Long noncoding RNA GAS5: a novel marker involved in glucocorticoid response

Long noncoding RNA GAS5: a novel marker involved in glucocorticoid response

Lucafò Marianna[1, 2], De Iudicibus Sara[2], Di Silvestre Alessia[3], Pelin Marco[3], Candussio Luigi[3], Martelossi Stefano[2], Tommasini Alberto[2], Piscianz Elisa[2], Ventura Alessandro[1, 2], Decorti Giuliana[3 *]

[1] Department of Medicine, Surgery and Health Sciences, University of Trieste - Trieste, Italy

[2] Institute for Maternal and Child Health - IRCCS "Burlo Garofolo" - Trieste, Italy

[3] Department of Life Sciences, University of Trieste - Trieste, Italy

[*] Corresponding Author: Prof. Decorti Giuliana, University of Trieste, Via Fleming 22, Trieste 34127 Italy; Tel +39 040 5588777, Fax +39 040 5587838, E-mail decorti@units.it

Running Title

GAS 5 in glucocorticoid response

Keywords

GAS5, Gene expression, Glucocorticoid receptor, Long noncoding RNA, Methylprednisolone, NR3C1 gene, Proliferation assay

Abstract

Glucocorticoids (GCs) exert their effects through regulation of gene expression after activation in the cytoplasm of the glucocorticoid receptor (GR) encoded by NR3C1 gene. A negative feedback mechanism resulting in GR autoregulation has been demonstrated, through the binding of the activated receptor to intragenic sequences called GRE-like elements, contained in GR gene.

The long noncoding RNA growth arrest–specific transcript 5 (GAS5) interacts with the activated GR suppressing its transcriptional activity. The aim of this study was to evaluate the possible role of GAS5 and NR3C1 gene expression in the anti-proliferative effect of methylprednisolone in peripheral blood mononuclear cells and to correlate the expression with the individual sensitivity to GCs. Subjects poor responders to GCs presented higher levels of GAS5 and NR3C1 in comparison with good responders. We suggest that abnormal levels of GAS5 may alter GC effectiveness, probably interfering with the mechanism of GR autoregulation.

Introduction

Glucocorticoids (GCs), in particular prednisone and methylprednisolone (MP) are commonly used in inflammatory and autoimmune disorders and in the treatment of leukaemia and lymphomas, and in the prevention of rejection in transplant patients [1, 2]; however considerable inter-individual differences in their efficacy and side effects have been reported [3, 4]. The mechanisms involved in GC resistance are scarcely understood and there is presently no means to predict the response in advance [5-7].

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 [8, 9]. 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, encoded by NR3C1 gene, presents a C-terminal ligand-binding domain (LBD), an N-terminal transcriptional regulatory region, and a central DNA binding domain (DBD) [10, 11]. Upon ligand binding, the receptor transmigrates to the

nucleus and binds to glucocorticoid responsive elements (GREs), palindromic DNA-binding sites in the promoter region of target genes, assembling a transcriptional activation complex, and inducing or repressing gene expression [12-14].

It has been shown that GR expression is regulated by the receptor itself after prolonged GC treatment: in 1986 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 (GREs-like), contained in the GR gene [15-17]. These observations have been subsequently confirmed by other authors [16, 17].

It was recently 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 [18]. This interaction is physiologically relevant as it occurs at concentrations of the GR ligand dexamethasone at 10^{-10} M, that area lower than that of physiological endogenous glucocorticoid, cortisol. Kino et al. observed that overexpression of GAS5 greatly inhibits the transcription of GR target genes, among which those that encode cellular inhibitor of apoptosis 2 (cIAP2) and serum- and glucocorticoid-regulated kinase 1 (SGK1); the reduced binding of the GR to the promoters was demonstrated by chromatin immune-precipitation analysis [18, 19].

The aim of the present investigation was to evaluate the possible role of GAS5 and NR3C1 gene expression in the anti-proliferative effect of methylprednisolone (MP) in peripheral blood mononuclear cells (PBMCs) obtained from healthy subjects and to correlate the expression with the individual sensitivity to GCs. PBMCs can be induced to proliferate in vitro using mitogens, and proliferation is inhibited by GCs, although the mechanism is still unclear [20]. It has been suggested that the in vitro test is useful for predicting GC responsiveness in rheumatoid arthritis [21], systemic lupus erythematosus [22], bronchial asthma [23], renal transplant rejection [24] and ulcerative colitis [25].

The results presented here indicate that abnormal levels of GAS5 may alter GC effectiveness probably interfering with the mechanism of GR autoregulation. 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.

Materials and methods

Subjects

Samples from 14 blood donors were collected between January 2013 and October 2013 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 the variability due to circadian rhythm, and immediately processed. All donors have signed an individual review-board-approved consent for blood sampling and use for research purposes. Blood samples were delivered to the University of Trieste with no individually identifiable information. A total of 9 ml of each buffy coat was used for the isolation of PBMCs.

In vitro proliferation assay

The effect of MP on the proliferation of PBMCs was determined as reported by Cuzzoni and colleagues [26]. Nonlinear regression of dose–response data was performed using Graph-Pad Prism version 4.00 for computing IC_{50}, the MP concentration required to reduce proliferation to 50%. $I_{250ng/ml}$ was also calculated and defined as the inhibition of the proliferation achievable at 250 ng/ml concentration of MP. Subjects were divided into two groups based on their

individual response to MP and considered good or poor responders if their $I_{250ng/ml}$ values were respectively above or below the median of the whole population.

Total RNA isolation

PBMCs were treated with MP at a concentration of 250 ng/ml and after 72 h the cells were collected and preserved in RNA*later*® solution (Ambion) at -20 °C. RNA extraction using the MagMaxTM-96 Total RNA Isolation Kit (Ambion, Applied Biosystems, Foster City, CA) was performed according to the manufacturer's instructions. The RNA concentration and purity were calculated by Nano Drop instrument (NanoDrop 2000, EuroClone®).

Quantitative real-time PCR (TaqMan®)

Expression levels of GAS 5 and NR3C1 genes were evaluated by real-time RT-PCR TaqMan® analysis using the CFX96 real-time system-C1000 Thermal Cycler (Bio-Rad Laboratories). The reverse transcription reaction was carried out with the High Capacity RNA-to-cDNA Kit (Applied Biosystem) and the real-time PCR was performed in triplicate using the TaqMan® Gene Expression Assay to assess GAS5 and NR3C1 mRNA expressions, according to the manufacturer's instructions. The thermal cycling conditions for TaqMan assays were as follows: 2 min at 50 °C and 10 min at 95 °C followed by 40 cycles at 95 °C for 15 s and 60 °C for 60 s.

The expression levels of GAS5 and NR3C1 were evaluated using the comparative Ct method (2-ΔΔCt method) [27]. Ct values were corrected based on PCR efficiencies using LinRegPCR [28]. The GAS5 and NR3C1 expression values were normalized using the 18S as housekeeping gene.

Western Blotting

Cells ($1x10^7$) were cultured as reported for gene expression analysis, collected, and after washing with cold PBS, lysed using a lysis buffer composed by Tris-HCl 10mM pH 7.4, EDTA 100 mM, NaCl 100 mM, SDS 0.1%, Protease inhibitor cocktail 1%. Samples were then run in a PAGErTM Mini-gel Chamber (Lonza, Milan, Italy) using a 10% acrylamide gels with a Trys-Glycine buffer and subsequently semi-dry blotted for 2 h with 50 mA current on PVDF membrane. After blocking for 1 h with 5% not-fat milk in PBS, membranes were incubated overnight at 4 °C with primary antibodies (anti-actin 1:20000, Millipore; anti-GRα 1:500, Thermo Scientific, Milan, Italy). Membranes were then washed in Tween/Tris buffered salt solution (TTBS) and incubated for 1 h at 37 °C with an anti-rabbit HRP-conjugated secondary antibody 1:50000 (Millipore, Milan, Italy). Chemiluminescence was developed using LiteAblot® TURBO (Euroclone, Milan, Italy) and exposed on Kodak Biomax film. GR protein expression was quantified on western blots images using the ImageJ software, version 1.45s and are reported as % with respect to actin.

$DiOC_6$/PI test

3,3′-Dihexyloxacarbocyanine ($DiOC_6$) dye (Molecular Probes, Montluçon, France) was used to discriminate viable and dying cells with flow cytometer (FACScan, Becton-Dickinson) as marker of decreased mitochondrial transmembrane potential (ΔY_m). 6 x 10^5 cells per well were seeded in 24-well plates. MP dissolved in culture media was added (final concentration: range from 0.019 ng/ml to 20 μg/ml), and plates were incubated at 37 °C for 72 h. Subsequently, $DiOC_6$ 10 μM was added, and the incubation continued for 20 additional min. Subsequently, the PBMCs, were washed and 0.1 mg/mL propidium iodide (PI) were added to each sample and incubated for 10 min at room temperature . Cells with compromised cellular membrane (necrotic and late apoptotic cells) were stained with PI. Flow cytometric measurements were analyzed by means of the FlowJo software.

Statistical analyses

Statistical analyses were performed using the R statistical software (version 2.9.1). The nonparametric Wilcoxon test was used for the analysis of gene expression for all subjects and between good and poor responders. Western blot results were analyzed using T-test and two-way analysis of variance (two-way ANOVA) was used for the flow cytometric analysis using the probes $DiOC_6$ and PI. P-values < 0.05 were considered statistically significant.

Results

Individual sensitivity to MP

The effect of MP on concavalin A-induced proliferation was assessed on PBMCs obtained from 14 healthy blood donors (mean age 49.5, range 21-57 years; 21.4% female and 78.6% male). Using nonlinear regression for proliferation data, a sigmoidal dose-response curve was extrapolated for each subject, and, in accordance with previous papers [29], a wide inter-individual variation in IC_{50} and $I_{250ng/ml}$ was observed (IC_{50} median value $1.16x10^{-6}$ M, range $2.75x10^{-9}$ M – $1.60x10^{-4}$ M; $I_{250ng/ml}$ median value 53%, range 14-85.5%).

Subjects were divided into two groups based on whether their $I_{250ng/ml}$ values were above the median (good responders, 7 subjects; median 72%, range 75-97%) or below (poor responders, 7 subjects; median 29%, range 14-52%).

GAS5 and NR3C1 pattern during MP treatment in PBMCs

To evaluate the role of transcriptional response in the variability in GC sensitivity, GAS5 and NR3C1 gene expression was evaluated in concanavalin A-stimulated PBMCs treated for 72 h with MP.

In all the subjects studied, in untreated cells, no differences in GAS5 and NR3C1 expression were observed between time 0 and after 72 h in culture (Mann-Whitney test; GAS5 median and range: time 0 1.8×10^{-4} vs 72 h 1.4×10^{-4} p-value= 0.39; NR3C1 median: time 0 $8.5x10^{-5}$ vs 72 h 4.2×10^{-5}, p-value= 0.16).

When all subjects were considered, treatment with MP 250 ng/ml for 72 h induced a slight reduction of GAS5 and NR3C1 gene expression (expressed as fold change of MP treated vs untreated controls, GAS5: median -1.45, min -40.9 max +11.0; NR3C1: median - 1.42, min -11.8 max +9.7).

The gene expression pattern was evaluated in good and poor responders: in the good response group a downregulation of both GAS5 and NR3C1 genes was evident in cells treated for 72 h with MP at 250 ng/ml in comparison with their untreated controls, (fold change: GAS5: median - 2.14, min -40.9 max +2.5; NR3C1: median - 5.72, min -11.8 max -1.2). On the contrary, the poor response group showed an upregulation of the same genes (fold change: GAS5: median + 1.98, min -1.8 max +11.0; NR3C1: median + 2.29, min -2.5 max +9.7). These differences between the two groups were statistically significant (Wilcoxon test; MP good response vs MP poor response: GAS5 p-value=0.011; NR3C1 p-value=0.017) (Fig.1).

To confirm the expression of the GR protein, western blot analysis was performed. The quantification, normalized to the structural protein actin, was carried out in 2 good and 2 poor responders after treatment with MP for 72 h at 250 ng/ml, confirming the same pattern observed by gene expression analysis. Indeed, a reduction of GR expression after MP treatment was evident in the good response group. In particular, the level of protein expression of the untreated controls (76±6 % GR expression with respect to actin) was significantly decreased in cells exposed to 250 ng/ml MP for 72 h (25±3 %; p<0.001; Fig. 2). On the contrary, in the poor response group, the level of GR was significantly higher in treated cells (91±13%) in comparison to untreated controls (68±4%; p < 0.01; Fig. 2).

GAS5 as a modulator of the response to MP

To exclude that the increase of GAS5, observed in our resistant subjects, was related to the apoptotic state, PBMCs were treated with MP for 72 h, stained with $DiOC_6$ and PI and then analyzed by flow cytometric technique. The combination of $DiOC_6$-PI allows evaluating mitochondrial depolarization-membrane damage.

In our experimental conditions, the increase of GAS5 was not related to the apoptotic cell death in PBMCs treated with MP, and flow cytometric analysis pointed out that, at 72 h, treatment with MP induced mitochondrial depolarization, that was more evident in good responder subjects (Fig. 4, poor *vs* good responders two-way ANOVA: $p<0.05$); on the contrary, no difference in PI fluorescence signal was evident between treated and untreated cells (data not shown) indicating that even high concentrations of MP did not induce cellular membrane damage.

Discussion

Our results indicate that GAS5 may alter GC effectiveness probably interfering with the mechanism of GR autoregulation.

We hypothesize that upregulation of GAS5, occurring in poor responder PBMCs after treatment with MP, prevents the activated GR from binding to intragenic control elements on the NR3C1 gene, thus preventing the transcriptional repression of the gene (Fig. 3). Conversely, downregulation of GAS5 occurring in good responders does not hamper the binding of the activated receptor to GRE-like sequences (Fig. 3). However, it should be remembered that other mechanisms, such as post transcriptional modification, may be involved in the downregulation of the GR [15].

Our observations strongly suggest that GAS5 could be important in the regulation of the response to GCs. Moreover it can be assumed that the altered expression of endogenous GAS5 is a glucocorticoid-mediated event, indeed in untreated cells, both of good and poor responders, no differences in GAS5 relative quantification were observed. The mechanisms through which this transcriptional modulation occurs is not yet clear; to date it is only known that the expression of GAS5 mRNA is regulated at the posttranscriptional level during growth arrest and at the transcriptional level in differentiated cells [30].

GAS5 was reported to act as a sensitizer of apoptosis [31-34]. Our data showed that in resistant subjects, in which GAS5 was upregulated, cells proliferation at 250 ng/ml of MP was higher compared to responders and this data was confirmed by flow cytometric analysis. Hence, in our experiments, GAS5 could be considered a key mediator of GC resistance mechanism in PBMCs as it does not act as a growth arrest–specific transcript.

Conclusion

The antiproliferative in vitro effect of GCs has been correlated with clinical response to these agents in various diseases [21-25]. Our results suggest that the evaluation of GAS5 and NR3C1 gene expression, integrated with a lymphocyte proliferation assay, could lead to the identification of GC resistant subjects. This is the first report about the functional effects of changes in GAS5 expression in GC resistance, although the molecular mechanisms involved in this phenomenon need further investigations.

In conclusion, the altered expression of endogenous GAS5 seems to be a GC-mediated event, leading to a different regulation of the NR3C1 gene. If these results are confirmed in a larger series and in patients by chronic inflammatory and autoimmune diseases, GAS5 should be considered as a candidate marker of GC resistance.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

Marianna Lucafò: conception, design, execution and interpretation of the data and article preparation; Sara De Iudicibus: design and interpretation of the data and article preparation; Alessia Di Silvestre: execution and interpretation of the data; Marco Pelin: execution and interpretation of the data; Luigi Candussio: article preparation; Stefano Martelossi: interpretation of the data and article preparation; Alberto Tommasini: interpretation of the data and article preparation; Elisa Piscianz: execution and interpretation of the data; Alessandro Ventura: interpretation of the data and article preparation, Giuliana Decorti: design and interpretation of the data and article preparation.

The work was funded within the research project supported by Italian Ministry of Health, No. 44/GR-2010-2300447. Sara De Iudicibus is recipient of a postdoctoral fellowship from IRCCS "Burlo Garofolo". The research was supported by Fondazione Benefica Alberto & Kathleen Casali, Trieste, Italy and Fondazione Beneficentia Stiftung, Vaduz, Lichtenstein.

References

[1] Riccardi C, Bruscoli S, Migliorati G. Molecular mechanisms of immunomodulatory activity of glucocorticoids. Pharmacol Res. 2002; 45(5): 361-8.

[2] Inaba H, Pui CH. Glucocorticoid use in acute lymphoblastic leukaemia. Lancet Oncol. 2010; 11(11): 1096-106.

[3] Barnes PJ, Adcock IM. Glucocorticoid resistance in inflammatory diseases. Lancet. 2009; 373(9678): 1905-17.

[4] Maranville JC, Baxter SS, Torres JM, Di Rienzo A. Inter-ethnic differences in lymphocyte sensitivity to glucocorticoids reflect variation in transcriptional response. Pharmacogenomics J. 2013; 13(2): 121-9.

[5] De Iudicibus S, Franca R, Martelossi S, Ventura A, Decorti G. Molecular mechanism of glucocorticoid resistance in inflammatory bowel disease. World J Gastroenterol. 2011; 17(9): 1095-108.

[6] Barnes PJ. Mechanisms and resistance in glucocorticoid control of inflammation. J Steroid Biochem Mol Biol. 2010; 120(2-3): 76-85.

[7] Farrell RJ, Kelleher D. Glucocorticoid resistance in inflammatory bowel disease. J Endocrinol. 2003; 178(3): 339-46.

[8] Beato M, Herrlich P, Schutz G. Steroid hormone receptors: many actors in search of a plot. Cell. 1995; 83(6): 851-7.

[9] Davies P, Rushmere NK. The structure and function of steroid receptors. Sci Prog. 1988; 72(288 Pt 4): 563-78.

[10] Giguere V, Hollenberg SM, Rosenfeld MG, Evans RM. Functional domains of the human glucocorticoid receptor. Cell. 1986; 46(5): 645-52.

[11] Zhou J, Cidlowski JA. The human glucocorticoid receptor: one gene, multiple proteins and diverse responses. Steroids. 2005; 70(5-7): 407-17.

[12] Biddie SC, Hager GL. Glucocorticoid receptor dynamics and gene regulation. Stress. 2009; 12(3): 193-205.

[13] Meijsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR. DNA binding site sequence directs glucocorticoid receptor structure and activity. Science. 2009; 324(5925): 407-10.

[14] Nordeen SK, Suh BJ, Kuhnel B, Hutchison CA, 3rd. Structural determinants of a glucocorticoid receptor recognition element. Mol Endocrinol. 1990; 4(12): 1866-73.

[15] Okret S, Poellinger L, Dong Y, Gustafsson JA. Down-regulation of glucocorticoid receptor mRNA by glucocorticoid hormones and recognition by the receptor of a specific binding sequence within a receptor cDNA clone. Proc Natl Acad Sci U S A. 1986; 83(16): 5899-903.

[16] Burnstein KL, Jewell CM, Cidlowski JA. Human glucocorticoid receptor cDNA contains sequences sufficient for receptor down-regulation. J Biol Chem. 1990; 265(13): 7284-91.

[17] Webster JC, Cidlowski JA. Downregulation of the glucocorticoid receptor. A mechanism for physiological adaptation to hormones. Ann N Y Acad Sci. 1994; 746: 216-20.
[18] Kino T, Hurt DE, Ichijo T, Nader N, Chrousos GP. Noncoding RNA gas5 is a growth arrest- and starvation-associated repressor of the glucocorticoid receptor. Sci Signal. 2010; 3(107): ra8.
[19] Garabedian MJ, Logan SK. Glucocorticoid receptor DNA binding decoy is a gas. Sci Signal. 2010; 3(108): pe5.
[20] Creed TJ, Lee RW, Newcomb PV, di Mambro AJ, Raju M, Dayan CM. The effects of cytokines on suppression of lymphocyte proliferation by dexamethasone. J Immunol. 2009; 183(1): 164-71.
[21] Kirkham BW, Corkill MM, Davison SC, Panayi GS. Response to glucocorticoid treatment in rheumatoid arthritis: in vitro cell mediated immune assay predicts in vivo responses. J Rheumatol. 1991; 18(6): 821-5.
[22] Seki M, Ushiyama C, Seta N, et al. Apoptosis of lymphocytes induced by glucocorticoids and relationship to therapeutic efficacy in patients with systemic lupus erythematosus. Arthritis Rheum. 1998; 41(5): 823-30.
[23] Hirano T, Homma M, Oka K, Tsushima H, Niitsuma T, Hayashi T. Individual variations in lymphocyte-responses to glucocorticoids in patients with bronchial asthma: comparison of potencies for five glucocorticoids. Immunopharmacology. 1998; 40(1): 57-66.
[24] Langhoff E, Ladefoged J, Jakobsen BK, et al. Recipient lymphocyte sensitivity to methylprednisolone affects cadaver kidney graft survival. Lancet. 1986; 1(8493): 1296-7.
[25] Hearing SD, Norman M, Probert CS, Haslam N, Dayan CM. Predicting therapeutic outcome in severe ulcerative colitis by measuring in vitro steroid sensitivity of proliferating peripheral blood lymphocytes. Gut. 1999; 45(3): 382-8.
[26] Cuzzoni E, De Iudicibus S, Bartoli F, Ventura A, Decorti G. Association between BclI polymorphism in the NR3C1 gene and in vitro individual variations in lymphocyte responses to methylprednisolone. Br J Clin Pharmacol. 73(4): 651-5.
[27] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001; 25(4): 402-8.
[28] Ramakers C, Ruijter JM, Deprez RH, Moorman AF. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci Lett. 2003; 339(1): 62-6.
[29] Hearing SD, Norman M, Smyth C, Foy C, Dayan CM. Wide variation in lymphocyte steroid sensitivity among healthy human volunteers. J Clin Endocrinol Metab. 1999; 84(11): 4149-54.
[30] Coccia EM, Cicala C, Charlesworth A, et al. Regulation and expression of a growth arrest-specific gene (gas5) during growth, differentiation, and development. Mol Cell Biol. 1992; 12(8): 3514-21.
[31] Williams GT, Mourtada-Maarabouni M, Farzaneh F. A critical role for non-coding RNA GAS5 in growth arrest and rapamycin inhibition in human T-lymphocytes. Biochem Soc Trans. 2011; 39(2): 482-6.
[32] Mourtada-Maarabouni M, Pickard MR, Hedge VL, Farzaneh F, Williams GT. GAS5, a non-protein-coding RNA, controls apoptosis and is downregulated in breast cancer. Oncogene. 2009; 28(2): 195-208.
[33] Mourtada-Maarabouni M, Hedge VL, Kirkham L, Farzaneh F, Williams GT. Growth arrest in human T-cells is controlled by the non-coding RNA growth-arrest-specific transcript 5 (GAS5). J Cell Sci. 2008; 121(Pt 7): 939-46.
[34] Pickard MR, Mourtada-Maarabouni M, Williams GT. Long non-coding RNA GAS5 regulates apoptosis in prostate cancer cell lines. Biochim Biophys Acta. 2013; 1832(10): 1613-23.

Figure

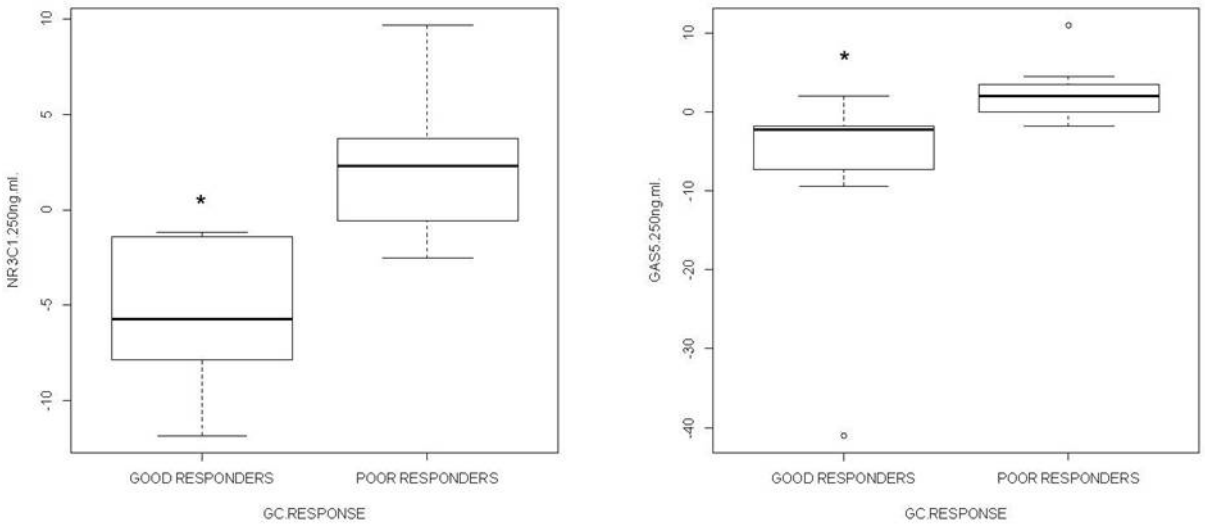

Fig. 1. Gene expression fold change of NR3C1 (on the left) and GAS5 (on the right) in good and poor responder subjects after treatment with MP for 72 h at 250 ng/ml compared to untreated controls. Wilcoxon test * p-value<0.05

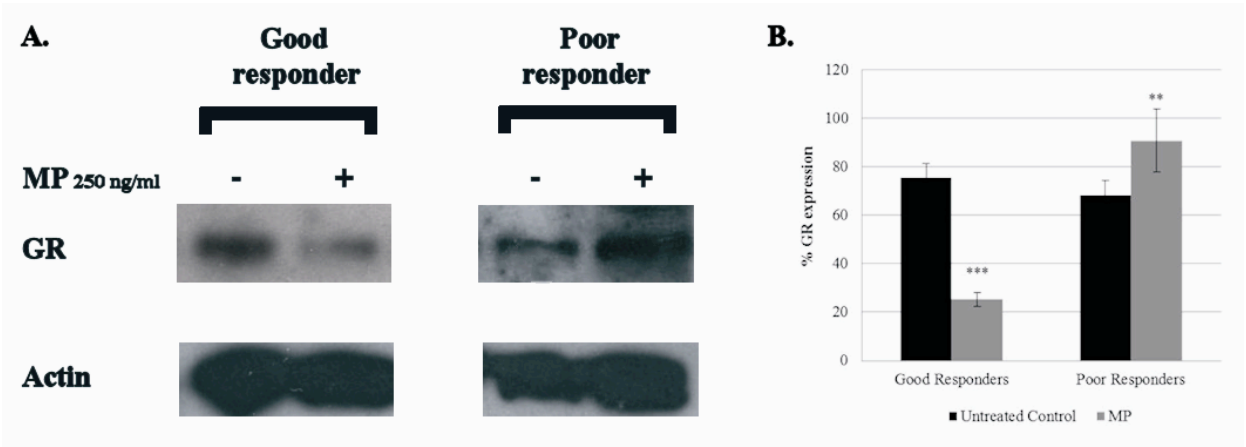

Fig. 2. A.) Protein expression of GR evaluated by western blot analysis on PBMCs in good and poor responder subjects treated (+) or untreated (-) after 72 h with MP; B.) Percentage of GR expression evaluated in PBMCs in good and poor responder subjects treated (grey bars) or untreated (black bars) for 72 h with MP in respect to actin; T-test analysis: MP treated cells vs untreated control ** p-value<0.01; *** p-value<0.001.

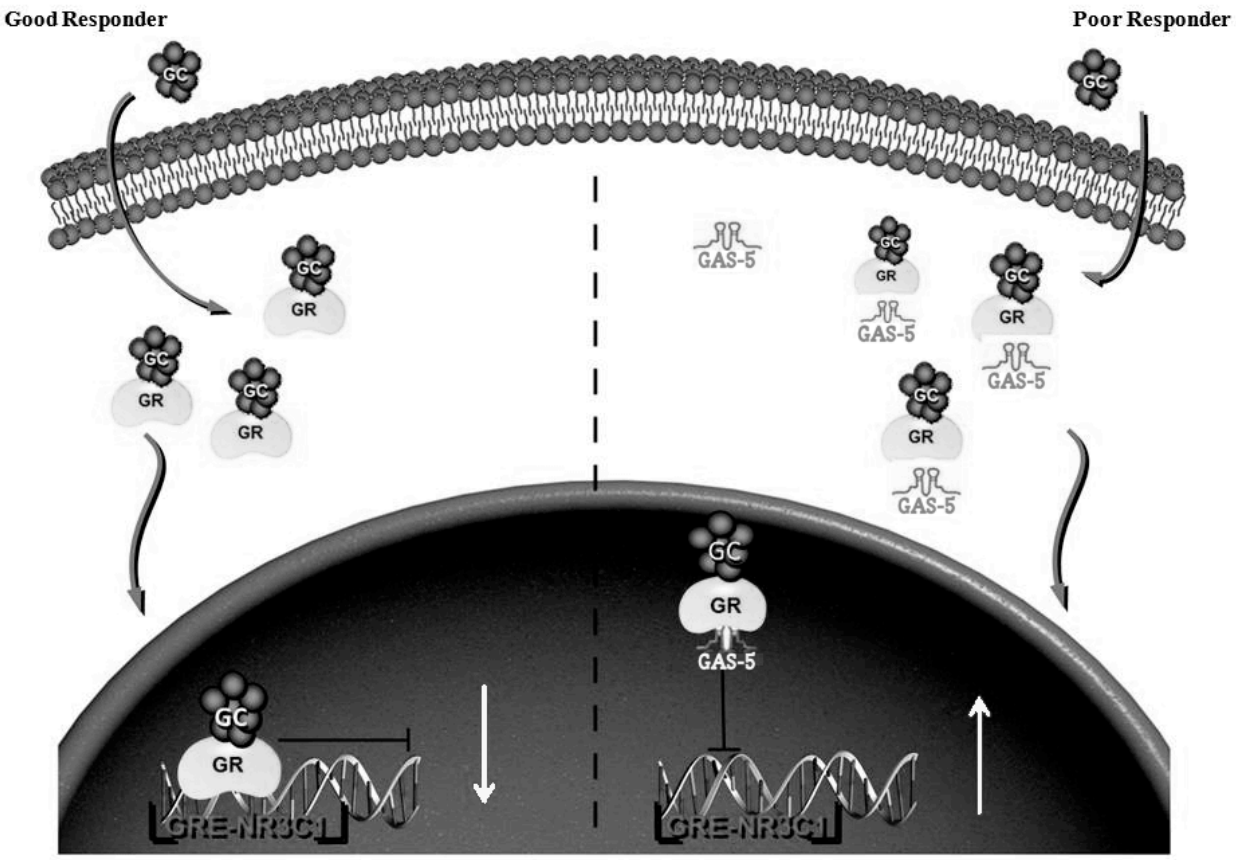

Fig. 3. Potential role of GAS5 in GC response and in the process of autoregulation of the GR.

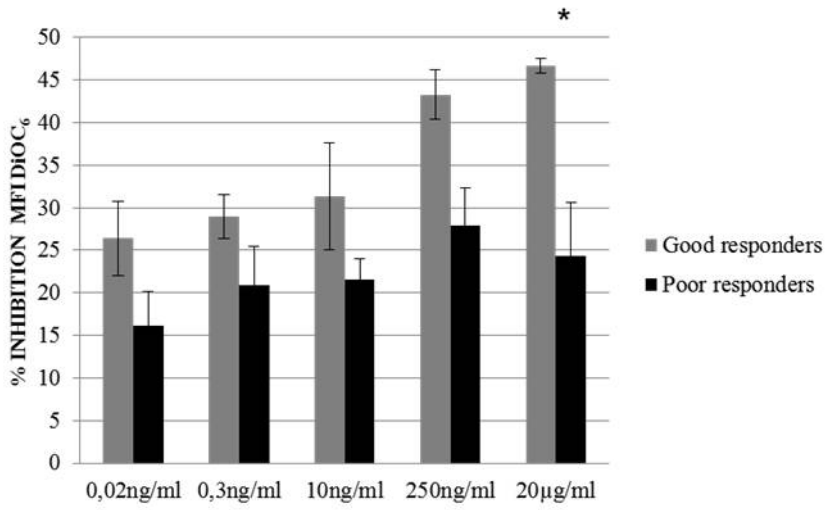

Fig. 4. Effect of 72 h incubation with MP on PBMCs obtained from two good and poor responder subjects: the histograms represent the percentage of inhibition of $DiOC_6$ fluorescence signal; Two way ANOVA: responder vs resistant interaction * p-value<0.05.