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Indirizzo medicina materno fetale perinatologica infantile

In vitro peripheral blood mononuclear cells sensitivity to steroids and identification of biomarkers for predicting clinical response in pediatric idiopathic nephrotic syndrome

Settore scientifico-disciplinare: **BIO14**

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CHAPTER 1

General Introduction

IDIOPATHIC NEPHROTIC SYNDROME (INS)

Nephrotic syndrome (NS) is a pathological condition characterized by a constellation of clinical signs resulting from abnormalities in the glomerular permeability. It is classically characterized by clinical features such as:

●Nephrotic range proteinuria − Urinary protein excretion greater than 50 mg/kg per day

●Hypoalbuminemia − Serum albumin concentration less than 3 g/dL (30 g/L)

●Edema

●Hyperlipidemia.

Of these features, the first two are used diagnostically while the last two may not be seen in all patients.

Massive proteinuria and hypoalbuminemia, as seen in NS, result from increased permeability of the glomerular filtration barrier to proteins. This barrier is composed of the fenestrated capillary endothelium, the glomerular basement membrane and the podocytes, epithelial cells of the visceral layer of a renal glomerulus having a number of footlike radiating processes (pedicles). In NS, podocytes show morphologic changes, including retraction and flattening of pedilcles (Figure 1).

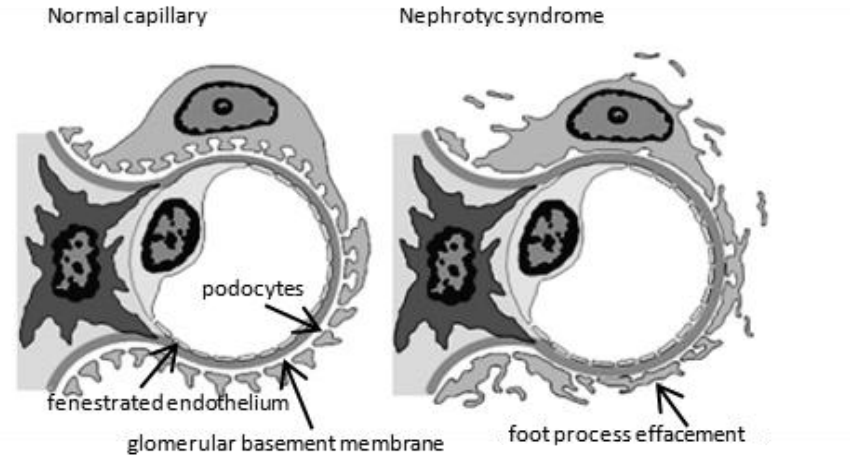

Figure 1: Schematic view of normal podocyte foot processes (left) and fused podocyte pedicles in nephrotic syndrome (right)

Classification

Childhood NS can be classified into 3 groups [1]:

1. The primary NS: which refers to NS in the absence of an identifiable systemic disease. Within this category are patients with Idiopathic Nephrotic Syndrome (INS), who have no glomerular inflammation on renal biopsy, and patients with primary glomerulonephritis, who have an urine sediment containing blood cells and glomerular inflammation on renal biopsy [2].

2. The secondary NS: which refers to NS associated with both inflammatory diseases (e.g.: lupus nephritis, acute post-infectious glomerulonephritis, IgA nephropathy) and non-inflammatory diseases (e.g.: syphilis, diabetes mellitus, hypertension and cancer).

3. The congenital and infantile NS: which occurs in children less than one year of age and can be either primary or secondary (mostly due to infection). Two-thirds of NS cases that occur during the first year of life, and as many as 85 percent of cases that occur during the first three months of life, have a poor outcome and a genetic basis that could be explained by mutations in one of the following four genes [3]:

●*NPHS1*, which encodes nephrin (a key component of the podocyte slit diaphragm) and is responsible for the Finnish-type congenital NS;

●*NPHS2*, which encodes podocin (a protein that interacts with nephrin at the slit diaphragm) and is responsible for familial focal segmental glomerulosclerosis;

●*WT1*, which encodes the transcription tumor suppressor (a protein involved in kidney and gonad development) and is responsible for the Denys-Drash syndrome;

●*LAMB2*, which encodes laminin beta 2 (a component of the glomerular basement membrane) and is responsible for the Pierson syndrome.

Epidemiology

INS is the most frequent form of NS in children [1, 4, 5] and is characterized by a wide variety of glomerular lesions, the most common type being the "minimal change NS" (MCN). MCN occurs in 90% of children under the age of six years and in 50% of children above this age, who underwent kidney biopsy. In the United States and Europe, the annual incidence of INS is of 2-7 cases per 100000 children per year, with a cumulative prevalence of 16 per 100000 pediatric subjects [4, 6]. In New Zealand, the incidence is higher, nearly 20 cases per 100000 children under 15 years of age [7]. A higher incidence of INS has been recorded also in Asian Indian and Japanese children (6 times the rate observed in European children) [8, 9]. The peak of incidence of the disease in children is at 3-4 years of age and, in 1/3 of children affected, there is a previous history of atopy (asthma, eczema, rhinitis). An unexplained male preponderance is observed, with male to female ratio’s ranging from 1.5 to 3.1. [10].

Clinical picture

The clinical onset is often preceded by an infection of the upper respiratory tract (~25-30% of cases) or by allergic reactions (~30%) [11]; an unusual event that precedes the onset of INS, as a reaction to an insect bite, has been reported in some cases [11].

Edema is present in about 95% of children with INS [12, 13]. Initially edema is intermittent and insidious, and its presence is not always appreciated. Edema can progress quickly or slowly, becoming more generalized and clear. In children it can typically remain confined in facial and periorbital regions, while in severe cases is associated with more or less conspicuous fluid in serous cavities, especially in pleural and peritoneal cavity. The pathogenetic mechanism of the edema is due to the massive loss of proteins in the urine, with the consequent decrease in oncotic pressure of the blood.

As regards to proteinuria, especially in children, it occurs for molecules with a molecular weight similar to that of albumin, transferrin and other plasma proteins (~70-80 kDa), with exclusion of larger molecules such as immunoglobulins. This results in hypoalbuminemia because of the albumin loss. Anorexia, irritability, fatigue, abdominal pain and diarrhea are other common symptoms [14].

In pediatric patients with these symptoms, renal biopsy is not necessary and a reliable diagnosis can be assessed based on clinical and laboratory parameters, reserving biopsy to patients who do not respond to pharmacological intervention.

Treatment and clinical outcome

Since the 1950's, glucocorticoids (GCs) represent the cornerstone of NS treatment, as they are able to induce remission of proteinuria in around 90% of patients [15-17]. In conjunction with the antibiotic therapy, GCs have caused a remarkable reduction of mortality from 35-50% to less than 3% [15].

The initial treatment of INS generally consists of high doses oral prednisone. Prednisone is inactive and is converted into its active metabolite prednisolone by hepatic enzymes. Systemic bioavailability of prednisolone is generally equal when oral administration of prednisone is compared to prednisolone [18].
Steroid responsiveness at diagnosis is of major prognostic importance in INS with regard to kidney function, which is generally well preserved in steroid sensitive NS (SSNS) [19, 20]. This is in contrast with steroid resistant NS (SRNS), occurring in about 10% of children with INS [1, 14] where patients are prone to progressive disease and renal failure [14].
Despite the high initial response rate to GCs (85-90%), relapses occur in 60-90% of the initial responders [15, 21]. Relapse frequency is highly variable among patients. Around 30-50% of patients develops frequent relapses (generally ≥2 relapses within six months after initial treatment, or ≥4 relapses per year) and half of them becomes steroid dependent [15, 21]. Children with INS form a heterogeneous and therapeutically challenging group as they suffer from relapses and GC toxicity to a varying degree. Those in need of numerous courses of GC therapy are at risk of serious infectious [22], as well as adverse effects on growth

and bone mineral density, obesity, hypertension, changes in behavior and cataract. These patients often need other immunomodulatory agents (cyclophosphamide, cyclosporine, mycophenolate mofetil, levamisol, rituximab) in order to reduce adverse effects of GC therapy. Children with INS who develop GC dependence or secondary resistance to therapy are likely to face a protracted disease course [11, 14].

To date, there are no worldwide common guidelines on the duration and dose of prednisone treatment for childhood INS. Different schedules are used across countries, regions and hospitals. The lack of common standardized protocols in Italy has led to the creation of a network of pediatric nephrologist from different Italian regions, called NEFROKID.

GLUCOCORTICOIDS

GCs mediate many essential physiological processes, including stress response, glucose metabolism and anti-inflammatory actions [23, 24]. The complex mechanisms of GC action give rise to the heterogeneity in GC sensitivity, which is known to exist in the general population [25, 26]. It is well known that patients differ in their clinical response to doses of prednisone. Many factors, both intracellular and extracellular, can influence the drug pharmacokinetics and pharmacodynamics, affecting treatment response and side-effects. Though GCs have been first choice treatment for INS for decades, surprisingly little is known about how patients will respond to therapy and which is the ideal dose children with INS need.

GC mechanism of action

GCs are involved in many processes in various tissues and organs [27, 28], ranging from glucose homeostasis and modulation of the immune and inflammatory responses to their important role in bone metabolism and their effects on mood, behavior and sleeping patterns. Approximately 10-20% of all genes are estimated to be positively or negatively regulated by GCs, illustrating the diversity of GC action [29-31]. The actions of GCs are mediated by the glucocorticoid receptor (GR), encoded by the *NR3C1* gene. The GR is one of the members of the nuclear receptor family, is expressed in virtually all cells and is essential for life. The *NR3C1* gene is located on chromosome 5 and consists of nine exons. As all other nuclear receptors, the GR has a N-terminal transactivation domain, a central DNA binding domain (DBD) and a C-terminal ligand binding domain. The nine exons comprising the *NR3C1* gene are subjected to alternative splicing, giving rise to alternative splice variants: the most common GC isoforms (GR-α and GR-β) are derived from alternative splicing of exon 9 [32, 33]. GR-α is the biologically active isoform while the GR-β isoform is not capable of binding GCs. Nevertheless, GR-β is thought to act as a dominant negative inhibitor of GR-α, hereby affecting its transcriptional activity [34-37].

In the cytoplasm, the ligand-free GR exists in a multimeric complex associated with various chaperones and co-chaperones, such as Hsp90, FKBP51, FKBP52, p23, Hsp70 and Hop [38], that keep the receptor in the correct folding for hormone binding [39] (Figure 2). Upon ligand binding, the GR undergoes conformational changes and exposes the DBD, which is otherwise hidden in the ligand-free conformation. Nuclear receptors are also provided of nuclear localization signals (NLSs) that interact with transporters located on nuclear membranes (the importins), thus mediating their translocation into the nucleus. Among these transporters, importin-13 (IPO13) has been functionally characterized as a primary regulator of the translocation of the GC-bound GR across the nuclear membrane [40].

In the nucleus, the GR-GC complex can mediate gene transcription via several different mechanisms. The first mode of transcriptional regulation requires the dimerization of GR-GC and the binding of the dimer on specific DNA sequences (the glucocorticoid-responsive elements, GREs). The subsequent recruitment of

several co-activators promotes remodeling of the chromatin and stimulates initiation of transcription by the RNA-polymerase II complex, thus resulting in a transactivation (transcription at higher rates) of downstream genes, mainly anti-inflammatory genes, such as *IL-10, IL-6,* and *IL-4.*

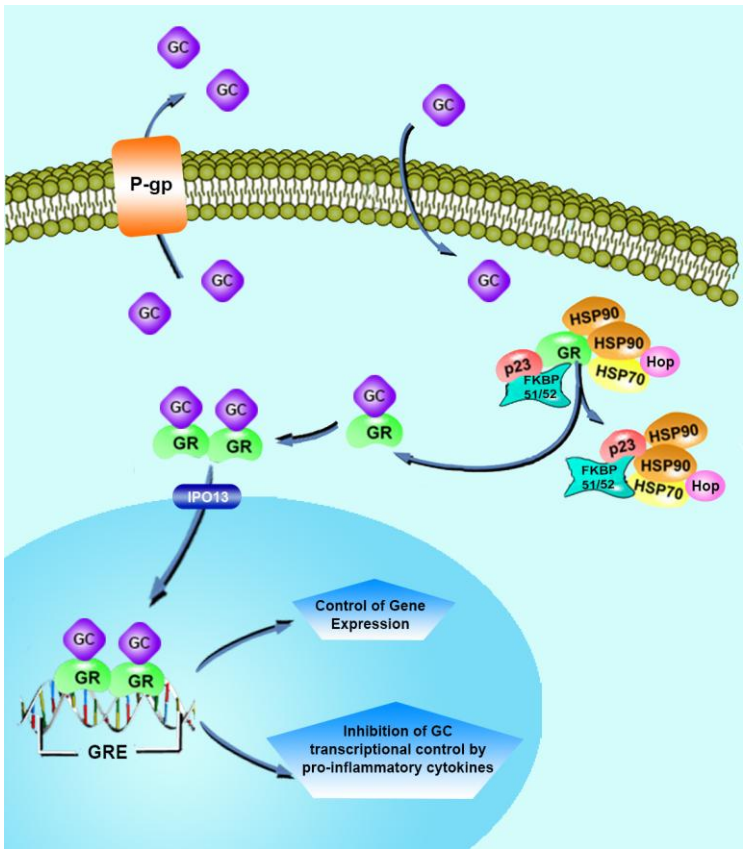

Figure 2: Schematic view of molecular mechanisms of action of glucocorticoids

Alternatively, GCs can bind to negative GREs in the promoter region of target genes, herewith inhibiting gene transcription (transrepression of pro-inflammatory cytokines enconding genes such as *IL-1, IL-12* and *TNF*). The GR monomer can also interfere with the transcriptional activity by means of direct protein-protein interactions with transcription factors [41].

Nongenomic mechanisms have also been described and are responsible for the GC-induced effects characterized by rapid onset and short duration [42]. These mechanisms are still not completely clear, but they likely involve non classical membrane-bound GR. In addition, at higher concentrations, GCs probably induce lipid peroxidation, with consequent alteration of the characteristics of plasma membranes and alteration in ion transport [42].

Clinical and biochemical predictors of response

The inter patients variability to GCs as well as the unpredictability of the clinical course of childhood NS called for better understanding of underlying mechanisms with the final aim of improvement of current treatment protocols.

Although many efforts have been made to predict response and relapse patterns in children with INS, it has been impossible to provide clinicians with a clear-cut set of risk indicators yet. Studies focusing on the prognostic value of genetic and biochemical factors, as well as demographic and clinical features, have yielded conflicting results [43-46].

The low incidence of NS complicates studying these parameters in a prospective setting; however demographic variables have been studied in correlation with clinical response to therapy [43].

Male gender has only occasionally been correlated to frequent relapses [43]. Most reports have not found a significant effect of gender on clinical course in terms of (frequent) relapses or other morbidity [44-46].

Age at onset has been proposed as an indicator of clinical outcome in NS. In adolescents, atypical features and steroid resistance are seen more often [47-50], whereas younger age at diagnosis (1-6 years of age) has been associated with frequent relapses, steroid dependency and/or a longer duration of disease [16, 43, 46]. A possible explanation for the higher incidence as well as the increased number of relapses in young patients could be a higher frequency of potentially triggering events, such as viral infections [10, 51]. Some reports however did not find any effect of age on clinical course [44, 51-53]).

Low birth weight has been associated with unfavorable clinical outcome. In children with low birth weight, steroid dependence and hypertension were observed more often compared to children with normal birth weight [54, 55]. A lower nephron number in patients with low birth weight has been suggested as part of the underlying mechanism for this relationship, though this requires further clarification.

A wide variety of genetic and biochemical factors have been put forward as biomarkers for the clinical course of INS in children. Several gene mutations have been associated to hereditary forms of disease, in particular variations in genes such as *NPHS1, NPHS2, PLCE1, WT1, CD2AP* and others (for a review see [56], encoding for glomerular proteins. Moreover variation in genes coding for proteins involved in the mechanism of action of GC (*NR3C1, FKBP4/5, IPO13, IL-10*), have been also correlated with clinical response to steroids (for a review see [57]). However these studies investigated small number of patients, treated with different protocols, giving not consistent results and could not provide reliable biomarkers to predict clinical outcome of INS patients.

Plasma levels of cytokines have not been largely investigated in a cohort of pediatric patients at onset of INS. Since INS is characterized by proteinuria and this condition is mediated by cytokines [58], cytokine plasma levels should be considered as potential biomarkers of INS clinical course. Recently IL-4, IL-13 and IL-18 levels have been found to be significantly higher during the active stage of SSNS compared to

remission and controls while IFN-γ level was found to be significantly lower [59, 60]. These results require further clarification comparing steroid sensitive, dependent and resistant patients.

AIMS AND OUTLINE OF THE THESIS

This thesis is focused on pediatric patients affected by INS, and on the possible prediction of response to GCs in these patients. Differences in GC sensitivity have been already reported among INS patients: a substantial proportion (approximately 10-15%) of them is resistant to GCs at the start of therapy whereas almost 50% of patients becomes steroid dependent over the treatment. Exploration of biomarkers able to predict GC sensitivity *a priori* could provide more insight in the treatment of INS and may stimulate the development of individualized therapy.

With these premises, the aim of this thesis was to identify cellular and molecular markers associated to and/or predictive of outcome in childhood INS. For this purpose, a number of studies have been performed, in healthy donors and pediatric patients, as described in the following chapters

Chapter 2 reviews the literature on GC pharmacogenetics in pediatric INS while **Chapters 3 to 9** describe the research project undertaken to study the mechanisms involved in glucocorticoid response. **Chapter 3** describes the development of a pharmacodynamic *in vitro* assay, performed on healthy donors for predicting GC sensitivity and investigates the association between variation in genes coding for protein involved in GC mechanism of action, and the *in vitro* PBMC responses to GCs.

Chapter 4 describes the correlation between the PBMC *in vitro* sensitivity to methyl-prednisolone and the clinical response in a cohort of pediatric INS patients.

Chapters 5 to 8 focus on research of other predictors that may explain the variability in clinical outcome in those patients. In particular, **Chapter 5** focuses on cytokines, investigating the correlation between patients clinical response and the cytokines plasma profiles; **Chapter 6** analyses whether polymorphisms of genes involved in the GC mechanism of action are related to steroid *in vitro* sensitivity in a cohort of healthy subjects; in **Chapter 7** the mRNA expression profile has been investigated as a novel *in vitro* tool for assessing sensitivity to steroid treatment in healthy donors while in **Chapter 8** the role of a long noncoding RNA GAS5 and of NR3C1 gene expression are investigated in correlation with clinical response of INS pediatric patients.

Finally the results of the different studies will be summarized and discussed in **Chapter 9**, including suggestions for further research.

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CHAPTER 2

Glucocorticoid pharmacogenetics in pediatric idiopathic nephrotic syndrome

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.

Introduction

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.

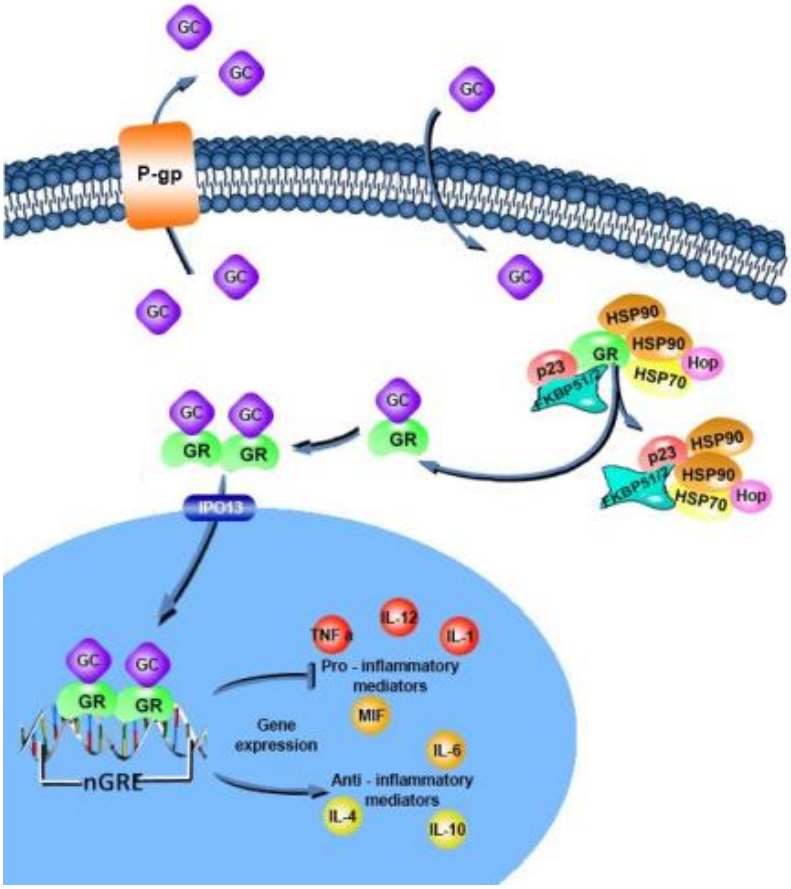

Figure 1: Molecular mechanisms of action of glucocorticoids.

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 *TthIII*I (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]. *TthIII*I 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, *TthIII*I and *Bcl*I polymorphisms in a well-defined cohort of 113 children with INS, showing that carriers of GR-9β+*TthIII*I 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.

Table 1. Summary of studies reporting genetic analysis of *NR3C1* in idiopathic nephrotic syndrome patients.

Study (year)	Ethnicity	Case/control	Age (mean)	Results	Ref.
Zalewski G *et al.* (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	[42]
Cho HY *et al.* (2009)	Asian (Korea)	190/100	4.95/NA	No correlation between the disease onset age, initial steroid responsiveness, renal pathologic findings or progression to end-stage renal disease and ER22/23EK, N363S and *Bcl*I polymorphisms	[43]
Teeninga N *et al.* (2014)	Caucasian (Holland)	113	4.1	Carriers of GR-9β + *Tth*III*I* mutated haplotype had a significantly higher incidence of steroid-dependence compared with noncarriers	[44]
Ye J *et al.* (2006)	Asian (China)	138	7.1	No association found with the studied polymorphisms	[52]

Table 1: Summary of studies reporting genetic analysis of NR3C1 in INS patients

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.

Moreover, in a recent study, Swierczewska et al. [108] investigated the role of seven other polymorphic variants of the *MIF* gene: two polymorphisms, rs2070767 (C>T) and rs2000466 (T>G), were found to have a significantly different distribution between 30 resistant and 41 sensitive INS patients (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 CI% 0.119-0.869, p=0.028); however, when linkage disequilibrium analysis was performed, the significance was lost.

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

Anti-inflammatory mediators

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

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

IL-4 signaling is mediated by the interaction of the cytokine with its receptor, mainly expressed in 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.

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 idiopathic nephrotic syndrome patients.

Study (year)	Ethnicity	Case/control	Age (mean)	Results	Ref.
IL-12					
Muller-Berghaus J *et al.* (2008)	Caucasian (Germany)	79	10.7	Significantly higher allele frequency of *IL-12Bpro-1* in steroid-dependent children compared with children without steroid dependency	[8]
TNF					
Kim SD *et al.* (2004)	Asian (Korea)	152/292	NA/NA	No association with TNF and IL-1β	[95]
Jafar T *et al.* (2011)	Asian (India)	150/569 115(SS)/35(SR)	4.8/NA	Association for *TNFA* (G308A) comparing patient with controls and steroid-resistant group with steroid-sensitive group	[96]
Tripathi G *et al.* (2008)	Asian (India)	115(SS)/35(SR)	4.8	The AA genotype of *TNFA* (G308A) was associated with lower steroid response	[97]
MIF					
Berdeli A *et al.* (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 steroid-resistance group	[105]
Vivarelli M *et al.* (2008)	Caucasian (Italian)	257/355	5.8/NA	Frequency of *MIF* -173*C allele was higher in patients than in controls and more frequent in steroid-resistant patients compared with steroid-responders	[106]
Choi HJ *et al.* (2011)	Asian (Korea)	170/100	5.17/NA	No association with *MIF* G-173C	[107]
Swierczewska M *et al.* (2014)	Caucasian (Poland)	71/30	10.1/10.1	*MIF* CT genotype of rs2070767C>T associated with the risk of steroid resistance, while the distribution of TG genotype of rs2000466T>G was higher in steroid-sensitive children compared with steroid-resistant	[108]
IL-4					
Jafar T *et al.* (2011)	Asian (India)	150/569	4.8/NA	Association for *IL-4* (C590T) polymorphism comparing patients with controls and steroid-resistant group with steroid-sensitive group	[96]
Tripathi G *et al.* (2008)	Asian (India)	115(SS)/35(SR)	4.8	The TT genotype of *IL-4* (C590T) polymorphisms associated with reduced steroid response	[97]
Ikeuchi Y *et al.* (2009)	Asian (Japan)	85/127	NA	*IL-4R alpha* (Ile50Val) mutated genotype less frequent in patients with four or more relapses compared with those who experienced fewer recurrences	[119]
IL-6					
Jafar T *et al.* (2011)	Asian (India)	150/569	4.8/NA	Association for *IL-6* (G174C) comparing patient with controls and steroid-resistant group with steroid-sensitive group	[96]
Tripathi G *et al.* (2008)	Asian (India)	115(SS)/35(SR)	4.8	The GG genotype of *IL-6* (G174C) polymorphism associated with reduced steroid response	[97]

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.

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.

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

Table 3: Summary of studies reporting genetic analysis on the role of P-gp in INS patients

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.

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CHAPTER 3

Association between *Bcl*I polymorphism in the *NR3C1* gene and *in vitro* individual variations in lymphocyte responses to methylprednisolone

Abstract

AIM

To evaluate the association between the in vitro sensitivity of peripheral blood mononuclear cells (PBMCs) to methylprednisolone (MP) and the presence of genetic polymorphisms involved in glucocorticoid (GC) response.

METHODS

In vitro MP inhibition of the proliferation of lymphocytes stimulated with concanavalin A was determined. Non linear regression of dose–response data was performed computing the MP concentration required to reduce proliferation to 50% (IC_{50}). The maximum inhibition achievable at the highest MP concentration (I_{max}) was also calculated. Moreover, the Taqman technique was used to analyze the *Bcl*I polymorphism in the NR3C1 gene and the Leu155His polymorphism in the NALP1 gene.

RESULTS

A significant association between the *Bcl*I mutated genotype and an increased in vitro sensitivity to GCs was observed.

CONCLUSIONS

The a priori evaluation of the *Bcl*I polymorphism, associated with a lymphocyte proliferation assay, could represent a useful diagnostic tool for the optimization of steroid treatment.

Introduction

Glucocorticoids (GCs) are a well-accepted therapy for inflammatory and autoimmune diseases in transplant patients and in the treatment of leukaemia and lymphomas [1]. However, despite their large clinical impact and justified use, the benefits of these agents are often narrowed by a great inter-individual variability that might potentially lead to treatment failure or drug induced toxicity.

Polymorphisms in genes involved in the molecular effects of these hormones could be important in the observed differences in efficacy. In recent studies conducted in our laboratory [2, 3], among various polymorphisms considered, the *Bcl*I polymorphism in the GC receptor gene (NR3C1), and the Leu155His polymorphism in the NALP1 gene (NLRP1: NACHT, LRR and PYD domain-containing protein 1), were associated with GC response. The *Bcl*I polymorphism consists in a C > G substitution 646 nucleotides downstream from exon 2, and the mutated allele has been associated with hypersensitivity to GCs [4, 5], and with a better response to these hormones in paediatric patients with inflammatory bowel disease (IBD) [2, 3]. NALP1 belongs to a group of cytoplasmic pattern recognition receptors that stimulate innate immunity and promote the maturation of cytokines [6]. Jin et al. [7] have recently shown that variants in the NALP1 gene, in particular Leu155His, confer susceptibility to autoimmune and auto-inflammatory diseases, probably related to an altered cytokine activation. Moreover, paediatric patients with IBD, carriers of the NALP1 homozygous variant, exhibit a higher probability of non response to GC therapy [2].

In vitro tests based on the proliferation of mononuclear cells exposed to GCs have been correlated with clinical response in different diseases such as rheumatoid arthritis [8], systemic lupus erythematosus [9], bronchial asthma [10], renal transplant rejection [11] and ulcerative colitis [12]. These findings, however, have not always been reproduced and a bioassay that could be used to predict GC responsiveness in clinical practice is still lacking. To evaluate individual response to GCs, a pharmacodynamic approach using patients' peripheral blood mononuclear cells (PBMC), together with a pharmacogenetic approach with the evaluation of polymorphisms involved in the GC response, could be an efficient strategy. The final goal of our study was therefore to set up a simple and reproducible assay to evaluate in vitro methylprednisolone (MP) individual sensitivity and to correlate this with the presence of the *Bcl*I and Leu155His polymorphisms.

Methods

Subjects

An in vitro proliferation assay with MP and genetic analyses were performed on PBMCs obtained from 42 blood donors.

Samples were obtained between September 2010 and March 2011 from the Transfusion Center, Azienda Ospedaliera Universitaria, Trieste. Blood was obtained by venipuncture between 08.00 a.m. and 10.00 a.m. to minimize any variability due to circadian rhythms, and immediately processed. Written informed consent was obtained from each subject and the local ethics commission gave permission for this study. A total of 9 ml of buffy coats was used for the isolation of PBMCs.

Drug/molecular target nomenclature conforms to the Guide to Receptors and Channels [13].

In vitro proliferation assay

The effect of MP on proliferation of PBMCs was determined by labelling metabolically active cells with [methyl-3H] thymidine (Perkin Elmer, Milan, Italy). PBMCs were collected by density gradient centrifugation on Ficoll Paque™ Plus (Healthcare, Milan, Italy), resuspended in complete RPMI-1640 medium containing concanavalin-A (5 μg ml−1) and seeded into a 96 well round bottom plate (2 × 105 cells/well) in the presence of MP (range from 54 μm to 0.05 nm). After 50 h of incubation, cells were pulsed with [methyl-3H] thymidine (2.5 μCi ml−1 well−1) and the incubation was continued for an additional 22 h. The radioactivity of the samples was determined by a liquid scintillation analyzer (Wallac 1450 Microbeta liquid scintillation counter, Perkin Elmer, Milan, Italy). Raw counts per minute (counts min−1) data were converted and normalized to percent of maximal survival for each experimental condition (counts min−1 MP/counts min−1 control × 100). Non linear regression of dose–response data was performed using GraphPad Prism version 4.00 for computing IC_{50}, the MP concentration required to reduce proliferation to 50%. I_{max} was also calculated and defined as the maximum inhibition achievable at the highest concentration of MP (54 μm).

The calculated coefficient of variation was 15% and the limit of determination of this assay was calculated at 1 nCi ml−1 of [methyl-3H] thymidine.

Genetic analysis

Total genomic DNA was isolated from peripheral blood using a commercial kit (Gene Elute Blood Genomic DNA kit, Sigma Aldrich, Milan, Italy) and genetic polymorphisms were determined using TaqMan® genotyping technologies (Applied Biosystems, Bedford, UK) on an ABI7900 HT sequence detection system device.

Statistical analysis

Any possible association between MP IC_{50} and I_{max} and the studied polymorphisms was investigated by the non-parametric Mann-Whitney and Kruskal-Wallis tests. On the basis of previous results [2, 3], *Bcl*I homozygous carriers were compared with a group of both heterozygous and wild type carriers. However a dose allele effect was also studied and results are presented in Figure S1.

Statistical analysis was performed using the software R.

Results and discussion

The in vitro lymphocyte sensitivity to MP was evaluated in 42 healthy blood donors (mean age 41.8, range 18–60 years; 16.7% female and 83.3% male) and a wide interindividual variation in IC_{50} and I_{max} was evident (IC_{50} median value 1.43 × 10−7m, range 7.43 × 10−10m–2.94 × 10−4m; I_{max} median value 91.5%, range 50.0–98.0%) and comparable with that reported in the literature [14, 15]. Preliminary work conducted in our laboratory revealed that, within a given individual, relatively little variation, both in IC_{50} and I_{max}, was observed. MP was employed in this in vitro study as it is one of the steroids of choice in chronic diseases.

Among the possible causes of a variable response to GCs, genetic polymorphisms can be important. Two variants in genes coding for proteins involved in the pharmacodynamics of these agents, the *Bcl*I polymorphism in the NR3C1 gene, and a polymorphism in the NALP1 gene, have been shown to be particularly relevant in previous studies from our laboratory [2, 3]. Therefore, the presence of these polymorphisms was evaluated in this study. The genotype distribution was in Hardy-Weinberg equilibrium (*Bcl*I P = 0.64, Leu155His P = 0.71) and is presented in Table 1.

Gene	Polymorphisms	Wild type	Heterozygous	Mutated	HW equilibrium (*P* value)
NR3C1	*Bcl*I (*n*° (%))	20 (47.6)	17 (40.4)	5 (12.0)	0.64
NALP1	Leu155His (*n*° (%))	8 (19.0)	22 (52.4)	12 (28.6)	0.71

Table 1: Frequencies of genotype polymorphisms involved in GC action and Hardy-Weinberg (HW) equilibrium

An increased GC in vitro sensitivity was observed in lymphocytes with the mutated *Bcl*I genotype. Indeed this genotype was associated with a lower MP IC_{50} (median 2.39 × 10^{-9}M, range 7.43 × 10^{-10}M–1.46 × 10^{-7}M) compared with non mutated carriers (wild-type and heterozygous; median 2.76 × 10^{-7}M, range 2.03 × 10^{-9}M–2.94 × 10^{-4}M, P = 0.0058 Mann-Whitney test; Figure 1 and Figure S1). Selected inhibition curves are presented in Figure S2. The mutated *Bcl*I genotype was also associated with significantly higher I_{max} values than non mutated (mutated: median 95%, range 95–98%; wild-type and heterozygous: median 90%,

range 50–98%, P = 0.0078 Mann Whitney test; Figure 1 and Figure S1), revealing the presence of a subgroup of unresponsive cells in non mutated patients. The present data confirm that the *Bcl*I polymorphism in the NR3C1 gene, already associated with a better GC response in pediatric patients with IBD [2, 3], is an important marker of increased sensitivity to GCs.

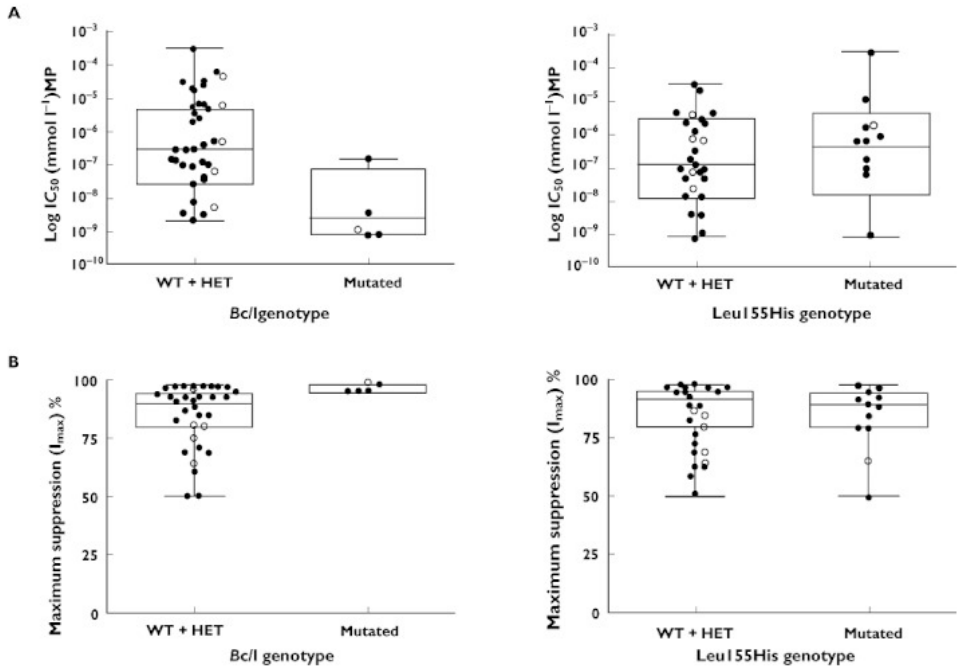

Figure 1: A) IC_{50} of non mutated genotype (wild type: WT and heterozygous: HET) compared with mutated genotype for *Bcl*I genotype in the NR3C1 gene (P = 0.0058) and for Leu155His in the NALP1 gene (P = 0.5544, Mann-Whitney test). Close circles indicate males and open circles indicate females. B) I_{max} of non mutated genotype (wild type: WT and heterozygous: HET) compared with mutated genotype for *Bcl*I genotype in the NR3C1 gene (P = 0.0078) and for Leu155His in the NALP1 gene (P = 0.56, Mann-Whitney test). Close circles indicate males and open circles indicate females

No association was observed in our study between the Leu155His polymorphism in the NALP1 gene with MP IC_{50} and I_{max} (IC_{50} mutated: median 3.78×10^{-7}M, range 7.60×10^{-10}M–2.93×10^{-4}M; wild-type and heterozygous: median 1.25×10^{-7}M, range 7.43×10^{-10}M–2.94×10^{-5}M, P = 0.5544; I_{max} mutated: median 89.5%, range 50–98%; wild-type and heterozygous: median 92.5%, range 50–98%, P = 0.56 Mann Whitney test; Figure 1 and Figure S1). We can hypothesize that, due to the role of NALP1 in the activation of cytokines, genetic polymorphisms of this gene become relevant only in inflammatory conditions such as IBD. Our study was, on the contrary, performed on lymphocytes obtained from healthy subjects, and this could explain this somewhat unexpected result.

Literature data [12, 14] indicate that measurement of in vitro PBMC steroid sensitivity is a predictor of response to treatment in inflammatory chronic diseases. Our results, on lymphocytes obtained from healthy donors, suggest that the evaluation of the *Bcl*I polymorphism, associated with a lymphocyte proliferation assay could represent a small step in the identification of subjects with a reduced probability

of response to GCs. The in vitro prediction of GC response before the start of treatment would have important clinical implications, allowing to adjust therapy a priori, avoiding the use of these agents in patients who would probably not respond and reducing dosages in those who are hypersensitive, and hence at risk of toxicity. A limitation of this study is that only 42 healthy subjects were enrolled, a low number for an association study with polymorphisms. Therefore further studies are needed to confirm these results in a larger number of subjects and also in patients affected by chronic diseases.

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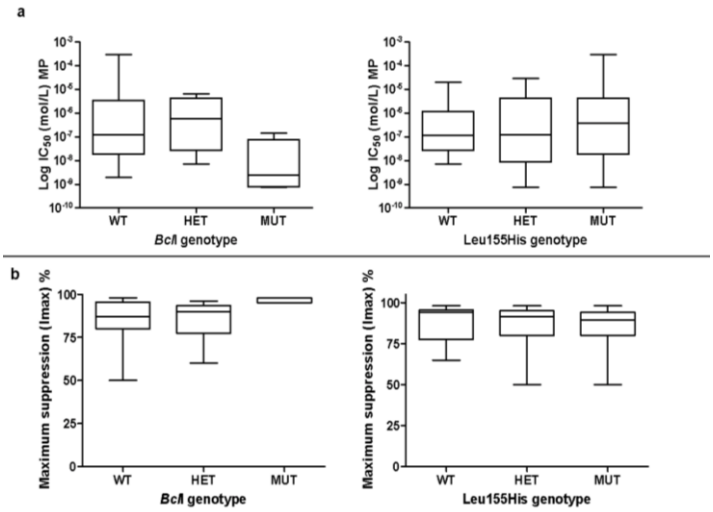
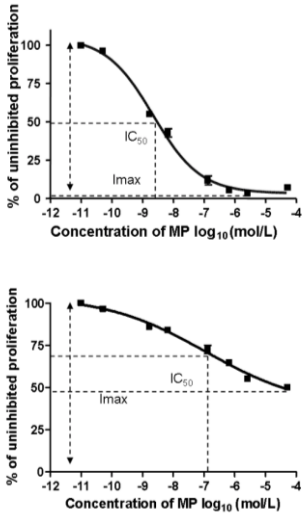
Supplementary material

Figure S1



A) IC_{50} of wild type (WT) genotype comparedwith heterozygous (HET) and mutated genotype for *Bcl*I genotype in the NR3C1 gene (P = 0.018) and forLeu155His in the NALP1 gene (P = 0.75, Kruskal-Wallistest). B) I_{max} of wild type (WT) genotype compared withheterozygous (HET) and mutated genotype for *Bcl*I genotype inthe NR3C1 gene (P = 0.02) and for Leu155His in theNALP1 gene (P = 0.77, Kruskal-Wallis test)

Figure S2



Selected inhibition curves in a steroid sensitive (*Bcl*Imutated) (top) and a steroid resistant patient (*Bcl*I wildtype) (bottom). Points represent the mean values of triplicate dataand vertical bars represent standard errors

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CHAPTER 4

In vitro sensitivity to methyl-prednisolone for predicting clinical response in pediatric idiopathic nephrotic syndrome

Abstract

The aim of this study was to evaluate the in vitro steroid sensitivity as predictor of clinical response to glucocorticoids in childhood idiopathic nephrotic syndrome (INS). Seventy-four patients (median age 4.33, IQR 2.82-7.23; 63.5% male) were enrolled in a prospective multicenter study: in vitro steroid inhibition of patients' peripheral blood mononuclear cell proliferation was evaluated by [methyl-3H] thymidine incorporation assay at disease onset (T0) and after 4 weeks (T4) of treatment. Steroid dependence was associated with increased in vitro sensitivity at T4 assessed both as maximum inhibition at the highest drug concentration (I_{max}; OR=1.13, 95%CI=1.02-1.31; p-value=0.017) and drug concentration inducing 50% of inhibition (IC_{50}; OR=0.48, 95%CI=0.24–0.85; p-value=0.0094). I_{max} < 92% at T4 was a good predictor for optimal clinical response. These results suggest that this test may be useful for predicting the response to glucocorticoid therapy in pediatric INS.

Introduction

Idiopathic nephrotic syndrome (INS) is a rare childhood kidney disease (2-7 cases per year per 100.000 age related population) (1-3). Steroids represent the best first-line therapeutic option, inducing remission in 90% of patients (steroid sensitive – SS) (1, 4, 5). Within those patients, after an initial response to prednisone, almost 40-50% show frequent relapses or become steroid dependent (FR-SD), while the rest of the patients will never relapse or will show infrequent relapses (NR-IR), presenting an optimal response to steroid treatment. Moreover 10% of patients will never respond and are therefore steroid resistant (SR). Steroid responsiveness is of major prognostic importance: patients with steroid dependence and resistance are at risk of more aggressive treatment and disease related complications (6, 7). Many efforts have been made to predict steroid response in children with INS, however, to date, no definite prognostic factor has been defined (1, 8-12).

Peripheral blood mononuclear cells (PBMCs), in particular T lymphocytes, are involved in the immunosuppressive effects of steroids and their in vitro sensitivity may reflect that of other tissues. Steroid-mediated inhibition of mitogen-stimulated PBMCs has been associated with clinical response in different diseases such as rheumatoid arthritis (13), systemic lupus erythematosus (14), bronchial asthma (15), renal transplant rejection (16) and ulcerative colitis (17). For this reason, a pharmacodynamic approach using patients' PBMCs was set up, with the aim of investigating whether steroid sensitivity in vitro was associated with clinical response to steroid therapy in a well characterized cohort of pediatric patients with INS at onset.

Results

Patients

Between August 2011 and February 2014, 184 children were recruited by the pediatric departments participating in the trial. One hundred fourteen patients were excluded from the study for different reasons: non-adherence to the therapeutic protocol, the parents did not give written informed consent, onset of the disease occurred at weekends or holidays when it was not possible to send blood samples to the collecting center in Trieste, insufficient number of PBMCs obtained and cells not viable at arrival. Therefore, 74 patients (median age 4.33, IQR 2.82-7.23; 63.5% male) were enrolled in the pharmacodynamic study; blood was available for 68 patients at T0 (11 steroid resistant (SR), 26 frequent relapse-steroid dependent (FR-SD) and 31 no relapse-infrequent relapse (NR-IR)) and for 54 at T4 (9 SR, 18 FR-SD and 27 NR-IR); for 48 patients (8 SR, 18 FR-SD and 22 NR-IR) the in vitro test was conducted at both time points (for definition of clinical classification see Table 1).

REMISSION	Urine protein <4 mg/m2/h or nil/trace for 3 consecutive early morning specimens.
TIME TO REMISSION	Time elapsed from treatment initiation and the first day of remission.
RELAPSES	Urine protein >40 mg/m2/h or 3+ or 4+ for 3 consecutive early morning specimens, having previously been in remission.
FREQUENT RELAPSES	Two or more relapses within 6 months of initial response or four or more relapses in any 12 month period.
STEROID DEPENDENCE	Two consecutive relapses during corticosteroid therapy or within 14 days of its discontinuation.
STEROID RESISTANCE	Absence of remission despite therapy with daily prednisolone at a dose of 60 mg/m^2/day per day for 4/6 weeks.

Table 1: Definition of clinical response used in the text.

In vitro sensitivity and clinical response to steroids

The in vitro lymphocyte sensitivity to methyl-prednisolone was evaluated and a wide interindividual variation in IC_{50} and I_{max} was evident at both T0 (IC_{50} median value 18.3 nM, IQR 4.5-79.7 nM; I_{max} median value 95.5%, IQR 87.0-98.2%) and T4 (IC_{50} median value 12.4 nM, IQR 1.4-205.2 nM; I_{max} median value 95%, IQR 88.5-98.7%).

No correlation was found between IC_{50} or I_{max} values and time to remission or gender. On the contrary, a significant correlation was evident between in vitro sensitivity to steroids and age at onset, with older patients showing higher in vitro resistance at T0 for methyl-prednisolone I_{max} (p-value Spearman = 0.043, r = -0.25; Figure 1); univariate logistic regression analysis, considering SS patients in comparison with SR subjects, showed that older patients at T0 were more resistant to steroid treatment (OR = 0.81, 95% CI = 0.67 - 0.98; p-value = 0.028; Figure 2).

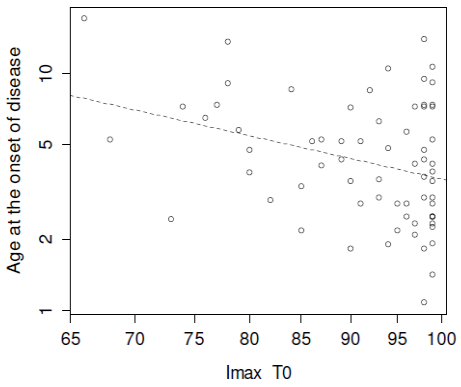

Figure 1. Scatter plot displaying drug sensitivity (I_{max}) at Time 0 (T0) and age at the onset of the disease. *In vitro* response and age at the onset of disease are plotted in Log10 scale. The correlation between continuous variables was assessed using Spearman tests. A significant correlation was found for I_{max} (p-value Spearman = 0.043, r = -0.25) data obtained at T0.

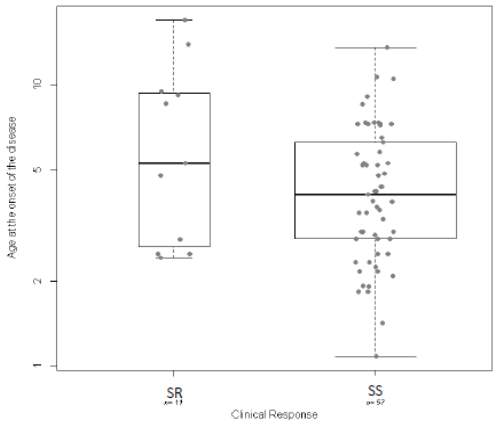

Figure 2: Box plot comparing age at disease onset and clinical response. Age at onset of disease is plotted in Log10 scale. Statistical significance was assessed by carrying out logistic regression analysis. A significant association was found (p-value = 0.028).

In vitro sensitivity, at T0 and T4, and clinical response to steroids

Univariate multinomial logistic regression showed a significant association between clinical and in vitro response at T4 comparing all groups (p-value I_{max} = 0.031; IC_{50} = 0.015; Figure 3).

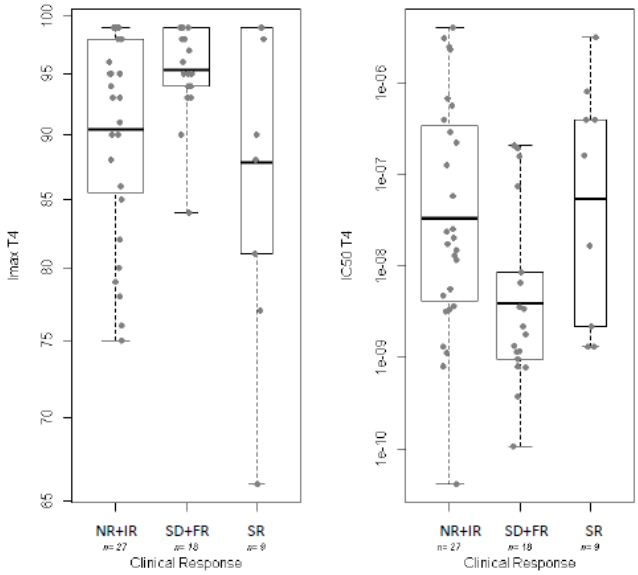

Figure 3: Box plot comparing *in vitro* and clinical response at T4 between the three groups of patients. *In vitro* response is plotted in Log10 scale. The bold horizontal line represents the distribution mean. Statistical significance was assessed by carrying out logistic regression analysis. A significant association was found for log-transformed I_{max} (p-value = 0.031) and for log-transformed IC_{50} values (p-value = 0.015)

The most significant result was found at T4 comparing FR-SD patients vs NR-IR: FR-SD showed higher log-transformed I_{max} values (OR = 1.13, 95% CI = 1.02 - 1.31; p-value = 0.017; Figure 4). A similar pattern was evident for in vitro sensitivity represented as log-transformed IC_{50} (OR = 0.48, 95% CI = 0.24 – 0.85; p-value = 0.0094; Figure 4). ROC curves were constructed to assign optimal cut-off values for in vitro parameters

significantly associated with clinical response. Only for I_{max} at T4 a unique optimal cut-off of 92.0% could be defined. Area under the ROC curves was 65.4% (Supplementary Figure). The test had a high sensitivity of 88.9% and a relatively low specificity of 44%. Logistic regression confirmed a higher proportion of FR-SD patients among those who reached the optimal cut-off point for I_{max} (OR = 6.4, 95% CI = 1.44 – 45.7; p-value < 0.013) in comparison with those who did not.

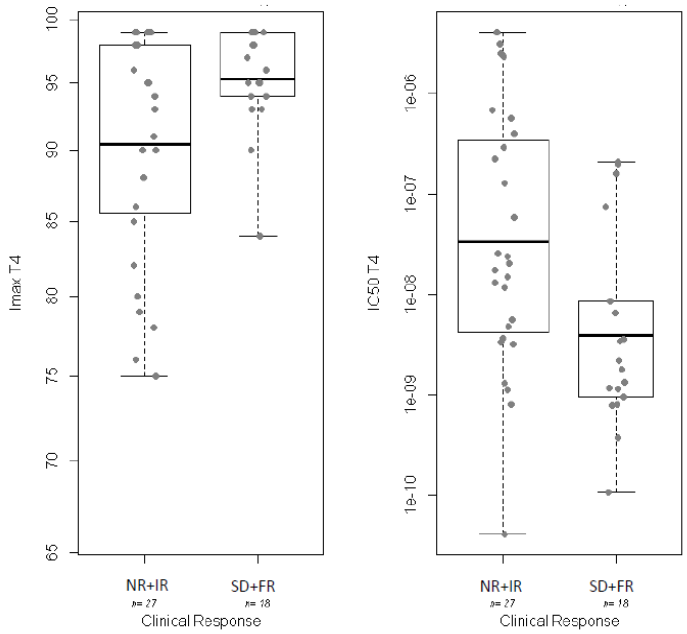

Figure 4: Box plot comparing *in vitro* and clinical response at T4 between the NR-IR group and the SD-FR group of patients. *In vitro* response is plotted in Log10 scale. The bold horizontal line represents the distribution mean. Statistical significance was assessed by carrying out logistic regression analysis. A significant association was found for log-transformed I_{max} values comparing SD-FR patients and NR-IR patients (p-value = 0.017) and for log-transformed IC_{50} values (p-value = 0.0094)

Moreover, at T0, a trend was observed considering resistant patients and all the other subjects (SS: NR-IR and FR-SD): lower log-transformed I_{max} values at T0 were significantly associated with clinical steroid resistance (OR = 1.07, 95% CI = 1.00 - 1.15; p-value logistic regression = 0.046; Figure 5).

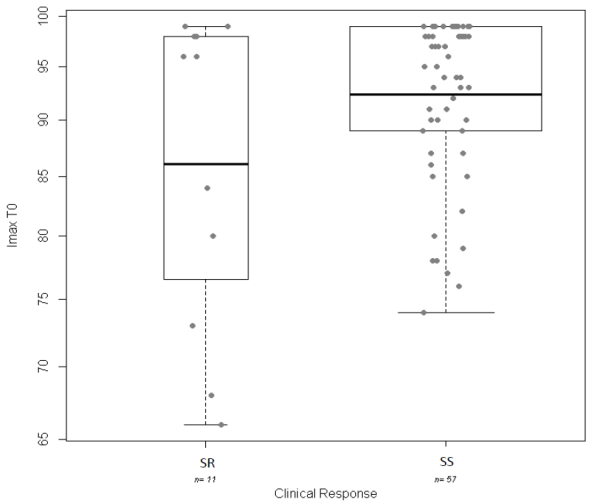

Figure 5: Box plot comparing *in vitro* and clinical response between steroid sensitive (SS) versus steroid resistant (SR) patients. *In vitro* response is plotted in Log10 scale. The bold horizontal line represents the distribution median. Statistical significance was assessed by carrying out logistic regression analysis. A correlation was found for log-transformed I_{max} values comparing SR vs SS (p-value = 0.046).

Discussion

This study was designed to investigate the possible association between in vitro response to methyl-prednisolone in PBMCs of pediatric patients with INS and their clinical response to steroids. The study was conducted prospectively, in a well characterized cohort of Italian pediatric patients treated with a shared therapeutic protocol, allowing for the evaluation of a large group of subjects, despite the relative rarity of the disease.

While INS is a rare disease, it is the most common primary glomerular disease affecting children. Steroids remain the mainstay in the management of INS, with about 80-90% of subjects achieving remission, however, variable degrees of steroid responsiveness and different patterns of disease relapse have been observed (19). Response to steroid treatment is an important indicator of outcome. It is well known that patients with steroid resistant disease represent a difficult therapeutic challenge for clinicians; however, to date, approximately 40-50% of SS patients relapse when therapy is discontinued, resulting in a steroid dependent course of the disease; these patients are at high risk of severe treatment related complications (6, 7) and there is still no means to predict this drug dependence. Many efforts have been made to evaluate steroid response in INS children, but providing a clear-cut set of risk indicators has proved to be impossible and studies focusing on the prognostic value of demographic and clinical features have yielded conflicting results. Among the prognostic indicators of clinical outcome, age at onset of the disease has been proposed; steroid resistance is seen more often in adolescents (20-23), whereas young age at diagnosis (1-6 years of age) has been associated with better steroid response (8, 9). In line with these studies, similar results were obtained in our cohort of patients. We did not find any association between

gender or clinical course of the disease, in terms of risk of relapses or steroid dependence, as reported by others (9, 24).

Immune mechanisms, rather than primary structural defects of the filtration barrier, play a prominent role in INS; mononuclear cells, in particular T lymphocytes, are involved in the immunosuppressive effects of steroids and several studies have demonstrated alterations in T lymphocytes in steroid resistant INS (25, 26). The density and binding affinity of steroids in mononuclear cells have been evaluated in various studies. A significant inverse correlation between the percentage of T lymphocytes expressing steroid receptors and the time interval from the start of steroid therapy to complete remission was demonstrated in 60 children with INS (27). Similar results were recently obtained by Zahran et al. (28) who found that the expression of steroid receptors in T lymphocytes was significantly higher in early steroid responders than in late responders. A decreased expression of steroid receptors in lymphocytes and monocytes of peripheral blood, obtained before therapy initiation and evaluated by flow cytometry was also observed in 51 children with steroid resistant disease (29). To our knowledge, no data on the expression of steroid receptors in steroid dependent patients with INS have been published.

Literature data (17, 30) show that in vitro PBMC sensitivity to dexamethasone could be considered a predictor of response to treatment in various diseases such as rheumatoid arthritis (13), systemic lupus erythematosus (14), bronchial asthma (15), renal transplant rejection (16), inflammatory bowel disease (17) and depression (31). Carlotti et al. also used this assay in INS patients, however, due to the small number of patients enrolled, no definitive data were obtained (32).

In this study, methyl-prednisolone was used instead of dexamethasone because prednisone, a prednisolone prodrug, is currently used in INS. Previous studies conducted in our laboratory have shown that the lymphocyte suppression test can be safely performed with methyl-prednisolone and that this agent gives more consistent results than prednisolone (18); moreover literature data showed that this test had a low inter- and intra-assay variation (30) allowing us to consider this assay useful for the study and prospectively for routine application in the clinical setting. In the context of a standardized study protocol, all patients were evaluated at diagnosis, before starting treatment and after 4 weeks of prednisone therapy. A considerable interindividual variability for in vitro steroid sensitivity was evident in our population; this is not surprising, given that it has already been reported in various diseases and in normal subjects (33).

We demonstrate an increased in vitro response to steroid treatment in FR-SD patients after 4 weeks of therapy, both in terms of I_{max} and IC_{50}. At T4 SR patients were not further considered in our analysis since those patients with lack of therapy response will switch to other drug therapy. For I_{max} a unique cut-off value of 92% was identified in this population; the high sensitivity of the test (88.9%) indicates that there is a very low incidence of false negatives and almost all the FR-SD patients (16/18)

could be identified. Unfortunately, specificity was 44%, therefore a high proportion of not FR-SD patients would be included (12/27). Almost all patients (15/17) with an I_{max} lower than 92% are NR-IR; moreover 16/28 of patients with an I_{max} higher than 92% are FR-SD: in those patients a longer steroid withdrawal could be hypothesized in order to reduce the number of relapses. This would however results in a longer therapy also in a percentage of NR-IR patients. The increased in vitro response at T4 observed in SD-FR patients was quite unexpected; a correlation between relapses and hypothalamic–pituitary–adrenal (HPA) axis suppression has been already demonstrated (34, 35). Relapses in INS are often triggered by infection (36). Viral infections induce the release of cytokines, in particular interleukin (IL)2, 4 and 13 (37), that are in part responsible for proteinuria. In patients who are extremely sensitive to these agents, and hence have an increased HPA suppression, the reduced endogenous steroid production when steroid therapy is discontinued could not be enough to reduce cytokine release; this would result in INS relapse and steroid dependency.

A correlation between I_{max} and the risk of steroid dependence or frequent relapses was not found at T0, but the data we collected underline an increase in in vitro sensitivity in SD-FR patients after four weeks of treatment, which was not present in the other groups. The molecular mechanisms responsible for this effect are not clear and further studies are needed.

A further outcome of this study was the lower in vitro sensitivity of SR patients, evaluated as I_{max} at disease onset: this information needs to be confirmed in a larger group of patients as it could be useful for the early identification of patients who will not respond to steroids, thus avoiding the initiation of ineffective treatment, as previously demonstrated in other diseases (13-17). However, due to the small number of SR patients, a significant cut off value for the in vitro sensitivity test was not found.

In conclusion the results of this study suggest that the in vitro steroid susceptibility test could be used, after four weeks of treatment, to clinically identify patients at increased risk of steroid dependence. These children could therefore benefit from slower steroid tapering or treatment with other immunosuppressive drugs. In addition, if confirmed in a larger group of patients, this test could be useful for identifying those patients who are already resistant at diagnosis, and could thus be considered for alternative treatments, avoiding steroid administration and the relative side effects. Results of this in vitro test could be obtained within 72 hours and would facilitate rapid decisions regarding alternative treatment regimes.

Methods

The pharmacodynamics of steroids was studied in a cohort of patients with INS at onset, recruited for a prospective multicenter Italian trial on the treatment of INS (ClinicalTrials.gov Id.: NCT01386957). In brief, children with a first episode of INS, presenting at 49 Pediatric and Pediatric Nephrology Units in 10 Italian regions, were treated with prednisone at a dose of 60 mg/m2/day for either 4 or 6 weeks, depending on whether time to remission was < or ≥ 10 days. Steroids were then tapered over a 16 weeks period. Total prednisone dosage was 2828 mg/m2 in subjects achieving remission within ten days, 3668 mg/m2 in the others. Patients were classified into 2 groups: steroid resistant (SR) and steroid sensitive (SS). SS subjects were further stratified into frequent relapse-steroid dependent subjects (FR-SD) and no relapse-infrequent relapse subjects (NR-IR), as defined in Table 1.

All the recruited children were admitted to hospital. The parents of all the participating children gave written informed consent before the study began. Ethics committee approval was obtained from all the participating centers.

Peripheral blood, anticoagulated with EDTA (8 ml), was collected before starting therapy (T0) and after 4 weeks of prednisone treatment (T4). Blood samples were sent at temperature of 4°C to the collecting center at the University of Trieste and processed within 24 hours from collection.

In vitro proliferation assay

The effect of methyl-prednisolone on the proliferation of PBMCs was determined by labeling metabolically active cells with [methyl-3H] thymidine (PerkinElmer, Milan, Italy) as previously reported (18). PBMCs were collected by density gradient centrifugation on Ficoll PaqueTM Plus (Healthcare, Milan, Italy), resuspended in complete RPMI-1640 medium containing Concanavalin-A (5 µg/ml) and seeded into 96 well round bottom plates (2×105 cells/well) in the presence of methyl-prednisolone (range from 0.05 nM to 54 µM). After 50 hours of incubation, cells were pulsed with [methyl-3H] thymidine (final concentration of 2.5 µCi/ml) and incubation was continued for an additional 22 hours. The radioactivity of the samples was determined by a Liquid Scintillation Analyzer (Wallac 1450 Microbeta liquid scintillation counter, PerkinElmer, Milan, Italy). Raw count per minute (cpm) data were converted and normalized to percent of maximal survival for each experimental condition (cpm methyl-prednisolone/cpm control *100). Non linear regression of dose–response data was performed using Graph-Pad Prism version 4.00 for computing IC_{50}, the methyl-prednisolone concentration required to reduce proliferation to 50%. I_{max} was also calculated and defined as the maximum inhibition achievable at the highest concentration of methyl-prednisolone (54 µM).

I_{max} and IC_{50} data at T0 and T4 were compared between subjects with different clinical responses to treatment (SR vs SS subjects) or with a different clinical outcome of the disease (NR, IR, FR and SD subjects). Moreover, gender, age at disease onset and time to remission were evaluated and compared with the pharmacodynamic data.

Statistical analysis

For continuous variables, normality of distribution was assessed by means of visual examination of the data plot and a Shapiro test. Logarithmic transformation was applied to normalize distribution and/or reduce variance. The correlation between continuous variables was assessed using the appropriate parametric (Pearson) and non parametric (Spearman) tests. Any possible association between methyl-prednisolone IC_{50}, I_{max} and clinical variables (response, time to remission, age at the onset of disease and sex) was investigated using univariate logistic regression models. Receiver operating characteristic (ROC) curves were constructed for the significant in vitro tests to determine the optimal cut-off value for discriminating between patients' clinical response to steroid treatment. Sensitivity, specificity, and the positive and negative predictive values (PPV, NPV, respectively) of the cut-off point were analyzed. Logistic regression, considering the proportion of patients achieving the predicted clinical response, comparing patients who reached the optimal cut-off point and those who did not, was used to confirm the significance of the cut-off values. Statistical analyses were performed using the software R.

P values lower than 0.05 were considered statistically significant. Odds Ratio (OR) and 95% confidence interval (95% CI) were calculated for all the analyses.

Study highlights

What is the current knowledge on the topic?
Children with INS are treated with steroids: some patients are initially steroid resistant and other became steroid dependent despite initial complete remission. To date, the mechanisms of steroid resistance and/or dependence are scarcely understood and there is no means to predict the response in advance.

What question did this study address?
In the present study, we investigated the in vitro steroid sensitivity in patients with INS, in order to elucidate whether this test could predict the efficacy of the treatments.

What this study adds to our knowledge?

The in vitro steroid susceptibility test at T4 shows a direct correlation between steroid dependence and in vitro response, while, at T0, an inverse correlation between steroid resistance and in vitro methyl-prednisolone response is evident.

How this might change clinical pharmacology and therapeutics?
Knowing in advance the response to steroid treatment is a field of particular interest, especially in young children to reduce ineffective treatments and side effects. This test could be useful to predict steroid response in pediatric patients with INS undergoing this treatment.

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CHAPTER 5

Cytokine plasma profile in children with idiopathic nephrotic syndrome

Abstract

Background and objectives: Childhood idiopathic nephrotic syndrome (INS) is probably the result of a primary immune disturbance. Steroid therapy is given to most children who present with INS: however considerable inter-individual differences in their efficacy and side effects have been reported. To date, the mechanisms of steroid resistance and/or dependence are scarcely understood and there is presently no means to predict the response in advance. Recently, it has been proposed that alterations in the cytokine profile of INS patients might contribute to proteinuria and glomerular damage. However, measurements of level of cytokines in INS patients have given conflicting results.

Design, setting, participants, and measurements: The cytokine plasma levels have been measured in plasma of INS children at diagnosis and after a 4-week treatment to investigate the possible correlation between cytokine pattern and clinical response to GCs. Twenty-one patients have been enrolled on the basis of their clinical response: 7 no relapse-infrequent relapse (NR-IR), 7 frequent relapse-steroid dependent (FR-SD) and 7 steroid resistant (SR).

Results: Our results show that, within the 48 cytokines analyzed, macrophage migration inhibitory factor (MIF) is the best predictor of steroid response before treatment in children with INS. Indeed patients non-responsive to GCs showed significantly higher MIF plasma levels compared with steroid sensitive ones ($p=0.022$) and MIF>473 pg/ml was a good predictor for SR patients. On the contrary, patients FR-SD showed lower MIF plasma levels compared to all the other patients ($p=0.01$) and MIF<351 pg/ml was a good predictor of steroid dependence.

Conclusion: Our data indicate that MIF plasma levels are able to predict steroid response and the clinical course of pediatric patients with INS.

INTRODUCTION

Idiopathic nephrotic syndrome (INS) is the most common primary glomerular disease affecting 16-17 per 100.000 children between the ages of 2 and 8 years, with a peak of incidence between 3 and 5 years. INS is characterized by an increase in permeability of the capillary walls of the glomerulus leading to proteinuria. Various studies have shown that proteinuria, which is the hallmark of this condition, is mediated by cytokines [1]. Relapses are often triggered by viral infections, which possibly result in the release of cytokines, causing immunoregulatory imbalances.

Glucocorticoids (GCs) are commonly used in inflammatory and autoimmune disorders, and represent the best first-line therapeutic option in INS inducing remission in 85-90% of patients. However, despite initial complete remission, almost 50% of the patients show recurrence of the proteinuria and are classified as frequent relapses and steroid dependent patients (FR-SD). Those patients, after a long steroid therapy, with the possibility of severe adverse effects, need to switch to other immunomodulating drugs. Moreover, 10-15% of patients are initially steroid resistant (SR) and do not respond to treatment [2-4]. Steroid responsiveness is of major prognostic importance: the mechanisms involved in GC dependence and resistance are scarcely understood and patients that do not respond to therapy are at risk of more aggressive treatment and disease related complications. Measurements of levels of cytokines, and of the soluble markers of immune activation that are products of cytokine activities, have been used as diagnostic and prognostic indicators in many diseases; however, in INS patients, there is presently no means to predict steroid response in advance [5, 6].

Changes in various plasma cytokine profiles prior to and after steroid treatment in INS patients have not been extensively examined. In the present study, we have investigated the plasma cytokines levels in patients with INS, who were treated with steroids, in order to elucidate whether there is any specific cytokine that could serve as biomarker that could predict the efficacy of the treatment.

MATERIALS AND METHODS

Study design and population

Between August 2011 and February 2014, 184 children were recruited in the prospective multicenter Italian trial on the treatment of INS (ClinicalTrials.gov Id.: NCT01386957). The parents of all the participating children gave written informed consent before the study began. Ethics committee approval was obtained from all the participating centers.

Briefly, patients were treated with prednisone at a dose of 60 mg/m^2 /day for either 4 or 6 weeks, depending on whether time to remission was < or ≥ 10 days. Steroids were then tapered over a 16 weeks period. Total prednisone dosage was 2828 mg/m^2 in subjects achieving remission within 10 days, 3668 mg/m^2 in the others. Patients were classified into 2 groups: steroid resistant (SR) and steroid sensitive (SS). SS subjects were further stratified into frequent relapse-steroid dependent subjects (FR-SD) and no relapse-infrequent relapse subjects (NR-IR), as defined in Table I.

REMISSION	Urine protein <4 mg/m2/h or nil/trace for 3 consecutive early morning specimens.
TIME TO REMISSION	Time elapsed from treatment initiation and the first day of remission.
RELAPSES	Urine protein >40 mg/m2/h or 3+ or 4+ for 3 consecutive early morning specimens, having previously been in remission.
FREQUENT RELAPSES	Two or more relapses within 6 months of initial response or four or more relapses in any 12 month period.
STEROID DEPENDENCE	Two consecutive relapses during corticosteroid therapy or within 14 days of its discontinuation.
STEROID RESISTANCE	Absence of remission despite therapy with daily prednisolone at a dose of 60 mg/m^2/day per day for 4/6 weeks.

Table I: Definition of clinical response used in the text.

Plasma was collected at the onset of the disease (t0) and after 4 weeks of treatment (t4) for 48 children (median age 4.33 years, interquartile range: 2.82-7.23 years; 63.5% male; 8 SR, 18 FR-SD and 22 NR-IR).

The first 7 consecutive patients for each group were characterized for the plasma cytokine levels; the sub-groups were representative of the entire group. Demographical characteristic of patients are reported in Table II.

Samples and cytokine measurements

The plasma cytokines levels were studied in a sub-cohort of 21 patients with INS. Peripheral blood, anticoagulated with EDTA, was collected before starting therapy (t0) and after 4 weeks of prednisone treatment (t4). Blood samples were sent to the Department of Life Sciences at the University of Trieste and plasma aliquots for cytokine measurement were stored at -80˚C until the assay. Each sample (20 µl) was studied by magnetic bead suspension array using the Bio-Plex Pro Human Cytokine 21- and 27-plex panels (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The 21-plex panel

measures interleukin 1α (IL-1α), IL-2 receptor α (IL-2Rα), IL-3, IL-12p40, IL-16, IL-18, cutaneous T-cell attracting chemokine (CTACK), growth-regulated oncogene α (GRO-α), hepatocyte growth factor (HGF), interferon α2 (IFN-α2), leukemia inhibitory factor (LIF), monocyte chemotactic protein 3 (MCP-3), macrophage colony-stimulating factor (M-CSF), macrophage migration inhibitory factor (MIF), monokine induced by IFN-γ (MIG), stem cell factor (SCF), stem cell growth factor β (SCGF- β), stromal cell–derived factor 1α (SDF-1α), tumor necrosis factor β (TNF-β) and TNF-related apoptosis inducing ligand (TRAIL). The 27-plex measures IL-1β, IL-1 receptor antagonist (IL-1Ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, basic fibroblast growth factor (FGF-basic), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-γ, IFN-γ-induced protein 10 (IP-10), monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, platelet-derived growth factor BB (PDGF-BB), regulated on activation normal T cell expressed and secreted (RANTES), TNF-α and vascular endothelial growth factor (VEGF). The samples were analyzed using the Bio-Plex 200 System, and the results were calculated using Bio-Plex Manager 6.0 software (Bio-Rad Laboratories). No measurable values for MCP-3, IL-15, IL-12p40 and MCP-1 were obtained, therefore those cytokines were excluded from data analyses.

Statistical analysis

For statistical purposes, out-of-range cytokine levels were assigned an arbitrary value corresponding to half of the minimum (or double of the maximum) detectable concentration. This was necessary to account for the low cytokine concentrations in samples.

For continuous variables, normality of distribution was assessed by means of visual examination of the data plot and a Shapiro test. Data not normally distributed were log transformed. Any possible association between cytokine levels and clinical response was investigated using univariate logistic regression models. Receiver operating characteristic (ROC) curves were constructed for the significant cytokine levels to determine the optimal cut-off value for discriminating between patients' clinical response to steroid treatment. Sensitivity, specificity, and the positive and negative predictive values (PPV, NPV, respectively) of the cut-off values were analyzed. Logistic regression, considering the proportion of patients achieving the predicted clinical response, comparing patients who reached the optimal cut-off point and those who did not, was used to confirm the significance of the cut-off values. Finally, to understand whether cytokine levels patterns reflect clinical response, we used heat maps to group patients based on the levels of the 48 cytokines. Statistical analyses were performed using the software R.

P values lower than 0.05 were considered statistically significant. Odds Ratio (OR) and 95% confidence interval (95% CI) were calculated for all the analyses.

Results

Patients

The studied population consists of 21 patients diagnosed with INS. Demographical characteristics of the 21 patients analyzed are reported in table II.

Characteristic	SR (n = 7)	SS (n =14)	
		SD-FR (n = 7)	NR-IR (n = 7)
Male, n (%)	5 (71%)	6 (86%)	4 (57%)
Mean age (range)	8.3 (2-14)	3.8 (3-7)	4.4 (2-11)

Table II: Demographical characteristic of the 21 patients.

Comparison of baseline plasma cytokine concentrations between the three groups of patients

Using the Bioplex assay, we assessed the concentration of 48 soluble plasma mediators at baseline and during treatment. Baseline was defined as the plasma sample collected before initiation of steroid treatment (t0). Comparison of baseline plasma concentration among SR, FR-SD and NR-IR patients was performed for each cytokine. Multinomial logistic regression analysis showed a significant difference only for 4 cytokines: IL-18, MIF, SCGF-b, G-CSF (figure 1). However when considering these cytokines in a multivariate analysis, none of them remained significant, likely because of the small number of patients considered in each group.

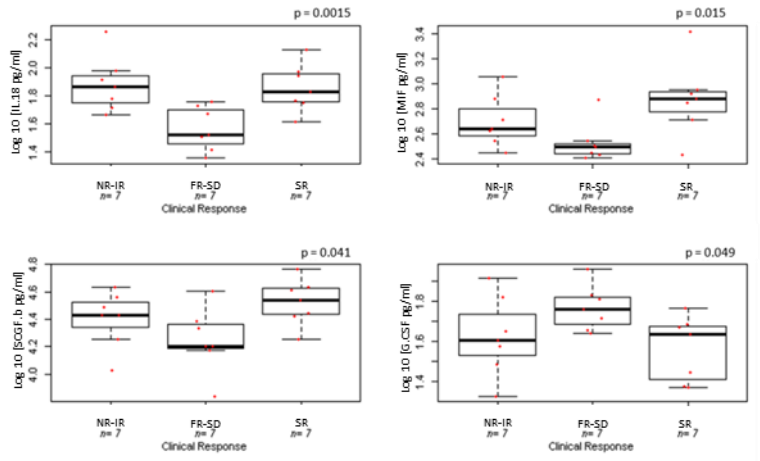

Figure 1: Box plots of cytokine concentration across the three groups of patients: SR, FR-SD and NR-IR

Comparison of baseline plasma cytokine concentrations between resistant and sensitive patients

Given the clinical interest in recognizing SR patients at diagnosis before starting treatment, any possible correlation between cytokine levels and the clinical response in the SS and SR groups of patients was analyzed. When analyzing the profile of each cytokine separately, univariate logistic regression models showed significantly elevated concentration of MIF (p=0.022) and SCGF.b (p=0.034) in SR patients

compared to SS (Figure 2). However when multivariate analysis was applied, only MIF was able to distinguish the two groups (p=0.022).

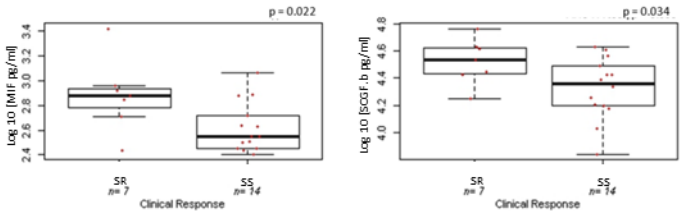

Figure 2: Box plots of cytokine concentration across the two groups of patients: SR and SS

To define if cytokine profiles were distinct between SR and SS patients, we performed heat maps and hierarchical clustering analyses. Figure 3 shows that all the SR patients (7/7) are grouped together, sharing a specific cytokine expression pattern (Fisher test, p = 0.011).

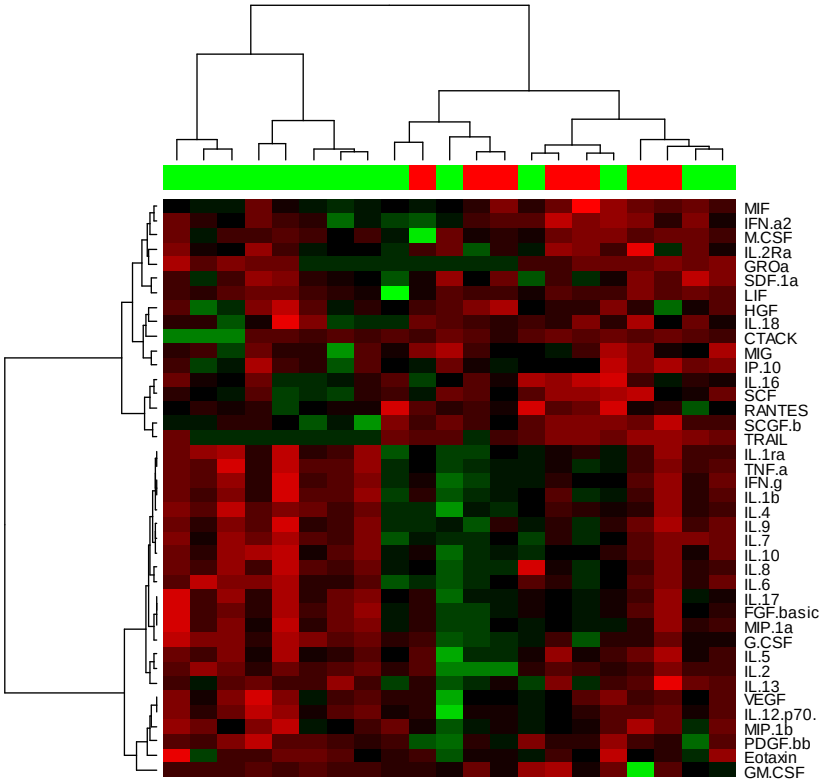

Figure 3: Heat map and cluster analysis of cytokine levels in SR (red) and SS (green) patients

Comparison of baseline plasma cytokine concentrations between frequent relapse-steroid dependent and all other patients

Treatment of FR-SD patients represents a major challenge for clinicians; for this reason we analyzed the differences in cytokine levels between FR-SD and all other patients (SR+NR-IR). Comparison of baseline plasma concentrations for the 5 significantly associated cytokines is shown in Figure 4. Significantly lower concentration of IL-18 (p=0.0003), MIF (p=0.010), and SCGF (p=0.030) and significantly higher concentration of IL-17 (p=0.031) and G-CSF (p=0.019) were observed in FR-SD patients as compared to all other patients. However when multivariate analysis was applied, only MIF and IL-18 were able to significantly distinguish the two groups (p=0.010 and p=0.00082, respectively).

Hierarchical clustering was performed to define cytokine profiles between FR-SD and all other patients. Figure 5 shows that 86% (6/7) of FR-SD patients are grouped together, indicating that the majority of these patients shared a specific cytokine pattern at baseline. The significant difference was confirmed by Fisher's exact test (p = 0.0015).

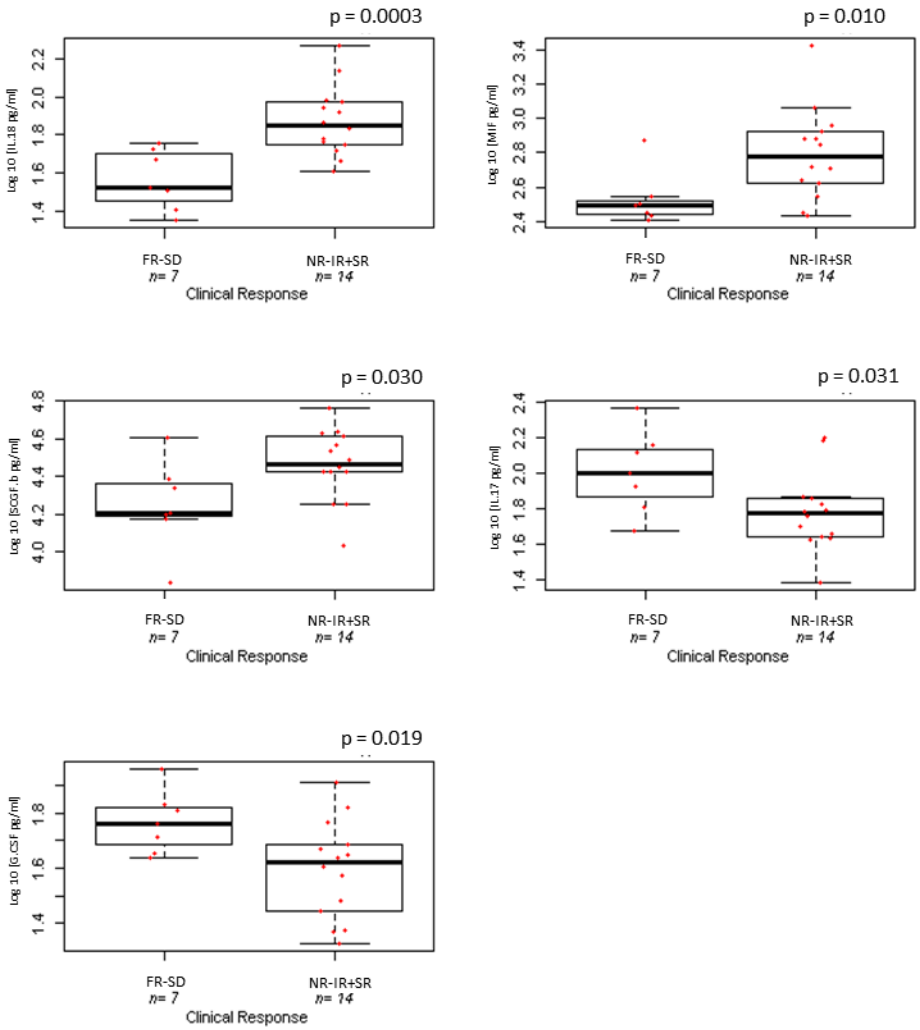

Figure 4: Box plots of cytokine concentration in FR-SD and all other patients (NR-IR+SR)

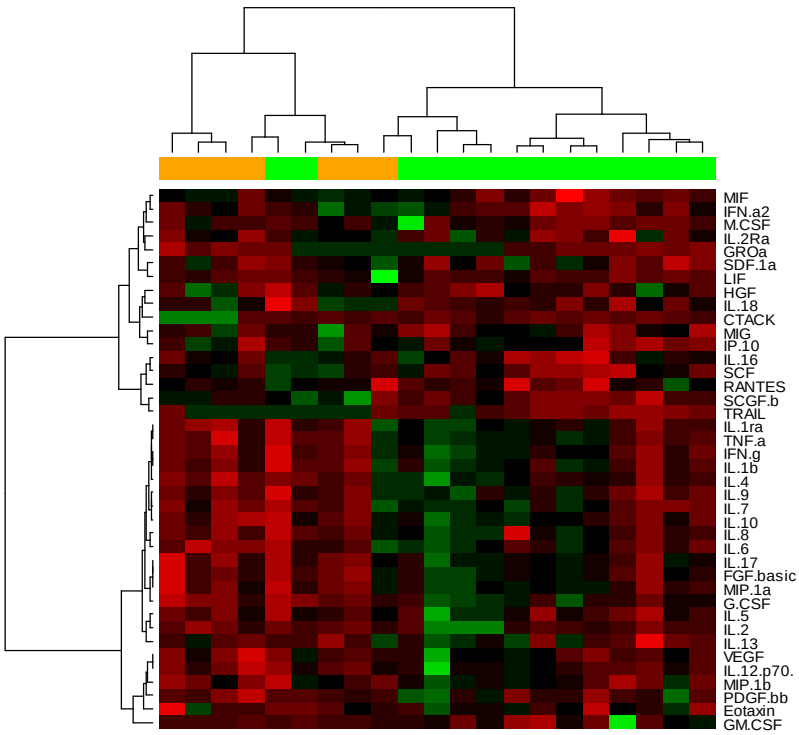

Figure 5: Heat map and cluster analysis of cytokine level in FR-SD (orange) and all other patients (NR-IR+SR, green)

Comparison of baseline plasma cytokine concentrations between no relapse-infrequent relapse and all other patients

In order to evaluate if NR-IR patients could be identified at the onset of the disease, we investigated the possible correlation between clinical response and plasma cytokine levels in NR-IR and all other patients. Univariate logistic regression models show a significant difference only for one cytokine: CTACK (p-value = 0.041; Figure 6).

Using hierarchical clustering, we found that NR-IR patients did not have a specific cytokine profile (data not shown).

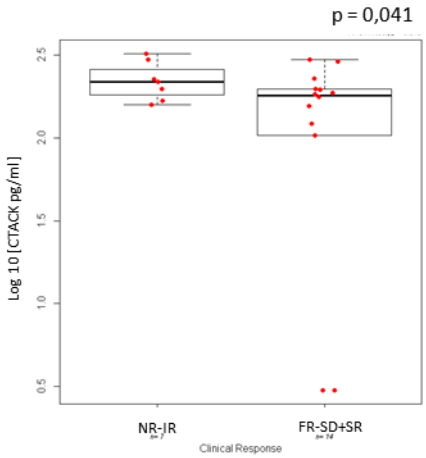

Figure 5: Box plot of CTACK concentration in NR-IR group and all other patients (FR-SD+SR)

Baseline plasma cytokine concentrations: MIF

Overall, these results show that patients with different clinical response have a distinct baseline (pretreatment) cytokine expression pattern; however, only MIF was able to discriminate NR-IR, FR-SD and SR patients in multivariate analysis (Figure 1b). ROC curves were constructed to assign optimal cut-off values for MIF level significantly associated with clinical response. For SR patients a unique cut-off of 473 pg/ml could be defined. Area under the ROC curves (AUC) was 76.0% (Figure 7a). The test had high sensitivity (71.4%) and specificity (81.7%). Logistic regression confirmed a higher proportion of SR patients among those who reached the optimal cut-off point (p-value = 0.024) in comparison with those who did not. Moreover for FR-SD patients a cut-off of 351pg/ml was found (AUC=83.2%, sensitivity=85.7%, specificity=83.2%; Figure 7b). Logistic regression confirmed higher proportion of FR-SD patients among those who did not reach the cut-off point (p-value = 0.011) in comparison with those who reach it.

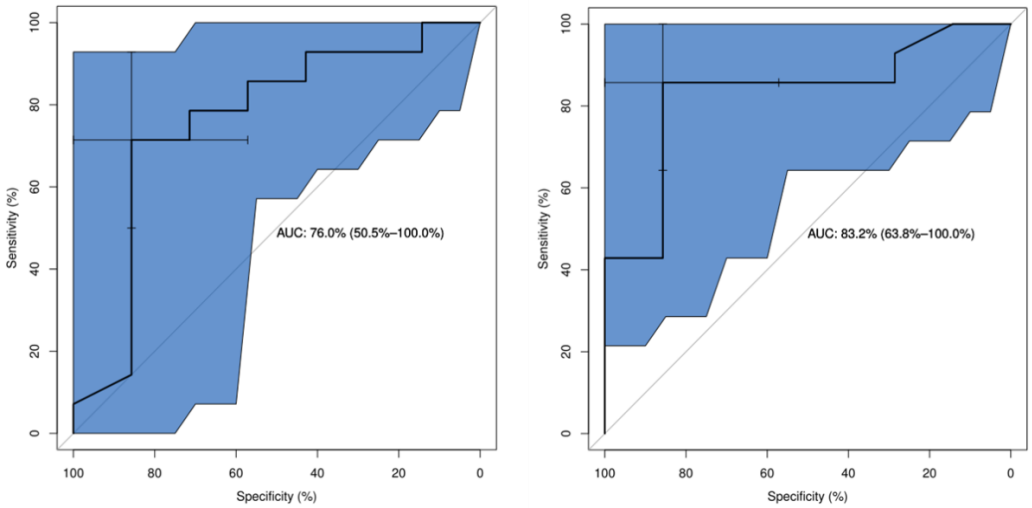

Figure 7: ROC curves for MIF in SR vs all other patients (left panel) and SD-FR and all other patients (right panel)

T4 analysis: No relapse-infrequent relapse and frequent relapse-steroid dependent patients

To define the impact of steroid therapy on cytokine expression profiles, we analyzed the plasma concentrations of cytokines after a 4-week treatment course (t4). Since at t4 SR patients have been already identified in clinics, we compared only NR-IR and FR-SD patients. When analyzing the profile of each cytokine separately, we identified 4 cytokines significantly different between the two groups of patients: IL18, MIG, SCF and IP10 (Figure 8); while, when performing a multivariate analysis none of the four remained significant.

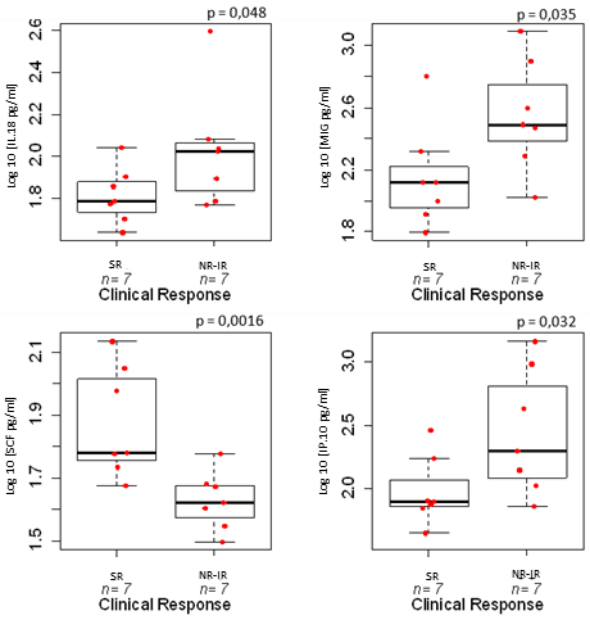

Figure 8: Box plots of cytokine concentration in NR-IR group versus FR-SD patients considering T4 analysis

Log Ratio T0/T4

To evaluate the change in cytokine levels after 4 weeks of treatment, we considered the log ratio between T0 and T4. Only two cytokines showed differences between NR-IR and FR-SD patients when analyzed with univariate logistic regression models: IL-12p70 and G-CSF (Figure 9). However when multivariate analysis was applied, none of the two cytokines was able to distinguish the two groups.

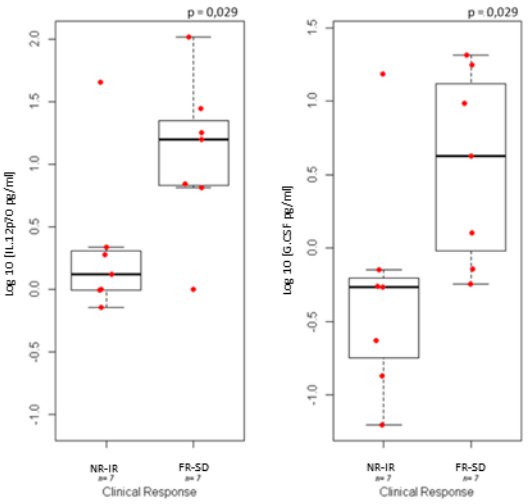

Figure 9: Box plots of cytokine concentration in NR-IR group versus FR-SD patients considering the Log ratio T0/T4

Discussion

In this study we evaluated the plasma level of 48 cytokines in INS patients responsive and non-responsive to GC treatment with the final aim of finding a biomarker useful to predict response. The study was conducted on 21 patients with the first episode of INS. Patients were selected from a well-characterized cohort of Italian pediatric patients treated with a shared therapeutic protocol.

INS is the most frequent primary glomerular disease in the pediatric population [3, 7]. 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 [3].

Since the 1950s steroid treatment is the most frequently used therapy of INS [8]. However, not all patients show positive response to this therapy. GCs are potent inhibitors of cytokines and prostaglandin production in immune and non-immune cells and are able to induce remission in about 85-90% of subjects, however, variable degrees of steroid responsiveness and different patterns of disease relapse have been observed [9]. Response to steroid treatment is an important indicator of outcome. It is well known that patients with steroid resistant disease represent a difficult therapeutic challenge for clinicians; moreover, to date, approximately 40-50% of SS patients relapse when therapy is discontinued, resulting in a steroid dependent course of the disease; these patients are at high risk of severe treatment related complications [3, 10] and there is still no means to predict this drug dependence.

Macrophage migration inhibitory factor (MIF) is a pleiotropic lymphocyte and macrophage cytokine; its inflammatory activities appear to be due to its effects on macrophages and T cells.

Although GCs inhibit the production of inflammatory molecules, they induce the secretion of MIF from macrophages [11] and T cells [12]. In turn, MIF counter-regulates the activity of GCs by suppressing the

inhibition of other pro-inflammatory cytokines [11, 13]. The mechanism by which MIF overrides this effect has not been fully clarified. One mechanism may be the interference with the effects of GCs on the transcription of cytokines, mediated by nuclear factor κB (NFκB), under inflammatory conditions [14]. NFκB is normally retained in the cytosol as a complex with IκBα (inhibitor of NFκB). Inflammatory stimuli activate IκBα kinase (IκK), resulting in phosphorylation and degradation of IκBα. Under these conditions, NFκB is free to enter the nucleus and function as a transcription factor. GCs interfere with this process by, in part, inducing the synthesis of IκBα, thereby maintaining a sufficient quantity of IκBα in the cytosol to keep NFκB from localizing into the nucleus. MIF, however, counter-regulates GC activities by decreasing IκBα levels in the cytosol and, consequently, increasing NFκB transcriptional activity in the nucleus [14]. Moreover, MIF stimulates the extracellular-signal-regulated kinase (ERK)-1 and ERK-2 pathway in a sustained fashion, leading to activation of the cytoplasmic isoform of phospholipase A2 (PLA2) and production of arachidonic acid [15]. GCs are normally potent inhibitors of PLA2 activation, but MIF counter-regulates this effect. In addition to repressing transcriptional activity of immune genes, GCs have been shown to down-regulate inflammatory responses by increasing the degradation of mRNAs of pro-inflammatory genes [16, 17]. Blocking this cytokine mRNA degradation was shown to be another mechanism by which MIF counterbalances the inhibitory effects of GCs [18] (Figure 10).

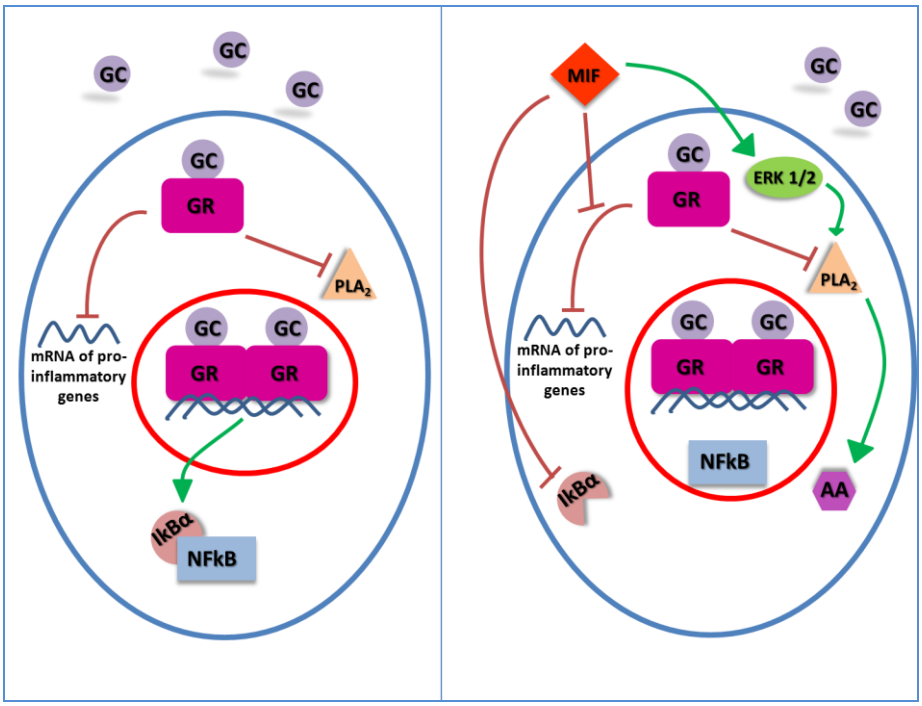

Figure 10: Pro-inflammatory mechanism of action of MIF

These mechanisms are probably insufficient to explain all the pro-inflammatory activities of MIF, but they do point to its specific antagonism on GC-mediated immunosuppression and also demonstrate how MIF promotes the expression of pro-inflammatory cytokines and prostaglandins.

MIF has been already investigated and proved to be implicated in a number of diseases including systemic lupus erythematous [19], rheumatoid arthritis [20] and chronic kidney disease [21].

Our results show that, within the 48 cytokines analyzed, MIF is the best predictor of steroid response before treatment in children with INS. Indeed patients non-responsive to GCs show significantly higher MIF plasma levels compared with steroid sensitive ones. These results are supported also by Wang et al. in patients with systemic lupus erythematosus [22]; these authors demonstrated that MIF serum expression was correlated whit steroid resistance. A clear cut-off value for serum MIF level could be identified at 473 pg/ml to distinguish SR and SS patients, with a high sensitivity of 71.4% and a high specificity of 81.7%. Considering patients achieving this cut-off, almost all the SR patients could be identified (6/7), however also a small proportion (4/14) of SS patients were included. This finding, if confirmed in a larger group of patients, could be useful for the early identification of patients who will not respond to steroids avoiding an ineffective treatment.

Moreover in this study we focus our attention also on FR-SD patients who show very low plasma MIF expression as compared with all other patients. This is the first study, to our knowledge, which investigate cytokine plasma levels in patients who show frequent relapses. Steroid dependent patients represent almost 40-50% of INS patients and are at risk of more aggressive treatment and disease related complications, representing a challenge for clinicians. Plasma MIF level in these patients was shown to be lower than in all other patients: a cut-off value of 351 pg/ml identified 6/7 FR-SD patients and only 2/14 of all other patients (test sensitivity 85.7%,specificity 83.2%).

In conclusion the results of this study suggest that, within the 48 cytokine considered, plasma MIF levels could be used to clinically identify patients at increased risk of steroid resistance at diagnosis that could thus be considered for alternative treatments, avoiding steroid administration and the relative side effects. Moreover, plasma MIF levels were able to identify patients with steroid dependence; these children could benefit of treatment with slower steroid tapering or of other immunosuppressive drugs.

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CHAPTER 6

In vitro response to methyl-prednisolone in pediatric patients with idiopathic nephrotic syndrome: role of genetic polymorphisms

Abstract

Idiopathic nephrotic syndrome (INS) is the most common primary glomerular disease in children and is characterized by massive proteinuria and hypoalbuminemia associated with dyslipidemia and generalized edema.

The response to glucocorticoid (GC) treatment is an important indicator for INS outcome indeed, most patients respond to GC therapy, while 10-20% of children fail to respond. Clinical experience has demonstrated that patients with poor response to steroids have unfavorable prognosis and often develop end stage renal failure. To date, no satisfactory explanation has been provided as to why some INS patients respond to GCs and others do not. GCs exert their biological effects through binding to the GC receptor (GR), which regulates either positively or negatively the expression of target genes. The GR is not self-standing in the cell and the receptor-mediated function are the result of a complex interplay of GR and many other cellular partners; the latter comprise several chaperonins of the large cooperative hetero-oligomeric complex that binds the hormone-free GR in the cytosol. Polymorphisms in the GR gene (NR3C1), such as *Bcl*I, have been described within the normal population and associated with GC response and toxicity; other polymorphisms in genes of proteins involved in molecular mechanisms of these hormones have also been suggested to play a role in the observed inter-individual differences in efficacy and toxicity. Moreover the inhibition of proliferation of peripheral blood mononuclear cells (PBMCs) by GCs has been correlated with clinical response in various diseases such as rheumatoid arthritis, systemic lupus erythematosus, bronchial asthma, renal transplant rejection and ulcerative colitis.

The aim of this study was to evaluate the relationship between individual variations in the anti-proliferative activity of methyl-prednisolone (MP), and several polymorphisms in genes of proteins involved in GC mechanisms of action.

Patients with INS were enrolled by an Italian pediatric nephrology network as part of a prospective study. Samples were collected before starting therapy and after 4 weeks, and processed within 24 hours. The effect of MP on the proliferation of PBMCs was determined by [methyl-h3]-thymidine incorporation. The drug concentration that would give 50% of lymphocyte inhibition (IC_{50}) and the maximum inhibition achievable at the highest MP concentration (I_{max}) were determined from the sigmoidal dose response curve. All subjects were genotyped for the selected polymorphism using TaqMan® genotyping technologies: *Bcl*I of NR3C1 gene, Leu155His of NALP1 gene, C3435T of MDR1 gene, rs1360780, rs2845597 and rs4713916 of FKBP5 gene, rs2282490 of STIP1 gene and rs2240447, rs2486014, rs4448553, rs199150, rs2301993 and rs1636879 of IPO13 gene. MP IC_{50} and I_{max} were determined for each subject from the dose response curves (IC_{50} median $5.43x10^{-9}$ M; mean $2.02x10^{-8}$ M; range $8.64x10^{-11}$ M - $2.40x10^{-7}$ M and I_{max} median 98; mean 95; range 74-99). A reduced MP in vitro response was observed in patients with the mutated *Bcl*I genotype (median IC_{50} 4.5 $X10^{-8}$ M, range $1.0x10^{-8}$ M – $2.4x10^{-7}$ M) compared to non-mutated carriers (median IC_{50} $4.6x10^{-9}$ M, range $8.6x10^{-11}$ M – $7.6x10^{-8}$ M; Wilcoxon test p-value=0.012) and a trend

was evident comparing the I_{max} of *Bcl*I mutated (median 86, range 80-99) and non-mutated (median 98, range 74-99) carriers (Wilcoxon test p-value=0.066). All other polymorphisms analyzed in this work were not significantly correlated with in vitro PBMC response.

Large scale clinical studies, together with the analyses of other polymorphisms of interest are necessary to confirm the role of in vitro proliferation test and genetic variability in GC response in children with INS.

Acknowledgment: this study was founded by “Associazione Sogno di Stefano”

Introduction

Idiopathic nephrotic syndrome (INS) is the most common primary glomerular disease in children and is characterized by massive proteinuria and hypoalbuminemia associated with dyslipidemia and generalized edema.

Glucocorticoids (GCs) remain the mainstay of childhood INS treatment, and response to initial oral prednisone determines disease prognosis. To date, no satisfactory explanation has been provided as to why some INS patients respond to GCs and other do not. Despite their broad therapeutic activity and effectiveness in remission induction, early markers that would allow optimization of the GC dose and of the duration of therapy could improve the management of nephrotic patients and reduce treatment failure or drug induced toxicity.

GCs interact with their cytoplasmic receptor, and are able to repress inflammatory gene expression through several distinct mechanisms. The GC receptor (GR) is therefore crucial for the effects of these agents and several polymorphisms of this gene (NR3C1) have been described and associated with GC response and toxicity. The GR is not self-standing in the cell and the receptor-mediated function are the result of a complex interplay of GR and many other cellular partners; the latter comprise several chaperonins of the large cooperative hetero-oligomeric complex that binds the hormone-free GR in the cytosol. Other polymorphisms in genes of proteins involved in the molecular mechanisms of these hormones have also been suggested to play a role in the observed inter-individual differences in efficacy and toxicity.

In vitro tests based on the proliferation of mononuclear cells exposed to GCs have been correlated with clinical response in different diseases such as rheumatoid arthritis, systemic lupus erythematosus, bronchial asthma, renal transplant rejection and ulcerative colitis (Hearing SD et al, 1999; Hirano T et al, 2002).

More recently we have associated the in vitro response to GCs with polymorphisms of genesinvolved in GC mechanisms of action in healthy donors (Cuzzoni E et al, 2011).

To evaluate a priori the individual response to GCs, a pharmacodynamic approach using patients's peripheral blood mononuclear cells (PBMC), together with a pharmacogenetic approach with the evaluation of polymorphisms involved in GC response, could be an efficient strategy.

Aim of the study

The aim of this study was, therefore, to evaluate the relationship between individual variations in the anti-proliferative activity of methyl-prednisolone (MP), and several polymorphisms in genes of proteins involved in GC mechanisms of action.

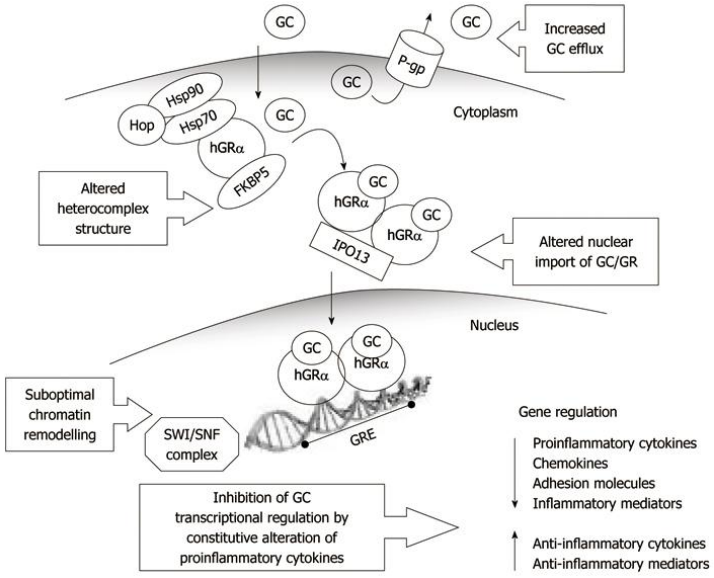


Figure 1: Mechanism of glucocorticoid resistance in inflammatory diseases

Methods

Subjects

PBMCs were obtained from 35 pediatric patients with INS (mean age 5.7; range 1-13; 42.9% females and 57.1% males). Patients were enrolled, between August 2011 and July 2012, by an Italian paediatric nephrology network as part of a prospective study. Samples were collected before starting therapy and after 4 weeks of prednisolone treatment, and processed within 24 hours. A total of 9 ml of blood anticoagulated with EDTA was used for the isolation of PBMCs.

In vitro proliferation assay

The effect of MP on the proliferation of PBMCs was determined by labelling metabolically active cells with [methyl-3H] thymidine (Perkin Elmer, Milan, Italy). PBMCs were collected by density gradient centrifugation on Ficoll PaqueTM Plus (Healthcare, Milan, Italy), resuspended in complete RPMI-1640 medium containing Concanavalin-A (5 µg/ml) and seeded into a 96 well round bottom plate (2×105 cells/well) in the presence of MP (range from 54 µM to 0.05 nM). After 50 hours of incubation, cells were pulsed with [methyl-3H] thymidine (2.5 µCi/ml/well), and the incubation was continued for additional 22 hours. The radioactivity of the samples was determined by a Liquid Scintillation Analyzer (Wallac 1450 Microbeta liquid scintillation counter, Perkin Elmer, Milan, Italy). Raw count per minute (cpm) data were converted and normalized to percent of maximal survival for each experimental condition (cpm MP/cpm control *100). Non linear regression of dose-response data was performed using GraphPad Prism version 4.00 for computing IC_{50}, the MP concentration required to reduce proliferation to 50%. I_{max} was also calculated and defined as the maximum inhibition achievable at the highest concentration of MP (54 µM).

The in vitro proliferation assay was applied also on PBMCs of 50 healthy control subjects.

Genetic analysis

Total genomic DNA was isolated from peripheral blood using a commercial kit (Gene Elute Blood Genomic DNA kit, Sigma Aldrich, Milan, Italy) and genotyped for the selected polymorphisms using TaqMan® genotyping technologies (Applied Biosystems, Bedford, UK) on an ABI7900 HT sequence detection system device.

Statistical analysis

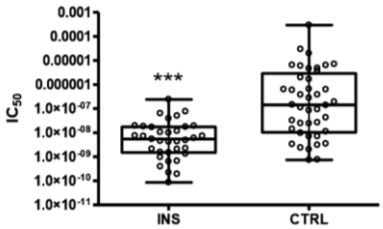
Any possible association between GC sensitivity and the polymorphisms in each gene was investigated by the non-parametric Mann-Whitney and Kruskall-Wallis tests. Statistical analysis was performed using the software R.

Gene	Mutation	WT (%)	HET (%)	MUT (%)	NA	P-HW
NR3C1	BclI rs41423247	13 (41.9%)	14 (45.1%)	4 (13.0%)	4	0.93
NALP	Leu155His rs12150220	9 (30.0%)	13 (43.3%)	8 (26.7%)	5	0.46
FKBP5	rs4713916	13 (41.9%)	14 (45.2%)	4 (12.9%)	4	0.94
STIP1	rs2845597	15 (51.7%)	9 (31.0%)	5 (17.3%)	6	0.11
	rs2282490	22 (68.8%)	9 (28.1%)	1 (3.1%)	3	0.94
IPO13	rs2240447	17 (53.1%)	14 (43.8%)	1 (3.1%)	3	0.35
	rs2486014	25 (80.6%)	6 (19.4%)	0	4	0.55
	rs4448553	17 (53.1%)	14 (43.8%)	1 (3.1%)	3	0.35
	rs199150	18 (60.0%)	11 (36.7%)	1 (3.3%)	5	0.66
	rs2301993	17 (53.1%)	14 (43.8%)	1 (3.1%)	3	0.35
	rs1636879	23 (76.7%)	7 (23.3%)	0	5	0.47
PGP	C3435T rs1045642	11 (37.9%)	15 (51.7%)	3 (10.4%)	6	0.52

Table I: distribution of genotype of the studied polymorphisms

Results

The IC_{50} median values of MP in INS patients and healthy controls are represented in Figure 2. The IC_{50} median value of INS patients was significantly lower than the IC_{50} median value of controls (Mann-Withney test CTRL-IC_{50} median $1.43x10^{-7}$; INS-IC_{50} median $5.43x10^{-9}$; P-value<0.0001). Similar results were obtained with the I_{max} values (Mann-Withney test CTRL-IC_{50} median 85; INS-IC_{50} median 98; P-value<0.0001). Furthermore, when median IC_{50} and I_{max} value of INS patients were analyzed before starting therapy and after 4 weeks of prednisolone treatment, no difference was evident (Mann-Withney test median INS-IC_{50}-t0 $5.43x10^{-9}$; median INS-IC_{50}-t4 $1.97x10^{-9}$ P-value=0.1402; Fig.3; median INS-I_{max}t0 98; INS-I_{max}t4 99; p-value=0.3942).



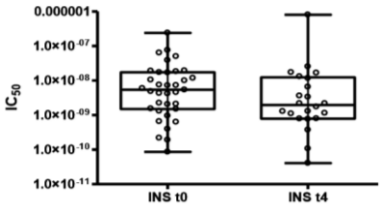


Figure 2 (left panel): Mann-Whitney test INS-IC_{50} and CTRL-IC_{50} median and Figure 3 (right panel): Mann-Whitney test INS-IC_{50} and CTRL-IC_{50} median

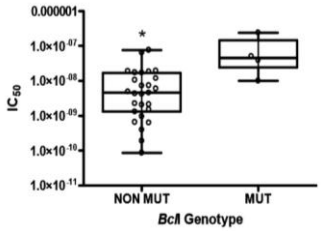
All patients were genotyped for polymorphisms involved in GC action (Table I). Any possible association between the studied polymorphisms with an increased or decreased GC sensitivity was investigated (Table II).

Gene	Mutation	IC_{50} median value (range)			P-value Kruskal-wallis
		WT (%)	HET (%)	MUT (%)	
NR3C1	BclI rs41423247	$4.18x10^{-9}$	$4.74x10^{-9}$	$4.47x10^{-8}$	**0.045**
NALP	Leu155His rs12150220	$1.05x10^{-8}$	$7.34x10^{-9}$	$3.12x10^{-9}$	0.195
FKBP5	rs4713916	$4.57x10^{-9}$	$1.02x10^{-8}$	$4.73x10^{-9}$	0.537
STIP1	rs2845597	$7.34x10^{-9}$	$5.96x10^{-9}$	$4.91x10^{-9}$	0.911
	rs2282490	$4.74x10^{-9}$	$1.18x10^{-8}$	$2.25x10^{-9}$	0.809
IPO13	rs2240447	$4.18x10^{-9}$	$1.02x10^{-8}$	$6.61x10^{-10}$	0.143
	rs2486014	$4.91x10^{-9}$	$8.63x10^{-9}$	-	0.920
	rs4448553	$4.32x10^{-9}$	$1.03x10^{-8}$	$6.61x10^{-10}$	0.292
	rs199150	$4.25x10^{-9}$	$1.05x10^{-8}$	$6.61x10^{-10}$	0.218
	rs2301993	$4.18x10^{-9}$	$1.02x10^{-8}$	$6.61x10^{-10}$	0.284
	rs1636879	$4.32x10^{-9}$	$1.05x10^{-8}$	-	0.292
PGP	C3435T rs1045642	$4.57x10^{-9}$	$1.05x10^{-8}$	$9.60x10^{-10}$	0.214

Table 2: IC_{50} median values in relation with genotype

Only an association between the *Bcl*I polymorphism in the NR3C1 gene with an increased GC resistance was observed. The mutated *Bcl*I genotype was associated with an higher IC_{50} compared to non-mutated carriers or wild type and heterozygoes subjects (Wilcoxon test: median 4.47x10-8 versus $4.57x10^{-9}$ respectively, p-value=0.012;Fig 4; Mann-Whitney test: median 4.18x10-9 versus $4.74x10^{-9}$ versus $4.47x10^{-8}$ respectively, p-value=0.045; Fig 5).

All other studied polymorphisms were not associated with *in vitro* response.



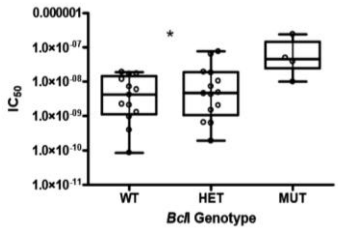


Figure 4 (left panel): *Bcl*I mutated genotype associated with an higher MP-IC_{50} and Figure 5 (right panel): *Bcl*I mutated genotype associated with an higher MP-IC_{50}

Conclusion

The *in vitro* prediction of GC response before the start of treatment would have important clinical implications, allowing to adjust therapy a priori, avoiding the use of these agents in patients who would probably not respond and reducing dosages in those who are hypersensitive, and hence at risk of toxicity.

In this study we have shown that patients with INS have an higher sensitivity to MP in comparison with healthy controls, and *in vitro* response is not modified by a four weeks treatment with these hormones. In addition, among the studied polymorphisms, only the *Bcl*I SNP of the NR3C1 gene was associated with a reduced response to GCs.

Further studies are needed to confirm these preliminary results in a larger number of patients: clinical data of these patients will be correlated with the *in vitro* results to confirm these data.

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CHAPTER 7

Evaluation of the mRNA expression response as a novel in vitro tool for assessing sensitivity to steroid treatment

ABSTRACT

Glucocorticoids (GC) are the most widely used anti-inflammatory and immunomodulatory drugs. Despite their large clinical impact and justified use, the benefits of these agents are often narrowed by a great inter-individual variability that might potentially lead to treatment failure or drug induced toxicity. A reliable way of predicting response to GC therapy by the patient would therefore be useful in clinical management.

Our aim was to establish an in vitro tool for evaluation of the intrinsic sensitivity to GC. For this, we have set up dose-response [3H]-thymidine incorporation assays of methylprednisolone (MP) treatment on inhibition of proliferation of stimulated peripheral blood mononuclear cells (PBMC). In addition, we have developed a means of measuring the action of MP based on its effect on the kinetics of mRNA transcription by the cells. We designed quantitative real-time PCR assays for a panel of mRNA transcripts, the levels of which may be affected downstream of GC treatment. This panel consists of pro-inflammatory mediators (IFNγ, TNF-α, IL- 2, IL-6, IL-8, CCL-2, CCL-3, CCL-5, CCL-13, CXCL-9, CXCL-10, CXCL-11), anti-inflammatory mediators (IL-10, FKBP5, DUSP1, SAP-30, TLR-7) and intracellular signalling molecules (NF-κB, STAT-3, STAT-6, MAPK). Expression levels are standardized to those of reference genes β-actine and GADPH. We have investigated PBMC from healthy donors at the Sanquin Blood bank in Leiden, the Netherlands.

Until now, we have analyzed MP effects on proliferation of phytohaemagglutinin (PHA)-stimulated PBMC in 17 different donors. Non-linear regression of dose–response curves was applied to compute the MP concentration required to reduce proliferation by 50% (IC_{50}). The data showed an high inter-individual variability in MP-IC_{50}, ranging between $1.5x10^{-9}$ and $1.1x10^{-5}$ M. To investigate the mRNA response, we first investigated which cell types in total PBMC fractions are stimulated by either PHA, concanavalin A, CD3/CD28 beads or lipopolysaccharide (LPS). The PBMC were labelled by carboxyfluorescein succinimidyl ester and stimulated for 72h. This was followed by flow cytometric staining for T cells (CD3+CD4+ and CD3+CD8+), B cells (CD19+), monocytes (CD14+), and NK cells (CD3-CD16+CD56+). Results were evaluated as proliferation index number (the sum of the cells in all generations divided by the calculated number of original parent cells). Stimulation with PHA resulted into the highest T cell proliferation index number (1.64) both of T helper cells (CD4+, 1.84) and cytotoxic T cells (CD8+, 1.44), while other cell types did not proliferate. LPS was the only stimulus that induced monocyte proliferation (1.24). Next, we studied in three donors the kinetics of mRNA expression of the different markers at different time periods of stimulation (6h, 12h, 24h, 48h, 72h), using both PHA and LPS. In general, the peak in expression levels was observed at 6 and 12 hours after stimulation. Further experiments will be performed for these two time points, and in combination with different concentrations of MP (10^{-4}M and 10^{-7}M).

In conclusion, we are establishing a novel in vitro tool, based on the mRNA response of different hematopoietic cell types, for quick evaluation of the intrinsic sensitivity to GC treatment. Data from this

test will be compared to the outcome of dose-response proliferation assays, and will eventually be placed in the context of clinical outcome in vivo.

Introduction

Glucocorticoids (GCs) are a well-accepted therapy for inflammatory and autoimmune diseases, in transplant patients and in the treatment of leukemia and lymphomas. However, despite their large clinical impact and justified use, the benefits of these agents are often narrowed by a great inter-individual variability that might potentially lead to treatment failure or drug induced toxicity. A reliable tool for predicting patient in vivo response would therefore be useful for clinical management of the disease.

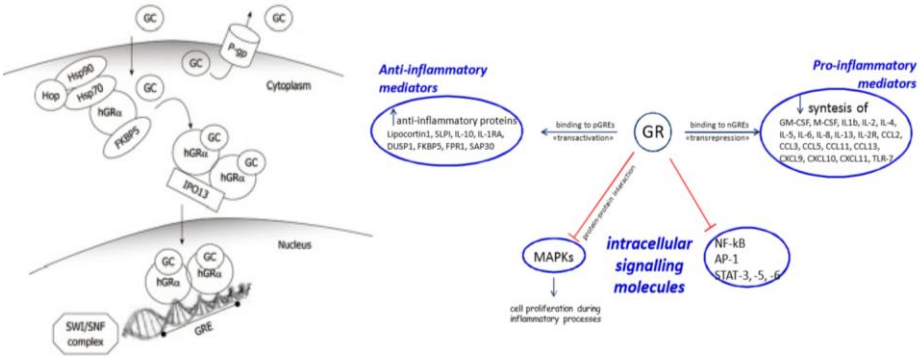

Fig. 1: Glucocorticoids mechanism of action

In vitro tests based on the proliferation of GC-exposed mononuclear cells have been correlated with clinical response in different diseases such as rheumatoid arthritis, systemic lupus erythematosus, bronchial asthma, renal transplant rejection and ulcerative colitis (Hearing SD, 1999). However these findings have not been always reproducible, and a bioassay that could be useful to predict GC responsiveness in clinical practice is still lacking. To evaluate individual response to GCs, a pharmacodynamic approach using patients peripheral blood mononuclear cells (PBMC), in combination with a pharmacogenetic approach that evaluates the mRNA expression of cytokines involved in glucocorticoid mechanism of action, could be an efficient strategy.

Several studies have demonstrated differences in the co-chaperonine gene expression profiles in steroid resistant in comparison with responder patients (Raddatz D, 2004; Charmandari E, 2010; Matysiak M, 2008). But no big study has been already performed on the changes in the gene expression occurring downstream of GC treatment to elucidate the mechanism of GC resistance

Aim of the study

The final goal of our study is to establish a simple and reproducible *in vitro* tool for evaluating the intrinsic sensitivity of PBMC to GC.

Methods and results

Subjects

The in vitro proliferation assay with methyl-prednisone (MP) and genetic analyses was performed on PBMC isolated from 17 blood donors at the Sanquin Blood bank in Leiden, the Netherlands.

In vitro proliferation assay

The effect of MP on PBMC proliferation was determined by labelling metabolically active cells with [methyl-^{3}H] thymidine. PBMCs were collected by density gradient centrifugation on Ficoll PaqueTM Plus, resuspended in complete RPMI-1640 medium containing PHA (1 µg/ml) and seeded into a 96 well round bottom plate (2×10^{5} cells/well) in the presence of MP (range from $1x10^{-3}$ M to $1x10^{-15}$ M). After 50 hours of incubation, cells were pulsed with [methyl-^{3}H] thymidine (2.5 µCi/ml/well), and the incubation was continued for additional 22 hours. The radioactivity of the samples was determined by a Liquid Scintillation Analyzer. Raw count per minute (cpm) data were normalized to control and expressed as percent of maximal survival for each experimental condition (cpmMP/cpmcontrol *100). Non linear regression of dose-response data was performed using GraphPad Prism for computing IC_{50}, the MP concentration required to reduce proliferation to 50%.

Data from proliferation assay showed an high inter-individual variability in MP-IC_{50}, ranging between $1.5x10^{-9}$ and $1.1x10^{-5}$ M. (Fig.2)

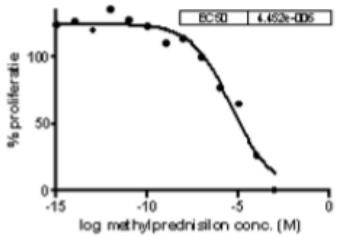

Fig. 2: Rapresentative inhibition curve of MP-treated PBMC

Cell proliferation

To investigate which cell types of the isolated PBMC were stimulated by PHA, concanavalin A, CD3/CD28 beads or lipopolysaccharide (LPS), the PBMC were labelled by carboxyfluorescein diacetate succinimidyl ester (CFSE), exposed to the 4 different stimuli and incubated at 37°C and 5% CO_2 . Flow cytometric staining for T cells (CD3+CD4+ and CD3+CD8+), B cells (CD19+), monocytes (CD14+), and NK cells (CD3-CD16+CD56+) was performed after 72h incubation .The flowcytometry data analysis was performed using FlowJo software. Data with 10,000 acquired events of CD3+CD4+ or CD3+CD8+ were analyzed

Results were evaluated using ModFit software calculating the proliferation index number (the sum of the cells in all generations divided by the calculated number of original parent cells).

Cytometric analysis show that stimulation with PHA resulted into the highest T cell proliferation index number (1.64) both of T helper cells (CD4+, 1.84) and cytotoxic T cells (CD8+, 1.44), while other cell types did not proliferate (Fig.3a). LPS was the only stimulus that induced monocyte proliferation (1.24) (Fig.3b).

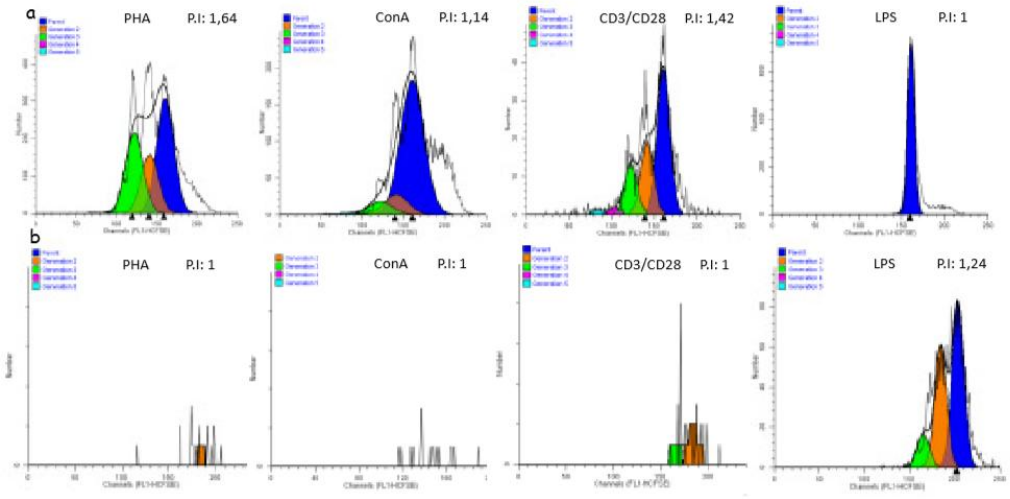

Fig. 3: Histograms show peaks of parental undivided PBMC and daughter cells for the 4 different stimuli (PHA,ConA, CD3/CD28 and LPS) and the proliferation index (P.I.). Cytometric analysis of a) T-cells and b) $CD14^+$ cells

mRNA expression

For mRNA detection, PBMC were cultured at $1x10^5$ cells per well for different incubation time in 96-well plates in the presence of PHA or LPS. Cells were harvested and preserved in RNAlater solution. RNA was extracted using the NucleoSpin® kit (Qiagen) following the manufacturer's instructions. RNA quantity was assessed with a spectrophotometer (Nanodrop Technologies). All samples showed A260/A280 ratios between 1.9 and 2.1.

cDNA synthesis from total RNA (100 ng) was carried out following the manufacturers' manual of SuperScript III RT (Invitrogen).

Quantitative real-time PCR assays was performed using SYBR Green supermix (Bio-Rad) for a panel of mRNA transcripts, known to be regulated by GC treatment. This panel consists of pro-inflammatory mediators (IFNγ, TNF-α, IL- 2, IL-6, IL-8, CCL-2, CCL-3, CCL-5, CCL-13, CXCL-9, CXCL-10, CXCL-11), anti-inflammatory mediators (IL-10, FKBP5, DUSP1, SAP-30, TLR-7) and intracellular signalling molecules (NF-κB, STAT-3, STAT-6, MAPK). The PCR was performed using an iCycler MyiQ (Bio-Rad). Expression levels were standardized to those of reference genes β-actin and GADPH.

Results from kinetics experiments at 6h, 12h, 24h, 48h and 72h are show in figure 4.

Time points of 6h and 12h showed the highest mRNA expression and were chosen for the further experiments.

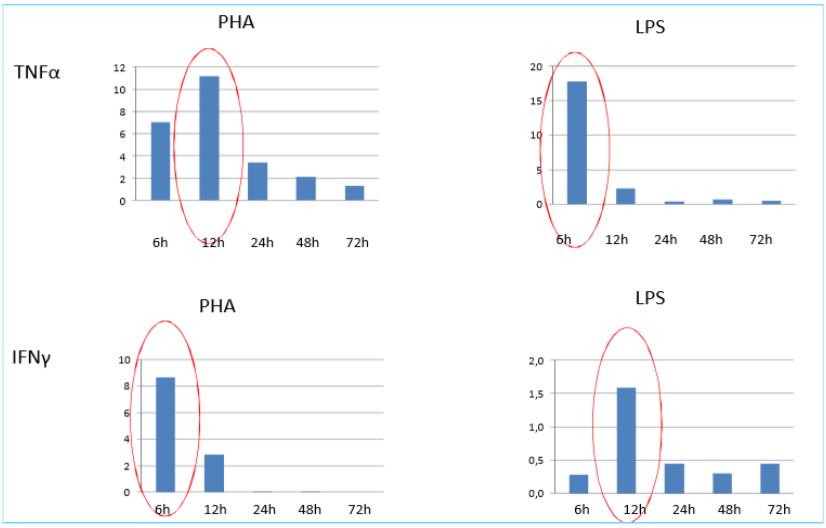

Fig. 4: mRNA expression of TNFα and IFNγ after stimulation of PBMC with PHA and LPS for 6h, 12h, 24h 48h and 72h

$CD3^+$ and $CD14^+$ cells of total isolated PBMC were separated by performing a magnetic sorting with the MIDIMACS Technique (Miltenyi Biotec) according to the manufacturer's instructions.

As shown in figure 5, LPS stimulation of total PBMC resulted in high TNFα mRNA expression. Comparison with separated CD3+ and $CD14^+$ cells revealed that the expression was only due to the monocytes (Fig.5a). On the contrary, PHA stimulation of total PBMC resulted in high IFNγ mRNA expression due only to T-cells population (Fig. 5b)

These results confirmed that total isolated PBMC could be used without any further subdivision.

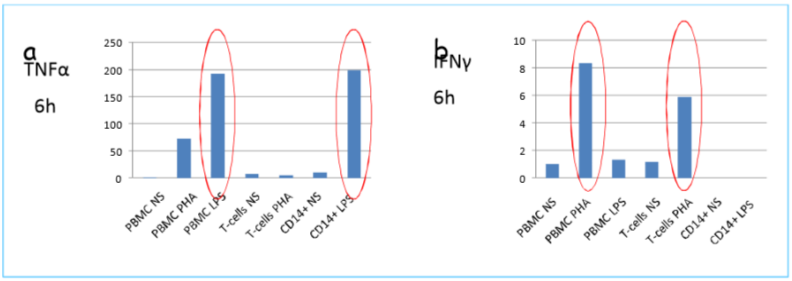

Fig. 5: Comparison of TNFα and IFNγ mRNA expression in total PBMC, $CD3^+$ and $CD14^+$ isolated subpopulation stimulated with PHA and LPS for 6h

mRNA expression in response to MP-treatment

Three different healthy subject were chosen on the basis of their different *in vitro* sensibility to MP (resistant, intermediate and sensitive).

Experiments were performed under optimized condition (6h and 12h, PHA and LPS stimolation on total isolated PBMC), in presence of two different concentration of MP (10^{-4}M and 10^{-7}M) to analyze their cytokine expression profile after steroid treatment.

Dose dependent variation were observed for all the cytokine of the panel, as representatively showed in figure 7 for TNFα and IFNγ.

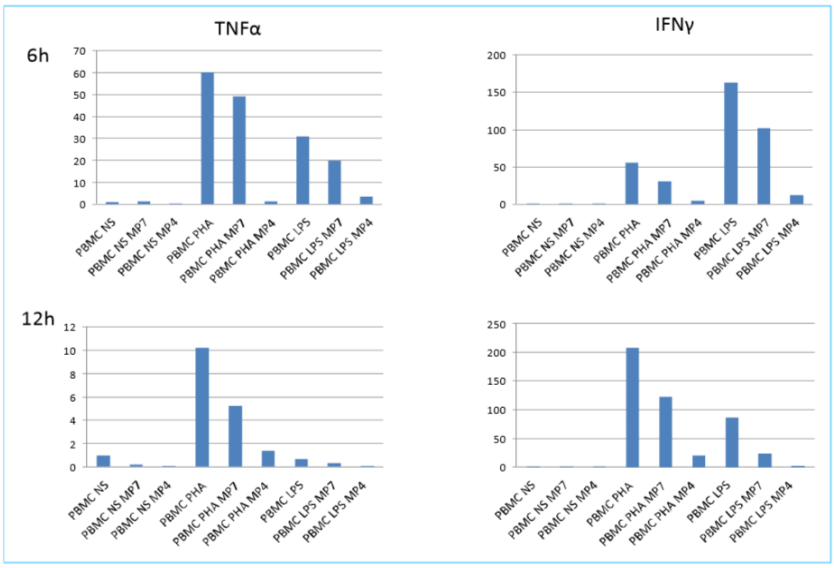

Fig. 7: mRNA expression of TNFα and IFNγ after stimulation of PBMC with PHA and LPS, and treatment with MP for 6h and 12h

Figure 8 clearly shows a different pattern of cytokines mRNA expression after MP treatment among the three subjects.

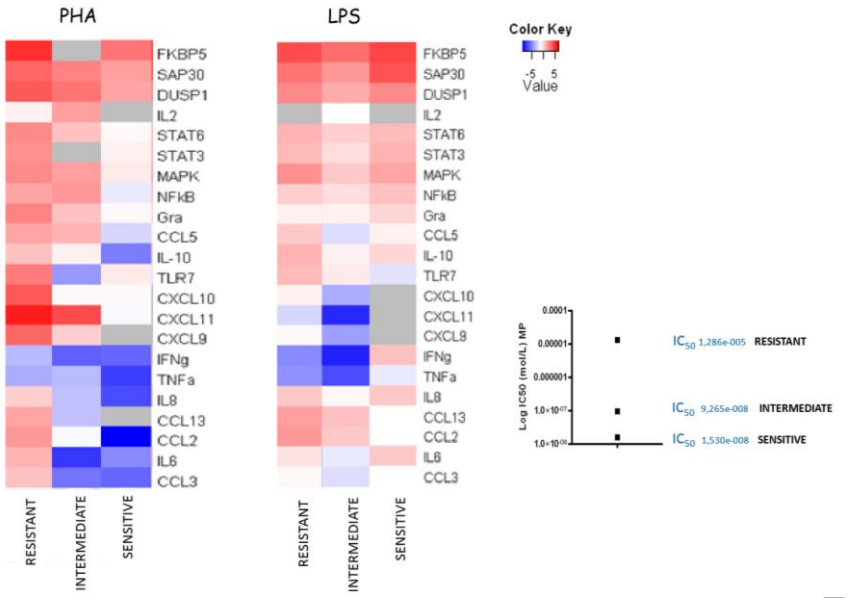

Fig. 8: Difference, in 3 different subject, in the mRNA expression after stimulation of PBMC with PHA and LPS for 12h

Conclusion

In conclusion, we are establishing a novel *in vitro* tool on MP-treated PBMC, integrating the results of dose-response proliferation assays with the cytokines mRNA level. These data are preliminary and need larger study population to draw any firm conclusion for translating this approach in patients.

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CHAPTER 8

Glucocorticoid resistance in pediatric idiopathic nephrotic syndrome patients: role of noncoding RNA GAS5 and NR3C1 gene

Abstract

Children diagnosed with idiopathic nephrotic syndrome (INS) are commonly treated with glucocorticoids (GC). These children show variation in their clinical response to standardized doses of systemic GCs, and 10-20% of patients fail to respond. Given the high incidence of suboptimal response, associated with a significant number of side effects, that are particularly severe in paediatric patients, the identification of subjects that are most likely to respond poorly to GCs is extremely important. A positive correlation between steroid resistance and reduction of glucocorticoid receptor (GR) in cells has been suggested (1).

Growth arrest-specific 5 (GAS5) is a long (~650 bases in humans) noncoding RNA (ncRNA), originally isolated during a screening for potential tumor suppressor genes, that is expressed at high levels in growth arrested cells (2) even though its functions are not yet well known. Recently, it was found that GAS5 interacts with the DNA binding domain of the ligand-activated GR and suppresses GR-induced transcriptional activity of glucocorticoid-responsive genes by inhibiting binding of GRs to target genes GREs (3).

The aim of this study was to evaluate the correlation between individual variability in GAS5 and GC receptor gene (NR3C1) expression in lymphocytes and clinical response in INS patients.

Between August 2011 and February 2014, 95 patients (median age 4.25 years, range 1-17) with INS were enrolled by an Italian pediatric nephrology network as part of a prospective study. Samples were collected before starting therapy and processed within 24 hours. All patients were treated with the same protocol and divided, on the bases of their clinical response, in three groups: corticosteroid sensitive (CS), dependent (CD) and resistant (CR).

A preliminary study was conducted on 9 patients (3 CS, 3 CD, 3 CR); peripheral blood mononuclear cells (PBMCs) were obtained and GAS5 and NR3C1 gene expression was evaluated using TaqMan® technology.

CR patients presented higher levels of GAS5 (QRmean 0,0072 ± 0.0009) in comparison with responder (QRmean 0,0017 ± 0.016) and dependent patients (QRmean 0,0015 ± 0.0010; Anova One Way (Kruskal Wallis test) p-value=0.06). In addition, the expression of NR3C1 gene in CR patients was undetectable in comparison with CS (QRmean 4,088E-05 ± 3,368E-05) and CD (QRmean 1,676E-05 ± 2,460E-05) confirming data reported in literature (1).

We hypothesize that, in CR patients, abnormally high levels of GAS5 expression, through the interaction with the DNA binding domain of the activated GR, results in the suppression of GC transcriptional activity, reducing their effectiveness.

If these results are confirmed in a larger number of subjects, GAS5 and NR3C1 should be considered as candidate markers of GC resistance.

Introduction

Children diagnosed with idiopathic nephrotic syndrome (INS) are commonly treated with glucocorticoids (GC). These children show variation in their clinical response to standardized doses of systemic GCs, and 10-20% of patients fail to respond. Patients with two recurrences during the decalage of GC or within 2 weeks from suspension were defined as GC dependent subjects. Given the high incidence of suboptimal response, associated with a significant number of side effects, that are particularly severe in paediatric patients, the identification of subjects that are most likely to respond poorly to GCs is extremely important. GCs exert their biological effects through binding to the GC receptor (GR), which translocates from the cytoplasm into the nucleus and binds, through its DNA-binding domain (DBD), to glucocorticoid response elements (GREs), the regulatory regions of GC responsive genes. Growth arrest-specific 5 (GAS5) is a long (~650 bases in humans) noncoding RNA (lncRNA) that was originally isolated during a screening for potential tumor suppressor genes expressed at high levels in growth arrest [1] even though its functions are not yet well known. It was found that GAS5 interacts with the DBD of the ligand-activated GR and suppresses GR-induced transcriptional activity of GC-responsive genes by inhibiting binding of GRs to target genes GREs [2]. Data recently obtained in our laboratory [3] showed that GAS5 may alter GC effectiveness probably interfering with the mechanism of GR autoregulation (Fig. 1). Our observations strongly suggest that GAS5 could have an important role in the regulation of the response to GCs.

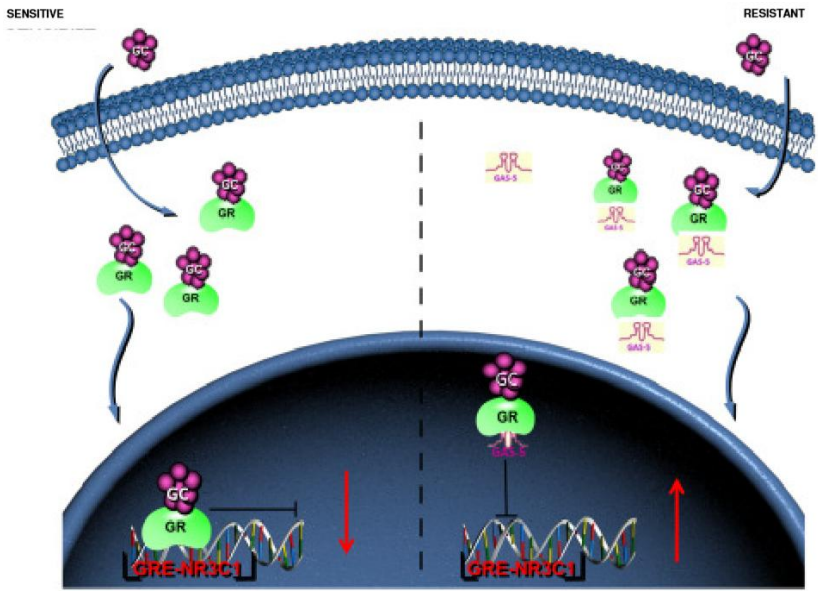

Fig. 1 Potential mechanism of resistance through the involvement of GAS5 in the process of autoregulation of GR.

Aim of the study

The aim of this preliminary study was to evaluate the correlation between individual variability in GAS5 and GC receptor gene (NR3C1) expression in lymphocytes and clinical response in INS patients, identifying potential markers involved in GC response.

Results

Between August 2011 and February 2014, 95 patients (median age 4.25 years, range 1-17) with INS were enrolled by an Italian paediatric nephrology network as part of a prospective study. Samples were collected before starting therapy and processed within 24 hours. All patients were treated with the same protocol and divided, on the basis of their clinical response, in three groups: corticosteroid sensitive (CS), dependent (CD) and resistant (CR). This preliminary study was conducted on 13 patients (6 CS, 3 CD, 4 CR); peripheral blood mononuclear cells (PBMCs) were obtained and GAS5 and NR3C1 gene expression was evaluated using TaqMan® technology. CR patients presented significantly higher levels of GAS5 in comparison with CS group (REmean 4.93 ± 0.99; One-way ANOVA test p value < 0.05, Fig. 2); conversely, CD patients showed the same relative GAS5 expression respect to CS patients (REmean 1.24 ± 0.49) (Fig. 2). In addition, the expression of NR3C1 gene in CR (REmean 0.66 ± 0,54) was lower, but non significantly, in comparison with CS, confirming data reported in literature [4]. Relative NR3C1 expression in CD patients resulted slightly higher compared to CS group (REmean 1.46 ± 1.20). We hypothesize that, in resistant PBMCs, as a consequence of GAS5 interaction, a reduced availability of the activated GR for binding to GREs target genes suppresses GC transcriptional activity. The low levels of relative NR3C1 expression observed in CR patients, suggest that abnormal levels of GR transcript may alter GC effectiveness too. GAS5 and NR3C1 gene may offer a diagnostic tool for early identification of nephrotic children who are unlikely to respond to conventional glucocorticoid therapy.

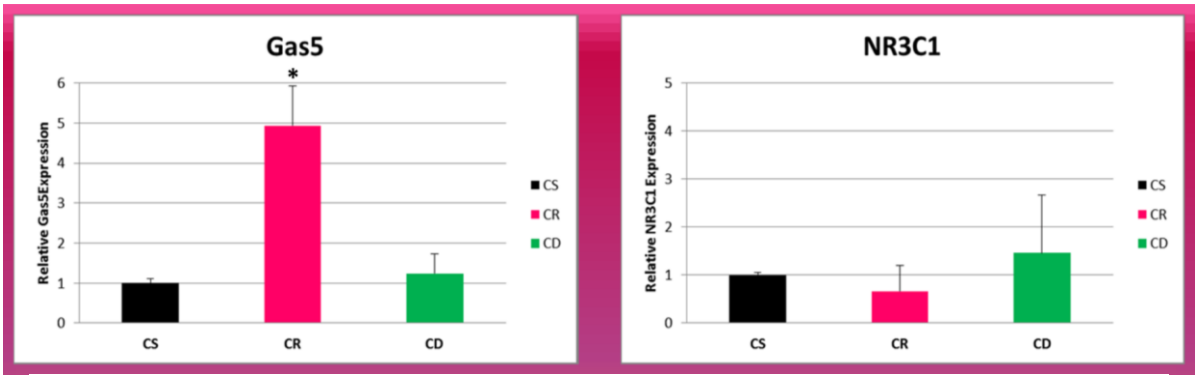

Fig. 2 The relative expression of GAS5 and NR3C1 in CR and CD patients vs CS patients was calculated and normalized using the 2−ΔΔ Ct method relative to 18S. One-way ANOVA and post Dunn's test * p value=0.05

Conclusion

In summary, the results presented here indicate that, in CR patients, abnormally high levels of GAS5 expression, through the interaction with the DNA binding domain of the activated GR, could results in the suppression of GC transcriptional activity, reducing their effectiveness. Moreover we confirmed that low expression of glucocorticoid receptor in INS patients, could represent a poor prognostic sign in childhood nephrotic syndrome. If these results will be confirmed in a larger number of subjects, GAS5 and NR3C1 should be considered as candidate markers of GC resistance in paediatric INS patients translating this to optimize the therapy and in particular to avoid an ineffective and potentially toxic treatment.

Acknowledgements

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CHAPTER 9

Summary and discussion

RATIONALE OF THE THESIS

Since their discovery in the late forties of the 20th century, and the recognition of their favorable effects on inflammation, GCs soon found their way into daily clinical practice. Nowadays, GCs are indispensable for the treatment of numerous disorders, ranging from inflammatory disease and classic autoimmune diseases, such as INS, inflammatory bowel diseases, rheumatoid arthritis and systemic lupus erythematosus to asthma and hematological malignancies. However, during more than 60 years use of these agents, no or little progress has been made in the development of tools to:

1) identify patients who will or will not benefit from GCs treatment, or to

2) adjust GCs dose according to an individual's GC sensitivity.

Indeed, a substantial proportion of patients experiences a lack of, or suboptimal, effect of GCs therapy, interfering with a favorable disease outcome. Determination of GC sensitivity prior to the beginning of therapy, allowing individual-dosed treatment schedules, could further optimize GC therapy in these patients. Alternatively, patients with proven GC resistance may be treated with alternative (more aggressive) immunomodulatory agents, which also increase the likelihood of successful initial treatment. Studies evaluating GC sensitivity in the very early phase of INS could contribute to solve this intriguing question in this population of patients.

The studies described in this thesis aim to increase our understanding of the clinical variability observed in childhood INS and to offer new insights for improving current therapy. Here, the results will be discussed and reviewed in light of the current literature. In addition, directions for future research will be given.

CHILDHOOD IDIOPHATIC NEPHROTIC SYNDROME

Children with INS are at high risk of relapsing disease [1]. Though steroid sensitive childhood INS is generally considered relatively benign in terms of renal function, the high burden and morbidity accompanying recurrent relapses should not be underestimated. Moreover in steroid resistant INS the prognosis is less favorable, and patients should be treated with alternative immunomodulatory agents.

For more than twenty years, many studies have investigated the right dose and duration of initial GC treatment in this syndrome and it has been suggested that prolonging initial GC treatment reduces the risk of relapses [2, 3]. The sometimes detrimental adverse effects occurring during these intensive regimes [4, 5], lead the International Study of Kidney Diseases in Children (ISKDC) to decide on a standard two-

month regime in 1966 [6]. Afterwards, no real consensus existed, since this regime was adopted but also adapted by many pediatric nephrologists.

Around twenty years later, prolonging initial treatment gained new interest when it was demonstrated that shortening initial treatment from two to one month increased the risk of relapses [2]. At the same time, results from a small Japanese study suggested that prolonged tapering of prednisolone following the ISKDC schedule successfully reduced the incidence of relapses [7]. Several other studies investigating the effect of prolonged, tapered treatment followed in the 1990s [8-11]. In 2000, the first meta-analysis summarizing the results of previous studies was published by Hodson and colleagues [3, 12], and several sequels appeared in the Cochrane Database of Systematic Reviews [3]. From the work by Hodson et al., it became clear that many of the existing studies showed methodological weaknesses, particularly those comparing three month treatment to longer treatment regimens [3]. In addition, no sufficient evidence ascribing the beneficial effect to either prolonged treatment duration or a higher cumulative dose of GCs existed. Considering the quality level of current evidence, it is hardly surprising that no worldwide consensus exists on the duration and dose of prednisolone treatment for childhood INS. Many different schedules are used across countries and even within countries.

For this reason in more than ten different Italian regions the NEFROKID group of pediatric nephrologists, has conducted a prospective multicenter Italian trial on the treatment of the first episode of INS (ClinicalTrials.gov Id.: NCT01386957), with the final goal to find a regimen that allows maximum savings of GCs limiting the frequency of relapses. This is particularly difficult in the beginning of the disease due to the lack of indicators that allow clinicians to distinguish patients who will respond to therapy from patients who will not or will be destined to relapse frequently.

Therefore, the cohort of patients explored in this thesis has been recruited from a clinical trial on children with a first episode of INS, presenting at Pediatric Units and Pediatric Nephrology Units in different Italian regions, that were all treated with the same protocol: prednisone at a dose of 60 mg/m^2/day for either 4 or 6 weeks, depending on whether time to remission was < or ≥ 10 days. Steroids were then tapered over a 16 weeks period as reported in the table I.

prednisone	40 mg/m^2	every 48 hours	4 weeks
	30 mg/m^2	every 48 hours	2 weeks
	22.5 mg/m^2	every 48 hours	2 weeks
	15 mg/m^2	every 48 hours	2 weeks
	7.5 mg/m^2	every 48 hours	2 weeks
	4.5 mg/m^2	every 48 hours	4 weeks

Table I: Scheme of steroids tapering after the first episode of INS

Each time a relapse occurs, induction therapy (60 mg/m^2 prednisone) is restarted until remission is achieved, then followed by the decalage reported in table I or in table II, depending on whether relapse has occurred after 3 months from suspension or during decalage/within 3 months from suspension.

prednisone	40 mg/m^2	every 48 hours	4 weeks
	30 mg/m^2	every 48 hours	2 weeks
	22.5 mg/m^2	every 48 hours	2 weeks
	15 mg/m^2	every 48 hours	12 months

Table II: Scheme of steroids tapering after a relapse during decalage/within 3 months from suspension occurs

A large cumulative amount of prednisone is therefore administered to patients with frequent relapses. Other immunosuppressive agents are considered when frequent relapses with or without steroid dependence occur, to limit the use of prednisone. Those immunosuppressive agents have limited efficacy and additional side effects [13].

The prospective multicenter Italian cohort enrolled 183 pediatric patients, ranging from 0 to 18 years, with the first episode of INS. The median age of all patients was 4.0 years, with around 66% of male patients and 34% of female. Patients showed a clinical spectrum ranging from no relapses at all to frequent relapses with steroid dependence. Only 21% of children remained free of relapses following treatment for the first episode. Forty-five percent of sensitive patients experience frequent relapses, over half of these being steroid dependent (Figure 1a and 2a).

To our laboratory of pharmacogenomics of the University of Trieste around 100 patients have been enrolled, from the larger cohort of NEFROKID, to investigate the possibility of predicting GCs response in advance. The subpopulation of patients was representative of the prospective cohort: patients ranged from 1 to 17 years with a median of 4,3. Around 61% were male and 39% female. Response to GCs was not achieved by 15% of patients (Figure 1b), from all sensitive patients only 26% never experienced relapses after the first episode; while 50% presented frequent relapses and half of them became dependent to steroid treatment (Figure 2b).

Therefore, improvement of current treatment strategies based on reliable, good quality trials are necessary.

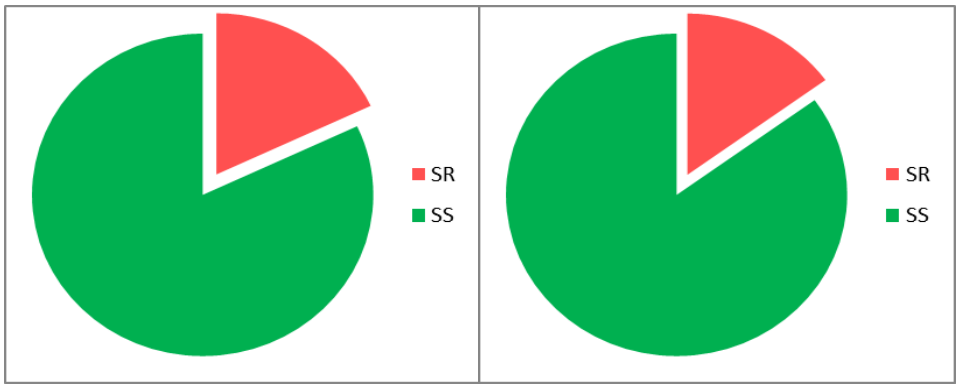

Figure 1: Therapeutic outcome in INS children from a) the prospective multicenter Italian cohort, and b) the cohort of patients enrolled for this thesis. SR steroid resistant; SS steroid sensitive.

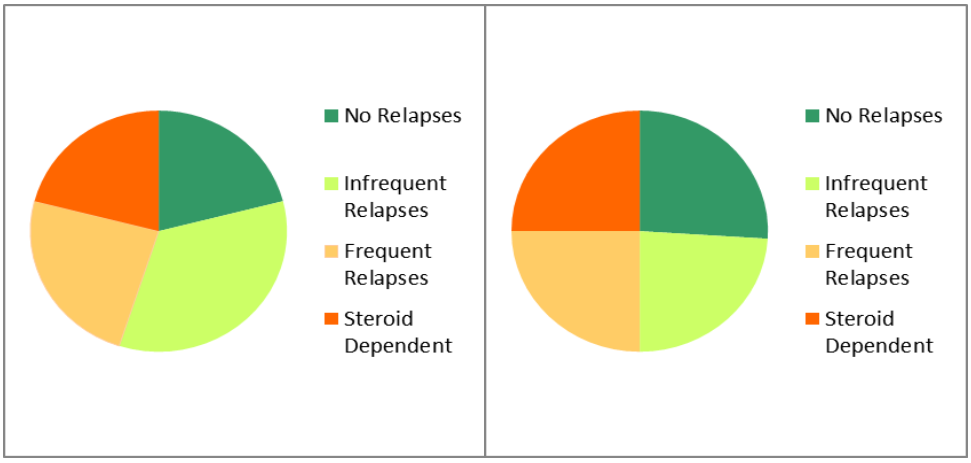

Figure 2: Therapeutic outcome in INS steroid sensitive children from a) the prospective multicenter Italian cohort, and b) the cohort of patients enrolled for this thesis.

GC SENSITIVITY: PREDICTION OF GC THERAPY EFFICACY

Genetic markers

Since patients' response to GCs is the best indicator for INS outcome, many studies have been made over the past years in order to understand 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 20-95% of variability in the efficacy of therapeutic agents [14]. Pharmacogenetics has therefore been considered to be promising in personalized medicine and many studies have investigated the possible role of this field of research. In **Chapter 2** we review the current knowledge about mechanisms of GC resistance in childhood INS and the role of pharmacogenetics. To induce apoptosis, GCs have to bind to the intracellular GC receptor (GR). One of the possible mechanisms for GC resistance is the presence of genetic mutations or polymorphisms in the GR gene. In the cytoplasm, the ligand-free GR exist in a multimeric complex associated with various chaperones and co-chaperons: variations on genes coding for those proteins could also lead to reduced response to drugs. Moreover

proteins involved in nuclear translocation, pro- and anti-inflammatory mediators in the downstream signaling pathway of GC-GR complex, the P-gp efflux transporter of GCs and the drug metabolizing enzyme CYP3A5 have been investigated to highlight their involvement in drug response.

However, despite the large amount of papers found in literature on the molecular mechanisms of GC anti-inflammatory action and the role of genetic polymorphisms in variable GC response in patients with INS, at present none of the potential pharmacogenetics markers can be considered strong enough to be used in clinical practice.

A pharmacodynamic assay to predict GC response: healthy subjects

In vitro tests based on the proliferation of mononuclear cells exposed to GCs have been correlated with clinical response in different diseases such as rheumatoid arthritis [15], systemic lupus erythematousus [16], bronchial asthma [17], ulcerative colitis [18, 19] and renal transplant rejection [20]. In **Chapter 3** a pharmacodynamic approach using healthy subjects' peripheral blood mononuclear cells, together with a pharmacogenetic approach with the evaluation of polymorphisms involved in GC response have been developed to evaluate individual response to GCs. We have shown that an increased GC *in vitro* sensitivity is evident in lymphocytes with the mutated *Bcl*I genotype. Our data confirm the present literature on the role of *Bcl*I polymorphism in the NR3C1 gene. *Bcl*I has already been associated with a better GCs response in pediatric patients with INS, when considered in haplotype with other two polymorphisms [21], and also in other disease such as IBD [22, 23].

The results presented in **Chapter 3**, on lymphocytes obtained from healthy donors, have some limitation: the main one is the limited number of healthy subjects enrolled, low for an association study with polymorphisms. Moreover, due to the high inter-individual variability of GC treatment and the high impact of inflammation on peripheral blood mononuclear cells of patients, further studies are needed to confirm these results also in patients affected by disease such as INS.

Genetic markers and pharmacodynamic assay in INS patients

In **Chapter 6** we addressed this issue in peripheral mononuclear cells of patients at diagnosis of INS. *In vitro* PBMC steroid sensitivity test developed in chapter 3, has been investigated in a small number of patients in relationship with different polymorphism found to be involved in GC mechanisms of action and also reported in Chapter 2. In those preliminary results an association between the *Bcl*I polymorphism in the NR3C1 gene with an increased GC resistance was observed *in vitro*; while none of the other polymorphisms investigated were linked to a favorable or unfavorable *in vitro* response to steroid. So far, only few studies have evaluated the role of *Bcl*I polymorphism on the response to GCs in patients affected

by INS and, as we reported in Chapter 2, outcome are not conclusive and often in contradiction. *Bcl*I studied in haplotype with other two polymorphisms [21] has been associated with a higher steroid sensitivity, determined by time to proteinuria resolution. However Cho HY et al. [24] as well as Teeninga N et al. [25] could not find an involvement of this polymorphism in GC response.

Our results are also not conclusive and still to be confirmed in a larger cohort. This will be discussed in the future perspective part of this chapter.

A pharmacodynamic assay to predict GC response: INS patients

The validated assay described in Chapter 3 enabled us to study the possible association between the *in vitro* response to methyl-prednisolone in PBMC's of pediatric patients with INS and their clinical response to steroid in **Chapter 4**.

To date different studies have been performed to assess *in vitro* steroid sensitivity in INS children investigating dexamethasone-mediated inhibition of concanavalin-A-stimulated peripheral blood mononuclear cell proliferation [26], or determining the steroid effect by means of the antibody-dependent cellular cytotoxicity method [27]. However all these studies included very few patients, and to our knowledge, none of these assays yielded reliable information on the effectiveness of prednisolone treatment. Researchers still need to find an assay that will be reproducible and effective in the prediction of sensitivity to steroid treatment in INS patients. In our work, performing the *in vitro* sensitivity test of PBMCs to methylprednisolone developed in Chapter 3, we have demonstrated an increased *in vitro* response to steroid treatment in dependent patients after four weeks of therapy and found a cut-off value that could be used to clinically identify patients at increased risk of steroid dependence: these children could therefore benefit from slower steroid tapering or treatment with other immunosuppressive drugs. A correlation between relapses and the suppression of hypothalamic–pituitary–adrenal (HPA) axis has already been demonstrated [28, 29]. Moreover it is well known that relapses in INS are often triggered by infection [30], that induces the release of cytokines, in particular interleukin (IL)2, 4 and 13 [31], that are in part responsible for proteinuria. A possible explanation for the increased *in vitro* response to steroid treatment in dependent patients could be that in patients who are extremely sensitive to these agents, and hence have an increased HPA suppression, the reduced endogenous steroid production, when steroid therapy is discontinued, could not be enough to reduce cytokine release; this would result in INS relapse and steroid dependency.

In addition this test could be useful for identifying those patients who are already resistant at diagnosis, and could thus be considered for alternative treatments, avoiding steroid administration and the relative side effects as previously demonstrated in other diseases [15-17, 19, 20], however these results still need to be confirmed in a larger group of resistant patients.

The results of this *in vitro* test could be obtained within 72 hours, this very short time would facilitate rapid decisions regarding alternative treatment regimens and may lead to safer and more effective treatment for children who will not respond or will be dependent to steroid treatment.

Cytokine levels to predict GC response in INS patients

There is strong evidence that proteinuria, which is the hallmark of INS condition, is mediated by cytokines [32]. Relapses are often triggered by viral infections, which possibly result in the release of cytokines, causing immunoregulatory imbalances. Evidence for a possible cytokine-mediated role in the pathogenesis of the INS includes clinical response of the nephrotic state to immunomodulating drugs which affect cytokine production, such as steroids. Several studies have demonstrated increased *in vitro* mitogen-stimulated production of cytokine [33, 34]. In **Chapter 5** we investigated whether cytokine plasma levels would be a good indicator of GC response: measurements of cytokine levels and of the soluble markers of immune activation that are products of cytokine activities has been used as diagnostic and prognostic indicator in many diseases [35, 36], however changes in various plasma cytokine profiles prior to and after steroid treatment in INS patients have not been examined. The results in Chapter 5 indicate that, within the 48 cytokines analyzed, MIF was the best predictor of steroid response before treatment in childhood INS: patients non-responsive to GCs had higher MIF plasma levels compared with steroid sensitive ones. Our results are in accordance with results by Wang et al. in patients with systemic lupus erythematosus [37] who showed that MIF serum expression was correlated with steroid resistance.

This new finding could be of particular relevance to children receiving steroids, as it encourages new studies aimed at drug prediction with non-invasive methods. Though confirmation of our results in a larger cohort is needed, these promising results justify further investigation on the use of cytokine measurement for prediction of GC response.

Cytokine mRNA expression to predict GC response

Cytokine mRNA expression was also considered. Therefore, in **Chapter 7** we have used the validated pharmacodynamic assay described in Chapter 3 to study the *in vitro* response to methyl-prednisolone in PBMC's of healthy subjects, and we have developed a means of measuring the action of methyl-prednisolone based on its effect on the kinetics of mRNA transcription by the cells. We performed quantitative real-time PCR assays for a panel of mRNA transcripts, the levels of which are known to be affected downstream by GC treatment. This panel consists of pro-inflammatory mediators, anti-inflammatory mediators and intracellular signaling molecules.

To date, many studies have investigated the role of mRNA expression of different protein in relation to steroid responsiveness in INS patients [38-41], but research aimed at uncovering predictors of clinical outcome are still not conclusive. In the preliminary study described in Chapter 7, we showed a

different pattern of mRNA expression in healthy subject with different *in vitro* sensitivity. The confirmation of our results is needed in higher number of subjects, and need to be translated to a cohort of patients to be correlated also to clinical response.

Role of noncoding RNA GAS5 and NR3C1 gene in GC resistance in childhood INS

As already mentioned, GCs exert their effects on target cells primarily through the regulation of gene expression after activation in the cytoplasm of the glucocorticoid receptor (GR), which acts as a transcription factor [42, 43]. The biological and molecular mechanisms involved in GR activity have been studied in details, but to date GR expression pattern does not represent a reliable predictive tool to explain the complex mechanism of GC resistance observed in clinical practice. The GR is encoded by NR3C1 gene and it has been shown that its expression is regulated by the receptor itself after prolonged GC treatment: Okret et al. observed a negative feedback mechanism enabling cells to attenuate the continuous signal evoked by chronic exposure to the ligand, resulting in GR downregulation, through the binding of the activated receptor to intragenic sequences called GRE-like elements, contained in the GR gene [44-46]. These observations have been subsequently confirmed by other authors [45, 46]. Recently, it has been demonstrated that growth arrest–specific transcript 5 (GAS5), a long noncoding RNA (lncRNA), interacts with the activated GR, preventing its association with GREs, and consequently suppressing its transcriptional activity [47]. This interaction is physiologically relevant as it occurs at concentrations of the GR ligand dexamethasone at 10^{-10} M, lower than that of physiological endogenous glucocorticoid [47].

The aim of the investigation described in **Chapter 8**, was to evaluate the possible role of GAS5 and NR3C1 gene expression as potential markers to predict clinical outcome in INS patients. The preliminary study indicate that, in steroid resistant patients, abnormal levels of GAS5 may alter GC effectiveness probably interfering with the mechanism of GR autoregulation: high levels of GAS5 expression, through the interaction with the DNA binding domain of the activated GR, could results in the suppression of GC transcriptional activity, reducing their effectiveness. Moreover we have shown that low expression of the GC receptor in INS patients could represent a poor prognostic sign in childhood INS. These preliminary results are supported by those described by Lucafò et al. [48] who recently investigated GAS5 and NR3C1 levels in healthy subjects correlating results with *in vitro* steroid sensitivity.

The altered expression of endogenous GAS5 is a GC-mediated event, leading to a different regulation of the NR3C1 gene. Our findings provide the basis for further studies, identifying a lncRNA as a potential marker involved in GC pathway and thus providing a new view upon its implication in the phenomenon of drug resistance: if these results will be confirmed in a larger cohort of patients with INS, GAS5 could be considered as a candidate marker of GC resistance to optimize the therapy and in particular to avoid an ineffective and potentially toxic treatment.

FUTURE PERSPECTIVES

In the last decades several methods to measure GC sensitivity have been developed [18, 49-56]. Nevertheless, most assays are labor-intensive and are so far used in the experimental setting only. Another major drawback of these assays is the relative poor correlation with clinical outcome parameters, which hamper their introduction in clinical practice. Thus, clinical applicability of an assay guiding individual GC treatment requires

1) accurate prediction of an individual's GC sensitivity,

2) a low degree of labor-intensity and

3) cost-effectiveness.

To date, a number of demographic and/or clinical markers have been examined in correlation with GC response also in INS, but results have not been consistently replicated. Genetic and epigenetic markers are likely to complement clinical and demographic predictors: phenotypes resulting from genetic changes, such as single nucleotide polymorphisms, and epigenetic modifications, such as DNA methylation, can markedly influence drug mechanism of action and alter efficacy profiles.

Exome sequencing analyses is a cost-effective approach and an innovative technique. To date, only few works have performed exome sequencing in small cohort of INS patients with non-conclusive results; moreover most studies have investigated the role of genetic variance in the development of the disease or have studied only disease-causing genes in the subpopulation of steroid resistant patients [57-59]. Therefore, sequencing of candidate genes known to be involved in GC mechanism of action such as the GC receptor (NR3C1), proteins of the heterocomplex (e.g. FKPB5, FKBP4, HSP90AA1, HSPA4, ST13), proteins involved in nuclear translocation (e.g. IPO13) and efflux transporters of GCs (e.g. ABCB1), in a large cohort of pediatric patients with INS, could provide important information useful to clinicians.

The understanding of the complex gene regulation mediated by GCs may shed light on the causes of the variable responses to these hormones. There is a lot of interest in identifying the role of miRNA in the modulation of drug response, but studies about this topic are still very limited, and the possible correlation between miRNAs expression and variability on GC response in INS patients have been examined only in small Asian cohort [60, 61]. Studies about miRNAs and pharmacogenomics may therefore represent a new and promising investigation topic that could increase the understanding of the pharmacology of steroids in pediatric INS.

Epigenomics is a rapidly growing field, and to date is known that changes in the DNA methylation profiles could lead to differences in gene expression patterns and thereby influence GC response in INS. Until now, only one work from Kobayashi et al. [62], have investigated the role of DNA methylation changes

between relapse and remission of minimal change nephrotic syndrome in a very small cohort of monocytes and T helper cells isolated from Japanese patients. However no data are available on the role of DNA methylation changes between steroid resistant, steroid dependent and steroid sensitive INS patients: studies in this field could provide new biomarker to predict clinical response in advance.

CONCLUDING REMARKS

The short-term advantages of prednisolone for childhood NS are obvious: remission is seen within days or weeks in most cases and, in two out of ten patients, no further treatment is needed after the first prednisolone course. Understandably, its position as first line treatment has been sturdy and virtually unquestioned until now.

Though the benefits of prednisolone with regard to morbidity and mortality in childhood INS were clearly recognized shortly after the discovery of this drug, idle progress has been made since. Though new agents have been introduced for the treatment of frequent relapses and steroid dependence in the last decades, it is highly unsatisfactory that so many patients develop frequent relapses. Much work needs to be done before current GC therapy can be actually replaced by better treatment strategies.

The studies in this thesis have described and set up several methods to predict GC response in children with INS. We believe those results, being based on strong methodology, could really cause a turnaround in current thinking about the treatment explaining clinical variability in childhood INS: the promise of non-invasive methods to predict GC response, could be highly relevant also to other pediatric populations. Altogether, this thesis has brought new information to the field of GC treatment in pediatric nephrology.

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Figure and text from chapter 2: "**Glucocorticoid pharmacogenetics in pediatric idiopathic nephrotic syndrome**"; Cuzzoni E, De Iudicibus S, Franca R, Stocco G, Lucafò M, Pelin M, Favretto D, Pasini A, Montini G, Decorti G.; *Pharmacogenomics. 2015 Sep;16(14):1631-48*; were reproduced with permission from Pharmacogenomics as agreed by Future Medicine Ltd.

Figure and text from chapter 3: "**Association between BclI polymorphism in the NR3C1 gene and in vitro individual variations in lymphocyte responses to methylprednisolone**"; Cuzzoni E, De Iudicibus S, Bartoli F., Ventura A., Decorti G.; *British Journal of Clinical Pharmacology. 2012 Apr;73(4):651-655*; were reproduced with permission from British Journal of Clinical Pharmacology as agreed by John Wiley and Sons.