In vitro sensitivity to methyl-prednisolone is associated with clinical response in pediatric idiopathic nephrotic syndrome.

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Idiopathic nephrotic syndrome, *in vitro* sensitivity, steroid response, peripheral blood mononuclear cells

Running title

***In vitro* and clinical response to methyl-prednisolone**

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-^{3}H] 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 drug concentration inducing 50% of inhibition (IC_{50}; OR=0.48, 95%CI=0.24–0.85; p-value=0.0094) and maximum inhibition at the highest drug concentration (I_{max}; OR=1.13, 95%CI=1.02-1.31; p-value=0.017). IC_{50} > 4.4nM and I_{max} < 92% at T4 were good predictors 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 the *in vitro* steroid-mediated inhibition of mitogen-stimulated PBMCs has been used to investigate the association 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 ten 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. Differences between the two groups of patients were analyzed: the group of patients enrolled in the pharmacodynamic study was representative of the larger group (Supplementary Table). 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).

In vitro sensitivity and clinical response to steroids

The *in vitro* lymphocyte sensitivity to methyl-prednisolone was evaluated and dose response curves obtained are shown in Figure 1. *In vitro* sensitivity was expressed as IC_{50} and I_{max} that displayed a wide interindividual variation both at 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%). A significant correlation was found between *in vitro* parameters (IC_{50} and I_{max}) at T0 and T4 (Spearman test IC_{50} T0 vs I_{max} T0 $p=2.9x10^{-6}$; Spearman test IC_{50} T4 vs I_{max} T4 $p=2.2x10^{-5}$; Figure 2). No correlation was found between IC_{50} or I_{max} values and gender, time to remission (39 patients within ten days and 35 after ten days) or total dose utilized. On the contrary, a 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); univariate logistic regression analysis, considering steroid sensitive (SS: NR-IR and FR-SD) patients in comparison with steroid resistant (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; Supplementary Figure 1)

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

At T0, a trend was observed comparing SR versus SS patients: 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; Supplementary Figure 2). However, this trend was not confirmed considering IC_{50} values.

Moreover, univariate multinomial logistic regression showed a significant association between clinical and *in vitro* response at T4 comparing all groups (p-value IC_{50} = 0.015; I_{max} = 0.031; Figure 3). The most significant result was found at T4 comparing FR-SD patients vs NR-IR: FR-SD showed lower log-transformed IC_{50} (OR = 0.48, 95% CI = 0.24 – 0.85; p-value = 0.0094; Figure 3). A similar pattern was evident for *in vitro* sensitivity represented as log-transformed I_{max}

values (OR = 1.13, 95% CI = 1.02 - 1.31; p-value = 0.017; Figure 3). ROC curves were constructed to assign optimal cut-off values for *in vitro* parameters significantly associated with clinical response. For IC_{50} at T4 an optimal cut-off of 4.4 nM could be defined. Area under the ROC curves was 74.1% (Figure 4). The test had a sensitivity of 66.6% and a specificity of 77.7% (PPV = 60.9%; NPV = 81.9%). Logistic regression confirmed a lower proportion of NR-IR patients among those who reached the optimal cut-off point for IC_{50} (OR = 0.17, 95% CI = 0.04 – 0.62; p-value = 0.009) in comparison with those who did not. A similar result was found also for I_{max} at T4: a unique optimal cut-off of 92.0% could be defined (AUC = 65.4%; sensitivity = 88.9%, specificity = 44%; PPV = 51.6%; NPV = 85.7%) (Figure 4). 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.

Differences between clinical groups considering the two time points pairwise were also analysed, however no significant correlation was found (Supplementary Figure 3).

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.

The main result of this study is the increased *in vitro* response to steroid treatment in FR-SD patients after 4 weeks of therapy, both in terms of IC_{50} and I_{max} and optimal cut-off values were identified in this population. These results could be clinically useful for understanding which subjects are at greater risk of becoming steroid dependent or relapse frequently, defining the severity of the steroid dependence during the initial treatment period and evaluating the need for

a second immunosuppressive drug. A further outcome of this study was the lower *in vitro* sensitivity of SR patients, evaluated as I_{max} at disease onset.

Literature data (17, 18) show that *in vitro* PBMC sensitivity to dexamethasone could be considered a predictor of response to treatment in various diseases (13-17, 19). Carlotti et al. also used this assay in INS patients, however, due to the small number of patients enrolled, no definitive data were obtained (20). 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 (21); moreover literature data showed that this test has a low inter- and intra-assay variation (18) allowing us to consider this assay useful for the study and prospectively for routine application in the clinical setting. A considerable interindividual variability for *in vitro* steroid sensitivity was evident in our population as already reported in various diseases and in healthy subjects (18,22). The increased *in vitro* response at T4 observed in FR-SD patients was quite unexpected; a correlation between relapses and hypothalamic–pituitary–adrenal (HPA) axis suppression has been already demonstrated (23,24). Relapses in INS are often triggered by infection (25). Viral infections induce the release of cytokines, in particular interleukin (IL)2, 4 and 13 (26), 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.

Apart from rare cases of severe steroid dependence, SD subjects are normally diagnosed only after several weeks of treatment. Indeed, glucocorticoid treatment of INS in this study involves 4 or 6 weeks of initial therapy with 60 mg/m^2/day, followed by a 16-weeks tapering. Moreover, other frequently used steroid regimens in the treatment of a first episode of INS (27, 28) also involve very long treatment (6 weeks of full-dose 60 mg/m^2 treatment + 6 weeks of alternate day

40 mg/m^2 treatment). Interestingly, the use of the described *in vitro* test could help in defining the severity of the steroid dependence after only 4 weeks of therapy, allowing to evaluate the need for a second immunosuppressive drug. For SR patients, reduced *in vitro* response at T0 could be used to shorten the duration of the steroid treatment necessary for the definition of steroid resistance (at least 8 weeks according the KDIGO guidelines) (3), thus resulting in the possibility to perform biopsy, genetic testing and introduce other immunosuppressive drugs earlier, as previously demonstrated in other diseases (13-17).

Among the prognostic indicators of clinical outcome, age at onset of the disease has been proposed by various authors; steroid resistance is seen more often in adolescents (29-32), 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. On the contrary, 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, 33).

A limitation of this study is the number of patients enrolled; however, pediatric INS is a relatively rare disease and this is a prospective study on a special population (children) that could provide important and innovative insights. Moreover, another limitation was the lack of a clear cut-off value for the *in vitro* sensitivity test for distinguish SR patients at T0: this is probably due to low frequency of SR patients, and results from a larger cohort are needed.

In conclusion, the results of the in vitro test, associated with other clinical and laboratory parameters, if confirmed, could help clinicians in the choice of more personalised steroid treatments and represent an incentive for the future exploration of steroid sensitivity in pediatric INS patients.

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/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 ten 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 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.

Sample size has been determined as described in the supplementary material.

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-^{3}H] thymidine (PerkinElmer, Milan, Italy) as previously reported (21). 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 (2×10^5 cells/well) in the presence of methyl-prednisolone (range from 0.05 nM to 54 μM) (34). After 50 hours of incubation, cells were pulsed with [methyl-^{3}H] thymidine (final concentration of 2.5 μCi/ml) and incubation was continued for an additional 22 hours. The radioactivity of the samples was

217 determined by a Liquid Scintillation Analyzer (Wallac 1450 Microbeta liquid scintillation counter,
218 PerkinElmer, Milan, Italy). Raw count per minute (cpm) data were converted and normalized to
219 percent of maximal survival for each experimental condition (cpm methyl-prednisolone/cpm
220 control*100). Non linear regression of dose–response data was performed using Graph-Pad
221 Prism version 4.00 for computing IC_{50}, the methyl-prednisolone concentration required to reduce
222 proliferation to 50%. I_{max} was also calculated and defined, according to previous studies on
223 glucocorticoids (18), as the maximum percentage inhibition of thymidine incorporation achieved
224 at the highest concentration of methyl-prednisolone (54 μM) tested.
225 I_{max} and IC_{50} data at T0 and T4 were compared between subjects with different clinical
226 responses to treatment (SR vs SS subjects) or with a different clinical outcome of the disease
227 (NR, IR, FR and SD subjects). Moreover, gender, age at disease onset and time to remission
228 were evaluated and compared with the pharmacodynamic data.
229
230 Statistical analysis
231 For continuous variables, normality of distribution was assessed by means of visual examination
232 of the data plot and a Shapiro test. Logarithmic transformation was applied to normalize
233 distribution and/or reduce variance. The correlation between continuous variables was
234 assessed using the appropriate parametric (Pearson) and non parametric (Spearman) tests.
235 Any possible association between methyl-prednisolone IC_{50}, I_{max} and clinical variables
236 (response, time to remission, age at the onset of disease and sex) was investigated using
237 univariate logistic regression models. Receiver operating characteristic (ROC) curves were
238 constructed for the significant *in vitro* tests to determine the optimal cut-off value for
239 discriminating between patients' clinical response to steroid treatment. Sensitivity, specificity,
240 and the positive and negative predictive values (PPV, NPV, respectively) of the cut-off point
241 were analyzed. Logistic regression, considering the proportion of patients achieving the
242 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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AUTHOR CONTRIBUTIONS

C.E., S.D.I., G.S., A.P., G.M. and G.D. wrote the manuscript; C.E., S.D.I., A.P., G.M. and G.D designed the research; C.E., S.D.I., D.F., and M.P. performed the research; C.E., S.D.I., G.S., and G.D. analyzed the data; G.M., L.G., E.M., A.P., and G.M. contributed to the enrolment of patients.

CONFLICT OF INTEREST

The authors declare no competing financial interests.

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FIGURES/TABLE LEGENDS

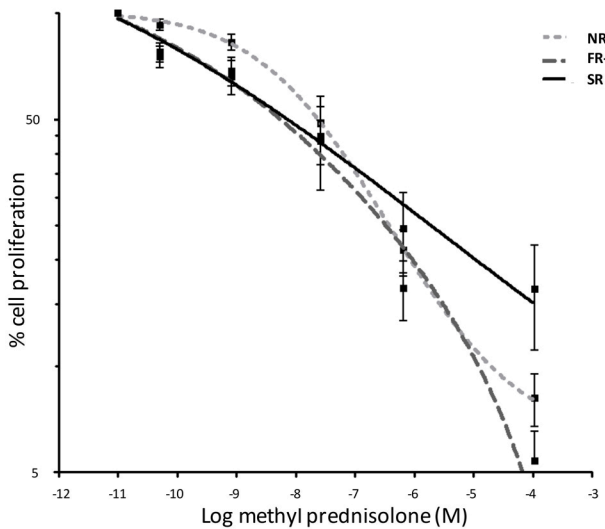
Figure 1: I*n vitro* dose-response curves for the various subpopulations : a) SR, FR-SD and NR-IR at T0; b) SR, FR-SD and NR-IR at T4. *In vitro* response is plotted in Log10 scale.

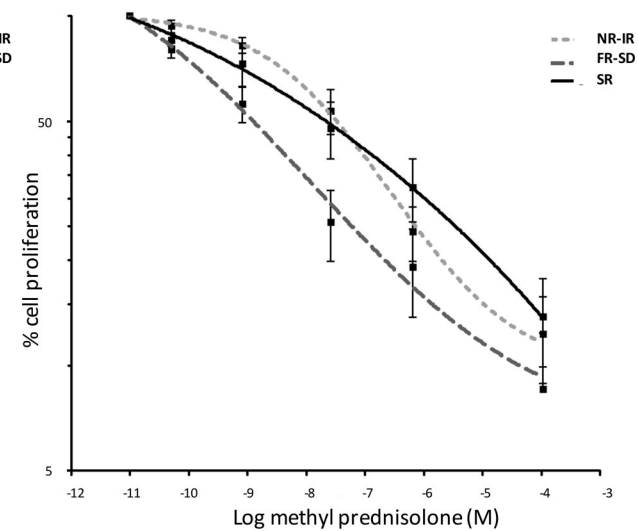
Figure 2. Scatter plot displaying drug sensitivity (IC_{50} and I_{max}) at Time 0 (T0) and T4. *In vitro* response is plotted in Log10 scale. The correlation between continuous variables was assessed using Spearman tests. A significant correlation was found between in vitro parameters (IC_{50} and I_{max}) at T0 and T4 (Spearman test IC_{50} T0 and I_{max} T0 p=2.9x10-6; Spearman test IC_{50} T4 and I_{max} T4 p=2.2x10-5.

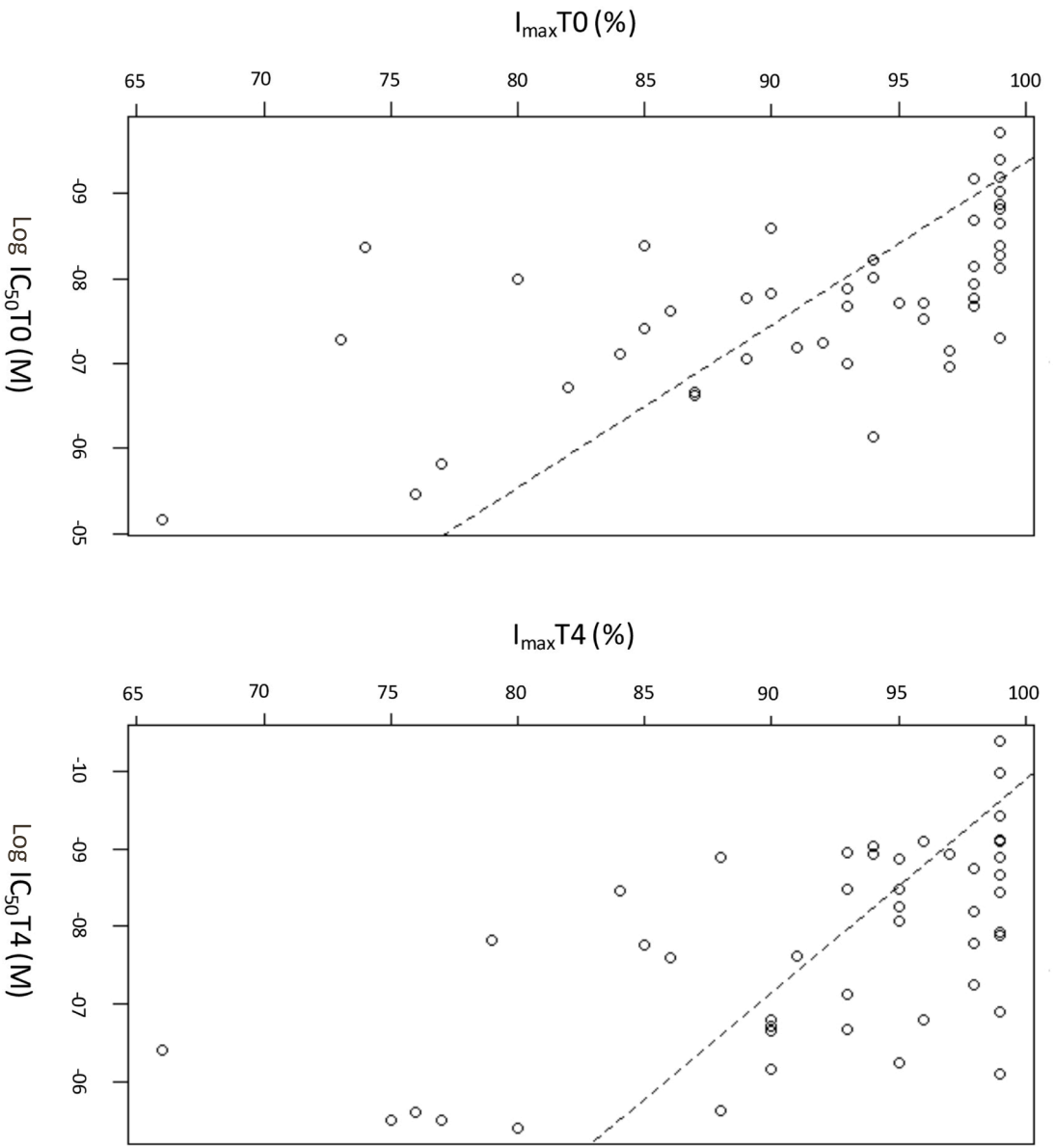
Figure 3: Box plot comparing *in vitro* and clinical response at T4 between the three groups of patients. *In vitro* response is plotted in Log10 scale. The bold horizontal line represents the distribution mean. Statistical significance was assessed by carrying out logistic regression analysis. A significant association was found for log-transformed IC_{50} values (p-value = 0.015) and for log-transformed I_{max} (p-value = 0.031). A significant association was also found for log-transformed IC_{50} values comparing FR-SD patients and NR-IR patients (p-value = 0.0094) and for log-transformed I_{max} values (p-value = 0.017)

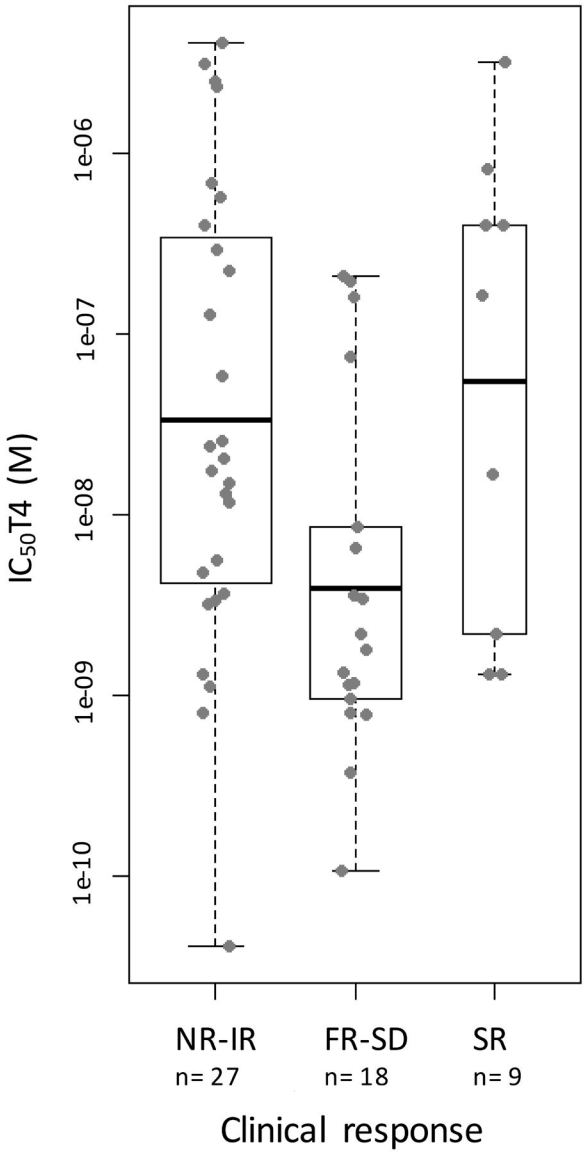
Figure 4: Areas under the ROC curves of IC_{50} (left panel) and I_{max} (right panel) among 27 NR-IR and 18 FR-SD patients after 4 weeks of treatment with prednisone for a first episode of INS; ROC, receiver operating characteristic. Optimal cut-off value was for IC_{50} 4.4 nM (sensitivity 66.6%, specificity 77.7%, positive predicting value (PPV) and negative predicting value (NPV) 60.9% and 81.9% respectively) and for I_{max} 92.0% (sensitivity 89%, specificity 44%, PPV 51.6% and NPV 85.7%).

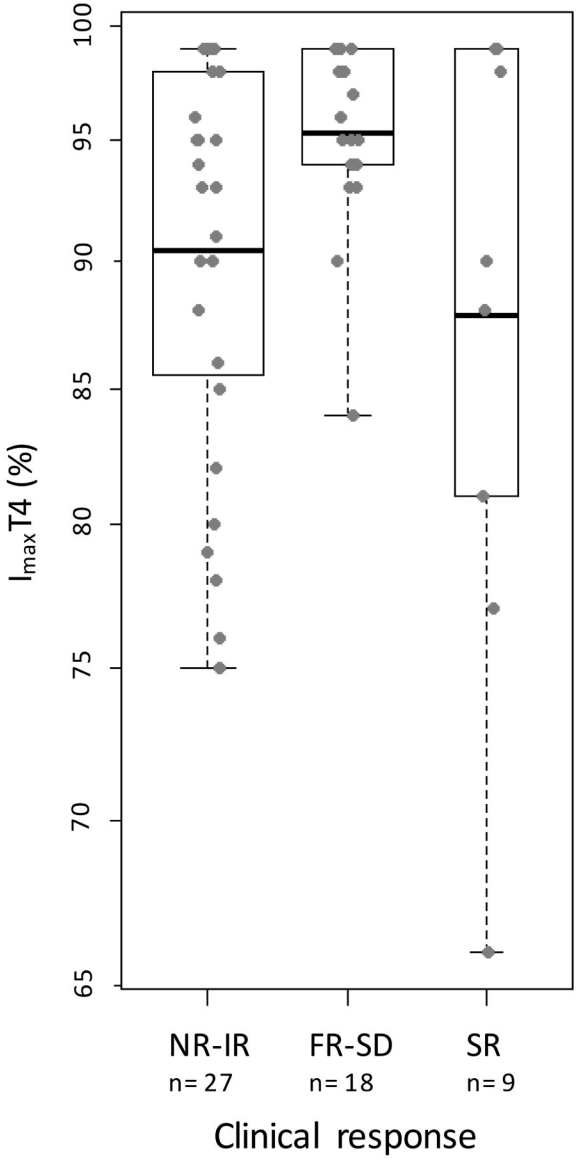
Table 1: Definition of clinical response used in the text.

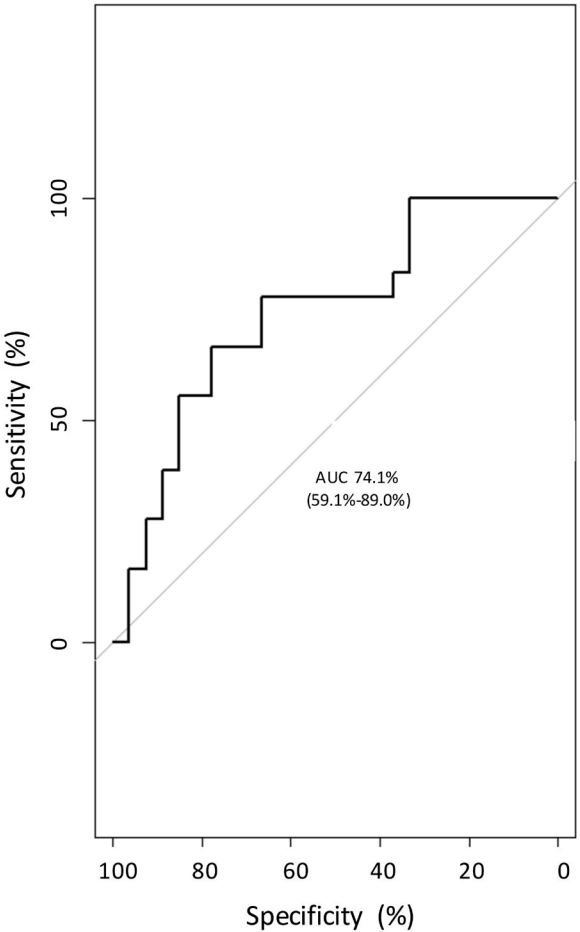

NR-IR
FR-SD
SR
50
5
% cell proliferation
-12
-11
-10
-9
-8
-7
-6
-5
-4
-3
Log methyl prednisolone (M)

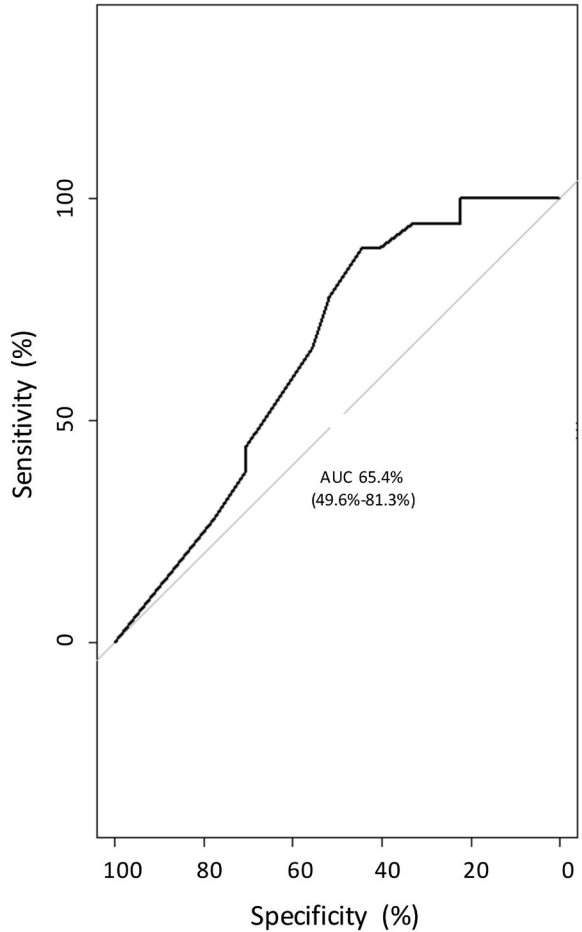

NR-IR
FR-SD
SR
50
5
% cell proliferation
-12
-11
-10
-9
-8
-7
-6
-5
-4
-3
Log methyl prednisolone (M)


I_{max}T0 (%)
65
70
75
80
85
90
95
100
Log IC_{50}T0 (M)
-05
-06
-07
-08
-09
I_{max}T4 (%)
65
70
75
80
85
90
95
100
Log IC_{50}T4 (M)
-06
-07
-08
-09
-10


IC50T4 (M)
1e-06
1e-07
1e-08
1e-09
1e-10
NR-IR
n= 27
FR-SD
n= 18
SR
n= 9
Clinical response


Imax T4 (%)
100
95
90
85
80
75
70
65
NR-IR
n= 27
FR-SD
n= 18
SR
n= 9
Clinical response


Sensitivity (%)
100
50
0
AUC 74.1%
(59.1%-89.0%)
100
80
60
40
20
0
Specificity (%)

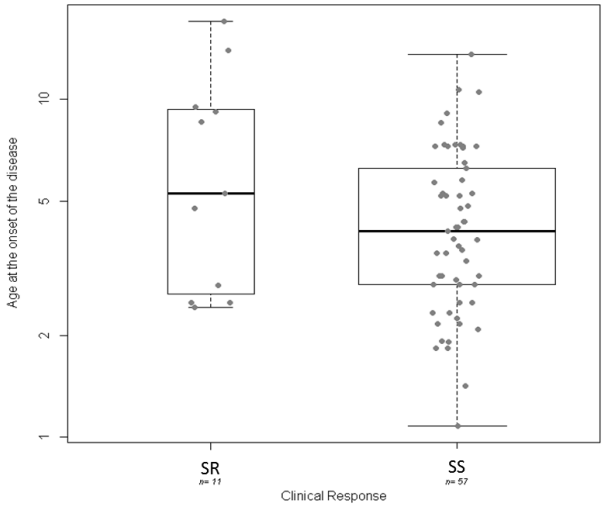

Sensitivity (%)
100
50
0
AUC 65.4%
(49.6%-81.3%)
100
80
60
40
20
0
Specificity (%)

Supplementary Table

	N° patients	M (%)	F (%)	M:F ratio	median age (range)	NR-IR (%)	CD-FR (%)	CR (%)
All patients	184	120 (65%)	64 (35%)	1,87	3,9 (0,6-17)	90 (49%)	73 (40%)	21 (11%)
Patients with in vitro sensitivity test available	74	47 (64%)	27 (36%)	1,74	4,3 (1,0-17)	36 (48)%	26 (35%)	12 (16%)

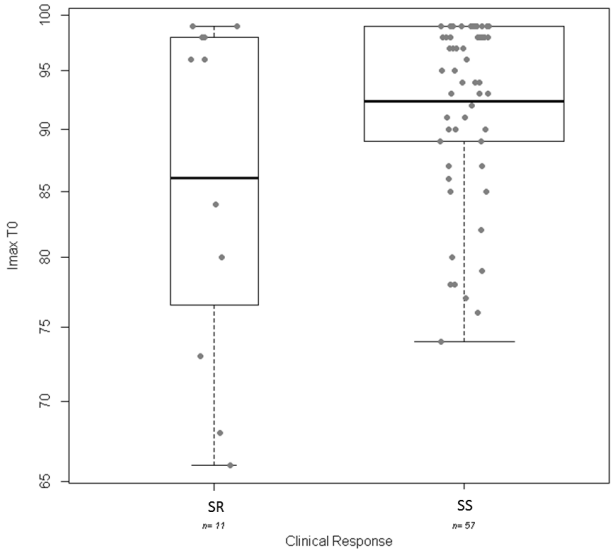
Supplementary Table: Differences between the whole group of patients enrolled and the patients enrolled in the pharmacodynamics study.

Supplementary Figure 1



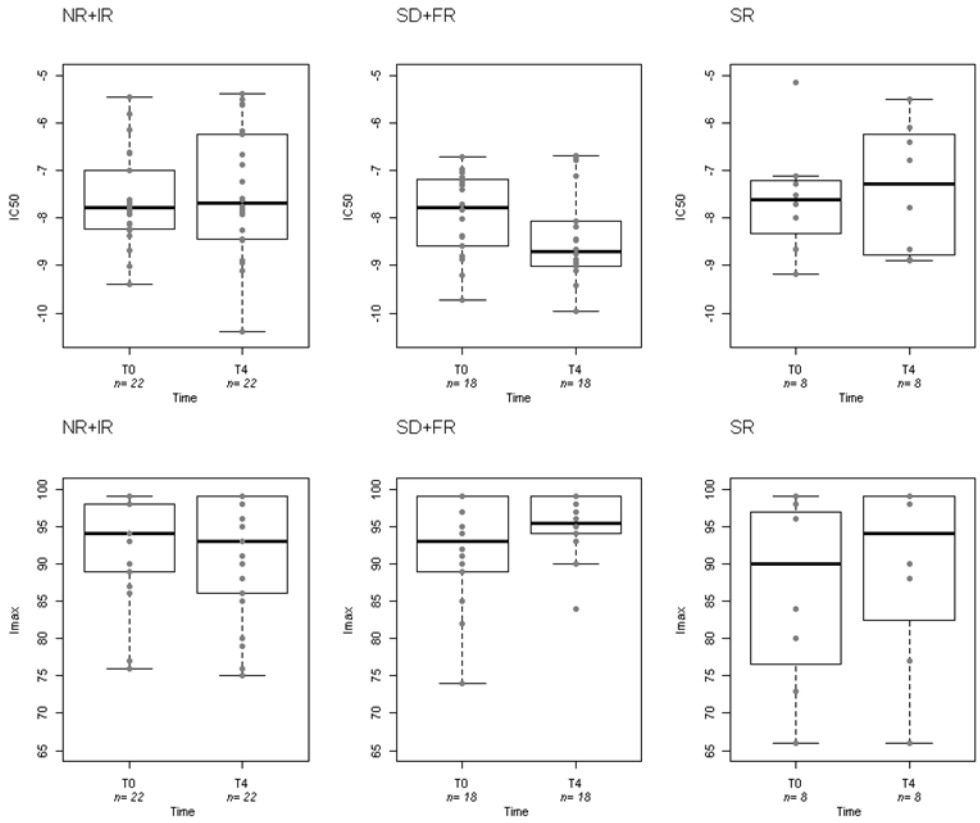
Supplementary Figure 1: Box plot comparing age at disease onset and clinical response. Age at onset of disease is plotted in Log10 scale. Statistical significance was assessed by carrying out logistic regression analysis. A significant association was found (p-value = 0.028).

Supplementary Figure 2



Supplementary Figure 2: 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).

Supplementary Figure 3



Supplementary figure 3: Box plot comparing differences between clinical groups (NR-IR, SD-FR and SR) considering the changes between T0 and T4. Any significant differences were found (IC50 NR-IR T0 vs T4 Wilcoxon test p-value = 0.61, Imax NR-IR T0 vs T4 Wilcoxon test p-value = 0.33; IC50 SD-FR T0 vs T4 Wilcoxon test p-value = 0.08, Imax SD-FR T0 vs T4 Wilcoxon test p-value = 0.063; IC50 SR T0 vs T4 Wilcoxon test p-value = 0.64, Imax SR T0 vs T4 Wilcoxon test p-value = 0.45)

Sample size determination

Sample size was determined by average enrollment of the NEFROKID consortium (60-80 patient/year). Considering that due to technical issues 50% of patients enrolled would be available for the pharmacodynamics analysis, with a study length of 2 years, around 80 samples would be analyzed. Moreover, considering a frequency of glucocorticoid resistant patients of 10-15%, this study, therefore enrolling 10 resistant and 70 sensitive patients, would be sufficiently powered (p = 0.05, power = 80%), to detect a difference of in vitro sensitivity parameters between sensitive and resistant patients of strong magnitude (ratio between difference of means and pooled standard deviation = 0.96). Considering a frequency of glucocorticoid dependence (SD+FR) of 50% of sensitive patients, this study, enrolling 35 SD+FR and 35 NR+IR patients, would be sufficiently powered (p = 0.05, power = 80%) to detect a difference of in vitro sensitivity parameters between SD+FR and NR+IR patients of at least medium magnitude (ratio between difference of means and pooled standard deviation = 0.67).