calcineurin inhibitor recommend as initial second-line therapy for children with steroid-resistant [46]. Due to side effects of cyclosporine began to be used in preventing acute rejection and long-term survival, besides being as effective as cyclosporine [65]. Both have the risk of nephrotoxicity and the different side effects can be used to guide which of these will be used.
- *Mycophenolate Mofetil* (MMF) is an immunosuppressive medication that blocks the proliferative response of T and B lymphocytes by inhibiting inosine monophosphate dehydrogenase, an important enzyme for the synthesis of purines. It can be used in patients with complicated NS, such as, for example, patients who have already used cyclosporine with unfavorable response. Although MMF can be extremely useful in NS secondary to systemic erythematosus lupus, scientific evidence for its use in SDNS and

SRNS is low [46]. The use of MMF, in these situations, is an attempt to reduce the side effects associated with other immunosuppressive medications. MMF is considered as a third-line agent [66]. MMF was generally well tolerated, with gastrointestinal symptoms being the main side effects, reported in 3–11% of patients [62].

- *Rituximab* (RTX) is a monoclonal antibody that targets CD20, which is a protein found in mature B cells and their precursors. It acts by binding to CD20, inducing destruction of B lymphocytes by direct cytotoxicity, apoptosis or phagocytosis, and consequent decrease in the production of antibodies. In addition, RTX can increase the number and function of regulatory T cells (Treg), which has been associated with maintaining remission in patients with SN [67]. It can be used in patients with SSNS with frequent relapses or SDNS. An insignificant response was seen in SRNS patients [46]. Relapses after RTX therapy have been associated with the reappearance of memory B lymphocytes [62]. RTX use is associated with a reduction in the number of children with recurrence in a period of 6 to 12 months. However, the effect of treatment is temporary, and many children will need additional doses [48]. The centers with more experience in the use of this medication have recommended doses of 375-750 mg/m² (maximum 1.000 mg), completing 2 to 4 infusions, with an interval of 1-2 weeks between doses, adopted from two previously used schedules [68]. The doubts related to the safety of the medication are due to the lack of knowledge about the long-term side effects. It is recommended that the administration of RTX should be performed only in centers specialized in pediatric nephrology, with administration in a hospital environment, under monitoring. The effects related to the drug are fever, high blood pressure, vomiting, diarrhea, skin rash, hypersensitivity reaction, bronchospasm and increased risk of infections [69]. Although the studies do not mention important adverse effects other than those related to the infusion of the drug, there are reports of serious reactions related to other indications for RTX which include: pneumonia, pulmonary fibrosis, gastrointestinal ulcerative disease, anaphylactic reaction, fulminant myocarditis, reactivation of hepatitis B and multifocal leukoencephalopathy [67]. The risk for prolonged hypogammaglobulinemia is controversial. It is recommended to follow up the patients with serial measurements.

In summary, current INS therapy is based on the use of corticosteroids, inducing complete remission in most pediatric patients [70]. However, the introduction of other immunosuppressive agents may be necessary in cases of resistance to corticosteroids in order

to achieve remission, as well as to reduce the side effects of corticosteroids. The morbidity of the disease is mainly related to the side effects of the medications [62]. Recently, clinical studies have shown satisfactory results with medications that directly target the immune system [71] and the signaling pathways of the podocytes [72,73]. Anti-CD20 antibodies, such as rituximab, are able to induce and/or maintain a long-term remission in INS patients, allowing the reduction of corticosteroids or other immunosuppressors in use [10,74].

There is no evidence-based consensus on the most effective therapeutic approach, especially for SSNS. In general, the response to immunosuppressive agents is limited and variable. CPA, levamisole, cyclosporine and MMF can be used in children with relapsing NS. Further studies, with larger samples and well designed are needed to compare the different treatments proposed, in order to determine how and when these medications should be used, the possible combinations and the benefits and harms of the treatment [48,64]. Currently, the drug protocol varies according to clinical situations and health system conditions [61].

COMPLICATIONS

Thromboembolism is a serious complication of INS, affecting up to 9% of pediatric patients. High risk of thromboembolic events has been associated with the presence of steroid-resistance [75]. Hypercoagulability results from changes in blood levels of various factors involved in the coagulation and in the fibrinolytic system, changes in platelet functions, increased blood viscosity due to hemoconcentration, high blood pressure and, probably, administration of steroids and/or diuretics [76]. The urinary loss of antithrombin III, plasminogen, protein C and protein S, physiological anticoagulants, has been associated with low serum levels of albumin [77]. However, the pathophysiological mechanisms of this complication in NS have not yet been fully elucidated.

The propensity to infection is another common complication in INS. Peritonitis, meningitis and cellulite are frequent conditions and the most common microorganisms are *Streptococcus pneumoniae*, *Haemophilus influenzae* and *E. coli*. Bacteriuria is common [26]. This predisposition is mainly attributed to the urinary loss of immunoglobulins, the loss of zinc and factors of the alternative pathway of the complement system (especially factor B) and the use of immunosuppressive therapy [1,26]. Specifically, virus infection (chickenpox, influenza) deserves attention given the potential severity in immunosuppressed individuals [1,3].

NS patients have lipid abnormalities including hyperlipidemia due to elevated total cholesterol, LDL and VLDL fractions, triglycerides, apolipoprotein B and A1 [1]. The studies did not show an increased risk for acute myocardial infarction (AMI) in pediatric patients with INS [6].

Despite this, the use of ARBs and/or ACEIs, in addition to statins in INS pediatric patients with risk factors can be recommended, in order to prevent premature atherosclerosis [1].

Lipid alterations can lead to dyslipidemia, a complication little described in SN patients. The loss of proteins in urine, especially those involved in essential transport such as albumin, lead to an increase in protein synthesis by the liver and lipid production [1]. Such cellular lipid overload contributes to renal and endothelial dysfunction due to the generation of ROS and expression of adhesion molecules, in addition to promoting changes in adipocyte metabolism, production of inflammatory cytokines and expression of metabolic markers, key components for decreased renal function [78].

Decompensated NS can lead to reduced diuresis due to hypovolemia. Some risk factors such as sepsis, diarrhea and the indiscriminate use of diuretics can trigger a reduction in blood volume. Patients with SRNS can present anemia, which results from urinary losses of iron, erythropoietin, transcobalamin, transferrin and copper [26].

Finally, hypothyroidism and vitamin D deficiency can be seen. Therefore, in order to avoid disturbances in the growth and formation of bone structure, thyroid function must be monitored and, eventually, nutritional supplementation with vitamin D must be prescribed [1,3,6].

CONCLUSION

In pediatric patients with INS, the therapeutic response to corticosteroids still remains the main predictive factor for the prognosis of the disease. A large interindividual variation is observed in INS patients, especially in regard to clinical course, response to treatment and side effects of the medications [51]. Children older than 4 years of age, who present remission at the beginning of treatment with corticosteroids, in the absence of microscopic hematuria, have fewer episodes of relapse. In contrast, SRNS patients that are refractory to other therapeutic strategies evolve to ESRD with several associated complications [6].

in relation to genetic tests, it is likely that they will become more and more necessary, as the number of works related to the screening of mutations associated with SN has increased exponentially. The increase in the availability of new techniques, such as Next-Generation Sequencing (NGS), can take on special importance in cases of difficult treatment. Genetic studies may help to identify patients who will benefit from immunosuppressive treatments from those who will only suffer from the adverse effects of these therapies [79,80]. Pharmacogenomics studies show that genetic factors can be responsible for 20 to 95% of the variability in the therapeutic response among INS patients in regard to efficacy and to adverse effects. Pharmacogenomics influences the pharmacokinetics and pharmacodynamics of

medications in patients. This understanding may allow individualized therapy in order to increase the effectiveness and to reduce the toxicity [51].

CONSENT FOR PUBLICATION

Not applicable.

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CONFLICT OF INTEREST

The authors declare the absence of conflict of interest.

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REFERENCES

1 Noone DG, Lijima K, Parekh R. Idiopathic nephrotic syndrome in children. The Lancet. 2018; 392(10141): 61-74.

2 Souto MFO, Teixeira MM, Penido MG, Simões e Silva AC. Physiopathology of nephrotic syndrome in children and adolescents. Arch Latin Nefr Ped. 2008; 8(1): 1-10.

3 Wang C, Greenbaum LA. Nephrotic Syndrome. Pediatr Clin North Am. 2019; 66(1), 73–85.

4 Chesney R. The changing face of childhood nephrotic syndrome. Kidney Int. 2004; 66(3): 1294-1302.

5 D'Agati VD, Kaskel FJ, Falk RJ. Focal segmental glomerulosclerosis. N Engl J Med. 2011; 365(25): 2398-2411.

6 Eddy AE, Symons JM. Nephrotic syndrome in childhood. The Lancet. 2003; 362(9384): 629-639.

7 Dossier C, Sellier-Leclerc AL, Rousseau A, Michel Y , Gautheret-Dejean A, Englender M, et al. Prevalence of herpesviruses at onset of idiopathic nephrotic syndrome. Pediatr Nephrol. 2014; 29(12): 2325-2331.

8 Soares SF, Donatti TL, Souto FJ. Serological markers of viral, syphilitic and toxoplasmic infection in children and teenagers with nephrotic syndrome: case series from Mato Grosso State, Brazil. Rev Inst Med Trop São Paulo. 2014; 56 (6): 499-504.

9 Ferrara P, Pierri F, Zenzeri L, Vena F, Ianniello F, Chiaretti A. Post-infectious glomerulonephritis with nephrotic syndrome secondary to rotavirus infection. Med Mal Infect. 2013; 43 (9): 398-400.

10 Colucci M, Corpetti G, Emma F, Vivarelli M. Immunology of idiopathic nephrotic syndrome. Pediatr Nephrol. 2018; 33 (4):573-584.

11 Hinkes BG, Mucha B, Vlangos CN, Gbadegesin R, Liu J, Hasselbacher K, et al. Nephrotic syndrome in the first year of life: two thirds of cases are caused by mutations in 4 genes (NPHS1, NPHS2, WT1 and LAMB2). Pediatrics. 2007; 119(4): 907-919.

12 Preston R, Stuart HM, Lennon R. Genetic testing in steroid-resistant nephrotic syndrome: why, who, when and how? Pediatr Nephrol. 2019; 34(2), 195-210.

13 Lama G, Luongo I, Tirino G, Borriello A, Carangio C, Salsano M. T-lymphocyte populations and cytokines in childhood nephrotic syndrome. Am J Kidney Dis. 2002; 39(5): 958-965.

14 Gbadegesin RA, Adeyemo A, Webb NJ, Greenbaum LA, Abeyagunawardena A, Thalgahagoda S, et al. HLA-DQA1 and PLCG2 are candidate risk loci for childhood-onset steroid-sensitive nephrotic syndrome. J Am Soc Nephrol. 2015; 26(7): 1701-1710.

15 Floege J, Amann K. Primary glomerulonephritides. Lancet. 2016; 387(10032): 2036-2048.

16 International Study of Kidney Disease in Children. Nephrotic syndrome in children: prediction of histopathology from clinical and laboratory characteristics at time of diagnosis. Kidney Int.1978; 13:159-165.

17 Kim SH, Park SJ, Han KH, Kronbichler A, Saleem MA, Oh J, et al. Pathogenesis of minimal change nephrotic syndrome: an immunological concept. Korean J Pediatr. 2016; 59(5): 205-211.

18 Guimarães FTL, Melo GEBA, Cordeiro TM, Feracin V, Vieira ER, Pereira WF, et al. T-lymphocyte-expressing inflammatory cytokines underlie persistence of proteinuria in children with idiopathic nephrotic syndrome. J Pediatr. 2018; 94(5): 546-553.

19 Zhao L, Cheng J, Zhou J, Wu C, Chen J. Enhanced steroid therapy in adult MCNS: a systematic review and meta-analysis. Intern Med. 2015; 54(17): 2101-2108.

20 Sprangers B, Meijers B, Appel G. FSGS: diagnosis and diagnostic work-up. Biomed Res Int. 2016; 2016:4632768.

21 Avner ED, Harmon WE, Niaudet P, Yoshikawa N, Emma F, Goldstein SL. Pediatr Nephrol. 7th ed. New York: Springer 2016; pp.1055-1077.

22 Elie V, Fakhoury M, Deschênes G, Jacqz-Aigrain E. Physiopathology of idiopathic nephrotic syndrome: lessons from glucocorticoids and epigenetic perspectives. Pediatr Nephrol. 2012; 27(8): 1249-1256.

23 Pereira WF, Brito-Melo GEA, Guimarães FTL, Carvalho TGR, Mateo EC, Silva ACS. The role of the immune system in idiopathic nephrotic syndrome: a review of clinical and experimental studies. Inflamm Res. 2014; 63(1): 1-12.

24 Schönenberger E, Ehrich JH, Haller H, Schiffer M. The podocyte as a direct target of immunosuppressive agents. Nephrol Dial Transplant. 2011; 26(1): 18-24.

25 Shimada M, Araya C, Rivard C, Ishimoto T, Johnson RJ, Garin EH. Minimal change disease: a "two-hit" podocyte immune disorder? Pediatr Nephrol. 2011; 26 (4): 645-649.

26 Kopp JB, Anders HJ, Susztak K, Podestà MA, Remuzzi G, Hildebrandt F, Romagnani P. Podocytopathies. Nature Reviews Disease Primers. 2020; 6(1), 1-24.

27 Goldwich A, Burkard H, Olke H, Daniel C, Amann K, Higo C, et al. Podocytes are nonhematopoietic professional antigen-presenting cells. J Am Soc Nephrol. 2013; 24(6): 906–916.

28 Novelli R, Benigni A, Remuzzi G. The role of B7-1 in proteinuria of glomerular origin. Nat Rev Nephrol. 2018; 14(9): 589-596.

29 Karp AM, Gbadegesin RA. Genetics of childhood steroid-sensitive nephrotic syndrome. Pediatr Nephrol. 2017; 32(9): 1481-1488.

30 Park E, Chang HJ, Shin JI, Lim BJ, Jeong HJ, Lee KB, et al. Familial IPEX syndrome: different glomerulopathy in two siblings. Pediatr Int. 2015; 57(2): 59-61.

31 Guimarães FTL, Ferreira RN, Brito-Melo GEA, Rocha-Vieira E, Pereira WF, Pinheiro SVB, et al. Pediatric patients with steroid-sensitive nephrotic syndrome have higher expression of t regulatory lymphocytes in comparison to steroid-resistant disease. Front Pediatr. 2019; 7:114.

32 Bertelli R, Bonanni A, Di Donato A, Cioni M, Ravani P, Ghiggeri GM. Regulatory T cells and minimal change nephropathy: in the midst of a complex network. Clin Exp Immunol. 2016; 183(2): 166-174.

33 Avner ED, Harmon WE, Niaudet P, Yoshikawa N, Emma F, Goldstein SL. Pediatr Nephrol 7th ed. New York: Springer. 2016; pp.705-750.

34 Shalaby SA, Al-Edressi HM, El-Tarhouny SA, Fath El-Bab M, Zolaly MA. Type 1/type 2 cytokine serum levels and role of interleukin-18 in children with steroid-sensitive nephrotic syndrome. Arab J Nephrol Transplant. 2013; 6(2): 83-88.

35 Davin JC. The glomerular permeability factors in idiopathic nephrotic syndrome. Pediatr Nephrol. 2016; 31(2): 207-215.

36 Simões e Silva AC, Lanza K, Palmeira, VA, Costa, LB, Flynn, JT. 2020 update on the renin–angiotensin–aldosterone system in pediatric kidney disease and its interactions with coronavirus. Pediatr Nephrol. 2021; 36(6) 1407-1426.

37 Filha RDS, Pinheiro SVB, Macedo e Cordeiro T, Feracin V, Vieira, ÉLM, Miranda, AS, Simões e Silva, AC. Evidence for a role of angiotensin converting enzyme 2 in proteinuria of idiopathic nephrotic syndrome. Bioscience Reports. 2019; 39(1).

38 Rheault MN, Gbadegesin RA. The genetics of nephrotic syndrome. J Pediatr Genet. 2016; 5(1): 15-24.

39 Rood IM, Deegens JK, Wetzels JF. Genetic causes of focal segmental glomerulosclerosis: implications for clinical practice. Nephrol Dial Transplant. 2012; 27 (3): 882-890.

40 Laurin LP, Lu M, Mottl AK, Blyth ER, Poulton CJ, Weck KE. Podocyte-associated gene mutation screening in a heterogeneous cohort of patients with sporadic focal segmental glomerulosclerosis. Nephrol Dial Transplant. 2014; 29(11): 2062-2069.

41 Kitzler TM, Kachurina N, Bitzan MM, Torban E, Goodyer PR. Use of genomic and functional analysis to characterize patients with steroid-resistant nephrotic syndrome. Pediatr Nephrol. 2018; 33(10): 1741-1750.

42 Świerczewska M, Ostalska-Nowicka D, Kempisty B, Nowicki M, Zabel M. Molecular basis of mechanisms of steroid resistance in children with nephrotic syndrome. Acta Biochim Pol. 2013; 60(3): 339-344.

43 Rheault MN, Zhang L, Selewski DT, Kallash M, Tran CL, Seamon M, et al. AKI in children hospitalized with neprhotic syndrome. Clin J Am Soc Nephrol. 2015; 10(12): 2110-2118.

44 Rumana J, Hanif M, Muinuddin G, Maruf-ul-Quader M. Correlation of fractional excretion of magnesium with steroid responsiveness in children with nephrotic syndrome. Saudi J Kidney Dis Transpl. 2014; 25(4): 830-836.

45 Mendonça AC, Oliveira EA, Fróes BP, Faria LD, Pinto JS, Nogueira MM, et al. A predictive model of progressive chronic kidney disease in idiopathic nephrotic syndrome. Pediatr Nephrol. 2015; 30(11): 2011-2020.

46 Kidney Disease: Improving Global Outcomes (KDIGO). In KDIGO Clinical Practice Guideline for Glomerulonephritis. Kidney Int Suppl. 2020.

47 Hahn D, Samuel SM, Willis NS, Craig JC, Hodson EM. Corticosteroid therapy for nephrotic syndrome in children. The Cochrane Database of Syst Rev. 2020; 8: 1-30.

48 Larkins NG, Liu ID, Willis NS, Craig JC, Hodson EM. Non-corticosteroid immunosuppressive medications for steroid sensitive nephrotic syndrome in children. The Cochrane Database of Syst Rev. 2020; 4: 1-42.

49 McCarthy HJ, Bierznska A, Wherlock M, Ognjanovic M, Kerecuk L, Hegde S, et al. Simultaneous sequencing of 24 genes associated with steroid-resistant nephrotic syndrome. Clin J Am Soc Nephrol. 2013; 8(4): 637-648.

50 Hahn D, Hodson EM, Willis NS, Craig JC. Corticosteroid therapy for nephrotic syndrome in children. The Cochrane Database of Syst Rev. 2015; 3: 1-30.

51 Schijvens AM, Ter Heine R, De Wildt SN, Schreuder MF. Pharmacology and pharmacogenetics of prednisone and prednisolone in patients with nephrotic syndrome. Pediatr Nephrol. 2018; 34(3): 389-403.

52 Hodson EM, Willis NS, Craig JC. Interventions for idiopathic steroid-resistant nephrotic syndrome in children. The Cochrane Database of Syst Rev. 2016; 10: 1-35.

53 van Husen M, Kemper MJ. New therapies in steroid-sensitive and steroid-resistant idiopathic nephrotic syndrome. Pediatr Nephrol. 2011; 26(6): 881-892.

54 Yamazaki M, Fukusumi Y, Kayaba M, Kitazawa Y, Takamura S, Narita I, et al. Possible role for glomerular-derived angiotensinogen in nephrotic syndrome. J Renin-Angiotensin-Aldosterone Syst. 2016; 17(4): 1-8.

55 Yi Z, Li Z, Wu X-C, He Q-N, Dang X-Q, He X-J. Effect of fosinopril in children with steroid-resistant idiopathic nephrotic syndrome. Pediatr Nephrol. 2006; 21: 967-972.

56 Gipson DS, Chin H, Presler TP, Jennette C, Ferris ME, Massengill S, et al. Differential risk of remission and ESRD in childhood FSGS. Pediatr Nephrol. 2006; 21(3): 344-349.

57 Hilgers KF, Dötsch J, Rascher W, Manm JFE. Treatment strategies in patients with chronic renal disease: ACE inhibitors, angiotensin receptor antagonists, or both? Pediatr Nephrol. 2004; 19(9): 956-961.

58 Bagga A, Mudigoudar BD, Hari P, Vasudev V. Enalapril dosage in steroid-resistant nephrotic syndrome. Pediatr Nephrol. 2004; 19(1): 45-50.

59 Khatibi SMH, Ardalan M, Abediazar S, Vahed SZ. The impact of steroids on the injured podocytes in nephrotic syndrome. J Steroid Biochem Mol Biol. 2020; 196 (suppl.1): 105490.

60 Fakhouri F, Bocquet N, Taupin P, Presne C, Gagnadoux MF, Landais P, Niaudet, P. Steroid-sensitive nephrotic syndrome: from childhood to adulthood. Am J Kid Dis. 2003; 41(3): 550-557.

61 Hui Z, Zheng W, Li Qun D, Yan Nan G. Children with steroid-resistant nephrotic syndrome: long-term outcomes of sequential steroid therapy. Biomed Environ Sci. 2016; 29(9): 650-655.

62 Zotta F, Vivarelli M, Emma F. Update on the treatment of steroid-sensitive nephrotic syndrome. Pediatric Nephrology 2021. https://doi.org/10.1007/s00467-021-04983-3

63 Mühlig AK, Lee JY, Kemper J, Kronbichler A, Yang JW, Lee JM., et al. Levamisole in children with idiopathic nephrotic syndrome: clinical efficacy and pathophysiological aspects. Journal of Clinical Medicine. 2019; 8(6): 860.

64 Liu ID, Willis NS, Craig JC, Hodson EM. Interventions for idiopathic steroid-resistant nephrotic syndrome in children. The Cochrane Database of Syst Rev. 2019; 11: 1-32.

65 Arslansoyu Camlar S, Soylu A, Kavukçu S. Cyclosporine in pediatric nephrology. Iran J Kidney Dis. 2018;12(6): 319-330.

66 Barletta GM, Smoyer WE, Bunchman TE. Use of mycophenolate mofetil in steroid-dependent and -resistant nephrotic syndrome. Pediatr Nephrol. 2003; 18: 833-837.

67 Iijima K, Sako M, Nozu K. Rituximab for nephrotic syndrome in children. Clin Exp Nephrol. 2016; 21(2): 193-202.

68 Chan EY, Tullus K. Rituximab in children with steroid sensitive nephrotic syndrome: in quest of the optimal regimen. Pediatr Nephrol. 2021; 36(6): 1397-1405.

69 Prytula A, Iijima K, Kamei K, Geary D, Gottlich E, Majeed A, et al. Rituximab in refractory nephrotic syndrome. Pediatr Nephrol. 2010; 25(3): 461–468.

70 Larkins N, Kim S, Craig J, Hodson E. Steroid-sensitive nephrotic syndrome: an evidence-based update of immunosuppressive treatment in children. Arch Dis Child. 2016; 101(4): 404-408.

71 Bagga A, Sinha A, Moudgil A. Rituximab in patients with steroid-resistant nephrotic syndrome. N Engl J Med. 2007; 356 (26): 2751-2752.

72 Greenbaum L, Benndorf R, Smoyer W. Childhood nephrotic syndrome: current and future therapies. Nat Rev Nephrol. 2012; 8(8): 445-458.

73 Fornoni A, Sageshima J, Wei C, Merscher-Gomez S, Aguillon-Prada R, Jauregui NA, et al. Rituximab targets podocytes in recurrent focal segmental glomerulosclerosis. Sci Transl Med. 2011; 3(85): 85ra46.

74 Sinha A, Bagga A. Rituximab therapy in nephrotic syndrome: implications for patients management. Nat Rev Nephrol. 2013; 9 (3): 154-169.

75 Kara A, Gurgoze MK, Serin HM, Aydin M. Cerebral arterial thrombosis in a child with nephrotic syndrome. Nigerian J of Clinical Practice. 2018; 21(7): 945-948.

76 Gera DN, Yadav DK, Kute VB, Patil SB, Trivedi HL. Cerebral venous sinus thrombosis in children with nephrotic syndrome. Indian J Nephrol. 2012; 22(6): 455-458.

77 Lee JM, Kronbichler A, Shin JI, Oh J. Current understandings in treating children with steroid-resistant nephrotic syndrome. Pediatric Nephrology 2020; 36 (suppl.1): 1-15.

78 Agrawal S, Zaritsky JJ, Fornoni A, Smoyer WE. Dyslipidaemia in nephrotic syndrome: mechanisms and treatment. Nat Rev Nephrol. 2018; 14(1):57-70.

79 Becherucci F, Mazzinghi B, Provenzano A, Murer L, Giglio S, Romagnani P. Lessons from genetics: is it time to revise the therapeutic approach to children with steroid-resistant nephrotic syndrome? J Nephrol. 2016; 29(4): 543-550.

80 Lovric S, Ashraf S, Tan W, Hildebrandt F. Genetic testing in steroid-resistant nephrotic syndrome: when and how? Nephrol Dial Transplant. 2016; 31(11): 1802-1813.

4 OBJETIVOS

O objetivo do presente estudo foi avaliar potenciais biomarcadores em pacientes pediátricos com SN primária. Para isso, as concentrações urinárias dos componentes do SRA [Ang II, Ang-(1-7), ECA e ECA2], das citocinas inflamatórias [IL-1β, IL-6, IL-8, IL-10, TNF, IL-12p70] e quimiocinas [IL-8/CXCL8, MIG/CXCL9, IP-10/CXCL10, MCP-1/CCL2 e RANTES/CCL5] e as concentrações séricas das adipocinas [adiponectina, resistina, leptina], dos hormônios do metabolismo energético [grelina, GLP-1, GIP)] e dos marcadores de função endotelial [sVCAM-1), PAI-1] foram dosadas em pacientes com SN primária e comparadas com mensurações em crianças e adolescentes saudáveis, pareados em sexo e idade (grupo controle).

Os **objetivos específicos** foram:

a. Mensurar os componentes do SRA e moléculas inflamatórias em amostras de urina de pacientes pediátricos com SN primária e indivíduos saudáveis, pareados por idade e sexo;
b. Mensurar as adipocinas, hormônios do metabolismo, sVCAM e PAI-1 em amostras de urina e plasma de pacientes pediátricos com SN primária e indivíduos saudáveis, pareados por idade e sexo;
c. Comparar as mensurações de componentes do SRA e moléculas inflamatórias em amostras de urina de pacientes que estão ou não em uso crônico de inibidores da ECA (iECA) e/ou antagonistas de receptores AT1 (BRA) e de acordo com a presença ou ausência de proteinúria.
d. Avaliar possíveis correlações entre os biomarcadores estudados e a proteinúria de 24 horas.
e. Avaliar as correlações entre adipocinas, hormônios do metabolismo, sVCAM e PAI-1 com os dados clínicos e laboratoriais dos pacientes pediátricos com SN primária.

5 METODOLOGIA

5.1 Delineamento e aspectos éticos

O delineamento da pesquisa foi de um estudo transversal, totalizando 33 pacientes pediátricos portadores de SN primária, acompanhados no ambulatório de Nefrologia Pediátrica do Hospital das Clínicas da Universidade Federal de Minas Gerais (HC-UFMG) no período compreendido entre março de 2017 a fevereiro de 2019. O critério utilizado para diagnóstico da doença foi baseado no KDIGO Clinical Practice Guideline for Glomerulonephritis (2012)[30].

O estudo recrutou crianças e adolescentes com idade variando entre 8 a 16 anos (n=31), sendo 19 do sexo masculino e 12 do sexo feminino, em um primeiro momento. Posteriormente, foram incluídos pacientes com idade entre 2 a 19 anos (n=33), totalizando 22 do sexo masculino e 11 do sexo feminino, em uso ou não de corticoides, imunossupressores e bloqueadores do SRA.

Este projeto de pesquisa foi aprovado pelo Comitê de Ética e Pesquisa da Universidade Federal de Minas Gerais (COEP-UFMG), sob os protocolos ETIC 572/07, ETIC 316/09 e CAAE-07513513.9.0000.5149. O termo de consentimento livre e esclarecido (TCLE) foi apresentado e assinado por todos os responsáveis e o termo de assentimento foi apresentado e assinado pelos participantes, quando pertinente. Nos termos em questão foram informados os objetivos do estudo e era assegurado o direito de recusa na participação, não havendo qualquer prejuízo no acompanhamento dos pacientes. Ressalta-se que o estudo não interferiu com nenhuma prescrição e recomendação médica destinada aos pacientes. Foram informados também que as informações e dados clínicos e laboratoriais coletados, por consulta aos prontuários, seriam mantidos em sigilo e usados exclusivamente para fins de pesquisa.

5.2 Critérios de inclusão e exclusão

5.2.1 Grupo SN

Foram incluídos os pacientes diagnosticados com SN primária (n=33) em tratamento clínico, acompanhados regularmente no ambulatório, de ambos os sexos, que aceitaram participar do estudo após os pais ou responsável legal concordar e assinar o TCLE. Os critérios adotados foram função renal preservada, diagnóstico claramente definido e se encontrarem em remissão completa ou parcial da doença no momento de coleta das amostras.

Os pacientes que apresentavam síndrome nefrótica congênita, formas secundárias de SN, estágios de 2 a 5 da DRC, recidivas clínicas e laboratoriais, ou infeções agudas e alérgicas no momento das coletas de urina foram excluídos.

5.2.2 Grupo controle

Crianças e adolescentes saudáveis (n=29) foram recrutados nos Ambulatórios de Atenção Primária Pediátrica do Hospital das Clínicas da UFMG, pareados em sexo e idade com os casos de SN. Após assinatura do TCLE, os sujeitos que não apresentavam alterações ao exame clínico e nem história pregressa de nefropatias ou quaisquer doenças crônicas ou agudas foram incluídos no estudo.

5.3 Protocolo de estudo

Os pacientes pediátricos com SN primária foram recrutados de acordo com a atividade da doença, conforme os valores de proteinúria e ausência de edema: pacientes com excreção de proteína em urina de 24 horas entre 150 e 1000 mg/dl e sem edema foram considerados em *remissão parcial* e aqueles que também não apresentavam edema, mas com excreção de proteína em urina de 24 horas abaixo a 150 mg/dL foram considerados em *remissão completa*[23].

A realização das coletas de material biológico para avaliação dos componentes do SRA, mediadores inflamatórios, adipocinas, hormônios do metabolismo, e dos marcadores de função endotelial, sVCAM e PAI-1 foram concomitantes à coleta de dados clínicos e laboratoriais do prontuário de cada paciente. Variáveis como idade, sexo, peso, altura, índice de massa corpórea (IMC), pressão sistólica e diastólica, dosagens séricas de creatinina, albumina, colesterol, triglicérides e dosagens urinárias de creatinina, proteinúria de 24 horas foram avaliadas. Além disso, os resultados de biopsia renal e as medicações em uso foram também verificados. O ritmo de filtração glomerular (RFG) foi estimado empregando a fórmula de Schwartz modificada[31]. A avaliação do estado nutricional foi baseada nos critérios da Organização Mundial de Saúde (OMS - SISVAN) que utiliza a classificação percentil do IMC segundo idade e sexo[32].

Os controles saudáveis realizaram as coletas de urina e plasma em uma única ocasião para avaliação das moléculas inflamatórios, dos componentes do SRA, das adipocinas, dos hormônios do metabolismo e dos marcadores de função endotelial, sVCAM e PAI-1. Da mesma maneira, as variáveis clínicas foram avaliadas no mesmo momento das coletas.

5.3.1 Coleta e processamento das amostras biológicas

Amostras de urina foram coletadas em recipientes estéreis, homogeneizadas e separadas em tubo tipo *falcon*. Foram centrifugadas a 3800 rpm, 4ºC por 5 minutos para decantação. Realizada a assepsia prévia, as amostras de sangue foram obtidas por meio de punção venosa periférica em tubos individualizados de heparina. O protocolo para separação do plasma consistiu em centrifugação a 3000 rpm, por 10 minutos, a 4 ºC.

Posteriormente ao processamento, as amostras biológicas foram aliquotadas (1 ml) em tubo tipo *eppendorf* e armazenadas em freezer -80 °C até o momento das análises de biomarcadores.

5.3.2 Estudo dos componentes do SRA e sVCAM-1 por imunoensaio enzimático

A determinação das moléculas do SRA [Ang II, Ang-(1-7), ECA e ECA2] e sVCAM-1 foi realizada pela técnica de ensaio imunoenzimático (ELISA). Seguindo as recomendações do fabricante (My Biosource, San Diego, CA, USA) e (R&D Systems, Minneapolis, EUA), respectivamente, a técnica de ELISA tipo sanduíche foi aplicada na determinação dos níveis urinários de Ang II, Ang-(1-7) e ECA2 e das concentrações plasmáticas e urinárias de sVCAM-1. O método de ELISA competitivo foi usado para a mensuração de ECA.

O procedimento é baseado em interações antígeno-anticorpo do marcador investigado e um substrato de detecção colorimétrico específico para enzima. A sensibilidade estimada do ensaio é de 1,0 pg/ml para ECA e ECA2, 2,0 pg/ml para Ang II e Ang-(1-7) e 1,26 ng/mL para sVCAM-1.

Brevemente, foi feita a adição das amostras de urina e padrões às microplacas revestidas com anticorpos específicos para cada marcador analisado e anticorpos específicos ligados à enzima. Após um período de incubação, as placas foram lavadas e um substrato específico para a enzima foi acrescido gerando cor, sendo a intensidade da cor diretamente proporcional ao marcador avaliado, exceto para a medida da ECA, que é inversamente proporcional. Finalmente, uma solução de parada foi adicionada para interromper a reação. As placas foram lidas a 450 nm em espectrofotômetro (Emax – Molecular Devices).

As concentrações dos marcadores nas amostras dos participantes foram obtidas interpolando os valores da absorbância em uma curva de calibração feita com padrões de concentração conhecida, fornecidos pelo fabricante, utilizando o software SoftMaxPro®.

5.3.3 Quantificação de citocinas e quimiocinas por CBA (Cytometric Beads Array)

A dosagem dos níveis urinários de moléculas inflamatórias, citocinas e quimiocinas, foi realizada pelo método de CBA, segundo informações do fabricante (BD Biosciences, San Jose, CA, USA), utilizando os kits CBA Human Inflammatory Cytokine e CBA Human Chemokine Kit. Os padrões e amostras foram adquiridos em citômetro de fluxo FACS Canto II (Becton, Dickinson and Company, San Jose, CA, USA) e os resultados analisados pelo programa FCAP Array (Soft Flow Inc., Pecs, Hungary) e representados em pg/ml.

5.3.4 Quantificação de adipocinas, hormônios metabólicos e PAI-1 por Milliplex/Luminex

Seguindo os protocolos do fabricante, os níveis plasmáticos e urinários de adiponectina, leptina, resistina, grelina, GLP-1, GIP, PAI-1 foram medidos pela plataforma Milliplex/Luminex xMAP® (Millipore Corporation, MA, EUA). Os padrões e amostras foram adquiridos no analisador de microesferas MAGPIX® (Luminex Corporation, Texas, EUA) e os resultados analisados no programa Milliplex Analyst (MilliporeSigma) e representados em pg/ml.

Sucintamente, microesferas de captura recobertas por anticorpos monoclonais específicos para cada analito são adicionados aos poços, juntamente com as amostras e padrões. Após incubação e lavagem, uma mistura de anticorpos biotinilados secundários é adicionada. Em seguida, a estreptavidina conjugada com a proteína fluorescente é incubada por um período breve. Após lavagem, o sobrenadante foi desprezado e o precipitado contendo as microesferas foi ressuspendido em uma solução tampão.

5.4 Análise estatística

Para cada grupo, os resultados obtidos foram expressos como médias e erro padrão da média (EPM) ou medianas e intervalo interquartil ou porcentagens, quando apropriado.

A associação entre variáveis categóricas foi avaliada por meio do teste qui-quadrado e a distribuição das variáveis quantitativas foi verificada pelo teste de Shapiro-Wilk. A comparação entre dois grupos foi feita pelo teste t de Student para dados não pareados para variáveis de distribuição normal e, para as variáveis sem distribuição gaussiana, o teste Mann-Whitney foi usado. As correlações entre as variáveis foram analisadas pelo teste de Spearman.

Todos os testes estatísticos foram bicaudais, usando um nível de significância de $p<0,05$. Para construção do banco de dados e realização das análises estatísticas, utilizamos os programas SPSS versão 22.0 (SPSS Inc., Chicago, IL, USA) e Prism 5.0 (GraphPad, La Jolla, CA, USA).

5.5 Referências Bibliográficas

30 Kidney Disease Improving Global Outcomes (KDIGO). Clinical practice guideline for glomerulonephritis. Kidney Int 2012; (suppl. 2): 139–274.

31 Schwartz GJ, Munoz A, Schneider MF, Mak RH, Kaskel F, Warady BA, et al (2009) New equations to estimate GFR in children with CKD. J Am Soc Nephrol 20:629-637. doi: 10.1681/ASN.2008030287

32 Brasil. Ministério da Saúde. Secretaria de Atenção à Saúde. Departamento de Atenção Básica. Protocolos do Sistema de Vigilância Alimentar e Nutricional – SISVAN na assistência à saúde / Ministério da Saúde, Secretaria de Atenção à Saúde. Departamento de Atenção Básica– Brasília: Ministério da Saúde, 2008. Disponível em: http://189.28.128.100/dab/docs/portaldab/publicacoes/protocolo_sisvan.pdf

6 MANUSCRITO ORIGINAL 1 (artigo já publicado no periódico Bioscience Reports)

Evidence for a role of angiotensin converting enzyme 2 in proteinuria of idiopathic nephrotic syndrome

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Introduction: Renin angiotensin system (RAS) plays a role in idiopathic nephrotic syndrome (INS). Most studies investigated only the classical RAS axis. Therefore, the aims of the present study were to evaluate urinary levels of RAS molecules related to classical and to counter-regulatory axes in pediatric patients with INS, to compare the measurements with levels in healthy controls and to search for associations with inflammatory molecules, proteinuria and disease treatment. **Subjects and methods:** This cross-sectional study included 31 patients with INS and 19 healthy controls, matched for age and sex. Patients and controls were submitted to urine collection for measurement of RAS molecules [Ang II, Ang-(1-7), ACE and ACE2] by enzyme immunoassay and cytokines by Cytometric Bead Array. Findings in INS patients were compared according to proteinuria: absent (<150 mg/dl, n=15) and present (≥150 mg/dl, n=16). **Results:** In comparison to controls, INS patients had increased Ang II, Ang-(1-7) and ACE, levels while ACE2 was reduced. INS patients with proteinuria had lower levels of ACE2 than those without proteinuria. ACE2 levels were negatively correlated with 24-h-proteinuria. Urinary concentrations of MCP-1/CCL2 were significantly higher in INS patients, positively correlated with Ang II and negatively with Ang-(1-7). ACE2 concentrations were negatively correlated with IP-10/CXCL-10 levels, which, in turn, were positively correlated with 24-h-proteinuria. **Conclusion:** INS patients exhibited changes in RAS molecules and in chemokines. Proteinuria was associated with low levels of ACE2 and high levels of inflammatory molecules.

INTRODUCTION

The pathophysiological mechanisms of idiopathic nephrotic syndrome (INS) support an underlying role for the immune system [1,2]. This hypothesis relies on the fact that the main treatment is based on corticosteroids and studies show different inflammatory profiles in patients with INS [3–6]. Experimental and clinical studies also suggest that renin angiotensin system (RAS) may play an important role on the pathogenesis of INS [7–10].

RAS is classically described as a circulating system that leads to the production of angiotensin II (Ang II), which binds to angiotensin type 1 receptors (AT1), promoting vasoconstriction, sodium retention, aldosterone release, inflammation and fibrosis [11]. However, RAS is now conceived as a dual acting system, mainly formed by two opposite axes [12–14] the classical one, including angiotensin converting enzyme (ACE), Ang II and AT1 receptor and the counter-regulatory axis formed by the enzyme homologue to ACE, named ACE2 [15,16], the heptapeptide angiotensin-(1-7) [Ang-(1-7)] and its receptor, Mas [17]. Ang-(1-7) is mainly produced by ACE2 that uses Ang II as the major substrate [18]. Ang-(1-7) exerts its effects via Mas receptor activation, leading to vasodilation, anti-inflammatory and anti-fibrogenic effects [12–14]. The unbalance between both RAS axes is thought to have an active role in renal diseases [12].

Experimental studies indicate that activation of the counter-regulatory RAS axis reduces renal inflammation, fibrosis and proteinuria. In a murine model of nephrotic syndrome (NS), the treatment with a Mas receptor agonist, the compound AVE0991, reduced proteinuria, renal levels of TGF-beta and renal tissue damage [8]. On the other hand, NS induced in Mas receptor knockout mice did not improved in response to the use of AT1 receptor blocker (ARB), suggesting that the presence of Mas receptor is critical for the therapeutic response to ARBs [8]. Despite clinical studies showing beneficial effects of ACE inhibitors and ARBs [6,7], there are no studies evaluating the role of the counter-regulatory RAS axis in INS patients, mostly in pediatric population. Therefore, the aims of the present study were to evaluate urinary levels of RAS molecules [(ACE, ACE2, Ang II and Ang-(1-7)] in pediatric patients with INS and to compare with the same measurements in healthy sex- and age-matched children. In addition, measurements of RAS molecules were correlated with proteinuria and with urinary levels of markers of inflammation in pediatric patients with INS.

SUBJECTS AND METHODS

Study design

This is a cross-sectional study with a sample of children and adolescents with remitted and

partially remitted INS, followed-up at the Pediatric Nephrology Unit (PNU) of our institution from 2017 to 2018. Our PNU has attended approximately 300 children with nephrotic syndrome, according to a systematic protocol that includes definition of disease etiology, assessment of clinical course and laboratory alterations, institution of treatment protocols and indication of renal biopsy based on clinical (corticosteroid unresponsiveness) and laboratory findings as detailed elsewhere [19]. The diagnostic criteria for INS were based on the KDIGO Clinical Practice Guideline for Glomerulonephritis (2012) [20].

Patients with INS

Thirty-one patients ranging from 8 to 16 years with INS in total or partial remission were included in the study. The inclusion criteria were still-preserved renal function, well-established diagnosis of INS and complete or partial remission of the disease. After parents' consent, urine samples were collected simultaneously to routine laboratory exams. Exclusion criteria were congenital nephrotic syndrome, secondary forms of nephrotic syndrome, INS patients at stages 2–5 of chronic kidney disease, INS patients during clinical and laboratory relapses and the presence of acute infections and allergies at the moment of urine collection.

Control group

The control group consisted of 19 healthy sex- and age-matched subjects from the Pediatric Primary Care Center. Healthy status was determined through the subjects' medical history and either a parental report or self-report to rule out the presence of chronic or acute diseases.

Ethical aspects

The Ethics Committee of our institution approved the present study under the protocol CAAE-07513513.9.0000.5149. Informed consent was obtained from the parents of all included subjects. The research protocol did not interfere with any medical recommendations or prescriptions. The follow-up of the INS patients and healthy controls was guaranteed even in cases of refusal to participate in the study.

Study protocol

The present study included only patients presenting complete or partial remission of INS. Patients with 24-h urine protein excretion above or equal to 150 mg/dl and absence of edema were considered in partial remission. Patients with 24 h urine protein excretion below 150mg/dl and absence of edema were considered in complete remission. Patients exhibiting relapses of

edema and intense proteinuria were excluded. The established reference for proteinuria was based on the KDIGO [20]. Clinical characteristics and casual measurements were obtained at the same time of urine collection. Clinical variables were age, gender, height, weight, body mass index, and systolic and diastolic blood pressure. In INS patients, serum levels of creatinine, albumin, cholesterol and triglycerides were assessed at the same time of urine collection obtained for the measurements of RAS molecules and immune mediators. Urinary determinations of creatinine and of 24-h protein excretion were also performed simultaneously to other measurements. Glomerular filtration rate (GFR) was estimated using the modified formula of Schwartz et al. [21]. Renal biopsy results and medications in use at the time of urine sampling were also analyzed.

INS patients were subdivided according to the values of proteinuria, which ranged from 48 mg/dl to 1220 mg/dl. Thereby, those with 24-h urinary protein excretion inferior to 150 mg/dl were allocated to the subgroup named absence of proteinuria (n=15) and when proteinuria was equal to or above 150 mg/dl patients were included in the subgroup called presence of proteinuria (n=16).

INS patients were also analyzed in regard to the use or not of medications that directly interfere with RAS as ACE inhibitors and/or ARBs and to the use or not of steroids. Therefore, 16 patients in use of ACE inhibitors and/ or ARBs were compared with 15 patients not receiving these medications and 18 patients in use of steroids were compared with 13 not under treatment at the time of urine sampling.

Urine samples

Urine specimens for the measurement of biomarkers were collected into sterile dry tubes. After homogenization, 15ml of the collected urine were centrifuged at 4◦C for 5 min and aliquoted into 1 ml tubes and stored at −80◦C until the measurements.

Renin angiotensin system (RAS) components

Urine levels of RAS molecules [Ang II, Ang-(1-7), ACE and ACE2] were measured by enzyme immunoassay (ELISA), according to procedures supplied by the manufacturer (MyBioSource, San Diego, CA, USA). All kits applied sandwich ELISA technique, except for ACE measurement whose kit applied competitive ELISA method. The sensitivity of the assays was 1.0 pg/ml for ACE and ACE2, 2.0 pg/ml for Ang II and Ang-(1-7) and reading the optical density at 450 nm. All biochemical assessments were performed blinded in regard to clinical diagnosis.

Cytokines and chemokines measurements

The urinary levels of multiple cytokines [interleukin (IL)-12p70, IL-6, IL-8, IL-10, IL-1β, tumor necrosis factor (TNF) and interferon gamma (IFN-γ)] and chemokines [induced protein 10 (IP-10/CXCL-10), monocyte chemoattractant protein-1 (MCP-1/CCL2), IL-8/CXCL8, monokine induced by gamma interferon (MIG/CXCL9), regulated on activation normal T cell expressed and secreted (RANTES/CCL5)] were assessed simultaneously using a Human FlexSet kit for Cytometric Bead Array (CBA, BD Bioscience, San Jose, CA,USA), following manufacture's instruction. The acquisition was performed using an FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The instrument has been checked for sensitivity and overall performance with Cytometer Setup & Tracking beads (BD Biosciences) prior to data acquisition. Quantitative results were generated using FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary). Urinary levels of all these biomarkers were expressed as concentrations standardized for urine creatinine and expressed as pictograms per milligram. Positive controls were also included in urine measurements of cytokines and chemokines to confirm the accuracy of the assays.

Statistical analysis

The softwares SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis. The results obtained were expressed as means and standard error of mean (SEM), medians and interquartile range or percentages, when appropriate. Categorical variables were compared by Qui-square. Gaussian distribution was checked by Shapiro–Wilk test. For variables without Gaussian distribution, Mann–Whitney test was used to compare two groups. For variables with normal distribution, comparisons between two groups were made by unpaired Student's t test. Spearman's correlation analyses examined the relationship between proteinuria, urinary levels of RAS components and measurements of inflammatory molecules in the same samples. All statistical tests were two-tailed with a significance level of $P < 0.05$.

RESULTS

Subject characteristics and casual measurements

Clinical and laboratory findings of INS patients and healthy controls are shown in Table 1. The control group (n=19) included 12 boys and 7 girls, ranging from 9 to 15 years old. All controls had clinical and laboratory parameters within normal range (Table 1). No statistical differences between INS patients (n=31) and controls were found in age, sex distribution, body mass index,

GFR, plasma levels of triglycerides, cholesterol, creatinine and albumin (P>0.05 for all comparisons, Table 1). As expected, the values of proteinuria were significantly higher in INS patients than in healthy controls (Table 1).

In regard to treatment, 6 among 31 (19%) INS patients were not receiving any medication at the time of urine collection, whereas the remaining patients needed at least one medication (Table 1). Sixteen among 25 (64%) patients of the INS group were receiving steroids and 9 (36%) cyclosporine, both isolated or in association with inhibitors of the RAS. In regard to histopathology, 15 (48.4%) patients were not submitted to renal biopsy, 5 (16.1%) presented focal segmental glomerulosclerosis, 5 (16.1%) had minimal change disease, 3 (9.7%) exhibited mesangial glomerulopathy and 3 (9.7%) membranous glomerulopathy.

Table 1. Clinical and laboratorial findings of patients with idiopathic nephrotic syndrome and healthy controls matched by sex and age.

Parameters	**Patients (n=31)**	**Controls (n=19)**	***P values***
Age (years)	11.3 ± 4.8	11.9 ± 1.8	0.912
Sex (male / female)	19 / 12	12 / 7	0.905
BMI (Kg/m^2)	19.3 ± 3.2	17.8 ± 2.6	0.094
Creatinine (mg/dL)	0.6 ± 0.2	0.6 ± 0.1	0.210
Triglycerides (mg/dL)	114.4 ± 79.6	-	-
Total cholesterol (mg/dL)	188.8 ± 92.9	-	-
Albumin (g/dL)	4.0 ± 0.7	4.5 ± 0.2	
Glomerular filtration rate†	109.2 ± 28.8	119 ± 12	0.254
Proteinuria ($mg/m^2/24h$)	304.2 ± 378.5	< 100 mg/dL	-
Medications in use			
No medication	6	19	***<0.001***
Only Steroids	2	-	
Steroids + ACEi or ARB	16	-	
Only ACEi or ARB	5	-	
Only cyclosporine	0	-	
Cyclosporine + ACEi or ARB	9	-	
Histopathology			
No biopsy	15	-	
MDC	5	-	
FSGS	5	-	
Membranous nephropathy	3	-	
Mesangial glomerulopathy	3	-	

Values are expressed as mean and standard deviation. Sex, medications in use and renal histopathology are expressed as absolute values. †Glomerular filtration rate was estimated using modified Schwartz's formula. Abbreviations: BMI = body mass index; ACEi = inhibitor of the angiotensin converting enzyme; ARB = angiotensin receptor type 1 blocker; MDC = minimal change disease; FSGS = focal segmental glomerulosclerosis.

The comparison between INS patients without proteinuria (absent) versus patients with proteinuria (present) is shown in Table 2. No differences in clinical and laboratorial findings were detected ($P>0.05$ for all comparisons, Table 2), except for 24-h proteinuria.

Table 3 shows the comparison between patients receiving (n=23) and not receiving ACE inhibitors and/or ARBs (n=8). The only difference between both subgroups was the medication in use. No significant differences were detected in regard to age, sex distribution, clinical data, laboratory measurements and renal histopathology ($P>0.05$ for all comparisons, Table 3).

Table 2. Clinical and laboratorial findings of patients with idiopathic nephrotic syndrome subdivided according to 24-hour urinary protein excretion: absence of proteinuria ≤ 150mg/dL (without proteinuria) and presence of proteinuria > 150 mg/dL (with proteinuria).

Parameters	**Without proteinuria (n=15)**	**With proteinuria (n=16)**	***P values***
Age (years)	10.6 ± 3.2	11.8 ± 5.4	0.223
Sex (male / female)	10 / 5	9 / 7	0.716
BMI (Kg/m^2)	19.1 ± 2.5	19.4 ± 3.7	0.782
Creatinine (mg/dL)	0.53 ± 0.10	0.65 ± 0.32	0.500
Triglycerides (mg/dL)	98.5 ± 59.7	132.7 ± 97.1	0.204
Total cholesterol (mg/dL)	186.1 ± 107.8	191.8 ± 77.9	0.400
Albumin (g/dL)	4.1 ± 0.76	3.8 ± 0.6	0.197
Glomerular filtration rate†	113.8 ± 25.7	104.9 ± 31.6	0.519
Proteinuria ($mg/m^2/24h$)	80.7 ± 25.4	543.7 ± 434.1	***<0.0001***
Medications in use			
No medication	5	1	0.083
Only Steroids	1	1	0.999
Steroids + ACEi or ARB	3	5	0.685
Only ACEi or ARB	1	5	0.172
Only cyclosporine	0	0	-
Cyclosporine + ACEi or ARB	1	0	0.484
Histopathology			0.598
No biopsy	6	9	
MDC	4	1	
FSGS	2	3	
Membranous nephropathy	1	2	
Mesangial glomerulopathy	2	1	

Values are expressed as mean and standard deviation. Sex, medications and renal histopathology in use are expressed as absolute values. †Glomerular filtration rate was estimated using modified Schwartz's formula. Abbreviations: BMI = body mass index; ACEi = inhibitor of the angiotensin converting enzyme; ARB = angiotensin receptor type 1 blocker; MDC = minimal change disease; FSGS = focal segmental glomerulosclerosis.

Table 3 - Clinical and laboratorial findings of patients with idiopathic nephrotic syndrome subdivided according to use or not of medications that directly interfere with RAS: ACEi and ARB.

Parameters	**ACEi or ARB (n=23)**	**No ACEi ou ARB (n=8)**	***P* values**
Age (years)	11.4 ± 4.6	10.6 ± 4.5	0.584
Sex (male / female)	14 / 9	5 / 3	0.957
BMI (Kg/m^2)	19.8 ± 3.3	13.9 ± 8.8	0.260
Creatinine (mg/dL)	0.61 ± 0.28	0.54 ± 0.12	0.964
Triglycerides (mg/dL)	117.9 ± 87.2	103.9 ± 55.9	0.852
Total cholesterol (mg/dL)	186.2 ± 68.8	197 ± 153.8	0.320
Albumin (g/dL)	3.9 ± 0.5	4.2 ± 0.9	0.181
Glomerular filtration rate†	108.1 ± 29.4	112.3 ± 28.6	0.910
Proteinuria ($mg/m^2/24h$)	380.2 ± 421.8	104.9 ± 44.6	0.118
Medications in use			
No medications	0	5	< ***0.0001***
Steroids + ACEi or ARB	8	0	***0.031***
Only ACEi or ARB	4	1	-
Only cyclosporine	0	0	0.777
Cyclosporine + ACEi or ARB	1	0	0.604
Histopatology			
No biopsy	10	5	
MDC	5	0	
FSGS	4	1	
Membranous nephropathy	2	1	
Mesangial glomerulopathy	2	1	

Values are expressed as mean and standard deviation. Sex, medications and renal histopathology in use are expressed as absolute values. †Glomerular filtration rate was estimated using modified Schwartz's formula. Abbreviations: BMI = body mass index; ACEi = inhibitor of the angiotensin converting enzyme; ARB = angiotensin receptor type 1 blocker; MDC = minimal change disease; FSGS = focal segmental glomerulosclerosis.

RAS components analyses

In comparison to healthy controls, INS patients presented increased urinary levels of Ang II, Ang-(1-7) and ACE ($P<0.05$ for all comparisons, Figure 1). On the other hand, urinary levels of ACE2 were significantly lower in INS patients than in controls ($P<0.0003$, Figure 1).

Correlations were evaluated between RAS components and proteinuria. In INS patients, urinary levels of ACE2 were negatively correlated with 24-urine protein excretion (rho=−0.508, P=0.005). Furthermore, INS patients with proteinuria had significantly lower levels of ACE2 in urine than INS patients without proteinuria (P=0.02, Table 4).

Regarding other RAS components [Ang II, ACE and Ang-(1-7)], there were no correlations with 24-urine protein excretion. Accordingly, no differences were found in the comparison of these molecules in INS patients without proteinuria versus those with proteinuria (Table 4).

Table 4 - Urinary levels of RAS components in idiopathic nephrotic syndrome patients without proteinuria (≤ 150 mg/dL/day) and with proteinuria (> 150 mg/dL/day).

RAS components	Without proteinuria (n=15)	With proteinuria (n=16)	*p* values
Ang II (pg/ml)	16.4 ± 8.4	14.7 ± 8.0	0.570
ACE (pg/ml)	98.4 ± 50.0	172.9 ± 162.2	0.247
Ang-(1-7) (pg/ml)	17.5 ± 7.5	16.0 ± 6.8	0.662
ACE2 (pg/ml)	13.5 ±10.7	2.9 ± 5.2	***0.023***

Values are expressed as mean ± standard deviation. Ang II = angiotensin II; ACE = angiotensin converting enzyme; Ang-(1-7) = angiotensin-(1-7); ACE2 = angiotensin converting enzyme 2.

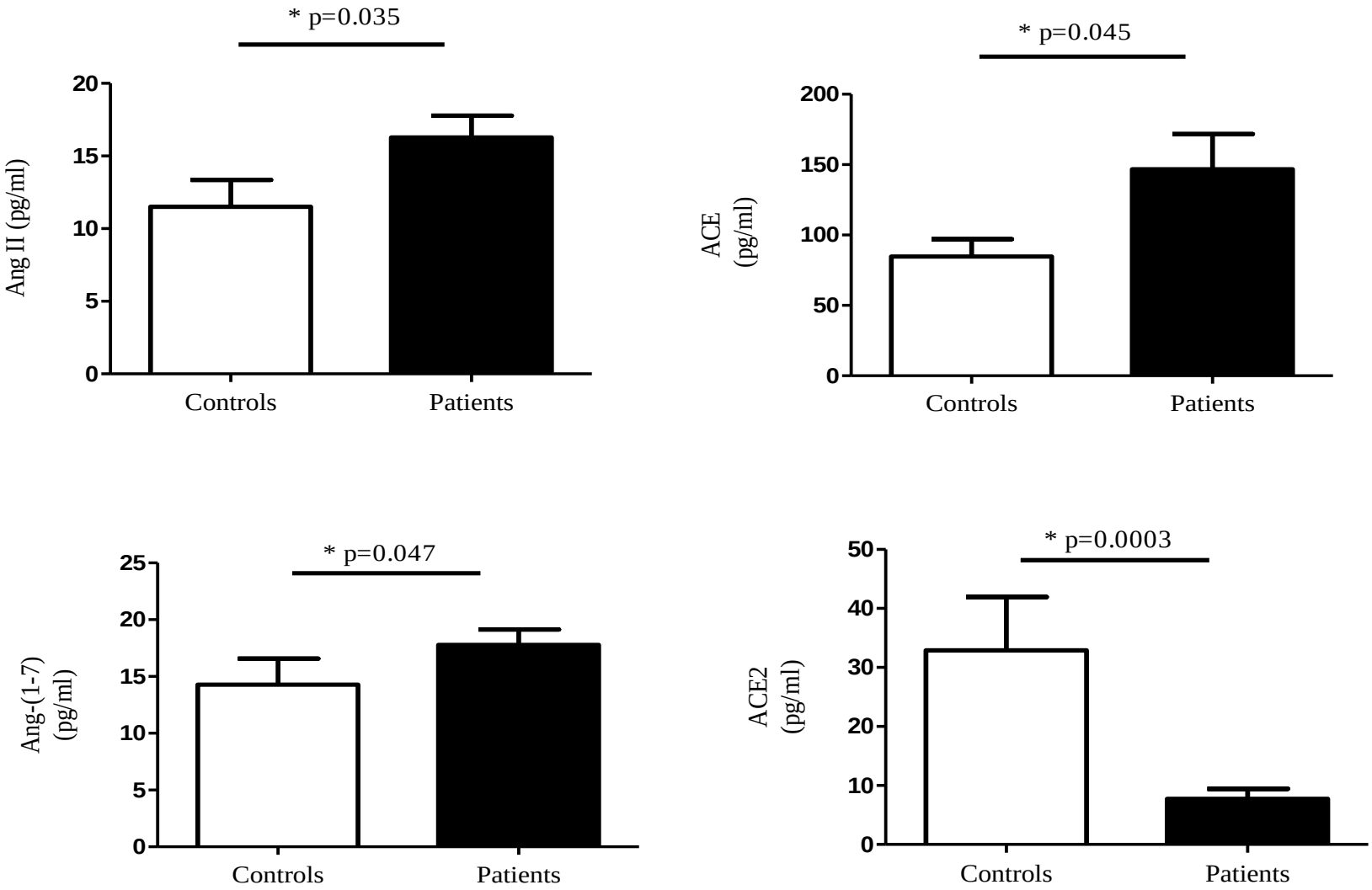

Figure 1. Levels of RAS components in urine samples of patients with INS and healthy sex and age-matched subjects (controls)
Patients with INS presented increased urinary levels of angiotensin (Ang) II, angiotensin-converting enzyme (ACE) and Ang-(1-7) in comparison with controls. On the other hand, ACE2 levels were lower in INS patients than in controls. Results are expressed as bar graphs with mean values and SEM. Differences were considered to be significant at $P<0.05$ (Mann–Whitney U test). Chemokines and cytokines analyses.

As shown in Figure 2, urinary concentrations of MCP-1/CCL2 were significantly higher in INS patients when compared with the control group (P=0.015). However, urinary levels of other chemokines (IP-10/CXCL-10, MIG/CXCL9, RANTES/CCL5, IL-8/CCCL8) and of cytokines (IL-12p70, TNF, IL-10, IL-6, IL-1β, IFN) did not significantly differ in the comparison of INS patients and healthy controls.

We also investigated the relation between urinary levels of chemokines and cytokines with 24-h urine protein excretion. Urinary levels of IP-10/CXCL-10 were positively correlated with 24-h urine protein excretion in INS patients (rho=0.3882, P=0.037). No correlations were found between 24-h urine protein excretion with other chemokines and cytokines.

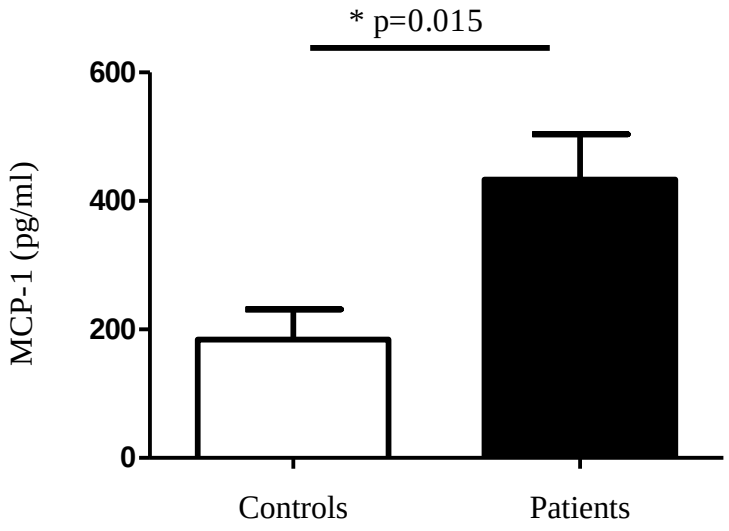

Figure 2. Urinary levels of MCP-1/CCL2 in patients with primary nephrotic syndrome (INS) and in healthy sex and age-matched subjects (controls). Patients with INS had increased urinary levels of MCP-1/CCL2. Results are expressed as bar graphs with mean values and SEM. Differences are considered to be significant at P<0.05 (Mann–Whitney U test). MCP-1/CCL2 = monocyte chemoattractant protein-1.

Correlations between RAS components and immune system molecules in INS patients

Urinary levels of chemokines and cytokines were also checked for correlations with RAS molecules in the same urine samples. In INS patients, levels of Ang II positively correlated with MCP-1/CCL2 levels (rho=0.424, P=0.017). On the other hand, as shown

in Figure 3, Ang-(1-7) levels negatively correlated with MCP-1/CCL2 (rho=−0.485, P=0.006, Figure 3 – panel superior) and MIG (rho=−0.379, P=0.035, Figure 3 – panel intermediate) and ACE2 levels were negatively correlated with IP-10/CXCL-10 (rho=−0.420, P=0.018, Figure 3 – panel inferior). No correlations were found between cytokines and RAS molecules.

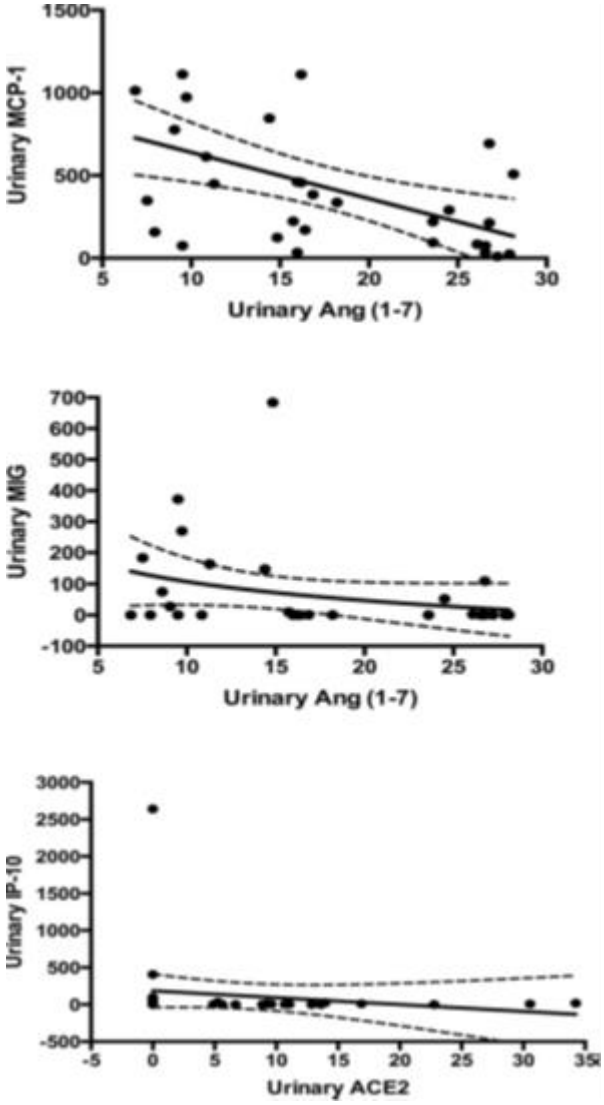

Figure 3. Correlations between RAS molecules and chemokines

Panel superior shows a negative correlation between urinary levels of Ang-(1-7) and urinary levels of MCP-1/CCL2 (rho=−0.485, P=0.006, Spearman correlation test). Panel intermediate displays a negative correlation between urinary levels of Ang-(1-7) and urinary levels of and MIG (rho=−0.379, P=0.035, Spearman correlation test). Panel inferior shows a negative correlation between urinary levels of ACE2 and urinary levels of IP-10/CXCL-10 (rho=−0.420, P=0.018, Spearman correlation test).

Effect of RAS inhibition on RAS components, proteinuria and immune system molecules

As shown in Table 3, 16 patients were receiving ACE inhibitors or ARBs at the time of urine collection. No differences were detected in urine levels of RAS molecules and in proteinuria when INS patients receiving RAS inhibitors were compared with those not receiving these medications. The same profile was not observed when inflammatory molecules were analyzed. INS patients receiving ACE inhibitors or ARBs had significantly higher urinary levels of IP-10/CXCL-10 and of IL-10 in comparison to patients not under RAS inhibition (Table 5).

Table 5 - Urinary levels of inflammatory molecules in patients with idiophatic nephrotic syndrome subdivided according to the use or not of medications that directly interfere with RAS: Angiotensin: ACEi and ARB.

Inflammatory molecules	Use ACEi and/or ARB (n = 16 / 11)	No use ACEi and/or ARB (n = 15 / 20)	*P* values
IP-10 pg/ml	134.8 ± 506.9	98.0 ± 448.1	***0.008***
MCP-1 pg/ml	454.8 ± 406.9	416.3 ± 384.5	0.675
MIG pg/ml	83.7 ± 158.6	55.3 ± 134.6	0.115
IL-8 pg/ml	26.7 ± 71.3	23.2 ± 64.6	0.187
IL-12p70 pg/ml	9.6 ± 9.0	6.7 ± 8.3	0.083
TNF pg/ml	10.6 ± 10.3	7.4 ± 9.3	0.095
IL-10 pg/ml	7.0 ± 6.7	4.6 ± 6.1	***0.042***
IL-6 pg/ml	12.1 ± 9.7	9.3 ± 9.5	0.056
IL-1β pg/ml	26.1 ± 22.6	18.4 ± 20.1	0.219

Values are expressed as mean ± standard deviation.

Effect of steroids administration on RAS components and immune system molecules

INS patients were also subdivided in regard to the use or not of steroids at the time of urinary measurements of RAS components, cytokines and chemokines. Urinary levels of ACE2 were significantly lower in patients under steroid therapy in comparison to those not receiving this medication (3.6±4.7 pg/ml versus 15.2±9.3 pg/ml, P=0.033). However, urinary levels of ACE, Ang II and Ang-(1-7) did not significantly differ in these subgroups. In regard to cytokines and chemokines, patients receiving steroids had higher urinary levels of IP-10/CXCL-10 (128.6±81.5 pg/ml versus 95.7±125.7 pg/ml, P=0.016)

than those not under this treatment at the time of urine sampling. Other molecules did not significantly differ in these subgroups.

Discussion

INS patients in complete or partial remission had significantly higher levels of ACE, Ang II and Ang-(1-7) in urine, while urinary concentrations of ACE2 were significantly lower than in healthy controls. In addition, ACE2 levels were significantly reduced in INS patients with proteinuria in comparison to those without proteinuria and urinary concentrations of this enzyme negatively correlated with 24-h urinary protein excretion. Acquired or genetic deficiency of ACE2 exacerbated kidney injury and proteinuria in many experimental models of renal diseases, possibly facilitating the deleterious effects of Ang II [22–27]. Renal expression of ACE2 was reduced in renal cortex of mice submitted to 5/6 nephrectomy and in a rat model of renal ischemia/reperfusion [27,28]. In a model of unilateral ureteral obstruction, the deletion of ACE2 gene resulted in a four-fold increase in the ratio of intrarenal Ang II/Ang-(1-7) and these changes were associated with tubulointerstitial fibrosis and high levels of TNF, IL-1β and MCP-1 [29]. Accordingly, we found that urinary concentrations of Ang II were elevated in INS patients when compared with healthy controls and were positively correlated with urinary levels of MCP-1. More recently, the daily administration of ethanol to pregnant rats resulted in glomerulosclerosis and interstitial fibrosis of the kidneys of adult offspring, accompanied by elevated levels of serum creatinine, proteinuria, total cholesterol and reduced concentrations of serum albumin [10]. These renal alterations compatible to NS were associated with increased serum levels of Ang II, high gene expression of ACE in renal tissue and reduced expression of ACE2 and of Mas receptor in the kidneys [10]. Taken together, these experimental studies indicate that the deficiency of ACE2promotes renal tissue lesion.

Most data regarding RAS components are obtained in experimental models. Few data from human samples corroborate our findings. Mizuiri et al. [30] showed that renal biopsies from patients with IgA nephropathy had significantly reduced glomerular and tubulointerstitial immunostaining for ACE2 when compared with healthy controls, while glomerular ACE staining was increased. These findings raise the possibility that an upward shift in the intrarenal ACE/ACE2 ratio favoring increased synthesis of Ang II and reduction in Ang-(1-7) might lead to progressive nephron loss in this condition [30]. Our research group recently detected lower urinary levels of ACE2 in children with sickle cell anemia (SCA) presenting persistent proteinuria in comparison to SCA patients with

normal albumin excretion in urine, also suggesting a role of reduced ACE2 protein in renal tissue in the emergence of proteinuria and nephropathy [31].

Another interesting finding of the present study was the elevation of Ang-(1-7) levels in urine of INS patients in comparison to controls. Experimental models of renal diseases showed a protective role for Ang-(1-7) [8,29,32–38]. In an experimental model of NS, the adriamycin-induced nephropathy, oral administration of AVE 0991, a Mas receptor agonist, improved renal function, reduced proteinuria and attenuated histological changes [8]. AVE 0991 or Ang-(1-7) administration also exerted renoprotective effects in experimental acute renal injury [35] and in chronic intermittent hypoxia [38]. These effects seem to be mediated, at least in part, by reducing inflammation, oxidative stress and fibrosis [38]. The infusion of Ang-(1-7) also prevented renal lesion in a model of unilateral ureteral obstruction by suppressing renal apoptosis, fibrosis, and possibly AT1 receptor expression [29]. On the other hand, Ang-(1-7) administration increased ACE2 expression [29]. We may speculate that the increase in urinary levels of Ang-(1-7) would be a compensatory response to renal damage elicited by the activation of ACE-Ang II-AT1 receptor axis in INS patients. A similar counter-regulatory response was reported in urine of fetuses with posterior urethral valves [39]. The urine of these fetuses showed intense increase in several cytokines and chemokines [40] and these changes may be opposed by a compensatory activation of ACE2-Ang-(1-7)-Mas receptor axis [39]. Another possible explanation for urinary elevation of Ang-(1-7) could be a dysfunction or reduced expression of Mas receptor at kidney tissue. Accordingly, Ng et al. [41] reported that Mas receptor expression is reduced in the kidneys of CKD rats.

In INS patients, inflammatory molecules may also contribute to disease activity and to RAS molecules changes. Urinary concentrations of MCP-1/CCL2 were significantly higher in INS patients when compared with control group, whereas levels of IP-10/CXCL-10 in urine were positively correlated with 24-hour proteinuria. Accordingly, Vianna and co-workers [42] previously found higher urinary levels of MCP-1/CCL2 in patients with CKD due to focal segmental glomerulosclerosis than in cases of congenital uropathies. More recently, Matsumoto and co-workers [9] showed that urinary level of MCP-1/CCL2 was significantly higher in steroid resistant INS than in steroid sensitive patients, supporting the idea that urinary MCP-1/CCL2 might contribute to the recruitment of macrophages into glomeruli. Remarkably, we found that urinary concentrations of Ang-(1-7) were negatively correlated with MCP-1/CCL2, whereas ACE2 levels negatively correlated with IP-10/CXCL-10. Therefore, we believe that Ang-(1-7) may oppose the glomerular damage induced by MCP-1/CCL2 attracted

macrophages. In turn, the reduced expression of ACE2 might result in altered release of IP-10/CXCL-10 in renal tissue, which affected glomerular permeability leading to proteinuria. To corroborate this hypothesis, Han et al. [43] previously reported that rats submitted to puromycin-induced nephropathy and to anti-nephrin antibody-induced nephropathy when treated with anti-IP-10/CXCL-10 function-blocking antibody displayed a decrease in the protein level of slit-diaphragm components and exacerbated proteinuria [43]. The authors also showed that the expression of CXCR3 increases in the injured podocyte in parallel with that of IP-10/CXCL-10, suggesting that IP-10 binds to CXCR3 in podocytes. The final conclusion is that IP-10/CXCL-10 may become a possible therapeutic target candidate in podocyte injury [43].

ACE inhibitors and ARBs exert renoprotective effects in glomerular diseases, mostly by reducing proteinuria [44]. However, scarce studies have investigated the effects of these medications in pediatric patients with INS [6,7]. It has also been suggested that the beneficial effects of these medications may be due, at least in part, to an activation of ACE2/Ang-(1-7)/Mas axis [45–47]. However, we did not find changes in urine concentrations of RAS components in INS patients receiving ACE inhibitors or ARBs. On the other hand, patients under treatment had higher levels of IP-10/CXCL-10 and IL-10. We did not know if changes in these cytokines are a consequence of the treatment or, alternatively, if increased levels of these molecules are related to INS itself. As already mentioned, experimental data support a role for IP-10/CXCL-10 in slit-diaphragm function [43], which might be altered in patients with more intense proteinuria. In turn, IL-10 is considered an anti-inflammatory cytokine, which may contribute to the beneficial effects of RAS blockers [48].

Our study has some limitations. First, the cross-sectional design precludes any conclusion regarding the cause of molecular changes. We probably would have obtained more conclusive results with longitudinal urine collections. Secondly, the use of immunosuppressive medications and/or RAS blockers at the time of urine collection may interfere with molecular measurements. Thirdly, we did not investigate the expression of Mas and AT1 receptors in renal tissue of INS patients. Fourthly, we did not use the traditional method, the radioimmunoassay, to measure RAS components. On the other hand, the inclusion of only relatively well controlled INS patients, exhibiting partial or total remission of the proteinuria, may increase the homogeneity of our sample and the results may represent more accurately changes related to disease itself rather than acute alterations due to relapses. Moreover, the utilization of a well-established protocol for molecular measurements may increase the strength of our findings [31,39,40].

In conclusion, our results support that ACE2 may exert renoprotective effects. Accordingly, genetic deficiency of ACE2 activity in mice fosters oxidative stress via AT1 dependent effect in the kidney [49]. In addition, daily treatment with recombinant ACE2 ameliorated renal fibrosis in apolipoprotein E-deficient mice via augmentation of Ang-(1-7)/Ang II ratio [50]. Taken together, studies indicated that the reduction in ACE2 levels at renal tissue might play a role in proteinuria and renal damage in glomerular diseases.

Clinical perspectives

In view of the advances of INS in the last years in the pediatric population and the lack of knowledge on pathophysiological mechanisms, different hypotheses have been suggested. There is evidence that RAS molecules and changes in inflammatory and cytokine expression are closely involved in the mechanisms of renal injury progression.

- ACE2 levels were significantly reduced in INS patients with proteinuria in comparison to those without proteinuria and urinary concentrations of this enzyme negatively correlated with 24-h urinary protein excretion.
- The chemokines MCP-1/CCL2 and IP-10/CXCL-10 may also contribute to proteinuria and to changes in RAS molecules in INS patients.
- The understanding on the interactions between RAS and immunoinflammatory molecules enables new strategies for diagnosis and therapeutic approach.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

ACSS, RSF, SVBP and ASM conceived research idea and study design; RSF, SVBP, TMC and VF collected clinical data and helped in statistical analysis; RSF, ELMV and ASM performed measurements of RAS molecules, cytokines and chemokines; ELMV,

ASM and ACSS performed statistical analysis; ACSS made general supervision. All authors approved the final version of the manuscript.

Abbreviations

ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; Ang-(1-7), angiotensin-(1-7); Ang II, angiotensin II; ARB, AT1 receptor blocker; AT1, angiotensin type 1; GFR, glomerular filtration rate; IFN-γ, interferon gamma; IL, interleukin; INS, idiopathic nephrotic syndrome; MCP-1/CCL2, monocyte chemoattractant protein-1; MIG/CXCL9, monokine induced by gamma interferon; NS, nephrotic syndrome; PNU, Pediatric Nephrology Unit; RANTES/CCL5, regulated on activation normal T cell expressed and secreted; IP-10/CXCL-10, induced protein 10; RAS, renin angiotensin system; SEM, standard error of the mean; TNF, tumor necrosis factor.

References

1 Mallory, L.D., Claire, G., Rulan, S.P. and Damien, G.N. (2017) Nephrotic syndrome in infants and children: pathophysiology and management. Paediatr. Int. Child Health 37, 248–258, https://doi.org/10.1080/20469047.2017.1374003

2 Pereira, W.F., Brito-Melo, G.E., Carneiro, C.M., Melo, D.S., Guimarães, F.L., Rocha-Vieira, E. et al. (2015) Increased migratory and activation cell markers of peripheral blood lymphocytes in an experimental model of nephrotic syndrome. Mediators Inflamm. 2015, https://doi.org/10.1155/2015/209764

3 Souto, M.F., Teixeira, A.L., Russo, R.C., Penido, M.G., Silveira, K.D., Teixeira, M.M. et al. (2008) Immune mediators in idiopathic nephrotic syndrome: evidence for a relation between interleukin 8 and proteinuria. Pediatr. Res. 64, 637–642, https://doi.org/10.1203/PDR.0b013e318186ddb2

4 Cara-Fuentes, G., Kairalla, J.A., Ishimoto, T., Rivard, C., Johnson, R.J. and Garin, E.H. (2014) Rituximab in idiopathic nephrotic syndrome: does it make sense? Pediatr. Nephrol. 29, 1313–1319, https://doi.org/10.1007/s00467-013-2534-4

5 Guimarães, F.T.L., Melo, G.E.B.A., Cordeiro, T.M., Feracin, V., Vieira, E.R., Pereira, W.F. et al. (2018) T-lymphocyte-expressing inflammatory cytokines underlie persistence of proteinuria in children with idiopathic nephrotic syndrome. J. Pediatr. 94, 546–553

6 Bagga, A., Mudigoudar, B.D., Hari, P. and Vasudev, V. (2004) Enalapril dosage in steroid-resistant nephrotic syndrome. Pediatr. Nephrol. 19, 45–50. https://doi.org/10.1007/s00467-003-1314-y

7 Zhuwen, Y., Zhihui, L., Xiao-Chuan, W., Qing-Nan, H., Xi-Qiang, D. and Xiao-Jie, H. (2006) Effect of fosinopril in children with steroid-resistant idiopathic nephrotic syndrome. Pediatr. Nephrol. 21, 967–972, https://doi.org/10.1007/s00467-006-0096-4

8 Silveira, K.D., Barroso, L.C., Vieira, A.T., Cisalpino, D., Lima, C.X., Bader, M. et al. (2013) Beneficial effects of the activation of the angiotensin-(1–7) Mas receptor in a murine model of adriamycin-induced nephropathy. PLoS One 8, e66082, https://doi.org/10.1371/journal.pone.0066082

9 Matsumoto, Y., Ikezumi, Y., Kondo, T., Nakajima, Y., Yamamoto, Y., Morooka, M. et al. (2018) Urinary monocyte chemotactic protein 1 as a predictive marker of steroid responsiveness in children with idiopathic nephrotic syndrome. Fujita Med. J. 4, 17–22

10 Zhu, Y., Zuo, N., Li, B., Xiong, Y., Chen, H., He, H. et al. (2018) The expressional disorder of the renal RAS mediates nephrotic syndrome of male rat offspring induced by prenatal ethanol exposure. Toxicology 400–401, 9–19, https://doi.org/10.1016/j.tox.2018.03.004

11 Forrester, S.J., Booz, G.W., Sigmund, C.D., Coffman, T.M., Kawai, T., Rizzo, V. et al. (2018) Angiotensin II signal transduction: an update on mechanisms of physiology and pathophysiology. Physiol. Rev. 98, 1627–1738, https://doi.org/10.1152/physrev.00038.2017

12 Simões e Silva, A.C. and Teixeira, M.M. (2016) ACE inhibition, ACE2 and Angiotensin-(1-7) in kidney and cardiac inflammation and fibrosis. Pharmacol. Res. 107, 154–162, https://doi.org/10.1016/j.phrs.2016.03.018

13 Prestes, T.R.R., Rocha, N.P., Miranda, A.S., Teixeira, A.L. and Simoes-E-Silva, A.C. (2017) The anti-inflammatory potential of ACE2/angiotensin-(1-7)/Mas receptor axis: evidence from basic and clinical research. Curr. Drug Targets 18, 1301–1313

14 Santos, R.A.S., Sampaio, W.O., Alzamora, A.C., Motta-Santos, D., Alenina, N., Bader, M. et al. (2018) The ACE2/angiotensin-(1–7)/Mas axis of the renin-angiotensin system: focus on angiotensin-(1–7). Physiol. Rev. 98, 505–553, https://doi.org/10.1152/physrev.00023.2016

15 Tipinis, S.R., Hooper, N.M., Hyde, R., Karran, E., Christie, G. and Turner, A.J. (2000) A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. J. Biol. Chem. 275, 33238–33243

16 Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N. et al. (2000) A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. Circ. Res. 87, E1–E9, https://doi.org/10.1161/01.RES.87.5.e1

17 Santos, R.A., Simoes e Silva, A.C., Maric, C., Silva, D.M., Machado, R.P., de Burhr, I. et al. (2003) Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. Proc. Natl Acad. Sci. U.S.A. 100, 8258–8263, https://doi.org/10.1073/pnas.1432869100

18 Vickers, C., Hales, P., Kaushik, V., Dick, L., Gavin, J., Tang, J. et al. (2002) Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. J. Biol. Chem. 277, 14838–14843, https://doi.org/10.1074/jbc.M200581200

19 Mendonc¸ a, A.C., Oliveira, E.A., Fro´ es, B.P., Faria, L.D., Pinto, J.S., Nogueira, M.M. et al. (2015) A predictive model of progressive chronic kidney disease in idiopathic nephrotic syndrome. Pediatr. Nephrol. 30, 2011–2020, https://doi.org/10.1007/s00467-015-3136-0

20 Kidney Disease Improving Global Outcomes (KDIGO) (2012) Clinical practice guideline for glomerulonephritis. Kidney Int. 82, 139–274

21 Schwartz, G.J., Munoz, A., Schneider, M.F., Mak, R.H., Kaskel, F., Warady, B.A. et al. (2009) New equations to estimate GFR in children with CKD. J. Am. Soc. Nephrol. 20, 629–637, https://doi.org/10.1681/ASN.2008030287

22 Oudit, G.Y., Herzenberg, A.M., Kassiri, Z., Wong, D., Reich, H., Khokha, R. et al. (2006) Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis. Am. J. Pathol. 168, 1808–1820, https://doi.org/10.2353/ajpath.2006.051091

23 Ye, M., Wysocki, J., William, J., Soler, M.J., Cokic, I. and Batlle, D. (2006) Glomerular localization and expression of Angiotensin-converting enzyme 2 and Angiotensin-converting enzyme: implications for albuminuria in diabetes. J. Am. Soc. Nephrol. 17, 3067–3075, https://doi.org/10.1681/ASN.2006050423

24 Soler, M.J., Wysocki, J., Ye, M., Lloveras, J., Kanwar, Y. and Batlle, D. (2007) ACE2 inhibition worsens glomerular injury in association with increased ACE expression in streptozotocin-induced diabetic mice. Kidney Int. 72, 614–623, https://doi.org/10.1038/sj.ki.5002373

25 Wong, D.W., Oudit, G.Y., Reich, H., Kassiri, Z., Zhou, J., Liu, Q.C. et al. (2007) Loss of angiotensin-converting enzyme-2 (ACE2) accelerates diabetic kidney injury. Am. J. Pathol. 171, 438–451, https://doi.org/10.2353/ajpath.2007.060977

26 Wysocki, J., Ye, M., Soler, M.J., Gurley, S.B., Xiao, H.D., Berntein, K.E. et al. (2007) ACE and ACE2 activity in diabetic mice. Diabetes 55, 2132–2139, https://doi.org/10.2337/db06-0033

27 Dilauro, M., Zimpelmann, J., Robertson, S.J., Genest, D. and Burns, K.D. (2010) Effect of ACE2 and angiotensin-(1-7) in a mouse model of early chronic kidney disease. Am. J. Physiol. Renal. Physiol. 298, F1523–F1532, https://doi.org/10.1152/ajprenal.00426.2009

28 Silveira, K.D., Pompermayer Bosco, K.S., Diniz, L.R., Carmoma, A.K., Cassali, G.D., Romero, O. et al. (2010) ACE2-angiotensin-(1-7)-Mas axis in renal ischaemia/reperfusion injury in rats. Clin. Sci. (Lond.) 119, 385–394, https://doi.org/10.1042/CS20090554

29 Liu, Z., Huang, X.R., Chen, H.Y., Penninger, J.M. and Lan, H.Y. (2012) Loss of angiotensin-converting enzyme 2 enhances TGF-beta/Smad-mediated renal fibrosis and NF-kappaB-driven renal inflammation in a mouse model of obstructive nephropathy. Lab. Invest. 92, 650–661, https://doi.org/10.1038/labinvest.2012.2

30 Mizuiri, S., Hemmi, H., Arita, M., Aoki, T., Ohashi, Y., Miyagi, M. et al. (2011) Increased ACE and decreased ACE2 expression in kidneys from patients with IgA nephropathy. Nephron Clin. Pract. 117, c57–66, https://doi.org/10.1159/000319648

31 Belisario, A.R., Vieira, E.L.M., Almeida, J.A., Mendes, F.G., Miranda, A.S., Rezende, P.V. et al. (2018) Low urinary levels of angiotensin-converting enzyme 2 may contribute to albuminuria in children with sickle cell anaemia. Br. J. Haematol., https://doi.org/10.1111/bjh.15439

32 Pinheiro, S.V., Ferreira, A.J., Kitten, G.T., da Silveira, K.D., da Silva, D.A., Santos, S.H.S. et al. (2009) Genetic deletion of the angiotensin-(1-7) receptor Mas leads to

glomerular hyperfiltration and microalbuminuria. Kidney Int. 75, 1184–1193, https://doi.org/10.1038/ki.2009.61

33 Zhang, J., Noble, N.A., Border, W.A. and Huang, Y. (2010) Infusion of angiotensin-(1-7) reduces glomerulosclerosis through counteracting angiotensin II in experimental glomerulonephritis. Am. J. Physiol. Renal. Physiol. 298, F579–588, https://doi.org/10.1152/ajprenal.00548.2009

34 Giani, J.F., Munoz, M.C., Pons, R.A., Cao, G., Toblli, J.E., Turyn, D. et al. (2011) Angiotensin-(1-7) reduces proteinuria and diminishes structural damage in renal tissue of stroke-prone spontaneously hypertensive rats. Am. J. Physiol. Renal. Physiol. 300, F272–282, https://doi.org/10.1152/ajprenal.00278.2010

35 Barroso, L.C., Silveira, K.D., Lima, C.X., Borges, V., Bader, M., Rachid, M. et al. (2012) Renoprotective effects of AVE0991, a nonpeptide Mas receptor agonist, in experimental acute renal injury. Int. J. Hypertens. 808726

36 Xue, H., Zhou, L., Yuan, P., Wang, Z., Ni, J., Yao, T. et al. (2012) Counteraction between angiotensin II and angiotensin-(1-7) via activating angiotensin type I and Mas receptor on rat renal mesangial cells. Regul. Pept. 177, 12–20, https://doi.org/10.1016/j.regpep.2012.04.002

37 Kim, C.S., Kim, I.J., Bae, E.H., Ma, S.K., Lee, J. and Kim, S.W. (2015) Angiotensin-(1-7) attenuates kidney injury due to obstructive nephropathy in rats. PLoS One 10, e0142664, https://doi.org/10.1371/journal.pone.0142664

38 Lu, W., Kang, J., Hu, K., Tang, S., Zhou, X., Yu, S. et al. (2017) Angiotensin-(1-7) relieved renal injury induced by chronic intermittent hypoxia in rats by reducing inflammation, oxidative stress and fibrosis. Braz. J. Med. Biol. Res. 50, e5594, https://doi.org/10.1590/1414-431x20165594

39 Rocha, N.P., Bastos, F.M., Vieira, E.L.M., Prestes, T.R.R., Silveira, K.D.D., Teixeira, M.M. et al. (2018) The protective arm of the renin-angiotensin system may counteract the intense inflammatory process in fetuses with posterior urethral valves. J. Pediatr., https://doi.org/10.1016/j.jped.2018.02.003

40 Vieira, E.L.M., Rocha, N.P., Bastos, F.M., Silveira, K.D., Pereira, A.K., Oliveira, A.E. et al. (2017) Posterior urethral valve in fetuses: evidence for the role of inflammatory molecules. Pediatr. Nephrol. 32, 1391–1400, https://doi.org/10.1007/s00467-017-3614-7

41 Ng, H.Y., Yisireyili, M., Saito, S., Lee, C.T., Adelibieke, Y., Nishijima, F. et al. (2014) Indoxyl sulfate downregulates expression of Mas receptor via OAT3/AhR/Stat3 pathway in proximal tubular cells. PLoS One 9, e91517, https://doi.org/10.1371/journal.pone.0091517

42 Vianna, H.R., Soares, C.M., Silveira, K.D., Elmiro, G.S., Mendes, P.M., de Sousa Tavares, M. et al. (2013) Cytokines in chronic kidney disease: potential link of MCP-1 and dyslipidemia in glomerular diseases. Pediatr. Nephrol. 28, 463–469, https://doi.org/10.1007/s00467-012-2363-x

43 Han, G.D., Suzuki, K., Koike, H., Suzuki, K., Yoneyama, H., Narumi, S. et al. (2006) IFN-inducible protein-10 plays a pivotal role in maintaining slit-diaphragm function by regulating podocyte cell-cycle balance. J. Am. Soc. Nephrol. 17, 442–453, https://doi.org/10.1681/ASN.2004090755

44 Rutkowski, B. and Tylicki, L. (2015) Nephroprotective action of renin angiotensin aldosterone system blockade in chronic kidney disease patients: the landscape after ALTITUDE and VA NEPHRON-D trails. J. Renal Nutr. 25, 194–200, https://doi.org/10.1053/j.jrn.2014.10.026

45 Azizi, M. and Menard, J. (2004) Combined blockade of the renin-angiotensin system with angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists. Circulation 109, 2492–2499, https://doi.org/10.1161/01.CIR.0000131449.94713.AD

46 Simoes e Silva, A.C., Diniz, J.S., Pereira, R.M., Pinheiro, S.V. and Santos, R.A. (2006) Circulating renin Angiotensin system in childhood chronic renal failure: marked increase of Angiotensin-(1-7) in end-stage renal disease. Pediatr. Res. 60, 734–739, https://doi.org/10.1203/01.pdr.0000246100.14061.bc

47 Kocks, M.J., Lely, A.T., Boomsma, F., de Jong, P.E. and Navis, G. (2005) Sodium status and angiotensin-converting enzyme inhibition: effects on plasma angiotensin-(1-7) in healthy man. J. Hypertens. 23, 597–602, https://doi.org/10.1097/01.hjh.0000160217.86597.b6

48 Baracho, NCDV, Silveira, K.D.D., Rocha, N.P., Cordeiro, T.M., Feracin, V., Pereira, R.M. et al. (2017) Urinary cytokine profiles according to the site of blockade of the renin-angiotensin system in nephrectomized rats. Braz. J. Nephrol. 39, 108–118

49 Wysocki, J., Ortiz-Melo, D.I., Mattocks, N.K., Xu, K., Prescott, J., Evora, K. et al. (2014) ACE2 deficiency increases NADPH-mediated oxidative stress in the kidney. Physiol. Rep. 2, e00264, https://doi.org/10.1002/phy2.264

50 Chen, L.-J., Xu, Y.-L., Song, B., Yu, H.M., Oudit, G.Y., Xu, R. et al. (2016) Angiotensin-converting enzyme 2 ameliorates renal fibrosis by blocking the activation of mTOR/ERK signaling in apolipoprotein E-deficient mice. Peptides 79, 49–57, https://doi.org/10.1016/j.peptides.2016.03.008

7 MANUSCRITO ORIGINAL 2 (artigo em revisão no periódico Pediatric Nephrology)

Metabolic changes and endothelial dysfunction in different remission stages in children with idiopathic nephrotic syndrome

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ABSTRACT

Background: Dyslipidemia in patients with idiopathic nephrotic syndrome (INS) can alter the production of adipokines and hormones and may lead to endothelial disfunction. This study aimed to assess adipokines, peptide hormones and endothelial markers at different stages of INS and define whether these molecules were associated with clinical and laboratorial data.

Methods: This cross-sectional study included thirty-three patients ranging from 2 to 19 years with INS in complete or partial remission and twenty-nine healthy sex- and age-matched subjects. We evaluated plasma and urinary levels of adiponectin, leptin, resistin, ghrelin, active GLP, GIP, PAI-1, and sVCAM-1, and their relation with clinical and laboratory parameters.

Results: INS patients showed lower urinary levels of adiponectin (p=0.022) and PAI-1 (p=0.043). Conversely, there was an increase resistin and decrease ghrelin, in plasma, compared to controls, in partial (p=0.012 and p=0.0003, respectively) and complete (p=0.002 and p=0.0001, respectively) remitted patients. Likewise, urinary leptin levels (p=0.004) and plasma GLP-1 (p=0.011) levels were significantly higher in patients with partial remission. In INS patients, plasma adiponectin was positively correlated with glomerular filtration rate (GFR) (p=0.023) and negatively with albumin (p=0.033) in complete remission state. Plasma ghrelin correlated positively with GFR in the partial

remission phase (p=0.041). The same was observed for urinary sVCAM-1 in complete remission (p=0.030).

Conclusion: INS patients exhibited changes in adipokines, peptide hormones and endothelial disfunction markers, suggesting their role in the pathophysiology of the disease.

Keywords: Biomarkers, Adipokines, Peptide Hormones, Pediatric, Nephrotic Syndrome

INTRODUCTION

The idiopathic nephrotic syndrome (INS) is one of the most common glomerulopathy in the pediatric population with an incidence rate that varies from 1.15 to 16.9/100,000 children depending on nationality and ethnicity [1,2]. INS is associated with loss or altered function of the podocytes, resulting in highly increased permeability of glomerular filtration barrier to plasma protein, which leads to severe proteinuria, hypoalbuminemia, and edema [3].

Among the various proteins lost in the urine in INS stands out key transport proteins such as apolipoproteins and albumin, which carries free cholesterol [3]. This triggers a compensatory increased synthesis of cholesterol, triglycerides and proteins related to triglyceride metabolism, resulting in hypercholesterolemia and hypertriglyceridemia [4]. Furthermore, in INS patients, there is an acquired lecithin-cholesteryl acyltransferase (LCAT) deficiency due to urinary losses, preventing the normal development of HDL [5]. The activity of lipoprotein lipase, which is produced by the adipose tissue, was also decreased. The lipoprotein lipase normally facilitates the maturation of LDL from VLDL. As a consequence of its decreased activity, there is an excess of LDL, which worsens the dyslipidemia. The magnitude of dyslipidemia in INS is directly associated with the severity of proteinuria [6]. Hence, children with relapsed INS have marked dyslipidemia that might increase the risk for future cardiovascular complications. However, longitudinal studies with adults who had INS during childhood are still lacking.

Despite the large number of metabolic changes caused by INS, little is known about the role of the adipose tissue in the pathophysiology of the disease. Since the adipose tissue is directly affected by the dyslipidemia state, it is reasonable to consider that hormonal regulatory mechanisms are aggravated in the context of INS [7]. Plasma levels of adipokines, including adiponectin [7], leptin [8,9] and ghrelin [8], were found significantly altered in patients with INS. However, other hormones that regulate the energy metabolism, in particular glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) were not measures in INS.

Moreover, dyslipidemia has the potential to cause endothelial damage. The exposure of endothelial cells to oxidized LDL *in vitro* releases vascular cell adhesion molecule (VCAM), which is considered a marker of endothelial cell injury [10]. Concurrently, the conversion of endothelial cell to procoagulant phenotype, as seen in patients with INS, may lead to upregulation of other endothelial adhesion molecules [11], including VCAM-1. Nonetheless, plasma or urinary levels of VCAM-1 were not still evaluated in patients with INS.

In this regard, the aim of the present study was to measure urinary and plasma concentrations of adipokines, energy metabolism hormones, sVCAM-1, and plasminogen activator inhibitor-1 (PAI-1) in pediatric patients with INS and to compare with values obtained in healthy children, age and gender matched. In addition, we also evaluated the associations between these molecules, clinical and laboratory findings of INS patients.

METHODS

This is a cross-sectional study with a sample of children and adolescents with remitted and partially remitted INS, followed-up at the Pediatric Nephrology Unit (PNU) of our institution from 2017 to 2019. Our PNU has attended approximately 300 children with nephrotic syndrome, according to a systematic protocol that includes definition of disease etiology, assessment of clinical course and laboratory alterations, institution of treatment protocols and indication of kidney biopsy based on clinical (corticosteroid unresponsiveness) and laboratory findings as detailed elsewhere [12]. The diagnostic criteria for INS were based on the KDIGO Clinical Practice Guideline for Glomerulonephritis (2012) [13].

Patients with INS

Thirty-three patients ranging from 2 to 19 years with INS in complete or partial remission were included in the study. The inclusion criteria were still-preserved kidney function, well-established diagnosis of INS and complete or partial remission of the disease. After parents' consent, urine samples were collected simultaneously to routine laboratory exams. Exclusion criteria were congenital nephrotic syndrome, secondary forms of nephrotic syndrome, INS patients at stages 2–5 of chronic kidney disease, INS patients during clinical and laboratory relapses and the presence of acute infections and allergies at the moment of urine collection.

Control Group

The control group consisted of 29 healthy sex- and age-matched subjects from the Pediatric Primary Care Center. Healthy status was determined through the subjects' medical history and either a parental report or self-report to rule out the presence of chronic or acute diseases.

Ethical aspects

The Ethics Committee of our institution approved the present study under the protocol CAAE-07513513.9.0000.5149. Informed consent was obtained from the parents of all included subjects. The research protocol did not interfere with any medical recommendations or prescriptions. The follow-up of the INS patients and healthy controls was guaranteed even in cases of refusal to participate in the study.

Study protocol

The present study included only patients presenting complete or partial remission of INS. Patients with 24-h urine protein excretion above or equal to 150 mg/dl but bellow 1000 mg/dL and the absence of edema were considered in partial remission. Patients with 24-h urine protein excretion below 150 mg/dl and absence of edema were considered in complete remission. Patients exhibiting relapses of edema and intense proteinuria were excluded. The established reference for proteinuria was based on the KDIGO (KDIGO, 2012). Clinical characteristics and casual measurements were obtained at the same time of plasma and urine collection. Clinical variables were age, gender, height, weight, body mass index, and systolic and diastolic blood pressure. In INS patients, serum levels of creatinine, albumin, cholesterol, and triglycerides were assessed at the same time of plasma and urine collection obtained for the measurements of energy metabolism hormones and markers of endothelial function. Urinary determinations of creatinine and of 24-h protein excretion were also performed simultaneously to other measurements. Glomerular filtration rate (GFR) was estimated using the modified formula of Schwartz et al. [14]. Kidney biopsy results and medications in use at the time of blood and urine sampling were also analyzed.

INS patients were also analyzed regarding the use or not of medications that could potentially alter energy metabolism hormones and markers of endothelial function levels, including ACE inhibitors and/or ARBs and to the use or not of steroids.

Urine samples

Urine specimens for the measurement of biomarkers were collected into sterile dry tubes. After homogenization, 15 ml of the collected urine were centrifuged at 20 °C for 5 min and aliquoted into 1 ml tubes and stored at −80°C until the measurements.

Plasma samples

Peripheral blood samples were collected in vacuum tubes with heparin, centrifuged twice at 3000 rpm for 10 minutes at 4 °C. Plasma samples were then obtained and stored at -80 °C until further processing.

Quantification of markers and sVCAM- 1 by immunoassay

Following the manufacturer's protocols, the plasma and urine levels of adiponectin, leptin, resistin, ghrelin, GLP-1, GIP, PAI-1 were measured by a Milliplex/Luminex xMAP® platform (Millipore Corporation, MA, USA).

Briefly, capture microspheres coated with specific monoclonal antibodies for each molecule were added to the wells, along with standards, plasma and urine samples. After incubation and washing, a mixture of secondary biotinylated antibodies was added. Then, streptavidin conjugated to the fluorescent protein was incubated for a brief period. After washing, the supernatant was discarded and the precipitate containing the microspheres was resuspended in a buffer solution. The standards and samples were acquired in the MAGPIX® microsphere analyzer (Luminex Corporation, Texas, USA) and the results were analyzed using the Milliplex Analyst program (MilliporeSigma) and represented in pg/ml.

All measurements were performed in single assays to avoid interassay variations. Our intra-assay variation was below 3%. The minimum detectable concentrations for each molecule were: 13 pg/ml for ghrelin; 41 pg/ml for leptin; 11 pg/ml for adiponectin; 2.2 pg/ml for resistin; 1.2 pg/ml for GLP-1; 0.6 pg/ml for GIP and 4.1 pg/ml for PAI-1.

Urine and plasma levels of sVCAM-1 were also measured by enzyme immunoassay (ELISA), according to procedures supplied by the manufacturer (R&D Systems, Minneapolis, USA). All kits applied sandwich ELISA technique. All molecular assessments were performed blinded regarding clinical diagnosis. All measurements were performed in a single assay to avoid interassay variations. Our intra-assay variation was below 5% and the minimum detection limit was 1.26 ng/mL ng/ml.

Statistical analysis

The software SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis. The results obtained were expressed as means and standard error of mean (SEM), medians and interquartile range or percentages, when appropriate. Categorical variables were compared by Qui-square. The Shapiro–Wilk test checked Gaussian distribution. For variables without Gaussian distribution, Mann–Whitney test was used to compare two groups and Kruskal-Wallis test for comparisons between more than two groups. For variables with normal distribution, comparisons between two groups were made by unpaired Student's t test and for more than two groups by analysis of variance followed by Bartlett's post-test. Pearson or Spearman tests were adopted to evaluate correlations according to the variables' distribution. All statistical tests were two-tailed with a significance level of $p < 0.05$.

RESULTS

Subject characteristics and casual measurements

Table 1 shows clinical and laboratory findings of INS patients and healthy controls. The control group (n=29) included 18 boys and 11 girls, ranging from 9 to 19 years old. The clinical and laboratory parameters of all controls were within normal range (Table 1). The comparison between INS patients (n=33), separated according to disease stage in partial (n=17) and complete remission (n=16), and controls did not show statistical differences regarding age, sex distribution, body mass index, systolic blood pressure (SBP), diastolic blood pressure (DBP), plasma levels of triglycerides, cholesterol, HDL-c, LDL-c, creatinine, and albumin ($p>0.05$ for all comparisons, Table 1).

The values of proteinuria were significantly higher in INS patients than in healthy controls as expected and of GFR were decreased (Table 1). Regarding histopathology, 12 (36.4%) patients were not submitted to kidney biopsy, 5 (15.2%) presented focal segmental glomerulosclerosis, 8 (24.2%) had minimal change disease, 5 (15.2%) exhibited mesangial glomerulopathy, and 3 (9.0%) membranous glomerulopathy.

At the time of blood and urine collections, among 33 INS patients, 12 (36.4%) were not receiving ACE inhibitors and/or ARBs, 24 (72.7%) were receiving steroids and 9 (27.3%) cyclosporine, both isolated or in association with inhibitors of the RAS. Based on WHO criteria (SISVAN), the nutritional diagnosis (score, BMI-age) of SNI patients subdivided by phases of remission showed that 20 patients were eutrophic, being 10 in partial remission and the other 10 in complete remission; 7 had overweight (3 patients with

partial remission and 4 with total remission); and 6 were classified as obese individuals (3 patients from each subgroup).

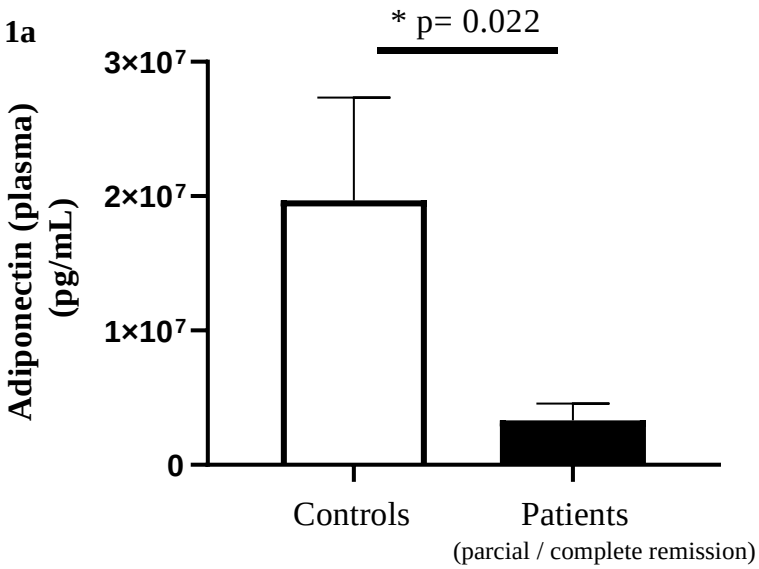
Table 1. Clinical and laboratorial findings of patients with idiopathic nephrotic syndrome and healthy controls matched by sex and age.

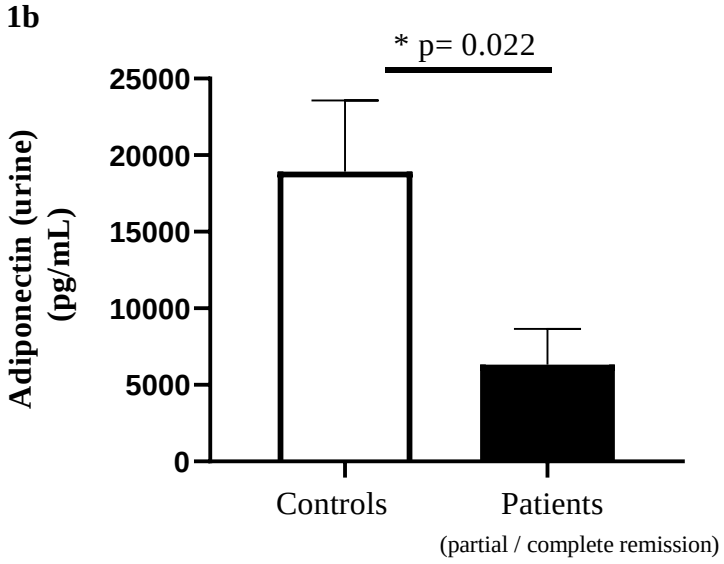
Parameters	Patients				
	Partial Remission (n = 17)	**Complete Remission (n = 16)**	**All patients (n = 33)**	**Controls (n = 29)**	***P* values***
Age (years)	12.2 ± 5.4	11.4 ± 2.9	11.8 ± 4.3	13.1 ± 2.6	0.39
Sex (male/female)	11 / 6	11 / 5	22 / 11	18 / 11	0.79
BMI (kg/m²)	19.4 ± 4.2	18.6 ± 2.9	19.0 ± 3.6	17.9 ± 1.1	0.34
SBP (mmHg)	101.5 ± 19.7	91.1 ± 13.6	96.3 ± 17.2	-	-
DBP (mmHg)	66.2 ± 18.1	56.9 ± 15.5	61.6 ± 17.2	-	-
Proteinuria (mg/m²/24h)	1790.0 ± 5003.0	365.3 ± 881.8	1077.0 ± 3607.0	<100 mg/dl	**< 0.05**
Creatinine (mg/dl)	0.7 ± 0.4	0.5 ± 0.1	0.6 ± 0.3	0.5 ± 0.07	0.30
Glomerular filtration rate†	105.1 ± 34.9	115.9 ± 22.5	110.3 ± 29.6	23.3 ± 12.2	**0.032**
Albumin (g/dl)	3.8 ± 0.6	3.9 ± 0.8	3.8 ± 0.7	-	-
Triglycerides (mg/dl)	**(n = 16)**	**(n = 16)**	**(n = 32)**		
	153.9 ± 109.0	105.4 ± 56.6	129.7 ± 88.9	-	-
Total cholesterol (mg/dl)	**(n = 16)**	**(n = 16)**	**(n = 32)**		
	194.3 ± 100.6	201.9 ± 109.8	198.1 ± 103.7	-	-
HDL-c (mg/dl)	**(n = 12)**	**(n = 16)**	**(n = 28)**		
	56.2 ± 13.1	54.2 ± 15.5	55.1 ± 14.3	-	-
LDL-c (mg/dl)	**(n = 12)**	**(n = 16)**	**(n = 28)**		
	87.8 ± 58.0	128.0 ± 99.4	110.8 ± 85.3	-	-

Values are expressed as mean and standard deviation. Sex is expressed as absolute value. *Comparison between the control group and the patients group using Student's t-test. †Glomerular filtration rate was estimated using modified Schwartz's formula. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, high-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol.

Adipokines analyses

The comparison between the entire group of INS patients and healthy controls showed significant decrease in plasma (Figure 1a) and urinary levels of adiponectin (Figure 1b). Conversely, there was an increase in plasma resistin levels, compared to controls in partial (Figure 1c) and in complete (Figure 1d) remission subgroups. Urinary levels of leptin were significantly higher in partial remission than in healthy controls (Figure 1e).





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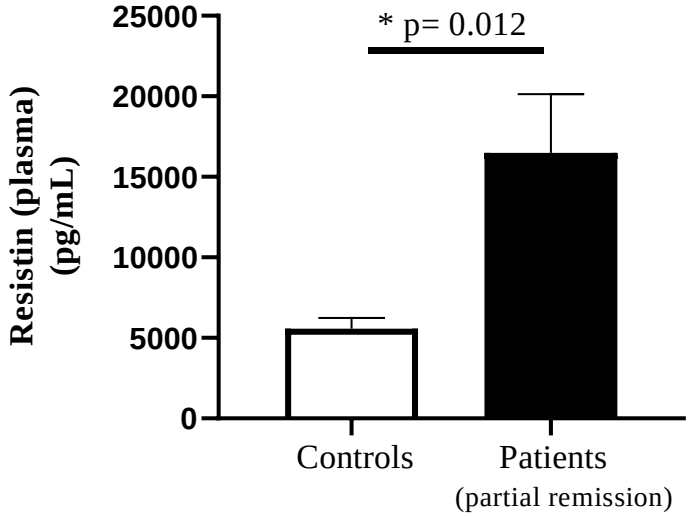

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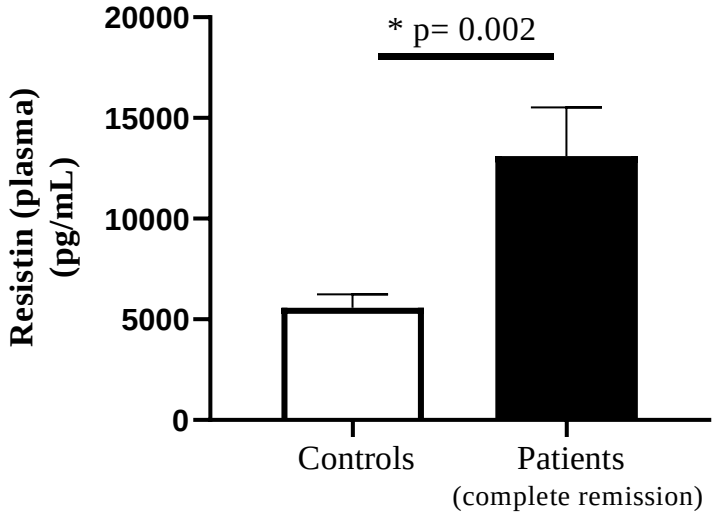

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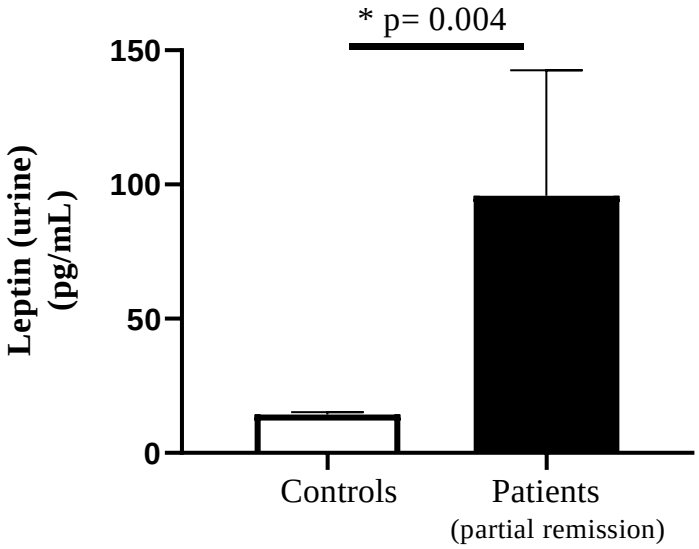


Figure 1. Levels of adipokines in plasma and urine samples from patients with primary nephrotic syndrome (INS) and controls. Patients with INS presented decreased plasma(1a) and urinary (1b) and levels of adiponectin in remission partial and complete in comparison with controls. In turn, resistin levels were increased in the plasma of INS patients than controls (1c, 1d). Urinary levels of leptin (1e) were significantly higher in partial remission than in healthy controls, Results are expressed as bar graphs with mean values and standard error of the mean (SEM). Differences were considered to be significant at $p < 0.05$ (Mann–Whitney U test).

Table 2 showed correlations between adipokines and laboratory findings. In INS patients, plasma adiponectin levels were positively correlated with GFR in complete and in partially remitted patients (Table 2). On the other hand, plasma levels of adiponectin were negatively correlated with albumin concentrations in complete remission (Table 2). No other correlation was found.

Table 2. Correlation between serum adiponectin and ghrelin levels and clinical parameters in patients with idiopathic nephrotic syndrome with complete and partial remission.

Parameters	Adiponectin (pg/ml)			Ghrelin (pg/ml)		
	Complete remission	**Partial remission**	**Remission**	**Complete remission**	**Partial remission**	**Remission**
Glomerular filtration rate†	**n = 7**	**n = 6**	**n = 13**	**n = 11**	**n = 8**	**n = 19**
r	0.857	0.942	0.906	-0.591	0.748	0.007
p	**0.023***	**0.016***	**<0.0001***	0.059	**0.041***	0.976
Albumin (g/dl)	**n = 6**	**n = 5**	**n = 11**	**n = 10**	**n = 7**	**n = 17**
r	-0.885	-0.700	-0.754	0.466	-0.103	0.278
p	**0.033***	0.233	**0.009***	0.184	0.823	0.263

*p < 0,05. †Glomerular filtration rate was estimated using modified Schwartz's formula.

Peptide hormones analyses

As shown in Figure 2, plasma levels of ghrelin in INS patients were significantly lower in partial (Figure 2a) and in complete remission subgroup (Figure 2b) when compared to the control group. Likewise, urinary levels of ghrelin were significantly decreased in partially remitted patients in comparison to controls (Figure 2c). In contrast, plasma GLP-1 levels were significantly higher in INS patients with partial remission than in controls (Figure 2d). However, urinary and plasma levels of GIP did not differ in any comparisons. Table 2 also showed the correlations between plasma and urinary levels of peptide hormones with laboratory data. In INS patients, plasma levels of ghrelin correlated positively with GFR in the partial remission subgroup (Table 2). No other correlations were found.

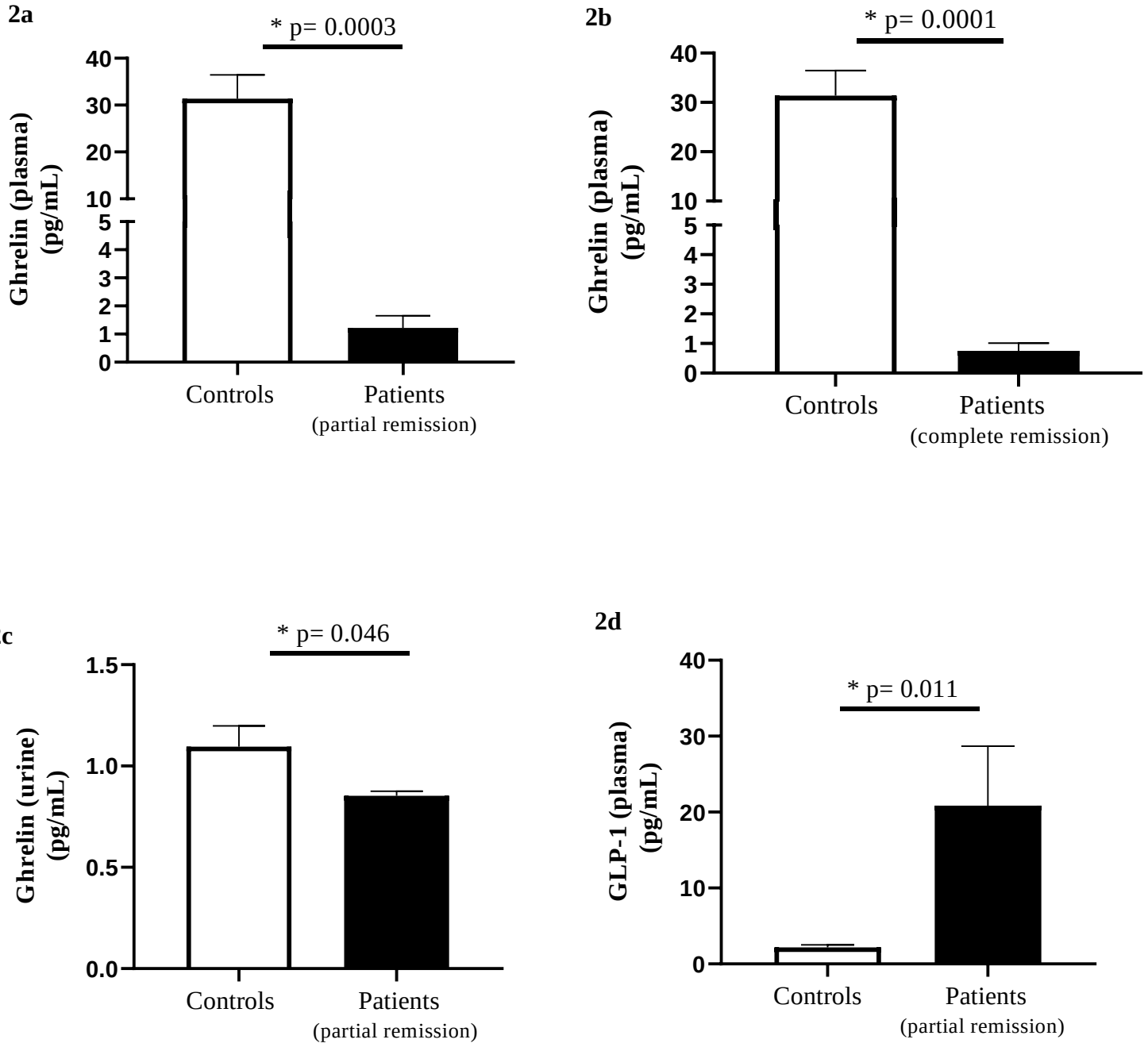
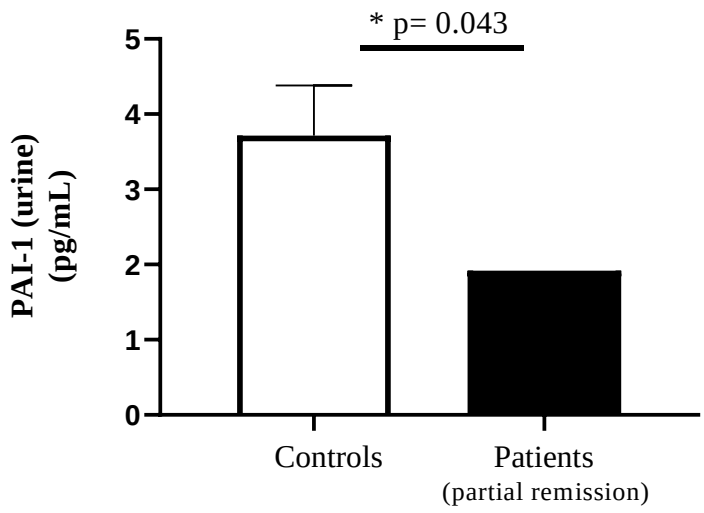


Figure 2. Levels of peptides hormones in plasma and urine samples from patients with primary nephrotic syndrome (INS) and controls. Patients with INS presented decreased plasma (2a, 2b) and urinary (2c) levels of ghrelin in comparison with controls. On the other hand, GLP-1 levels were increased in the plasma of INS patients than controls (2d). Results are expressed as bar graphs with mean values and standard error of the mean (SEM). Differences were considered to be significant at $p < 0.05$ (Mann–Whitney U test).

Markers of endothelial function

Urinary and plasma levels of sVCAM-1 did not differ in any comparison. On the other hand, urinary levels of PAI-1 were lower in patients in partial (Figure 3a) and complete remission (Figure 3b) when compared to healthy controls. The patients in partial remission tended to have an increased, however not statistically significant, plasma concentration of PAI-1 in comparison with the control group.

3a



3b

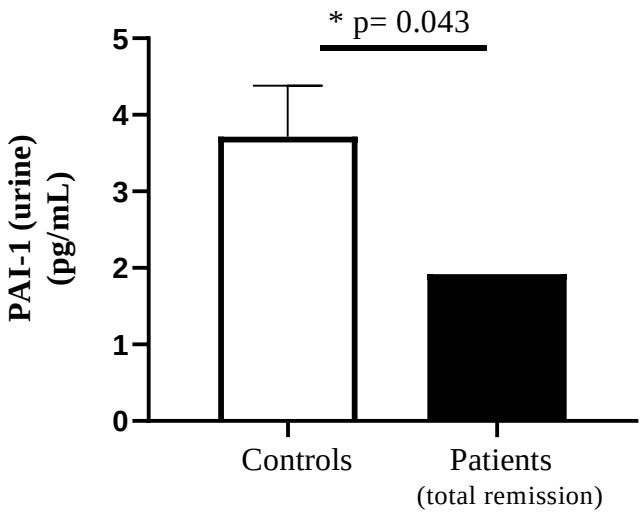


Figure 3. Levels of PAI-1 in urine samples from patients with primary nephrotic syndrome (INS) and controls. In INS patients in partial (Figure 3a) and complete remission (Figure 3b) the urinary levels of PAI-1 were lower when compared to healthy controls. Results are expressed as bar graphs with mean values and standard error of the mean (SEM). Differences were considered to be significant at $p < 0.05$ (Mann–Whitney U test)

Table 3 shows the correlations between markers of endothelial function with the laboratory findings of INS patients. Urinary levels of sVCAM-1 were positively correlated with GFR in complete remission subgroup (Table 3). No other correlations were found.

Table 3. Correlation between urinary ghrelin and vascular cell adhesion molecule 1 (sVCAM-1) levels and clinical parameters in patients with idiopathic nephrotic syndrome with complete and partial remission.

Parameters	Ghrelin (pg/ml)			sVCAM-1 (pg/ml)		
	Complete remission	**Partial remission**	**Remission**	**Complete remission**	**Partial remission**	**Remission**
Glomerular filtration rate†	**n = 16**	**n = 17**	**n = 31**	**n = 16**	**n = 17**	**n = 32**
r	-0.027	-0.414	-0.375	0.547	0.416	0.348
p	0.922	0.098	**0.037***	**0.030***	0.097	0.050
Proteinuria (g/dl)	**n = 16**	**n = 15**	**n = 30**	**n = 16**	**n = 15**	**n = 32**
r	0.391	-0.181	-0.234	-0.319	-0.517	-0.379
p	0.135	0.518	0.211	0.226	0.051	**0.032***

*p < 0,05. †Glomerular filtration rate was estimated using modified Schwartz's formula.

Body mass index classification and biomarkers

Figure 4 shows the comparison between patients' nutritional diagnosis (score, BMI-age) in regard to adipokines, peptide hormones markers of endothelial function. Plasma levels of leptin were higher in INS patients with excess body weight, classified as overweight (p=0.007) and obesity (p=0.006) (Figure 4a) when compared to eutrophic patients. However, higher levels of ghrelin were detected in the urine of patients with obesity (p=0.009) (Figure 4b) when compared to eutrophic patients. No significant differences were detected in relation to the other markers in regard to BMI classification.

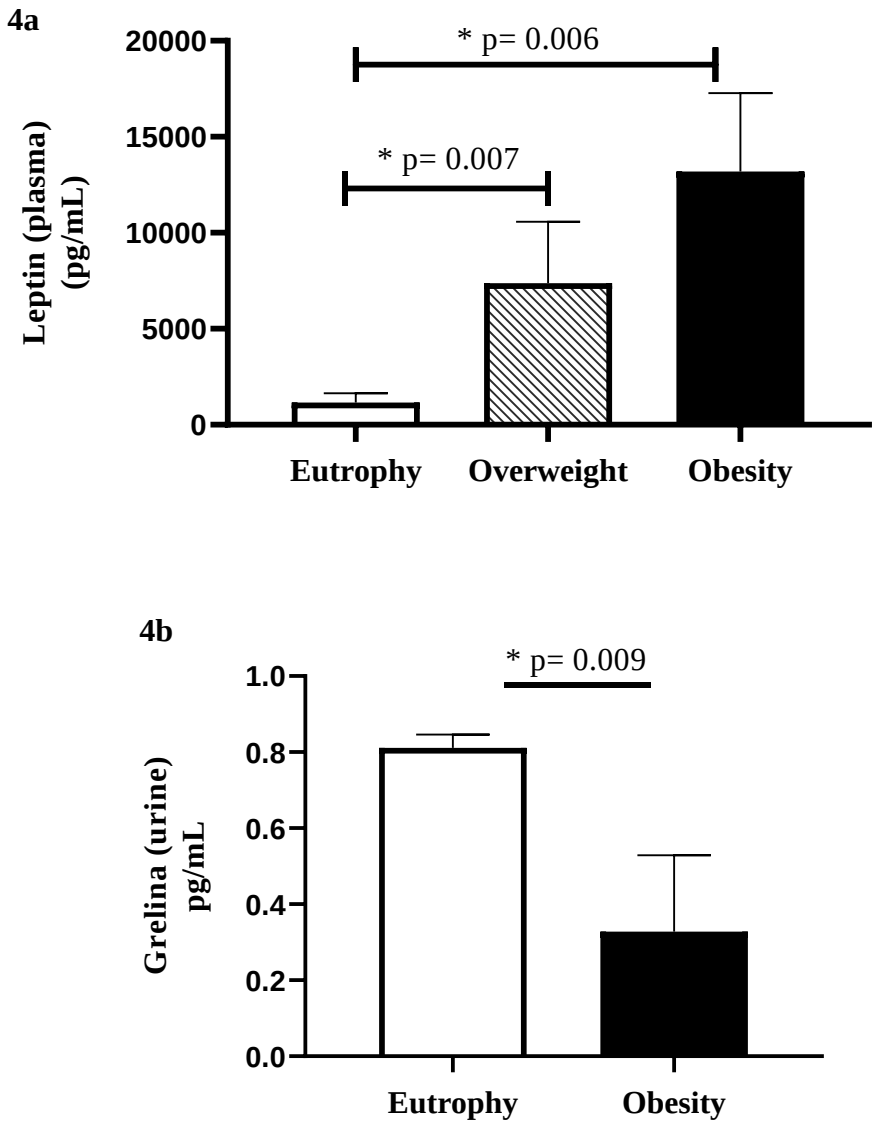


Figure 4. Comparison of adipokines and peptide hormones levels according to patients' nutritional status. The plasma levels of leptin were higher in INS patients with overweight (p=0.007) and obesity (p=0.006) (Figure 4a) when compared to eutrophic patients. However, higher levels of ghrelin were detected in the urine of patients with obesity (p = 0.009) (Figure 4b) when compared to eutrophic patients.

DISCUSSION

One of the main consequences of the INS podocytopathy is massive proteinuria, with loss of cholesterol-carrying proteins and consequent compensatory increased synthesis of cholesterol and triglycerides, resulting in dyslipidemia [15]. In response to the lipid imbalance and to the massive protein loss, different organs activate several compensatory mechanisms. Among

them, adipose tissue produces adipokines, which are proteins with endocrine, metabolic and immunological actions. Regarding the pathophysiology of kidney diseases, some adipokines have been described for their potential role, including adiponectin [16,17].

Our data point to a strong association of adiponectin and biochemical characteristics of INS. In relapsed INS patients, plasma adiponectin levels were significantly lower and independently related to proteinuria. These findings suggest a counter-regulatory action of adipokine on the inflammatory and metabolic effects caused by proteinuria, as previously described in the literature [18]. By inhibiting the expression of tumor necrosis factor-alpha (TNF-α), adiponectin can reduce the expression of endothelial adhesion molecules and the transformation of macrophage to foam cells [19]. By doing so, adiponectin decreases the atherogenic effect of dyslipidemia, one of the deleterious proteinuria's consequences. The lower concentration of adiponectin among patients with INS and its relationship with proteinuria suggest that the low plasma levels of adipokine may be one of the multiple factors associated with dyslipidemia in this population. Another factor that may be related to the low levels of this adipokine is corticosteroid therapy, since Agrawal et al. [20] suggested that the use of corticosteroids is associated with lower levels of adiponectin in steroid-sensitive nephrotic syndrome patients. In that sense, there is a possibility of the use of adiponectin as a predictive marker of therapeutic response [20].

Another adipokine possibly associated with the pathophysiology of INS is resistin since the INS children presented higher plasma levels during the relapse and remission states. Resistin, unlike adiponectin, has proinflammatory effects [21], being increased in several studies with CKD patients [22]. Although in the present study resistin did not show any correlation with other laboratory data, other studies reported an important increase of this adipokine in line with a decrease of GFR [23]. This difference is possibly due to the fact that most of our patients had normal levels of GFR even when compared to healthy controls and were clinically stable.

As with the other above mentioned adipokines, the INS patients showed altered levels of leptin. Patients with partial remission had significantly higher urinary levels of this adipokine when compared to healthy controls, similarly to the results obtained by Wasilewska et al [24]. In patients with remnant proteinuria, the urinary excretion of leptin becomes even greater [25,26] and may be influenced by the introduction of steroid therapy [9], resembling the therapy's effect on adiponectine levels. Therefore, leptin, as well as adiponectin and resistin, may play an important role in the INS pathophysiology and may be influenced by the treatment of the disease.

Together with the change in adipokine concentrations, some peptide hormones seem to exert a compensatory effect on the metabolic imbalance of INS. Among them, stands out ghrelin. Ghrelin is a hormone that possesses GH-releasing, cardiovascular and metabolic activities. For its biological activity, the acylation of the ghrelin is necessary, and it happens mainly in the stomach and in the kidney [27]. Moreover, ghrelin receptor genes are expressed in human kidney cells [28], indicating that this hormone performs endocrine and/or paracrine functions in the kidney. Diseases that impair renal function may alter ghrelin actions. Such change in ghrelin levels can be detected among patients with INS in our study, as they presented significantly lower plasma and urinary levels of this hormone when compared to controls. Another fact that corroborates the hypothesis of the relationship of this hormone with renal function is the fact that there was a positive correlation of this molecule with GFR. However, studies that investigate in depth how this hormone is related to INS are still lacking.

The change in peptide hormone levels in INS is not restricted to ghrelin. INS patients showed higher levels of plasma GLP-1 when compared to healthy controls. This finding might reflect a compensatory mechanism as a consequence of kidney inflammation, since one of GLP-1 actions is to modulate the inflammatory response in the kidneys [29]. By interacting with GLP-1R, a transmembrane G protein-coupled receptor present in kidney tissue, GLP-1 plays different renoprotective actions, including the reduction of glomerular hypertrophy [29, 30]. Thus, high levels of GLP-1 in the context of INS may be an intrinsic attempt toward kidney function preservation. Nevertheless, further studies are needed, as this is the first study that evaluated plasma and urinary levels of GLP in patients with INS.

Alongside the hormonal changes detected in INS, the impairment of renal function and consequent alterations in lipid and protein metabolism may lead to functional modifications in endothelial cells. As stated before, dyslipidemia, one of the main features of the disease, has the potential of causing endothelial dysfunction, which is an early phase of atherogenesis [31]. Hence, the study of endothelial function markers in patients with INS is essential for a better understanding of the disease and its long-term consequences. Accordingly, we evaluated plasma and urinary levels of PAI-1 and VCAM-1.

Regarding PAI-1, INS patients showed lower urinary levels of this marker, while plasma levels tended to be increased when compared to controls. This finding resembles the study of Tkaczyk et al. [32], in which patients with NS showed higher plasma PAI-1 concentrations when compared to healthy individuals. Despite this, the reason why there is a greater amount of this marker in INS patients is not well understood, but it is likely that this finding occurs due to a sum of different mechanisms associated with kidney and hormonal alterations. Since the liver and adipose tissue are primary sources of plasma PAI-1 [33], the production of this marker may be increased as a response of these organs to dyslipidemia. Another possibility is endothelial damage secondary to dyslipidemia, which could lead to greater release of this marker into the bloodstream [31]. Moreover, altered levels of PAI-1 may be related to the affected kidney function, since PAI-1 gene expression is transcriptionally regulated by the renin-angiotensin–aldosterone system (RAAS) [34]. This last hypothesis is especially interesting, as previous studies have already showed altered levels of components of RAAS in patients with INS [35].

Like PAI-1, VCAM-1 may play a role in the inflammatory process and kidney dysfunction of INS. Nevertheless, data between INS and VCAM-1 patients are scarce and no study has evaluated this molecule at different stages of the disease. Only one studied mentioned that, in patients with focal segmental glomerulosclerosis (FSGS), even at complete remission, plasma levels of sVCAM-1 remained unchanged [36], similar to our results. Notwithstanding the unchanged concentration of this marker, urinary levels of sVCAM-1 showed a positive correlation with GFR in patients at complete remission. The reason behind this association is still not understood.

Our study has several limitations. First, the cross-sectional design precludes any conclusion regarding the cause of molecular changes. We probably would have obtained more conclusive results with longitudinal urine collections. Secondly, the use of immunosuppressive medications and/or RAS blockers at the time of urine collection may interfere with molecular measurements. On the other hand, the inclusion of only relatively well controlled INS patients, exhibiting partial or total remission of the proteinuria, may increase the homogeneity of our sample and the results may represent more accurately changes related to disease itself rather than acute alterations due to relapses.

In conclusion, our findings suggest that adipokines, peptide hormones and endothelial markers may take part in the pathophysiology of INS. Unfortunately, we still not known the mechanisms related to these molecular changes. However, our data support a potential role of these molecules as biomarkers of disease activity and response to treatment.

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Conflict of Interest: The authors declare that there are no conflicting interests associated with the manuscript.

Availability of data and material: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions: ACSS, RSF, SVBP conceived the research idea and the study design; RSF, PASVC collected clinical data and performed analysis statistics; RSF, PASVC and RCM were performed as measurements of adipokines, metabolism and endothelial function markers; ACSS supervised the entire manuscript. All authors approved the final version of the manuscript.

Ethics approval: The Ethics Committee of our institution approved the present study under the protocol CAAE-07513513.9.0000.5149.

Consent to participate: Informed consent was obtained from the parents of all included subjects. The follow-up of the INS patients and healthy controls was guaranteed even in cases of refusal to participate in the study.

Consent for publication: All authors agree with the publication of the manuscript.

Acknowledgments: None

Disclosures: None to declare

References

1 Banh TH, Hussain-Shamsy N, Patel V et al (2016) Ethnic Differences in Incidence and Outcomes of Childhood Nephrotic Syndrome. Clin J Am Soc Nephrol 11(10):1760-1768. https://doi.org/10.2215/CJN.00380116

2 Chanchlani R, Parekh RS (2016) Ethnic Differences in Childhood Nephrotic Syndrome. Front Pediatr 4:39. https://doi.org/10.3389/fped.2016.00039.

3 Noone DG, Iijima K, Parekh R (2018) Idiopathic nephrotic syndrome in children. Lancet 392:61–74. https://doi.org/10.1016/S0140-6736(18)30536-1

4 Vaziri ND (2016) HDL abnormalities in nephrotic syndrome and chronic kidney disease. Nat Rev Nephrol 12: 37–47. https://doi.org/10.1038/nrneph.2015.180

5 Downie ML, Gallibois C, Parekh RS, Noone DG (2017) Nephrotic syndrome in infants and children: pathophysiology and management. Paediatr Int Child Health 37(4):248-258. https://doi.org/10.1080/20469047.2017.1374003

6 Hari P, Khandelwal P, Smoyer WE (2020) Dyslipidemia and cardiovascular health in childhood nephrotic syndrome. Pediatr Nephrol 35(9):1601-1619. https://doi.org/10.1007/s00467-019-04301-y

7 Zoccali C, Mallamaci F, Panuccio V et al (2003) Adiponectin is markedly increased in patients with nephrotic syndrome and is related to metabolic risk factors. Kidney Int Suppl (84):S98-S102. https://doi.org/10.1046/j.1523-1755.63.s84.49.x

8 Dinleyici M, Yildiz B, Cetin N, Kural N, Alatas O (2013) Serum and urinary leptin and ghrelin in children with nephrotic syndrome. Neuro Endocrinol Lett 34(5):388-394.

9 Zhou J, Shi F, Xun W (2018) Leptin, hs-CRP, IL-18 and urinary protein before and after treatment of children with nephrotic syndrome. Exp Ther Med 15(5):4426-4430. https://doi.org/10.3892/etm.2018.5923

10 Ross R (1998) Atherosclerosis—an inflammatory disease. N Engl J Med 340:115-26. https://doi.org/10.1056/NEJM199901143400207

11 Kaukinen A, Kuusniemi AM, Lautenschlager I, Jalanko H (2008) Glomerular endothelium in kidneys with congenital nephrotic syndrome of the Finnish type (NPHS1). Nephrol Dial Transplant 23(4):1224-1232. https://doi.org/10.1093/ndt/gfm799

12 Mendonça AC, Oliveira EA, Fróes BP, Faria LD, Pinto JS, Nogueira MM et al (2015) A predictive model of progressive chronic kidney disease in idiopathic nephrotic syndrome. Pediatr Nephrol 30:2011–2020.

13 Kidney Disease Improving Global Outcomes (KDIGO) (2012) KDIGO Clinical practice guideline for glomerulonephritis. Kidney Int 82:139–274.

14 Schwartz GJ, Munoz A, Schneider MF, Mak RH, Kaskel F, Warady BA et al (2009) New equations to estimate GFR in children with CKD. J Am Soc Nephrol 20: 629-637https://doi.org/10.1681/ASN.2008030287

15 Agrawal S, Zaritsky JJ, Fornoni A, Smoyer WE (2018) Dyslipidaemia in nephrotic syndrome: mechanisms and treatment. Nature reviews. Nephrology, 14(1):57–70. https://doi.org/10.1038/nrneph.2017.155

16 Booth A, Magnuson A, Fouts J, Foster M (2015) Adipose tissue, obesity and adipokines: role in cancer promotion. Hormone Molecular Biology and Clinical Investigation 21(1): 57-74. https://doi.org/10.1515/hmbci-2014-0037

17 D'Elia JA, Roshan B, Maski M, Weinrauch LA (2009) Manifestation of renal disease in obesity: pathophysiology of obesity-related dysfunction of the kidney. International Journal of Nephrology and Renovascular Disease, 2:39–49. https://doi.org/10.2147/ijnrd.s7999

18 Oguz Y, Yilmaz MI, Acikel C, Eyileten T, Caglar K, Oktenli C, Yenicesu M, Vural A (2009) The relationship between adiponectin levels and degree of proteinuria in patients with nephrotic and non-nephrotic proteinuria. Ren Fai 31(1):29-35. https://doi.org/10.1080/08860220802546461

19 Wang Y, Wang X, Lau WB et al (2014) Adiponectin inhibits tumor necrosis factor-α-induced vascular inflammatory response via caveolin-mediated ceramidase recruitment and activation. Circ Res 28;114(5):792-805. https://doi.org/10.1161/CIRCRESAHA.114.302439.

20 Agrawal S, Merchant ML, Kino J, Li M, Wilkey DW, Gaweda AE, Brier ME, Chanley MA, Gooding JR, Sumner SJ, Klein JB, Smoyer WE, Midwest Pediatric Nephrology Consortium (2019) Predicting and defining steroid resistance in pediatric nephrotic syndrome using plasma proteomics. Kidney International Reports, 5(1):66–80. https://doi.org/10.1016/j.ekir.2019.09.009

21 Bokarewa M, Nagaev I, Dahlberg L, Smith U, Tarkowski A (2005) Resistin, an adipokine with potent proinflammatory properties. J Immunol 2005; 174(9):5789–95. https://doi.org/10.4049/jimmunol.174.9.

22 Kawamura R, Doi Y, Osawa H, Ninomiya T, Hata J, Yonemoto K, Tanizaki Y, Iida M, Makino H, Kiyohara Y (2010) Circulating resistin is increased with decreasing renal function in a general Japanese population: the Hisayama Study. Nephrol Dial Transplant;25(10):3236-40. https://doi.org/10.1093/ndt/gfq155.

23 Menzaghi C, Salvemini L, Fini G, Thompson R, Mangiacotti D, Di Paola R, Morini E, Giorelli M, De Bonis C, De Cosmo S, Doria A, Trischitta V (2012) Serum resistin and kidney function: a family-based study in non-diabetic, untreated individuals. PLoS One;7(6):e38414. https://doi.org/10.1371/journal.pone.0038414.

24 Wasilewska A, Tomaszewska B, Zoch-Zwierz W, Biernacka A, Klewinowska K, Koput A (2005) Serum and urine leptin concentration in children with nephrotic syndrome. Pediatr Nephrol;20(5):597-602. https://doi.org/10.1007/s00467-004-1772-x.

25 Dinleyici M, Yildiz B, Cetin N, Kural N, Alatas O (2013) Serum and urinary leptin and ghrelin in children with nephrotic syndrome. Neuro Endocrinol Lett 34(5):388-394.

26 Schroth M, Groschl M, Dorr H, Blum W, Rascher W, Dotsch J (2001) Renal loss of leptin in patients with nephrotic syndrome. European Journal of Endocrinology 145(4):463–468. https://doi.org/10.1530 / eje.0.1450463

27 Mori K, Yoshimoto A, Takaya K, Hosoda K, Ariyasu H, Yahata K, Mukoyama M, Sugawara A, Hosoda H, Kojima M, Kangawa K, Nakao K (2000) Kidney produces a novel acylated peptide, ghrelin. FEBS Lett 2000;486(3):213-6. https://doi.org/10.1016/s0014-5793(00)02308-5.

28 Camiña JP, Lodeiro M, Ischenko O, Martini AC, Casanueva FF (2007) Stimulation by ghrelin of p42/p44 mitogen-activated protein kinase through the GHS-R1a receptor: role of G-proteins and beta-arrestins. J Cell Physiol ;213(1):187-200. https://doi.org/10.1002/jcp.21109.

29 Muskiet MHA, Tonneijck L, Smits MM, van Baar MJB, Kramer MHH, Hoorn EJ, Joles JA, van Raalte DH (2017) GLP-1 and the kidney: from physiology to pharmacology and outcomes in diabetes. Nat Rev Nephrol 13(10):605-628. https://doi.org/10.1038/nrneph.2017.123.

30 Katsurada K, Nandi SS, Zheng H, Liu X, Sharma NM, Patel KP (2020) GLP-1 mediated diuresis and natriuresis are blunted in heart failure and restored by selective afferent renal denervation. Cardiovascular Diabetology 19(1):57. https://doi.org/10.1186/s12933-020-01029-0

31 Sharma B, Saha A, Dubey NK, Kapoor K, Anubhuti, Batra VV, Upadhayay AD (2014) Endothelial dysfuntion in children with idiopathic nephrotic syndrome. Atherosclerosis 233 (2):704–706. https://doi.org/10.1016/j.atherosclerose.2014.01.055

32 Tkaczyk M, Czupryniak A, Owczarek D, Lukamowicz J, Nowicki M. (2007) Markers of endothelial dysfunction in children with Idiopathic Nephrotic Syndrome. American Journal of Nephrology 28(2):197–202. https://doi.org/10.1159/000110088

33 Pandey M, Loskutoff DJ, Samad F (2005) Molecular mechanisms of tumor necrosis factor-alpha-mediated plasminogen activator inhibitor-1 expression in adipocytes. Faseb Journal 19:1317–1319. https://doi.org/10.1096/fj.04-3459fje

34 Masuda Y, Emoto N, Nonaka H, et al Role of angiotensin and the clock system in the circadian regulation of plasminogen activator inhibitor-1 (2009) Kobe J Med Sci;54(6):E264–E271

35 Filha RDS, Pinheiro SVB, Macedo E Cordeiro T, Feracin V, Vieira ÉLM, Miranda AS, Simões E Silva AC (2019) Evidence for a role of angiotensin converting enzyme 2 in proteinuria of idiopathic nephrotic syndrome. Biosci Rep;39(1):BSR20181361. https://doi.org/10.1042/BSR20181361

36 Zhang Q, Zeng C, Fu Y, Cheng Z, Zhang J, Liu Z. Biomarkers of endothelial dysfunction in patients with primary focal segmental glomerulosclerosis (2012) Nephrology, 17(4):338-45. https://doi.org/10.1111/j.1440-1797.2012.01575.x.

8 CONCLUSÕES GERAIS

Os pacientes com síndrome nefrótica primária em remissão parcial ou total apresentaram as seguintes alterações:

- Aumento nas concentrações urinárias de Ang II, Ang-(1-7) e ECA, e diminuição dos níveis de ECA2 em relação ao grupo controle.
- Aumento dos níveis urinários da quimiocina MCP-1/CCL2 em comparação ao grupo controle.
- Aumento dos níveis plasmáticos de resistina, GLP-1 e dos níveis urinários de leptina e grelina em relação ao grupo controle.
- Diminuição nos níveis plasmáticos e urinários de adiponectina, níveis plasmáticos de grelina e níveis urinários de PAI-1 em relação ao grupo controle.
- Correlação negativa entre a excreção de proteínas na urina de 24 horas e os níveis urinários de ECA2.
- Correlação positiva entre a excreção de proteínas na urina de 24 horas e os níveis urinários de de IP-10/CXCL-10.
- Correlação negativa de MCP-1/CCL2 com Ang-(1-7), IP-10/CXCL-10 com ECA2 e MIG com Ang-(1-7).
- Correlações positivas entre adiponectina, grelina, sVCAM-1 e o RFG e negativa entre adiponectina e albumina sérica.
- Pacientes que receberam inibidores de ECA e/ou BRA apresentaram um aumento significativo nos níveis urinários de IP-10/CXCL-10 e de IL-10.
- Pacientes com sobrepeso/obesidade apresentaram aumento dos níveis plasmáticos de leptina e dos níveis urinários de grelina em comparação aos eutróficos.

9 CONSIDERAÇÕES FINAIS

Ressalta-se que a compreensão da fisiopatologia SN primária são de suma importância pela possibilidade de progressão para a DRC e pelo aumento do número de pacientes corticorresistentes[1,2]. Além disso, a SN representa um desafio para o sistema de saúde, afeta negativamente o crescimento e desenvolvimento de crianças e adolescentes e compromete aspectos sociais e emocionais. Deste modo, há vários fatores que necessitam ser investigados que vão desde as complicações decorrentes da própria doença à toxicidade do uso prolongado de corticoides.

Os achados desse estudo fornecem evidências preliminares de que as citocinas, quimiocinas, moléculas do SRA, adipocinas, hormônios metabólicos e marcadores endoteliais podem ter um papel na atividade da doença e nas complicações relacionadas à SN primária, constituindo-se em potenciais alvos terapêuticos. Embora os mecanismos de alteração na permeabilidade/seletividade da barreira de filtração glomerular e, consequentemente, proteinúria ainda não estejam bem definidos, há indícios de uma importante atuação do sistema imunológico e do SRA no desencadeamento e manutenção da SN primária[3-7]. A produção exacerbada de mediadores inflamatórios pode induzir alterações nos podócitos, ocasionando um eventual aumento na permeabilidade da proteína[5,8-10] que provavelmente é atenuada pela eficácia da terapia imunossupressora[7,9,11].

O potencial risco de evolução para a DRC nas doenças renais pode ser influenciado pelo papel do SRA, cuja ativação da via clássica pode desencadear uma cascata de eventos lesivos com exacerbação da proteinúria, inflamação e fibrose renal[12]. Evidências clínicas em nosso estudo e em alguns modelos experimentais apontam para uma interação entre os eixos do SRA e a SN primária[3,13-15]. Além disso, o uso de iECA e/ou BRA, como terapia alternativa, exerce efeitos renoprotetores ao controlar a proteinúria[16-18]. No entanto, poucos estudos investigaram os efeitos renoprotetores dos inibidores do SRA em pacientes pediátricos com SN primária[19-21].

A dislipidemia é uma complicação bem conhecida da SN em função do metabolismo lipídico alterado, que, por si só, pode causar lesão renal[21]. É possível que o desequilíbrio lipídico gerado principalmente pela perda exacerbada de proteínas na urina resulta na liberação de adipocinas, hormônios peptídicos e marcadores de função endotelial. Os mecanismos relacionados a esses biomarcadores e a SN não estão claros e poucos dados estão descritos na literatura[22-27]. No entanto, a investigação do papel dessas moléculas pode auxiliar na

compreensão dos efeitos metabólicos da dislipidemia, do ganho de peso e da corticoterapia prolongada.

Nosso estudo possui limitações. Primeiramente, o desenho transversal do estudo não permite estabelecer fatores de risco ou avaliar causalidade. Além disso, não foi possível avaliarmos mudanças dinâmicas nas concentrações dos biomarcadores investigados. Os resultados poderiam fornecer informações adicionais caso as coletas de amostras biológicas fossem longitudinais. Em segundo lugar, as mensurações obtidas podem ter sofrido interferência pelo uso de medicamentos imunossupressores e/ou bloqueadores de SRA. Em terceiro lugar, não investigamos a expressão dos biomarcadores ou seus respectivos receptores no tecido renal. Em contrapartida, a avaliação dos pacientes com SN primária em diferentes estágios da doença e os protocolos rigorosos de acompanhamento clínico e de medidas laboratoriais ampliaram a força de nossos achados, pois aumentaram a homogeneidade de nossa amostra.

Dessa forma, embora já tenhamos detectado potenciais biomarcadores na SN, são necessários estudos longitudinais para investigar mudanças dinâmicas dessas moléculas conforme a abordagem terapêutica, o estágio da doença e suas complicações.

10 REFERÊNCIAS BIBLIOGRÁFICAS

1 Uwaezuoke SN (2017) The role of novel biomarkers in childhood idiopathic nephrotic syndrome: a narrative review of published evidence. International J Neph and Reno Disease 2017; 10:123-128. doi: 10.2147/PHMT.S100803

2 Preston R, Stuart HM, Lennon R (2019) Genetic testing in steroid-resistant nephrotic syndrome: why, who, when and how? Pediatr Nephrol. 34(2), 195-210. doi: 10.1007/s00467-017-3838-6

3 Filha RDS, Pinheiro SVB, Macedo E Cordeiro T, Feracin V, Vieira ÉLM, Miranda AS, Simões E Silva AC (2019) Evidence for a role of angiotensin converting enzyme 2 in proteinuria of idiopathic nephrotic syndrome. Biosci Rep 39(1):BSR20181361. doi: https://doi.org/10.1042/BSR2018136116

4 Matsumoto Y, Ikezumi Y, Kondo T, Nakajima Y, Yamamoto Y, Morooka M, et al (2018) Urinary monocyte chemotactic protein 1 as a predictive marker of steroid responsiveness in children with idiopathic nephrotic syndrome. Fujita Medical Journal 4(1):17-22. doi: https://doi.org/10.20407/fmj.4.1_17

5 Kim SH, Park SJ, Han KH, Kronbichler A, Saleem MA, Oh J, et al (2016) Pathogenesis of minimal change nephrotic syndrome: an immunological concept. Korean J Pediatr 59(5): 205-211. doi: 10.3345/kjp.2016.59.5.205

6 Vianna HR, Soares CMBM, Silveira KD, Elmiro GS, Mendes PM, Tavares MS, et al (2013) Cytokines in chronic kidney disease: potential link of MCP-1 and dyslipidemia in glomerular diseases. Pediatr Nephrol 28(3):463-469. Doi: 10.1007/s00467-012-2363-x

7 Souto MF, Teixeira AL, Russo RC, Penido MGMG, Silveira KD, Teixeira MM, Silva ACS (2008) Immune mediators in idiopathic nephrotic syndrome: evidence for a relation between interleukin 8 and proteinuria. Pediatr Res64(6):637-642. doi: 10.1203/PDR.0b013e318186ddb2

8 Noone DGK, Iijima K, Parekh R (2018) Idiopathic nephrotic syndrome in children. Lancet 392(10141):61-74. doi: https://doi.org/10.1016/S0140-6736(18)30536-1

9 Lama G, Luong I, Tirino G, Borrrielo A, Carangio C, Salsano ME (2002) T-lymphocyte populations and cytokines in childhood nephrotic syndrome. Am J Kidney Dis 39(5):958-965. doi: 10.1053/ajkd.2002.32769

10 Guimaraes FTL, Melo GEBA, Cordeiro TM, Feracin V, Vieira ER, Pereira WF et al (2018) T-lymphocyte-expressing inflammatory cytokines underlie persistence of proteinuria in children with idiopathic nephrotic syndrome. J Pediatr 94(5):546-553. doi: https://doi.org/10.1016/j.jped.2017.08.005

11 Coutinho AE, Chapman KE (2011) The anti-inflammatory and immunosuppressive effects of glucocorticoids, recent developments and mechanistic insights. Mol Cell Endocrinol 335(1):2-13. doi: 10.1016/j.mce.2010.04.005

12 Remuzzi G, Perico N, Macia M, Ruggenenti P (2005) The role of renin-angiotensin-aldosterone system in the progression of chronic kidney disease. Kidney International 68(99):57-65. doi: https://doi.org/10.1111/j.1523-1755.2005.09911.x.

13 Bhardwaj R, Dosani I, Clark BA (2012) Steroid-responsive nephrotic syndrome and bilateral renal artery stenosis: a possible role for angiotensin-mediated podocyte injury. Case Rep Nephrol Urol 2(1):59-64. doi: 10.1159/000339407

14 Tabel Y, Berdeli A, Mir S, Serdaroglu E, Yilmaz E (2005) Effects of genetic polymorphisms of the renin-angiotensin system in children with nephrotic syndrome. J Renin Angiotensin Aldosterone Syst 6(3):138-144. doi: 10.3317/jraas.2005.020

15 Oktem F, Sirin A, Bilge I, Emre S, Agachan B, Ispir T (2004) ACE I/D gene polymorphism in primary FSGS and steroid-sensitive nephrotic syndrome. Pediatr Nephrol 19(4):384-389. doi: 10.1007/s00467-003-1398-4

16 Kemper MJ, Lemke A (2018) Treatment of genetic forms of nephrotic syndrome. Front Pediatr 6:72. doi: 10.3389/fped.2018.00072

17 Wühl, E, Schaefer F (2008) Therapeutic strategies to slow chronic kidney disease progression. Pediatric Nephrology 23(5):705-716. doi: 10.1007/s00467-008-0789-y

18 Nakao N, Yoshimura A, Morita H, Takada M, Kayano T, Ideura T (2003) Combination treatment of angiotensin-II receptor blocker and angiotensin-converting-enzyme inhibitor in non-diabetic renal disease (COOPERATE): a randomised controlled trial. Lancet 361(9352): 117-124. doi: 10.1016/S0140-6736 (03) 12229-5

19 Supavekin S, Surapaitoolkorn W, Tancharoen W, Pattaragarn A, Sumboonnanonda A (2012) Combined renin angiotensin blockade in childhood steroid-resistant nephrotic syndrome. Pediatr Int 54(6):793-797. Doi: 10.1111/j.1442-200X.2012.03668.x

20 Soliman NA, Francis M, Heeringa SF, Chernin G (2009) Complete remission of nephrotic syndrome in an infant with focal segmental glomerulosclerosis: is it renin–angiotensin blockade?" Pediatric Nephrology 24(5): 1065-1070. doi: 10.1007/s00467-008-1026-4

21 Bagga A, Mudigoudar BD, Hari P, Vasudev V (2004) Enalapril dosage in steroid-resistant nephrotic syndrome. Pediatr Nephrol 19(1): 45-50. doi: https://doi.org/10.1007/s00467-003-1314-y

21 Agrawal S, Zaritsky JJ, Fornoni A, Swoyer WE (2018) Dyslipidaemia in nephrotic syndrome: mechanisms and treatment. Nephrology 14:57-70. doi: https://doi.org/10.1038/nrneph.2017.155

22 Varal IG, Civilibal M, Duru NS, Elevli M (2020) A prospective study of serum concentrations of leptin, homocysteine and insulin resistance in children with steroid-sensitive nephrotic syndrome. Exp Biomed Res 3(2):79-89. doi: 10.30714/j-ebr.2020258647

23 Gubina NV, Kupnovytska IH, Mishchuk VH, Markiv HD (2020) Ghrelin levels and decreased kidney function in patients with early stages of chronic kidney disease against the background of obesity. Journal of medicine and life, 13(4):530–535. doi.org/10.25122/jml-2020-0152

24 Ghosh AK, Vaughan DE (2012) PAI-1 in tissue fibrosis. J Cell Physiol 227(2):493-507. doi: 10.1002/jcp.22783

23 Zhang J, Gu C, Lawrence DA, Cheung AK, Huang Y (2014) A PAI-1 mutant retards diabetic nephropathy in db/db mice through protecting podocytes. Exp Physiol 99 (5):802-815. doi: 10.1113/expphysiol.2013.077610

24 Stinghen AE, Gonçalves SM, Martines EG, Nakao LS, Riella MC, Aita CA, Pecoits-Filho R. (2009) Increased plasma and endothelial cell expression of chemokines and adhesion molecules in chronic kidney disease. Nephron Clin Pract 111(2):c117-26. doi: 10.1159/000191205.

25 Axelsson J, Bergsten A, Qureshi AR, Heimbuguer U, Bárány P, Lonnqvist P, Lindholm B, Nordfors L, Alvestrand A, Stenvinkel P (2006) Elevated resistin levels in chronic kidney disease are associated with decreased glomerular filtration rate and inflammation, but not with insulin resistance. Kidney International 69(3):596–604. doi: 10.1038/sj.ki.5000089

26 Wasilewska A, Tomaszewska B, Zoch-Zwierz W, Biernacka A, Klewinowska K, Koput A (2005) Serum and urine leptin concentration in children with nephrotic syndrome. Pediatr Nephrol 20(5):597-602. doi: 10.1007/s00467-004-1772-x.

27 Hamano K, Iwano M, Akai Y, Sato H, Kubo A, Nishitani Y, Dohi K (2002) Expression of glomerular plasminogen activator inhibitor type 1 in glomerulonephritis. American Journal of Kidney Diseases, 39(4):695–705. doi:10.1053/ajkd.2002.31986

APÊNDICE

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

(responsável legal)

"Interação de marcadores de inflamação com moléculas do Sistema Renina Angiotensina na progressão da Síndrome Nefrótica Primária em pacientes pediátricos"

Por meio deste termo de consentimento, informamos que estamos desenvolvendo uma pesquisa no Hospital das Clínicas da UFMG para estudarmos substâncias do sangue, da urina chamadas pelos médicos de angiotensinas, citocinas e quimiocinas. Estas substâncias podem participar da piora do funcionamento dos rins, o que pode ocorrer em crianças e adolescentes com doença renal crônica. Já se sabe que essas substâncias participam de uma série de doenças dos rins e do coração e também devem participar da progressão da doença renal crônica.

Além disso, já existem alguns medicamentos capazes de mudar a quantidade dessas substâncias que aparecem na urina e no sangue, como, por exemplo, os corticóides, o captopril, o enalapril, o losartan e outros remédios usados normalmente para tratamento de doenças inflamatórias e da pressão alta. Alguns estudos acreditam que esses medicamentos podem melhorar alguns pacientes com doença renal porque diminuem essas substâncias que queremos estudar.

Este estudo quer, então, saber a quantidade de angiotensinas, citocinas e quimiocinas que está no sangue e na urina das crianças e dos adolescentes com doença renal crônica, bem como nos fragmentos de biópsia, quando esta for indicada para o diagnóstico da causa da doença renal crônica, tentando ver se isso tem relação com a forma da doença (se é mais branda ou mais forte), a resposta ao tratamento e a piora do funcionamento dos rins.

Os exames para determinação dessas substâncias no sangue serão coletados através de punção de veia periférica durante a mesma coleta que é feita para outros exames de rotina do paciente. A dosagem dessas substâncias na urina será feita pegando uma parte da urina de 24 horas que é colhida de rotina para medir a proteinúria dos pacientes com doença renal crônica. Naqueles pacientes submetidos à biópsia renal ou do trato urinário por indicação médica, serão examinadas também as sobras do material colhido, após o diagnóstico do patologista. Não serão colhidas amostras de tecido além daquela necessária para o diagnóstico patológico, bem como de pacientes que não tenham indicação médica para procedimentos de biópsia renal e/ou do

trato urinário. Estamos garantindo que a realização destes exames só será autorizada após assinatura deste termo de consentimento pós-informado pelo paciente e por um de seus responsáveis.

Ao assinar este formulário, você autoriza o Comitê de Ética em Pesquisa da UFMG (telefone 34094592) de Belo Horizonte e outras autoridades regulamentadoras a consultar seus registros médicos a fim de checar os dados coletados neste estudo com o que está escrito nos registros. Sua identidade não será revelada e as leis regulando tais procedimentos serão seguidas.

Este estudo seguirá as diretrizes e normas regulamentadoras de pesquisa envolvendo seres humanos – Resolução no 196/96 e 215/97 do Conselho Nacional de Saúde.

Garantimos ainda que a identidade e a privacidade do paciente serão mantidas. Os resultados desse estudo somente serão utilizados para aumentar os conhecimentos da medicina. Os resultados dos parâmetros avaliados nas amostras do seu filho serão informados ao final do estudo por meio impresso. Finalmente, será resguardado o direito de recusa em participar do trabalho em qualquer etapa do mesmo, sabendo-se que o paciente continuará a receber o acompanhamento médico convencional adequado à idade, tendo assim garantida sua assistência médica.

Eu, _____, mãe, (ou pai ou responsável) pelo paciente_____entendi tudo que foi explicado sobre a pesquisa e concordo que meu filho (ou minha filha ou outro grau de parentesco) participe do estudo sobre a quantidade de angiotensinas, citocinas e quimiocinas no sangue e na urina.

Este estudo será feito pelo Dr. Sérgio Veloso Brant Pinheiro (telefones 34099772 ou 88197208), Dra. Ana Cristina Simões e Silva (telefone 34099772) e Dr. Eduardo Araújo Oliveira (telefone 34099772), do Hospital das Clínicas da UFMG, e pelo Dr. Marco Aurélio Romano-Silva (telefone 34099785) e Dr. Robson Augusto Souza dos Santos (telefones 34092928), professores da UFMG. Dou meu consentimento para que seja coletado sangue e urina de meu filho (minha filha ou outro grau de parentesco) para medir as quantidades de angiotensinas, citocinas e quimiocinas. Confirmo que meu filho (minha filha ou outro grau de parentesco) foi selecionado de forma voluntária para participar dessa pesquisa. Eu assinei e recebi uma cópia dessa autorização.

Belo Horizonte, _____ de _____ de _____.

Assinatura do responsável: _____

Assinatura do pesquisador: _____

ANEXOS

ANEXO 1 - PRODUÇÕES TÉCNICAS E PARTICIPAÇÃO EM EVENTOS - TEMA RELACIONADO À TESE

1 **FILHA RS**, PINHEIRO SVB, MACEDO E CORDEIRO T, FERACIN V, VIEIRA ÉLM, MIRANDA AS, SIMÕES E SILVA AC. Evidence for a role of angiotensin converting enzyme 2 in proteinuria of idiopathic nephrotic syndrome. Bioscience Report 39(1):BSR20181361. https://doi.org/10.1042/BSR20181361 (Manuscrito original da tese)

2 **FILHA RS**, CASTRO PASV, MAGALHÃES RC, PINHEIRO SVB, SIMÕES E SILVA AC. Metabolic changes and endothelial dysfunction in different remission stages in children with idiopathic nephrotic syndrome. Submetido ao periódico Pediatric Nephrology (Manuscrito original da tese).

3 **FILHA RS,** BURINI K, PIRES LG, PINHEIRO SVB, SIMÕES E SILVA AC. Idiopathic Nephrotic Syndrome in Pediatrics: an up-to-date. Submetido ao periódico Current Pediatric Review (Manuscrito de revisão da tese).

4 **FILHA RS**, CASTRO PASV, MAGALHÃES RC, PINHEIRO SVB. Níveis séricos de adipocinas em crianças com síndrome nefrótica idiopática. I Simpósio do Laboratório Interdisciplinar de Investigação Médica, 2021, Belo Horizonte.

5 **FILHA RS**, BASTOS FM, PINHEIRO, PINHEIRO SVB; VIEIRA, VIEIRA ÉLM, MIRANDA AS, SIMÕES E SILVA AC. The potencial role of IP-10/CXCL10 and MCP-1/CCL2 at different stages of the Idiopathic Nephrotic Syndrome. XII Internacional Symposium on Vasoactive Peptides, 2019, Belo Horizonte.

6 **FILHA RS**, PINHEIRO SVB, MACEDO E CORDEIRO T, FERACIN V, VIEIRA ÉLM, MIRANDA AS, SIMÕES E SILVA AC. Evaluation of urinary levels of chemokines in Idiopathic Nephrotic Syndrome: a potential role of MCP-1 and IP-10/CXCL10. Inflamma IV, 2018, Belo Horizonte.

ANEXO 2 - PARECER COEP/UFMG

UNIVERSIDADE FEDERAL DE MINAS GERAIS
COMITÊ DE ÉTICA EM PESQUISA - COEP

Parecer nº. ETIC 316/09

Interessado(a): Prof. Sérgio Veloso Brant Pinheiro
Departamento de Pediatria
Faculdade de Medicina - UFMG

DECISÃO

O Comitê de Ética em Pesquisa da UFMG – COEP aprovou, no dia 02 de setembro de 2009, o projeto de pesquisa intitulado **"Doença Renal Crônica: avaliação, evolução, fatores prognósticos, marcadores de inflamação, fibrose e apoptose"** 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. Maria Teresa Marques Amaral
Coordenadora do COEP-UFMG

Av. Pres. Antonio Carlos, 6627 – Unidade Administrativa II - 2º andar – Sala 2005 – Cep:31270-901 – BH-MG
Telefax: (031) 3409-4592 - e-mail: coep@prpq.ufmg.br

ANEXO 3 – ARTIGO 1 – PUBLICAÇÃO NO PERIÓDICO BIOSCIENCE REPORTS

Bioscience Reports (2019) 39 BSR20181361
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Research Article

Evidence for a role of angiotensin converting enzyme 2 in proteinuria of idiopathic nephrotic syndrome

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Introduction: Renin angiotensin system (RAS) plays a role in idiopathic nephrotic syndrome (INS). Most studies investigated only the classical RAS axis. Therefore, the aims of the present study were to evaluate urinary levels of RAS molecules related to classical and to counter-regulatory axes in pediatric patients with INS, to compare the measurements with levels in healthy controls and to search for associations with inflammatory molecules, proteinuria and disease treatment. **Subjects and methods:** This cross-sectional study included 31 patients with INS and 19 healthy controls, matched for age and sex. Patients and controls were submitted to urine collection for measurement of RAS molecules [Ang II, Ang-(1-7), ACE and ACE2] by enzyme immunoassay and cytokines by Cytometric Bead Array. Findings in INS patients were compared according to proteinuria: absent (<150 mg/dl, $n = 15$) and present (≥150 mg/dl, $n = 16$). **Results:** In comparison to controls, INS patients had increased Ang II, Ang-(1-7) and ACE, levels while ACE2 was reduced. INS patients with proteinuria had lower levels of ACE2 than those without proteinuria. ACE2 levels were negatively correlated with 24-h-proteinuria. Urinary concentrations of MCP-1/CCL2 were significantly higher in INS patients, positively correlated with Ang II and negatively with Ang-(1-7). ACE2 concentrations were negatively correlated with IP-10/CXCL-10 levels, which, in turn, were positively correlated with 24-h-proteinuria. **Conclusion:** INS patients exhibited changes in RAS molecules and in chemokines. Proteinuria was associated with low levels of ACE2 and high levels of inflammatory molecules.

Introduction

The pathophysiological mechanisms of idiopathic nephrotic syndrome (INS) support an underlying role for the immune system [1,2]. This hypothesis relies on the fact that the main treatment is based on corticosteroids and studies show different inflammatory profiles in patients with INS [3–6]. Experimental and clinical studies also suggest that renin angiotensin system (RAS) may play an important role on the pathogenesis of INS [7–10].

RAS is classically described as a circulating system that leads to the production of angiotensin II (Ang II), which binds to angiotensin type 1 receptors (AT_1), promoting vasoconstriction, sodium retention, aldosterone release, inflammation and fibrosis [11]. However, RAS is now conceived as a dual acting system, mainly formed by two opposite axes [12–14] the classical one, including angiotensin converting enzyme (ACE), Ang II and AT_1 receptor and the counter-regulatory axis formed by the enzyme homologue to ACE, named ACE2 [15,16], the heptapeptide Angiotensin-(1-7) [Ang-(1-7)] and its receptor, Mas [17]. Ang-(1-7) is mainly produced by ACE2 that uses Ang II as the major substrate [18]. Ang-(1-7) exerts its effects via Mas receptor activation, leading to vasodilation, anti-inflammatory and anti-fibrogenic effects [12–14]. The unbalance between both RAS axes is thought to have an active role in renal diseases [12].

Experimental studies indicate that activation of the counter-regulatory RAS axis reduces renal inflammation, fibrosis and proteinuria. In a murine model of nephrotic syndrome (NS), the treatment with a Mas receptor agonist, the compound AVE0991, reduced proteinuria, renal levels of TGF-beta and renal tissue damage [8]. On the other hand, NS induced in Mas receptor knockout mice did not improved in response to the use of AT_1 receptor blocker (ARB), suggesting that the presence of Mas receptor is critical for the therapeutic response to ARBs [8]. Despite clinical studies showing beneficial effects of ACE inhibitors and ARBs [6,7], there are no studies evaluating the role of the counter-regulatory RAS axis in INS patients, mostly in pediatric population. Therefore, the aims of the present study were to evaluate urinary levels of RAS molecules [(ACE, ACE2, Ang II and Ang-(1-7)] in pediatric patients with INS and to compare with the same measurements in healthy sex- and age-matched children. In addition, measurements of RAS molecules were correlated with proteinuria and with urinary levels of markers of inflammation in pediatric patients with INS.

Subjects and methods

Study design

This is a cross-sectional study with a sample of children and adolescents with remitted and partially remitted INS, followed-up at the Pediatric Nephrology Unit (PNU) of our institution from 2017 to 2018. Our PNU has attended approximately 300 children with nephrotic syndrome, according to a systematic protocol that includes definition of disease etiology, assessment of clinical course and laboratory alterations, institution of treatment protocols and indication of renal biopsy based on clinical (corticosteroid unresponsiveness) and laboratory findings as detailed elsewhere [19]. The diagnostic criteria for INS were based on the KDIGO Clinical Practice Guideline for Glomerulonephritis (2012) [20].

Patients with INS

Thirty-one patients ranging from 8 to 16 years with INS in total or partial remission were included in the study. The inclusion criteria were still-preserved renal function, well-established diagnosis of INS and complete or partial remission of the disease. After parents' consent, urine samples were collected simultaneously to routine laboratory exams. Exclusion criteria were congenital nephrotic syndrome, secondary forms of nephrotic syndrome, INS patients at stages 2–5 of chronic kidney disease, INS patients during clinical and laboratory relapses and the presence of acute infections and allergies at the moment of urine collection.

Control group

The control group consisted of 19 healthy sex- and age-matched subjects from the Pediatric Primary Care Center. Healthy status was determined through the subjects' medical history and either a parental report or self-report to rule out the presence of chronic or acute diseases.

Ethical aspects

The Ethics Committee of our institution approved the present study under the protocol CAAE-07513513.9.0000.5149. Informed consent was obtained from the parents of all included subjects. The research protocol did not interfere with any medical recommendations or prescriptions. The follow-up of the INS patients and healthy controls was guaranteed even in cases of refusal to participate in the study.

Study protocol

The present study included only patients presenting complete or partial remission of INS. Patients with 24-h urine protein excretion above or equal to 150 mg/dl and absence of edema were considered in partial remission. Patients with 24 h urine protein excretion below 150 mg/dl and absence of edema were considered in complete remission. Patients exhibiting relapses of edema and intense proteinuria were excluded. The established reference for proteinuria was based on the KDIGO [20]. Clinical characteristics and casual measurements were obtained at the same time of urine collection. Clinical variables were age, gender, height, weight, body mass index, and systolic and diastolic blood pressure. In INS patients, serum levels of creatinine, albumin, cholesterol and triglycerides were assessed at the same time of urine collection obtained for the measurements of RAS molecules and immune mediators. Urinary determinations of creatinine and of 24-h protein excretion were also performed simultaneously to other measurements. Glomerular filtration rate (GFR) was estimated using the modified formula of Schwartz et al. [21]. Renal biopsy results

and medications in use at the time of urine sampling were also analyzed.

Table 1 **Clinical and laboratorial findings of patients with idiopathic nephrotic syndrome and healthy controls matched by sex and age.**

Parameters	Patients ($n = 31$)	Controls ($n = 19$)	*P* values
Age (years)	11.3 ± 4.8	11.9 ± 1.8	0.912
Sex (male / female)	19 / 12	12 / 7	0.905
BMI (kg/m^2)	19.3 ± 3.2	17.8 ± 2.6	0.094
Creatinine (mg/dl)	0.6 ± 0.2	0.6 ± 0.1	0.210
Triglycerides (mg/dl)	114.4 ± 79.6	–	–
Total cholesterol (mg/dl)	188.8 ± 92.9	–	–
Albumin (g/dl)	4.0 ± 0.7	4.5 ± 0.2	
Glomerular filtration rate*	109.2 ± 28.8	119 ± 12	0.254
Proteinuria (mg/m^2/24 h)	304.2 ± 378.5	<100 mg/dl	–
Medications in use			–
No medication	4	19	*<0.001*
Only steroids	2	–	–
Steroids + ACEi or ARB	16	–	–
Only ACEi or ARB	0	–	–
Only cyclosporine	0	–	–
Cyclosporine + ACEi or ARB	9	–	–
Histopathology			
No biopsy	15	–	–
MDC	5	–	–
FSGS	5	–	–
Membranous nephropathy	3	–	–
Mesangial glomerulopathy	3	–	–

Values are expressed as mean and standard deviation. Sex, medications in use and renal histopathology are expressed as absolute values.
*Glomerular filtration rate was estimated using modified Schwartz's formula. Abbreviations: ACEi, inhibitor of the angiotensin converting enzyme; ARB, angiotensin receptor type 1 blocker; BMI, body mass index; FSGS, focal segmental glomerulosclerosis; MDC, minimal change disease.

INS patients were subdivided according to the values of proteinuria, which ranged from 48 mg/dl to 1220 mg/dl. Thereby, those with 24-h urinary protein excretion inferior to 150 mg/dl were allocated to the subgroup named absence of proteinuria (n=15) and when proteinuria was equal to or above 150 mg/dl patients were included in the subgroup called presence of proteinuria (n= 16).

INS patients were also analyzed in regard to the use or not of medications that directly interfere with RAS as ACE inhibitors and/or ARBs and to the use or not of steroids. Therefore, 16 patients in use of ACE inhibitors and/ or ARBs were compared with 15 patients not receiving these medications and 18 patients in use of steroids were compared with 13 not under treatment at the time of urine sampling.

Urine samples

Urine specimens for the measurement of biomarkers were collected into sterile dry tubes. After homogenization, 15 ml of the collected urine were centrifuged at 4˚C for 5 min and aliquoted into 1 ml tubes and stored at 80˚C until the measurements.

Renin angiotensin system (RAS) components

Urine levels of RAS molecules [Ang II, Ang-(1-7), ACE and ACE2] were measured by enzyme immunoassay (ELISA), according to procedures supplied by the manufacturer (MyBioSource, San Diego, CA, USA). All kits applied sandwich

ELISA technique, except for ACE measurement whose kit applied competitive ELISA method. The sensitivity of the assays was 1.0 pg/ml for ACE and ACE2, 2.0 pg/ml for Ang II and Ang-(1-7) and reading the optical density at 450 nm. All biochemical assessments were performed blinded in regard to clinical diagnosis.

Cytokines and chemokines measurements

The urinary levels of multiple cytokines [interleukin (IL)-12p70, IL-6, IL-8, IL-10, IL-1β, tumor necrosis factor (TNF) and interferon gamma (IFN-γ)] and chemokines [induced protein 10 (IP-10/CXCL-10), monocyte chemoattractant protein-1 (MCP-1/CCL2), IL-8/CXCL8, monokine induced by gamma interferon (MIG/CXCL9), regulated on activation normal T cell expressed and secreted (RANTES/CCL5)] were assessed simultaneously using a Human FlexSet kit for Cytometric Bead Array (CBA, BD Bioscience, San Jose, CA, USA), following manufacture's instruction. The acquisition was performed using an FACSCanto II flow cytometer (BD Biosciences, San Jose, CA, USA). The instrument has been checked for sensitivity and overall performance with Cytometer Setup & Tracking beads (BD Biosciences) prior to data acquisition. Quantitative results were generated using FCAP Array v1.0.1 software (Soft Flow Inc., Pecs, Hungary). Urinary levels of all these biomarkers were expressed as concentrations standardized for urine creatinine and expressed as pictograms per milligram. Positive controls were also included in urine measurements of cytokines and chemokines to confirm the accuracy of the assays.

Table 2 **Clinical and laboratorial findings of patients with idiopathic nephrotic syndrome subdivided according to 24-h urinary protein excretion: absence of proteinuria ≤150 mg/dl (without proteinuria) and presence of proteinuria >150 mg/dl (with proteinuria)**

Parameters	Without proteinuria ($n = 15$)	With proteinuria ($n = 16$)	*P* values
Age (years)	10.6 ± 3.2	13.1 ± 4.4	0.385
Sex (male / female)	10 / 5	7 / 7	0.078
BMI (kg/m^2)	19.1 ± 2.5	19.7 ± 3.9	0.981
Creatinine (mg/dl)	0.53 ± 0.10	0.70 ± 0.32	0.162
Triglycerides (mg/dl)	98.5 ± 59.7	132.7 ± 97.1	0.205
Total cholesterol (mg/dl)	186.1 ± 107.8	186.4 ± 78.3	0.519
Albumin (g/dl)	4.1 ± 0.76	3.9 ± 0.5	0.285
Glomerular filtration rate*	113.8 ± 25.7	102.1 ± 32.8	0.326
Proteinuria (mg/m^2/24 h)	80.7 ± 25.4	543.7 ± 434.1	***<0.0001***
Medications in use			
Only steroids	1	1	0.859
Steroids + ACEi or ARB	4	10	0.076
Only ACEi or ARB	0	0	0.125
Only cyclosporine	0	0	0.806
Cyclosporine + ACEi or ARB	5	4	0.806
No medications	5	1	0.093
Histopathology			0.535
No biopsy	6	7	
MDC	4	1	
FSGS	2	3	
Membranous nephropathy	1	2	
Mesangial glomerulopathy	2	1	

Values are expressed as mean and standard deviation. Sex, medications and renal histopathology in use are expressed as absolute values.
*Glomerular filtration rate was estimated using modified Schwartz's formula. Abbreviations: ACEi, inhibitor of the angiotensin converting enzyme; ARB, angiotensin receptor type 1 blocker; BMI, body mass index; FSGS, focal segmental glomerulosclerosis; MDC, minimal change disease.

Statistical analysis

The softwares SPSS version 22.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA, USA) were used for statistical analysis. The results obtained were expressed as means and standard error of mean (SEM), medians and interquartile range or percentages, when appropriate. Categorical variables were compared by Qui-square. Gaussian distribution was checked by Shapiro–Wilk test. For variables without Gaussian distribution, Mann–Whitney test was used to compare two groups. For variables with normal distribution, comparisons between two groups were made by unpaired Student's t test. Spearman's correlation analyses examined the relationship between proteinuria, urinary levels of RAS components and measurements of inflammatory molecules in the same samples. All statistical tests were two-tailed with a significance level of $P < 0.05$.

Results

Subject characteristics and casual measurements

Clinical and laboratory findings of INS patients and healthy controls are shown in Table 1. The control group (n =19) included 12 boys and 7 girls, ranging from 9 to 15 years old. All controls had clinical and laboratory parameters within

Table 3 **Clinical and laboratorial findings of patients with idiopathic nephrotic syndrome subdivided according to use or not of medications that directly interfere with RAS: ACE inhibitors (ACEi) and ARB.**

Parameters	ACEi or ARB (*n* = 25)	No ACEi or ARB (*n* = 6)	*P* values
Age (years)	11.4 ± 4.6	10.7 ± 4.5	0.585
Sex (male / female)	14 / 9	5 / 3	0.957
BMI (kg/m^2)	19.8 ± 3.3	13.9 ± 8.8	0.260
Creatinine (mg/dl)	0.61 ± 0.28	0.54 ± 0.12	0.964
Triglycerides (mg/dl)	117.9 ± 87.2	103.9 ± 55.9	0.852
Total cholesterol (mg/dl)	186.2 ± 68.8	197 ± 153.8	0.320
Albumin (g/dl)	3.9 ± 0.5	4.2 ± 0.9	0.181
Glomerular filtration rate*	108.1 ± 29.4	112.3 ± 28.6	0.910
Proteinuria (mg/m^2/24 h)	380.2 ± 421.8	104.9 ± 44.6	0.118
Medications in use		–	
Only steroids	0	2	*0.032*
Steroids + ACEi or ARB	16	0	–
Only ACEi or ARB	0	0	–
Only cyclosporine	0	0	–
Cyclosporine + ACEi or ARB	9	0	–
No medications	0	4	*<0.0001*
Histopatology		0.920	
No biopsy	10	5	
MDC	4	0	
FSGS	4	1	
Membranous nephropathy	2	1	
Mesangial glomerulopathy	2	1	

Values are expressed as mean and standard deviation. Sex, medications and renal histopathology in use are expressed as absolute values.
*Glomerular filtration rate was estimated using modified Schwartz's formula. Abbreviations: ACEi, inhibitor of the angiotensin converting enzyme; ARB, angiotensin receptor type 1 blocker; BMI, body mass index; FSGS, focal segmental glomerulosclerosis; MDC, minimal change disease.

normal range (Table 1). No statistical differences between INS patients (n=31) and controls were found in age, sex distribution, body mass index, GFR, plasma levels of triglycerides, cholesterol, creatinine and albumin ($P > 0.05$ for all comparisons, Table 1). As expected, the values of proteinuria were significantly higher in INS patients than in healthy controls (Table 1).

In regard to treatment, 6 among 31 (19%) INS patients were not receiving any medication at the time of urine collection, whereas the remaining patients needed at least one medication (Table 1). Sixteen among 25 (64%) patients of the INS group were receiving steroids and 9 (36%) cyclosporine, both isolated or in association with inhibitors of the RAS. In regard to histopathology, 15 (48.4%) patients were not submitted to renal biopsy, 5 (16.1%) presented focal segmental glomerulosclerosis, 5 (16.1%) had minimal change disease, 3 (9.7%) exhibited mesangial glomerulopathy and 3 (9.7%) membranous glomerulopathy.

The comparison between INS patients without proteinuria (absent) versus patients with proteinuria (present) is shown in Table 2. No differences in clinical and laboratorial findings were detected ($P > 0.05$ for all comparisons, Table 2), except for 24-h proteinuria.

Table 3 shows the comparison between patients receiving (n = 16) and not receiving ACE inhibitors and/or ARBs (n = 15).The only difference between both subgroups was the medication in use. No significant differences were detected in regard to age, sex distribution, clinical data, laboratory measurements and renal histopathology ($P > 0.05$ for all comparisons, Table 3).

RAS components analyses

In comparison to healthy controls, INS patients presented increased urinary levels of Ang II, Ang-(1-7) and ACE ($P < 0.05$ for all comparisons, Figure 1). On the other hand, urinary levels of ACE2 were significantly lower in INS patients than in controls ($P < 0.0003$, Figure 1).

Correlations were evaluated between RAS components and proteinuria. In INS patients, urinary levels of ACE2 were negatively correlated with 24-urine protein excretion (rho = −0.508, $P = 0.005$). Furthermore, INS patients with proteinuria had significantly lower levels of ACE2 in urine than INS patients without proteinuria ($P = 0.02$, Table 4).

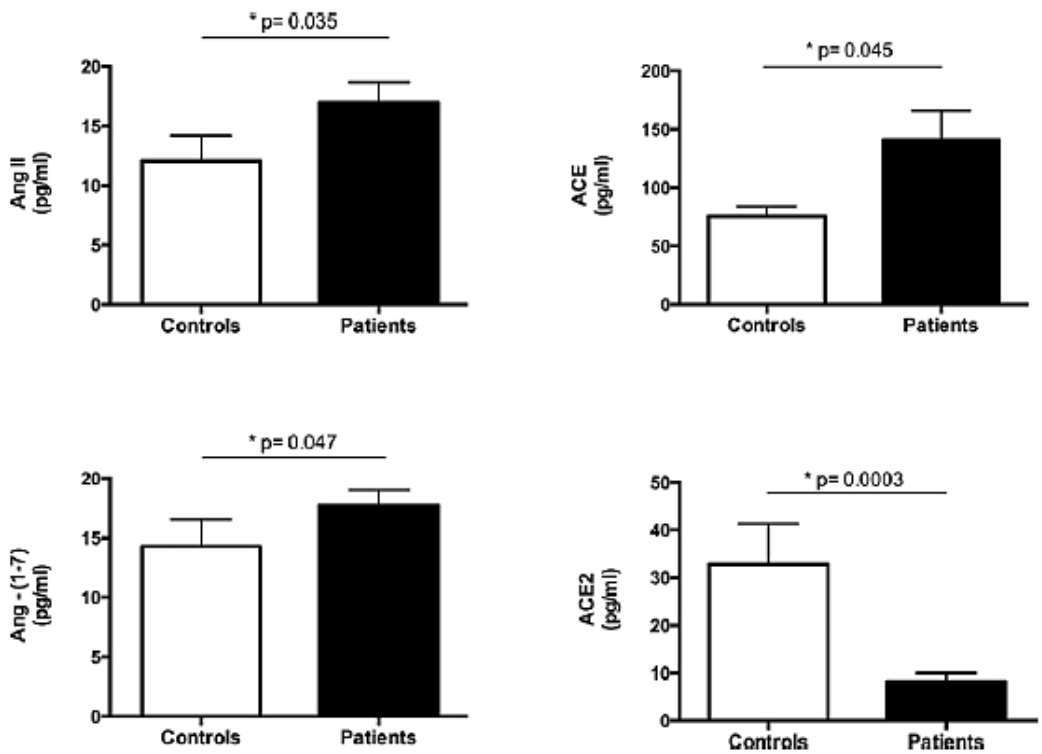

Figure 1. Levels of RAS components in urine samples of patients with INS and healthy sex and age-matched subjects (controls)

Patients with INS presented increased urinary levels of angiotensin (Ang) II, angiotensin-converting enzyme (ACE) and Ang-(1-7) in comparison with controls. On the other hand, ACE2 levels were lower in INS patients than in controls. Results are expressed as bar graphs with mean values and SEM. Differences were considered to be significant at $P < 0.05$ (Mann–Whitney U test).

Table 4 Urinary levels of RAS components in idiopathic nephrotic syndrome patients without proteinuria (≤150 mg/dl/day) and with proteinuria (>150 mg/dl/day)

RAS components	Without proteinuria ($n = 15$)	With proteinuria ($n = 16$)	*P* values
Ang II (pg/ml)	16.4 ± 8.4	14.7 ± 8.0	0.570
ACE (pg/ml)	98.4 ± 50.0	172.9 ± 162.2	0.247
Ang-(1-7) (pg/ml)	17.5 ± 7.5	16.0 ± 6.8	0.662
ACE2 (pg/ml)	13.5 ±10.7	2.9 ± 5.2	***0.023***

Values are expressed as mean ± standard deviation. Abbreviations: ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; Ang II, angiotensin II; Ang-(1-7), angiotensin-(1-7).

Regarding other RAS components [Ang II, ACE and Ang-(1-7)], there were no correlations with 24-urine protein excretion. Accordingly, no differences were found in the comparison of these molecules in INS patients without proteinuria versus those with proteinuria (Table 4).

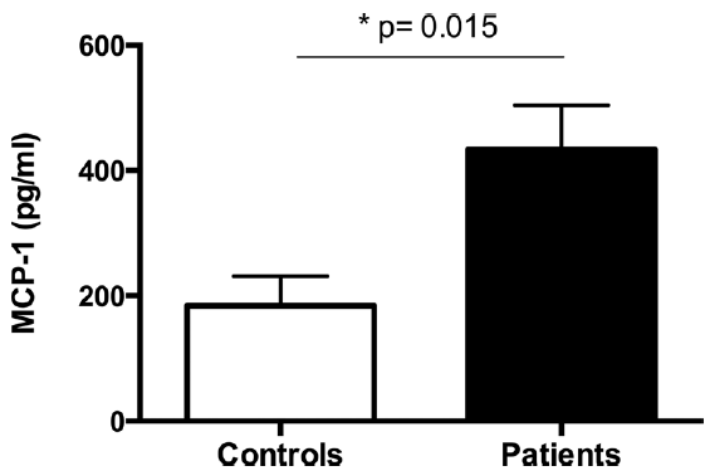

Figure 2. Urinary levels of MCP-1/CCL2 in patients with primary nephrotic syndrome (INS) and in healthy sex and age-matched subjects (controls)

Patients with INS had increased urinary levels of MCP-1/CCL2. Results are expressed as bar graphs with mean values and SEM. Differences are considered to be significant at $P < 0.05$ (Mann–Whitney *U* test). MCP-1/CCL2 = monocyte chemoattractant protein-1.

Chemokines and cytokines analyses

As shown in Figure 2, urinary concentrations of MCP-1/CCL2 were significantly higher in INS patients when compared with the control group (P = 0.015). However, urinary levels of other chemokines (IP-10/CXCL-10, MIG/CXCL9, RANTES/CCL5, IL-8/CCCL8) and of cytokines (IL-12p70, TNF, IL-10, IL-6, IL-1β, IFN) did not significantly differ in the comparison of INS patients and healthy controls.

We also investigated the relation between urinary levels of chemokines and cytokines with 24-h urine protein excretion. Urinary levels of IP-10/CXCL-10 were positively correlated with 24-h urine protein excretion in INS patients (rho= 0.3882, P= 0.037). No correlations were found between 24-h urine protein excretion with other chemokines and cytokines.

Correlations between RAS components and immune system molecules in INS patients

Urinary levels of chemokines and cytokines were also checked for correlations with RAS molecules in the same urine samples. In INS patients, levels of Ang II positively correlated with MCP-1/CCL2 levels (rho=0.424, P=0.017). On the other hand, as shown in Figure 3, Ang-(1-7) levels negatively correlated with MCP-1/CCL2 (rho=-0.485, P=0.006, Figure 3 – panel superior) and MIG (rho=-0.379, P=0.035, Figure 3 – panel intermediate) and ACE2 levels were negatively correlated with IP-10/CXCL-10 (rho=-0.420, P=0.018, Figure 3 – panel inferior). No correlations were found between cytokines and RAS molecules.

Effect of RAS inhibition on RAS components, proteinuria and immune system molecules

As shown in Table 3, 16 patients were receiving ACE inhibitors or ARBs at the time of urine collection. No differences were detected in urine levels of RAS molecules and in proteinuria when INS patients receiving RAS inhibitors were compared with those not receiving these medications. The same profile was not observed when inflammatory molecules were analyzed. INS patients receiving ACE inhibitors or ARBs had significantly higher urinary levels of IP-10/CXCL-10 and of IL-10 in comparison to patients not under RAS inhibition (Table 5).

Effect of steroids administration on RAS components and immunesystem molecules

INS patients were also subdivided in regard to the use or not of steroids at the time of urinary measurements of RAS components, cytokines and chemokines. Urinary levels of ACE2 were significantly lower in patients under steroid therapy in comparison to those not receiving this medication (3.6 ±4.7 pg/ml versus 15.2 ± 9.3 pg/ml, P= 0.033).

However, urinary levels of ACE, Ang II and Ang-(1-7) did not significantly differ in these subgroups. In regard to cytokines and chemokines, patients receiving steroids had higher urinary levels of IP-10/CXCL-10 (128.6 ± 81.5 pg/ml versus 95.7 ± 125.7 pg/ml, P= 0.016) than those not under this treatment at the time of urine sampling. Other molecules did not significantly differ in these subgroups.

Table 5 **Urinary levels of inflammatory molecules in patients with idiophatic nephrotic syndrome subdivided according to the use or not of medications that directly interfere with RAS: ACEi and ARBs.**

Inflammatory molecules	Use ACEi and/or ARB (n = 16/11)	No use ACEi and/or ARB (n = 15/20)	P values
IP-10 (pg/ml)	134.8 ± 506.9	98.0 ± 448.1	*0.008*
MCP-1 (pg/ml)	454.8 ± 406.9	416.3 ± 384.5	0.675
MIG (pg/ml)	83.7 ± 158.6	55.3 ± 134.6	0.115
IL-8 (pg/ml)	26.7 ± 71.3	23.2 ± 64.6	0.187
IL-12p70 (pg/ml)	9.6 ± 9.0	6.7 ± 8.3	0.083
TNF (pg/ml)	10.6 ± 10.3	7.4 ± 9.3	0.095
IL-10 (pg/ml)	7.0 ± 6.7	4.6 ± 6.1	*0.042*
IL-6 (pg/ml)	12.1 ± 9.7	9.3 ± 9.5	0.056
IL-1β (pg/ml)	26.1 ± 22.6	18.4 ± 20.1	0.219

Values are expressed as mean ± standard deviation.

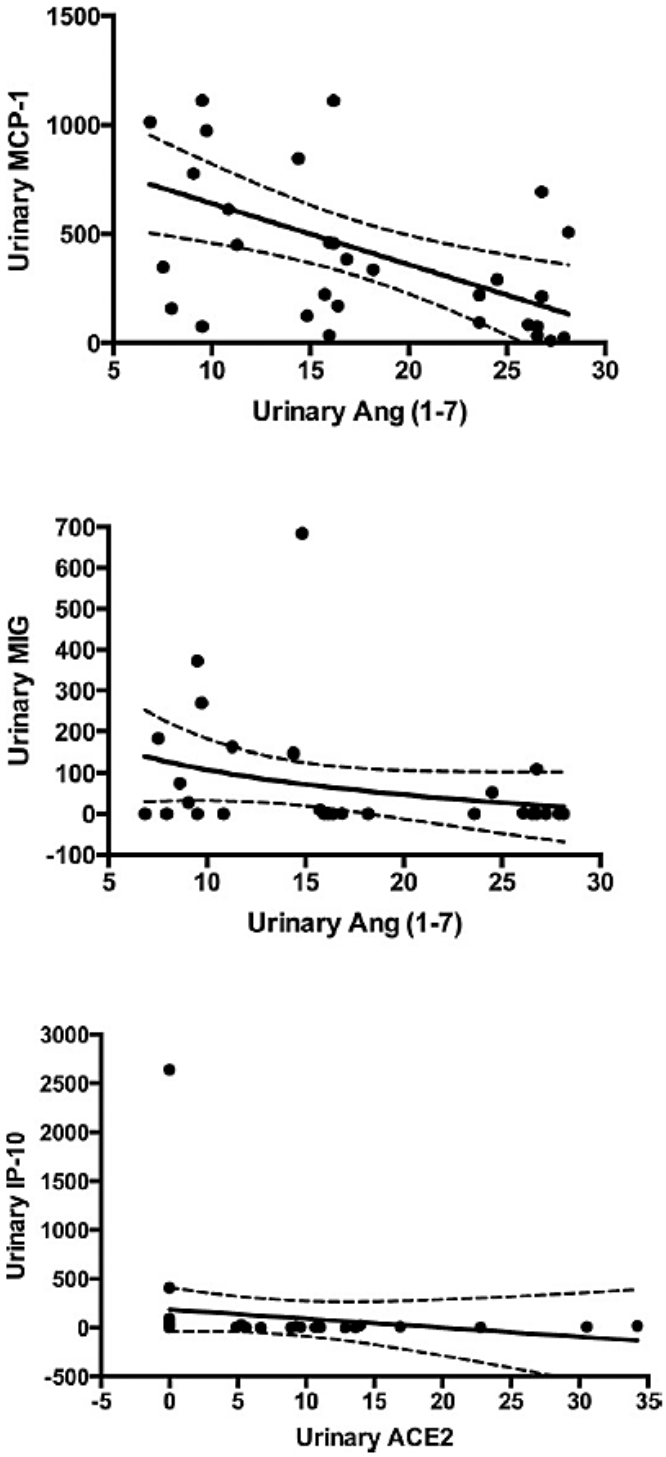

Figure 3. Correlations between RAS molecules and chemokines

Panel superior shows a negative correlation between urinary levels of Angiotensin-(1-7) [Ang-(1-7)] and urinary levels of MCP-1/CCL2 (rho = −0.485, $P = 0.006$, Spearman correlation test). Panel intermediate displays a negative correlation between urinary levels of Ang-(1-7) and urinary levels of and MIG (rho = −0.379, $P = 0.035$, Spearman correlation test). Panel inferior shows a negative correlation between urinary levels of Angiotensin Converting Enzyme 2 (ACE2) and urinary levels of IP-10/CXCL-10 (rho = −0.420, $P = 0.018$, Spearman correlation test).

Discussion

INS patients in complete or partial remission had significantly higher levels of ACE, Ang II and Ang-(1-7) in urine, while urinary concentrations of ACE2 were significantly lower than in healthy controls. In addition, ACE2 levels were significantly reduced in INS patients with proteinuria in comparison to those without proteinuria and urinary concentrations of this enzyme negatively correlated with 24-h urinary protein excretion.

Acquired or genetic deficiency of ACE2 exacerbated kidney injury and proteinuria in many experimental models of renal diseases, possibly facilitating the deleterious effects of Ang II [22–27]. Renal expression of ACE2 was reduced in renal cortex of mice submitted to 5/6 nephrectomy and in a rat model of renal ischemia/reperfusion [27,28]. In a model of unilateral ureteral obstruction, the deletion of ACE2 gene resulted in a four-fold increase in the ratio of intrarenal Ang II/Ang-(1-7) and these changes were associated with tubulointerstitial fibrosis and high levels of TNF, IL-1β and MCP-1 [29]. Accordingly, we found that urinary concentrations of Ang II were elevated in INS patients when compared with healthy controls and were positively correlated with urinary levels of MCP-1. More recently, the daily administration of ethanol to pregnant rats resulted in glomerulosclerosis and interstitial fibrosis of the kidneys of adult offspring, accompanied by elevated levels of serum creatinine, proteinuria, total cholesterol and reduced concentrations of serum albumin [10]. These renal alterations compatible to NS were associated with increased serum levels of Ang II, high gene expression of ACE in renal tissue and reduced expression of ACE2 and of Mas receptor in the kidneys [10]. Taken together, these experimental studies indicate that the deficiency of ACE2 promotes renal tissue lesion.

Most data regarding RAS components are obtained in experimental models. Few data from human samples corroborate our findings. Mizuiri et al. [30] showed that renal biopsies from patients with IgA nephropathy had significantly reduced glomerular and tubulointerstitial immunostaining for ACE2 when compared with healthy controls, while glomerular ACE staining was increased. These findings raise the possibility that an upward shift in the intrarenal ACE/ACE2 ratio favoring increased synthesis of Ang II and reduction in Ang-(1-7) might lead to progressive nephron loss in this condition [30]. Our research group recently detected lower urinary levels of ACE2 in children with sickle cell anemia (SCA) presenting persistent proteinuria in comparison to SCA patients with normal albumin excretion in urine, also suggesting a role of reduced ACE2 protein in renal tissue in the emergence of proteinuria and nephropathy [31].

Another interesting finding of the present study was the elevation of Ang-(1-7) levels in urine of INS patients in comparison to controls. Experimental models of renal diseases showed a protective role for Ang-(1-7) [8,29,32–38]. In an experimental model of NS, the adriamycin-induced nephropathy, oral administration of AVE 0991, a Mas receptor agonist, improved renal function, reduced proteinuria and attenuated histological changes [8]. AVE 0991 or Ang-(1-7) administration also exerted renoprotective effects in experimental acute renal injury [35] and in chronic intermittent hypoxia [38]. These effects seem to be mediated, at least in part, by reducing inflammation, oxidative stress and fibrosis [38]. The infusion of Ang-(1-7) also prevented renal lesion in a model of unilateral ureteral obstruction by suppressing renal apoptosis, fibrosis, and possibly AT_1 receptor expression [29]. On the other hand, Ang-(1-7) administration increased ACE2 expression [29]. We may speculate that the increase in urinary levels of Ang-(1-7) would be a compensatory response to renal damage elicited by the activation of ACE-Ang II-AT_1 receptor axis in INS patients. A similar counter-regulatory response was reported in urine of fetuses with posterior urethral valves [39].

The urine of these fetuses showed intense increase in several cytokines and chemokines [40] and these changes may be opposed by a compensatory activation of ACE2-Ang-(1-7)-Mas receptor axis [39]. Another possible explanation for urinary elevation of Ang-(1-7) could be a dysfunction or reduced expression of Mas receptor at kidney tissue. Accordingly, Ng et al. [41] reported that Mas receptor expression is reduced in the kidneys of CKD rats.

In INS patients, inflammatory molecules may also contribute to disease activity and to RAS molecules changes. Urinary concentrations of MCP-1/CCL2 were significantly higher in INS patients when compared with control group, whereas levels of IP-10/CXCL-10 in urine were positively correlated with 24-hour proteinuria. Accordingly, Vianna and co-workers [42] previously found higher urinary levels of MCP-1/CCL2 in patients with CKD due to focal segmental glomerulosclerosis than in cases of congenital uropathies. More recently, Matsumoto and co-workers [9] showed that urinary level of MCP-1/CCL2 was significantly higher in steroid resistant INS than in steroid sensitive patients, supporting the idea that urinary MCP-1/CCL2 might contribute to the recruitment of macrophages into glomeruli. Remarkably, we found that urinary concentrations of Ang-(1-7) were negatively correlated with MCP-1/CCL2, whereas ACE2 levels negatively correlated with IP-10/CXCL-10. Therefore, we believe that Ang-(1-7) may oppose the glomerular damage induced by MCP-1/CCL2 attracted macrophages. In turn, the reduced expression of ACE2 might result in altered release of IP-10/CXCL-10 in renal tissue, which affected glomerular permeability leading to proteinuria. To corroborate this hypothesis, Han et al. [43] previously reported that rats submitted to puromycin-induced nephropathy and to anti-nephrin antibody-induced nephropathy when treated with anti-IP-10/CXCL-10 function-blocking antibody displayed a decrease in the protein level of slit-diaphragm com-

ponents and exacerbated proteinuria [43]. The authors also showed that the expression of CXCR3 increases in the injured podocyte in parallel with that of IP-10/CXCL-10, suggesting that IP-10 binds to CXCR3 in podocytes. The final conclusion is that IP-10/CXCL-10 may become a possible therapeutic target candidate in podocyte injury [43]. ACE inhibitors and ARBs exert renoprotective effects in glomerular diseases, mostly by reducing proteinuria [44]. However, scarce studies have investigated the effects of these medications in pediatric patients with INS [6,7]. It has also been suggested that the beneficial effects of these medications may be due, at least in part, to an activation of ACE2/Ang-(1-7)/Mas axis [45–47]. However, we did not find changes in urine concentrations of RAS components in INS patients receiving ACE inhibitors or ARBs. On the other hand, patients under treatment had higher levels of IP-10/CXCL-10 and IL-10. We did not know if changes in these cytokines are a consequence of the treatment or, alternatively, if increased levels of these molecules are related to INS itself. As already mentioned, experimental data support a role for IP-10/CXCL-10 in slit-diaphragm function [43], which might be altered in patients with more intense proteinuria. In turn, IL-10 is considered an anti-inflammatory cytokine, which may contribute to the beneficial effects of RAS blockers [48].

Our study has some limitations. First, the cross-sectional design precludes any conclusion regarding the cause of molecular changes. We probably would have obtained more conclusive results with longitudinal urine collections. Secondly, the use of immunosuppressive medications and/or RAS blockers at the time of urine collection may interfere with molecular measurements. Thirdly, we did not investigate the expression of Mas and AT_1 receptors in renal tissue of INS patients. Fourthly, we did not use the traditional method, the radioimmunoassay, to measure RAS components. On the other hand, the inclusion of only relatively well controlled INS patients, exhibiting partial or total remission of the proteinuria, may increase the homogeneity of our sample and the results may represent more accurately changes related to disease itself rather than acute alterations due to relapses. Moreover, the utilization of a well-established protocol for molecular measurements may increase the strength of our findings [31,39,40].

In conclusion, our results support that ACE2 may exert renoprotective effects. Accordingly, genetic deficiency of ACE2 activity in mice fosters oxidative stress via AT_1 dependent effect in the kidney [49]. In addition, daily treatment with recombinant ACE2 ameliorated renal fibrosis in apolipoprotein E-deficient mice via augmentation of Ang-(1-7)/Ang II ratio [50]. Taken together, studies indicated that the reduction in ACE2 levels at renal tissue might play a role in proteinuria and renal damage in glomerular diseases.

Clinical perspectives

In view of the advances of INS in the last years in the pediatric population and the lack of knowledge on pathophysiological mechanisms, different hypotheses have been suggested. There is evidence that RAS molecules and changes in inflammatory and cytokine expression are closely involved in the mechanisms of renal injury progression.

ACE2 levels were significantly reduced in INS patients with proteinuria in comparison to those without proteinuria and urinary concentrations of this enzyme negatively correlated with 24-h urinary protein excretion.

- The chemokines MCP-1/CCL2 and IP-10/CXCL-10 may also contribute to proteinuria and to changes in RAS molecules in INS patients.
- The understanding on the interactions between RAS and immunoinflammatory molecules enables new strategies for diagnosis and therapeutic approach.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Author Contribution

ACSS, RSF, SVBP and ASM conceived research idea and study design; RSF, SVBP, TMC and VF collected clinical data and helped in statistical analysis; RSF, ELMV and ASM performed measurements of RAS molecules, cytokines and chemokines; ELMV, ASM and ACSS performed statistical analysis; ACSS made general supervision. All authors approved the final version of the manuscript.

Abbreviations

ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; Ang-(1-7), angiotensin-(1-7); Ang II, angiotensin II; ARB, AT1 receptor blocker; AT1, angiotensin type 1; GFR, glomerular filtration rate; IFN-γ, interferon gamma; IL, interleukin; INS, idiopathic nephrotic syndrome; MCP-1/CCL2, monocyte chemoattractant protein-1; MIG/CXCL9, monokine induced by gamma interferon; NS, nephrotic syndrome; PNU, Pediatric Nephrology Unit; RANTES/CCL5, regulated on activation normal T cell expressed and secreted; IP-10/CXCL-10, induced protein 10; RAS, renin angiotensin system; SEM, standard error of the mean; TNF, tumor necrosis factor.

References

1 Mallory, L.D., Claire, G., Rulan, S.P. and Damien, G.N. (2017) Nephrotic syndrome in infants and children: pathophysiology and management. *Paediatr. Int. Child Health* **37**, 248–258, https://doi.org/10.1080/20469047.2017.1374003

2 Pereira, W.F., Brito-Melo, G.E., Carneiro, C.M., Melo, D.S., Guimarães, F.L., Rocha-Vieira, E. et al. (2015) Increased migratory and activation cell markers of peripheral blood lymphocytes in an experimental model of nephrotic syndrome. *Mediators Inflamm.* **2015**, https://doi.org/10.1155/2015/209764

3 Souto, M.F., Teixeira, A.L., Russo, R.C., Penido, M.G., Silveira, K.D., Teixeira, M.M. et al. (2008) Immune mediators in idiopathic nephrotic syndrome: evidence for a relation between interleukin 8 and proteinuria. *Pediatr. Res.* **64**, 637–642, https://doi.org/10.1203/PDR.0b013e318186ddb2

4 Cara-Fuentes, G., Kairalla, J.A., Ishimoto, T., Rivard, C., Johnson, R.J. and Garin, E.H. (2014) Rituximab in idiopathic nephrotic syndrome: does it make sense? *Pediatr. Nephrol.* **29**, 1313–1319, https://doi.org/10.1007/s00467-013-2534-4

5 Guimarães, F.T.L., Melo, G.E.B.A., Cordeiro, T.M., Feracin, V., Vieira, E.R., Pereira, W.F. et al. (2018) T-lymphocyte-expressing inflammatory cytokines underlie persistence of proteinuria in children with idiopathic nephrotic syndrome. *J. Pediatr.* **94**, 546–553

6 Bagga, A., Mudigoudar, B.D., Hari, P. and Vasudev, V. (2004) Enalapril dosage in steroid-resistant nephrotic syndrome. *Pediatr. Nephrol.* **19**, 45–50, https://doi.org/10.1007/s00467-003-1314-y

7 Zhuwen, Y., Zhihui, L., Xiao-Chuan, W., Qing-Nan, H., Xi-Qiang, D. and Xiao-Jie, H. (2006) Effect of fosinopril in children with steroid-resistant idiopathic nephrotic syndrome. *Pediatr. Nephrol.* **21**, 967–972, https://doi.org/10.1007/s00467-006-0096-4

8 Silveira, K.D., Barroso, L.C., Vieira, A.T., Cisalpino, D., Lima, C.X., Bader, M. et al. (2013) Beneficial effects of the activation of the angiotensin-(1–7) Mas receptor in a murine model of adriamycin-induced nephropathy. *PLoS One* **8**, e66082, https://doi.org/10.1371/journal.pone.0066082

9 Matsumoto, Y., Ikezumi, Y., Kondo, T., Nakajima, Y., Yamamoto, Y., Morooka, M. et al. (2018) Urinary monocyte chemotactic protein 1 as a predictive marker of steroid responsiveness in children with idiopathic nephrotic syndrome. *Fujita Med. J.* **4**, 17–22

10 Zhu, Y., Zuo, N., Li, B., Xiong, Y., Chen, H., He, H. et al. (2018) The expressional disorder of the renal RAS mediates nephrotic syndrome of male rat offspring induced by prenatal ethanol exposure. *Toxicology* **400–401**, 9–19, https://doi.org/10.1016/j.tox.2018.03.004

11 Forrester, S.J., Booz, G.W., Sigmund, C.D., Coffman, T.M., Kawai, T., Rizzo, V. et al. (2018) Angiotensin II signal transduction: an update on mechanisms of physiology and pathophysiology. *Physiol. Rev.* **98**, 1627–1738, https://doi.org/10.1152/physrev.00038.2017

12 Simões e Silva, A.C. and Teixeira, M.M. (2016) ACE inhibition, ACE2 and Angiotensin-(1-7) in kidney and cardiac inflammation and fibrosis. *Pharmacol. Res.* **107**, 154–162, https://doi.org/10.1016/j.phrs.2016.03.018

13 Prestes, T.R.R., Rocha, N.P., Miranda, A.S., Teixeira, A.L. and Simoes-E-Silva, A.C. (2017) The anti-inflammatory potential of ACE2/angiotensin-(1-7)/Mas receptor axis: evidence from basic and clinical research. *Curr. Drug Targets* **18**, 1301–1313

14 Santos, R.A.S., Sampaio, W.O., Alzamora, A.C., Motta-Santos, D., Alenina, N., Bader, M. et al. (2018) The ACE2/angiotensin-(1–7)/Mas axis of the renin-

angiotensin system: focus on angiotensin-(1–7). *Physiol. Rev.* **98**, 505–553, https://doi.org/10.1152/physrev.00023.2016

15 Tipinis, S.R., Hooper, N.M., Hyde, R., Karran, E., Christie, G. and Turner, A.J. (2000) A human homolog of angiotensin-converting enzyme. Cloning and functional expression as a captopril-insensitive carboxypeptidase. *J. Biol. Chem.* **275**, 33238–33243

16 Donoghue, M., Hsieh, F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N. et al. (2000) A novel angiotensin-converting enzyme-related carboxypeptidase (ACE2) converts angiotensin I to angiotensin 1-9. *Circ. Res.* **87**, E1–E9, https://doi.org/10.1161/01.RES.87.5.e1

17 Santos, R.A., Simoes e Silva, A.C., Maric, C., Silva, D.M., Machado, R.P., de Burhr, I. et al. (2003) Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc. Natl Acad. Sci. U.S.A.* **100**, 8258–8263, https://doi.org/10.1073/pnas.1432869100

18 Vickers, C., Hales, P., Kaushik, V., Dick, L., Gavin, J., Tang, J. et al. (2002) Hydrolysis of biological peptides by human angiotensin-converting enzyme-related carboxypeptidase. *J. Biol. Chem.* **277**, 14838–14843, https://doi.org/10.1074/jbc.M200581200

19 Mendonça, A.C., Oliveira, E.A., Fróes, B.P., Faria, L.D., Pinto, J.S., Nogueira, M.M. et al. (2015) A predictive model of progressive chronic kidney disease in idiopathic nephrotic syndrome. *Pediatr. Nephrol.* **30**, 2011–2020, https://doi.org/10.1007/s00467-015-3136-0

20 Kidney Disease Improving Global Outcomes (KDIGO) (2012) Clinical practice guideline for glomerulonephritis. *Kidney Int.* **82**, 139–274

21 Schwartz, G.J., Munoz, A., Schneider, M.F., Mak, R.H., Kaskel, F., Warady, B.A. et al. (2009) New equations to estimate GFR in children with CKD. *J. Am. Soc. Nephrol.* **20**, 629–637, https://doi.org/10.1681/ASN.2008030287

22 Oudit, G.Y., Herzenberg, A.M., Kassiri, Z., Wong, D., Reich, H., Khokha, R. et al. (2006) Loss of angiotensin-converting enzyme-2 leads to the late development of angiotensin II-dependent glomerulosclerosis. *Am. J. Pathol.* **168**, 1808–1820, https://doi.org/10.2353/ajpath.2006.051091

23 Ye, M., Wysocki, J., William, J., Soler, M.J., Cokic, I. and Batlle, D. (2006) Glomerular localization and expression of Angiotensin-converting enzyme 2 and Angiotensin-converting enzyme: implications for albuminuria in diabetes. *J. Am. Soc. Nephrol.* **17**, 3067–3075, https://doi.org/10.1681/ASN.2006050423

24 Soler, M.J., Wysocki, J., Ye, M., Lloveras, J., Kanwar, Y. and Batlle, D. (2007) ACE2 inhibition worsens glomerular injury in association with increased ACE expression in streptozotocin-induced diabetic mice. *Kidney Int.* **72**, 614–623, https://doi.org/10.1038/sj.ki.5002373

25 Wong, D.W., Oudit, G.Y., Reich, H., Kassiri, Z., Zhou, J., Liu, Q.C. et al. (2007) Loss of angiotensin-converting enzyme-2 (ACE2) accelerates diabetic kidney injury. *Am. J. Pathol.* **171**, 438–451, https://doi.org/10.2353/ajpath.2007.060977

26 Wysocki, J., Ye, M., Soler, M.J., Gurley, S.B., Xiao, H.D., Berntein, K.E. et al. (2007) ACE and ACE2 activity in diabetic mice. *Diabetes* **55**, 2132–2139, https://doi.org/10.2337/db06-0033

27 Dilauro, M., Zimpelmann, J., Robertson, S.J., Genest, D. and Burns, K.D. (2010) Effect of ACE2 and angiotensin-(1-7) in a mouse model of early chronic kidney disease. *Am. J. Physiol. Renal. Physiol.* **298**, F1523–F1532, https://doi.org/10.1152/ajprenal.00426.2009

28 Silveira, K.D., Pompermayer Bosco, K.S., Diniz, L.R., Carmoma, A.K., Cassali, G.D., Romero, O. et al. (2010) ACE2-angiotensin-(1-7)-Mas axis in renal ischaemia/reperfusion injury in rats. *Clin. Sci. (Lond.)* **119**, 385–394, https://doi.org/10.1042/CS20090554

29 Liu, Z., Huang, X.R., Chen, H.Y., Penninger, J.M. and Lan, H.Y. (2012) Loss of angiotensin-converting enzyme 2 enhances TGF-beta/Smad-mediated renal fibrosis and NF-kappaB-driven renal inflammation in a mouse model of obstructive nephropathy. *Lab. Invest.* **92**, 650–661, https://doi.org/10.1038/labinvest.2012.2

30 Mizuiri, S., Hemmi, H., Arita, M., Aoki, T., Ohashi, Y., Miyagi, M. et al. (2011) Increased ACE and decreased ACE2 expression in kidneys from patients with IgA nephropathy. *Nephron Clin. Pract.* **117**, c57–66, https://doi.org/10.1159/000319648

31 Belisário, A.R., Vieira, E.L.M., Almeida, J.A., Mendes, F.G., Miranda, A.S., Rezende, P.V. et al. (2018) Low urinary levels of angiotensin-converting enzyme 2 may contribute to albuminuria in children with sickle cell anaemia. *Br. J. Haematol.*, https://doi.org/10.1111/bjh.15439

32 Pinheiro, S.V., Ferreira, A.J., Kitten, G.T., da Silveira, K.D., da Silva, D.A., Santos, S.H.S. et al. (2009) Genetic deletion of the angiotensin-(1-7) receptor Mas leads to glomerular hyperfiltration and microalbuminuria. *Kidney Int.* **75**, 1184–1193, https://doi.org/10.1038/ki.2009.61

33 Zhang, J., Noble, N.A., Border, W.A. and Huang, Y. (2010) Infusion of angiotensin-(1-7) reduces glomerulosclerosis through counteracting angiotensin II in experimental glomerulonephritis. *Am. J. Physiol. Renal. Physiol.* **298**, F579–588, https://doi.org/10.1152/ajprenal.00548.2009

34 Giani, J.F., Munoz, M.C., Pons, R.A., Cao, G., Toblli, J.E., Turyn, D. et al. (2011) Angiotensin-(1-7) reduces proteinuria and diminishes structural damage in renal tissue of stroke-prone spontaneously hypertensive rats. *Am. J. Physiol. Renal. Physiol.* **300**, F272–282, https://doi.org/10.1152/ajprenal.00278.2010

35 Barroso, L.C., Silveira, K.D., Lima, C.X., Borges, V., Bader, M., Rachid, M. et al. (2012) Renoprotective effects of AVE0991, a nonpeptide Mas receptor agonist, in experimental acute renal injury. *Int. J. Hypertens.* 808726

36 Xue, H., Zhou, L., Yuan, P., Wang, Z., Ni, J., Yao, T. et al. (2012) Counteraction between angiotensin II and angiotensin-(1-7) via activating angiotensin type I and Mas receptor on rat renal mesangial cells. *Regul. Pept.* **177**, 12–20, https://doi.org/10.1016/j.regpep.2012.04.002

37 Kim, C.S., Kim, I.J., Bae, E.H., Ma, S.K., Lee, J. and Kim, S.W. (2015) Angiotensin-(1-7) attenuates kidney injury due to obstructive nephropathy in rats. *PLoS One* **10**, e0142664, https://doi.org/10.1371/journal.pone.0142664

38 Lu, W., Kang, J., Hu, K., Tang, S., Zhou, X., Yu, S. et al. (2017) Angiotensin-(1-7) relieved renal injury induced by chronic intermittent hypoxia in rats by reducing inflammation, oxidative stress and fibrosis. *Braz. J. Med. Biol. Res.* **50**, e5594, https://doi.org/10.1590/1414-431x20165594

39 Rocha, N.P., Bastos, F.M., Vieira, E.L.M., Prestes, T.R.R., Silveira, K.D.D., Teixeira, M.M. et al. (2018) The protective arm of the renin-angiotensin system may counteract the intense inflammatory process in fetuses with posterior urethral valves. *J. Pediatr.*, https://doi.org/10.1016/j.jped.2018.02.003

40 Vieira, E.L.M., Rocha, N.P., Bastos, F.M., Siveira, K.D., Pereira, A.K., Oliveira, A.E. et al. (2017) Posterior urethral valve in fetuses: evidence for the role of inflammatory molecules. *Pediatr. Nephrol.* **32**, 1391–1400, https://doi.org/10.1007/s00467-017-3614-7

41 Ng, H.Y., Yisireyili, M., Saito, S., Lee, C.T., Adelibieke, Y., Nishijima, F. et al. (2014) Indoxyl sulfate downregulates expression of Mas receptor via OAT3/AhR/Stat3 pathway in proximal tubular cells. *PLoS One* **9**, e91517, https://doi.org/10.1371/journal.pone.0091517

42 Vianna, H.R., Soares, C.M., Silveira, K.D., Elmiro, G.S., Mendes, P.M., de Sousa Tavares, M. et al. (2013) Cytokines in chronic kidney disease: potential link of MCP-1 and dyslipidemia in glomerular diseases. *Pediatr. Nephrol.* **28**, 463–469, https://doi.org/10.1007/s00467-012-2363-x
43 Han, G.D., Suzuki, K., Koike, H., Suzuki, K., Yoneyama, H., Narumi, S. et al. (2006) IFN-inducible protein-10 plays a pivotal role in maintaining slit-diaphragm function by regulating podocyte cell-cycle balance. *J. Am. Soc. Nephrol.* **17**, 442–453, https://doi.org/10.1681/ASN.2004090755
44 Rutkowski, B. and Tylicki, L. (2015) Nephroprotective action of renin angiotensin aldosterone system blockade in chronic kidney disease patients: the landscape after ALTITUDE and VA NEPHRON-D trails. *J. Renal Nutr.* **25**, 194–200, https://doi.org/10.1053/j.jrn.2014.10.026
45 Azizi, M. and Menard, J. (2004) Combined blockade of the renin-angiotensin system with angiotensin-converting enzyme inhibitors and angiotensin II type 1 receptor antagonists. *Circulation* **109**, 2492–2499, https://doi.org/10.1161/01.CIR.0000131449.94713.AD
46 Simoes e Silva, A.C., Diniz, J.S., Pereira, R.M., Pinheiro, S.V. and Santos, R.A. (2006) Circulating renin Angiotensin system in childhood chronic renal failure: marked increase of Angiotensin-(1-7) in end-stage renal disease. *Pediatr. Res.* **60**, 734–739, https://doi.org/10.1203/01.pdr.0000246100.14061.bc
47 Kocks, M.J., Lely, A.T., Boomsma, F., de Jong, P.E. and Navis, G. (2005) Sodium status and angiotensin-converting enzyme inhibition: effects on plasma angiotensin-(1-7) in healthy man. *J. Hypertens.* **23**, 597–602, https://doi.org/10.1097/01.hjh.0000160217.86597.b6
48 Baracho, NCDV, Silveira, K.D.D., Rocha, N.P., Cordeiro, T.M., Feracin, V., Pereira, R.M. et al. (2017) Urinary cytokine profiles according to the site of blockade of the renin-angiotensin system in nephrectomized rats. *Braz. J. Nephrol.* **39**, 108–118
49 Wysocki, J., Ortiz-Melo, D.I., Mattocks, N.K., Xu, K., Prescott, J., Evora, K. et al. (2014) ACE2 deficiency increases NADPH-mediated oxidative stress in the kidney. *Physiol. Rep.* **2**, e00264, https://doi.org/10.1002/phy2.264
50 Chen, L.-J., Xu, Y.-L., Song, B., Yu, H.M., Oudit, G.Y., Xu, R. et al. (2016) Angiotensin-converting enzyme 2 ameliorates renal fibrosis by blocking the activation of mTOR/ERK signaling in apolipoprotein E-deficient mice. *Peptides* **79**, 49–57, https://doi.org/10.1016/j.peptides.2016.03.008

ANEXO 4 – COMPROVANTE DE SUBMISSÃO DO ARTIGO DE REVISÃO

Current Pediatric Reviews

Dear Dr. Roberta Silva Filha,

This is with reference to an article entitled: "Idiopathic Nephrotic Syndrome in Pediatrics: an up-to-date" which has been submitted for possible publication in Current Pediatric Reviews, and in which you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Ana Cristina Simões E Silva who is listed as the main author and who will be authorized to track the status of the paper after login.

If you have any objections to this submission, then please contact the editorial office as soon as possible by replying to this email. If we do not hear back from you within one week, we will assume you agree with your co-authorship.

Thank you very much.

Editorial Office
Current Pediatric Reviews
Bentham Science Publisher

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ANEXO 5 – COMPROVANTE DE SUBMISSÃO DO ARTIGO ORIGINAL 2

Pediatric Nephrology

Submission ID: PNEP-D-21-00460

Re: "Metabolic changes and endothelial dysfunction in children with idiopathic nephrotic syndrome"
Full author list: Roberta Silva Filha; Pedro Alves Soares Vaz de Castro; Rafael Coelho Magalhães; Sergio Veloso Brant Pinheiro; Ana Cristina Simões e Silva

Dear Dr Roberta Silva Filha,

We have received the submission entitled: "Metabolic changes and endothelial dysfunction in children with idiopathic nephrotic syndrome" for possible publication in Pediatric Nephrology, and you are listed as one of the co-authors.

The manuscript has been submitted to the journal by Dr. Prof. Ana Cristina Simões e Silva who will be able to track the status of the paper through his/her login.

If you have any objections, please contact the editorial office as soon as possible. If we do not hear back from you, we will assume you agree with your co-authorship.

Thank you very much.

With kind regards,

Springer Journals Editorial Office
Pediatric Nephrology