

ARTICLE

Hypomethylation of NLRP3 gene promoter discriminates glucocorticoid-resistant from glucocorticoid-sensitive idiopathic nephrotic syndrome patients

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Abstract

To assess whether NLRP3 gene promoter methylation was able to discriminate glucocorticoid (GC)-resistant from GC-sensitive idiopathic nephrotic syndrome (INS), patients with minimal change disease (MCD) or focal segmental glomerulosclerosis (FSGS), we measured the methylation level of NLRP3 promoter in DNA from peripheral blood cells of 10 adult patients with GC-resistant FSGS already in hemodialysis and 18 patients with GC-sensitive INS (13 MCD/5 FSGS) and in 21 pediatric patients with INS with MCD/FSGS before starting any treatment. Association of NLRP3 inflammasome with GC resistance was recapitulated in vitro in monocytic cell lines (THP-1 and U937). In both adults and pediatric patients, NLRP3 promoter methylation was significantly reduced in GC-resistant compared with GC-sensitive patients. Indeed, NLRP3 methylation distinguished GC-resistant and GC-sensitive patients (area under the receiver operating characteristic curve [AUROC] 86.7% in adults, $p = 0.00019$, and 73.5% in children, $p = 0.00097$). NLRP3 knock-down augmented sensitivity to GCs in THP-1 cells, whereas NLRP3 inflammasome activation lowered GC receptor concentration, increasing GC resistance in U937 cells. Our results uncovered a new biological mechanism by which patients with INS may acquire GC resistance, that could be used in future as a novel noninvasive diagnostic tool.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

Approximately 80% of patients with idiopathic nephrotic syndrome (INS) respond to glucocorticoids, with the remaining 20% being steroid-resistant.

WHAT QUESTION DID THIS STUDY ADDRESS?

Whether NLRP3 gene promoter methylation was able to discriminate glucocorticoid-resistant from glucocorticoid (GC)-sensitive INS.

Marianna Lucafò and Simona Granata authors contributed equally to the study.

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WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

In both adults and children, NLRP3 promoter methylation was significantly reduced in leukocytes of patients with GC-resistant compared with GC-sensitive INS. NLRP3 inflammasome activation lowered GC receptor concentration and augmented GC resistance, whereas NLRP3 knockdown increased sensitivity to GCs in cell lines representative of monocytes (U937 and THP1).

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

Our findings uncovered a new biological mechanism whereby patients with INS may develop resistance to GCs that could be used in the future as a novel noninvasive diagnostic tool.

INTRODUCTION

The idiopathic nephrotic syndrome (INS) is a well-defined chronic clinical condition characterized by hypoalbuminemia, edema, and hyperlipidemia, that affects both pediatric and adult patients with an incidence rate of 1.5–16.9 per 100,000 each year.^{1,2} Several primary glomerular disorders can be associated with this condition. However, minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS) are the most frequent, especially in children.³

MCD is characterized by only minimal mesangial prominence or no obvious glomerular changes detectable by light microscopy, low-intensity staining for C3 and IgM or absent staining on immunofluorescence microscopy, and diffuse foot processes effacement but no electron-dense deposits on electron microscopy,⁴ whereas FSGS is a histological lesion displaying focal and segmental glomerular sclerosis.⁵ However, there are patients in which the clinical-pathology differences between the two aforementioned diseases are less obvious and renal lesions develop from initial pathological findings of minimal lesions to FSGS. The presence of clinical and pathology overlaps between the two diseases has led several pathologists to propose MCD and FSGS as two phases of the same disease.^{6,7} Some pathologists have therefore introduced the term “MCD-FSGS spectrum.”^{8,9} However, most of time these pathological features are indistinguishable at the time of diagnosis.

INS is characterized by augmented permeability of the capillary walls of glomeruli, causing proteinuria. Therapy is aimed at reducing the causes of increased permeability and clinical response is evaluated by resolution of proteinuria (disappearance of proteinuria for at least 3 consecutive days).¹⁰

Several pharmacological strategies can be undertaken to treat these disorders, but corticosteroids are considered the first line treatment worldwide. Glucocorticoids (GCs) are steroid hormones that regulate various homeostatic functions and physiological processes, such as inflammation,

immunity, metabolism, and other. They cause their effects by binding to the glucocorticoid receptor (GR; encoded by *NR3C1* gene), determining its activation as a transcription factor, translocation to the nucleus, leading to transcriptional changes that bring reduced proliferation and may trigger apoptosis of lymphocytes.¹¹

Based on the clinical response to GC therapy, patients with INS are classified as steroid-sensitive (when GC induces remission) or steroid-resistant (when treatment fails to induce remission). Approximately 80% of patients with INS respond to GCs, with the remaining 20% being steroid-resistant,³ but the underlying mechanism of resistance remains largely unknown.

Steroid resistant nephrotic syndrome (SRNS) presents significant heterogeneity in its onset and clinical course and neither the clinical features nor the histological trait predicts therapy response.¹² SRNS is also more likely to present resistance to other immunosuppressants,¹³ resulting in being more difficult to treat, with up to 50% of patients with SRNS progressing to end-stage renal disease within 10 years.^{14–16} Children with SRNS developing end-stage renal disease present lowered life expectancy, 20 years on average, after dialysis initiation.¹⁷

Persistent nephrotic syndrome is also related to poor patient-reported quality of life, thromboembolic events, and other complications, such as peritonitis, hypertension, dyslipidemia, and death.^{18–21}

At present, several biological factors have been implicated in SRNS and numerous molecular pathways are reported to be deregulated.^{22,23} Data obtained in animal models suggest that the NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome can be deregulated in a wide variety of glomerular diseases, including those causing INS²⁴ and we have previously shown that it is activated in white blood cells of patients with kidney disease undergoing dialysis.²⁵ NLRP3 inflammasome is a multiprotein complex composed of apoptosis-associated speck-like protein containing a CARD domain (adipose-derived stem

cell [ASC]) and procaspase-1²⁶ that mediates activation of caspase 1, which, in turn, promotes secretion of the pro-inflammatory cytokines interleukin 1 β (IL-1 β) and IL-18.²⁷ Moreover, whereas NLRP3 promoter methylation has not been clearly associated with a particular disease, some reports indicate that inflammation may lead to hypomethylation of this gene.^{28,29}

A potential role of this multiprotein complex in the mechanism of GC resistance in other clinical settings has been recently suggested. Paugh and collaborators have documented that increased expression of NLRP3, due to hypomethylation of its promoter, causes GC-resistant acute lymphoblastic leukemia. Lymphoblasts from GC resistant cases have higher expression of caspase 1 and its activator NLRP3 compared with sensitive cases, leading to increased activation of caspase 1 and its cleavage of the GR.³⁰

We therefore assessed whether the analysis of NLRP3 methylation could be able to explain, at least in part, the biological machinery associated with GC resistance in patients with INS and potentially used as a noninvasive clinical tool.

METHODS

Patients

Adult patients

A total of 28 adult patients with a history of INS in clinical follow-up at the Renal Unit of Hospital/University of Verona were enrolled in the study. Eighteen of them (13 with MCD and 5 with FSGS) were classified as INS GC-sensitive patients in our clinical records (all of them were in clinical remission and out of corticosteroids/immunosuppressive treatment for more than 6 months). Remission was

defined as the disappearance of proteinuria for at least 3 consecutive days.

All the 10 GC-resistant patients were in hemodialysis (out of any immunosuppressive therapy for more than 6 months). These patients received a kidney histological diagnosis of FSGS (80% NOS variants, 10% tip variants, and 10% collapsing).

At diagnosis, INS was defined according to our standard clinical protocol and according to Kidney Disease Improving Global Outcomes (KDIGO) 2012 guidelines.³

To avoid confounding factors, all adult patients with secondary glomerulonephritis, concomitant infectious diseases, diabetes, chronic lung diseases, neoplasm, and patients receiving antibiotics or nonsteroidal anti-inflammatory agents were excluded. We also excluded patients with less than 12 months of follow-up.

Pediatric patients

To exclude potential confounding effects on NLRP3 promoter methylation due to previous immunosuppressive treatments and/or hemodialysis, we enrolled a control group of children with a first episode of INS, presenting at 49 Pediatric and Pediatric Nephrology Units in 10 Italian regions.

At the time of diagnosis and before treatment, in all pediatric patients, whole blood for NLRP3 promoter methylation analysis was obtained. Subsequently, they 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 less than or greater than or equal to 10 days. Remission was defined as the disappearance of proteinuria for at least 3 consecutive days, as in the adult patients. Prednisone was then tapered over a 16-week period. As previously described,³¹ patients were then classified into the two study groups: GC-resistant if they did not achieve remission after therapy with daily prednisolone at a dose of 60 mg/m²/day for 4–6 weeks, whereas those patients achieving remission were

TABLE 1 Demographic and clinical features of all patients included in the study

	Discovery cohort (adults)			Validation cohort (children)		
	Glucocorticoid resistant	Glucocorticoid sensitive	p value	Glucocorticoid resistant	Glucocorticoid sensitive	p value
Number	10	18	/	7	14	/
Age, y	54.1 \pm 15.5	53.8 \pm 17.9	0.96	7.3 \pm 3.9	10.5 \pm 2.8	0.08
Creatinine, mg/dl	/	1.0 \pm 0.4	/	0.6 \pm 0.2	0.4 \pm 0.1	0.21
Total cholesterol, mg/dl	207.8 \pm 26.2	215.8 \pm 23.4	0.37	365.8 \pm 83.1	404.2 \pm 117.2	0.49
Proteinuria, g/24 h	/	0.1 \pm 0.2	/	4.7 \pm 2.5	4.4 \pm 5.9	0.90
Systolic BP, mmHg	130.4 \pm 10.3	124.4 \pm 16.8	0.25	124 \pm 19.0	107.6 \pm 13.1	0.07
Diastolic BP, mmHg	81.4 \pm 8.6	76.4 \pm 8.0	0.09	61.0 \pm 6.4	64.6 \pm 7.6	0.30
Disease: FSGS, MCD	10, 0	5, 13	/	2, 5	0, 14	/

Note: Values are expressed as mean \pm SD. The *p* values calculated by *t*-test.

Abbreviations: BP, blood pressure; FSGS, focal segmental glomerulosclerosis; MCD, minimal change disease.

considered GC-sensitive. Seven patients resulted GC-resistant, whereas 14 children were GC-sensitive (ages 4–18 years).

The main demographic and clinical features of both study groups are summarized in Table 1.

A cohort of 52 healthy subjects (41 adults, 11 women, mean age 44.5 ± 11.8 years, and 11 children, 5 girls, age 10.2 ± 5.5 years) was also enrolled.

The study was carried out according to the Declaration of Helsinki. The patients or parents of all participating children gave written informed consent before being enrolled in the study. Ethics committee approval was obtained from all the participating centers.

NLRP3 promoter methylation analysis

DNA was extracted from whole blood as previously described³² (Supplementary Information).

NLRP3 promoter methylation was measured by SNuPE reaction after bisulfite treatment and bisulfite-specific polymerase chain reaction (PCR). This assay interrogated NLRP3 promoter methylation CpG sites previously linked to GC resistance in acute lymphoblastic leukemia cells,²⁵ in particular cg21991396 (Supplementary Information).

Peripheral blood mononuclear cells isolation

To assess differences in the level of expression and activation of the NLRP3 inflammasome between GC-resistant versus GC-sensitive patients, we collected 15 ml of peripheral blood from 3 GC-resistant and 3 GC-sensitive adult patients with FSGS with INS at the time of diagnosis. Peripheral blood mononuclear cells (PBMCs) were isolated by density separation over a Ficoll–Paque (GE Healthcare, Chicago, IL) gradient (460 g for 30 min) and washed 3 times with phosphate-buffered saline (PBS) pH 7.4/1 mM EDTA (Sigma-Aldrich, St. Louis, MO). Due to the difficulty in obtaining biological samples of GC-resistant patients, we considered to compare at least three resistant versus three sensitive patients.

Confocal microscopy

Freshly isolated PBMC from 3 GC-resistant and 3 GC-sensitive adult patients with FSGS with INS were spotted on poly-L-lysine-coated slides and fixed with 4% paraformaldehyde (PFA). After blocking with 1% bovine serum albumin in PBS, the slides were incubated with anti-NLRP3 and anti-ASC antibodies (Abcam) for 1 h. The slides were then extensively washed in PBS and incubated with Alexa Fluor 488 Goat Anti-Rabbit and Alexa Fluor 594 anti-mouse.

Nuclei were stained with DAPI. Images were collected using the SP5 confocal microscope from Leica Microsystems (Wetzlar, Germany).

Corrected total cell fluorescence and Pearson's correlation coefficient were calculated using Fiji software (1 = perfect colocalization, 0 = no colocalization).

Cell culture

Human monocytic (U937 and THP-1) cell lines were purchased from ATCC (Manassas, VA). Cells were cultured in suspension in RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. Where indicated, U937 cells were stimulated with lipopolysaccharides (LPS; 10 ng/ml; Sigma-Aldrich) for 4 h followed by adenosine triphosphate (ATP; Sigma-Aldrich) 1 mM for 15 min, in RPMI 1640 without FBS, to induce the activation of the NLRP3 inflammasome pathway.

NLRP3 shRNA knockdown

Five different MISSION pLKO.1-puro clones of shRNA-NLRP3 expression plasmid bacterial glycerol stocks (Sigma-Aldrich: TRCN00000627223, TRCN0000062724, TRCN0000062725, TRCN0000062726, and TRCN0000062727) and MISSION shRNA non-targeting control (Sigma-Aldrich: SHC002 V) were plated on petri dishes (Supplementary Information). The extracted and purified plasmid DNAs were subsequently packaged into lentiviral particles. The lentiviral particles were then used to transduce the above-mentioned human monocytes and selected with puromycin. After several weeks of culture in selection medium, the NLRP3 inflammasome protein expression and sensitivity to GCs (prednisolone [PRED]) were tested by Western blot, MTT, or CellTiter glo, respectively, as described below.

Immunoblotting

Equal amounts of proteins (20 μ g) were separated on 4–12% NuPAGE Bis-Tris gels (ThermoFisher, Waltham, MA) and then transblotted to polyvinylidene difluoride (PVDF) membrane. After blocking in non-fat dry milk, membranes were incubated with primary antibodies (Supplementary Information). Blots were subsequently incubated with the appropriate secondary antibodies horseradish peroxidase (HRP)-conjugated (Jackson Immuno, West Grove, PA). Immunocomplexes were detected by chemiluminescence using Odyssey Imager (Li-Cor, Lincoln, NE). The signal

intensity was normalized to internal control proteins, such as beta-actin or GAPDH.

Total RNA isolation

Total RNA from the U937 human monocytes cell line was extracted using TRIzol reagent (ThermoFisher) according to manufacturer's instructions. The RNA concentration and purity were calculated by NanoDrop instrument (NanoDrop 2000; EuroClone, Milan, Italy).

Quantitative real-time PCR (TaqMan)

Expression levels of the glucocorticoid-induced leucine zipper (*GILZ*) gene were evaluated by real-time PCR TaqMan analysis using the CFX96 real-time system-C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA). The reverse transcription reaction was carried out with the High Capacity RNA-to-cDNA Kit (Applied Biosystem, Carlsbad, CA), and the real-time PCR was performed in triplicate using the TaqMan Gene Expression Assay, according to the manufacturer's instructions. The expression levels of the selected transcript were determined using the comparative Ct method ($2^{-\Delta\Delta C_t}$ method). *GILZ* expression values were normalized using GAPDH as the reference gene. Results are provided as the mean and standard error of four replicates.

In vitro proliferation assay

The effect of PRED on the proliferation of the U937 cells was determined by labeling metabolically active cells with [methyl- ^3H] thymidine (PerkinElmer, Milan, Italy). Cells were seeded into 96-well plates (10,000 cells/well) and treated with or without LPS/ATP and then exposed to PRED (range from 0.5 μM to 100 μM) for 24 hours. After 19 hours of incubation, cells were pulsed with [methyl- ^3H] thymidine (final concentration of 2.5 $\mu\text{Ci/ml}$) and the incubation was continued for an additional 5 h. The radioactivity of the cells was determined by a liquid scintillation analyzer (Wallac 1450 Microbeta liquid scintillation counter; PerkinElmer). Raw count per minute (cpm) data were converted and normalized to percent of maximal survival for each experimental condition (cpm PRED/cpm control*100).

MTT cell viability assay

Cell suspensions were plated with PRED to a final drug concentration of 0.01–250 $\mu\text{g/ml}$. The plate was then

incubated for 72 h at 37°C. Ten μL of 5 mg/ml MTT solution was then added to the plate and incubated for another 4 h (Supplementary Information).

Statistical analysis

The association between methylation at the *NLRP3* CpG sites and demographic and clinical covariates was evaluated by generalized linear models considering methylation as the dependent variable and the demographic and clinical covariates as the independent variables. Normality of methylation level was assessed by the Shapiro test. Receiver operating characteristic (ROC) curves were constructed for the significant in vitro tests to determine the optimal cutoff value for discriminating between patients' clinical response to GC treatment. Sensitivity, specificity, and the positive and negative predictive values (PPV, NPV, respectively) of the cutoff point were calculated. Logistic regression, considering the proportion of patients achieving the predicted clinical response, comparing patients who reached the optimal cutoff point and those who did not, was used to establish the level of significance of the cutoff values. Statistical analyses were performed using the software R.

For the in vitro studies, statistical analyses were performed using GraphPad Prism version 4.00. Two-way analysis of variance (ANOVA) with Bonferroni post-test and *t*-test were used for the analysis of inhibition of proliferation and gene expression; $p < 0.05$ values were considered statistically significant.

RESULTS

NLRP3 promoter methylation in adult patients

Methylation of the *NLRP3* promoter was consistently detectable but variable, with a median methylation value of 0.32 (inter quartile range 0.2–0.35; Figure 1a).

A statistically significant difference was observed between the level of *NLRP3* promoter methylation in the 18 GC-sensitive patients compared with the 10 GC-resistant patients, with significantly lower methylation in GC-resistant patients (median and interquartile range in sensitive patients 0.33, 0.30–0.38 vs. 0.2, 0.18–0.23 in resistant patients, generalized linear model $p = 0.00024$; Figure 1a). Within the adult population, there was no significant association of the extent of *NLRP3* methylation and patient age or sex (generalized linear model p values of 0.57 and 0.55, respectively; Supplementary Data, Figure S1A,B).

ROC curves were constructed to determine *NLRP3* methylation level for distinguishing GC-resistant patients from GC-sensitive patients, yielding an optimal cutoff of

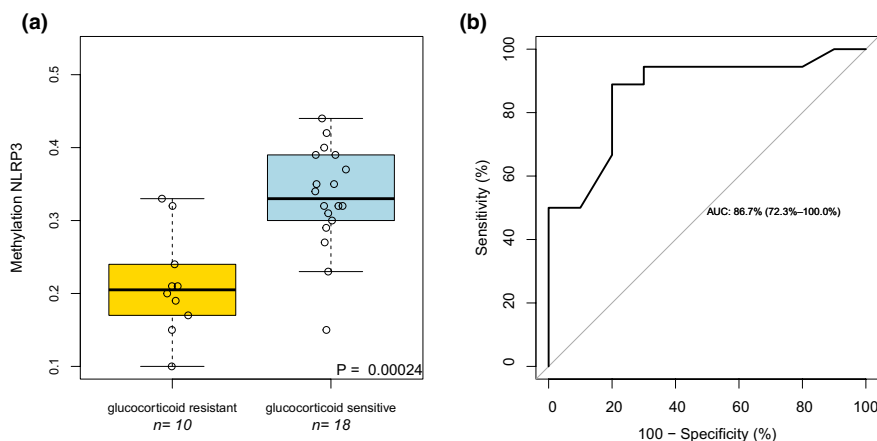


FIGURE 1 Methylation of NLRP3 promoter and ROC curve in adults. (a) Boxplot showing higher leukocyte DNA methylation of NLRP3 in glucocorticoid (GC)-sensitive compared with GC-resistant (generalized linear model $p = 0.00024$) adult patients with nephrotic syndrome. (b) ROC curve of NLRP3 methylation in adult patients revealed an optimal cutoff of 0.255, able to significantly discriminate GC-resistant from GC-sensitive patients. AUC, area under the curve; ROC, receiver operating characteristic

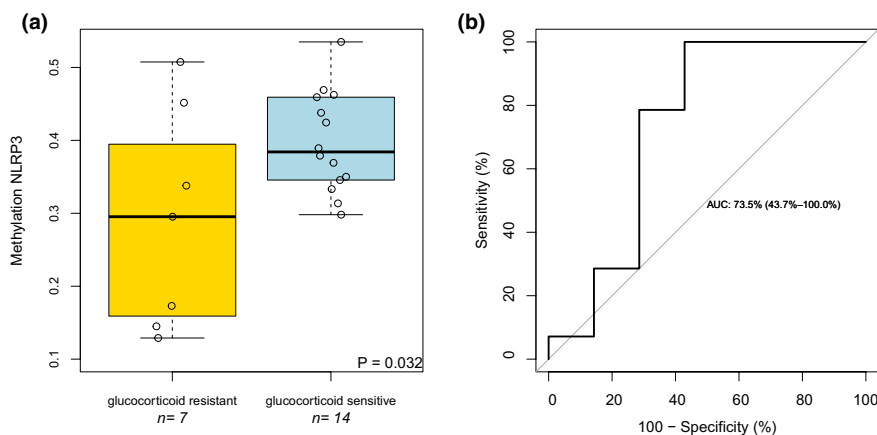


FIGURE 2 Methylation of NLRP3 promoter and ROC curve in children. (a) Boxplot showing higher leukocyte DNA methylation of NLRP3 in glucocorticoid (GC)-sensitive compared with GC-resistant pediatric patients (generalized linear model $p = 0.032$) with nephrotic syndrome. (b) ROC curve of NLRP3 methylation in pediatric patients revealed an optimal cutoff of 0.297, able to significantly discriminate GC-resistant from GC-sensitive patients. AUC, area under the curve; ROC, receiver operating characteristic

0.255 in adult patients. The area under the ROC curves (AUC) was 86.7% (Figure 1b), with a sensitivity of 88.9% and a specificity of 80.0% for identifying GC-resistant patients.

The 80.0% of patients with low NLRP3 promoter methylation (i.e., < 0.255) were resistant to GC, whereas among those with higher NLRP3 methylation (≥ 0.255), the frequency of GC resistance was significantly lower (2/16; 12.5%, $p = 0.00019$, logistic regression).

NLRP3 promoter methylation and glucocorticoid resistance in pediatric patients

In pediatric patients, as observed in adults, methylation of NLRP3 promoter exhibited varying degrees of methylation, with a median methylation value of 0.37 (interquartile range 0.31–0.45) (Figure 2a).

A statistically significant difference was observed in the level of NLRP3 promoter methylation in the GC-sensitive compared to the GC-resistant patients, with lower methylation in GC-resistant patients (median and inter quartile range in sensitive patients 0.38, 0.35–0.45 vs. in resistant patients, 0.30, 0.16–0.29, generalized linear model $p = 0.032$; Figure 2a). Within the pediatric population, there was no significant association of the extent of NLRP3 methylation and patient age or sex (generalized linear model p values of 0.24 and 0.20, respectively; Supplementary Data, Figure S1A,B).

The ROC curve in children revealed an optimal cut-off of 0.297 for distinguishing GC-sensitive and GC-resistant pediatric patients. The AUROC curve was 73.5% (Figure 2b), yielding a sensitivity of 100% and a specificity of 57.1% for identifying GC-resistant patients. All 4 patients (100%) with NLRP3 methylation below 0.297 were resistant to GCs, whereas among those with NLRP3

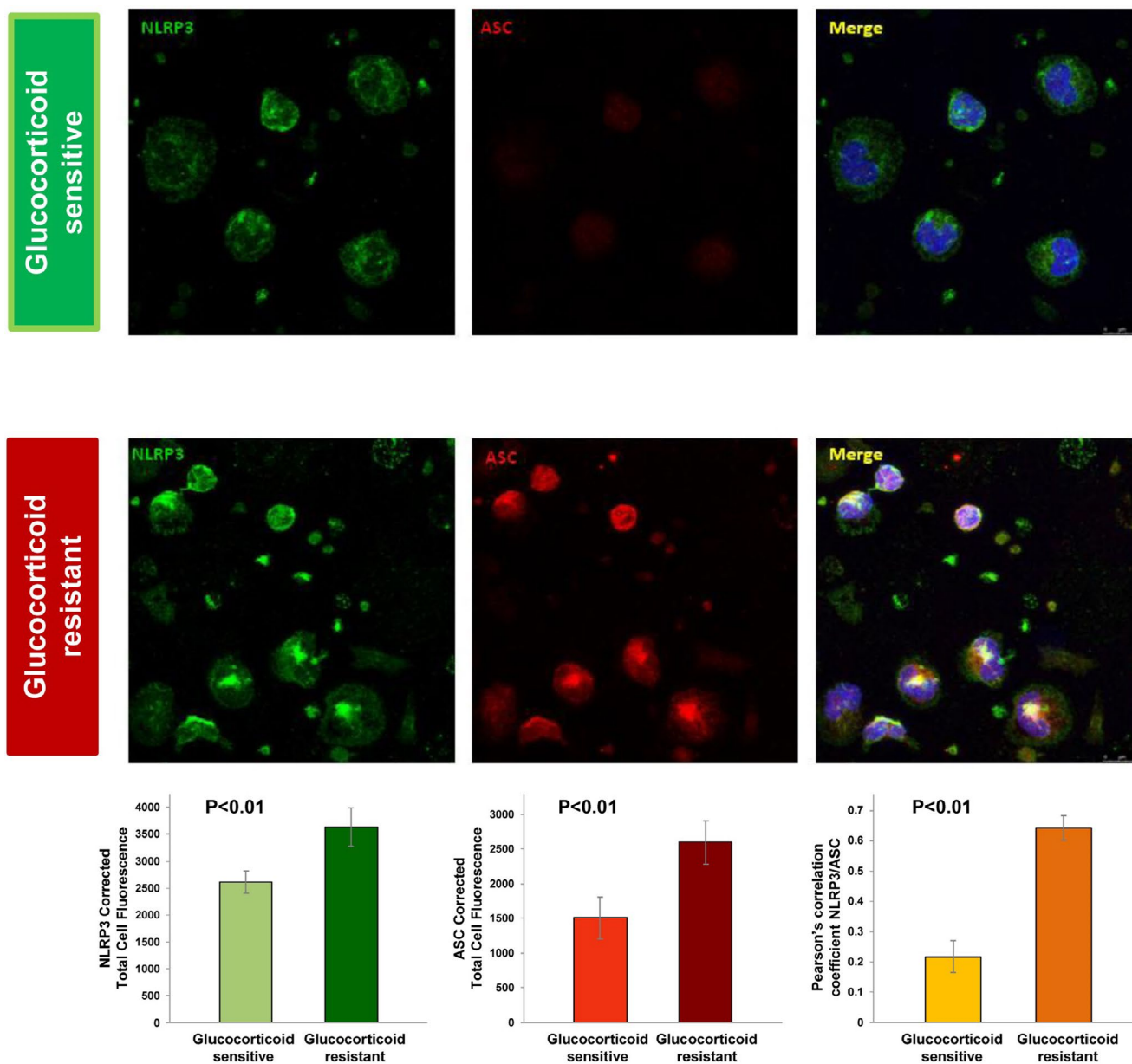


FIGURE 3 Colocalization of NLRP3 and ASC in PBMCs from glucocorticoid (GC)-resistant patients. The figure depicts representative confocal images for NLRP3 and ASC in PBMCs isolated from (upper) one adult GC-resistant patient and (lower) one adult GC-sensitive patient. NLRP3 protein co-localizes with ASC in PBMCs from GC-resistant patients suggesting a possible activation of the NLRP3 inflammasome, whereas it remains in cytoplasmic granular structure in PBMCs from GC-sensitive patients. The histograms represent the quantification of NLRP3 (green bars), ASC (red bars), and the Pearson's correlation coefficient NLRP3/ASC (orange bars) measured by Fiji. For NLRP3 and ASC, values are expressed as mean \pm SD of fluorescence intensity in PBMCs isolated from three GC-resistant and three GC-sensitive patients. The p value was calculated by t -test. ASC, adipose-derived stem cell; PBMCs, Peripheral blood mononuclear cells

methylation above 0.297 the number of GC-resistant patients was significantly lower (3/17, 17.6%; $p = 0.00097$, logistic regression).

To further assess age-related differences in NLRP3 methylation, leukocytes from 52 healthy donors were analyzed (41 adults and 11 children), revealing no association between NLRP3 methylation and age in healthy

donors (Supplementary Data, Figure S2). Comparison of NLRP3 methylation in healthy donors and patients with INS revealed significantly lower NLRP3 methylation in the leukocytes of patients with INS (median and interquartile range in healthy donors 0.44, 0.38–0.51 vs. 0.33, 0.24–0.39 in patients with INS, generalized linear model $p = 2.2 \times 10^{-7}$).

Activation of NLRP3 inflammasome in PBMC from glucocorticoid-resistant patients

Confocal microscopy was used to determine whether lower NLRP3 methylation in adult patients with GC-resistant INS corresponds to increased activation of the NLRP3 inflammasome.

NLRP3 and ASC were significantly more abundant in PBMC from GC-resistant compared with GC-sensitive patients. Moreover, their colocalization suggested a possible activation of the NLRP3 inflammasome in this group of patients (Figure 3).

In vitro sensitivity of NLRP3 knock-down cells and changes in inflammasome protein expression

NLRP3 was knocked down in human THP-1 monocyte cells. This cell line was chosen because it is GC-resistant. Five different shRNAs targeting NLRP3 caused significant knockdown of the encoded protein (NALP3), with clone #27 (TRCN0000062727) showing the highest knockdown (79.3%) and therefore selected for further experiments (Figure 4a,b).

The knockdown of NLRP3 in the THP1 monocyte cell line significantly increased sensitivity to GCs ($p < 0.0001$), decreasing the PRED lethal concentration 50% (LC_{50}) from 998.9 to 833 μ M (Figure 4c, Figure S3).

NLRP3 inflammasome activation contributes to glucocorticoid resistance in U937 monocytes

To evaluate the role of the inflammasome activation on GC sensitivity, an in vitro proliferation assay was performed on U937 monocyte cell line treated with or without LPS/ATP to activate the inflammasome, then incubated with PRED for 24 h. As shown in Figure 5a, NLRP3 inflammasome activation significantly increased resistance to PRED (178 μ M vs. 275 μ M, $p = 0.027$) confirming the involvement of NLRP3 inflammasome in the mechanism of resistance to GCs.

Consistent with this finding, the levels of GC receptor diminished significantly after the activation of the NLRP3 inflammasome and the treatment with PRED ($p < 0.05$; Figure 5b). Moreover, as shown in Figure 5c, the ability of GC to induce GILZ expression was markedly lower after activation of the NLRP3 inflammasome compared to the monocytes without NLRP3 activation ($p = 0.0045$), results consistent with reduced GC transcriptional effect after NLRP3 activation.

DISCUSSION

The pathophysiology of INS associated with MCD and FSGS is complex and still not completely defined.³³ However, a systemic activation of the inflammatory machinery with an enhanced release of a variety of cytokines, eicosanoids and complement components (e.g., C5b-9, TNF-alpha, and IL-1 beta) seems to have a role in triggering or maintaining this condition.^{34,35}

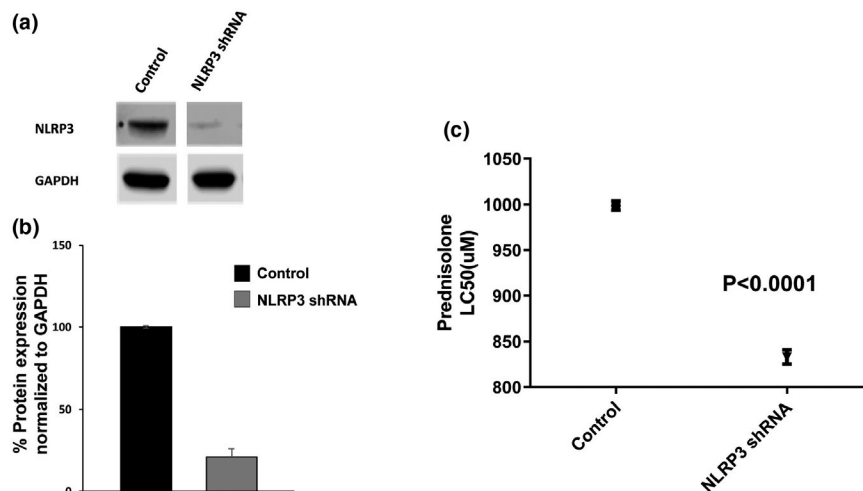


FIGURE 4 Inflammation proteins and corresponding variations of glucocorticoid sensitivity in THP1 cells. (a) Representative western blotting for NLRP3 in NLRP3 knockdown cells (shNLRP3) versus non-target control cells (NTCs). GAPDH was used as the loading control. (b) The histograms show mean \pm SD of protein expression level normalized to GAPDH. (c) THP-1-NLRP3 knockdown cells tested for prednisolone (PRED) sensitivity with MTT. A statistically significant decrease of lethal concentration 50% (LC_{50}) and increase in the sensitivity compared with the LC_{50} for the control was observed (833 vs. 998.9 μ M, $p < 0.0001$)

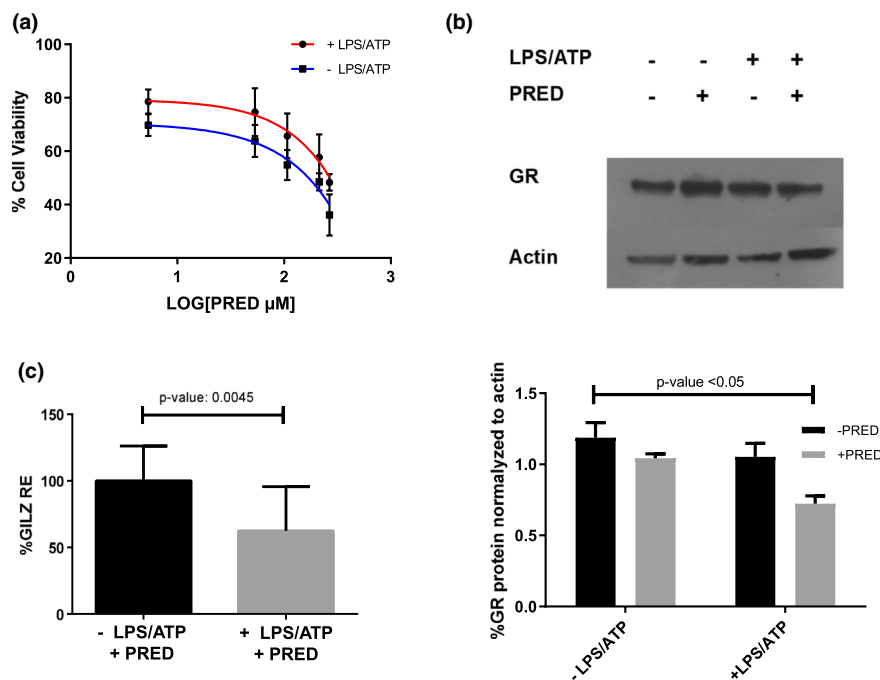


FIGURE 5 Prednisolone (PRED) sensitivity in U937 cell line in response to activation of NLRP3 inflammasome. (a) Dose response curve showing a reduction in PRED sensitivity after NLRP3 inflammasome activation (+LPS/ATP, red line) compared with the U937 cells without NLRP3 inflammasome activation (-LPS/ATP, blue line). Two-way analysis of variance (ANOVA), column factor $p = 0.023$. Results are mean values \pm SD from four independent experiments. (b) Levels of glucocorticoid receptor (GR) diminished significantly after the activation of the NLRP3 inflammasome and the treatment with PRED ($p < 0.05$). (c) Relative expression (RE) of GILZ after treatment with or without LPS/ATP and exposure to PRED (2 $\mu\text{g}/\text{mL}$) for 3 h. For the t -test analyses ($p = 0.0045$), the data are reported as means \pm SD of four independent experiments performed in triplicate. ATP, adenosine triphosphate; GILZ, glucocorticoid-induced leucine zipper; LPS, lipopolysaccharides

Additionally, recent *in vivo* animal models suggest that the NLRP3 inflammasome can be deregulated in these diseases,²⁴ and, as reported by our group in another clinical setting, is potentially involved in the mechanism of GC resistance.³⁰

Our data, although performed in a small dataset and, in part, in adult patients already treated for their clinical condition, indicated that methylation of the NLRP3 gene promoter was able to clearly discriminate those with INS who have responded to corticosteroid treatment (GC-sensitive patients) from those who have lost their kidney function and undergone hemodialysis treatment (GC-resistant patients) confirming previous findings obtained in other clinical settings.³⁰ In the pediatric cohort, however, there is significant overlap between the sensitive and resistant patients, reducing the specificity of the assay, possibly because of the contribution of age that seems to independently increase NLRP3 methylation.

Interestingly, although the degree of NLRP3 promoter methylation was similar in both GC-resistant adults and children, the cutoff of methylation for discriminating GC-resistant patients was somewhat higher in children, consistent with the age-related differences in NLRP3 methylation in children and adults with INS, perhaps contributing to the higher sensitivity to GCs in children. This has been described for other gene loci,

especially those involved in inflammation.³⁶ Additionally, we cannot exclude that adult patients have high levels of inflammation compared with children due to the presence of additional comorbidities and treatments (including dialysis). However, at the moment, the effects of these factors on DNA epigenetics has not been assessed. On these bases, it is important to include age, together with NLRP3 methylation in a prediction model for GC resistance in patients with INS. Further studies to investigate the association between age and NLRP3 methylation, considering in particular age-stratified groups of pediatric patients could be of importance.

Adult patients with INS indeed showed markedly reduced NLRP3 methylation in comparison with healthy adult controls, whereas pediatric patients with INS did not differ from pediatric healthy controls, perhaps because of a different disease mechanism or less prolonged inflammation. So far, this is the first report of an association of NLRP3 methylation with a particular disease.

Our hypothesis is that an epigenetic change that causes the activation of NLRP3 inflammasome, a multiprotein complex that recruits pro-caspase-1 via ASC and then proceeds to cleave the cytokine precursors pro-IL-1 β and pro-IL-18 into mature IL-1 β and IL-18,³⁷ may cause biological changes leading to an impaired GC receptor function.³⁸ Over the past few decades, evidence has accumulated

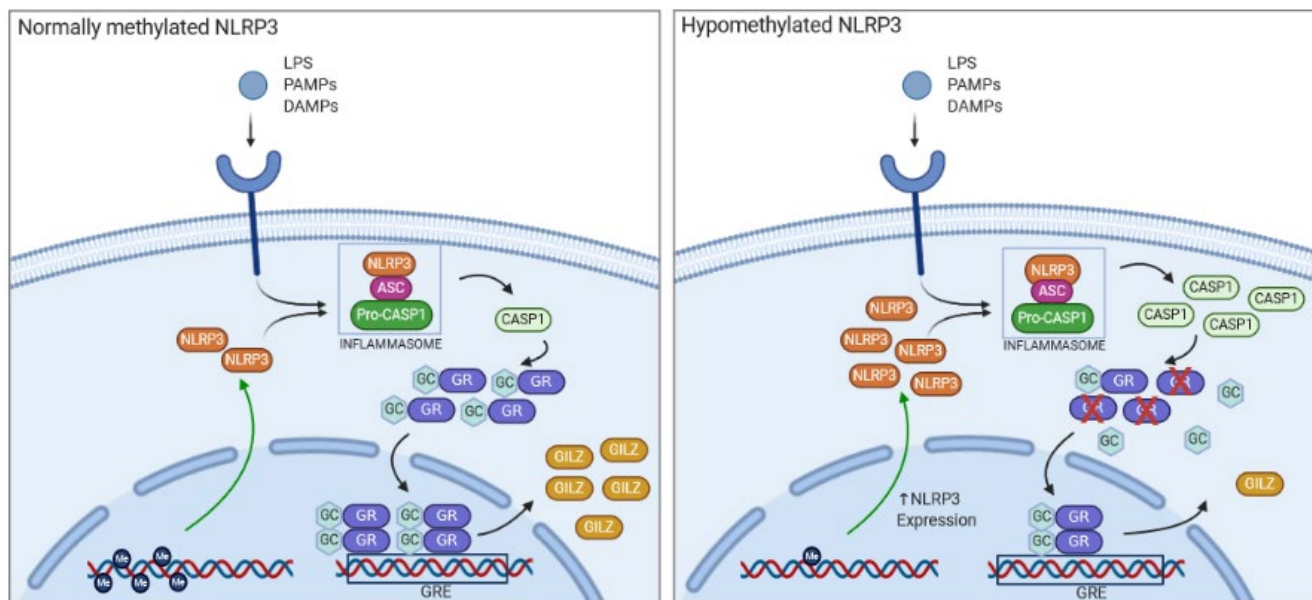


FIGURE 6 Mechanism of glucocorticoid resistance in patients with INS induced by a hypomethylation of the NLRP3 gene. Adaptor molecule apoptosis-associated speck-like protein containing a CARD (ASC), caspase-1 (CASP1), damage-associated molecular pattern (DAMP), glucocorticoid (GC), glucocorticoid receptor (GR), glucocorticoid responsive elements (GRE), glucocorticoid-induced leucine zipper (GILZ), lipopolysaccharides (LPS), methyl group (Me), NLR family pyrin domain containing 3 (NLRP3), pathogen associated molecular patterns (PAMP), and pro caspase-1 (pro-CASP1). INS, idiopathic nephrotic syndrome

that GC-resistance in several chronic diseases may be the result of impaired GC receptor function secondary to the direct long-term exposure to inflammatory cytokines (such as IL-1 β).³⁸

In fact, mechanisms include reduced expression of GC receptor, reduced nuclear translocation of GC receptor, and/or GC receptor-DNA binding due to the interactions of inflammatory mediators with the receptor itself as well as alterations in its phosphorylation status.³⁹

A variety of stimuli, including danger-associated molecular patterns (DAMPs, such as silica and uric acid crystals) and pathogen-associated molecular patterns (PAMPs) can activate NLRP3 inflammasome. Therefore, in INS, NLRP3 inflammasome can be activated by the inflammatory status that characterizes these patients, contributing to the development of steroid resistance.

However, due to the low number of patients enrolled and the choice to include adult patients not at the time of diagnosis and in clinical follow-up (some of them already on hemodialysis) cannot allow us to draw definitive conclusions.

Nonetheless, currently, no data have reported a large impact of dialysis treatment on global DNA methylation.⁴⁰ Additionally, to further confirm our hypothesis and to exclude potential confounding effects on NLRP3 promoter methylation due to previous immunosuppressive treatments and/or hemodialysis, we performed the same experiments in 21 pediatric patients with MCD/FSGS-induced INS at the time of diagnosis and before starting any treatment.

Furthermore, the absence in our dataset of GC-resistant adult patients with MCD and the absence of GC-sensitive children with FSGS may reveal a potential disease-correlation effect. In the future, a larger clinical study could define this association.

Taken together, our results uncovered a new biological mechanism whereby patients with INS may develop resistance to GCs via leukocyte epigenetic changes of NLRP3 (Figure 6). The consequent overexpression of NLRP3 and diminished cellular response to GCs identified a potential noninvasive diagnostic tool that could be used in daily clinical practice if validated in a larger cohort of patients. Additionally, evaluation of NLRP3 methylation, performed before initiation of GC therapy, and the assessment of the expression profile of NLRP3 in PBMC could represent important future research objectives. In particular, this could be useful to avoid kidney biopsy in patients with INS at high risk of bleeding complications (including those with solitary native kidney, uncorrectable bleeding diathesis, severe hypertension that cannot be controlled with antihypertensive medications, anatomic abnormalities of the kidney, active renal, or perirenal infection and uncooperative) and to choose therapy in fragile patients. Avoiding corticosteroids in low/unresponsive patients, based on NLRP3 promoter methylation analysis, could reduce the risk of onset of severe unfavorable side effects (e.g., worsening of diabetes, severe osteoporosis, glaucoma, and recurrent infections). These patients could be treated with alternative therapies, including calcineurin inhibitors (cyclosporine and tacrolimus), mycophenolate mofetil, cyclophosphamide, rituximab,

angiotensin-converting enzyme inhibitors, and angiotensin II receptor blockers.^{10,41,42}

Finally, these findings provide the foundation for future efforts to identify small molecule inhibitors of NLRP3 that can reverse this mechanism of GC resistance and thereby improve the treatment of INS and potentially other disorders treated with GCs.

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CONFLICT OF INTEREST

All authors declared no competing interests for this work.

AUTHOR CONTRIBUTIONS

M.L., S.G., G.Z., G.S., G.G., G.D., and W.E. wrote the manuscript. W.E. and G.Z. designed the research. M.L., S.G., E.B., R.M., D.S., A.C., E.C., C.C., and C.Z. performed the research. M.L., S.G., G.S., W.E., G.Z., A.P., and G.M. analyzed the data.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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