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Role of the Long Non-Coding RNA Growth Arrest-Specific 5 in Glucocorticoid Response in Children with Inflammatory Bowel Disease

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Abstract: Glucocorticoids (GCs) are widely employed in inflammatory, autoimmune and neoplastic diseases, and, despite the introduction of novel therapies, remain the first-line treatment for inducing remission in inflammatory bowel disease (IBD). 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. Recent evidence suggests that the long non-coding RNA (lncRNA) GAS5 could be a potential marker of GC resistance. To address this issue, we evaluated the association between the lncRNA GAS5 and the efficacy of steroids, in terms of inhibition of proliferation, in two cell lines derived from colon and ovarian cancers, to confirm the sensitivity and specificity of these lncRNAs. These cells showed a different sensitivity to GCs and revealed differential expression of GAS5 after treatment. GAS5 was up-regulated in GC-resistant cells and accumulated more in the cytoplasm compared to the nucleus in response to the drug. The functions of GAS5 were assessed by silencing, and we found that GAS5 knock-down reduced the proliferation during GC treatment. Furthermore, for the first time, we measured GAS5 levels in 19 paediatric IBD patients at diagnosis and after the first cycle of GCs, and we demonstrated an up-regulation of the lncRNA in patients with unfavourable steroid response. Our preliminary results indicate that GAS5 could be considered a novel pharmacogenomic marker useful for the personalization of GC therapy.

Due to their anti-inflammatory and immunosuppressive properties, glucocorticoids (GCs) are widely used in the treatment of many inflammatory and autoimmune diseases [1,2]. In particular, they play a critical role in the treatment of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), where they are used to induce remission in patients with moderate to severe disease [3]. However, a considerable interindividual variability in GC response has been documented [4,5]: close to 20% of patients are resistant to these agents, while 40% of patients become dependent from GCs for maintaining clinical remission. Presently, there are no means to predict patients' response to GCs in advance [6,7].

GCs diffuse across the cell membrane and exert their biological effects primarily by binding to the cytoplasmic GC receptor (GR) [8,9], which translocates into the nucleus and interacts, through its DNA-binding domain (DBD) [10,11], with steroid-responsive genes promoter regions known as GC responsive elements (GREs) [12–14].

Recent reports have shown that the growth arrest-specific 5 (GAS5) gene encodes for a long non-coding RNA (lncRNA) which can act as a riborepressor of the GR [15]. In particular, GAS5 exon 12-derived sequence has been shown to structurally mimic the GREs, preventing the binding of the activated GR complex to its target DNA sequences [16].

In previous studies conducted in our laboratory, peripheral blood mononuclear cells (PBMCs) obtained from healthy donors were *in vitro*-treated with methylprednisolone (MP) and two groups of subjects (good and poor responders) could be identified on the basis of the antiproliferative effect of the steroid. In poor responders, higher levels of GAS5 were evident in comparison with good responders, suggesting that this lncRNA could be involved in GC resistance [17,18]. The current investigation was therefore stimulated by the demonstration of the key role played by GAS5 in modulating GC response.

As the molecular basis of GCs resistance remains poorly understood, the goal of this study was to clarify the potential role of GAS5 in GC sensitivity. In this context, we chose two different human immortalized epithelial cell lines which endogenously express the GR and the lncRNA GAS5 to examine the correlation between GAS5 levels and the response to GCs, in terms of inhibition of proliferation. Preliminary data on the clinical significance of GAS5 in paediatric IBD patients treated with GCs were also obtained. The analysis of the mechanism of action of GAS5 confirmed that this lncRNA could be considered a biomarker to personalize therapy with GCs and a candidate therapeutic target for the development of new pharmacological approaches to restore GC sensitivity.

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Materials and Methods

Cell lines. The HeLa human cervical carcinoma (ATCC, CCL-2) and LoVo colorectal cancer (ATCC, CCL-229) cell lines were grown in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS, 1% L-glutamine 200 mM, 1% penicillin 10,000 UI/mL and streptomycin 10 mg/mL. Cell cultures were maintained according to standard procedures in a humidified incubator at 37°C and with 5% CO_2, and cell passage was performed once a week.

In vitro *viability assays.* The effect of MP (Sigma-Aldrich, St. Louis, MO, USA) on the proliferation of HeLa and LoVo cells was determined by labelling metabolically active cells with [methyl-^{3}H] thymidine (Perkin Elmer, Milan, Italy). Cells were seeded into a 96-well round bottom plate (2×10^5 cells/well) in the presence of MP (range from 0.019 ng/mL to 20 μg/mL). After 50 hr of incubation, cells were pulsed with [methyl-^{3}H] thymidine (2.5 μCi/mL) and the incubation was continued for an additional 22 hr. The radioactivity of the samples was determined by a liquid scintillation analyser (MicroBeta^2 plate counter, mod 2450-0010, Perkin Elmer, Milan, Italy). Raw counts per minute (cpm) data were converted and normalized to per cent of maximal proliferation for each experimental condition (cpm MP/cpm control*100).

Propidium iodide (PI; Sigma-Aldrich, St. Louis, MO, USA) was used to evaluate the integrity of the cell membrane and assess cell viability. Cells were seeded and treated as described above. After incubation, the cells were washed with PBS and then 10 μL of 0.1 mg/mL PI were added to each sample and incubated for 10 min. at room temperature. The fluorescence intensity was read by a FluoroCount Microplate Fluorometer (Packard, Germany) at an excitation length of 530 nm and emission length of 590.

Total RNA isolation. Total RNA was extracted using TRIzol reagent (Thermo Scientific, Carlsbad, CA, USA) according to manufacturer's instructions. The RNA concentration and purity were calculated by Nano Drop instrument (NanoDrop 2000, EuroClone®, Milan, Italy).

Quantitative real-time PCR (TaqMan®). Expression levels of GAS5 gene were evaluated by real-time RT-PCR TaqMan® analysis using the CFX96 real-time system-C1000 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). The reverse transcription reaction was carried out with the High Capacity RNA-to-cDNA Kit (Applied Biosystem, Carlsbad, CA, USA), and the real-time PCR was performed in triplicate using the TaqMan® Gene Expression Assay to assess GAS5 mRNA expression, according to the manufacturer's instructions. The expression levels of the selected transcripts were determined using the comparative Ct method ($2^{-\Delta C_t}$ method) [19]. GAS5 expression values were normalized using 18S as reference gene. The results are provided as the mean and standard error (SE) of three replicates.

Subcellular distribution of lncRNAs. The experiments were conducted on HeLa and LoVo cell lines treated with MP at the final concentration of 250 ng/mL. Cells were seeded (density of 1.5×10^5 cells for HeLa and 3.0×10^5 cells for LoVo) and incubated for 72 hr at 37°C and 5% of CO_2; after this time, cytoplasmic and nuclear fractions were obtained by the following protocol [20]. Cells were washed with cold PBS and resuspended in hypotonic buffer A: 20 mM Tris-HCl [pH 7.5], 10 mM NaCl, 3 mM $MgCl_2$, 10% glycerol and the protease inhibitors cocktail (Roche, Basel, Switzerland). After 1 min., NP-40 was added at 0.1% v/v final concentration for 5 min., and the cytoplasmic fraction was collected by centrifugation at 1500×g for 5 min. at 4°C. The pellet was washed with buffer A, and the nuclei were collected by centrifugation. The cytoplasmic fraction and nuclei were subjected to RNA extraction using TRIzol according to the manufacturer's protocol (Thermo scientific, Carlsbad, CA, USA). To verify optimal fractionation, Western blot of cytoplasmic (tubulin) and nuclear (PARP1) proteins was performed. The abundance and the correct migration of the GR into the nucleus after treatment with GC was also evaluated by Western blot.

Western blot. Cells (1×10^5) were cultured as reported above, collected and after washing with cold PBS, lysed using a lysis buffer composed of Tris-HCl 10 mM pH 7.4, EDTA 100 mM, NaCl 100 mM, SDS 0.1%, protease inhibitor cocktail 1%. The protein concentration of each sample was determined using the Pierce BCA Protein Assay (ThermoFisher, Carlsbad, CA, USA) to allow an equal loading of total proteins.

Western blot image acquisition was performed using the ECL detection kit (Amersham, Little Chalfont, UK) and the Alliance 4.7 software (UVITECH, Cambridge, UK). Quantifications were performed using the UVIband imager software (UVITECH).

The following primary antibodies were used in Western blot analysis: rabbit monoclonal anti-PARP 1/10 000 (Abcam Cat. No ab-32138, Cambridge, UK) mouse monoclonal antitubulin 1/1000 (Abcam Cat. No ab-5667, Cambridge, UK) and rabbit polyclonal anti-GR 1/300 (ThermoFisher Cat. No PA1-511A, Carlsbad, CA, USA).

RNA interference. Depletion of endogenous GAS5 was performed by RNA interference using HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) and siRNA specific for GAS5 (Qiagen; target sequence 5′-aacaagcaagcatgcagctta-3′). Shortly before transfection, 7×10^5 cells were seeded in six-well plates in 1.4 mL of complete medium. A total of 3 μL of 40 μM siRNA GAS5 was diluted in 100 μL of Opti-MEM (Life Technologies, Carlsbad, CA, USA), and 5 μL of HiPerFect Transfection Reagent (Qiagen, Hilden, Germany) was added to the diluted siRNA. After 10 min. of incubation, the complexes were added drop-wise onto the cells. After 24 hr, the same procedure was performed, with the exception that the cells were detached from the six-well plates and reseeded shortly before transfection. Three siRNA transfections were performed for each experiment. The siRNA against the firefly luciferase gene was used as control (Dharmacon Non-Targeting siRNA #2, Lafayette, AL, USA). The analysis of specific silencing of GAS5 expression was carried out after 48 hr after reseeding, using real-time RT-PCR, and transfection efficiencies (after 48 hr) were 70–80%.

Clinical samples. Nineteen IBD paediatric patients (mean age at enrolment 12.9 years, 16 UC and 3 CD, 9 males and 10 females) were enrolled at the Paediatric Clinic of IRCCS Burlo Garofolo in Trieste and treated with prednisone 1–2 mg/kg/day for 30 days according to standard clinical protocol. Peripheral blood was obtained from these patients at diagnosis (T0) and after 4 weeks of steroid treatment (T4).

RNA was extracted from patients' PBMCs at T0 and T4 and used to analyse the levels of the lncRNA GAS5. Clinical activity, inclusive of clinical and inflammatory markers evaluation, was assessed by 'Pediatric Crohn's Disease Activity Index' (PCDAI) for patients with CD, and by 'Pediatric Ulcerative Colitis Activity Index' (PUCAI) for patients with UC: clinical remission was defined as PCDAI < 10 or PUCAI < 10, while clinical improvement was defined as a reduction of at least 15 points from baseline score for PCDAI and of at least 20 points from baseline for PUCAI. Patients were classified on the basis of their clinical response into three groups: steroid-resistant (SR), patients who have active disease despite treatment with prednisone 2 mg/kg/day (max 50 mg/day) for 4 weeks; steroid-sensitive (SS) patients who did not relapse when therapy was discontinued after tapering and did not need GCs for at least 1 year, and steroid-dependent (SD) patients, who experienced disease relapse during steroid tapering or within 3 months after the steroid was stopped [21].

Statistical analyses. Statistical analyses were performed using GraphPad Prism version 4.00. Two-way ANOVA with Bonferroni post-test and *t*-test were used for the analysis of inhibition of proliferation and gene expression. The nonparametric Kruskal–Wallis test with Dunn's multiple comparison test was used for the analysis of gene expression in SS, SD and SR patients; *p*-values <0.05 were considered statistically significant.

Ethical considerations. Local ethical committee approval for the study (Prot 2198) was provided: all patients participated in this study in accordance with the principles outlined in the Declaration of Helsinki, and the parents of all the participating children gave written informed consent before the study began.

Results

Sensitivity to GCs.
To evaluate the effect of GCs on HeLa and LoVo cell lines, the [methyl-^{3}H] thymidine incorporation assay was performed after MP treatment at different concentrations (0.019 ng/mL–20 μg/mL) for 72 hr.

As shown in fig. 1, MP induced a concentration-dependent reduction in cell proliferation more evident on HeLa (I_{250ng} = 73%) than in LoVo cells (I_{250ng} = 16%) and these differences were statistically significant (fig. 1). These data demonstrated that LoVo cells can be considered a GC-resistant cell line while HeLa cells a GC-sensitive cell line.

To analyse the mechanism underlying the reduction of cell proliferation, observed during the treatment with MP, we performed an experiment on HeLa and LoVo cells using the PI dye. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI (necrotic and late apoptotic cells). No difference in PI fluorescence signal was evident between treated and untreated cells indicating that high concentrations of MP did not induce cellular membrane damage (data not shown).

Expression pattern of GAS5 during GC treatment.
To analyse the role of GAS5 in the variability of GC sensitivity, gene expression of this lncRNA was evaluated in HeLa and LoVo cells before and after treatment for 72 hr with MP at the concentration of 10 and 250 ng/ml. These concentrations were chosen on the basis of the results of the sensitivity assay.

In untreated HeLa and LoVo cells, no significant difference in basal expression levels of GAS5 was observed between the two cell lines (RE HeLa = 1.00; RE LoVo = 1.33; p = 0.265). After treatment with MP, a down-regulation of GAS5 in HeLa cells (GC sensitive) in comparison with untreated cells, and an up-regulation in LoVo cells (GC-resistant), was evident (fig. 2). The differences observed between the two cell lines were statistically significant at both concentrations.

Intracellular localization of GAS5 in response to MP.
To evaluate GAS5 distribution in cytoplasmic and nuclear compartments, subcellular fractionation was performed after treatment for 72 hr with MP (250 ng/ml) on HeLa and LoVo cells. In LoVo cells, a significant accumulation of GAS5 was evident in the cytoplasm compared to nucleus in which the levels were unchanged after treatment (RE citosol = 1.80; RE nucleus = 1.03); no difference between the two compartments was detected on HeLa cells (RE citosol = 0.87; RE nucleus = 0.84; fig. 3A). Yields of purified fractions of cytosolic and nuclear proteins were validated by Western blot (fig. 3B).

We also fractionated the HeLa and LoVo cells and measured the levels of GR proteins in the nucleus and in the cytoplasm. As expected (fig. 3B,C), in both cell lines GR expression significantly increased in the nuclear compartment after MP treatment.

GAS5 silencing induced reversion of GC resistance.
RNAi experiments were performed to knock down the lncRNA with a specific small interfering RNA (siRNA) for GAS5 in order to confirm its regulatory role in GCs responsiveness.

LoVo cells were transfected with siRNA for luciferase (siLUCI) as non-target control or GAS5 siRNA and incubated with MP at three different concentrations (10, 250 ng/mL and 20 μg/mL) for 72 hr: the cell lines transfected with GAS5

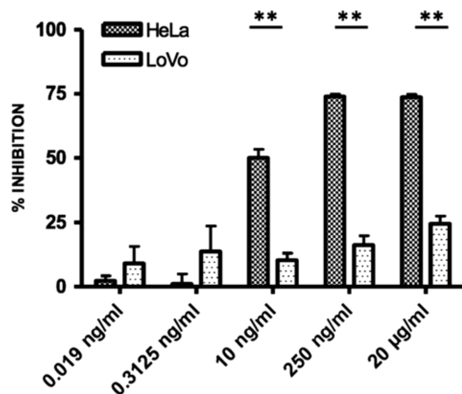

Fig. 1. Effect of MP on the HeLa and LoVo cells. Cells were exposed for 72 hr to MP at different concentrations, and cell proliferation was evaluated by the [methyl-3H] thymidine incorporation assay. Two-way ANOVA ($p < 0.0001$) and Bonferroni post-test **p-value<0.001. The data are reported as means ± SE of three independent experiments performed in triplicate.

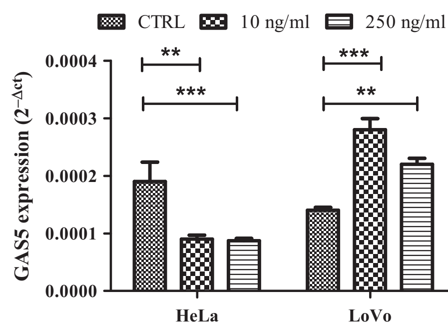

Fig. 2. Expression profile of GAS5 during GC treatment. GAS5 expression (values are expressed as $2^{-\Delta C_t}$) in HeLa and LoVo cells before (CTRL) and after treatment with MP for 72 hr at 10 and 250 ng/mL. Two-way ANOVA ($p < 0.0001$) and Bonferroni post-test ***p-value<0.001; **p-value<0.01. The data are reported as means ± SE of three independent experiments performed in triplicate.

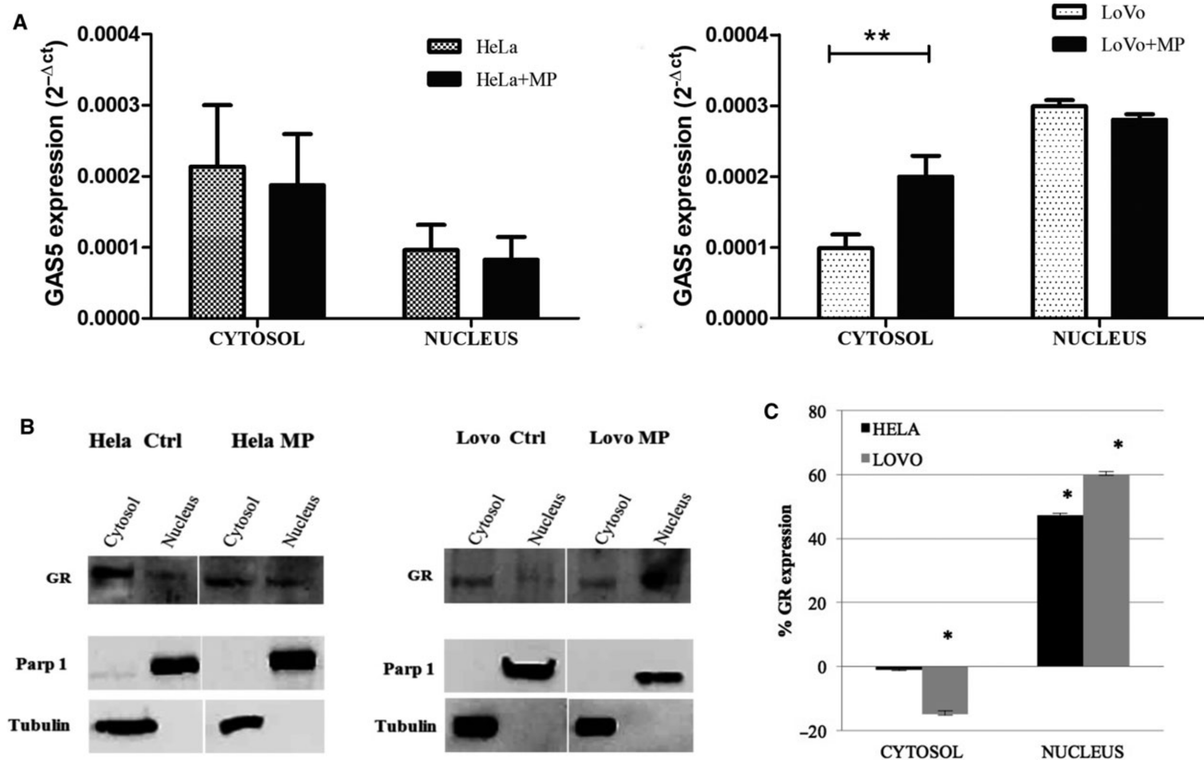

Fig. 3. Intracellular localization of GAS5 in response to MP. (A) Endogenous GAS5 expression values before and after treatment for 72 hr with MP (250 ng/mL). Two-way ANOVA (LoVo $p = 0.0120$) and Bonferroni post-test **p-value<0.01. (B) Protein expression of GR evaluated by Western blot analysis on subcellular fractions of HeLa and LoVo cells treated with MP (250 ng/ml) for 72 hr. (C) Percentage of GR expression evaluated in cytoplasm and nuclear compartments of HeLa and LoVo cells treated for 72 hr with MP in comparison with PARP1 and tubulin, respectively: GR expression in treated *versus* untreated cells in both compartments; Two-way ANOVA and Bonferroni post-test **p-value<0.01; *p-value<0.05. The data are reported as means ± SE of three independent experiments performed in triplicate.

siRNA showed an improved response to the drug. In particular, an inhibition of proliferation at 250 ng/ml and 20 µg/ml of 30% and 40%, respectively, was observed in LoVo cells siGAS5 compared to, respectively, 4% and 29% in siLUCI cells, demonstrating a key role of GAS5 in GC resistance (fig. 4). The same experiment was conducted on HeLa cells, and the results showed a similar trend: the inhibition of proliferation at 10 and 250 ng/mL MP in HeLa SiGAS5 was of 53% and 82% compared to HeLa SiLUCI (32% and 76%), confirming, once again that GAS5 could interfere with GC activity.

GAS5 as a marker of GC resistance in paediatric IBD patients.

The baseline characteristics of children enrolled in this prospective study are shown in table 1. GAS5 transcript levels were assayed by real-time RT-PCR analysis in PBMCs obtained from patients at diagnosis and after 4 weeks of treatment with prednisone 1–2 mg/kg/day. Among 19 enrolled patients, four were SR, eight SD and seven SS. No difference was observed in GAS5 levels among the three groups of patients at diagnosis (data not shown). Considering the relative expression after 4 weeks of treatment respect to diagnosis, SR patients presented significantly higher increase of GAS5 in comparison with SS and SD groups (fig. 5). In addition, the SD group presented higher levels of GAS5 in comparison with SS patients even though the results were not significant in the post-test analysis (fig. 5). Interestingly, patients with unfavourable steroid response (SD + SR) presented higher GAS5 level, further supporting a contribution of GAS5 to steroid ineffectiveness.

Discussion

GCs are effective inhibitors of cytokine secretion and T-cell activation, and are consequently largely employed in different inflammatory conditions, including IBD. In these diseases, GC resistance or dependence is particularly frequent, and the interindividual variability in response to therapy can limit their clinical use. Several distinct molecular mechanisms contributing to GC resistance have been identified, so that there is heterogeneity of mechanisms even within a single disease [22,23].

LncRNAs are major epigenetic regulators of gene expression at transcriptional and post-transcriptional levels, and they have also been reported to play a role in chemo-resistance by impairing the response to anticancer drugs [24].

The role of lncRNAs in GC resistance is poorly understood. Recent investigations in this field were conducted in our laboratory: in particular, we have demonstrated a correlation between the lncRNA GAS5 levels and the sensitivity to GCs *in vitro* [17,18].

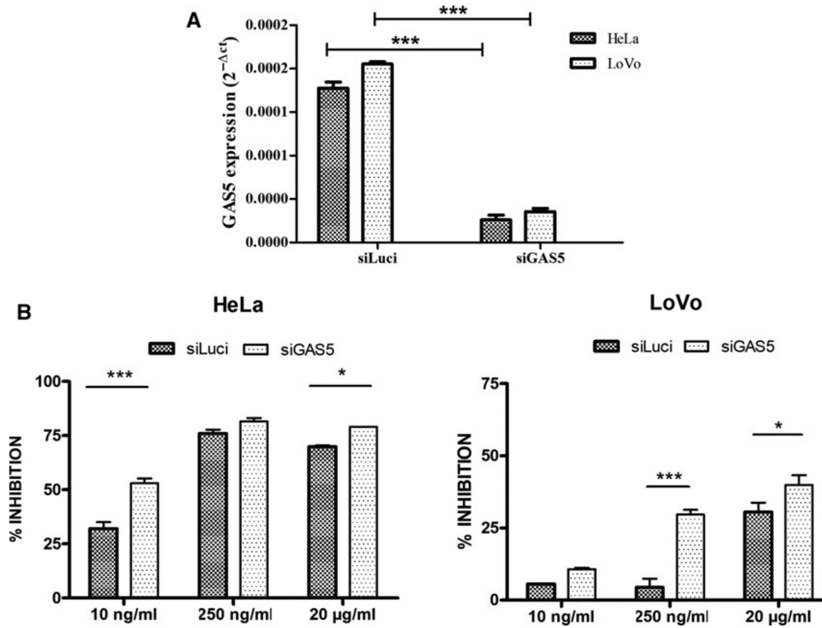

Fig. 4. GAS5 silencing sensitizes LoVo and HeLa cells to MP treatment. (A) GAS5 expression levels (values are expressed as $2^{-\Delta C_t}$) after silencing in HeLa and LoVo cells (Two-way ANOVA $p = 0.0056$ and Bonferroni post-test ***$p < 0.001$). (B) Effect of MP on cell proliferation of LoVo and HeLa cells transfected with control (siLuci) or GAS5-selective (siGAS5) siRNA. Two-way ANOVA (LoVo $p = 0.0026$; HeLa $p = 0.0033$) and Bonferroni post-test ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. The data are reported as means $\pm$ SE of three independent experiments performed in quadruplicate.

Table 1.

Clinical characteristics of the patients.

Age, mean (SD)	12.9 (3.8)					
Male (%)	9 (47.4)					
	10 (52.6)					
Female (%)	T0			T4		
	SS	SD	SR	SS	SD	SR
PCDAI score, mean (SD)	–	32.5 (3.5)	45 (0)	–	5 (0)	32.5 (0)
PUCAI score, mean (SD)	30.7 (23.1)	39.4 (5.5)	23.3 (11.5)	2.8 (3.9)	5.2 (7.1)	31.6 (33.3)
Laboratory indexes						
C-reactive protein, mean (SD), mg/dL	0.2 (0.3)	0.9 (1.4)	1.2 (0.8)	0.04 (0.1)	0.1 (0.1)	0.4 (0.2)
Erythrocyte sedimentation rate, mean (SD), mm/hr	24.7 (19.2)	54.6 (33.7)	46.5 (25.1)	10.7 (7.3)	28.3 (36.4)	26.1 (17.5)
Faecal calprotectin, mean (SD), µg/g	1813.6 (748.7)	1359.3 (1130.7)	1986 (0)	173.4 (267.2)	495.6 (836.6)	2085.5 (1502.6)
Haemoglobin, mean (SD), g/dL	12.6 (1.5)	10.1 (1.2)	11.5 (2.9)	13.7 (1.5)	12.1 (1.9)	13.1 (2.4)
Albumin (SD), g/dL	3.3 (0.6)	3.5 (0.4)	3.4 (0.7)	3.9 (0.5)	3.7 (0.4)	2.9 (0.6)

T0 = diagnosis; T4 = after 4 weeks of treatment; SS = steroid-sensitive, SD = steroid-dependent, SR = steroid-resistant.

In this report, we analysed the role of GAS5 in modulating GC response on HeLa and LoVo cell lines, which endogenously express the GR and the lncRNA GAS5. In our experimental conditions, MP was more effective on HeLa cells in comparison with LoVo cells. Assuming that GAS5 could be considered a key mediator of GC resistance, we examined the levels of GAS5 in HeLa and LoVo cells before and after treatment with MP.

Our studies revealed that, in untreated cell lines, no difference was evident in GAS5 levels between the two cell lines; as already demonstrated in our laboratory in PBMCs [17,18], suggesting therefore that the levels of GAS5 cannot predict the response to GCs in advance.

Treatment with MP caused changes in the balance of the lncRNA levels as GAS5 expression decreased in HeLa cells after treatment, whereas it was up-regulated in LoVo cells in the same experimental condition. It has been already reported that the up-regulation of GAS5 occurring in PBMCs from poor responders after treatment with MP could prevent the activated GR from binding to GRE elements [17,18]. The present data suggest that, even in LoVo cells, which are resistant to GCs, higher levels of GAS5 may alter GC effectiveness

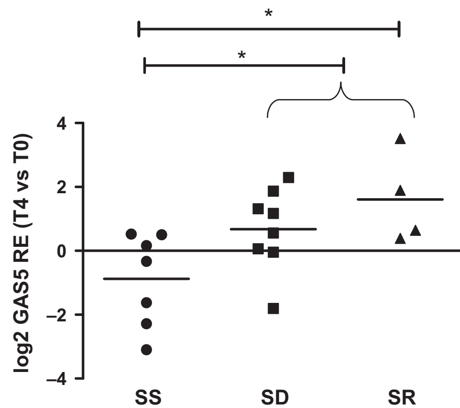

Fig. 5. GAS5 levels in paediatric patients with IBD during GC treatment. Relative expression (RE) of GAS5 in SS, SR and SD patients after treatment with prednisone 1–2 mg/kg/day for 4 weeks (T4) with respect to the diagnosis (T0). Overall GAS5 expression was different among GC responder groups (Kruskal–Wallis test $p = 0.033$). In particular, SR patients displayed higher levels of GAS5 than SS patients (Dunn's multiple comparison test $p < 0.05$). Moreover, analysis grouping patients with unfavourable response (SD + SR) showed higher GAS5 expression than in SS patients, Mann–Whitney test $p = 0.016$. *$p < 0.05$.

through the inhibition of their activity by inhibiting the binding of the activated GR to GREs.

Cellular localization is an important determinant in understanding the functional roles of lncRNAs [25]. To clarify whether the changes in the level of GAS5 mRNA were transcriptionally regulated, we have analysed the expression of this lncRNA considering its cellular localization before and after treatment with GCs in the two cellular models. Endogenous GAS5 was present in the cytoplasm and nucleus in both cell lines and, in untreated cells, no differences in its expression were observed between the cytoplasmic and nuclear compartments. After treatment with MP, levels of GAS5 were unchanged in the two compartments in HeLa cells. These results are in contrast to a previous report in which the authors described that dexamethasone increased GAS5 copy numbers in the nucleus in HeLa cells [15]. However, this discrepancy could be explained by the differential experimental setting; indeed, in this paper, we considered the endogenous levels of GAS5 on HeLa cells in contrast to Kino and colleagues, who examined the effect of the exogenous lncRNA. Furthermore, in Kino's study cells were treated with dexamethasone, a steroid with a higher potency and duration of action in comparison with MP [26].

A different profile was observed in LoVo cells, as treatment with MP caused an accumulation of GAS5 in the cytoplasm, the environment in which the GR is activated.

In the cytoplasm, lncRNAs play crucial roles in modulating mRNA stability, regulating mRNA translation and mediating protein functions. The increase in the amount of GAS5 in the cytoplasm during GC treatment could depend on many causes: for instance, expression of the GAS5 gene could be under post-transcriptional regulation in treated cells by another lncRNA or miRNA. In this context, a role of the lncRNA GAS5-AS1 could be suggested, a natural antisense transcript localized on the opposite strand and arranged tail-to-tail with GAS5 for a partial overlap of 40 terminal nucleotides. In agreement with this hypothesis, several ncRNAs target RNA transcripts and modulate their stability in the cytoplasm: among these, the antisense transcript for β-secretase 1 (BACE1-AS) [27] that positively regulates BACE1 mRNA, and the terminal differentiation-induced lncRNA (TINCR) that binds target mRNAs through a 25 nucleotide motif named the *TINCR box* [28].

To confirm that GAS5 could be considered a key mediator of GCs resistance, RNA interference experiments were performed to knock down the lncRNA with a specific siRNA. In LoVo cells (GC-resistant), transfection with GAS5 siRNA was associated with an increased response to MP after incubation with the drug at different concentrations, confirming that GAS5 interferes with GC effect.

Notably, levels of GAS5 were altered also in paediatric patients with IBD after treatment with GCs, while no difference could be detected before treatment. In fact, GAS5 levels differentiated patients with good or poor response to GCs. Nineteen paediatric IBD patients were treated with GCs and classified on the basis of their clinical response as steroid-resistant (SR), steroid-sensitive (SS) and steroid-dependent (SD); after 4 weeks of treatment, SR and SD patients presented higher levels of GAS5 expression in comparison with the SS group. We suggest that, similarly to *in vitro* data, in SR and SD patients, an altered expression of GAS5, through the interaction with the DNA-binding domain of the activated GR, may result in the suppression of GC transcriptional activity, thereby reducing their effectiveness.

In consideration of the complexity of GC mechanisms of action, we cannot exclude that other factors could be involved in GC resistance, particularly in some SD and SR patients which did not show an increase in GAS5 expression levels after treatment with MP. Until now, different candidates of resistance have been identified in IBD patients [7], even though reported genetic associations have not yet shown consistent or robust results, and for most of them, results are controversial [7].

In summary, our results demonstrate that GAS5 is differently expressed in sensitive and resistant cells and positively correlates with drug resistance, directly or indirectly modulating the drug's efficacy. In particular, the altered expression of endogenous GAS5 affects GC effectiveness, probably because of its accumulation in the cytoplasm and alteration at the post-transcriptional level. Further prospective studies should evaluate whether the levels of GAS5 in PBMCs, obtained from patients at diagnosis and *in vitro*-treated with GCs for 72 hr, may be predictive of clinical response to treatment. This easy and rapid *in vitro* assay could in fact help clinicians in predicting the effectiveness of treatment with GCs eventually suggesting early switching to other approaches. Moreover, inhibition of GAS5 by a specific molecule could be considered as a strategy to restore GC response in refractory patients.

In conclusion, if these results are confirmed in a larger number of patients affected by IBD or other chronic diseases, GAS5 expression in PBMCs should be considered a candidate marker of GC resistance.

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Conflicts of interest

All authors have read the journal's authorship agreement and policy on conflict of interests and have none to declare.

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