Glucocorticoid pharmacogenetics in pediatric idiopathic nephrotic syndrome

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Abstract

Idiopathic nephrotic syndrome (INS) represents the most common type of primary glomerular disease in children: glucocorticoids (GCs) are the first line therapy, even if considerable inter-individual differences in their efficacy and side effects have been reported. Immunosuppressive and anti-inflammatory effects of these drugs are mainly due to the GC-mediated transcription regulation of pro- and anti-inflammatory genes. This mechanism of action is the result of a complex multi-step pathway that involves the glucocorticoid receptor and several other proteins, encoded by polymorphic genes. Aim of this review is to highlight the current knowledge on genetic variants that could affect GC response, particularly focusing on children with INS.

Keywords

Glucocorticoids; idiopathic nephrotic syndrome; polymorphisms; glucocorticoid receptor; glucocorticoid receptor heterocomplex; inflammatory mediators; P-glycoprotein.

Idiopathic nephrotic syndrome (INS) is the most frequent primary glomerular disease in the pediatric population, and affects 16 - 17 per 100.000 children. The onset of the disease occurs usually between the ages of 2 and 8 years, with a peak of incidence between 3 and 5 years [1, 2]. The physiopathologic mechanisms of INS have not been completely clarified yet; however, the disease is triggered by an increase in glomerular permeability caused by an abnormal immunologic response, that results in an alteration of the capillary structure and of the integrity of the glomerular membrane [1].

Glucocorticoids (GCs) are the mainstay of INS therapy. Response to GCs is highly correlated to histological subtypes of the disease, and is poor in genetic forms that occur either as isolated kidney disease or as syndromic disorders. Several gene mutations have been associated to these hereditary forms, in particular variations in genes encoding for glomerular proteins such as nephrin (*NPHS1*), podocin (*NPHS2*), phospholipase C epsilon-1 (*PLCE1*), Wilms Tumor gene (*WT1*), CD2-associated protein (*CD2AP*) and others (for a review see [3]).

Also in non-genetic forms of INS, patients' response to GCs is the best indicator for outcome: indeed, those who respond poorly to these drugs and do not achieve remission have an unfavourable prognosis and often develop end-stage renal failure [4]. In minimal change nephrotic syndrome, the most common histopathological pattern in children, accounting for 70-80% of cases [2], after an initial response to prednisone, around 80% children relapse and some become steroid-dependent, while others never respond to GC therapy and are therefore steroid resistant (10%). These patients often require intensified immunosuppression with cyclophosphamide and/or cyclosporin A [1] [5].

This variable response to GCs is likely not attributable to the characteristics of the disease, and is clinically difficult to predict. Significant advances have been made over the past years in understanding the molecular basis of inter-patient variability: recent investigations have led to the hypothesis that genetic factors influencing the patient pharmacokinetic or pharmacodynamic profiles may account for 20% to 95% of variability in the efficacy and side effects of therapeutic agents [6]. Pharmacogenetics has therefore a promising role in personalized medicine, hopefully allowing the identification, a priori, of treatment sensitive and resistant patients and ensuring the right drug and right dose for each of them. In the context of INS, little is known about the impact of genetic polymorphisms on steroid response. Nonetheless, identification of predictive genetic biomarkers would be extremely beneficial, in particular for children with a steroid resistant disease, preventing their exposure to ineffective drug courses.

This review describes the mechanisms of GC action and discusses the molecular and genetic basis of GC resistance, with particular reference to non-genetic forms.

MOLECULAR MECHANISM OF GC ACTION (Figure 1)

GCs are anti-inflammatory and immunosuppressive drugs that exert their molecular action through both genomic and non-genomic mechanisms. Depending on whether or not they modulate gene transcription, GC induced effects could be delayed in onset but long-lasting or, vice versa, of more rapid onset and shorter duration.

Genomic mechanisms

Exogenous and endogenous GCs are lipophilic substances that diffuse across plasma membranes, thus interacting with a cytosolic receptor (the glucocorticoid receptor, GR), expressed in virtually all tissues. This receptor is a member of the large nuclear receptor superfamily, which includes receptors for steroid hormones and other hydrophobic molecules [7]; all these receptors are highly homologous to each other and have a common modular domain organization with a transactivation domain at the N-terminal part (NTD), a central zinc finger DNA-binding domain (DBD) and a ligand-specific binding domain (LBD) at the C-terminus. In the cytoplasm, the ligand-free GR exists in a multimeric complex associated with various chaperones and co-chaperones, such as the heat-shock proteins Hsp90, FKBP51, FKBP52, p23, Hsp70 and Hsp70/Hsp90 organizing protein (Hop) [8], that keep the receptor in the correct folding for hormone binding [9]. Upon binding, the receptor undergoes conformational changes and exposes the DBD and the nuclear localization signals, both hidden in the ligand-free conformation. The nuclear localization signals interact with transporters located on nuclear membranes (the importins), thus mediating the GR translocation into the nucleus. Once there, the DBD interacts, through its zinc finger motifs, with specific DNA sequences located within regulatory regions of GC-responsive genes, the GC-responsive elements (GRE), [10] [11]. The GR homodimerizes on GREs and recruits transcriptional co-activators and basal transcription machinery to the transcription start site. These co-activators, that include CREB (cAMP response element-binding) binding protein (CBP), steroid receptor co-activator-1 (SRC-1), GR-interacting protein (GRP-1) and the transcription factors p300 and switching/sucrose non fermenting (SWI/SNF), induce histone acetylation and thus the transactivation of GC-responsive genes (mediated by positive GREs). Through the induction of anti-inflammatory genes, such as interleukin (*IL*)*10*, *annexin 1* and the inhibitor of nuclear factor (*I-κB*),

transactivation is responsible for some of the GCs anti-inflammatory effects [12, 13]; however, transactivation enhances mainly the expression of genes involved in metabolic processes [14, 15], and is therefore responsible for the majority of side effects related to GC administration [16, 17]. In contrast, negative GREs [18] mediate downregulation of transcription of responsive genes and transrepression is responsible for the majority of the beneficial anti-inflammatory effects of GCs [16, 19-21]. Furthermore, GRE-independent mechanisms of transrepression also exist: the GR physically interacts and inhibits AP-1 [22] and nuclear factor (NF)-κB [23], two important transcription factors involved in the pro-inflammatory mechanism.

Non genomic mechanisms

Non genomic mechanisms have been also described and are responsible for the effects induced by GCs characterized by rapid onset and short duration. The mechanisms are still not completely clear, but likely involve non-classical membrane-bound GC receptors. In addition, at higher concentrations, GCs probably induce lipid peroxidation, with consequent alteration of the characteristics of plasma membranes and alteration in ion transport [24].

MOLECULAR MECHANISM OF GC RESISTANCE

The precise molecular mechanism conferring dependence or resistance to GCs in INS and in other diseases is still unclear; likely, the mechanism is not unique and probably occurs after impairments at different levels such as: 1) the GR receptor heterocomplex and proteins involved in nuclear translocation; 2) the pro- and anti-inflammatory mediators in the downstream signalling pathway of the GC-GR complex; 3) the P-glycoprotein (P-gp), an efflux transporter of GCs, and the drug-metabolizing enzyme CYP3A5.

1. The GR heterocomplex and proteins involved in nuclear translocation

The GR

The *NR3C1* gene, encoding for the human GR, is located on chromosome 5q31.3 and includes nine exons [25]. Several polymorphic sites have been described in this gene and have been supposed to affect, at least partially, the inter-patient variability in GCs response because they might alter the formation and the dynamic of the GC–GR complex and hence the downstream gene expression regulation [26]. However, only few variants have been associated with differences in metabolic parameters, body composition and altered

endogenous cortisol levels and are functionally relevant [26-37]. Single nucleotide polymorphisms (SNPs) such as *Tth*III (rs10052957), ER22/23EK (rs6189/rs6190) and GR-9β (rs6198), have been related to a reduced sensitivity to endogenous and exogenous GCs, while other *NR3C1* SNPs such as N363S (rs6195) and *Bcl*I (rs41423247) have been related to an increased sensitivity [26, 37]. *Tth*III is a C>T change in the *NR3C1* promoter region, located 3807 bp upstream of the GR start site [9]; the ER22/23EK polymorphisms involve two nucleotides changes (GAGAGG to GAAAAG) in codon 22 and 23 of *NR3C1* exon 2, which change the amino acid sequence of the NTD domain from glutamic acid-arginine (E-R) to glutamic acid-lysine (E-K) [38]; the GR-9β polymorphism is located in the 3'-untranslated region of exon 9β, where an ATTTA sequence is changed into GTTTA [39]. The N363S polymorphism consists of an AAT>AGT nucleotide change at position 1220 in exon 2, resulting in an asparagine to serine change in codon 363 [40], the *Bcl*I polymorphism was initially described as a polymorphic restriction site inside intron 2, and the nucleotide alteration was subsequently identified as a C>G substitution, 646 nucleotides downstream from exon 2 [41].

So far, only few studies have evaluated the role of the *NR3C1* polymorphisms on the response to exogenous GCs in patients affected by INS. The distribution of *Bcl*I and of two other SNPs, rs33389 and rs33388, (respectively a C>T and A>T substitution, 76889 and 80093 nucleotides downstream from exon 2) also located in intron B of the GR receptor gene, as well as the three-marker haplotype, has been studied in 136 healthy children and 118 INS pediatric patients who initially responded to oral GC therapy. The GTA haplotype was associated with a higher steroid sensitivity, determined by time to proteinuria resolution, and was more prevalent in early (response ≤ 7 days) than late (response > 7 days) prednisone responders (27.7 vs 14.5%, hap-score = -2.22, p = 0.05) [42]. The *Bcl*I polymorphism has been also analysed by Cho and co-workers [43] in 190 Korean children with INS and 100 controls, but no correlation with the development of INS, onset age, initial steroid responsiveness, renal pathologic findings and the progression of renal disease was found. The authors have also examined two other SNPs, namely ER22/23EK and N363S, but no variant allele was found in any of the patients or control subjects. Recently, Teeninga et al. [44] have evaluated GR-9β, *Tth*III and *Bcl*I polymorphisms in a well-defined cohort of 113 children with INS, showing that carriers of GR-9β+*Tth*III mutated haplotype had a significantly higher incidence of steroid dependence compared with non-carriers (52% vs 25%, OR = 3.04 95% CI 1.37–6.74, log rank test p = 0.003).

Several GR protein isoforms are generated through an alternative splicing: the most abundant and functionally active isoform is GRα, whereas GRβ is the inactive protein, unable to bind the ligand that exerts

a dominant negative effect on GRα. The GR-9β polymorphism has been associated with increased expression of the mature GR-β protein and implicated in steroid resistance in several diseases [45-49]. In patients with INS, an increased expression of GRβ has been demonstrated in peripheral blood mononuclear cells (PBMCs) of steroid resistant patients [50], while the expression of the functional isoform GRα was correlated with a positive steroid response (steroid responders vs partial- and non-responders $p < 0.01$) [51].

In 2006, Ye et al. [52] sequenced candidate exons of *NR3C1* gene and examined all the genetic variations in 138 Chinese children with sporadic steroid resistant and sensitive INS, founding no significant association between the SNPs analysed in the study and steroid response; however the analysis excluded the above mentioned polymorphisms that are located in *NR3C1* introns and regulatory regions.

The GR heterocomplex

Beside the proper functioning of the receptor itself, also the activity of all other components in the GR heterocomplex is essential for an adequate response to GCs. Altered levels of heterocomplex proteins, such as Hsp90, Hsp70, FKBP51, FKBP52, p23 and Hop, may contribute to altered GC cellular sensitivity [53] [54]. In INS, Ouyang et al. [55] have shown that the expression level of Hsp90 mRNA was significantly higher in adult patients than in healthy controls (1.09 ± 0.17 vs 0.98 ± 0.14, $p < 0.05$), and both the expression and nuclear distribution of Hsp90 were increased in PBMCs obtained from GC-resistant patients in comparison to GC-sensitive ones (1.28 ± 0.25 vs 1.13 ± 0.21; $p < 0.05$). The same authors have subsequently explored the interaction between Hsp90 and the GR in the nucleus as well as the DNA binding activity of the GR, showing that the nuclear enrichment rather than total cellular expression of Hsp90 might contribute to GC resistance and that the DNA binding activity of the GR was significantly ($p < 0.05$) decreased in GC resistant patients, hindering transactivation [56].

Clinical studies on the association between variants in genes coding for GR heterocomplex proteins and the GC response have been already carried out in several GC-treated diseases. In inflammatory bowel disease Maltese et al. [57] analyzed the role of *FKBP5* genetic variants (rs3800373, rs1360780 and rs4713916) and evidenced that the variant rs4713916 polymorphism was significantly associated with resistance to GC treatment in Crohn's disease (responders = 17% vs resistants = 35%; $p = 0.0043$). Moreover, in a cohort of asthmatic patients, Hawkins et al. [58] analyzed the role of *FKBP5* genetic variants in response to GCs, however the studied polymorphisms (rs3800373, rs9394309, rs938525, rs9470080, rs9368878 and rs3798346) were not correlated with response to these drugs. In the same study, genetic

variations in the *STIP1* gene (rs4980524, rs6591838, rs2236647, rs2236648), which codes for Hop, have been investigated and shown to have a role in identifying asthmatic subjects who were more responsive to GC therapy. An association with improved lung function, evaluated as baseline FEV1 (rs4980524, p = 0.009; rs6591838, p = 0.0045; rs2236647, p = 0.002; and rs2236648; p = 0.013) was found [58]. To date, no data on these polymorphisms and therapeutic outcome in INS are available. Pharmacogenetic studies are therefore required in order to understand the importance of these genetic variants in identifying resistant patients in this condition.

Nuclear transport factors

Upon binding with the receptor, the GR-GC nuclear translocation is essential to exert the GC pharmacological function, and this step is mediated by several nuclear receptors known as importins. [59] [60]. Importin 13 (IPO13) has been functionally characterized as a primary regulator of GC-bound GR across the nuclear membrane [10]. Altered levels of this protein might affect the therapeutic responsiveness to GCs and it has been demonstrated that *IPO13* silencing prevents GC transport across the cytoplasmic-nuclear membrane in airway epithelium and abrogates GC-induced anti-inflammatory responses [61]. SNPs in the *IPO13* family have been associated with neonatal respiratory outcomes after maternal antenatal corticosteroid treatment (SNP impact on fetal bronchopulmonary dysplasia: rs4448553; OR 0.01; 95% CI 0.00-0.92, p = 0.04; SNP impact on surfactant maternal therapy: rs2428953 OR, 13.8; 95% CI 1.80-105.5, p= 0.01 and rs2486014 OR 35.5; 95% CI 1.71-736.6, p = 0.02) [62]. Polymorphisms of *IPO13* (rs6671164, rs4448553, rs1990150, rs2240447, rs2486014, rs2301993, rs2301992, rs1636879, rs7412307 and rs2428953) have been investigated in children with mild to moderate asthma in relation with clinical response to GCs evidencing that *IPO13* variants could increase the nuclear bioavailability of endogenous GCs (subjects harboring minor alleles demonstrate an average 1.51–2.17 fold increase in mean PC_{20} at 8-months post-randomization that persisted over four years of observation: p = 0.01–0.005) [63]. To date, no study on *IPO13* genetic variants are available in INS patients, therefore investigation in this population is required.

2. The pro- and anti-inflammatory mediators in the downstream signaling pathway of the GC–GR complex

INS was proposed as a T cell dysfunction disorder [64], although mechanisms by which T cells affect the course of the disease are still unclear. Cytokines are released from activated T cells and play a crucial

role in the pathogenesis of INS [65] [66]; imbalances in T cells phenotypes, response and cytokines have been found between steroid sensitive and resistant INS patients [67] as well as between those who relapse and those in remission [68] [64].

Endogenous GCs are involved in the balance of pro- and anti-inflammatory mediators: a complex circular interplay between GCs and cytokines takes place, with GCs downregulating pro-inflammatory cytokines and cytokines limiting GC action [69] [70-72].

Basal cytokine expression levels are fine-tuned by genetic profile. Polymorphisms in the cytokine genes involved in the pathogenesis of INS (among which *IL1*, *IL12*, tumor necrosis factor (*TNFA*), macrophage migration inhibitory factor (*MIF*), *IL4*, *IL6* and *IL10*) and in glucocorticoid-induced transcript 1 gene (*GLCCI1*) might in part be responsible of inter-individual variations in therapy.

Pro-inflammatory mediators

IL-1: IL-1 family is a group of 11 cytokines among which IL-1α and IL-1β are the most studied. In glomeruli affected by several forms of INS, podocytes are capable of producing IL-1α/β [73]; however, the role of IL-1 in the immunopathogenesis of INS is still controversial. Saxena et al. found that, in supernatants of phytohaemagglutinin activated lymphocyte cultures obtained from patients with minimal change nephrotic syndrome, IL-1 levels were increased when compared to controls [74], while other studies did not confirm such finding. Chen and co-workers showed an overexpression of IL-1 at the protein and mRNA level in glomerular mesangial cells of patients affected by IgM mesangial nephropathy but not in those with minimal change nephrotic syndrome [75], and Suranyi et al. could not find differences between INS patients and controls in IL-1β levels measured in plasma, urine and culture supernatant of mitogen-stimulated PBMCs [76].

Several polymorphisms in *IL1* genes have been described [77] and associated with altered levels of the cytokine level [78]: T-31C (rs1143627) SNP results in the loss of the first T in TATA box and has been observed to cause a paradoxical increase in IL-1β in the presence of steroids in PBMCs under acute inflammation [79]. The C-511T SNP (rs16944) has been correlated to loss of the binding site for the transcription factor AP-2. Carriers of the haplotype composed of IL-1β -31C allele and -511T allele have showed a 2-3 fold increase in LPS-induced IL-1β secretion measured by an ex-vivo blood stimulation assay, the association was observed in two independent population ($p = 0.0084$ and $p = 0.0017$) [80, 81]; these

SNPs might therefore be of relevance in the modulation of GC response. So far, no data are available for INS and studies that investigate this association should be carried out.

IL-12: IL-12 has also been implicated in the pathogenesis of INS; this cytokine is produced by antigen presenting cells and regulates the growth and development of natural killer (NK) and T cells; in addition, it is the major inducer of interferon (IFN)-γ [82].

IL-12 serum levels have been investigated in different cohorts of patients: Lin and Chien [83] studied 20 INS patients and found a significant increase of the cytokine in relapsed patients as compared to patients in remission and to normal controls. The amount of IL-12 was also increased during the active phase of the disease as compared to the remission and was reported to upregulate the production of vascular permeability factor, a clinical index of INS [84, 85]. On the contrary, Stefanovic et al. did not find difference in terms of IL-12 production between concanavalin A-stimulated PBMCs of 20 children with steroid sensitive INS and 17 healthy control subjects [86].

Genetic variations in *IL12* gene have been investigated: a complex bi-allelic polymorphism in the promoter region of the gene, coding for the p40 subunit (IL12B) has been described (IL-12Bpro, CTCTAA/GC polymorphisms; rs17860508). IL-12Bpro allele 1 has been related to a reduced IL-12 secretion in dendritic cells [8, 87]. Surprisingly, this allele had a high frequency in 45 steroid dependent INS children (46.7%) compared to 34 non dependent (17.6 %; p = 0.016) [8].

TNF: TNF is a potent pro-inflammatory protein released by monocytes upon stimulation, being almost undetectable in resting conditions [88]. The *TNFA* gene is located on chromosome 6p21.3, in the class III region of the major histocompatibility complex within the human leukocyte antigen [89, 90], which contains many genes involved in inflammatory and immune responses [91]. An increase in *TNFA* gene expression, higher serum TNF levels and TNF production by monocytes has been demonstrated in INS patients with active disease, in comparison with patients in remission and controls [92]. TNF was the only cytokine found to be increased in plasma and urine in INS patients affected by segmental glomerulosclerosis and membranous nephropathy, but not in those with minimal change nephropathy [76].

Among *TNFA* polymorphisms, the G-308A (rs1800629) is one of the best documented [93]. This SNP lies in a binding site for the transcription factor AP-1 and the A allele has been shown to have higher transcriptional activity than the G allele, increasing TNF production *in vitro* [94]. Conflicting results have been

reported for this polymorphism in patients with INS. A study by Kim and colleagues, on 152 patients with childhood INS and 292 healthy adult controls, investigated the association between cytokine polymorphisms, among which *TNFA* G-308A, and disease susceptibility, and did not find significant differences in allele frequencies between the two populations [95]. This study is in contrast with other results that found a significant association, both at genotypic and allelic level, with susceptibility and with steroid resistance. Indeed, on comparing 115 GC sensitive and 35 GC resistant patients, the AA genotype was suggested as a causative factor of non responsiveness to steroid therapy among INS children (responsive vs non-responsive patients: at genotypic level OR = 14.71, 95% CI = 1.59-136.46, p = 0.0121; and at allelic level OR = 2.251, 95% CI = 1.09-4.66, p = 0.0433) [96, 97].

MIF: MIF is also a pro-inflammatory cytokine with a pathogenic role in kidney diseases [98]. MIF is produced by several cell types, particularly T cells but also monocytes, macrophages, glomerular epithelial cells, tubular epithelial cells and vascular endothelial cells. Due to its regulatory properties on innate and adaptive immune responses, MIF is considered a critical mediator in various immune and inflammatory diseases [99-102]: its expression has been found to be increased in all forms of glomerulonephritis although not in minimal change nephrotic syndrome [98].

MIF has the ability to override the inhibitory effects of GCs on the immune system: when present at low levels, GCs up-regulate MIF, while at higher GC concentrations, a counter-regulatory mechanism is observed and GCs down-regulate this cytokine expression [103, 104]. The *MIF* gene is located on chromosome 22q11, and recently a G-173C (rs755622) polymorphism, that involves a G to C substitution at base pair 173 of the 50-flanking region, was found to be strongly associated with higher MIF expression *in vitro* [101]. Berdeli et al. [105] and Vivarelli et al. [106] have investigated this polymorphism in Turkish and Italian children with INS (214 and 257 respectively) and found that the frequency of the C allele was higher in patients than in controls (19 vs 8%, OR=2.5, 95 CI% 1.4–4.2, p = 0.0007 [105] and 32 vs 22% OR=1.67, 95% CI 1.16–2.41; p = 0.006 [106]); in addition, the polymorphism was significantly more frequent in steroid resistant patients than in sensitive ones (33 vs 12% OR=3.6, 95 CI% 2.2–6.0, p < 0.0001 [105] and 44 vs 23% OR 2.61, 95% CI 1.52–4.47; p = 0.0005 [106]). Interestingly Choi et al. [107], investigating the same SNP in 170 Korean children with INS could not find any association between the G-173C polymorphism and clinical parameters, renal histological findings and steroid responsiveness.

313 Moreover, in a recent study, Swierczewska et al. [108] investigated the role of seven other
314 polymorphic variants of the *MIF* gene: two polymorphisms, rs2070767 (C>T) and rs2000466 (T>G), were
315 found to have a significantly different distribution between 30 resistant and 41 sensitive INS patients
316 (rs2070767, CT vs CC, OR=3.00, 95 CI% 1.043-8.627, p=0.047; rs2000466, TG+GG vs TT, OR=0.321, 95
317 CI% 0.119-0.869, p=0.028); however, when linkage disequilibrium analysis was performed, the significance
318 was lost.

319 Finally, a recent meta-analysis of Tong and colleagues [109], considering all the articles cited before,
320 confirmed that *MIF* G-173C polymorphism may increase the risk of renal disease and may be associated
321 with GCs resistance in INS, especially in children. The pooled results, considering eight case–control studies
322 and 2755 participants, indicated a significant association between MIF −173G/C polymorphism and renal
323 disease risk (CC+CG vs GG, OR = 1.77, P < 0.01; C vs G, OR = 3.94, P < 0.01).

324

325 *Anti-inflammatory mediators*

326 **IL-4:** IL-4 is a potent anti-inflammatory [110] and a key cytokine involved in the development of allergic
327 diseases, being required, together with other cytokines, for the class switching of B cells to immunoglobulin
328 E (IgE) production [111]. INS is frequently associated with allergic symptoms and elevated serum IgE levels
329 [112]. Increased serum IL-4 levels have been observed in patients with INS [113] and in particular in steroid
330 sensitive patients in active stage compared with those in remission (p=0.033) and with healthy controls,
331 (p=0.011) [68]; similar results were obtained by Prizna et al. in INS patients with active stage in comparison
332 with patients in remission on steroids (p < 0.0001), in remission off steroids (p < 0.0001) and controls (p <
333 0.0001) [114].

334 Genetic variants in *IL4* may be associated with predisposition to INS, and to the clinical course of the
335 disease [115-117]. A C>T exchange at position 590 upstream from the open reading frame of the *IL4* gene
336 (rs2243250) has been shown to be associated with elevated levels of IgE [118]. Tripathi et al. [97]
337 demonstrated that this polymorphism influences the prognosis of the disease: indeed, the TT genotype was
338 more frequent in 35 children with steroid resistant INS as compared to 115 steroid sensitive (OR = 7.29, 95%
339 CI = 1.26-41.69, p = 0.0386). This observation was subsequently confirmed by Jafar et al. in a cohort of 150
340 INS children (OR = 6.46, 95 CI% 1.11–37.66, p = 0.020) [96].

341 IL-4 signaling is mediated by the interaction of the cytokine with its receptor, mainly expressed in
342 hematopoietic cells. The distribution of the IL-4 receptor α chain genetic polymorphism Ile50Val (rs1805010)

was studied in 85 Japanese INS patients grouped according to the number of relapses: the mutated genotype was significantly less frequent in patients who experienced four or more relapses (3.3%) compared to those who experienced three or less recurrences (29.8%, p = 0.007) [119]. However, these data were not confirmed by Tenbrock et al. [120] who could not find an association between patient genotypes and INS clinical courses (measured as frequent relapses (29 children) and steroid dependence (35) or resistance (11)).

IL-6: IL-6, a multifunctional cytokine that plays a central role in host defenses [121], and has both pro- and anti-inflammatory effects. In INS, plasma levels of this cytokine were associated to disease susceptibility, being increased in patients compared to controls [122], and to treatment responsiveness, being enhanced in steroid resistant patients compared to steroid sensitive and controls ($p < 0.05$) [123].

The *IL-6* gene, located on chromosome 7p21-24, presents different polymorphisms. Among these, the common G>C SNP at position -174 in the promoter region, influences the transcriptional regulation and the cytokine plasma levels in different renal diseases [124, 125]. Tripathi et al. [97] found that the GG genotype was more frequent in 35 INS steroid resistant children (11.4%), as compared with 115 steroid sensitive patients (0.9%; OR = 14.71, 95% CI = 1.59-136.46, p = 0.0121). These results have been confirmed by Jafar et al. [96] (OR = 31.40, 95% CI = 3.62–272.3, $p < 0.001$) suggesting that this polymorphism could be a causative factor for non-responsiveness toward steroid therapy among INS children.

IL-10: IL-10, known as human cytokine synthesis inhibitory factor, is produced primarily by monocytes and to a lesser extent by lymphocytes. IL-10 has pleiotropic effects in immunoregulation and inflammation [126] [127]; it inhibits the production of inflammatory mediators, and can be considered as a natural immunosuppressant of TNF [128].

GCs upregulate the expression of IL-10 [69], that in turn acts synergistically with GCs, as demonstrated in whole-blood cell cultures where the presence of IL-10 improved the ability of dexamethasone to reduce IL-6 secretion. In addition, the cytokine increased the concentration of dexamethasone-binding sites in these cells, with no effect on the binding affinity [126].

IL-10 expression was significantly reduced in T regulatory cells from adult INS patients (10.3 ± 3.4 pg/ml) compared to healthy donors (19.3 ± 5.9 pg/ml; $p < 0.01$) [129]; similar results were obtain by Araya

and colleagues; p<0.0191) [130], while no significant difference was found between IL-10 serum levels of INS pediatric patients in nephrotic phase (heavy proteinuria) and in remission [111].

The human *IL10* gene is located on chromosome 1q31–q32. Previous studies have demonstrated that an A>G polymorphism at nucleotide position –1082 in the promoter region (rs1800896) influences the IL-10 transcriptional levels. The mutated genotype has been associated with significantly higher cytokine plasma levels in acute lymphoblastic leukemia patients [131], as well as with a positive prednisone response in childhood acute lymphoblastic leukemia [33, 131] and in patients with rheumatoid arthritis [132].

To authors' knowledge, association of *IL10* polymorphisms and the response to steroid therapy in INS has never been investigated; in a pharmacogenetic study on rs1800896, the GA/GG genotypes have been associated, in 191 patients, with the progression of the disease in both IgA nephropathy and focal segmental glomerulosclerosis (the GA/AA genotypes was over-represented in fast progressors: OR = 1.25, 95% CI 1.07–1.47, p = 0.012) [133].

GLCCI1: GLCCI1 was initially identified as a transcript rapidly up-regulated in response to GC treatment in cells derived from a thymoma [134]. In the kidney, it is expressed specifically in mesangial cells and podocytes and knockdown of the transcript impairs the glomerular filtration barrier in developing zebrafish [135]. Recently in a genome-wide association study, which examined the response to inhaled GCs in 1041 asthmatic patients, two SNPs (rs37972 and rs37973) in complete linkage disequilibrium in the promoter region of *GLCCI1* have been associated with a poorer response to steroid treatment (OR = 1.52, 95% CI = 1.13 - 2.03) [136].

Cheong and colleagues [137] genotyped 211 pediatric patients with INS and 102 controls for the rs37972 and rs37973, and did not found any statistically significant associations between the SNPs analyzed and either the development of INS, or initial response to steroid therapy.

3. P-glycoprotein (P-gp) and drug metabolizing enzyme CYP3A5

P-glycoprotein

P-gp is a 170-kDa ATP dependent membrane transporter, an efflux pump responsible for resistance to a number of structurally and functionally unrelated drugs, including natural and synthetic GCs [138], that are actively exported from cells against the concentration gradient [139]. Several studies have been conducted to evaluate the association of P-gp expression with the responsiveness to GCs in many diseases among

which INS: Wasilewska et al. [140] found that P-gp expression in CD3 positive lymphocytes was significantly higher in patients with INS than in controls ($p = 0.0004$). A significant difference was also observed between controls (1.24 ± 0.58) and both steroid dependent (7.00 ± 3.09, $p = 0.0001$), and the frequent relapsing group (5.56 ± 4.07, $p = 0.0002$); while the difference with the non frequent relapsing group was smaller ($p < 0.05$). Moreover a significant difference was observed between non frequent relapsing (3.02 ± 3.46) and both steroid dependent ($p < 0.001$) and frequent relapsing group ($p < 0.001$) [141]. P-gp mRNA expression levels in PBMCs were found to be variable in patients with INS prior to remission, but decreased after complete remission ($p < 0.003$) [142]. In another study by Stachowski et al. [143], mRNA expression in peripheral lymphocytes of patients with steroid, cyclophosphamide or cyclosporine resistant INS was higher than in lymphocytes from patients who were sensitive to these drugs ($p < 0.001$). Moreover, in a recent work, Prasad et al. [68] found that steroid therapy in INS decreased P-gp expression in peripheral blood lymphocytes (absolute P-gp expression at baseline 66.59 ± 21.13 vs remission 35.84 ± 22.26, $p < 0.05$) .

P-gp is encoded by the ATP-Binding Cassette, sub-family B (*ABCB1;* multi drug resistant protein 1 *MDR1*) gene, located on human chromosome 7q21.12 [144], and several studies have demonstrated that genetic polymorphisms in this gene lead to functional alterations and are associated with altered drug disposition [145, 146]. A synonymous SNP in exon 26 (C3435T, rs1045642) was the first variation to be associated with altered protein expression [145]. SNPs at exons 12 (C1236T, rs1128503), 21 (G2677T/A, rs2032582) and 1b (T-129C, rs3213619) may also be associated with altered transport function or expression [147].

In 108 pediatric INS patients, Wasiliewska et al. [148] have studied the association between C1236T, G2677T/A and C3435T polymorphisms and the clinical course and treatment response. All individual polymorphisms were strongly associated with time to response to initial prednisone therapy (OR = 6.79, 95% CI: 1.96-23.54, $p < 0.001$ for 1236 T/T, OR = 13.7, 95% CI: 2.78–67, $p < 0.001$ for 2677 T/T and OR = 9.92, 95% CI: 3.01–32.71, $p < 0.001$ for 3435 T/T), and the frequencies of the mutated allele were higher in late responders (53%, 52%, 66% for the C1236T, G2677T/A and C3435T polymorphisms respectively) than in early responders (24%, 19%, 32%). The TTT haplotype was also significantly associated with late steroid response compared to early response (49% vs. 19%, $p = 0.0003$).

More recently, Choi et al. [107] have investigated the same polymorphisms (C1236T, G2677T/A and C3435T) in 170 Korean children with INS, finding that the frequencies of the TGC haplotype was significantly lower in the initial steroid responders (115 children) than in non-responders (35) (15.8 vs 29.0%; OR 0.46,

95% CI 0.27–0.78, p = 0.004). Jafar at al. [149], in 216 patients with INS and 216 controls, found that the homozygous mutations of G2677T/A SNP was associated with steroid resistance (18% steroid resistant vs 6% steroid responsive OR = 3.39, 95% CI 1.29–8.93, p = 0.011) and that the combination of mutated genotype of SNP G2677T/A and C3435T synergistically increased the risk of developing steroid resistance in patients with INS (5% in steroid resistant patients, 2% in steroid responsive and 1% in controls, p = 0.038).

Chiou et al. [150] also investigated in 74 children with INS the same polymorphisms. They could find only a significant association of C1236T polymorphism with steroid resistance: the frequency of the T allele was significantly higher in steroid resistant patients than in sensitive ones (81 vs. 62%; OR = 2.65, 95 % CI 1.01-6.94; p = 0.042).

In a recent study Youssef et al. [151] evidenced that the mutated and heterozygous G2677T/A variants were significantly more frequent in 46 non-responders INS patients (28%) than in 92 responders (20%; OR = 2.9, 95% CI 0.95–9.21, p = 0.016). Finally Cizmarikova et al. [152] also found in 46 INS patients a significantly increased chance of therapeutic response in children carrying the 3435CT genotype (OR = 5.13, 95% CI 1.18-22.25, p = 0.022).

As shown in Table 1, P-gp has been largely studied in INS patients, and the results seem to be the most coherent among the polymorphisms studied in this disease.

CYP3A5

The human cytochrome P450 (CYP) family comprises a number of CYP isoforms that have important functions in the reductive and oxidative metabolism of many endogenous and exogenous compounds, among which steroids. CYP3A5*3 is an A to G transition (A6986G) within intron 3 of *CYP3A5* gene that creates an alternative splice site in the pre-mRNA, producing an aberrant mRNA with a premature stop codon. CYP3A5*3 homozygotes (GG genotype) lack CYP3A5 expression, while individuals with at least one CYP3A5*1 wild-type allele (AA and AG genotypes) express the protein [153]. In a recent study of Chiou and colleagues, authors investigated polymorphic expression of CYP3A5 in 74 children with INS: the frequency of the G allele (A6986G SNP) was relatively higher in steroid resistant subjects than in steroid sensitive ones showing a trend of association, that however did not reach statistical significance (OR 2.63, 95 % CI 0.94–7.37; p=0.059) [150] .

Genetic polymorphisms of *CYP3A5* and *ABCB1* could have a role on the pharmacokinetics of prednisolone; in particular, intestinal CYP3A5 and P-glycoprotein are important in the absorption, systemic drug distribution and cellular accumulation of glucocorticoids. However, a study of Miura et al. [154] found only a small effect of *CYP3A5* and *ABCB1* genetic polymorphism on prednisolone pharmacokinetics. Intracellular accumulation of GCs within lymphocytes, influenced by the expression of P-gp on these cells, is probably more important and could influence steroid response in INS.

CONCLUSION

GCs are used in the treatment of active INS to induce remission of proteinuria, but inter-individual differences in their efficacy and side effects have been reported. A main goal for clinicians is therefore to improve the efficacy and safety of these agents and, when possible, to reduce steroid exposure. This is particularly important in patients that do not respond and will suffer considerable steroid side effects without any clinical gain, or in patients that will be dependent to steroid treatment and will not be able to withdraw the drug, in whom switching to other therapy as soon as possible could be very important. Molecular mechanisms involved in variability in GC response are still not completely known, but advance in pharmacogenomics could contribute to the optimization and personalization of therapy.

This review is about the current literature on the molecular mechanisms of GC anti-inflammatory action and the role of genetic polymorphisms in variable GC response in patients with INS. Results of reported papers are not conclusive and often in contradiction, and at present none of the potential pharmacogenetic markers is strong enough to be used in clinical practice.

FUTURE PERSPECTIVES

In the future, beside candidate gene approach it would be necessary to perform sequencing of all the genes involved in the GC mechanism of action, to obtain new comprehensive information. Recently, genetics have focused the attention on copy number variation (CNV) and DNA methylation analyses. CNVs are genomic alterations that result in the cell having an abnormal number of copies of one or more sections of the DNA. Some CNVs have already been associated with susceptibility to diseases or response to drug therapy but, until now, no data are available for GCs in relation to clinical response. In addition, DNA methylation of gene promoters has been associated with transcriptional inactivation: changes in DNA methylation can lead to differences in gene expression levels and thereby influence drug response. All these

approaches need to be performed in larger and well-characterized patient cohorts, uniformly treated and systematically evaluated, and subsequently validated in other independent cohorts.

In conclusion, these new strategies for the identification of pharmacogenetic determinants associated with GC response in paediatric INS patients, and the consequent personalization of therapy based on this information, will result in higher quality and less toxic treatment of children, avoiding inadequate regimens or time wasting and reducing overall health costs.

Executive Summary

- INS is the most frequent primary glomerular disease in the pediatric population and GCs are the first line therapy in these patients. However there is a considerable inter-individual variability in response to GCs that is clinically difficult to predict.
- Genetic factors could influence GC response, therefore pharmacogenetics has a promising role in personalized medicine even if, to date, not conclusive results have been reported for steroid clinical response.
- Several polymorphisms in genes involved in GC molecular mechanism (GR heterocomplex, pro- and anti-inflammatory mediators and P-gp) could affect GC response in INS patients.

GR heterocomplex

- The *NR3C1 Bcl*I, rs33389 and rs33388 SNPs have been associated with a higher steroid sensitivity while GR-9β and *TthIII*I haplotype was associated with steroid dependence.
- The expression level of Hsp90 mRNA was increased in PBMCs obtained from GC-resistant patients in comparison to GC-sensitive ones. On the contrary, to date, no data on *Hsp90, FKBP51, FKBP52, p23, Hop* and *IPO13* gene polymorphisms and therapeutic outcome in INS are available; pharmacogenetic studies are therefore still required.

Pro- and anti-inflammatory mediators involved in INS pathogenesis

- A complex bi-allelic polymorphism in the promoter region of the gene coding for the p40 subunit of *IL-12* gene has a higher frequency in steroid dependents compared to steroid responders.
- The *TNF-α* G-308A polymorphism has also been investigated and the AA genotype has been suggested to be a causative factor of non responsiveness to GC therapy.
- *MIF* G-173C polymorphism may increase the risk of renal disease and may be associated with GCs resistance risk especially in children.
- The *IL-4* C590T mutated genotype has been associated with steroid resistance in children with INS.
- The wild type genotype of G-174C polymorphism in *IL-6* gene has been suggested to be a causative factor for GC non-responsiveness.

P-glycoprotein (P-gp)

- Variant genotypes in *ABCB1* gene (C3435T,G2677T/A, C1236T) alone and in haplotype have been correlated with steroid resistance.

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Legend to the figure

Figure 1: Molecular mechanisms of action of glucocorticoids.

Table 1

Summary of studies reporting genetic analysis of NR3C1 in INS patients.

Table 2

Summary of studies reporting genetic analysis of pro- and anti-inflammatory mediators in the downstream signaling pathway of the GC-GR complex in INS patients.

Table 3

Summary of studies reporting genetic analysis on the role of P-gp in INS patients.

First author	Year	Ethnicity	Case/Control	Age (mean)	Results
Results for genetic analysis of NR3C1 in INS patients					
Zalewski G et al. [42]	2008	Caucasian (Poland)	118/136	5.1/NA	*Bcl*I (G>C), rs33389 (C>T) and rs33388 (A>T) GTA aplotype was associated with a higher steroid sensitivity.
Cho HY et al. [43]	2009	Asian (Korea)	190/100	4.95/NA	No correlation between the INS onset age, initial steroid responsiveness, renal pathologic findings, or progression to end-stage renal disease and ER22/23EK, N363S, and *Bcl*I polymorphisms.
Teeninga N et al. [44]	2014	Caucasian (Holland)	113	4.1	Carriers of GR-9β + *TthIII*I mutated haplotype had a significantly higher incidence of SD compared with non-carriers.
Ye J et al. [52]	2006	Asian (China)	138	7.1	No association found with the studied polymorphisms.

SD: steroid dependant; FR: frequent relapser; NFR: non frequent relapse; SS: steroid sensitive; SR: steroid resistant; NR: non responder

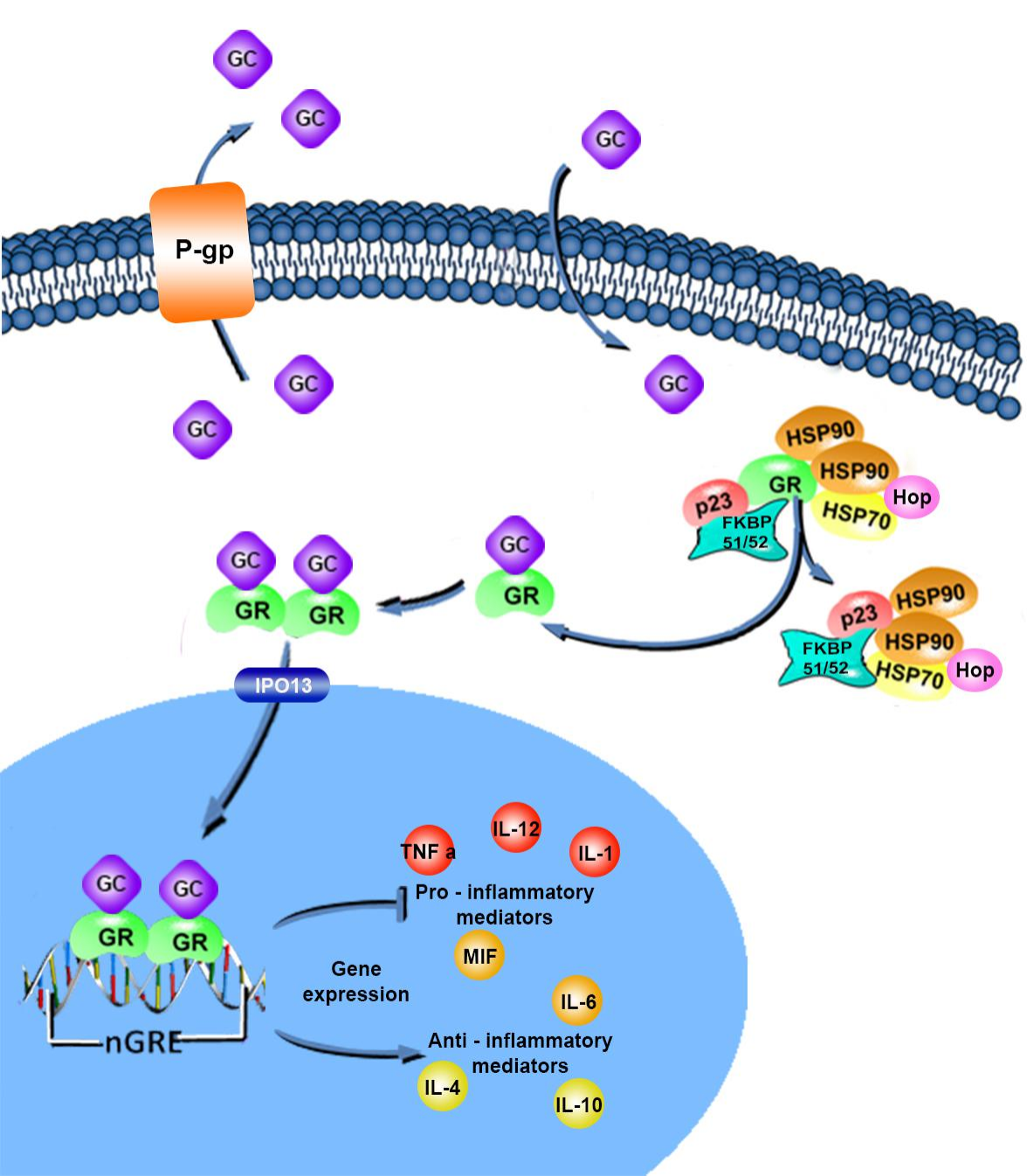
First author	Year	Ethnicity	Case/Control	Age (mean)	Results
Results for genetic analysis of pro- and anti-inflammatory mediators in the downstream signaling pathway of the GC-GR complex in INS patients					
IL-12					
Muller-Berghaus J *et al.* [8]	2008	Caucasian (Germany)	79	10.7	Significantly higher allele frequency of IL12Bpro-1 in steroid-dependent children compared to children without SD.
TNF					
Kim SD *et al.* [95]	2004	Asian (Korea)	152/292	NA/NA	No association with TNF and IL-1beta.
Jafar T *et al.* [96]	2011	Asian (India)	150/569 115(SS)/35(SR)	4.8/NA	Association for *TNFA* (G308A) comparing patient with controls and SR group with SS group.
Tripathi G *et al.* [97]	2008	Asian (India)	115(SS)/35(SR)	4.8	The AA genotype of *TNFA* (G308A) was associated with lower steroid response.
MIF					
Berdeli A *et al.* [105]	2005	Caucasian (Turkish)	214/103 137(SS)/77(SR)	3.5/NA	Significant increase in *MIF* G-173C GC genotype and C allele frequency in INS and higher frequency of CC genotype in the SR group.
Vivarelli M *et al.* [106]	2008	Caucasian (Italian)	257/355	5.8/NA	Frequency of *MIF* -173*C allele was higher in patients with INS than in controls and more frequent in SR patients compared with steroid responders.
Choi HJ *et al.* [107]	2011	Asian (Korea)	170/100	5.17/NA	No association with *MIF* G-173C.
Swierczewska M *et al*. [108]	2014	Caucasian (Poland)	71/30	10.1/10.1	*MIF* CT genotype of rs2070767C>T associated with the risk of SR, while the distribution of TG genotype of rs2000466T>G was higher in SS children compared to SR.
IL-4					
Jafar T *et al.* [96]	2011	Asian (India)	150/569	4.8/NA	Association for *IL-4* (C590T) polymorphism comparing patients with controls and SR group with SS group.
Tripathi G *et al.* [97]	2008	Asian (India)	115(SS)/35(SR)	4.8	The TT genotype of *IL-4* (C590T) polymorphisms associated with reduced steroid response.
Ikeuchi Y *et al.* [119]	2009	Asian (Japan)	85/127	NA	*IL-4R alpha* (Ile50Val) mutated genotype less frequent in patients with 4 or more relapses compared to those who experienced fewer recurrences.
IL-6					

First author	Year	Ethnicity	Case/Control	Age (mean)	Results
Jafar T *et al.* [96]	2011	Asian (India)	150/569	4.8/NA	Association for *IL-6* (G174C) comparing patient with controls and SR group with SS group.
Tripathi G *et al.* [97]	2008	Asian (India)	115(SS)/35(SR)	4.8	The GG genotype of *IL-6* (G174C) polymorphism associated with reduced steroid response.

SD: steroid dependant; FR: frequent relapser; NFR: non frequent relapse; SS: steroid sensitive; SR: steroid resistant; NR: non responder

First author	Year	Ethnicity	Case/Control	Age (mean)	Results
Results for P-gp expression analysis					
Wasilewska A *et al.* [141]	2006	Caucasian (Poland)	88/18	10.0/9.18	Expression of P-gp higher in SD and FR than in NFR.
Wasilewska A *et al.* [140]	2006	Caucasian (Poland)	18/18	5.75/6.50	Expression of P-gp higher in patients in relapse than in controls and decreased in remission.
Funaki S *et al.* [142]	2008	Asian (Japan)	14	10.4	mRNA levels decrease in complete remission in SS.
Stachowski J *et al.* [143]	2000	Caucasian (Poland)	39	(range 3-8)	Higher expression of P-gp mRNA in SR than in SS.
Prasad N *et al.* 68]	2015	Asian (India)	26/10	8.0/NA	Expression of P-gp higher at baseline and at the time of relapse compared to remission.
Results for genetic analysis of SNPs C1236T, G2677T/A, C3435T					
Wasilewska A *et al.*[148]	2007	Caucasian (Poland)	108/135	11.13/6.23	SNPs associated with time to response, TTT haplotype associated with late steroid response.
Choi HJ *et al.*[107]	2011	Asian (Korea)	170	5.17	Frequencies of 1236CC and CT higher in initial steroid responders than in NR, frequency of TGC haplotype lower in the initial steroid responders than in NR.
Jafar T *et al.*[149]	2011	Asian (India)	216/216	5.0/6.0	Frequency of 2677GG/AA higher in SR than in SS. Combination of 3435TT and 2677TT/AA increased the risk of SR.
Chiou YH *et al.*[150]	2012	Asian (Taiwan)	74	3.9(SS), 7.2(SR)	1236 T allele associate with SR.
Youssef DM *et al.*[151]	2013	African (Egypt)	138/140	2.7(SS), 4.6(SR)	Frequency of mutated and heterozygous G2677T/A higher in SR.
Cizmarikova M *et al.*[152]	2015	Caucasian (Slovakia)	46/100	6.42/7.89	3435TC was associated with SS.

SD: steroid dependant; FR: frequent relapser; NFR: non frequent relapse; SS: steroid sensitive; SR: steroid resistant; NR: non responder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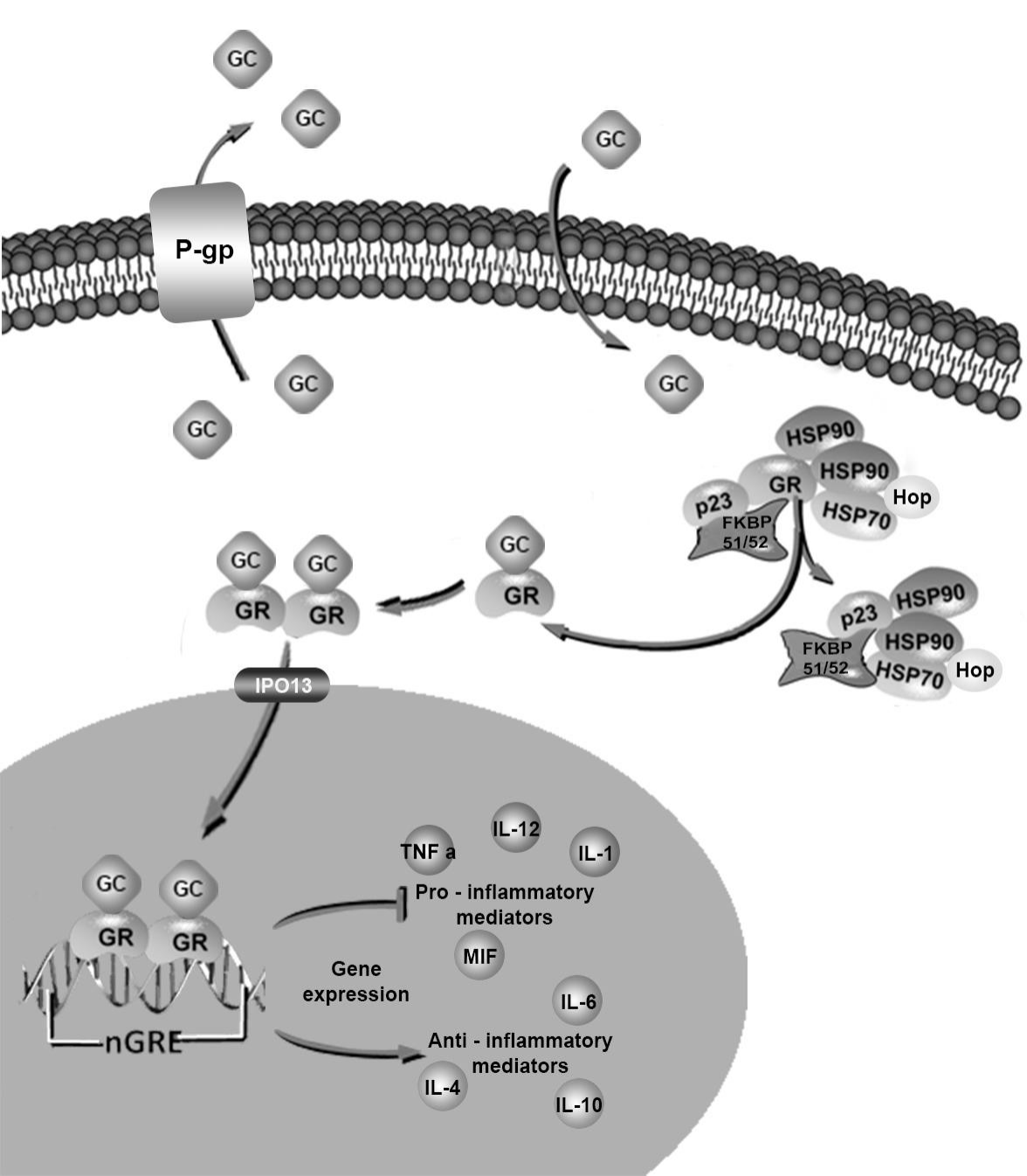
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