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Review

Molecular basis of mechanisms of steroid resistance in children with nephrotic syndrome

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Steroid therapy, due to a wide range of anti-inflammatory properties of steroids, is a basic field of treatment in many human diseases including the nephrotic syndrome in children. However, not all patients respond positively to therapy which divides them into steroid sensitive (SS) and steroid resistance (SR) individuals. Many potential factors associated with steroid resistance have been identified so far. It seems that genetic factors associated with glucocorticoid receptor α (GRα), the structure of heterocomplex of GR as well as glycoprotein P or cytochrome P450 may play a role in the induction of glucocorticoid resistance. Here we described several of the molecular mechanisms, which can regulate glucocorticoid sensitivity and resistance. Moreover, we presented genetic defects, which can lead to various effects of treatment and, in a longer perspective, enable clinicians to individualize therapies.

Key words: nephrotic syndrome, glucocorticoid receptor, steroid resistance, polymorphisms

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INTRODUCTION

Nephrotic syndrome (NS) is the most common kidney disease in childhood. In about 90% of cases it involves primary glomerulopathy while the remaining 10% encompass secondary glomerulopathies due to systemic, metabolic or infectious diseases (Banaszak *et al*., 2011). An idiopathic nephrotic syndrome (INS) is a clinical manifestation of histological variants such as the minimal change nephrotic syndrome (MCNS), in which no significant lesions are detected in renal morphology (52-78%) (Banaszak *et al*., 2011; Taraszkiewicz *et al*,. 2009). The remaining two variants, namely diffuse mesangial proliferation (DMP) and focal segmental glomerulosclerosis (FSGS), do not account for more than 10% of nephrotic syndrome diagnosed in children (Banaszak *et al*., 2011).

The treatment of primary NS is based on administration of synthetic glucocorticoids (Banaszak *et al*., 2011; Jaroniec *et al*., 2010). Unfortunately, it is not always effective and, therefore, cases of steroid sensitivity can be distinguished, generally associated with MCNS (about 80% of all children suffering from primary nephrotic syndrome), as well as cases of steroid resistance, linked more frequently to MesPGN or FSGS (characterizing about 20% of patients) (Banaszak *et al*., 2011). The frequently employed synthetic glucocorticoids include prednisone (encorton), methylprednisolone (medrol, metypred), prednisolone (encortolon).

The action of glucocorticoids may involve genomic and non-genomic mechanisms.

GENOMIC MECHANISMS OF GLUCOCORTICOID ACTIVITY

Genomic mechanisms of glucocorticoid activity involve mediation of glucocorticoid receptor (GR) function. GR belongs to the family of intracellular nuclear receptors, which also includes receptors for mineralocorticoids, androgens, estrogens and thyroid hormones (Barnes, 1998; Smoak *et al*., 2004). Two isoforms of human GR, GRα and GRβ, arise due to alternate splicing of a single gene. GRα consists of 777 amino acids, binds hormones and activates genes reacting to glucocorticoids. GRβ, in contrast to GRα, contains just 742 amino acids in its C-terminal domain and it does not bind glucocorticoids. The function of GRβ has not been fully clarified but it may play a role of a negative controller of GRα and may mediate resistance to glucocorticoids during inflammatory response (Barnes, 1998; De Iudicibus *et al*., 2011; Lu *et al*., 2004; Smoak *et al*., 2004) (Fig. 1).

GRα consists of three domains. Its N-terminal transactivation domain contains a region capable of activating transcription of AF-1 genes. The DNA-binding domain, a highly conserved domain among the nuclear receptors, contains a zinc finger structural motif, which participates in dimerization of the receptor and its translocation to the cell nucleus. The final segment contains the C-terminal domain, responsible for binding of the ligand (Gross *et al*., 2009; Heitzer *et al*., 2007; Yang *et al*., 2012) (Fig. 1). In the cytoplasm, GR is present in its inactive form in a multimeric complex with various proteins, such as heat shock proteins 90 (Hsp90), Hsp70, Hsp56, Hsp40, p23 protein and Src (steroid receptor coactivator). Binding of glucocorticoids causes dissociation of the proteins and the receptor becomes active. The active receptor undergoes translocation to the cell nucleus, dimerization,

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Abbreviations: NS idiopathic nephrotic syndrome, MesPGN mesangial proliferative glomerulonephritis, FSGS focal segmental glomerulosclerosis, GR glucocorticoid receptor, Hsp heat shock protein, GRE glucocorticoid response element, NR3C1, nuclear receptor subfamily 3, group C, member 1, SNP single nucleotide polymorphisms, IPO13 importin 13, MIF macrophage migration inhibitory factor, JIA juvenile idiopathic arthritis, ALU acute lung injury, CYP cytochrome P, IBD inflammatory bowel disease, IL interleukin, ABCB1 ATP-binding cassette subfamily B member 1, GLCCI1 glucocorticoid-induced transcript 1 gene

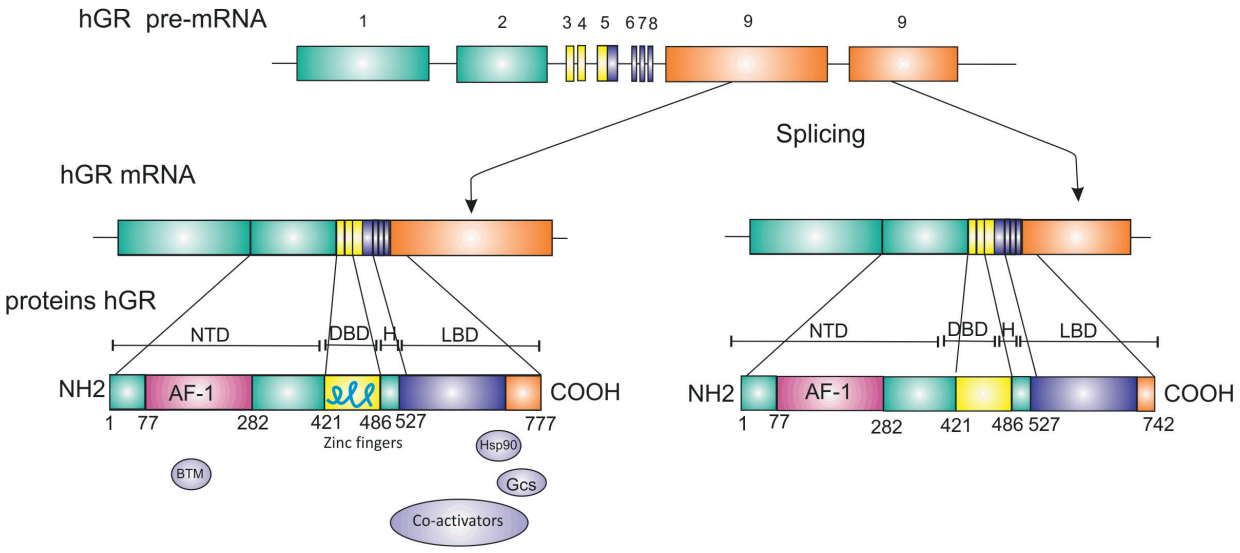

Figure 1. Structure of GR and formation of its two isoforms, α and β due to alternate splicing.
NTD, N-terminal transactivation domain; H, hinge; DBD, DNA binding domain; LBD, C-terminal ligand binding domain; GCs, glucocorticoids; hGR mRNA, human glucocorticoid receptor mRNA; hGR pre-mRNA, human glucocorticoid receptor pre-mRNA; AF-1, activation function-1.

and binds to specific DNA regions, the so called GREs (glucocorticoid response elements) or, as a monomer, to other transcription factors (a direct protein-protein interaction) (Croxtall *et al*., 2002; De Iudicibus *et al*., 2011; Lu *et al*., 2004) (Fig. 2).

Binding of the recetor to GRE may induce activation of genes, leading to an increased transcription of several anti-inflammatory genes, such as those encoding annexins, interleukin 10 (IL10), inhibitor of nuclear factor kappa B (ikB). The process is termed transactivation (Lu *et al*., 2004). Processes involving inhibition of gene activity, such as silencing of osteocalcin or prolactin genes due to binding to negative GRE are also possible but they are of no key importance for the anti-inflammatory role of glucocorticoids (Alangari, 2010). Reichardt *et al*. (1998) demonstrated that the activity of GR is not restricted to binding with GRE. They provided evidence for a direct action of GR. In the experiment with $GR^{dim/dim}$ mice, carrying a A458T point mutation within the dimerization-driving loop, the absence of dimerization and, thus, the absence of binding to GRE did not prevent GR from entering into functional reactions with transcription factors, such as AP-1 or NFκB (nuclear factor kappa B). This resulted in the attainment of maturity by the mice (Barnes, 1998; Croxtall *et al*., 2002).

NON-GENOMIC MECHANISMS OF GLUCOCORTICOID ACTIVITY

The non-genomic activity of glucocorticoids is manifested by a rapid effect (within seconds or minutes) of short duration (60–90 minutes), as compared to their genomic activity, but the time relationship is dependent on the dose of glucocorticoids (Alangari, 2010). The mechanism involves an interaction with cell membrane, effects resulting from binding of the cytosolic glucocorticoid receptor to the cell membrane and from binding of glucocorticoids to the receptor present in the cell membrane (Alangari, 2010; Stahn *et al*., 2008).

In higher concentrations glucocorticoids, through lipid peroxidation, may affect the properties of membranes leading to their increased permeability. This may be followed by alterations in ion transport across the membrane and changes in cellular production of ATP. The effects result in the inhibition of inflammatory cell function (Alangari, 2010). Interaction of glucocorticoids with membrane-incorporated cytosolic GR may be followed by inhibition of the release of arachidonic acid from

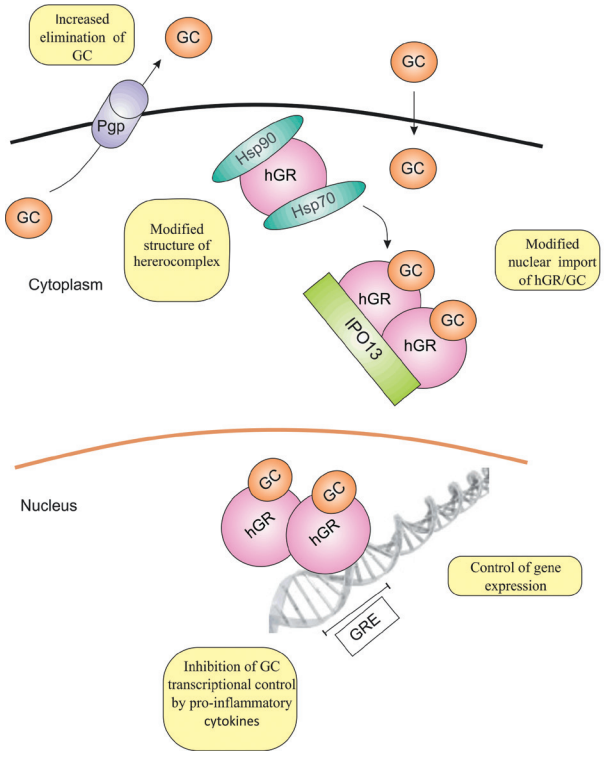

Figure 2. Mechanism of glucocorticoid action and factors engaged in development of steroid resistance according to De Iudicibus *et al*. (2011) modified.
IPO13, importin 13; hGR, human glucocorticoid receptor; GC, glucocorticoid; GRE, glucocorticoid response element; Pgp, glycoprotein P; Hsp90, heat shock protein 90.

membrane phospholipids; arachidonic acid acts as a mediator of cell growth and inflammatory reactions (Alangari, 2010; Stahn *et al*., 2008). Effects of non-genomic activity of glucocorticoids are also linked to their interaction with membranous GR (mGR), supposed to represent an alternative form of GR, arising due to alternate splicing, post-translational processing or a distinct promoter (Bartholome *et al*., 2004).

GENETIC FACTORS

The mechanisms of glucocorticoid action sheds some light on the potential causes due to which steroid resistance may develop in treatment of autoimmune diseases. Currently, extensive attention is devoted to the search for genetic variables which might explain ineffective steroid therapy in idiopathic nephrotic syndrome (Table 1).

The key element in the molecular mechanism of glucocorticoid action seems to involve GR. Mutations in the GR gene (*NR3C1*, nuclear receptor subfamily 3, group C, member 1) may result in steroid resistance. Also several polymorphisms were identified, which were linked to the disease (De Iudicibus *et al*., 2011; Mwinyi *et al*., 2010; Ouyang *et al*., 2012). In association with this, in recent years the term "Chrousos syndrome" appeared: a rare, familial or sporadic syndrome linked to mutation in the *NR3C1* receptor gene, characterized by insensitivity to glucocorticoids due to reduced expression or absence of functional GR, and by elevated levels of adrenocorticotropic hormone (ACTH) (De Iudicibus *et al*., 2011). Among the examined polymorphisms in the *NR3C1* gene, three proved to be functionally significant: they were linked to reduced sensitivity to both endogenous and exogenous glucocorticoids and could be potential causes of steroid resistance. They included the following polymorphisms: TthIII (rs10052957) — with a substitution of C>T in the promoter region, ER22/23EK (rs6189/rs6190) in the N-terminal transactivation domain, a two nucleotide substitution in codons 22 and 23 of exon 2, GR-9β (rs6198) in 3'UTR (3'-untranslated region), and an ATTTA sequence altered to GTTTA in exon 9β. On the other hand, two polymorphisms, N363S (rs6195) — exon 2, a substitution of AAT> AGT, resulting in a substitution of Asp-> Ser in codon 363, and BclI (rs41423247) — intron 2, with a substitution of C>G, were linked to an increased sensitivity to glucocorticoids (De Iudicibus *et al*., 2011; Gross *et al*., 2009; Mwinyi *et al*., 2010; van Rossum & Lamberts, 2006).

Components of the GR heterocomplex were also examined, including heat shock proteins 90 (Hsp90), which act as important molecular chaperones for GR and are assumed to be of key importance for the control of glucocorticoid effects (Gross *et al*., 2009; Ouyang *et al*., 2012). Earlier studies demonstrated that both expression and nuclear distribution of Hsp90 were augmented in steroid-resistant patients with idiopathic nephrotic syndrome. Ouyang *et al*. (2012) activated the expression of total Hsp90 in peripheral blood mononuclear cells by exposing them to interleukin 6 (IL6) *in vitro* and showed that their nuclear level of Hsp90, the binding of GR to DNA, and the activity of apoptotic cells remained unaltered. Moreover, an increased level of nuclear Hsp90 was mainly due to its binding to GR in the cell nucleus while GR binding to DNA decreased markedly in patients with steroid resistant nephrotic syndrome. This indicates that accumulation of nuclear Hsp90 potentially decreases the ability of GR to bind DNA and activate transcription, which may induce resistance to steroid therapy in patients with nephrotic syndrome (Ouyang *et al*., 2012). An altered level of Hsp90 was identified also in peripheral blood mononuclear cells in patients with steroid-resistant asthma or multiple sclerosis (Gross *et al*., 2009). Single nucleotide polymorphisms (SNPs) were also examined in the Hsp90 encoding gene (*HSPCA* coding for hsp90-1α, *HSPCB* coding for hsp90-1β). However, in adults with asthma, no correlation could be demonstrated between SNPs in *HSPCA* (3'-UTR+307, rs3736807, rs4906178, rs3809386, promoter –32) or *HSPCB* (rs504697 and rs3757286) genes on one hand and the response to treatment on the other (De Iudicibus *et al*., 2011).

In the mechanism of glucocorticoid action a significant role is played also by nuclear translocation recep-

Table. 1. Factors engaged in development of steroid resistance.

Factor	Mechanism
GR	Mutations and polymorphism of *NR3C1* gene
Heterocomplex of GR	Polymorphism of *HSP90* gene
	Expression of *HSP90* altered by coactivators
Nuclear receptors of translocation	Polymorphisms of importin 13 gene (*IPO13*)
Pro- and anti-inflammatory factors	Involvement of cytokines, including *MIF, IL4,IL6,TNF-α*
Glucocorticoid-induced transcript 1 protein	Polymorphisms in *GLCCI1*gene
Glycoprotein P	Polymorphisms of *ABCB1 (MDR1)* gene
	Altered expression of *ABCB1* under effect of pro-inflammatory cytokines
Cytochrome P450	Polymorphisms in *CYP3A5, CYP3A7,CYP3A4 i CYP3A43* genes

tors, which are responsible for the effective transport of GR to the cell nucleus. They include importin-13 (IPO13), which mediates the import of GR to cell nucleus. Raby *et al.* (2009) examined genetic variants of IPO13 in children with benign or moderate asthma treated with inhaled anti-inflammatory agents, including glucocorticoids, such as budesonid or nedocromil. The detected polymorphisms in the *IPO13* gene were found to affect the reactivity of the respiratory tract, influencing the bioavailability of glucocorticoids in childhood asthma (Raby *et al.*, 2009).

A significant involvement of several pro-inflammatory cytokines, including macrophage migration inhibitory factor (MIF) was also documented. MIF is produced mainly by T lymphocytes, but also by epithelial and endothelial cells and it plays an important role in securing innate immune resistance of the organism (Berdeli *et al.*, 2005). However, due to the broad range of action of the cytokine it also represents a critical mediator in many inflammatory and autoimmune diseases, such as septic shock, juvenile idiopathic arthritis (JIA), ulcerative colitis, pneumonia, type I diabetes mellitus, glomerulonephritis or tumours (Berdeli *et al.*, 2005; Stosic-Grujicic *et al.*, 2009; Vivarelli *et al.*, 2008). In contrast to other pro-inflammatory cytokines, which — in general — are suppressed by glucocorticoids, expression and secretion of MIF increase in response to physiological concentrations of glucocorticoids. On the other hand, during treatment of autoimmune diseases, MIF counteracts the effects of glucocoricoids by re-establishing production of cytokines by macrophages and by activating T lymphocytes. Based on this, it is concluded that MIF represents a factor which is induced by glucosteroids and, in parallel, leads to inhibition of their efficacy (Bucala, 2012; Stosic-Grujicic *et al.*, 2009). In this way inhibition of MIF seems to represent an effective tool in the treatment of many autoimmune diseases, including patients manifesting resistance to standard therapy with glucocorticoids (Berdeli *et al.*, 2005, Stosic-Grujicic *et al.*, 2009). Since it is assumed that polymorphisms in immune response genes may be a cause of many inflammatory and autoimmune diseases and that they may exhibit a prognostic value, polymorphisms of the *MIF* gene were examined. Baugh *et al.* (2002) identified a polymorphism in short tandem repeats within the *MIF* gene and found it to be linked to reduced activity of its promoter; a homozygous form of a specific genotype was demonstrated to be associated with a lowered risk of rheumatoid arthritis. The polymorphism involved the number of repeats of the CATT sequence in position –791 of the promoter, where the allele of 5 CATT repeats manifested the lowest activity of the promoter and it was the least effective in stimulating MIF promoter *in vitro*. Low expression of the allele with 5 CATT repeats correlated with low intensity of inflammation in rheumatoid arthritis. The presence of the –173G/C SNP (rs755622) in the *MIF* gene promoter was also analysed but no significant association with the disease was found (Baugh *et al.*, 2002). Shiroeda *et al.* (2010) examined functional polymorphisms in the *MIF* gene promoter in ulcerative colitis. In this case the 5/5-CATT genotype was found to be linked to a lower risk of ulcerative colitis (UC). In addition, the 7/7-CATT genotype was found to be strongly linked to UC phenotype in the distal colon and to chronically persistent disease. No such relationship could be detected in the case of the G-173-C polymorphism. Therefore, the authors suggested that the four nucleotide CATT repeat within the *MIF* gene promoter may be linked to development of UC and to advancement of inflammation in UC patients (Shiroeda *et al.*, 2010). Gao *et al.* (2007) examined MIF in acute lung injury (ALI), studying patients with sepsis or ALI-induced sepsis. In reference to Donn *et al.* (2002), who detected association between the –173C allele and JIA, Gao *et al.* (2007) also found a significant linkage between the CC genotype of the rs755622 polymorphism and an increased risk of sepsis development and pointed to the correlation between the polymorphism and microsatellite CATT repeats. Berdeli et al. (2005) examined the –173G/C polymorphism in children with an idiopathic nephrotic syndrome who failed to react to steroid therapy. The C allele proved to be strongly linked to steroid resistance in INS and a correlation was disclosed with histopathology characteristic for FSGS and IgM nephropathy. Moreover, Vivarelli *et al.* (2008) demonstrated a relationship between polymorphism of the –173C allele in the *MIF* gene promoter in steroid-resistant children with INS. The studies on MIF related to steroid resistance suggest that in such cases alternative therapies should be attempted, including treatment with MIF-specific antibodies (Berdeli *et al.*, 2005; Bucala, 2012; Vivarelli *et al.,* 2008).

It was shown that SNPs in the promoter region of the glucocorticoid-induced transcript 1 gene (*GLCCI1*) were responsible for steroid resistance in asthmatic patients and were associated with a decreased level of expression in lymphoblastoid B cells. Because of reports that *GLCCI1* is expressed in podocytes and mesangial cells in the kidney and suggestions that the loss of this protein may be correlated with the dysfunction of the filtration barrier, Cheong *et al.* (2012) examined *GLCCI1* gene polymorphisms in pediatric NS, including SR and SS patients, in the Korean population. They analysed two SNPs in the promoter region: rs37972 and rs3797, which were correlated with renal biopsy. However, they did not observe any association with steroid-responsiveness. It may suggest limitations of studies conducted on patients of one nationality and it shows that the function of GLCC1 at the cellular level is still not well determined (Cheong *et. al.*, 2012).

FARMACOKINETICS

Metabolizing enzymes and cellular pumps are fully engaged in pathways leading to elimination of several drugs. The most important of them include glycoprotein-P, the principal transporter eliminating xenobiotics, exporting drugs from the cell, and cytochrome P450 (CYP), which metabolizes several exo- and endogenous components. Both glycoprotein P and cytochrome P450 are expressed in lymphocytes, in cells of intestinal epithelium, in liver. They are engaged in transport and metabolism of glucocorticoids (De Iudicibus *et al.*, 2011).

A significant expression of glycoprotein P was detected in circulating lymphocytes and in epithelial cells in patients with Crohn disease (CD), in patients with UC with resistance to glucocorticoids and in patients with inflammatory bowel disease (IBD) but the relationship between administration of glucocorticoids and expression of glycoprotein P was described only in monocytes of patients with IBD. Therefore, it remains unknown whether increased glycoprotein P expression is linked to IBD or if it represents a secondary effect of therapy with glucocorticoids (De Iudicibus *et al.*, 2011). Glycoprotein P is encoded by the *MDR1* gene (*ABCB1*ATP — binding cassette, sub-family B, member 1) located on human chromosome 7q21.12. Polymorphisms of the *MDR1* gene were examined in SR and SS children with INS.

Jafar *et al.* (2011) examined three exon polymorphisms in the *MDR1* gene: G3435T, G2677T/A and C1236T in a population of Indian children with nephrotic syndrome and found that the homozygosity for the polymorphisms in exon 26 G3435T (TT *versus* CC) and in exon 26 G2677T/A (TT+AA *versus* GG) was observed much more frequently in nephrotic children than in the control and, in addition, that these polymorphisms were linked to the development of SRNS. In a similar manner Choi *et al.* (2011) and Wasilewska *et al.* (2006, 2007) presented results indicating involvement of the polymorphisms in the development of steroid resistance in children with NS. Krupoves *et al.* (2011) pointed to the relationship between the G3435T polymorphism in the ABCB1 gene and results of treatment with corticoids in patients with CD. In addition, Yossef *et al.* (2011) examined the relationship between serum IL2 content and the level of *MDR1* gene expression and found that high IL2 content correlated with high expression of the *MDR1* gene in lymphocytes of patients with SRNS. This pointed to elimination of glucocorticoids by glycoprotein P as a possible cause of steroid resistance. This finding is in agreement with a previous report by Stachowski (2000), which showed that the activity of Th1 cells, based on the production of IL2 and IFN-γ, was higher in steroid resistant children from the Polish population with NS. Furthermore, the high expression of the *MDR1* gene was observed in SRNS patients. This mechanism resulted in higher activity of the P-gp export pump in steroid therapy, whereas in the SSNS, CSNS (cyclophosphamide sensitive nephrotic syndrome) and CsASNS (cyclosporine sensitive nephrotic syndrome) groups the expression and activity of MDR1 were at a low level compared to SRNS and controls. Chiou *et al.* (2012) documented the link between the C1236T polymorphism in the *ABCB1* gene and steroid resistance in the Chinese population.

Human cytochrome P450 (CYP) comprises several isoforms. In humans, the CYP family includes CYP1, CYP2, CYP3, CYP4, CYP5, CYP7, CYP8, CYP11 and CYP17. The subfamily 3A of human cytochrome P450 (CYP3A) plays a significant role in drug metabolism. The four genes of CYP3A: *CYP3A5, CYP3A7,CYP3A4* and *CYP3A43* are located on chromosome 7q22.1 (De Iudicibus *et al.*, 2011, Chiou *et al.*, 2012). Chiou *et al.* (2012) analysed the A6986G polymorphism in the *CYP3A5* gene in INS patients, both steroid-sensitive (SS) and steroid-resistant (SR), subjected to therapy with prednisolone. Even if the frequency of the G allele was relatively higher in SR than in SS patients (the difference proved to be insignificant), the result may point to the potential involvement of the polymorphism in the development of steroid resistance.

Among potential factors engaged in the development of steroid resistance, polymorphisms in cytokine genes should be mentioned, especially in IL-4, IL-6, TNF-α (tumor necrosis factor-alpha) genes, where TNF-α represents a pro-inflammatory cytokine while IL-4 and IL-6 are anti-inflammatory cytokines. Pro-inflammatory cytokines are important in T and B lymphocytes as they initiate immune responses (Barnes, 1998; Jafar *et al.*, 2011; Yang *et al.*, 2012). Jafar *et al.* (2011) demonstrated correlations between polymorphisms in IL-6, G174C (rs1800795), IL-4, C590T(rs2243250), TNF-α, G308A (rs1800629) and NS as well as their marked role in diagnosis of SRNS. In addition, the IL-4 polymorphism correlated with predisposition to the minimal change nephrotic syndrome (Jafar *et al.*, 2011; Kobayashi *et al.*, 2003).

SUMMARY

As indicated by many literature data, patients with classical syndrome of resistance to treatment with corticosteroids manifest a quite variable phenotypic pattern. In addition, the clinical scope of the syndrome is very broad. Apart from its correlation with nephrotic syndrome it accompanies multiple diseases, such as asthma, rheumatoid arthritis, Crohn disease, Cushing disease, autoimmune hepatitis, blood neoplastic diseases, sepsis. This makes the diagnosis difficult and it requires additional tests as well as searches for heritable links. High hopes are linked to genetics and pharmacogenomics. Until now, the investigators succeded in examining several polymorphisms in key genes of molecular pathways linked to glucocorticoid actions, such as *NR3C1, HSPCA, HSPCB, IPO13, CYP, MIF, ABCB1, IL6, IL4, TNF,* but the results point only to the potential background of the syndrome. Data obtained so far indicate the need for additional investigations, search for new mutations and, perhaps, for epigenetic studies.

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