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SOCS3 and *SOCS5* mRNA expressions may predict initial steroid response in nephrotic syndrome children

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Abstract: Suppressors of Cytokine Signaling (SOCS) inhibit Signal Transducers and Activators of Transcription (STATs) phosphorylation by binding and inhibiting Janus Kinases (JaKs). The aim of the present study was to evaluate the influence of glucocorticosteroids on the JaK/STAT signaling pathway in the leukocytes of nephrotic syndrome (NS) patients. The study group was composed of 34 steroid sensitive NS (SSNS) children and 20 steroid resistant NS (SRNS) subjects. Gene expression was assessed by real-time PCR using pre-designed human JaK/STAT PCR array. Protein expression was evaluated using ELISA assay (plasma concentration) and immunofluorescence (in situ protein expression). In SSNS children, the initial increased expression of *JaK1, JaK2, JaK3, STAT1, STAT2, STAT6, TYK2, SOCS1, SOCS2, SOCS3, SOCS4* and *SOCS5* was reduced back to the control limits. Similarly, in SRNS patients the increased levels of almost all mRNA expressions for the abovementioned genes were decreased, with the exceptions of *SOCS3* and *SOCS5* expressions. These mRNA expressions were still significantly increased and correlated with early unfavorable course of nephrotic syndrome in children. Plasma levels of SOCS3, SOCS5, IL-6 and IL-20 were significantly increased in SRNS subjects after six weeks of steroids medication compared to SSNS and control participants. We conclude that *SOCS3* and *SOCS5* increased mRNA expressions might predict initial resistance to steroids in NS patients. (*Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 4, pp. 719–728*)

Key words: JaK/STAT signaling pathway, leukocytes, nephrotic syndrome, resistance to steroids, children

Introduction

Nephrotic syndrome (NS) is a complex and non-specific disease characterized by severe proteinuria (> 3.5 g/day), hypoproteinemia, generalized edema, hyperlipidemia and, occasionally, hypertension [1]. Generally, NS base might reflect multiple immunological disorders [2], a critical filtration barrier breakage or could follow diabetes mellitus [3, 4], obesity [5], sarcoidosis [6] as well as different malignancies [7, 8].

Regardless of its primary or secondary cause, the gold standard in the induction of NS therapy is the administration of glucocorticosteroids (GCS) [1].

GCS-dependent suppression of both cell-mediated and humoral immunity is already well established in NS [9]. On the other hand, GCS influence all types of inflammatory events, no matter what their cause. One of the most interesting GCS impacts is that of JaK/STAT signaling pathway suppression in NS patients.

The JaK-STAT system is a major signaling pathway alternative to the second messenger transmission [10]. It directs information from chemical signals out-

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side the cell, through the cell membrane, and into gene promoters on the DNA in the cell nucleus, which causes DNA transcription and activity in the cell [10]. The JaK/STAT system consists of three main components: a receptor, a Janus Kinase (JaK) membrane receptor protein, and a Signal Transducer and Activator of Transcription (STAT) which transduces the signal from the receptor-JaK complex to the DNA in the cell nucleus. JaKs, which have tyrosine kinase activity, bind to some cell surface cytokine receptors. The binding of the ligand to the receptor triggers activation of JaKs. With increased kinase activity, they form phosphorylate tyrosine residues on the receptor and create sites for interaction with proteins (i.e. STATs) that contain a phosphotyrosine-binding SH_2 domain [11].

The pathway is negatively regulated on multiple levels. Protein tyrosine phosphatases remove phosphates from cytokine receptors as well as activated STATs [10]. More recently identified Suppressors of Cytokine Signaling (SOCS) inhibit STAT phosphorylation by binding and inhibiting JaKs or competing with STATs for phosphotyrosine binding sites on cytokine receptors. STATs are also negatively regulated by Protein Inhibitors of Activated STATs (PIAS), which act in the nucleus through several mechanisms [10, 12].

Interestingly, GCS are indicated as an essential part of the inductive treatment in all NS patients, including subjects in whom NS is the result of something other than immune disorders [1, 9]. The lack of effective response to GCS enables physicians to identify a group of patients with definitive resistance to steroids (steroid resistant NS, SRNS) and perform additional laboratory tests (i.e. biopsy of the kidney, DNA sequencing) to estimate the non-immunological base of the disorder.

The question is whether the abovementioned subsequent analyses could be executed earlier. Is it possible to select a leukocyte genes pattern predicting resistance to steroids or the presence of key mutations, regardless of NS type? Recently, a very few mechanisms have been proposed by which activation of JaK/STAT signaling might be involved in the progression of glomerular diseases with proteinuric state. They have been reported both in an animal model [13] and in humans [14]. Nakajima et al. suggested an overload proteinuria could activate inflammatory cascades as well as oxidative stress and, in part, activate JaK/STAT signaling pathway in renal proximal tubular cells *in vitro* [14]. On the other hand, hypoalbuminemia has been reported to cause an overexpression of SOCS family in peripheral white blood cells [11].

The aim of the present study was to: (1) evaluate GCS influence on the JaK/STAT signaling pathway in the segregated leukocytes of all NS patients; and (2) try to separate a gene pattern recruited from the JaK/STAT system which might predict resistance to steroids.

Material and methods

Patients. The study group was composed of children diagnosed with NS, who were treated between 2008 and 2010 in the Department of Pediatric Cardiology and Nephrology, Poznan University of Medical Sciences. One hundred and fifty-five children were referred to our clinic, and evidence of proteinuria was found in 78 of them. Ten subjects were excluded from the study because at least one of the following criteria was present: (1) age < 3 yr or > 18 yr, (2) end stage renal disease, (3) administration of erythropoetin, (4) administration of granulocyte colony stimulating factor or (5) multi-drug therapy influencing immunological system. Thirty-four patients (19 boys and 15 girls), following International Study for Kidney Diseases in Children (ISKDC) recommendations, underwent a biopsy [15]. The indications included: SRNS (n = 20) and secondary glomerulonephritis (n = 14, who were excluded from further analysis). According to World Health Organization definitions [15, 16], histological evaluation revealed: minimal change disease (MCD), n = 6; diffuse mesangial proliferation (DMP), n = 5; and focal segmental glomerulosclerosis (FSGS), n = 9.

Taken together, the study group comprised 54 subjects: 34 patients — steroid sensitive nephrotic syndrome (SSNS — no kidney biopsy performed) and 20 children with a diagnosis of SRNS. SSNS was defined as an absence of all NS symptoms in those patients who reached remission with steroid therapy alone. On the other hand, those patients in whom remission was not achieved within eight weeks of steroid therapy were labeled as SRNS subjects [16, 17]. The relevant data is presented in Table 1.

The Ethics Committee of Poznan University of Medical Sciences approved the research protocol and the parents of all study participants gave informed consent for the investigation.

Treatment protocol. Standard ISKDC regime for the first NS episode included administration of prednisolone — 60 mg/m^2/day in three divided doses for four weeks followed by 40 mg/m^2/day in a single dose on every alternate day for four weeks [17]. After a six week period of medication, when proteinuria and decreased serum albumin level were still found to be present, progressive steroid cancellation was performed and the introduction of other immunosuppressive drugs (cyclosporine, cyclophosphamide, mycophenolate mofetil) was recommended. The relevant data is summarized in Table 1.

Controls. Ten age- and sex-matched children with a normal renal function and urinalysis who were hospitalized in our university due to surgical reasons served as a control group.

Table 1. Clinical and biochemical characteristics of the study population at the beginning of treatment

Characteristics	Study population	
	SSNS — subgroup 1	SRNS — subgroup 2
Number of patients	34	20
Age (years)	10.5 (4–16)	11.3 (4–17)
Male/female	18/16	11/9
Proteinuria [mg/kg/24 h]	44.0 ± 12.0	92.0 ± 33.0*
Erythrocyturia (% of patients)	23.5	20.0
GFR [ml/min/1.73 m^2]	97.0 ± 7.0	89.0 ± 7.0
Serum albumin [g/dl]	3.0 ± 0.7	2.3 ± 0.8*
CRP [mg/dl]	< 0.5	< 0.5
Creatinine [mg/dl]	0.9 ± 0.2	0.8 ± 0.2
Urea [mg/dl]	25 ± 6.0	29 ± 8.0
Lipids [mg/dl]	158 ± 25	165 ± 21
Cholesterol [mg/dl]	182 ± 25	201 ± 24
WBC total [G/l]	5.6 ± 1.2	5.7 ± 2.3
Lymphocyte (% of WBC)	28.0 ± 12.0	32.0 ± 16.0

SSNS — steroid sensitive nephrotic syndrome; SRNS — steroid resistant nephrotic syndrome; erythrocyturia — number of red blood cells in urine > 2 cell per high power field; GFR — glomerular filtration rate; CRP — c reactive protein; WBC — white blood cells count; *statistical significance ($p < 0.05$)

Blood samples and isolation of peripheral leukocytes. Blood was sampled directly into EDTA-anticoagulated tubes. It was collected from all the patients comprising the study (n = 54) and control (n = 10) groups. Plasma was directly isolated and stored at –80°C for analysis of albumins, lipids, CRP, glucose, creatinine, and urea, using routine laboratory methods. It was also assayed for the presence of biologically active SOCS3, SOCS5, IL-6 and IL-20 using the ELISA technique. Glomerular filtration rate was calculated using the Schwartz formula [18].

Isolation of mononuclear cells. Ten milliliters of EDTA blood were centrifuged at 1,250 × g for 90 s to separate cells from the platelet-rich plasma. The mononuclear cells (monocytes and lymphocytes) were isolated on a Ficoll (Ficoll separating solution density 1.077; 17-1440-02; Amersham Ficoll-Paque) adhering to the manufacturer's instructions. Monocytes were consecutively isolated using indirect magnetic labeling system using biotinylated antibody against CD68 (MACS®, Miltenyi Biotec, Krakow, Poland). Non-monocytes i.e. lymphocytes (T cells, natural killer cells, B cells and dendritic cells) as well as basophilic granulocytes were indirectly magnetically labeled using a mixture of biotinylated antibodies against CD3, CD7, CD16, CD19, CD56, CD123, glycophorin A, and Anti-Biotin MicroBeads (MACS®, Miltenyi Biotec). Since lymphocytes formed the majority of non-monocytic mononuclear cells, we refer to this fraction as lymphocytes.

Blood from all the patients was sampled twice. Firstly, at the time of diagnosis — i.e. before treatment commencement, and secondly, after a six-week period of GCS medication. It was performed to enable comparison of gene expression variation during GCS treatment and set a diagnostic gene expression pattern in SSNS and SRNS study participants.

Quantitative PCR. RNA was isolated using TRIzol® (Gibco, Invitrogen, Carlsbad, CA, USA) [19]. One microgram of RNA was respectively reverse transcribed (iScript® cDNA synthesis Kit, Bio-Rad) to cDNA.

Gene expression was assessed by real-time PCR (Eppendorf, Poznan, Poland) using pre-designed human JaK/STAT Signaling Pathway PCR array (Sabiosciences, Qiagen, Wroclaw, Poland). The array represented all known JaK and STAT family members, the receptors that activate them, nuclear co-factors and co-activators associated with the STAT proteins, STAT-inducible genes, and negative regulators of the pathway (Table 2; the complete list of studied genes is also available at www.sabiosciences.com/rt_pcr_product/HTML/PAHS-039A.html).

Expression of each target gene in each subject was normalized to endogenous controls GAPDH+18S (ΔCT) and related to a reference (ΔΔCT), being RNA from untreated peripheral blood leukocytes of the controls.

ELISA reagents. The following colorimetric ELISA kits were used in the study: (1) SOCS-3 human ELISA system, ABIN366182 (antibodies-online GmbH, Aachen, Germany); (2) SOCS-5 human ELISA system, ABIN 422766 (antibodies-online GmbH); (3) human high sensitivity IL-6

Table 2. Functional gene grouping in human JaK/STAT Signaling Pathway PCR Array (according to http://www.sabiosciences.com/rt_pcr_product/HTML/PAHS-039A.html)

No.	Group	Analyzed genes
1	JaKs	JaK1, JaK2, JaK3, TYK2
2	STAT family	STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B, STAT6
3	Receptors that bind and activate JaK proteins	CSF1R, CSF2RB, EGFR, EPOR, F2R, GHR, IFNAR1, IFNGR1, IL10RA, IL2RA, IL2RG, IL4R, IL6ST, INSR, MPL, PDGFRA, SH2B1
4	SH3/SH2 adaptor protein activity	SH2B2 (APS), CRK, SIT1, SLA2, SRC, STAM
5	STAT protein nuclear translocation	F2, F2R, STAT1
6	Tyrosine phosphorylation of STAT protein	F2, F2R, IL20, PPP2R1A, PRLR, STAT1
7	Transcription factors or regulators that interact with STAT proteins	Positive regulation of transcription: HMGA1, SMAD3 Negative regulation of transcription: SLA2, SMAD3, SPI1, STAT3 RNA Polymerase II transcription factor activity: JUNB, SP1, USF1 Transcription coactivator activity: JUNB, YY1 Transcription corepressor activity: JUNB, PIAS1, YY1 Transcriptional activator activity: SMAD1, SMAD5, SP1 Other transcription factors and regulators: CEBPB, CRK, GATA3, IRF1, ISGF3G, JUN, MYC, NFKB1, NR3C1, PPP2R1A, SMAD2, SMAD4
8	Genes induced by STAT proteins	Stat1: CXCL9, IRF1, JUNB, NOS2A Stat3: A2M, BCL2L1, CDKN1A, CRP, FAS (TNFRSF6), IRF1, MMP3, MYC, SOCS1 Stat4: FCGR1A, IFNG, IRF1, MYC Stat5: CCND1, CDKN1A, IL2RA, IRF1, OSM Stat6: FCER1A, GATA3, IL4, IL4R Stat1/Stat1/p48: GBP1 Stat1/Stat2/p48: ISG15 (G1P2), OAS1
9	Negative regulators of the JaK/STAT pathway	PIAS1, PIAS2, PTPN1, PTPRC, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5
10	Apoptosis	BCL2L1, CDKN1A, F2, F2R, FAS (TNFRSF6), IL2RA, NFKB1, PPP2R1A, PRLR, STAT1
11	Cell differentiation	IL20, IL4, NOS2A, PPP2R1A, SOCS2, SOCS5
12	Cell growth and/or maintenance	Cell cycle: CCND1, CDKN1A, EGFR, F2, F2R, IL2RA, IRF1, JaK2, MYC, PPP2R1A, STAT1 Cell growth: A2M, EGFR, GHR, IFNG, IL4, INSR, OSM, PPP2R1A, SOCS1, SOCS2, SOCS3, SOCS4, SOCS5 Cell proliferation: CSF1R, IL2RG, IL4, MPL, OSM, PDGFRA, SMAD3
13	Immune response	Acute-phase response: CEBPB, CRP, F2, STAT3 Antigen processing: FCER1A, FCGR1A, PPP2R1A Humoral immune response: SH2B2 (APS), CSF1R, CSF2RB, IL4, NFKB1, YY1 Inflammatory response: CEBPB, CRP, CXCL9, IL10RB, IL20, NFKB1, NOS2A, NR3C1 Lymphocyte activation: IL4, PRLR, SIT1, SLA2 Other genes involved in the immune response: CXCL9, FAS (TNFRSF6), ISG15 (G1P2), GBP1, IFNG, IL2RA, IL2RG, IL4, IL4R, IL6ST, IRF1, ISGF3G, OAS1, OSM, STAT5A, STUB1

ELISA Kit, ab46042 (Abcam, Cambridge, UK); (4) human IL-20 ELISA Kit, ABIN415142 (antibodies-online GmbH).

Biological assay for SOCS3, SOCS5, IL-6 and IL-20. Defrosted supernatants were again clarified by centrifugation (4,000 rpm for 2 min) and than assayed for the presence of biologically active SOCS3, SOCS5, IL-6 and IL-20. The following steps of ELISA were performed according to the manufacturer's instructions. The estimation of an average SOCS3, SOCS5, IL-6 and IL-20 concentrations in each patient from both study (before and after GCS medication) and control groups were founded on double cytokine evaluation by reading in a spectrophotometer at 450 nm.

Immunohistochemistry. In order to demonstrate SOCS3 and SOCS5 expressions in certain isolated smeared mononuclear cells, an indirect immunocytochemical procedure was performed. The endogenous activity of peroxidase was blocked by 30 min pre-incubation in 3% hydrogen peroxide. The immunofluorescence investigation was based on the initial incubation with anti-SOCS3 (diluted 1:10; rabbit polyclonal anti-human; ab16030, Abcam) and anti-SOCS5

(diluted 1:10; rabbit polyclonal anti-human; ab3695, Abcam) at 4°C overnight, followed by goat anti-rabbit Alexa Fluor 555 secondary antibody (A-21429; diluted 1:200; Molecular Probes, MobiTec, Goettingen, Germany) at 37°C for 30 min. These reactions were observed using an Olympus BX60 microscope. Photographs were taken using a CCD camera connected to a soft-imaging analysis system (Soft Imaging System, Olympus, Muenster, Germany). Both analyses were performed blind on coded samples.

Based on the results of the hematological staining, which were analyzed using MicroImage (Olympus) morphometric software, the content of reaction-positive cells was determined by comparing the number of immunopositive cells to the total number of nucleated cells. A percentage of positive cells of < 5% was classified as a negative result.

Statistical analysis. The analysis was performed blind on coded samples. Continuous or interval-related variables were expressed as mean ± SD. Data was compared using Student's *t*-test, or one-way analysis of variance test (ANOVA) where appropriate. The analysis of different gene expressions among the study participants for assessing whether two independent samples of observations have equally large values, were exercised using Mann–Whitney U test (Wilcoxon–Mann–Whitney test). A general method of alpha adjustment for all the multiple comparisons performed in the study was Bonferroni correction. Statistical analysis of quantitative genes expression pattern was performed using web-based RT^2 Profiler™ PCR Array Data Analysis software available at www.sabiosciences.com/pcrarraydataanalysis.php (Sabiosciences).

This integrated web-based software package for the PCR Array System automatically performed all $\Delta\Delta C_t$ based fold-change calculations from uploaded raw threshold cycle data; $p < 0.05$ was considered as statistically significant [20].

Results

Clinical observation

In the subsequent analysis and discussion, the term 'study group' refers to all 54 study participants who were analyzed prior to commencement of the treatment. During the study protocol, 34 subjects responded to steroids (SSNS patients) and 20 children had no positive response to steroids (SRNS subjects). SSNS and SRNS children were consequently defined as subgroup 1 and subgroup 2 respectively.

Before treatment commencement, all the patients comprising subgroups 1 and 2 did not differ according to basic biochemical values (concentration of creatinine, urea, lipids and cholesterol); they had a similar white blood cells count, percentage of lymphocytes in peripheral blood as well as c-reactive protein (CRP) and glomerular filtration rate (GFR) levels. Similarly, the percentage of subjects with erythrocyturia was comparable. The only significant difference referred to serum albumin concentration (3.0 ± 0.7 mg/dl vs. 2.3 ± 0.8 mg/dl in subgroups 1 and 2 respectively; $p < 0.05$) and proteinuria (44 ± 12 mg/kg/24 h vs. 92 ± 33 mg/kg/24 h; $p < 0.05$).

After the six-week period of medication, proteinuria and decreased serum albumin level were still present in subgroup 2. This resulted in renal biopsy performance, progressive steroid cancellation and introduction of other immunosuppressive drugs (cyclosporine, cyclophosphamide, mycophenolate mofetil). The relevant data is summarized in Table 1.

Expression of JaK/STAT system components in peripheral monocytes and lymphocytes

The JaK/STAT signaling pathway expression in circulating leukocytes (monocytes and lymphocytes) of all the patients comprising the study group was analyzed twice: once before treatment commencement and again, after six weeks, when the response to steroid treatment enabled differentiation of SSNS from SRNS subjects. The results of the initial analysis were compared to mRNA expression in the control group (Table 3). This allowed us to separate the list of 'gene — candidates' including: *JaK1, JaK2, JaK3, STATl, STAT2, STAT6, TYK2, SOCS1, SOCS2, SOCS3, SOCS4* and *SOCS5*. Interestingly, mRNA expression of all these genes had increased more than ten-fold compared to the controls and did not differ between monocyte and lymphocyte populations. The rest of the analyzed genes (as described in 'Methods') did not differ significantly compared to the controls' mRNA expression and were excluded from subsequent study.

The results of the second analysis of the abovementioned 'gene — candidates' were statistically compared to the initial resistance to steroids as well as compared between SSNS and SRNS patients (Table 3). In SSNS children (subgroup 1), the initial increased expression of all the 'gene — candidates' was reduced back to the control limits. Similarly, in SRNS patients (subgroup 2) the increased levels of almost all mRNA expressions for 'gene — candidates' were decreased, except for *SOCS3* and *SOCS5* expressions. Expression of *SOCS3*, but not *SOCS5,* was increased in circulating monocytes of subgroup 2 patients versus subgroup 1 subjects ($p = 0.0005$). In contrast, expression of *SOCS5*, but not *SOCS3*, was increased in lymphocytes of SRNS patients *vs.* SSNS study participants ($p = 0.0086$). These mRNA expres-

Table 3. Expression of 'gene — candidates' of JaK/STAT signaling pathway in peripheral leukocytes of children comprising the study (subgroups 1 and 2) and control groups at the time of diagnosis and after six-week steroid treatment

Gene	Gene expression variation							
	Study group at the time of diagnosis vs. controls		Subgroup 1 at the time of diagnosis vs. after 6-week treatment		Subgroup 2 at the time of diagnosis vs. after 6-week treatment		Subgroup 1 after 6-week treatment vs. Subgroup 2 after 6-week treatment	
	Expression alteration	p	Expression alteration	p	Expression alteration	p	Expression alteration	p
JaK1	22.3 (↑)	0.0006	28.3 (↓)	0.0004	29.1 (↓)	0.0005	4.7 (↓)	NS
JaK2	12.8 (↑)	0.0052	12.8 (↓)	0.0048	17.4 (↓)	0.0086	3.8 (↓)	NS
JaK3	27.1 (↑)	0.002	13.8 (↓)	0.0042	16.3 (↓)	0.0038	8.6 (↓)	NS
STATl	19.4 (↑)	0.0016	21.6 (↓)	0.0035	14.9 (↓)	0.0018	9.2 (↓)	NS
STAT2	48.1 (↑)	< 0.0001	34.1 (↓)	0.0001	32.3 (↓)	0.0001	7.4 (↓)	NS
STAT6	11.3 (↑)	0.0079	15.9 (↓)	0.0174	15.8 (↓)	0.0086	6.6 (↓)	NS
TYK2	17.5 (↑)	0.0413	21.6 (↓)	0.0064	16.3 (↓)	0.0058	3.7 (↓)	NS
SOCS1	24.8 (↑)	0.0413	14.7 (↓)	0.0064	12.1 (↓)	0.0214	8.2 (↓)	NS
SOCS2	26.1 (↑)	0.0027	32.9 (↓)	0.0018	16.2 (↓)	0.0024	7.4 (↓)	NS
SOCS3	32.7 (↑)	0.0021	29.1 (↓)	0.0005	2.4 (↑)	NS	22.5 (↑)	0.0005
SOCS4	18.3 (↑)	0.0005	28.8 (↓)	0.0006	29.3 (↓)	0.0004	3.9 (↓)	NS
SOCS5	19.6 (↑)	0.0085	22.7 (↓)	0.0086	3.5 (↑)	NS	13.6 (↑)	0.0086

Expression alteration — times of gene expression value alteration; ↑ — increase of gene expression; ↓ — decrease of gene expression; NS — not significant

sions have also correlated to the evidences of proteinuria and decreased serum albumin in the nephrotic syndrome patients.

Moreover, *SOCS3* and *SOCS5* expressions were significantly increased in all SRNS patients after the six-week period of GCS medication, regardless of the histological type of the glomerulopathy (MCD, DMP or FSGS). *SOCS3* as well as *SOCS5* expressions did not differ significantly among MCD, DMP or FSGS subjects at the beginning of the treatment or after the six-week steroid administration.

Expression of other genes involved in the immune response and cell differentiation in peripheral monocytes and lymphocytes

Since JaK/STAT signaling pathway PCR array represented not only known JaK and STAT family members, the receptors that activate them and nuclear co-factors and co-activators associated with the STAT proteins, but also STAT-inducible genes as well as genes involved in the immune response, we were able to reliably analyze the expression of a focused panel of the abovementioned genes.

SSNS children and control patients did not reveal increased expression of any other genes as listed in the paragraph above. However, SRNS patients, both before treatment commencement and after the six-week period of GCS administration, performed a significantly higher expression of *IL-20, IL-4R, IL-6ST* and several transcription factors: *JUN, MPL, MYC, SP1* and *SRC*. The level of their expression did not differ significantly in SRNS subjects during the study protocol.

IL-20 and all the transcription factors were expressed exclusively in circulating monocytes. On the other hand, *IL-4R* and *IL-6ST* were expressed both in circulating monocytes and lymphocytes and the level of their expression did not differ between the groups.

Interestingly, *IL-20, IL-4R, IL-6ST, JUN, MPL, MYC, SP1* and *SRC* expressions did not differ significantly among MCD, DMP or FSGS subjects at the beginning of the treatment or after six-week steroid administration. Detailed information is summarized in Table 4.

Increased plasma concentrations of SOCS3, SOCS5, IL-6 and IL-20 in SRNS and SSNS patients before GCS medication

Plasma levels of SOCS3, SOCS5, IL-6 and IL-20 were significantly increased both in SRNS and SSNS children before GCS medication compared to control

Table 4. Expression of *IL-20, IL-4R, IL-6ST, JUN, MPL, MYC, SP1* and *SRC* in peripheral leukocytes of children comprising the study (subgroups 1 and 2) and control groups at the time of diagnosis and after six-week steroid treatment

Gene	Gene expression variation							
	Study group at the time of diagnosis vs. controls		Subgroup 1 at the time of diagnosis vs. after 6-week treatment		Subgroup 2 at the time of diagnosis vs. after 6-week treatment		Subgroup 1 after 6-week treatment vs. controls	
	Expression alteration	p	Expression alteration	p	Expression alteration	p	Expression alteration	p
IL-20	0.8 (↑)	NS	0.4 (↑)	NS	167.8 (↑)	< 0.0001	172.1 (↑)	< 0.0001
IL-4R	2.1 (↑)	NS	1.9 (↑)	NS	34.5 (↑)	< 0.0001	67.3 (↑)	< 0.0001
IL-6ST	1.1 (↑)	NS	1.3 (↑)	NS	16.9 (↑)	< 0.0001	12.9 (↑)	< 0.0001
JUN	1.9 (↓)	NS	1.1 (↓)	NS	109.2 (↑)	< 0.0001	121.5 (↑)	< 0.0001
MPL	1.6 (↓)	NS	0.9 (↓)	NS	99.8 (↑)	< 0.0001	78.3 (↑)	< 0.0001
MYC	0.3 (↑)	NS	0.5 (↑)	NS	110.7 (↑)	< 0.0001	119.3 (↑)	< 0.0001
SP1	0.8 (↓)	NS	0.6 (↓)	NS	39.2 (↑)	< 0.0001	38.2 (↑)	< 0.0001
SRC	0.9 (↑)	NS	1.3 (↑)	NS	32.7 (↑)	< 0.0001	29.7 (↑)	< 0.0001

Expression alteration — times of gene expression value alteration; ↑ — increase of gene expression; ↓ — decrease of gene expression; NS — not significant

participants. The average plasma SOCS3 concentration in these subjects was 4.8 ± 2.9 pg/ml and 5.2 ± ± 2.5 respectively ($p < 0.001$ as compared to controls). The average plasma SOCS5 concentration in SRNS patients equalled 6.6 ± 4.2 pg/ml and in SSNS children 6.1 ± 1.8 pg/ml ($p < 0.0001$ as compared to controls). IL-6 was also significantly increased in SRNS and SSNS study participants and was estimated at an average level of 8.8 ± 5.3 pg/ml and 10.2 ± 5.2 pg/ml respectively ($p < 0.0001$ as compared to controls). Finally, average IL-20 plasma concentration was estimated at the level 6.2 ± 4.3 pg/ml in SRNS patients and 16.5 ± 3.2 pg/ml in SSNS children ($p < 0.005$ comparing SRNS to SSNS; $p < 0.0001$ comparing SSNS to controls). This information is presented in Figure 1.

Increased plasma concentrations of SOCS3, SOCS5, IL-6 and IL-20 in SRNS patients after six-week GCS medication

Plasma levels of SOCS3, SOCS5, IL-6 and IL-20 were significantly increased in SRNS subjects after six-week GCS medication compared to SSNS and control participants.

The average plasma SOCS3 concentration in SRNS subjects was 4.3 ± 0.6 pg/ml ($p < 0.05$ compared to SSNS subjects after six-week GCS treatment; $p < 0.0001$ compared to controls).

The average plasma SOCS5 concentration in SRNS patients equalled 6.2 ± 2.1 pg/ml ($p < 0.05$ compared to SSNS subjects after six-week GCS treatment; $p < 0.0001$ compared to controls).

IL-6 was also significantly increased in SRNS study participants and was estimated at the average level of 2.3 ± 0.4 pg/ml ($p < 0.05$ compared both to SSNS subjects after six-week GCS medication and controls.

Finally, an average IL-20 plasma concentration was estimated at the level 17.2 ± 5.0 pg/ml ($p < 0.0001$ compared to SSNS study participants and controls). This data is summarized in Figure 2.

Immunohistochemical evaluation of SOCS3 and SOCS5 in isolated monocytes and lymphocytes

Immunocytochemical expressions of SOCS3 and SOCS5 were performed on the smears comprising isolated blood mononuclear cells but not segregated into monocytes and lymphocytes. Identification of both populations of cells (monocytes and lymphocytes) was based on the size of the cells: monocytes were defined as cells of a diameter ranging from 15 to 20 μm. The rest of the cells, with an average diameter not exceeding 12 μm, were regarded as lymphocytes. SOCS3 expression was identified exclusively in the monocyte population (Figure 3). Indirect immunofluorescence assay indicated the linear binding of SOCS3 in the monocyte cytoplasm. SOCS3 expression was not detected in lymphocytes (Figure 3). On the other hand, SOCS5 expression was detected in the lymphocyte population (Figure 4). A strong gran-

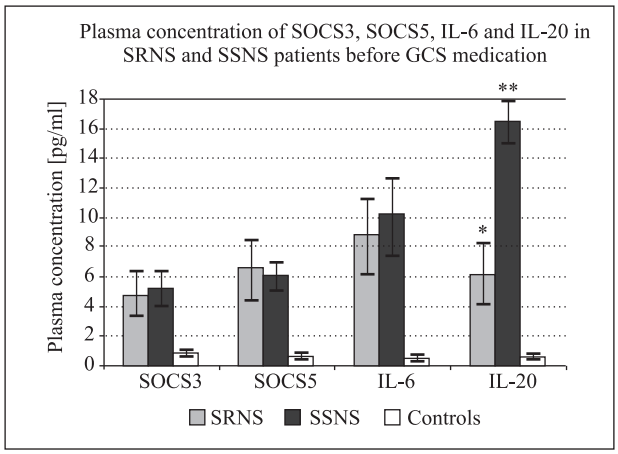

Figure 1. Plasma concentration of SOCS3, SOCS5, IL-6 and IL-20 in steroid resistant (SRNS) and steroid sensitive (SSNS) patients before glucocorticosteroids (GCS) medication

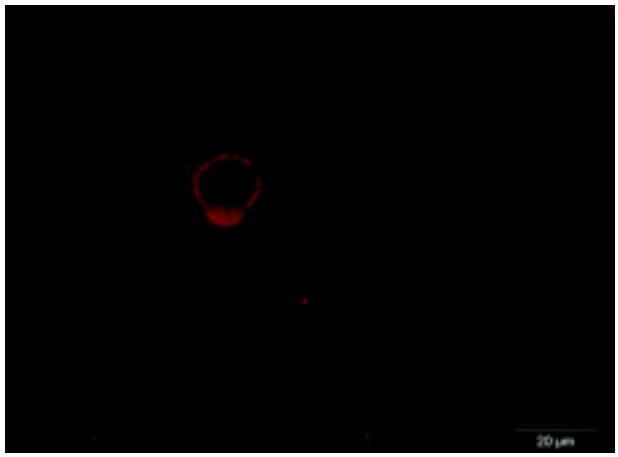

Figure 3. Expression of Suppressors of Cytokine Signaling 3 (SOCS3) evaluated by indirect immunofluorescence in a nine year-old SRNS child. Notice a linear binding of SOCS3 antibodies within the cytoplasm of a single monocyte; scale bar: 20 μm

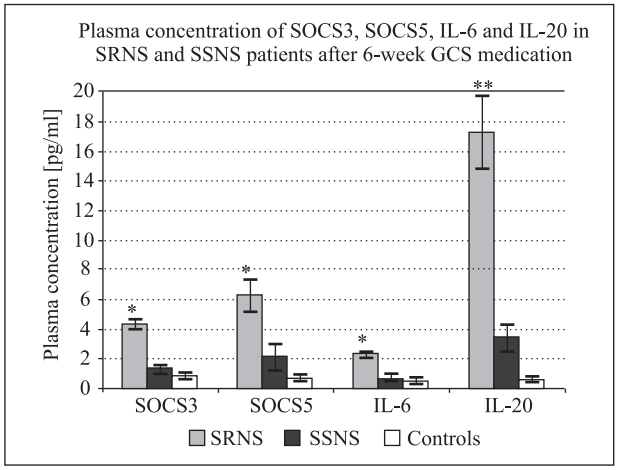

Figure 2. Plasma concentration of SOCS3, SOCS5, IL-6 and IL-20 in steroid resistant (SRNS) and steroid sensitive (SSNS) patients after six-week glucocorticosteroids (GCS) medication

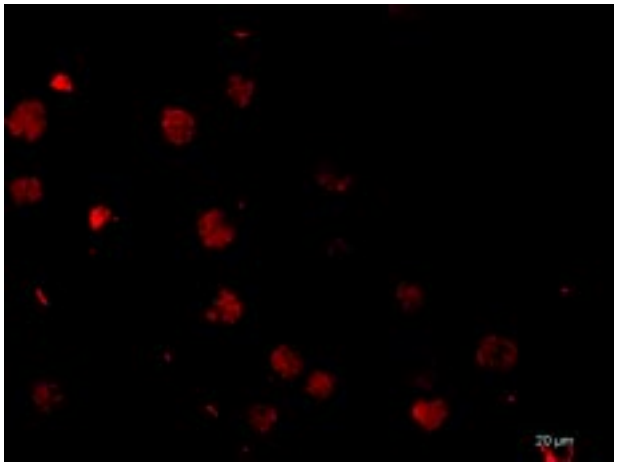

Figure 4. Suppressors of Cytokine Signaling 5 (SOCS5) evaluated by indirect immunofluorescence in a seven year-old SRNS child. The strong granular pattern of SOCS5 covering the territory of the nucleus is detected in the whole cytoplasm of lymphocyte population; scale bar: 20 μm

ular pattern of SOCS5 binding, covering also the territory of the nucleus, was detected in the whole lymphocyte cytoplasm.

Discussion

The question as to what causes nephrotic syndrome in children still remains open. Is the syndrome mostly related to immunological disorders and subsequent filtration barrier breakage, or does it follow gene mutations and glomerular protein modifications which result in increased glomerular permeability? Should we prioritize DNA sequencing in all the nephrotic syndrome patients to optimize medication costs and decrease steroid side-effects in those subjects in whom they seem to have no therapeutic effect?

The results of the present study reveal the immunological shift in leukocyte JaK/STAT signaling pathway expression in NS patients. *JaK1, JaK2, JaK3, STAT1, STAT2, STAT6, TYK2, SOCS1, SOCS2, SOCS3, SOCS4* and *SOCS5* mRNA expression values were increased more than ten-fold in all the studied patients. This certainly does not mean that all the study participants developed NS secondary to immunological system alterations.

In our opinion, immunological variations may be secondary or appear prior to protein modification in

the filtration barrier. Analyzing the JaK/STAT system before the commencement of treatment cannot anticipate the outcome. However, when the subsequent gene expression profile is performed after the steroid inductive treatment (usually, it appears between the sixth and the eighth weeks of treatment course) one could see a significant difference between SSNS and SRNS children. At this moment of disease process, *JaKs, STATs* as well as *TYK2, SOCS1, SOCS2* and *SOCS4* expressions are reduced to control limits. The only gene alterations are restricted to *SOCS3* and *SOCS5* expressions, exclusively in SRNS patients. Interestingly, *SOCS3* and *SOCS5* altered expressions in these subjects could be proved both on mRNA and protein levels. It is also followed by significantly increased IL-6 plasma expression in SRNS patients.

SOCS3 has now been shown to be a key regulator for the divergent activity of IL-6 and IL-20 (a member of IL-10 family) following toll-like receptor (TLR) stimulation [21]. IL-6 is a pro-inflammatory cytokine that assumes a progressive role in many inflammatory diseases including nephrotic syndrome; while IL-20, produced by activated monocytes, regulates proliferation and differentiation of other monocytes and lymphocytes [21]. In addition, IL-20 causes cell expansion of multipotential hematopoietic progenitor cells [21].

SRNS subjects, as demonstrated in the present report, revealed a significantly higher plasma expression of both IL-6 and IL-20 after six weeks of steroid treatment. It corresponded not only to the higher *SOCS3* and *SOCS5* expressions in circulating mononuclear cells, but also followed a significant loss of protein subsequent to the filtration barrier failure.

Our observations seem to prove that overload proteinuria might activate not only inflammatory cascades as well as oxidative stress and, in part, JaK/STAT signaling pathway in renal proximal tubular cells [14], but also in selected peripheral white blood cells.

Moreover, SOCS3 protein is strongly induced by both IL-6 and IL-10 in the presence of lipopolysaccharide (LPS), but IL-6 signaling is selectively inhibited due to the binding of SOCS3 to the IL-6ST subunit gp130 (Tyr759), but not to the IL-10 receptor (IL-10R). Modulation of the gp130/JaK/STAT pathway therefore represents a reasonable strategy for new anti-inflammatory drug development [21].

Little is known about SOCS5 function. When overexpressed, SOCS5 suppresses IL-6 and leukemia inhibitory factor-induced signaling, although to a lesser extent than SOCS1 or SOCS3 [22]. Human SOCS5 is expressed in many tissues, including the heart, brain, placenta, and skeletal muscle, but its expression is especially high in lymphoid organs such as the spleen, lymph nodes (LN), thymus, and bone marrow (BM) [23], indicating that SOCS5 might play a role in lymphocyte development or function. Recently, SOCS5 was reported to be a potential regulator of IL-4 signaling [24], an effect mediated through an SH2 domain-independent interaction between SOCS5 and the IL-4 receptor α chain.

The problem of JaK/STAT system signification in nephrotic syndrome pathophysiology has not been widely discussed in the literature. Individual publications suggest activation of JaK/STAT signaling is involved in the progression of glomerular diseases with proteinuric state [11]. These studies, however, were performed on an animal model and took advantage of only a few genes, including *JaK2, STAT1* and *STAT3*.

In line with the above, it seems that initial steroids administration in all types of NS is still desirable. Even if NS was following a primary filtration barrier failure resulting from gene mutations, it is accompanied by over-expression of JaK/STAT system. Steroids are then expected to put a brake on the immunological response and prepare a background for the introduction of other immunosuppressive drugs.

In SRNS patients, the hypothetical effect, described above, might be secondary to a persistent non-immunological breakage of the filtration barrier (i.e. an overload proteinuria secondary to gene mutations).

In our opinion, *SOCS3* and *SOCS5* increased mRNA expressions not only may predict initial resistance to steroids in pediatric nephrotic syndrome patients, but also should justify more detailed molecular investigations, including DNA sequencing.

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Conflict of interests

The corresponding author (on behalf of all authors in the paper) reveals any: (1) financial interests or arrangements with a company whose product was used in a study or is referred to in a manuscript; (2) any financial interests of arrangement with a competing company; (3) any direct payment to an author(s) from any source for the purpose of writing the manuscript; and (4) any other financial connections, direct or indirect, or other situations that might raise the question of bias in the work reported or the conclusions, implications, or opinions stated — in-

cluding pertinent commercial or other sources of funding for the individual author(s) or for the associated department(s) or organization(s), personal relationships, or direct academic competition.

The results presented in this paper have not been published previously in whole or in part, except in abstract format.

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