UNIVERSITÀ DEGLI STUDI DI TRIESTE

XXX CICLO DEL DOTTORATO DI RICERCA IN SCIENZE DELLA RIPRODUZIONE E DELLO SVILLUPPO

Identification of new molecular targets for personalized therapy in pediatric patients with inflammatory bowel disease (IBD)

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ABSTRACT

Inflammatory bowel disease (IBD) is a chronic immune-mediated condition of the gastrointestinal tract that includes Crohn disease and ulcerative colitis. Pediatric IBDs are of particular interest since their incidence is rising and, even if different pharmacological strategies are used, the optimal treatment is far from being achieved. Glucocorticoid (GCs) are prescribed for inducing remission but there is a high risk of adverse effects especially in subjects that poorly respond to these agents and require long treatments.

The long non-coding RNA (lncRNA) growth arrest-specific 5 (GAS5) interacts with the activated glucocorticoid receptor (GR), inhibiting the transcription of GCs responsive genes. The first part of my thesis project is focused on the study of GAS5 as a molecular marker of GCs resistance. We evaluated the association between the lncRNA and the efficacy of steroids, in terms of inhibition of proliferation, in two immortalized cell lines from colon and ovarian cancers, a GC-resistant and GC-sensitive model, respectively. After GCs treatment in the GC-resistant cells GAS5 upregulation was observed and, in response to the drug, the lncRNA accumulated more in the cytoplasm compared to the nucleus. Furthermore, we evaluated GAS5 levels in the peripheral blood mononuclear cells of pediatric IBD patients at diagnosis and after 4 weeks of GCs administration. Gene expression analysis have shown an upregulation of the lncRNA in patients with unfavourable steroid response. These preliminary results suggest that GAS5 could be considered a novel pharmacogenomic marker useful for the personalization of GC therapy.

GAS5 expression was also measured in IBD patients' colon biopsies and its levels have been evaluated with respect to the gene and protein expression of two metalloproteinases (MMP-2, MMP-9) involved in tissue damage in IBDs. The GAS5 downregulation observed in inflamed tissues compared with the non-inflamed one is inversely related to MMPs expression suggesting a role of this lncRNA in controlling the activity of these molecules.

In the second part of my thesis project we evaluated the role of the tristetraprolin (TTP) protein in IBDs. TTP is a zinc finger protein able to interact and inhibit pro-inflammatory cytokines through the binding with AU-rich elements on the 3' untranslated region on mRNA. The role of phosphorylation on TTP activity was also evaluated, since this post-translational modification could impair protein activity and consequently the stabilization of cytokines levels. TTP protein expression was studied in pediatric IBDs patients' colon tissues and in macrophages differentiated from peripheral blood mononuclear cells. An upregulation of TTP expression in both inflamed colon tissues and in macrophages of IBD patients was observed, and was closely related to the phosphorylation of the protein. These preliminary results, if confirmed with further

experiments, could open new perspectives in the study of IBDs and in the investigation of new target therapy based on the modulation of TTP phosphorylation by phosphatases to favour pro-inflammatory cytokines degradation.

RIASSUNTO

Le malattie infiammatorie corniche intestinali (MICI) sono un gruppo di malattie infiammatorie immunomediate che comprendono il morbo di Crohn e la rettocolite ulcerosa. Nella popolazione pediatrica le MICI sono di particolare interesse a causa della aumentata incidenza della malattia e, sebbene siano stati sviluppati diversi approcci terapeutici, è molto difficile individuare il trattamento ottimale. I glucocorticoidi (GC) sono farmaci prescritti per indurre la remissione ma alcuni pazienti risultano resistenti al trattamento o richiedono terapie prolungate e tali pazienti sono soggetti a numerosi reazioni avverse.

Il long non-coding RNA (lncRNA) growth-arrest specific 5 (GAS5) interagisce con il complesso GC-recettore dei glucocorticoidi (GR) inibendo l'attività trascrizionale dei geni responsivi ai GC. La prima parte del mio progetto di tesi si occupa di studiare il ruolo di GAS5 come marker molecolare della resistenza farmacologica ai GC. L'associazione tra il lncRNA e l'efficacia degli steroidi, espressa in termini di inibizione della proliferazione, è stata valutata su due linee cellulari tumorali di colon e ovaio che sono state identificate rispettivamente come modello di resistenza e sensibilità farmacologica ai GC. Inoltre, il ruolo di GAS5 è stato osservato nelle cellule mononucleate del sangue periferico di pazienti pediatrici affetti da MICI sia alla diagnosi che dopo 4 settimane di trattamento con GC; una maggiore espressione di GAS5 è stata osservata nei pazienti con una risposta sfavorevole agli steroidi. Questi risultati preliminari indicano che GAS5 potrebbe essere considerato un nuovo biomarker di resistenza farmacologica ai GC.

I livelli di espressione di GAS5 sono stati valutati anche nelle biopsie di colon di pazienti pediatrici affetti da MICI anche rispetto ai livelli di espressione proteica e genica di due metalloproteasi (MMP) coinvolte nel danno tissutale. La downregolazione di GAS5 osservata nei tessuti infiammati rispetto ai tessuti non infiammati è inversamente correlata all'espressione delle MMP suggerendo che il lncRNA potrebbe controllare l'attività di queste proteine.

Nella seconda parte del mio progetto di tesi abbiamo valutato i livelli di espressione proteica della tristetraprolin (TTP) nelle MICI. La TTP e una proteina zinc finger capace di interagire e inibire le citochine pro-infiammatorie attraverso il legame con gli elementi ricchi di AU sul 3' UTR degli mRNA target. Abbiamo considerato anche il ruolo della sua fosforilazione, poiché questa modificazione post-traduzionale interferisce con l'attività della TTP che in questo stato è responsabile della stabilizzazione delle citochine d'interesse. L'espressione proteica della TTP è stata valutata nei tessuti di colon e nei macrofagi dei pazienti pediatrici affetti da MICI. L'espressione della TTP risulta upregolata sia nei tessuti di colon che nei macrofagi. I risultati inoltre confermano il coinvolgimento della fosforilazione nell'attività della TTP. Questi risultati

preliminari, se confermati con ulteriori esperimenti, potrebbero aprire nuove prospettive nello studio delle IBD e nella formulazione di una nuova terapia farmacologica mirata in grado di modulare la fosforilazione della TTP attraverso l'uso di fosfatasi e favorire così la degradazione delle citochine pro-infiammatorie.

1.INTRODUCTION

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) is a chronic immune-mediated condition of the gastro-intestinal tract, IBD is considered a heterogeneous group of diseases that includes both ulcerative colitis (UC) and Crohn disease (CD) [1,2]. The incidence and prevalence of IBD is increasing, it is most often diagnosed in adolescent and young adult, with a rising incidence in pediatric population [3]. Indeed, approximately 25% of patients with IBD are before age of 20 years, among children with IBD, 4% before age of 5 years and 18% before age of 10 years [4,5]. The IBD study is of particular interest in children because of the negative consequence on growth, development and psychosocial function [6].

CD can affect any part of the gastrointestinal tract, from the mouth to the anus. Most commonly, in children, it involves the terminal ileum and colon. Endoscopic feature of CD showed a discontinuous inflammation and aphthous ulcers often in an irregular distribution [7]. CD typically demonstrates transmural inflammation that could cause disease complications such as fistulae and intra-abdominal or perianal abscess formation. Only in CD patients, perianal abscesses causing the formation of anal skin tags, fissures, fistulae and abscesses [2].

UC is characterized by continuous mucosal inflammation of the colon starting from the rectum and extending proximally [2,3]. In UC patients, the small bowel is not involved in the histopathology and it is possible observe the presence of backwash ileitis. The inflammation in UC patients, is much more superficial and largely limited to the mucosa [7] (Figure 1). Pediatric IBDs present a wide variety of symptoms, both gastrointestinal and extraintestinal. UC symptoms are more evident than on CD. In fact, UC patients initially present commonly abdominal pain and bloody diarrhea. A child with CD, when affected by colitis, may have bloody diarrhea or, otherwise, indistinct symptoms such as no-bloody diarrhea, weight loss or growth failure, fatigue, anemia and fever [6,8]. Extraintestinal manifestations are more common in CD patients and can involve the dermatologic, musculoskeletal, hepatic, ophthalmologic, renal, pancreatic or hematologic systems [2]. IBD is a multifactorial disease that involves a series of interactions between genetics, environmental factors, gut microbiota and the immune response [9]. In recent years, genome-wide association studies have identified more than 200 risk-associated loci with IBD [10]; many of the genes identified code for proteins involved in innate and adaptive immunity, autophagy and mucosal barrier integrity.

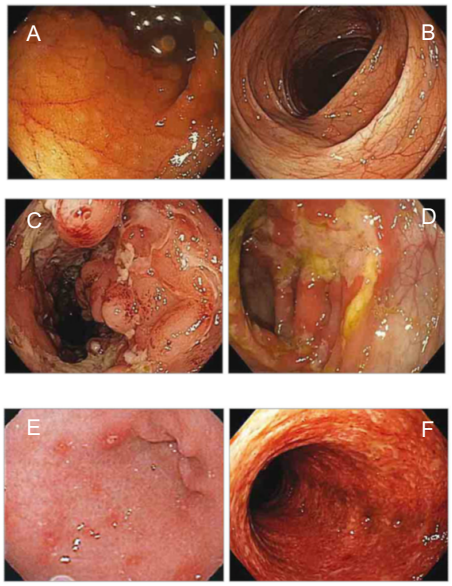


Figure 1 A. Normal vascular pattern, villous epithelium, and lymphoid nodularity. B. Normal mucosa and delicate vascular network. C. Terminal ileum in a child with CD. D. Linear ulcer directly adjacent to normal colon mucosa in a young child with CD. E. stomach antrum of a child with CD. F. An adolescent with UC [3].

This immune dysregulation could alter the intestinal microbial composition causing the chronic inflammation [11,12]. The tissue injury observed in IBD has a major role in the progression of the disease. On this perspective, an interesting role is attributed to a family of proteins called matrix metalloproteinases (MMPs). MMPs are a group of almost 20 proteins that are involved in the breakdown and reconstitution of extracellular matrix in physiological processes, like remodelling during development, growth and wound repair, or in pathological conditions as observed in arthritis or in tumor progression [13]. Among MMPs, the gelatinases MMP-2 and MMP-9 play a role in particular on the degradation of basement membrane type IV collagen, and in addition on collagen type I, V, VII, X, elastin, laminin and fibronectin [14]. MMP-2 and MMP-9 are involved in different mechanisms, among which intestinal tissue injury mediated by T cells in IBD [15]. Different studies demonstrated that MMP-9 and MMP-2 participate actively in the inflammatory and remodelling processes of IBD and a significantly increase of their expression was observed in inflamed tissues compared with non-inflamed colon mucosa of IBD patients [16,17]. Expression of MMPs was also evaluated in pediatric IBD patients and a prominent increase of expression was observed in the urine of CD and UC patients respect to a control group, demonstrating that MMPs could be considered non-invasive biomarkers in the evaluation of IBDs [18].

1.2 Role of cytokines in IBD

Genetic and environmental factors have a crucial role in the pathogenesis of IBD since a combination of both seems to initiate an alteration in epithelial barrier. An important role is covered by an aberrant and excessive cytokine response that causes subclinical or acute mucosal inflammation. In particular, mucosal immune cells (macrophages, T cells and innate lymphoid cells) produce cytokines that can promote chronic inflammation of the gastrointestinal tract [19]. Cytokines drive intestinal inflammation and associated symptoms in particular in progressive and destructive forms where intestinal stenosis, rectal bleeding, abscess and fistula formation are present [20,21]. Since 1980 it is known that in circulating immune cells and immune cells from intestinal lamina propria of IBD patients there is an altered cytokines production, however the functional relevance of these alterations is still unclear [22]. Lamina propria dendritic cells (DCs) and macrophages are key antigen-presenting cells (APCs) that have an important role in inflamed mucosa in IBDs. For instance, members of interleukin (IL)-12 family of heterodimeric cytokines (IL-12, IL-23, IL-27 and IL-35) are produced by APCs during intestinal inflammation. In fact, an increased expression of IL-12 in CD but not in UC has been observed [23]; similar results were described in CD patients for IL-23, a cytokine able to perpetuate local T helper 17 cells response and suppress regulatory T cells activity [24]. A significant decrease of IL-1RA was found in both CD and UC patients compared to control subjects, indicating an increased activation of IL-1 family of cytokines in IBD [25]. Furthermore, experiments on mice lacking IL-1β-converting enzyme (also known as caspase 1), that cleaves IL-1β and IL-18 into the active form, showed an improvement of DSS-induced colitis mice, suggesting that IL-1 family could be a target for therapy of chronic intestinal inflammation [26]. IL-6 production by lamina propria macrophages is also increased in experimental colitis and in IBD patients. IL-6 can regulate different pro-inflammatory functions trough the activation of multiple target cells such as APCs and T cells [27]. Interestingly, antibody-mediated blockade of IL-6 signalling suppressed chronic intestinal inflammation in mouse models and led to clinical response in CD patients. These effects were associated with a reduction of T cell apoptosis and reduction of other cytokines, such as interferon (INF) γ, tumor necrosis factor alpha (TNF-α) and IL-1β [28,29]. However, further studies are warranted to determine the therapeutic potential of this approach in IBDs. The well-studied cytokine TNF-α is produced both in membrane-bound and soluble form by lamina propria mononuclear cells and is markedly increased in IBD patients and also in macrophages, adipocytes, fibroblasts and T cells [30–32]. TNF-α induces different pro-inflammatory effects in colitis, binding to its receptors TNFR1 and 2 and activating the transcription factor nuclear factor-κB (NF-κB); as a consequence, angiogenesis, production of metalloproteinases by

myofibroblasts, activation of macrophages and effector T cells are induced [33–35]. Treatment of IBDs with antibodies that neutralize both soluble and membrane-bound TNF-α (infliximab and adalimumab) is highly effective, improving mucosa healing: indeed, anti TNF-α monoclonal antibodies are well established therapies in IBDs [36]. Moreover, other studies have been conducted on the use of new recombinant antibodies able to neutralize pro-inflammatory cytokines or on the administration of recombinant anti-inflammatory cytokines. Indeed, other cytokines in addition to the TNF-α have a fundamental role in controlling mucosal inflammation in IBD [19] (Figure 2).

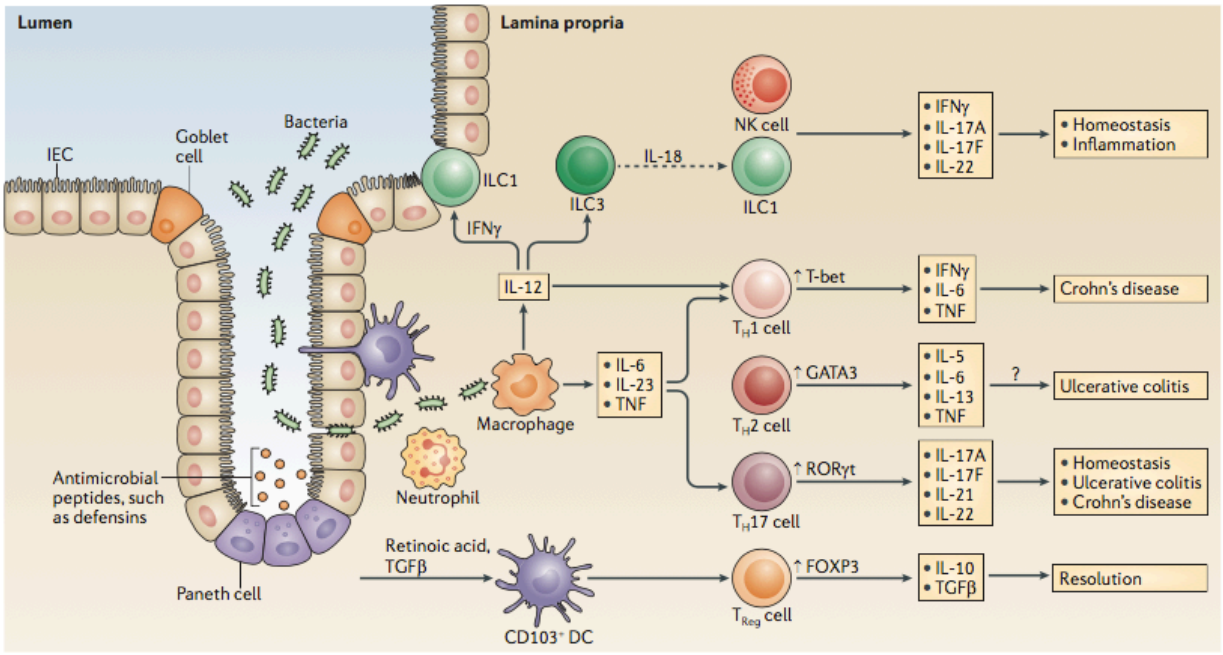


Figure 2: Cytokines in the pathogenesis of IBDs [19].

1.2.1 Tristetraprolin and cytokines regulation

Cytokines, chemokines and other proteins involved in the inflammatory response are encoded by relatively short-lived messenger RNA (mRNA). These transcripts usually contain *cis*-acting elements in the 3-untraslated region (3'UTR) rich in adenosine and uridine rich elements (AREs) that contribute to the regulation of mRNA, directing the rapid degradation of the transcript or its stability [37]. These effects on post-transcriptional fate of mRNA depend on the interaction with *trans*-acting RNA binding proteins that recognize the sequence UUAUUUAUU in the 3'UTR [38]. Several ARE-binding proteins have been described, but only for a few of them a clear evidence of a role in the mRNA stability has been demonstrated, among them a protein belonging to the TPA-inducible sequence 11 (TIS11) family called tristetraprolin (TTP) has

been largely studied [39]. TTP is a member of a family of zinc finger proteins of the unusual Cys-Cys-Cys-His (CCCH) class (Figure 3).

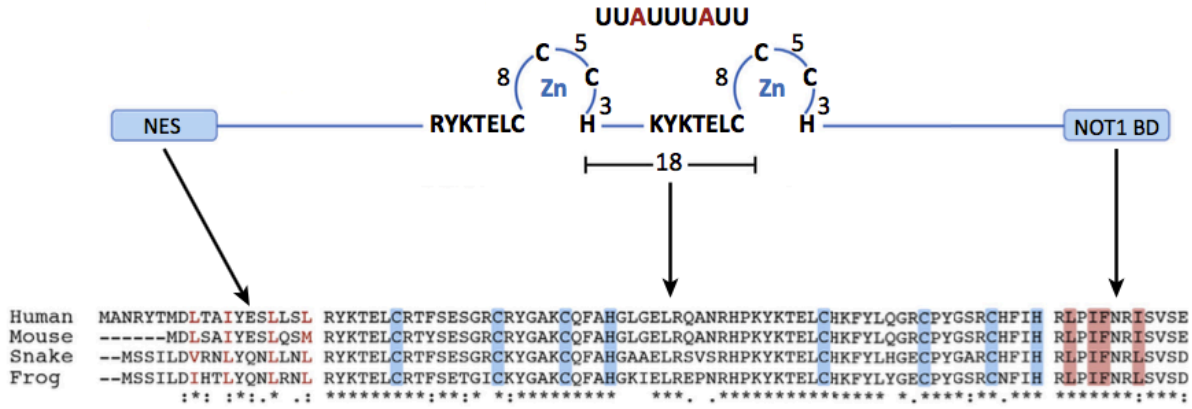


Figure 3: A schematic diagram of three critical domains of TTP: The N terminal nuclear export sequence (NES), the central tandem CCCH zinc finger domain, the C terminal NOT1-binding domain (NOT1 BD), the key cysteines in each finger and the conserved sequences of the Zinc finger domain [40].

The protein, also known as 12-O-tetradecanoyl phorbol 13 acetate-inducible sequence 11a, G0/G1 switch gene 24, nuclear protein 475, is encoded by the zinc finger protein of 36 kDa (*Zfp-36*) in mouse cells and *ZFP36* in human cells, which map to chromosomes 7 and 19q13.1, respectively [41]. TTP is widely distributed in the spleen, lymph nodes and thymus [42]. First studies demonstrated the role of TTP in both quiescent and serum-stimulated fibroblast; in subsequent researches a rapid translocation from the nucleus to the cytoplasm upon specific stimulations is observed [43,44]. Afterwards, more in-depth studies have demonstrated an exclusively high expression of TTP in the cytosolic compartment of human macrophage cell lines and in primary mouse macrophages [45]. Studies in TTP-deficient mice have suggested, for the first time, a link with cytokine TNF-α, indeed these animals appeared normal at birth but rapidly showed a severe syndrome of growth retardation, cachexia, arthritis, inflammation, autoimmunity, together with an over-expression of TNF-α in macrophages [46]. Since this behaviour suggests a role in controlling the TNF-α expression, the ability of TTP to destabilize mRNA through the ARE sequence found in 3'UTR of TNF-α was investigated. Indeed, a direct binding between the TTP zinc finger domain and ARE sequence on TNF-α was observed to which a series of steps follow that start from the remove of the poly(A) tail to the subsequent mRNA degradation [47]. Zinc finger proteins are able to recruit several proteins or protein complexes that participate to mRNA regulation [48]. These include: deadenylases, that shorten the 3'-poly(A) tail; decapping enzymes, that remove the 7-methylguanylate cap from 5'-end of mRNA; and exonucleases, that enhance the mRNA degradation from either the 5' or 3'end [49]. Therefore, the binding of TTP to AREs

on the 3'UTR of mRNA targets recruits the Ccr4/Caf1/Not1 deadenylases complex that shortens the poly(A) tail (Figure 4).

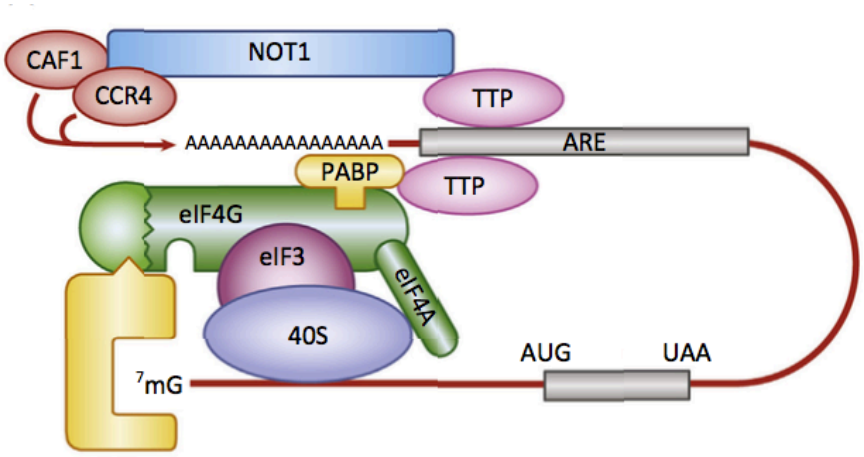


Figure 4: Model of the TTP mechanism of action. Tristetraprolin (TTP) binds to AU-rich elements (ARE) sequence of its target mRNAs and interacts with both polyA-binding protein (PABP) and NOT1, which can bring its associated deadenylases the chromatin assembly factor-1 (CAF1) and C-C chemokine receptor type 4 (CCR4) into proximity, resulting in the destabilization and decreased translation of the mRNA [40].

When the poly(A) tail becomes too short, the decappining enzymes and exonucleases guide a rapid degradation of mRNA transcripts. The poly(A) tail not only protects mRNA from degradation, but also promotes mRNA translation through the interaction between poly(A)-binding proteins and 5'-cap-binding proteins [50]. A number of papers describe different TTP targets among which many are cytokines involved in inflammatory response such as TNF-α, IL-1β, IL-2, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and many others [51].

1.2.2 Role of phosphorylation in TTP activity

The TTP protein is subject to extensive post-translational modifications, particularly phosphorylation. After a pro-inflammatory stimulus, the p38 mitogen-activated protein kinase (MAPK p38) plays a central role in the expression of different mediators of inflammatory response. The kinase operates through the downstream kinase MAPK-activated protein kinase 2 (MK2). TTP is phosphorylated by this pathway at mouse serines (Ser)-52 and Ser-178 (Ser-60 and Ser-186 in human), abrogating its destabilizing activity on mRNA transcripts and, as a consequence, a more pro-inflammatory cytokines expression is evident [52–54]. Furthermore, in primary macrophages and in a murine macrophages cell line (RAW264.7) the addition of

MAPK p38 inhibitor leads to a rapid degradation of TTP through the proteasome complex, demonstrating that phosphorylation is also necessary to protect TTP from its-self destruction [55]. This effect could be explained by the interaction of TTP with 14-3-3 proteins, a family of dimeric proteins that modulate the localization and function of the many phosphoproteins with which they interact. 14-3-3 not only changes the structure of TTP in a more stable one, but also inhibits the recruitment of Ccr4/Caf1/Not1 complex, improving protein translation [56,57] (Figure 5).

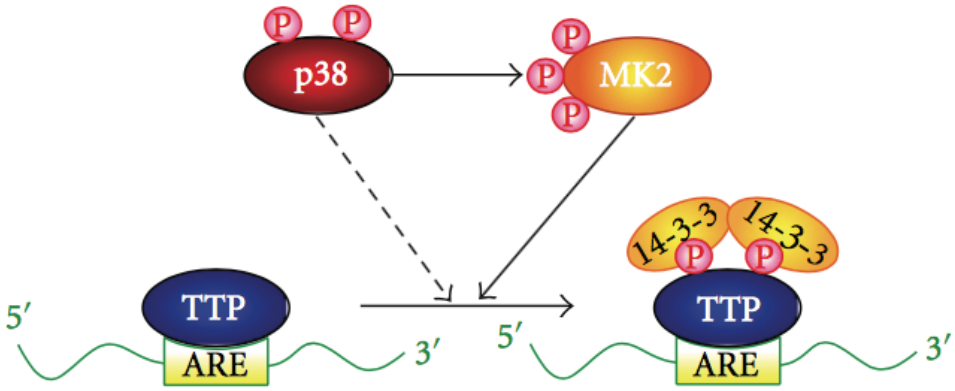


Figure 5: Role of phosphorylation in TTP activity. Unphosphorylated tristeraprolin (TTP) binds to the AU-rich elements (ARE), promoting degradation of the mRNAs. p38 mitogen-activated protein kinase (p38), trough MAPK-activated protein kinase 2 (MK2), phosphorylates TTP at two serine residues, allowing the interaction with 14-3-3 adaptor proteins and stabilizing its target mRNAs and protecting itself from proteasome activity [58].

These data open new perspectives on the control of inflammatory conditions. Indeed, a strong TTP protein expression has been demonstrated in inflamed synovium of rheumatoid arthritis (RA) patients. The protein was co-localized with the active MK2 in the cytoplasm of RA synovial macrophages, corroborating the hypothesis that inactive/phosphorylated TTP can be involved in a prolonged inflammation [59]. Moreover, the link between the MAPK cascade and TTP activity was also demonstrated in primary cells obtained from asthmatic patients, indeed an over expression of phosphorylated TTP was observed concurrently with MAPK p38 peak levels of activation [60]. These findings highlighted a role of TTP in orchestrating the response to pro-inflammatory stimuli even if a lot remain unknown about its regulation.

1.3 Therapeutic approaches in pediatric IBD

The main purpose in the pharmacological treatment of pediatric IBDs is to induce and maintain clinical remission and minimize adverse effects as much as possible. In the last decades a number of drugs have been used for the treatment of IBDs [61]. The choice of the appropriate

treatment for the IBD patient may be done using a "top-down" or a "step-up" approach. The step-up therapy starts with locally active agents, including aminosalicylates and antibiotics, followed by prednisone or budesonide and then escalates to immunomodulators, biologics (anti-TNF agents), or surgical intervention if the disease worsens. The top-down therapy, used principally in patients with a moderate to severe disease, initiates with biologics to ensure a rapid induction of the mucosal healing and then downgraded to other drugs [62,63] (Figure 6). The therapy depends on the disease location and severity. The treatment could induce the clinical remission or maintain the remission. The induction therapy includes the exclusive enteral nutrition, corticosteroids, antibiotics, 5-aminosalicylates, only for mild to moderate cases, and biologics; the maintenance therapy, instead, comprises the 5-aminosalicylates, immunomodulators (thiopurines and methotrexate) and biologics, used in patients that not respond to other treatments or when the step-up approach failed [6,61].

The principal goal of ongoing studies is to identify clinical markers that can help to provide a personalized therapy for pediatric IBD patients that could improve the mucosal healing and reduce the side effects observed after prolonged therapy.

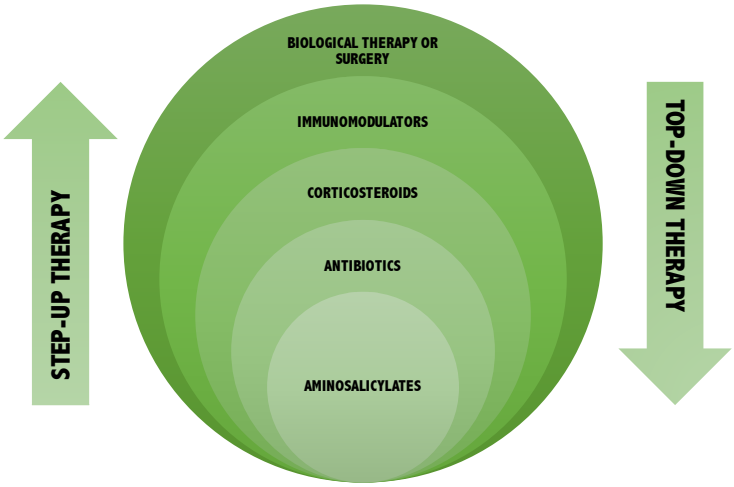


Figure 6: Step-up versus top-down treatment approach for IBD

1.3.1 Personalized therapy in IBD

The principal aim in IBDs treatment is to face the great inter-individual variability in pharmacological response. The study and the discovery of new biomarkers related to the disease could be useful in developing new strategies for future drug targeting.

On this panorama pharmacogenetics and pharmacogenomics studies have a primary role in discovering new markers for drug response. Pharmacogenetics analyze associations between patients' response and variants located on genes involved for instance in the metabolism of the drugs [64,65].

To date, different genetic variations have been associated with the pharmacological response in IBD for almost all classes of drugs, but only few of them are approved in clinical practice [66]. In particular, genetic test for thiopurine-S-methyltransferase (TPMT) is a normal procedure in many centres before starting thiopurines treatment. TPMT is responsible for the metabolism of drugs such as thiopurines (6-mercaptopurine and 6-thioguanine) and azathioprine, determining their pharmacological efficacy and toxicity [67,68]. Variations on patients genome are associated with a different enzymatic activity [66]. In fact, population studies have shown a trimodal distribution of TPMT enzymatic activity: 0.3% of the Caucasian population has a low-to-absent activity, around 10% an intermediate activity and 90% a normal-to-high enzyme activity [69]. Patients with low-to-absent activity produce high thioguanine nucleotide levels leading to an increased risk of developing bone marrow toxicity; in this case low doses of thiopurines are prescribed [70]. Many other genetic variations, such as that of glutathione-S-transferase and inosine-triphosphate-pyrophosphatase genes, are also involved in the thiopurines metabolism but their importance in the clinical practice remains controversial [66].

The introduction of anti-TNF therapy has improved the outcome of patients with IBD. Despite this, almost one third of the patients fail to respond to the treatment [71]. Different pharmacogenetics studies have been conducted to investigate variations that could interfere with anti-TNF therapy: in particular genes involved on the TNF-α pathway and drugs response, such as the TNF receptor superfamily 1A and 1B, TNF-α-induced protein 3 gene, interleukin-23, nucleotide-binding oligomerization domain-containing protein 2 and others have been studied. Nevertheless, studies on these variants have not demonstrated a sufficient sensitivity or specificity to be introduced in daily clinical management [66]. The personalized medicine for the treatment of IBD still need further investigation that could be improved with a genome-wide association and a deepen pharmacogenomics approach.

1.4 Glucocorticoids in the treatment of IBD

Few many studies report the use of corticosteroids in pediatric IBD and for this reason most of the clinical strategies in children are extrapolated from the experience in adults. In children, glucocorticoids (GCs) are prescribed for inducing remission with moderate to severe disease when the exclusive enteral nutrition is not possible. In addition, in children with mild to moderate ileo-cecal CD, budesonide or systemic corticosteroids are used to induce remission [12].

However during GC treatment there is a high risk for adverse effects principally related to the dose and the length of treatment [72] and no biomarkers are still available to predict the response to corticosteroids and reduce the risk of developing adverse events [73,74].

1.4.1 GCs: synthesis and release

Natural GCs are cholesterol-derived hormones generated through an enzymatic process termed steroidogenesis [75]. Cortisol is the biologically active GC in humans and is secreted by the adrenal gland and activated by the hypothalamic-pituitary-adrenal axis after a stressor stimulus, cytokine and endocrine signals [76]. The hypothalamus leads to the secretion of the corticotropin-releasing hormone, responsible, in the anterior pituitary, of the adrenocorticotropic hormone (ACTH) release [77] (Figure 7A). In turn, ACTH induces the synthesis and secretion of cortisol from the cortex of the adrenal glands into the bloodstream, resulting in a systemic effect [78]. Endogenous GCs regulate different biological functions that involve the development, growth, metabolism, behaviour and apoptosis [79]. Due to their multiplicity of roles, synthetic GCs are largely used in inflammatory, autoimmune and proliferative diseases [80] (Figure 7B).

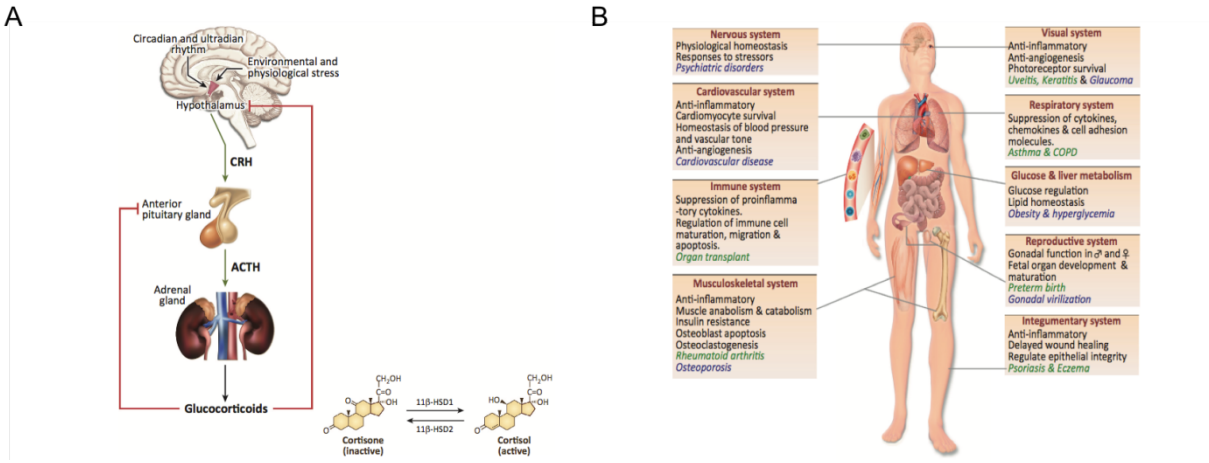


Figure 7: A. Schematic representation of the synthesis of cortisol by the hypothalamic–pituitary–adrenal (HPA) axis. Corticotropin-releasing hormone (CRH); adrenocorticotropic hormone (ACTH); 11b-hydroxysteroid dehydrogenase type 1 (11β-HSD1); 11b-hydroxysteroid dehydrogenase type 2 (11β-HSD2). B. Role of glucocorticoids in major organ systems (black text), beneficial roles of glucocorticoids in the clinic (green text), and adverse outcomes in patients with elevated glucocorticoid levels (blue text) [81].

Synthetic GCs, such as the commonly prescribed prednisone, dexamethasone (DEX), methylprednisolone (MP) and budesonide chemically differ from natural GCs to improve their activity. Indeed, the 11β-hydroxysteroid dehydrogenases (11β-HSDs) is involved in transformation active hydroxy- to inactive oxo- of cortisol but not of synthetic GCs [81]. Other

chemical modifications have potentiated the anti-inflammatory activity, like the double bond between the carbon atoms 1 and 2 of prednisolone or the methyl group added in position 6α of the methylprednisolone. DEX and betamethasone derive from modifications of the prednisolone structure with a fluorine atom in position 9α and a methyl group on carbon 16 [82], resulting in a 25-fold increase of the anti-inflammatory potency in comparison to cortisol, and a longer plasmatic and biological half-life [78].

1.4.2 GCs mechanism of action

The classic genomic pathway of GCs mechanism of action starts with the entering of GCs into the cell through passive diffusion, facilitated by their relatively small size and lipophilic nature and, once in the cytoplasm, bind the glucocorticoid receptor (GR) a ligand-inducible transcription factor, member of the nuclear receptor superfamily. In the inactivated form the GR resides in the cytoplasm in a multimeric complex that, thanks to the binding with different chaperones like the heat-shock proteins (Hsp) 90 and Hsp 70, immunophilins and others, is able to maintain the right folding for GCs recognition, preventing also its degradation [83]. When the GCs enter in the cytoplasm they can bind to the GR which, through conformational changes, dissociates from chaperones and becomes active. The complex GC-GR enters into the nucleus where it interacts with specific DNA sequences, the so called GC-responsive elements (GREs), regulating the transcription of GC target genes [84] (Figure 8). The GR is composed of three major domains: a N-terminal transactivation domain (NTD), a central DNA-binding domain (DBD), and a C-terminal ligand binding domain (LBD). LBD consists of 11 α-helices and 4 β-strands that fold into a three-layer helical domain that forms a cavity housing the GC molecule. The DBD is responsible of the recognition and binding with GRE regions and of the recruitment of another GR protein for dimerization that enhances the transcriptional activity of the receptor [85]. Among GCs target genes there are most encoding for anti-inflammatory proteins, including lipocortin-1, IL-10, IL-1 receptor antagonist (IL-1RA) and neutral endopeptidase [86]. GCs mechanism of action could have also a negative effect on gene target transcription. Different studies demonstrated the presence of a negative glucocorticoid-responsive element (nGRE), that differs from the positive GREs for few nucleotides, that mediates glucocorticoid-dependent repression of target genes by recruiting corepressors (nuclear receptor corepressor 1 and silencing mediator of retinoid and thyroid hormone receptor) and histone deacetlyases (Figure 8). GCs exert most of the anti-inflammatory activity through protein-protein interactions, with DNA-bound transcription factors, such as the Jun subunit of the activator protein-1 and the p65 subunit of NF-kB, interfering with their activity (Figure 8). Additionally, GR can interact with

members of the signal transducer and activator of transcription (STAT) family, to enhance transcription of certain target genes (Figure 8) [87,86].

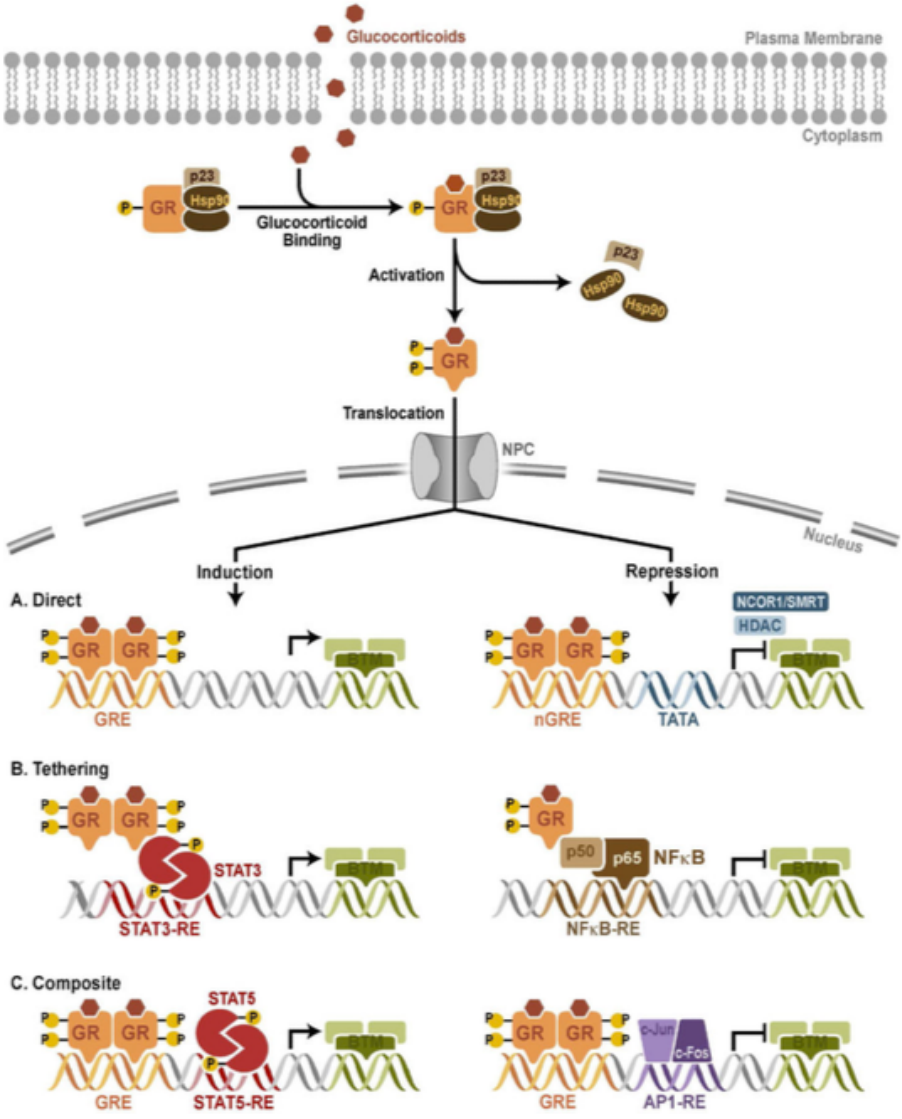


Figure 8: GCs mechanism of action by the binding with cytoplasmic GR that undergoes a conformation change, dissociates from multi-protein complex (heat-shock proteins (Hsp) 90 and Hsp 70), and translocates into the nucleus, where it regulates gene expression. GR activates or represses transcription of target genes by direct GC-responsive elements (GRE) binding, by tethering itself to other transcription factors apart from DNA binding, or in a composite manner by both direct GRE binding and interactions with transcription factors bound to neighboring sites. NPC = Nuclear pore complex; HDAC= histone deacetlyases; NCOR1= nuclear receptor corepressor 1; SMRT1= silencing mediator of retinoid and thyroid hormone receptor; STAT= signal transducer and activator of transcription; nGRE = negative GRE; AP1= activator protein-1; RE = response element [87].

1.4.3 GCs therapy in IBD and pharmacogenetics

The first clinical use of GCs dates on the late 1940s, when the Nobel Prize Philip Hench used cortisone to treat symptoms of rheumatoid arthritis [88]. Since then GCs have revolutionized the

field of medicine, indeed, they are being prescribed for chronic inflammatory conditions and for their autoimmune and anti-proliferative activity [89]. GCs are largely used in IBD for inducing remission in patients with moderate to severe disease, but a great inter-individual variability have been observed both in adults and in children. Indeed, up to 90% of pediatric patients have a rapid improvement of symptoms after GCs treatment, however, after 1 year only the 55% of these patients are still in remission (steroid-responsive), almost the 40% have an increase in disease activity when the dose is reduced (steroid-dependent) and 10% of pediatric patients do not respond to GC therapy (steroid-resistant) [90,91]. A number of severe side effects are evident, in particular in steroid dependent patient, who need prolonged treatment with these drugs, such as Cushing syndrome, psychologic disturbance, osteoporosis, metabolic disease, increased risk of cardiovascular disease and others. In the literature, different SNPs on genes involved in the GCs response were studied among which the glucocorticoid receptor gene nuclear receptor subfamily 3, group C, member 1 (*NR3C1)*, the ATP-binding cassette subfamily B (*ABCB1*) encoding the P-glycoprotein, the co-chaperone FKB51 encoding by the *FKB51* gene, the *TNF* and the gene for the multiprotein complex NACHT leucine-rich-repeat protein 1 (*NALP1*) [92]. In particular, one of the most studied polymorphism, the *Bcl*I on *NR3C1* gene, has been related to an increased response to GCs in a cohort of pediatric patients with IBD treated with prednisone 1-2 mg/kg/day for 2–4 weeks, suggesting that patients with this mutation are less likely to need additional courses of steroid treatment [93]. Despite these knowledges, the mechanism behind the GC resistance is still not clear. The study of GR regulation in terms of protein-protein, DNA-protein and RNA-protein (such as microRNA and long non-coding RNA) interactions could be of particular interest [85].

1.5 Non-coding RNAs

1.5.1 Definition and biogenesis of ncRNA

In the past two decades new technologies such as next generation sequences have revealed that most of the genome is transcribed into RNAs even if only 2% codes for proteins; the remaining is called non-coding RNA (ncRNA) [94]. NcRNAs can be divided in two classes, the first one ranges from few to 200 nt is called small non-coding RNAs and the other one, longer than 200 nt to several kilobases, is called long non-coding RNA (lncRNA). Among the small ncRNA, the microRNAs (miRNAs) are the most known and studied group [95]. miRNAs are expressed endogenously and during biogenesis they pass a multistep process that includes: transcription, nuclear maturation, export and cytoplasmic processing. Most miRNAs are transcribed as polycistrons by the RNA polymerase II (Pol II) [96]. Primary miRNAs (pri-miRNA) exhibit

modifications similar to that observed in mRNA, like the 5' capping and the 3' polyadenylation [97]. The pri-miRNAs are recognized and cleaved by the enzyme RNAse III endonuclease Drosha forming an hairpin precursor of 60 nucleotides (pre-miRNA) [98], the exportin-5 and RAN-GTP mediate the transfer of pre-miRNAs from the nucleus to the cytoplasm [99]. The RNase III endonuclease Dicer then cleaves the precursor, releasing an RNA duplex of 22 nucleotides. The mature miRNAs are obtained through the combined action of Argonaute proteins associated to the miRNA-induced silencing complex (miRISC) action [100] (Figure 9). The class of lncRNAs is of emerging interest among the non-coding transcriptome. Regarding the biogenesis they are quite similar to protein synthesis [101], in fact for example a large proportion on lncRNAs is capped and polyadenylated [102]. Even if they are present in lower amounts respect the other classes, their expression is more restricted to specific cell types [103].

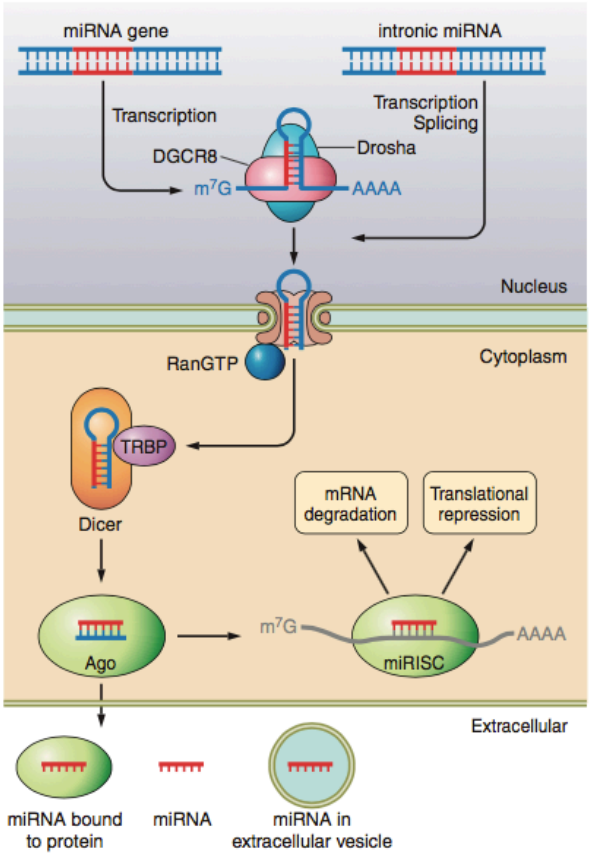


Figure 9: Biogenesis and function of miRNAs. miRNAs are transcribed as longer precursors or are derived from introns, and mature via endonucleolytic processing. Mature miRNAs are incorporated into the RNA-induced silencing complex (RISC) and regulate target transcript expression by degradation or translational repression. miRNAs can be secreted into the extracellular space, stabilized in vesicles or protein binding complexes. DGCR8= DiGeorge syndrome chromosomal region 8; RanGTP= Ras-related nuclear protein-GTP; TRBP= TAR RNA binding protein; Ago= Argonaute protein [101].

The biogenesis occurs in the nucleus and, like miRNAs, are transcribed by the Pol II. lncRNA

can be found in different compartments of the cell, indeed, after biogenesis and processing, several lncRNA are released in the cytoplasm but most of them are retained in the nucleus and are recruited to the chromatin [104]. It is currently unknown the lncRNA functions and how lncRNA primary sequences translate into lncRNA secondary-structure motifs, so deciphering the functional roles of the lncRNA language is more difficult than with the miRNA language [85]. The most important features of lncRNAs is their stable form that determines their function, in fact they act as regulators of the translation, transcription, mRNA processing and also at post-transcriptional level [101] (Figure 10).

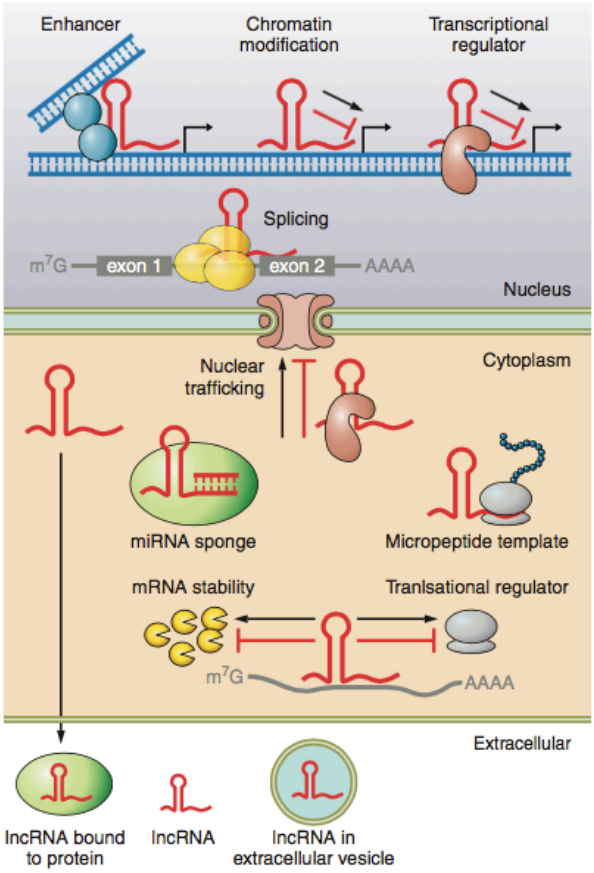


Figure 10: Biogenesis and function of lncRNAs. lncRNAs control the expression of genes in the nucleus by interacting with DNA, chromatin modifying complexes, and/or various transcriptional regulators. Cytoplasmic lncRNAs act as sponges for other transcripts or proteins or regulate mRNA degradation and translation [101].

1.5.2 ncRNAs in diseases

To date, few ncRNAs are used in clinical practice to predict drug response, but several *in vitro* studies have identified ncRNAs as biomarkers in cancer, cardiovascular diseases, autoimmune diseases, neurological disorders and infectious diseases [105,106]. The most important lncRNA used

in diagnostic is the prostate cancer 3 (PCA3) which is highly overexpressed in prostate cancer [107]. Of interest is also the lncRNA HOX transcript antisense RNA (HOTAIR) which is highly expressed in colorectal cancer tissues compared with adjacent uninvolved tissues [108] and it is also associated with prognosis and metastasis in breast cancer [109], hepatocellular carcinoma [110] and gastrointestinal stromal tumors [111]. The metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) not only is related to the onset of metastasis in non-small cell lung cancer (NSCLC) [112] and colorectal cancer [113] but is also considered a predictor of response to chemotherapy; indeed it is highly expressed in patients with osteosarcoma who had a poor response to COSS-96 polychemotherapy (doxorubicin, methotrexate, cisplatin and ifosfamid) [114]. miR-21 has been also related to chemoresistance in pancreatic cancer cells [115], glioblastoma multiforme [116], bladder cancer cells [117] and head and neck squamous cell carcinoma cells [118]. In breast cancer, miR-210 is related to sensitivity to trastuzumab [119] whereas miR-125b is predictive of chemoresistance [120]. miRNAs are also involved in autoimmune disease like systemic lupus erythematosus (SLE) (e.g. miR-21, miR-125a, miR-146a and miR-148a), rheumatoid arthritis (e.g., miR-124a, miR-146a and miR-155) and multiple sclerosis (e.g., miR-17-5p, miR-20a, miR- 34a, miR-155 and miR-326) [121]. Regarding IBD, experiments on serum from pediatric patients, analysed before and after treatment with prednisone from 3 to 18 weeks, showed a significant downregulation of miR-146a, miR-320a and miR-486 in response to the GC treatment. A higher expression of miR-146a has been demonstrated in inflamed mucosa of IBD pediatric patients in comparison to the normal mucosa[122].

1.6 The lncRNA growth-arrest specific 5

1.6.1 Gene structure and regulation

Almost 30 years ago Schneider *et al.* constructed a cDNA library enriched for RNA sequences preferentially expressed in growth-arrested cells and among the genes taken in consideration the gene of the growth-arrest specific 5 (GAS5) was characterized for the first time [123].
GAS5 is localized at 1q25.1 chromosome and comprises 650 base pairs, 12 exons and 11 introns. The gene encodes for small nucleolar RNAs (snoRNAs), and PIWI-interacting RNAs (piRNAs) as well as for the lncRNA [124]. Exons contain only a short open reading frame (ORF) that not encode a functional protein. GAS5 could exist in two mature lncRNAs trough the splicing of exon 7 forming GAS5a and GAS5b. Furthermore, GAS5 encodes within its introns ten box C/D snoRNAs involved in the 2'-O-methylation of rRNA [125,126]. Even if the importance of snoRNAs is not completely clear, recently it was demonstrated that the U44 snoRNA is able to modify the 18S rRNA whereas all the other snoRNAs direct the modification of 28S rRNA. Among these

snoRNAs the U44, U74 and U78 could be processed by the endonuclease Dicer and silencing mRNA molecules. These kind of snoRNAs are termed sno-miRNAs [127]. GAS5 is transcribed as 5'-terminal oligopyrimidine (5'-TOP) RNA, a class of transcripts that generally encode ribosomal proteins and other molecules involved in protein synthesis, but in GAS5 it serves to control transcript levels, conferring stability to the lncRNA [126,128]. 5'-TOP transcripts are subject to growth-dependent translational control which explains the accumulation of GAS5 mRNA in growth-arrested cells [125]. The 5'-TOP and the 12th exon on the 3' unsure the activity of the transcript, for this reason both splicing products (GAS5a and GAS5b) are functional [129], even if the GAS5b variant appears to be the predominant transcript in most cell lines [126] (Figure 11). Of emerging importance is the presence, in the 3' terminal, of a partial overlap of 40 nucleotides of another non-protein coding gene, the GAS5-antisense-1 (GAS5-AS1), which is encoded on the opposite strand and arranged tail-to-tail with GAS5 [126]. To date its function is still unknown but, in a work by Wu *et al.,* a downregulation of GAS5-AS1 in NSCLC tumors when compared to adjacent normal lung tissues was described; these findings indicate that GAS5-AS1 may function as a tumor suppressor [130]. Because of the small ORF, GAS5 may be under the control of the nonsense-mediated decay (NMD) that is considered a RNA quality control system to eliminate aberrant transcripts or regulate abundance of the same transcript. Indeed, in growing cells, the active translation of GAS5 through its 5'-TOP leads to a rapid degradation by the NMD; on the contrary, in growing arrest, due for example for serum starvation, a decrease of NMD activity was observed with an accumulation of the GAS5 transcripts. Experiments of NMD modification demonstrated that this pathway is fundamental for the physiologic regulation of GAS5 and then for the normal cell growth [128,131]. GAS5 regulation plays a role also in apoptosis and in the control of the cell cycle. In T-cells in which a specific plasmid was used to overexpress the lncRNA, an increase in apoptosis and a deceleration of the cell cycle was observed; opposite results were showed when GAS5 was silenced, indicating that this lncRNA is necessary for normal growth arrest in T-cells, opening new frontiers in the study of GAS5 in different diseases [132].

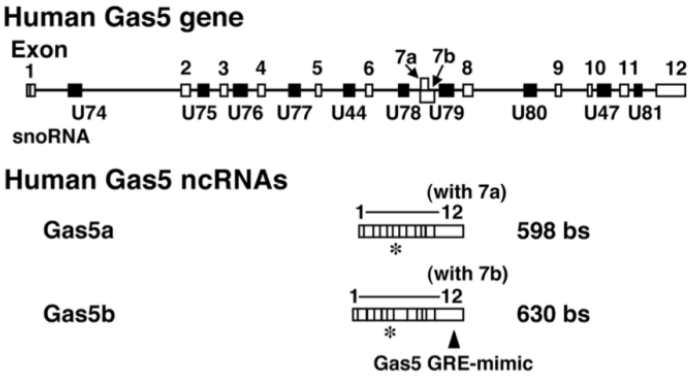


Figure 11: Human GAS5 gene. In white boxes the 12 exons and in black boxes 10 snoRNAs. On the bottom the two spliced isoforms Gas5a and Gas5b with alternative use of exon 7a and b [129].

1.6.2 Role of GAS5 in diseases

In recent years, progresses have been made in understanding the molecular mechanism of GAS5 in different diseases including cancer and autoimmune diseases. Further studies have revealed a crucial role of low GAS5 expression related to a poor patients survival in breast [133], hepatocellular [134], gastric [135], colorectal [136] and cervical cancers [137].

An interesting result was observed in patients with diffuse large B-cell lymphoma; in these patients, a translocation of GAS5 t(1;3)(q25;q27) has been described and a fusion transcript is created between GAS5 5'-TOP to exon 3 and the ORF of BCL6. This chimeric sequence is not recognized by the NMD and for this reason an aberrant transcript is produced [138]. In addition, Chen and collaborators demonstrated a role of GAS5 in mediating melanoma metastasis trough the regulation of MMP-2 and MMP-9 both *in vitro* that *in vivo*, but the mechanism behind the MMPs regulation by the lncRNA is still unknown [139].

As regard the role of GAS5 in autoimmune diseases, preliminary results in murine model system revealed a link between GAS5 and disease susceptibility to SLE [140] in addition, in this patients and in rheumatoid arthritis (RA) patients reduced levels of GAS5 in CD4 T-cells and B-cells [141] were also observed. Mayama *et al*. have analyzed the expression of GAS5 in different autoimmune, inflammatory, and infectious diseases: altered GAS5 levels were observed in patients with sarcoidosis, tuberculosis, HIV1 or severe N1H1 influenza virus infection, or bacterial sepsis, suggesting that GAS5 appears to be under the regulation of the immune system [141]. An important feature of GAS5 was discovered in 2010, Kino and collaborators have described GAS5 as repressor of the GR, influencing the GCs activity as observed also in healthy donors' peripheral blood mononuclear cells (PBMCs) and in IBD pediatric patients [129,142,143].

1.6.3 Role of GAS5 in GC activity

In 2010 Kino and collaborators described the interaction of GAS5 with the GR. In particular, a decoy RNA "GRE" on ncRNA sequence is responsible of the interaction between GAS5 and the DBD of the ligand-activated GR [129]. The sequence involved in the GR modulation is comprised between nucleotide 400 and 598; this portion contains 6 hairpin structures. UNAFold, a software that simulate folding, hybridization, and melting pathways for one or two single-stranded nucleic acid sequences [144], revealed that, on hairpin 5, two sequences form the so called "GRE-mimics" to create a binding site similar to GRE sequences on GC responsive genes. GRE-mimic sequences include nucleotides 539-544 (GRE-1) and 553-559 (GRE-2) that, thanks to RNA specific Wobble base paring between a U and a G, complement each other forming the complete hairpin structure. The binding with the GR occurs with hydrogen bond between the G540 in 5' strand and C554 in 3' strand, in GAS5 sequence, and between the K442 and R447 of the DBD [85,129]. GR does not discriminate between G-U and G-C pairs as demonstrated by mutagenesis experiments [145] (Figure 12).

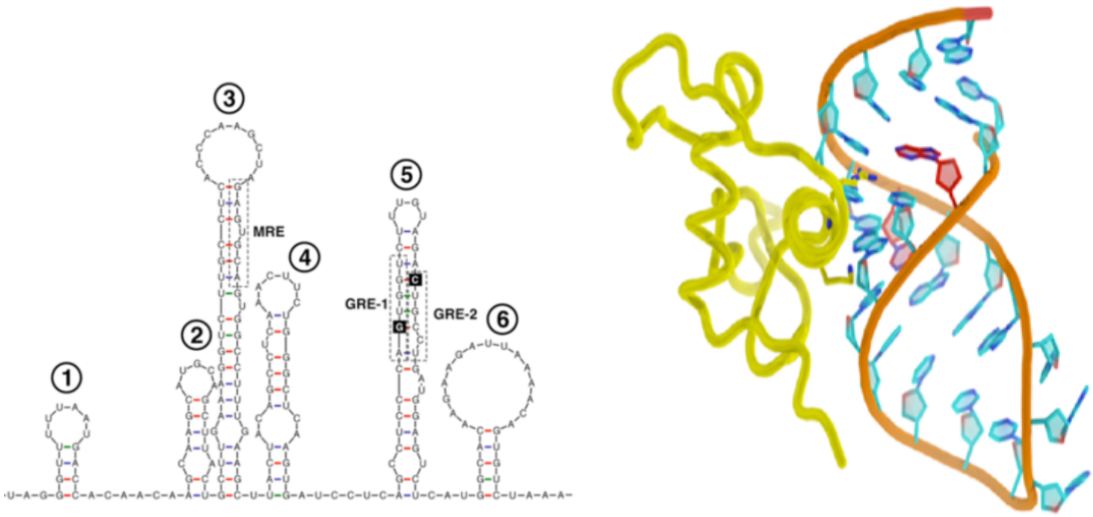


Figure 12: 2-dimensional structure of GAS5 hairpins, hairpin #5 contains two "GRE" sequences at nucleotides 539–544 ("GRE-1") and 553–559 ("GRE-2") which form a double-stranded hairpin structure responsible of the binding with the DBD of GR, as illustrated in the 3-dimensional structure [129].

Indeed, this interaction was demonstrated with a yeast two-hybrid assay, that identifies all the protein-protein and ligand-protein interactions, on Jurkat cell line. GR DBD was used as bait and different clones were screened, but only 2 independent clones that contained the GAS5 sequence gave a positive signal, demonstrating that the lncRNA interacts with the GR DBD. To confirm the direct binding, Kino *et al.* performed an RNA and protein coimmunoprecipitation

assay in HeLa cell line, which endogenously expresses both molecules. HeLa cells were also treated with DEX at a concentration of 10^{-6} M to observe if steroid treatment could influence the GAS5 GR interaction. Results have confirmed the binding between GAS5 and GR and an increase of this interaction after treatment with DEX. This interaction is physiologically relevant since the concentration of DEX used was more than 10-times lower than the physiologic levels of circulating cortisol. Furthermore, it was also demonstrated that GAS5 binding occurs in a DBD-dependent way, in fact, no interaction was observed when a GR chimera replaced the normal DBD sequence. GAS5 was localized both in the cytoplasm and in the nucleus and *in situ* hybridization experiments showed a more prominent presence in the former compartment [146]. After DEX treatment, a higher fraction of GAS5 was observed in the nucleus than in the cytoplasm indicating that GAS5 interacts with GR DBD in the cytoplasm and migrate together in the nucleus, this finding was established in HeLa cells with a plasmid containing a GRE-1 mutant sequence that instead failed to translocate [129]. At this stage GAS5 exerts its role in inhibiting the binding of GR to specific GREs and a decreased expression of GC target genes, such as leucine zipper protein, serum/glucocorticoid regulated kinase 1, phosphoenolpyruvate carboxykinase, glucose 6 phosphatase and above all the cellular inhibitor apoptosis 2 genes, is observed as demonstrated by Kino *et al*. [129]. These data confirm the role of GAS5 in controlling GC activity through the GR binding. In previous studies in our laboratory, the role of GAS5 was investigated in the pharmacological response after GCs treatment. Indeed, a study was conducted on PBMCs obtained from healthy donors, *in vitro* treated with different concentrations of MP. On the basis of the antiproliferative effects of steroids the population was divided in good and poor responders and higher levels of GAS5 were recorded in the latter group compared with the good responders. These results suggest that GAS5 could interfere with the activated GR-DBD in the binding to the GRE sequences of GC gene targets, opening new frontiers in the study of GC resistance [142,147] (Figure 13).

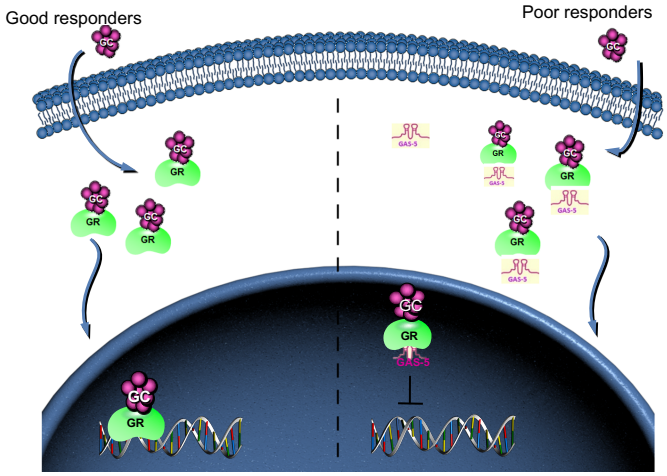


Figure 13: Potential role of GAS5 in GCs response [142].

2.AIMS OF THE PROJECT

IBD, including CD and UC, is an uncontrolled and multifactorial disorder characterized by chronic, relapsing or progressive inflammatory condition that may involve the entire or only part of the gastrointestinal tract [9]. The incidence of IBDs is increasing, in particular in the pediatric population where the potential negative effects are even more prominent [6]. The onset of the disease is a combination of genetics, environmental factors, gut microbiota and immune response. To date different pharmacological strategies are used in IBDs treatment with the purpose of inducing and maintain clinical remission, achieve normal growth and minimize adverse effects [61]. GCs are largely used in inflammatory, autoimmune and proliferative diseases and in IBD are prescribed to induce remission in patients with moderate-to-severe disease. Despite the multiple use in different pathologies, the therapeutic benefits of these agents are often narrowed by inter-individual variability; some patients are resistant to GCs, and do not respond to therapy, other are dependent and require high doses of GCs. This group of patients risks a series of side effects including Cushing syndrome, psychologic disturbance, osteoporosis, metabolic disease and increased risk of cardiovascular disease and others [84]. Therefore, due to the currently limited comprehension of a such a complicated disease the optimal treatment is far from being achieved and so it is necessary to identify biomarkers predictive of the pharmacological response. Researches on ncRNAs as regulators of gene expression could open new perspectives in the study of IBD and response to drugs. Recent papers described the role of the lncRNA GAS5 as a repressor of GR activity. This lncRNA presents an hairpin structure that mimics the GRE sequences of GC gene targets, competing with activated GR for the binding on DNA sequences [129]. In our laboratory, PBMCs obtained from healthy donors treated with different concentration of MP were divided in two groups, good and poor responders, based on *in vitro* pharmacological response. In poor responders, higher levels of GAS5 were evident in comparison with good responders, suggesting that this lncRNA could be involved in GC resistance [142]. Since GAS5 seems to be promising in the study of GCs resistance, the first part of my thesis work aimed to study in deep GAS5 activity and mechanism of action on IBD patients. The main purposes were to:

- Identify a cellular model to study *in vitro* the role of GAS5 in the GC pathway. Different immortalized cell lines were analyzed after GC treatment, using a proliferation assay were selected sensitive and resistant cell lines to GCs. The chosen cell lines were used to study GAS5 molecular functions by modulating its expression.
- Evaluate GAS5 expression in PBMCs of pediatric IBD patients enrolled at the diagnosis and after GCs administration for 4 weeks. Pediatric patients were divided in poor and good responders by standard clinical index of CD and UC diseases. These data could be useful to

confirm the proposed activity of GAS5 in GCs resistance.

Recently, Chen et al. demonstrated that the lncRNA GAS5 controls the metastasis phenotype via downregulation of MMP-2 and -9 [139]. MMP-9 and MMP-2 also participate actively in the inflammatory and remodelling processes of IBD and a significantly increase of their expression was observed in inflamed tissues of patients; however no data are published about the role of GAS5 as regulator of MMP-9 and MMP-2. The principal goal was to:

- Study the role of GAS5 as a negative regulator of MMP-2 and -9, whose activity has been implicated in the pathogenesis of IBD [16,17]. Through a gene expression analysis the expression of GAS5 and two gelatinases MMP-2 and MMP-9 was evaluated in tissue samples obtained from colon mucosa of pediatric patients with IBD. These studies could provide new perspectives for lncRNA-directed diagnostics and drug targets.

Twenty years ago, the first description of TTP knockout mouse underlined the importance of this protein in reducing systemic inflammation [54]. The role of TTP in controlling cytokines involved in the inflammatory response not only was studied in different strains of mice but its activity was investigated in different diseases such as cardiovascular disease, asthmatic inflammation and RA. In the synovia of RA patients, the role of phosphorylation in modulating TTP activity, enhancing pro-inflammatory cytokines expression, was described [59]. Recent studies associated the activity of phosphorylated TTP to the protein complex 14-3-3 that protects TTP and prevents the degradation of the transcript target stabilizing pro-inflammatory cytokine mRNAs [57]. Since there are no evidences in the literature of the role of TTP in IBD patients' tissues and cells, the aim of this project is to determine whether the amount of phosphorylated protein, therefore the inactivated form, is differently expressed in inflamed and non-inflamed IBD's samples.
To fulfil this aim, the second part of my thesis project has been carried out as follows:

- Quantification of TTP in pediatric IBD patients' macrophages and colon tissues. Since TTP is very stable protein in macrophages, protein expression was evaluated in macrophages differentiated from PBMCs of IBD patients and healthy donors. Colon tissues analysis was performed comparing inflamed tissues *versus* non-inflamed ones.
- Study of the role of phosphorylation in the activity of TTP. Based on the assumption that phosphorylated and inactivated TTP forms a complex with 14-3-3 proteins, a co-immunoprecipitation assay was performed on protein lysates from macrophages and colon

tissues to demonstrate not only the protein-protein binding complex but also indirectly the phosphorylation of the protein.

The results obtained in this thesis could clarify some mechanisms of such complicated disease, and developing new strategies for a future target therapy.

3.MATERIALS AND METHODS

3.1 Cell lines

The HeLa human cervical carcinoma (ATCC, CCL-2) and RAW264.7 murine macrophages (ATCC, TIB-71) cell lines were grown in Dulbecco's modified Eagle's medium (DMEM, EuroClone®); LoVo colorectal cancer (ATCC, CCL-229) and THP-1 human monocyte (ATCC, TIB-202) cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich). Both media were supplemented with 10% (v/v) fetal calf serum, 1% (v/v) L-glutamine 200 mM, 1% (v/v) penicillin 10,000 UI/mL and streptomycin 10 mg/mL, all purchased by Sigma-Aldrich. Cell cultures were maintained according to standard procedures in a humidified incubator at 37 °C with 5% CO_2, and cell passage was performed once a week.

3.2 Macrophages differentiation from THP-1 monocyte cell line

To differentiate macrophages from the immortalized cell line THP-1, $0.5X10^6$ cells were seeded in a six-well plate. Phorbol 12-myristate 13-acetate (PMA, Thermo Fisher), at 5 ng/mL was added to stimulate the differentiation in macrophages. Cells were maintained in the humidified incubator at 37 °C for 48 hours. At the end of this period cells were treated with bacterial lipopolysaccharide (LPS, Thermo Fisher) at a concentration of 1 μg/mL, as pro-inflammatory stimulus, for 3 hours. Plates were directly treated with 1 ml in TRIzol® reagent (Thermo Fisher) for RNA isolation and stored at -80°C for further analysis.

3.3 Clinical samples

Nineteen IBD pediatric patients (mean age at enrolment 12.9 years, 16 UC and 3 CD, 9 males and 10 females) were enrolled at the Pediatric Clinic of IRCCS Burlo Garofolo in Trieste and treated with prednisone 1–2 mg/kg/day for 30 days according to standard clinical protocol. PBMCs were obtained from these patients at diagnosis (T0) and after 4 weeks of steroid treatment (T4). In addition, PBMCs from four IBDs pediatric patients (mean age at enrolment 14.68 years, 3 males and 1 females) were treated with GM-CSF (50 ng/ml, Millipore) for 1 week to differentiate in macrophages.

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 based on 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.

Thirty-four IBD pediatric patients (mean age at enrolment 12.6 years, 16 UC and 18 CD, 18 males and 16 females) were enrolled at diagnosis at the Gastroenterology department of Pediatric Clinic of IRCCS Burlo Garofolo in Trieste. For each patient, during a colonoscopy, two biopsies (inflamed and non-inflamed) were collected. TRIzol® reagent was used for RNA isolation. Additionally, protein analysis was also performed on four biopsies (mean age at enrolment 13.8 years, 2 UC and 2 CD, 3 males and 1 females). For each patient, the inflamed and non-inflamed biopsies were immediately frozen on dry ice.

Both endoscopic and histologic evaluations were performed for all patients enrolled in the study. During each colonoscopy inflamed and non-inflamed segments was taken. Samples were fixed in 10% neutral formalin and stained with haematoxylin and eosin. The preparation of the histologic specimen was standardized using a kit for the orientation of the gastrointestinal biopsies (Bio-Optica®). The non-inflamed biopsies show a visible vascular design, absence of hyperemia, lack of mastitis or ulcers, while inflamed biopsies show a discontinuous or a completely loss of vascular pattern, hyperemia and deep ulcerations. The histologic inflammatory score was described in the table below with a score range from 0 (absence of inflammatory activity) to 5 (maximal inflammatory activity) (Table 1):

HISTOLOGIC FINDINGS	CRITERIA	SCORE
Crypt abscess	Present Absent	1 0
Erosions and ulcerations	Present Absent	1 0
Active inflammation	Severe Moderate Mild Normal	3 2 1 0

Table 1: Histologic inflammatory score for IBDs. Active inflammation was considered in term of neutrophils with or without eosinophils aggressive toward the glandular structures (i.e. in phase of penetration into the glandular structure) [148].

PBMCs samples from four blood donors were collected 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 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. PBMCs, isolated from blood samples, were treated with GM-CSF (50 ng/ml, Life Technologies) for 1 week to differentiate into macrophages.

3.4 Ethical considerations

Local ethical committee approval for the study (Protocol 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. The inclusion criteria: pediatric patients with a diagnosis of CD or UC. The exclusions criteria: a) patients already treated with immunosuppressive drugs at the diagnosis; b) disease requiring immediate surgical intervention; c) severe ulcerative colitis or toxic megacolon; d) any of the following conditions: active infection, stool culture positive for enteric pathogens, tumors, HIV, transplanted organ or non-controlled disease of the kidney, liver, endocrine system, heart, blood, nervous system or brain.

3.5 *In vitro* viability assays

The effect of MP (Sigma-Aldrich) on the proliferation of HeLa and LoVo cells was determined by labelling metabolically active cells with [methyl-3H] thymidine (Perkin Elmer). This assay utilizes the radioactive nucleoside which is incorporated into new strands of chromosomal DNA during mitotic cell division. A scintillation beta-counter (1450 Microbeta Trilux, Perkin Elmer) is used to measure the radioactivity in DNA recovered from the cells in order to determine the extent of cell division. Cells were seeded into a 96-well plate in the presence of MP (range from 0.019 ng/mL to 20 μg/mL) (Table 2).

METHYLPREDNISOLONE	
Concentration 1	20 μg/ml
Concentration 2	250 ng/ml
Concentration 3	10 ng/ml
Concentration 4	0,3125 ng/ml
Concentration 5	0,019 ng/ml

Table 2: Methylprednisolone concentrations used.

After 50 hours of incubation, cells were pulsed with [methyl-3H] thymidine (2.5 μCi/mL) and the incubation was continued for an additional 22 hours. After this time cells were transferred in a 96-well filter plate (MultiScreenHTS FB 1.0/0.6 μm) to capture the radioactive labeled DNA. Then the radioactivity of the samples was determined by the beta counter after adding the liquid scintillation cocktail (Optiphase, "Super Mix", Perkin Elmer). 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) was used to evaluate the integrity of the cell membrane and assess cell viability. After incubation, cells were washed with PBS and then 10 μL of 0.1 mg/mL PI were added to each sample and incubated for 10 minutes at room temperature. The fluorescence intensity was read by a FluoroCount Micro-plate Fluorometer (Packard) at an excitation length of 530 nm and emission length of 590 nm.

3.6 Total RNA isolation

Total RNA was extracted using TRIzol® reagent (Thermo Scientific) from HeLa and Lovo cell lines and from PBMCs and colon biopsies of IBD pediatric patients. All the samples were incubated with TRIzol® for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. 200 μL of chloroform (Sigma-Aldrich) were added and after 3 minutes of incubation at room temperature a centrifugation at 12,000 × g for 15 minutes at 4°C permits the separation in a lower red phenol-chloroform phase, an interphase and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase that is transferred into a new tube to proceed with the RNA isolation procedure. After precipitation with 500 μL of 100% isopropanol (Sigma-Aldrich) and a wash step with 1 mL of 75% ethanol (Sigma-Aldrich), RNA pellet was resuspended in RNase-free water (Gibco-Life Technologies) and incubated in a water

bath at 55–60 °C for 15 minutes. Then, the RNA concentration and purity were calculated by Nano Drop instrument (NanoDrop 2000, EuroClone®).

3.7 Quantitative real-time PCR

mRNA expression levels of different genes studied 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) using up to 2 μg of total RNA per 20 μL of reaction containing 10 μL of 2x RT Buffer, 1 μL of 20x RT Enzyme Mix. Then real-time PCR was performed in triplicate using the TaqMan® Gene Expression Assay (Applied Biosystem). Real-time PCR technique could be divided in three steps:

1) At the start of real-time PCR, the temperature is raised to denature the double-stranded cDNA. During this step, the signal from the fluorescent dye on the 5' end of the TaqMan® probe is quenched by the non-fluorescent quencher (NFQ) on the 3' end.
2) In the next step, the reaction temperature is lowered to allow the primers and probe to anneal to their specific target sequences.
3) DNA polymerase synthesizes new strands using the unlabeled primers and the template. When the polymerase reaches a TaqMan® probe, its endogenous 5' nuclease activity cleaves the probe, separating the dye from the quencher.

With each cycle of PCR, more dye molecules are released, resulting in an increase in fluorescence intensity proportional to the amount of amplicon synthesized. We used the 6-carboxyfluorescein (FAM) as fluorescent dye on 5’ end.

Probes used are summarized in Table 3:

PROBE	CODE	COMPANY
18S	Hs99999901_s1	Thermo Fisher
RPLP0	Hs99999902_m1	Thermo Fisher
GAS5	Hs03464472_m1	Thermo Fisher
GAS5-AS1	Hs04232243_s1	Thermo Fisher
MMP-9	Hs00957562_m1	Thermo Fisher
MMP-2	Hs01548727_m1	Thermo Fisher
Zfp36	Hs00185658_m1	Thermo Fisher

Table 3: TaqMan® probes used in real time PCR

To normalize the expression levels of gene targets it is necessary to use an endogenous control gene, the housekeeping gene. For the immortalized and primary cell lines the ribosomal RNA 18s was used, while for biopsies the RPLP0 that codified for a ribosomal protein of 60S subunit,

was employed. For each sample a mix was prepared with 2 μL of cDNA, 5 μL of 2X TaqMan® Universal Master Mix II, no UNG, 0,5 μL of 20X TaqMan® Gene Expression Assay and 2.5 μL of H_2O RNasi Free.
The expression levels of the selected transcripts were determined using the Livak method for relative expression (RE) and relative expression of ΔCt [149]. The results are provided as the mean and standard error (SE) of three replicates.

3.8 RNA interference

Depletion of endogenous GAS5 was performed by RNA interference (RNAi) using HiPerFect Transfection Reagent (Qiagen) and a small interfering RNA (siRNA), specific for GAS5 (target sequence 5'-AACAAGCAAGCATGCAGCTTA-3', Qiagen). Shortly before transfection, $7X10^5$ cells were seeded in six-well plates in 1.4 mL of complete medium. A total of 3 μL of siRNA GAS5 at 40 μM was diluted in 100 μL of Opti-MEM (Life Technologies), and 5 μL of HiPerFect Transfection Reagent (Qiagen) were added to the diluted siRNA. After 10 minutes of incubation, the complexes were added drop-wise onto the cells. After 24 hours, 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). The analysis of specific silencing of GAS5 expression was carried out after 48 hours from the reseeding, using real time PCR transfection efficiencies (after 48 hours) were 70–80%.

3.9 Subcellular distribution of lncRNAs

The experiment was conducted on HeLa and LoVo cell lines treated with MP at the final concentration of 250 ng/mL. Cells were seeded (density of $1.5\ X10^5$ cells for HeLa and $3.0X10^5$ cells for LoVo) and incubated for 72 hours at 37 °C and 5% of CO_2. After this time, cytoplasmic and nuclear fractions were obtained by the following protocol. Cells were washed with cold PBS and resuspended in hypotonic buffer A: 20 mM Tris-HCl [pH 7.5], 10 mM NaCl, 3 mM MgCl2, 10% glycerol and the protease inhibitors cocktail (Roche). After 1 minute, NP-40 was added at 0.1% v/v final concentration for 5 minutes, and the cytoplasmic fraction was collected by centrifugation at 150,000 × g for 5 minutes 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). 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.

3.10 Protein isolation

Depending by the starting sample, two different methods were used for the isolation of proteins:

1) HeLa and LoVo cell lines: after being cultured as reported above, cells were collected and washed with cold PBS. To lyse the membrane 100 µL of 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% was used, followed by sonication for 30 seconds and centrifugation at 10,000 x g for 10 minutes. The supernatant contains the whole protein lysate.
2) Patients' colon frozen biopsies and macrophages differentiated from blood from IBD patients and healthy donors: a co-immunoprecipitation kit (Abcam) protocol was used. Macrophages were washed with cold PBS and later non-denaturating lysis buffer, complete of protease inhibitor cocktail and inhibitor tablets for phosphatase (PhosSTOP™, Sigma-Aldrich), was added in a volume dependent from the size of the plate used for seeding cells (100-200 µL/well for 24-well plate, 250- 400 µL/well for 6-well plate, 250-500 µL for 100 x 60 mm dish or 500-1000 µL for 100 x 100 mm dish). Cells were scraped and transferred into a chilled microcentrifuge tube. The cell extract was mixed in the rotary mixer for 30 minutes at 4 °C and then centrifuged at 10,000 x g for 10 minutes at 4 °C. The supernatant consists of the protein lysate. Frozen biopsies were immediately grinded into a fine powder using a mortar and pestle in dry ice. The grounded tissue was transferred in a pre-weighed chilled tube and weighed again. 300 µL of non-denaturating lysis buffer with protease inhibitor cocktail and PhosSTOP™ were added per 5 mg of tissue powder and mixed on a rocker at 4 °C for an hour. The lysate was further cleaned by a 25-gauge needle and centrifuged at 10,000 x g for 5 minutes at 4 °C to remove cell debris. The supernatant was transferred in a fresh tube.

3.11 Co-immunoprecipitation

Co-immunoprecipitation assay was performed on protein lysate from inflamed and non-inflamed frozen biopsies, on proteins obtained from human macrophages differentiated from PBMCs of IBD pediatric patients and healthy donors and on RAW264.7 mice macrophages cell line. Human and mice macrophages were treated with bacterial lipopolysaccharide (LPS) 10 ng/mL for four and two hours, respectively. Immunoprecipitation (IP) is a technique used to enrich a specific protein from a heterogeneous cell or tissue extract using a target specific antibody. Co-immunoprecipitation (Co-IP) is the pull down of intact protein complexes. IP and co-IP are valuable and widely used techniques to identify protein-protein interactions and novel

members of protein complexes. TTP antibody (1:1000) was mixed with the protein lysates, volume was make up to 500 µL with the non-denaturating lysis buffer with protease inhibitor cocktail and PhosSTOP™ and the solution was maintained in the rocker for 4 hours at 4 °C. In the meantime, protein A/G Sepharose® beads (40 µL/reaction) were washed twice with 1 mL of wash buffer (supplied by the kit), centrifuging at 2,000 x g for 2 minutes and aspirating the supernatant in between washes. After antibody binding to protein lysate, 40 µL of Protein A/G Sepharose® beads were added and incubated for an hour at 4 °C. Three steps of washes with wash buffer interspersed by low speed centrifugation at 4 °C precede the elution of the complex with the 2X SDS-PAGE loading buffer. At this step samples are ready for Western blot detection of the TTP immunopreciptate protein and the TTP-14-3-3 co-immunoprecipitate protein complex.

3.12 Western blot

The protein concentration of each sample was determined using the Pierce BCA Protein Assay (ThermoFisher) to allow an equal loading of total proteins. Samples were then run in a PAGEr™ Mini-gel Chamber (Lonza) using 10% acrylamide gels with a Tris-Glycine buffer and subsequently semi-dry blotted for 2 hours with 50 mA current on PVDF membrane. After blocking for 1 hour with 5% not-fat milk in Tween/Tris buffered salt solution (T-TBS), membranes were incubated overnight at 4 °C with primary antibodies (Table 4). Membranes were then washed in T-TBS and incubated for 1 hour at 37 °C with secondary antibodies (Table 4). Chemiluminescence was developed using LiteAblot® TURBO (EuroClone®) and exposed on Kodak Biomax film. Protein expression was quantified on Western blot images using the ImageJ software and are reported in percentage with respect to loading control proteins.

ANTIBODY	HOST	FINAL DILUTION	COMPANY
PARP1 loading control	Rabbit	1:10000	Abcam
Tubulin loading control	Mouse	1:1000	Abcam
Actin loading control	Rabbit	1:20000	Millipore
Glucocorticoid receptor	Rabbit	1:300	Thermo Fisher
Tristetraprolin	Rabbit	1:1000	Millipore
14-3-3	Rabbit	1:1000	Abcam
Metalloproteinase 9	Rabbit	1:1000	Sigma-Aldrich
Anti-rabbit secondary antibody	Goat	1:50000	Millipore
Anti-mouse secondary antibody	Horse	1:40000	Cell Signaling

Table 4: Primary and secondary antibodies used for Western blot.

3.13 ELISA

Supernatants were collected from human macrophages (IBD patients and healthy donors) and from RAW264.7 murine macrophages cells after treatment with LPS (10 ng/mL) for four and two hours, respectively. Each sample (20 µl) was analyzed by magnetic beads suspension array using for human macrophages sample the Bio-Plex Pro Human Cytokine 17-plex panels (Bio-Rad Laboratories). The panel measures IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12(p70), IL-13, IL-17, granulocyte colony-stimulating factor (G-CSF), GM-CSF, IFN-γ, monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1 β (MIP-1β), TNF-α. Analysis for mice macrophages supernatant was performed using the Bio-Plex Pro Mouse Cytokine 23-plex panels. The panel measures IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-17, eotaxin, G-CSF, GM-CSF, IFN-γ, keratinocyte chemoattractant (KC), MCP-1, MIP-1α, MIP-1β, regulated on activation normal T cell expressed and secreted (RANTES) and TNF-α. Samples were run on a Bio-Plex 200 System (Bio-Rad Laboratories, Hercules, CA, USA), and the results were calculated using Bio-Plex Manager 6.0 software (Bio-Rad Laboratories).

3.14 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, gene expression and protein 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. Wilcoxon signed rank test (paired test) was used for gene expression analysis in colon biopsies

of IBD patients. One-way ANOVA with Dunn's multiple comparison test was used in THP-1 experiments; p-values <0.05 were considered statistically significant.

4.RESULTS AND DISCUSSION PART I

4.1 Sensitivity to GCs in immortalized cell lines

4.1.1 Evaluation of response to GCs

In our previous work, for the first time a likely mechanism of GCs resistance, that involves GR and GAS5, was described. By the [methyl-3H] thymidine assay PBMCs, isolated from healthy donors, were divided in good and poor responders, evaluating the inhibition of proliferation achievable at 250 ng/ml of MP ($I_{250ng/ml}$). This concentration was chosen as it inhibits cell proliferation without compromising cellular characteristics for subsequent analysis. A different GAS5 gene expression was observed after MP treatment: downregulation in good responders and upregulation in poor responders. Our hypothesis is that GAS5 could hamper the binding of the activated receptor trough the GRE-like sequences, preventing transcriptional activity [142,147]. To better understand the molecular basis of the key role played by GAS5 in modulating GC response, two different human immortalized epithelial cell lines, which endogenously express the GR and the lncRNA GAS5, were chosen to evaluate the correlation between GAS5 expression and GR activity [142]. HeLa and LoVo cell lines were treated for 72 hours with different concentrations of MP (Table 2) and the inhibition of cell proliferation was evaluated by the [methyl-3H] thymidine viability assay. In detail, a prominent inhibition of proliferation in HeLa cell line was recorded ($I_{250ng/mL}$=73%), on the contrary in the LoVo cell line only a moderate inhibition was observed ($I_{250ng/mL}$=16%) (Figure 14).

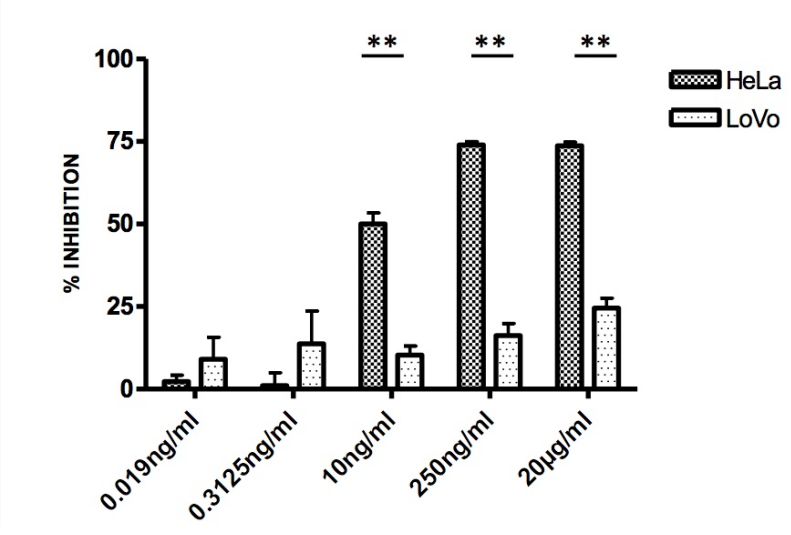


Figure 14: Effect of MP on HeLa and LoVo cells. Cells were exposed for 72 hours to MP at different concentrations, and cell proliferation was evaluated by the [methyl-3H] thymidine incorporation assay. Two-way ANOVA ($p < 0.0001$) and Bonferroni post-test **p-value<0.001. The data are reported as means ± SE of three independent experiments performed in triplicate.

These data demonstrated that LoVo cells can be considered a GC-resistant cell line, while HeLa cells a GC-sensitive cell line.

4.1.2 Evaluation of GCs treatment in cell membranes

To analyse the mechanism underlying the reduction of cell proliferation, observed during the treatment (Figure 14), we performed an experiment to evaluate if MP induces cellular membrane damage 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). HeLa and LoVo cell lines treated and untreated with MP were used. After incubation for 72 hours, 0.1 mg/mL of PI were added to the cells suspension and fluorescence was recorded. No differences in PI signal was observed between the treated and untreated samples, indicating that even high concentrations of MP did not affect cellular membrane integrity (data not shown). Moreover, this result excluded that the increase of GAS5, observed in LoVo cells, was related to the apoptotic state as observed in previous papers [132,133].

4.2 Gene expression analysis

4.2.1 GAS5 gene expression analysis in HeLa and LoVo cell lines

The role of GAS5 in the different sensitivity to GCs in HeLa and LoVo cells was assessed. Real time PCR technology was used to quantify gene expression of the lncRNA GAS5 both in untreated cells and cells treated with 10 and 250 ng/mL of MP. These concentrations were chosen on the basis of the results obtained from the sensitivity assay (Figure 14). In untreated HeLa and LoVo cells, basal expression levels of GAS5 did not shown significant differences. After treatment for 72 hours with MP significant differences were observed both in HeLa and LoVo cell lines. In HeLa GC-sensitive cells, a downregulation of GAS5 in comparison to untreated samples was evident ($2^{-\Delta Ct}_{\text{Untreated}} = 0.000190$; $2^{-\Delta Ct}_{\text{10 ng/mL}} = 0.000090$; $2^{-\Delta Ct}_{\text{250 ng/mL}} = 0.00008719$). On the contrary, expression analysis on LoVo GC-resistant cell line showed an upregulation of GAS5 in comparison to untreated samples ($2^{-\Delta Ct}_{\text{Untreated}} = 0.000140$; $2^{-\Delta Ct}_{\text{10 ng/mL}} = 0.000280$; $2^{-\Delta Ct}_{\text{250 ng/mL}} = 0.000220$). The differences observed in the cell lines were statistically significant at both MP concentrations (Figure 15).

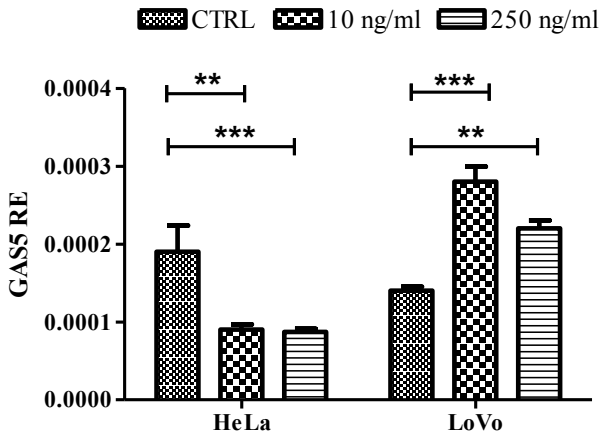


Figure 15: Expression profile of GAS5 during GC treatment. GAS5 releative expression (RE, values are expressed as $2^{-\Delta Ct}$) in HeLa and LoVo cells before (CTRL) and after treatment with MP for 72 hours at 10 and 250 ng/mL. Two-way ANOVA ($p < 0.0001$) and Bonferroni post-test ***p-value<0.001; **p-value<0.01. The data are reported as means ± SE of three independent experiments performed in triplicate.

These data revealed that in untreated HeLa and LoVo cell lines no difference in GAS5 expression is evident, as already demonstrated in healthy donors' PBMCs [142,147], suggesting that the levels of GAS5 cannot predict the response to GCs. However, after MP treatment, changes in GAS5 expression were observed. The lncRNA was downregulated in HeLa cells, whereas it was increased in LoVo cells. This pattern of GAS5 expression has been already described in PBMCs from good and poor responders after MP treatment [142,147]. These results suggest that, even in the immortalized GC-resistant cell lines, higher levels of GAS5 can inhibit GCs activity, competing with the GRE elements for the binding with the activated GR.

4.2.2 GAS5-AS1 gene expression analysis on HeLa and LoVo cell lines

In a recent work a partial overlap of 40 base pair in the 3' terminal of GAS5 with another non-protein coding gene, the GAS5-AS1 was described. This ncRNA is encoded on the opposite strand and arranged tail-to-tail with GAS5. Little it is known about the functions of GAS5-AS1, above all the impact of its transcription on GAS5 expression is unknown [126]. GAS5-AS1 expression was therefore evaluated in HeLa and LoVo cell lines. The two cell lines were treated with 10 ng/mL and 250 ng/mL of MP for 72 hours and real time PCR analysis was performed. In LoVo cells an increase of the antisense ncRNA was observed after treatment with both concentrations of MP ($RE_{GAS5\text{-}AS1\ 250\ ng/mL}=2.32$; $RE_{GAS5\text{-}AS1\ 10\ ng/mL}=1.22$); on the contrary, GAS-AS1 expression in the HeLa cell line ($RE_{GAS5\text{-}AS1\ 250\ ng/mL}=0.37$; $RE_{GAS5\text{-}AS1\ 10\ ng/mL}=0.27$) was significantly reduced after MP treatment (Figure 16).

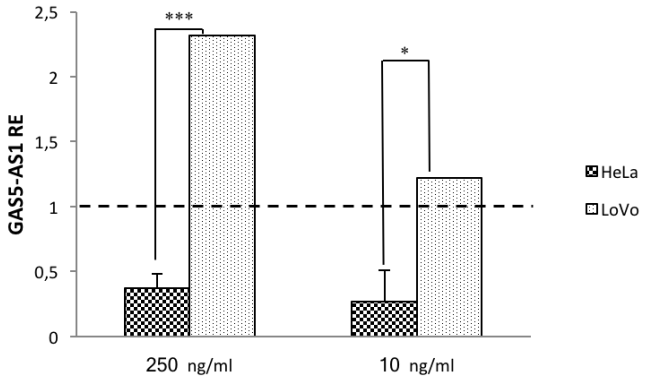


Figure 16: Expression profile of GAS5-AS1 in HeLa and LoVo cells treated with MP (250 ng/ml and 10 ng/ml) for 72 hours. GAS5-AS1 relative expression (RE, values are expressed as $2^{-\Delta\Delta Ct}$) respect to the housekeeping 18s was calculated according to the Livak method . Values > 1 stand for upregulation, values < 1 stand for downregulation. Two-way ANOVA $p = 0.0241$ and Bonferroni post-test ***$p < 0.001$, *$p < 0.05$. The data are reported as means ± SE of three independent experiments performed in triplicate.

Recent evidences show that antisense transcripts could have a role in almost all stages of gene expression, from transcription and translation to RNA degradation. Furthermore, antisense lncRNAs can regulate the expression of different molecules with various mechanism [150]. The increased levels of GAS5-AS1 gene expression in the LoVo cell line, resistant to GCs, could have a role in post-transcriptional regulation of GAS5. We speculate that the GAS5-AS1 is able to bind GAS5 and that this interaction protects the lncRNA from the degradation physiologically caused by the NMD pathway, [128] determining its accumulation in the cell (Figure 17).

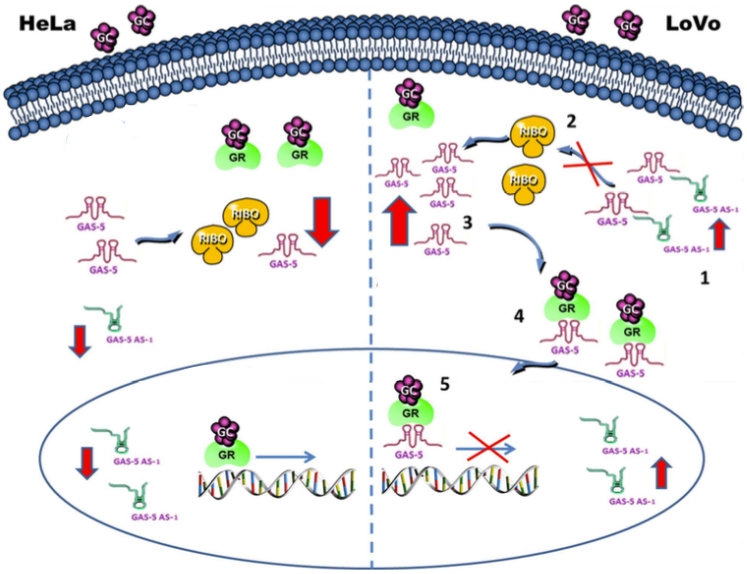


Figure 17: Schematic representation of the proposed GAS5-GAS5-AS1 mechanism of action. 1) in GC-resistant cell line, after treatment with MP, an upregulation of GAS5-AS1 was shown. GAS5-AS1 binds to 40 base pairs of GAS5 transcript. 2-3) the binding with GAS5 protects it from the ribosomal (RIBO) degradation guided by the NMD mechanism. 4) the accumulation of GAS5 promotes the binding with the activated GR in the cytoplasm. 5) Inhibition of the binding and transcription of GC responsive gene.

4.3 Intracellular localization of GAS5 in response to MP

Subcellular fractionation is useful for studying the molecular role of lncRNAs [151]. Since few information are available on the mechanism of action and regulation of GAS5, we have analysed the expression of this lncRNA, considering its cellular localization before and after treatment with GCs in the two cellular models. HeLa and LoVo cells were treated for 72 hours with 250 ng/mL of MP and then subcellular fractionation was performed. Real time PCR results have shown no differences in endogenous GAS5 levels between the cytoplasm and the nucleus in untreated HeLa and LoVo cell lines. After treatment with MP in the HeLa cell line, GAS5 expression was unchanged in the two compartments of the cell ($RE_{cytoplasm} = 0.87$; $RE_{nucleus} = 0.84$). On the contrary, in the LoVo cell line, after MP treatment for 72 hours, a significant accumulation of endogenous GAS5 was observed in the cytoplasm compared to the nucleus ($RE_{cytoplasm} = 1.80$; $RE_{nucleus} = 1.03$) (Figure 18).

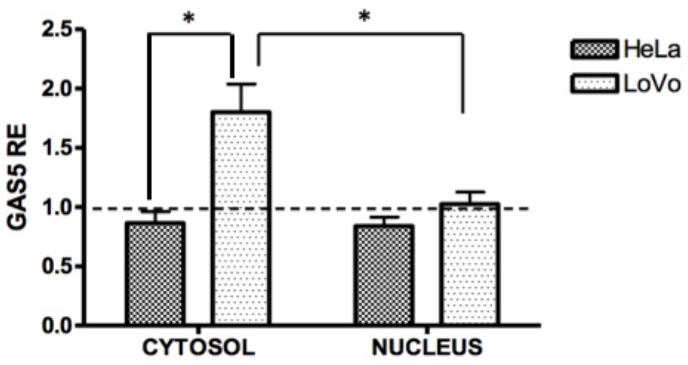


Figure 18: Intracellular localization of GAS5 in response to MP. Endogenous GAS5 relative expression (RE, values are expressed as $2^{-\Delta\Delta Ct}$) in HeLa and LoVo cytoplasmatic and nuclear compartment. GAS5 expression was calculated with respect to the housekeeping 18s gene. Cells were treated for 72 hours with MP (250 ng/ml). Two-way ANOVA ($p=0.0300$) and Bonferroni post-test * p-value<0.05. Data are reported as means ± SE of three independent experiments performed in triplicate.

Western blot analysis was performed with protein samples of the cytoplasmic and nuclear compartments of both HeLa and LoVo cells. Tubulin and PARP1 control antibodies were used to confirm that subcellular fractionation was successful, since tubulin protein is detectable only in the cytoplasm while PARP1 only in the nucleus. GR protein expression was evaluated before and after treatment with MP at 250 ng/mL. As expected, in both cell lines GR expression was significantly increased in the nuclear compartment after MP treatment (Figure 19).

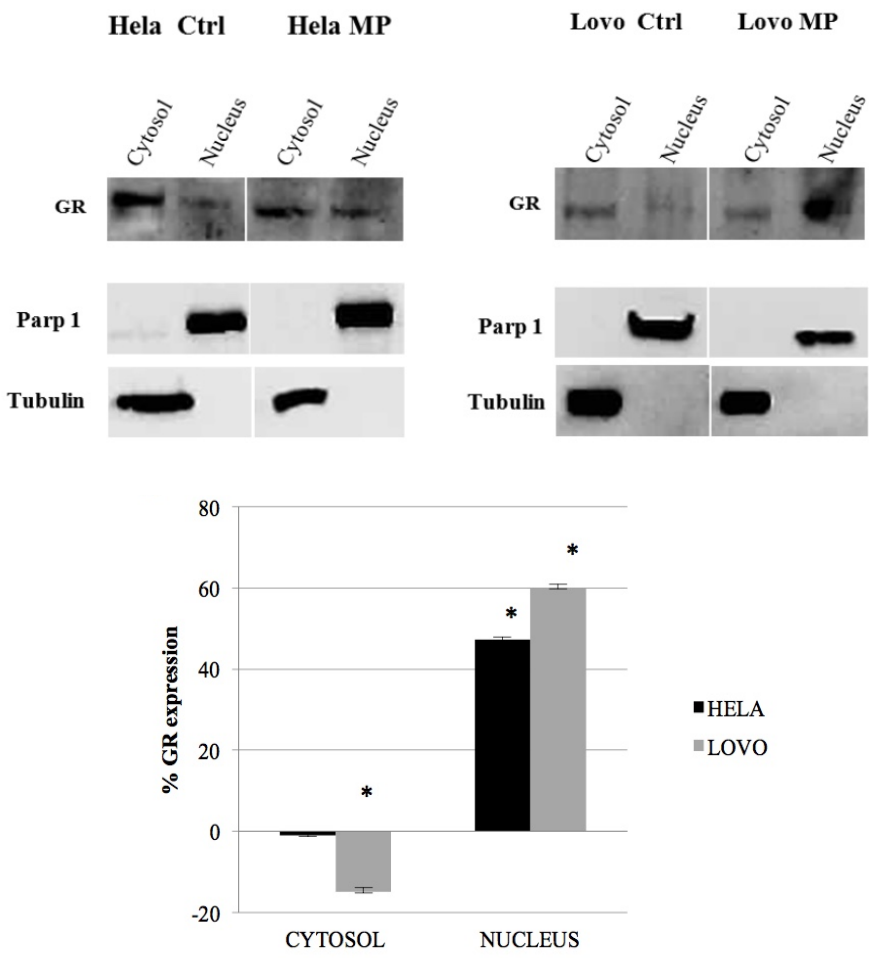


Figure 19: On the top, protein expression of GR evaluated by Western blot analysis on subcellular fractions of HeLa and LoVo cells treated with MP (250 ng/ml) for 72 hours. On the bottom, the percentage of GR expression evaluated in cytoplasm and nuclear compartments of HeLa and LoVo cells treated for 72 hours with MP in comparison with PARP1 and tubulin, respectively, GR expression was calculated as the ratio of 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.

In a previous work, Kino and collaborators have already analysed GAS5 expression in HeLa cells and described an increase of GAS5 levels in the nucleus after DEX treatment [129]. These results are in contrast with our findings, but this difference could be explained by the different experimental setting. In our study, MP was used, while in Kino's paper cells were treated with DEX, a steroid with a higher potency and duration of action in comparison with MP [152]. In addition, Kino and colleagues have examined the effect of the overexpression of GAS5, while we have considered endogenous levels.

A different pattern was shown in LoVo cell line: after MP treatment GAS5 was increased in the cytoplasm. We suggest that the accumulation of GAS5 in the cytoplasm could inhibit the activated GR before entering in the nucleus.

The upregulation of GAS5 in the cytoplasm does not depend from a transcriptional mechanism

but probably from other factors. Indeed, our results suggest that GAS5-AS1 could have a critical role in regulating GAS5 expression. Since we have already demonstrated an upregulation of GAS5-AS1 after MP treatment, the binding of the antisense with GAS5 could promote its accumulation in the cytoplasm of GC-resistant cells, as already described for other lncRNA in the same compartment [153,154].

4.4 GAS5 silencing in HeLa and LoVo cell lines

Studies on the modulation of GAS5 expression are useful to confirm its role in GCs mechanism of action. RNAi technology for gene knock down has become an important tool for gene function studies. Indeed, a small interfering RNA (siRNA) for GAS5 was used to silence the lncRNA and GCs responsiveness was observed in HeLa and LoVo cell lines. HeLa and LoVo cell lines were transfected with the control siRNA for luciferase (siLUCI) or with the siRNA for GAS5 and incubated for 72 hours with MP at three concentrations (10 ng/mL, 250 ng/mL and 20 μg/mL). Real time PCR was conducted on cell lines after 48 hours from siRNAs transfection; transfection efficiency was higher than 70-80%. Then, GCs activity was assessed by the [methyl-3H] thymidine viability assay to evaluate the inhibition of proliferation. After 72 hours in both cell lines, differences in proliferation were recorded. In particular, in GC-resistant cell line transfected with siGAS5 an inhibition of proliferation of 30% at 250 ng/mL and of 40% at 20 μg/mL was observed; these results were statistically significant when compared with the control siLUCI ($I_{250ng/mL}$= 4% and $I_{20\mu g/mL}$=29%). In HeLa cell lines, results have shown a similar trend with significant results at 10 ng/mL and 20 μg/mL (siGAS5, $I_{10ng/mL}$= 53 % and $I_{20\mu g/mL}$=82%; siLUCI, $I_{210ng/mL}$= 32% and $I_{20\mu g/mL}$=76%); at 250 ng/mL, HeLa cells maintained the same trend but the differences were not perceived probably because siLUCI and siGAS5 transfected cells exhibit a very high sensitivity (Figure 20).

These results confirm a key role of GAS5 in GCs resistance. More interestingly, in GC-resistant cell line, 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.

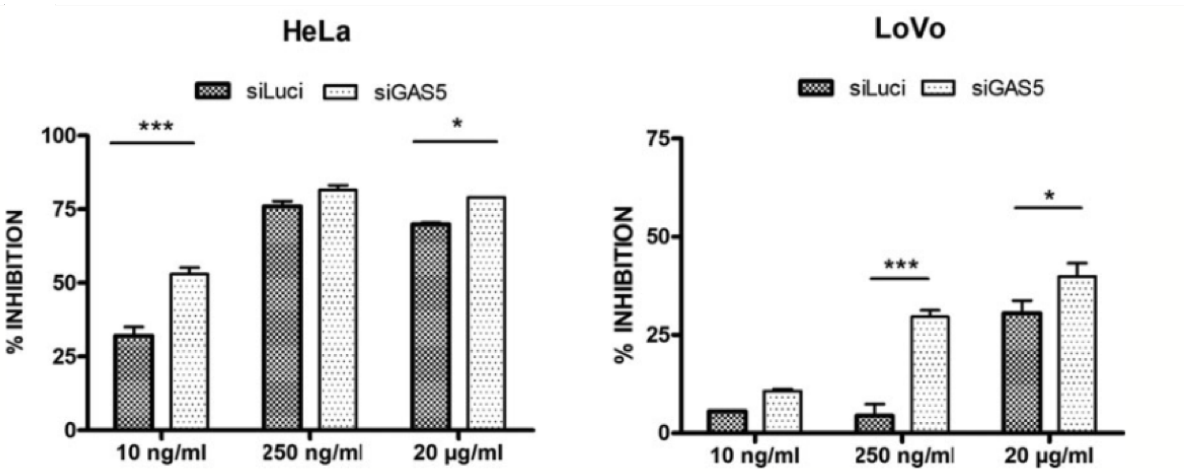


Figure 20: GAS5 silencing sensitizes LoVo and HeLa cells to MP treatment. Effect of MP on cell proliferation of LoVo and HeLa cells transfected with control (siLuci) or GAS5-selective (siGAS5) siRNA. Two-way ANOVA (LoVo $p = 0.0026$; HeLa $p = 0.0033$) and Bonferroni post-test ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. The data are reported as means ± SE of three independent experiments performed in quadruplicate.

4.5 GAS5 expression in pediatric patients with IBD

4.5.1 GAS5 gene expression analysis on PBMCs of pediatric patients with IBD

A prospective study was conduct on a cohort of nineteen children with IBD. For each patient, clinical characteristics were provided at diagnosis (T0) and after 4 weeks (T4) of treatment with prednisone 1-2 mg/kg/day (Table 5). Patients enrolled were classified based on their clinical response in three groups: four were SR, eight SD and seven SS.

Age, median (IQR)	13.36 (11.9–16.12)					
Male (%)	9 (47.4)					
Female (%)	10 (52.6)					
	T0			**T4**		
	SS	SD	SR	SS	SD	SR
PCDAI, score, median (IQR)	-	32.5 (30 - 35)	45 (*)	-	5 (5-5)	32.5 (*)
PUCAI, score, median (IQR)	25 (10-45)	40 (35–42.5)	30 (10 - 30)	0 (0-5)	0 (0-12.5)	15 (10-70)
Laboratory indexes						
C-reactive protein, median (IQR), mg/dL	0.12 (0.04–0.22)	0.35 (0.04–1.49)	1.17 (0.5–2.08)	0.04 (0.03–0.07)	0.07 (0.06–0.1)	0.42 (0.24–0.57)
Erythrocyte sedimentation rate, median (IQR), mm/hr	22 (6-47)	50 (33–80)	55.5 (20.25–63.75)	8 (5-19)	15 (8-35)	25 (10.5–42.5)
Faecal calprotectin, median (IQR), µg/g	1643 (1333-2380)	1800 (160.8-2338)	1986 (*)	63 (40-362)	164 (105–760.5)	2086 (1023-3148)
Haemoglobin, median (IQR), g/dL	12.4 (11.2–13.9)	10.7 (8.7–10.8)	10.8 (9.17–14.6)	13.8 (12.6-15)	12.2 (10.8–13.3)	12.15 (11.4–15.6)

Table 5: Clinical characteristics of the patients; T0 = diagnosis; T4 = after 4 weeks of treatment; SS = steroid-sensitive, SD = steroid-dependent, SR = steroid-resistant, (*) = data for only one patient.

GAS5 gene expression was evaluated by real time PCR analysis in PBMCs obtained at diagnosis (T0) and after 4 weeks (T4) of treatment. Results at T0 did not show relevant differences among the three groups. On the contrary, data obtained after 4 weeks of prednisone treatment have shown statistically significant differences among groups. Indeed, considering the relative expression of GAS5 at T4 compared to T0, an increase in GAS5 levels was observed in SR patients respect to SS and SD groups. Moreover, comparing GAS5 expression between SS and SD patients, an increase of the transcript levels was observed in the dependent group, even if this result is not significant. Interesting results were observed when the two groups with an unfavourable steroid response (SD+SR) were compared to SS patients. GAS5 expression was higher in SD+SR patients respect to the SS children (Log2 GAS5 $RE_{SS} = -0.878$; Log2 GAS5 $RE_{SD} = 0.679$; Log2 GAS5 $RE_{SR} = 1.611$) (Figure 21).

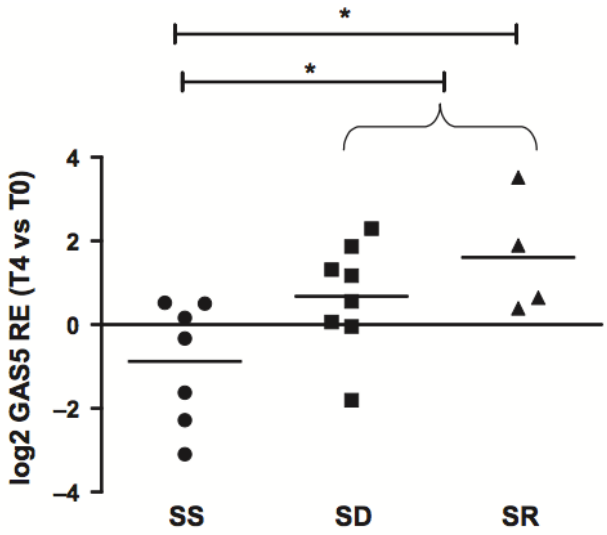


Figure 21: GAS5 levels in pediatric patients with IBD during GC treatment. Relative expression (RE, values are expressed as $2^{-\Delta\Delta Ct}$) of GAS5 in SS, SR and SD patients after treatment with prednisone 1–2 mg/kg/day for 4 weeks (T4) with respect to the diagnosis (T0). Overall GAS5 expression was different among GC responder groups (Kruskal–Wallis test $p = 0.033$). 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 $p = 0.016$. *$p < 0.05$.

These results describe, for the first time, a different trend in GAS5 expression in pediatric IBD patients treated with GCs. The experiments, already published, conducted in our laboratory on PBMCs from healthy donors [142,147] and data on immortalized cell lines [143] are in agreement with results of GAS5 gene expression on PBMCs obtained from children affected by IBDs. Indeed, no alterations were observed before treatment on basal GAS5 levels, but only after treatment with GCs a difference was evident. In fact, on the basis of GAS5 expression it is possible to

discriminate between good and poor responders to GCs. The results observed in pediatric patients have shown a higher expression of GAS5 in the group of patients with unfavourable response to GCs (SD+SR) compared to SS group, supporting the role of GAS5 in steroid ineffectiveness. In the two groups of pediatric patients, steroid-dependent and -resistant, the increase expression of GAS5 could interfere, through the binding with the DBD of the activated GR, with the transcription of GCs responsive genes, repressing its activity.
These results are promising, however GCs mechanism of action is extremely complex and other factors could be involved in GCs resistance [84,85].

4.6 Role of GAS5 in pediatric IBD mucosal biopsies and its activity in the tissue damage

In IBD pediatric patients, genetic and environmental factors are responsible of the alteration in epithelial barrier that involves also the activity of pro-inflammatory elements released from macrophages, T cells and innate lymphoid cells [19]. To date, GAS5 gene expression analysis in the mucosa of pediatric patients affected by IBDs has never been performed. For this reason, real time PCR methodology was applied to study GAS5 expression in inflamed and non-inflamed mucosa of IBD patients. Thirty-four patients at diagnosis were enrolled in a prospective study; for each patient, during a colonoscopy, an inflamed and a non-inflamed biopsy was collected in TRIzol® reagent. GAS5 gene relative expression were evaluated, showing a statistically significant difference comparing non-inflamed to inflamed mucosa from the same patient. Lower GAS5 gene expression levels were observed in inflamed mucosa respect to non-inflamed one (Log2 GAS5 RE $\Delta Ct_{Inflamed}$ = -4.436; Log2 GAS5 RE $\Delta Ct_{Non\text{-}inflamed}$ = -4.001) (Figure 22).

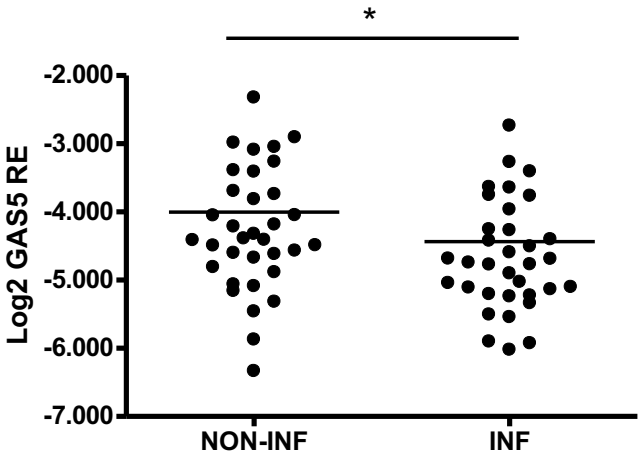


Figure 22: GAS5 levels in colon biopsies of IBD pediatric patients. Expression was evaluated in inflamed (INF) and non-inflamed (NON-INF) tissues from the same patient. GAS5 expression was calculated with respect to the housekeeping RPLP0 gene. Expression reported in Log_2 of GAS5 releative expression (RE, values are expressed as $2^{-\Delta Ct}$). Wilcoxon signed rank test, *p < 0.05.

Emerging data have shown that GAS5 is also involved in the regulation of important pro-inflammatory mediators such as MMP-2 and MMP-9 [139]. Chen et al. have shown that the expression of MMP-2 and MMP-9 is inversely correlated with the levels of the lncRNA GAS5 in melanoma cells. Overexpression of GAS5 reduced the levels of the gelatinases whereas the knockdown of GAS5 increased their expression. Until now, no data are published about the potential role of GAS5 in the regulation of MMP-2 and MMP-9 in inflamed colonic tissue of IBD patients [139]. Different studies have demonstrated that, in IBD patients, the expression of MMP-9 and MMP-2 is increased in inflamed tissues in comparison with non-inflamed ones [16,17]. In this scenario, it could be of interest to understand the involvement of MMP-2 and -9 and the role of GAS5 in their regulation.

4.6.1 Role of MMP-2, MMP-9 and GAS5 in the regulation of tissue damage in colon biopsies of pediatric IBD

Gene expression studies and preliminary protein expression analysis were performed to study the relation between gelatinases and GAS5 in colon biopsies of pediatric patients with IBD at diagnosis. Real time PCR results demonstrated an increased expression of both gelatinases in inflamed tissues (Log2 MMP-9 RE $\Delta Ct_{Inflamed}$ = -2.944; Log2 MMP-9 RE $\Delta Ct_{Non\text{-}inflamed}$ = -5.197; Log2 MMP-2 RE $\Delta Ct_{Inflamed}$ = -3.039; Log2 MMP-2 RE $\Delta Ct_{Non\text{-}inflamed}$ = -5.352) (Figure 23).

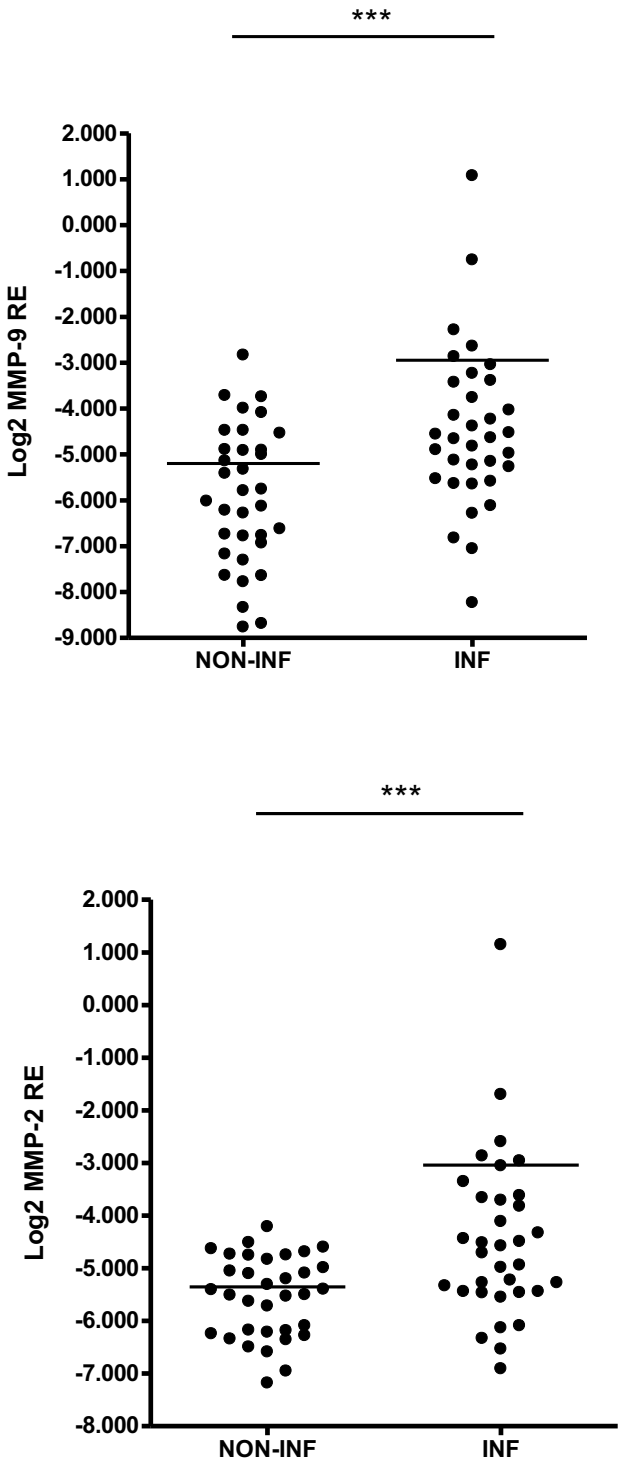


Figure 23: MMP-9 (on the top) and MMP-2 (on the bottom) levels in colon biopsies of IBD pediatric patients. Expression evaluated in inflamed (INF) and non-inflamed (NON-INF) tissues from the same patient. Expression was calculated with respect to the housekeeping RPLP0 gene. Expression reported as in Log_2 of MMP-9 and MMP-2 relative expression (RE, values are expressed as $2^{-\Delta Ct}$). Wilcoxon signed rank test, ***$p < 0.001$.

WB analysis of the MMP-9 protein was also performed. Preliminary results on protein extracted from inflamed and non-inflamed tissues of pediatric patients with IBD, have revealed an

increase in MMP-9 protein expression in inflamed tissues compared to non-inflamed ones (Figure 24).

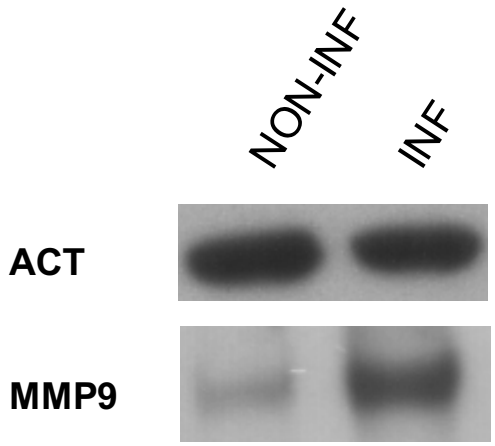


Figure 24: Representative image of three Western blot experiments on inflamed (INF) and non-inflamed (NON-INF) pediatric biopsies of patients affected by IBD. Actin (ACT) antibody used as loading control.

In the literature, MMP-2 was associated to infiltration processes of leukocytes in inflamed tissue, while MMP-9 was associated to an enhanced inflammation because of the activation of growth factors and pro-inflammatory cytokines [155]. More evidences are available about the role of MMP-9 in IBD, since an increase of gene and protein expression in IBD patients' inflamed tissues, serum, urine and fecal samples has been reported [16,18,156–158]. MMP-9 has been also proposed as a promising therapeutic target and a monoclonal antibody against this metalloproteinase is under study [159].

Comparing these results with GAS5 expression results on colon biopsies, an inverse correlation was observed since GAS5 is downregulated and MMP-2 and MMP-9 are upregulated in inflamed tissues.

4.7 GAS5, MMP-2 and MMP-9 gene expression in THP1 cell line

Infiltration of inflammatory cells in the epithelial barrier of colon mucosa is one of the first step in the onset of IBDs. Monocyte, macrophages and T-cell enhance the production of cytokines that can promote chronic inflammation in the gastrointestinal tract [19]. The THP-1 monocyte cell line was chosen to study the molecular mechanism by which GAS5 can regulate MMP-2 and MMP-9 expression. Experiments were conducted at different stages of differentiation, from monocyte to macrophages. RNA gene expression analysis was performed on control unstimulated cells, on cells stimulated with PMA (macrophages) or LPS, and on cells treated with PMA and then with LPS. Real time PCR results on GAS5 expression have shown a downregulation of endogenous levels in treated cells (GAS5 RE ΔCt_{ctrl}= 3.13e-004; RE ΔCt

$_{LPS}$= 1.03e-004; RE ΔCt $_{PMA}$= 0.67e-004; RE ΔCt $_{PMA+LPS}$= 0.48e-004) in comparison to controls. The decrease of GAS5 is more evident in PMA differentiated macrophages respect to the control (p=0.047) (Figure 25).

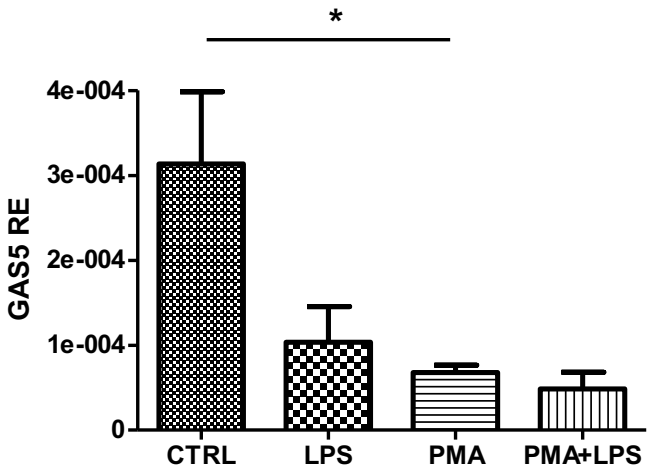


Figure 25: GAS5 gene expression levels in THP-1 cells treated with PMA and LPS. Relative expression (RE, values are expressed as $2^{-\Delta Ct}$) in THP-1 untreated cells (CTRL), stimulated with LPS (1 μg/mL), with PMA (5 ng/mL) and with both stimuli (PMA+LPS). Expression was calculated with respect to the housekeeping 18S gene. One-way ANOVA (p = 0.0471) and Dunn's Multiple Comparison Test, *p < 0.05. The data are reported as means ± SE of three independent experiments performed in triplicate.

Only a slight increase in monocytes after LPS stimulation was observed in MMP-2 expression levels (MMP-2 RE ΔCt $_{ctrl}$= 0.40e-005; RE ΔCt $_{LPS}$= 0.50e-005) while in PMA differentiated macrophages an increase of MMP-2 was recorded (MMP-2 RE ΔCt $_{PMA}$= 1.86e-005). The upregulation of the gelatinase was not maintained after the treatment of macrophages with the pro-inflammatory stimulus (MMP-2 RE ΔCt $_{PMA+LPS}$= 1.1e-005) (Figure 26).

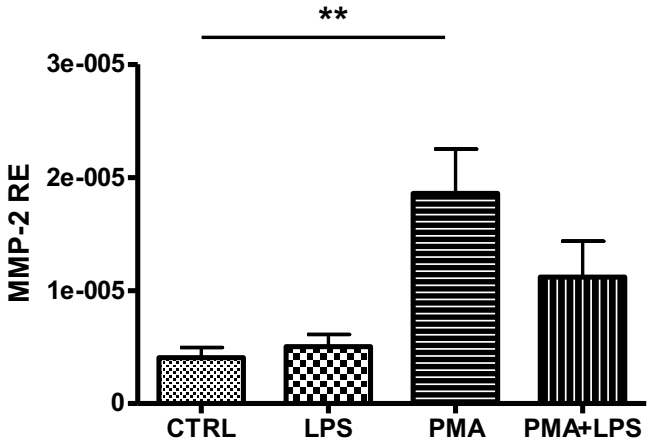


Figure 26: MMP-2 gene expression levels in THP-1 cells treated with PMA and LPS. Relative expression (RE, values are expressed as $2^{-\Delta Ct}$) in THP-1 untreated cells (CTRL), stimulated with LPS (1 μg/mL), with PMA (5 ng/mL) and with both stimuli (PMA+LPS). Expression was calculated with respect to the housekeeping 18S gene. One-way ANOVA (p = 0.0135) and Dunn's Multiple Comparison Test, ** p < 0,01. The data are reported as means ± SE of three independent experiments performed in triplicate.

MMP-9 expression levels in THP-1 monocyte stimulated with LPS have shown the same trend observed for MMP-2 (MMP-9 RE ΔCt_{ctrl}= 0.12e-006; RE ΔCt_{LPS}= 1.07e-006). However, differently from MMP-2, an upregulation of MMP-9 levels was evident in PMA-differentiated macrophages, that increased further after the stimulation with LPS (MMP-9 RE ΔCt_{PMA}= 1.87e-004; RE $\Delta Ct_{PMA+LPS}$= 1.95e-004) (Figure 27).

These are interesting results since THP-1 cell line differentiated in macrophages with 48 hours of PMA treatment could represent a good cellular model to better understand the mechanism by which GAS5 could have a role in mediating gut barrier function in IBD patients by regulating the effect of MMPs. These findings could improve the knowledge on IBD pathogenesis.

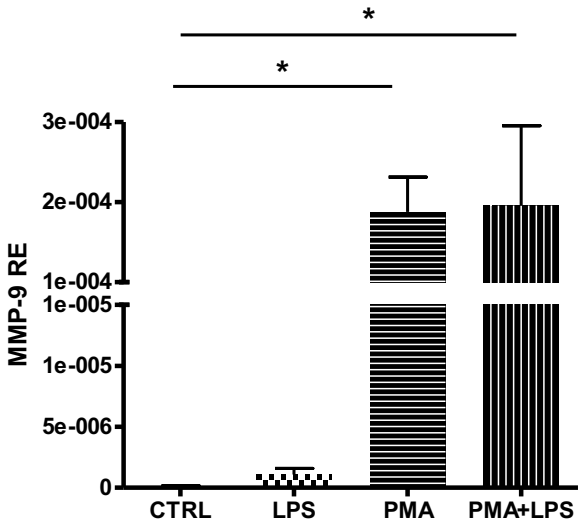


Figure 27: MMP-9 gene expression levels in THP-1 cells treated with PMA and LPS. Relative expression (RE, values are expressed as $2^{-\Delta Ct}$) in THP-1 untreated cells (CTRL), stimulated with LPS (1 µg/mL), with PMA (5 ng/mL) and with both stimuli (PMA+LPS). Expression was calculated with respect to the housekeeping 18S. One-way ANOVA ($p = 0.0140$) and Dunn's Multiple Comparison Test, * $p < 0,05$. The data are reported as means ± SE of three independent experiments performed in triplicate.

5.RESULTS AND DISCUSSION PART II

5.1 TTP expression in colon tissues of IBD patients

5.1.1 TTP gene expression analysis in the colonic mucosa of children with IBD

TTP protein is encoded by the *ZFP36* gene in human cells with a particular transcriptional regulation. Indeed, the TTP mRNA expression *in vitro* is transient since the transcript becomes detectable only under some condition (i.e. insulin or LPS stimulation) in certain cell types (fibroblast and macrophages) and only for a limited time, depending from the stimulation; subsequently TTP mRNA levels return to baseline [42,45]. Little it is known on the regulation of TTP mRNA transcripts in inflammatory diseases. For this reason, real time PCR analysis was performed on colon tissues of IBD patients, since TTP is detectable in the large intestine [160]. Gene expression analysis was performed on thirty-four pediatric patients enrolled at diagnosis, for each patient an inflamed and a non-inflamed biopsy was collected. TTP mRNA expression results, comparing inflamed and non-inflamed biopsies, have shown no statistically significant differences (TTP ΔCt_{INF}= 4.682, TTP $\Delta Ct_{NON\text{-}INF}$= 4.384) (Figure 28).

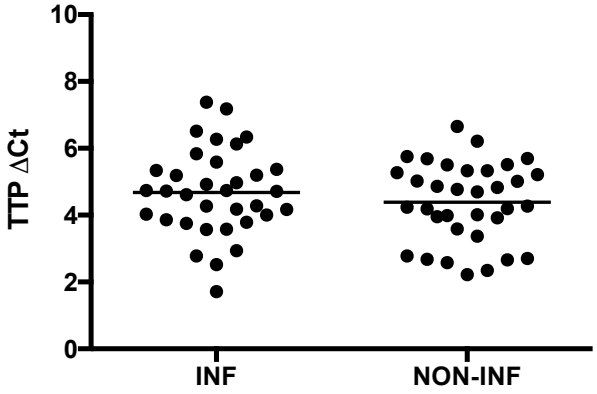


Figure 28: TTP levels in colon biopsies of IBD pediatric patients. Expression evaluated in inflamed (INF) and non-inflamed (NON-INF) tissues from the same patient. TTP expression was calculated with respect to the housekeeping RPLP0. Expression reported as $_{\Delta}$Ct. Wilcoxon signed rank test, p=0.1566.

Similar data were obtained by Suzuki and collaborators studies, who could not find differences in TTP mRNA expression in the synovium of patients with RA and control patients with osteoarthritis [161]. Therefore TTP was analysed at the protein level, since the protein undergoes extensive post-translational modifications, particularly phosphorylations, that influence its stability and activity [160].

5.1.2 TTP and 14-3-3 protein expression on pediatric IBD colon mucosa

TTP protein has been studied in different cell lines however little it is known on the behaviour of the protein in tissues of patients affected by pathologies were inflammation has a predominant role. This lack of information is in part due to the difficulty to detect TTP protein, since in most cells and tissues its expression is very low [162,163]. The protein is detectable in spleen, thymus, lung, and liver, and in large intestine [160]. In addition, TTP is a very unstable protein and requires particular attention in the isolation and detection. To set the best procedure to study TTP expression from protein extracted from pediatric IBD patients' biopsies, different methods were tested. We chose to apply IP analysis, since this technique gave the best results, allowing to enrich the protein of interest in tissue extracts. The experiments were conducted on of four IBD pediatric patients at different phases of the diseases. For each patient, after a colonoscopy, an inflamed and a non-inflamed portion of the colon was collected and immediately frozen in dry ice. Patients were classified on the basis of the histologic inflammatory score (range from 0 to 5) of the inflamed biopsy (Table 6).

Age, median (IQR)	13.93 (12.49 – 15.12)		
Male (%)	3 (75)		
Female (%)	1 (25)		
Patients	SCORE	DISEASE	DISEASE PHASE
Patient 1	3	CD	active phase
Patient 2	4	CD	active phase
Patient 3	1	UC	remission phase
Patient 4	4	UC	diagnosis

Table 6: Histological characteristic of inflamed colon mucosa of pediatric IBD patients; the active phase of the disease is described with a discontinuous or a complete loss of vascular pattern, hyperemia and deep ulcerations. In the remission phase an improvement of the vascular pattern was observed.

After IP of TTP a Western blot analysis was performed to quantify the protein expression in the tissues. Results of TTP protein levels have shown an increased expression in inflamed mucosa respect the non-inflamed (fold induction $_{inf/non\text{-}inf}$ = 2.67), and these results are statistically significant (Figure 29).

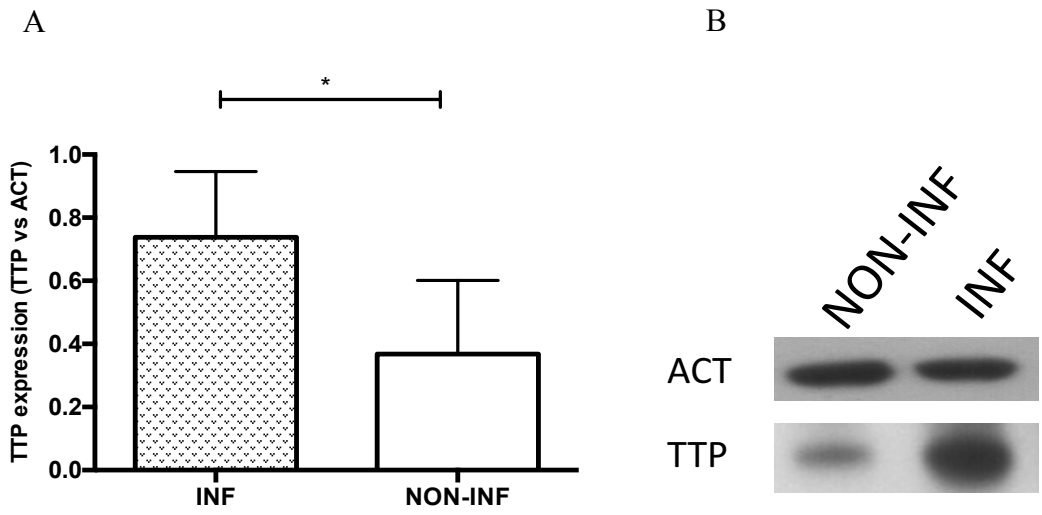


Figure 29: A. TTP expression in colon mucosa of pediatric IBD patients. IP for TTP on inflamed and non-inflamed frozen colon IBD tissues. Expression was calculated with respect to Actin (ACT), used as load control. Parametric T-test: * $p < 0,05$. B. Representative image of Western blot experiments for TTP protein on inflamed (INF) and non-inflamed (NON-INF) biopsies of pediatric patients affected by IBD.

Phosphorylations could be the cause of the higher expression of TTP in inflamed tissues, since it was demonstrated that after phosphorylation of two serine residues (Ser-60 and Ser-186) the recruitment of the deadenylation complex is impaired and TTP is stabilized. This alteration of TTP expression and activity was related to the role of a protein complex, the 14-3-3, that recognizes and controls phosphorylated proteins [57]. For this reason, the Co-IP assay, with an antibody for 14-3-3, was performed on protein lysates of colon biopsies immunoprecipitated for TTP, since this technique permits the recognition of protein-protein complexes. Western blot analysis was used to quantify 14-3-3 protein expression in inflamed and non-inflamed colon mucosa of IBD patients. Results have shown the same trend observed in TTP expression, confirming the interaction of the 14-3-3 with the zinc finger protein, but the data are not statistically significant because of the great variability observed in the non-inflamed tissues (Figure 30).

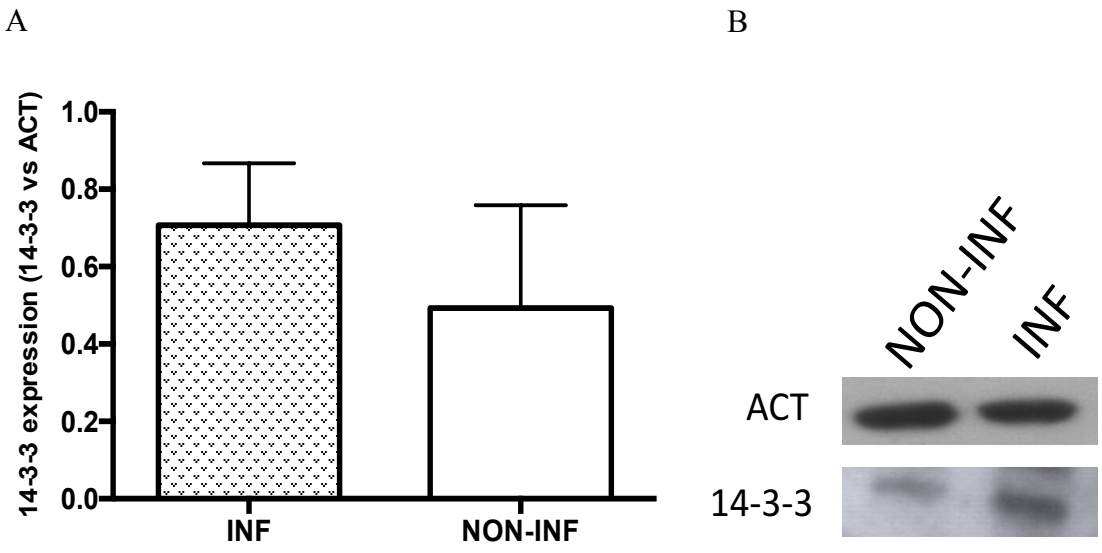


Figure 30: A. 14-3-3 expression in colon mucosa of pediatric IBD patients. Co-IP for 14-3-3 on inflamed and non-inflamed frozen colon IBD tissues. Expression was calculated with respect to Actin (ACT), used as load control. Parametric T-test: p=0.189. B. Representative image of Western blot experiments for 14-3-3 protein on inflamed (INF) and non-inflamed (NON-INF) biopsies of pediatric patients affected by IBD.

Only few studies analysed the role of TTP directly in the site of inflammation [59,164,165], and this is the first time that TTP expression was evaluated in patients affected by IBDs. The results described in inflamed and non-inflamed colon mucosa are in agreement with what was observed by other authors, indeed, TTP is significantly increased in inflamed samples respect the non-inflamed ones. Ross and collaborators demonstrated an increased TTP expression in synovial tissue of patients with RA compared with non-inflamed controls using an immunostaining approach. Furthermore the authors demonstrated a higher TTP signal in CD68+ macrophages of patients synovia and the co-localization of TTP with activated MK2 in the cytoplasm of macrophages, confirming a role of phosphorylation in the highest expression of TTP in inflamed tissues [59]. Also in our work we have demonstrated, in an indirectly, way the importance of phosphorylation in TTP activity. Indeed, the analysis of protein-protein interaction with the Co-IP assay was performed to demonstrate the formation of the TTP-14-3-3 complex, that occurs only if TTP is phosphorylated. We have shown the same trend of expression between TTP and 14-3-3 but, due to the small number of samples and the great variability observed, in particular in non-inflamed tissues, our results are not statistically significant.

5.2 TTP expression in mice and human macrophages

5.2.1 TTP and 14-3-3 expression in the RAW264.7 cell line

The RAW264.7 macrophages cell line produce much more TTP than the other cell types tested [160]. Experiments conducted on this cell line have shown that, after LPS stimulation, TTP was detectable on Western blot, but barely visible in the unstimulated cells [166]. Experimental studies showed that in LPS-stimulated RAW264.7 cells, TTP protein accumulated and reached steady-state levels after 120 minutes of stimulation [160]. For this project, we used this cell line as a model for studying TTP expression in macrophages. In further experiments on human macrophages differentiated from IBD patients, IP and Co-IP were performed because of the low amount of protein available; for this reason, also on RAW264.7 cells the same approaches were used. Indeed, after LPS stimulation for 2 hours, the protein lysate from RAW264.7 cells was obtained and IP with the antibody for TTP was performed. Western blot results showed an increase of the protein expression after LPS stimulation as observed in Figure 30 (fold induction $_{\text{LPS 4h/uns}}$= 30.22). Co-IP results with the 14-3-3 antibody showed the same trend of expression observed for TTP protein (fold induction $_{\text{LPS 4h/uns}}$= 5.53; Figure 31).

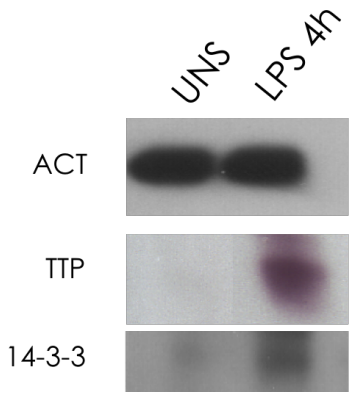


Figure 31: Representative image of three Western blot experiments. Western blot was performed on IP for TTP and Co-IP for 14-3-3 on RAW264.7 cells unstimulated (UNS) and 4 hours of LPS stimulation. Actin (ACT) used as load control.

These results have confirmed what it is already known in the literature: in RAW264.7 cells after LPS stimulation (10 ng/mL) a high expression of TTP was detectable and the pro-inflammatory stimulus promote the phosphorylation of the TTP protein that, in this conformation, it is protected by the 14-3-3 protein complex [57,160].

TTP mRNA targets are mostly pro-inflammatory cytokines such as TNF-α, IL-1β, IL-2 and IL-6 [51]. To evaluate if the high expression of TTP was linked to a variation in protein activity, cytokines expression was assessed through the Mouse Cytokine 23-plex panels. Only levels of cytokines mentioned above are reported in the present thesis. The values were expressed as pg of cytokines for μg of total protein. Results showed an increase of expression levels in all cytokines, particularly evident in TNF-α and IL-6 (TNF-α $_{uns}$= 0.0695 pg/μg, TNF-α $_{LPS\ 4h}$= 2.125 pg/μg; IL-6 $_{uns}$=0.0065 pg/μg, IL-6 $_{LPS\ 4h}$=94.77 pg/μg), while IL-1β and IL-2 are at the limit of significance (IL-1β $_{uns}$= 0.4060 pg/μg, IL-1β $_{LPS\ 4h}$= 0.5965 pg/μg; IL-2 $_{uns}$=0.0245 pg/μg, IL-2 $_{LPS\ 4h}$=0.0325 pg/μg; Figure 32).

This analysis has shown an increase expression of pro-inflammatory cytokines after LPS stimulation in RAW264.7 cells. These data have confirmed the role of the phosphorylated inactive TTP in stabilizing the mRNA transcripts through the binding with the ARE sequence on TNF-α, IL-1β, IL-2 and IL-6 cytokines enhancing their expression after a pro-inflammatory stimulus [46,167,168].

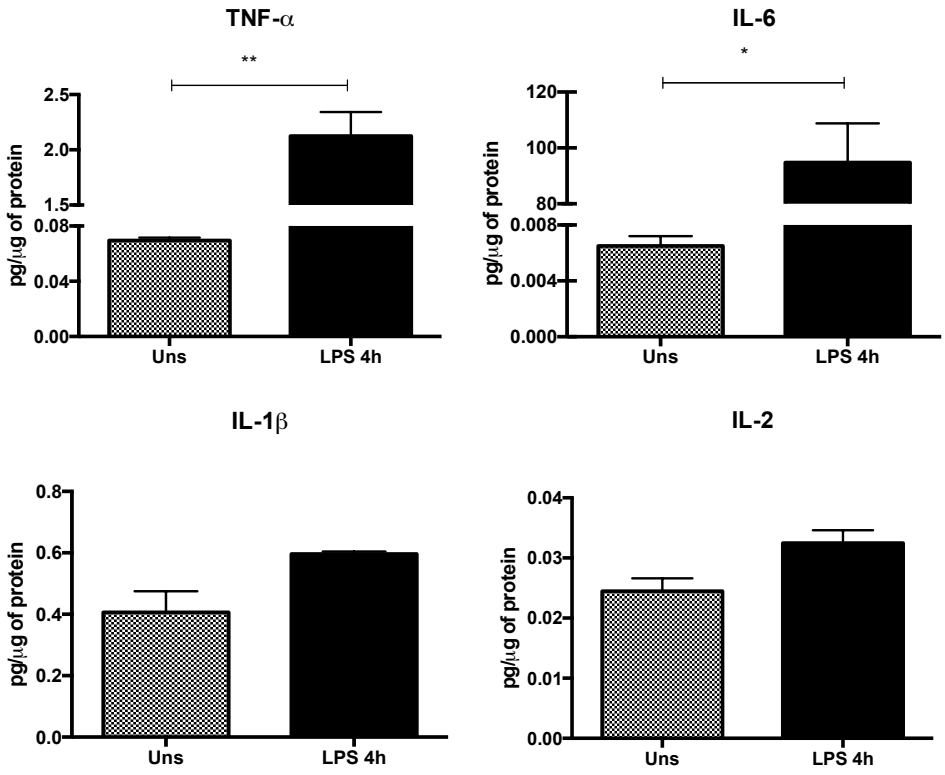
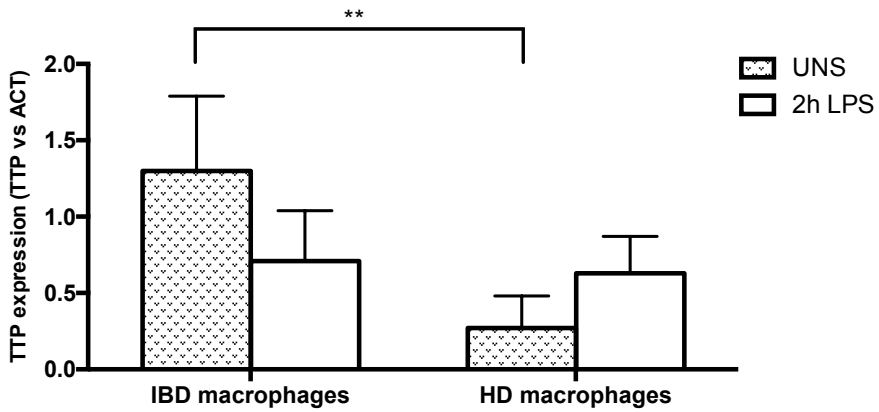


Figure 32: Mouse Cytokine 23-plex panels analysis. Supernatant of RAW264.7 cells unstimulated (uns) or stimulated with LPS (10 ng/mL) for 4 hours. Most common pro-inflammatory cytokines related to TTP activity were considered (TNF-α, IL-1β, IL-2 and IL-6). Values were expressed in pg of cytokines on μg of total protein. Parametric t-test TNF-α p= 0.0056; Parametric t-test IL-6 p= 0.0108; Parametric t-test IL-1β p= 0.0609; Parametric t-test IL-2 p= 0.0637. The data are reported as means ± SE of two independent experiments performed in duplicate.

5.2.2 TTP and 14-3-3 expression in human macrophages

TTP is a stable cytoplasmic protein, once induced by pro-inflammatory stimuli, in macrophages and in fibroblast [160]. Most of the studies were conducted on immortalized or primary mouse macrophages and little it is known about the TTP expression in human macrophages and above all in macrophages differentiated from patients affected by inflammatory diseases [51]. In this work macrophages were differentiated from PBMCs of four IBDs pediatric patients with active disease and from PBMCs of four healthy donors used as control. Differentiation of macrophages from blood of pediatric IBDs patients was difficult because of the poor initial cells number and above all for their disease status. For this reason, IP assay was necessary to detect TTP expression. Western blot results have shown a different trend of TTP expression between macrophages of IBDs patients and healthy donors. The zinc finger protein, as expected, in healthy donors is upregulated after LPS treatment for 2 hours ($HD_{uns\ (TTP/ACT)}$= 0.27; $HD_{2h\ LPS\ (TTP/ACT)}$=0.63); on the contrary TTP expression in IBDs patients was higher in unstimulated macrophages compared to macrophages stimulated with LPS ($IBD_{uns\ (TTP/ACT)}$= 1.3; $IBD_{2h\ LPS\ (TTP/ACT)}$=0.71). Interestingly, expression pattern of TTP was significantly different in unstimulated macrophages between IBDs patients and healthy donors (Figure 33).

A



B

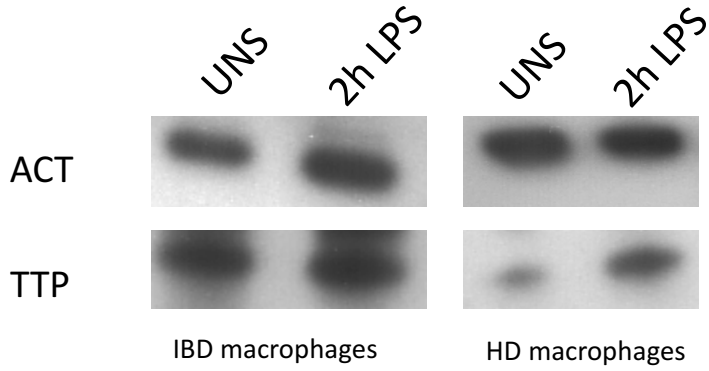
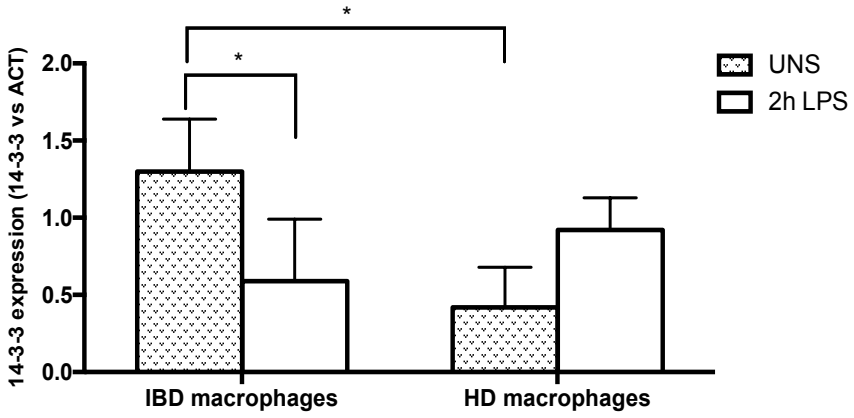


Figure 33: A. TTP expression of IBD and healthy donors (HD) macrophages. IP for TTP on unstimulated (uns) and LPS (2h LPS) treated macrophages differentiated from IBD patients and HD. Expression was calculated respect the Actin (ACT), used as load control. Two-way Anova: p=0.015; Bonferroni post-test: **p<0.01. B. Representative image of Western blot experiments for TTP protein.

Co-IP with an antibody against 14-3-3 protein was performed to demonstrate if the differences observed in unstimulated macrophages are correlated to the phosphorylation state of TTP and hence to an increased stability. Western blot results of 14-3-3 protein have shown the same trend of TTP expression with significant differences between unstimulated macrophages ($HD_{uns\ (14\text{-}3\text{-}3/ACT)}$= 0.42; $IBD_{uns\ (14\text{-}3\text{-}3/ACT)}$= 1.3) and between IBD unstimulated and LPS stimulated macrophages ($IBD_{uns\ (14\text{-}3\text{-}3/ACT)}$= 1.3; $IBD_{2h\ LPS\ (14\text{-}3\text{-}3/ACT)}$= 0.59; Figure 34).

A



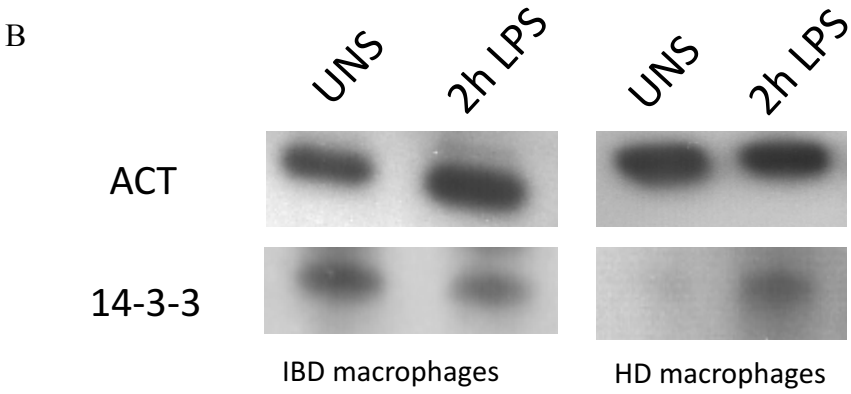


Figure 34: 14-3-3 expression of IBD and healthy donors (HD) macrophages. Co-IP for 14-3-3 on unstimulated (uns) and LPS treated macrophages differentiated from IBD patients and HD. Expression was calculated respect the Actin (ACT), used as load control. Two-way Anova: $p=0.0022$; Bonferroni post-test: $*p<0.05$. B. Representative image of Western blot experiments for 14-3-3 protein.

Results of TTP expression in macrophages derived from healthy donors confirm data already reported in literature [169], indeed an increase of TTP expression after LPS stimulation was observed even if at the limit of significance ($p=0.0595$). However, interesting results were described in macrophages differentiated from IBDs patients: in fact, in immunoprecipitate macrophages higher levels of the zinc finger protein were observed in unstimulated samples compared to macrophages treated with LPS. These data are not statistically significant because of the variability observed among samples and the poor number of patients. In addition, comparing unstimulated macrophages from IBDs patients and healthy donors an increase of TTP expression was evident in favour of IBDs patients. Hence, levels of TTP protein in IBD patients would be indicative of active inflammation, though these data must be confirmed in a larger patients' cohort.
Western blot analysis on 14-3-3 protein expression has shown the same trend of TTP expression confirming indirectly the involvement of phosphorylation in the activity and expression of TTP.

Macrophages derived from IBDs patients have shown a decrease of TTP expression after LPS stimulation. In the literature, a downregulation of TTP expression has been demonstrated after a prolonged exposure to pro-inflammatory stimuli, but the exact switching between the activated and inactivated state of protein is still unknown [170]. On the basis of the results obtained in this work we can hypothesize that the stimulation with LPS in IBD samples could cause a decrease of TTP activity and an increase of its degradation through the proteasome activity. Further studies are needed to clarify this mechanism of action on TTP stabilization.

To confirm the role of TTP in controlling the stability of pro-inflammatory cytokines, the Bio-

Plex Pro Human Cytokine 17-plex panels was performed to quantify cytokines expression on supernatant of human macrophages differentiated from IBDs patients and healthy donors. Due to the great variability and small number of patients, no significant differences could be observed between unstimulated and LPS stimulated IBD macrophages (Figure 35A). In healthy donors, instead, statistically significant differences were observed only for TNF-α (p= 0.0084) and IL-1β (p= 0.0062) (Figure 35B).

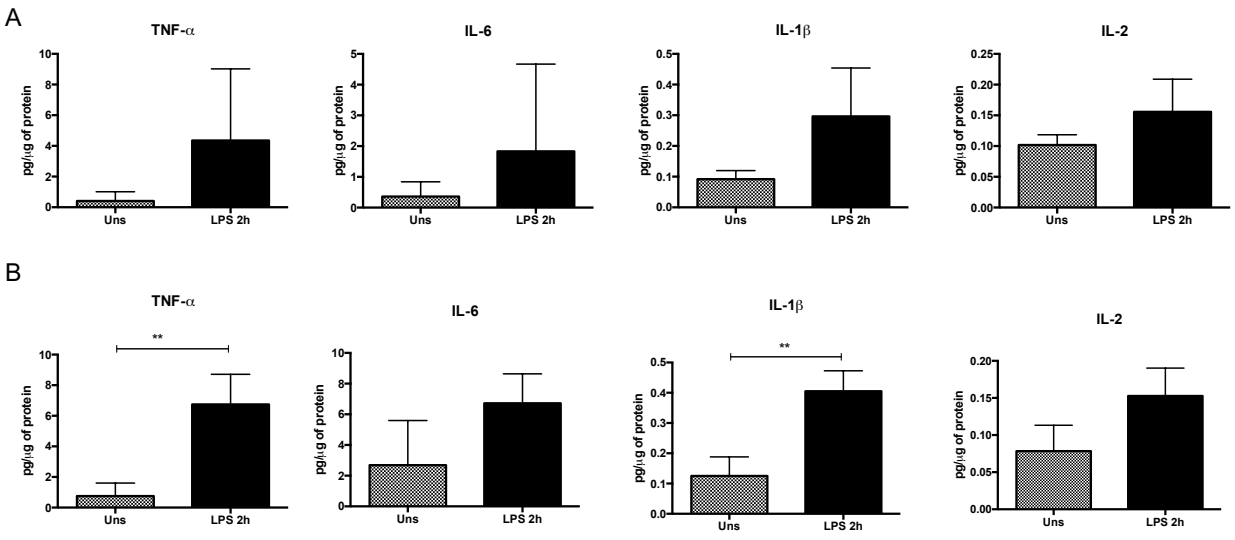


Figure 35: Bio-Plex Pro Human Cytokine 17-plex panels. A. Supernatant of macrophages differentiated from PBMCs of IBDs patients unstimulated (uns) or stimulated with LPS (10 ng/mL) for 2 hours. Most common pro-inflammatory cytokines related to TTP activity were considered (TNF-α, IL-1β, IL-2 and IL-6). Values were expressed in pg of cytokines on μg of total protein. Parametric t-test TNF-α p= 0.2195; Parametric t-test IL-6 p= 0.4242; Parametric t-test IL-1β p= 0.0915; Parametric t-test IL-2 p= 0.1693. B. Supernatant of macrophages differentiated from PBMCs of healthy donors unstimulated (uns) or stimulated with LPS (10 ng/mL) for 2 hours. Values were expressed in pg of cytokines on μg of total protein. Parametric t-test TNF-α p= 0.0084; Parametric t-test IL-6 p= 0.1148; Parametric t-test IL-1β p= 0.0062; Parametric t-test IL-2 p= 0.0664. The data are reported as means ± SE of two independent experiments performed in duplicate.

The cytokines expression results of unstimulated macrophages differentiated from IBDs patients in comparison to healthy controls did not show any significant difference. These results do not support the hypothesis of a role of phosphorylated TTP in controlling pro-inflammatory cytokines expression. The evaluation of cytokines expression in differentiated macrophages does not represent a useful tool to study the involvement of TTP protein in IBD pathogenesis.

6.CONCLUSION

IBD is a multifactorial disorder that is under the control of a series of interactions among genetics, environmental factors, gut microbiota and immune response. The chronic, relapsing or progressive inflammatory conditions can involve the entire gastrointestinal tract [9]. To date, a curative pharmacological therapy for IBD does not exist and the therapeutic approach is mainly aimed at the treatment and control of inflammation. Despite the introduction of novel biological therapies, GCs remain widely used for inducing remission in IBD, in particular for UC. Given the high incidence of suboptimal response, associated with a significant number of side effects, particularly severe in children, the identification of patients that are most likely to respond poorly to GCs is extremely important. The mechanisms of this variability are scarcely understood and there is presently no means to predict the response in advance. In this context, the role of the lncRNA GAS5 and the possible correlation between its expression and variability in GC response was the topic of the first part of my thesis.

The expression of GAS5, a molecule able to interact directly with the GR and impair its transcriptional activity, was evaluated both in immortalized cell lines treated with GCs and in PBMCs of patients before and after the administration for 4 weeks of GCs. Our results demonstrate that GAS5 is differently expressed in sensitive and resistant immortalized cells and positively correlates with drug resistance. Interestingly, the same profile was observed in PBMCs of IBDs pediatric patients in which an upregulation of GAS5 in subjects with unfavourable steroid response was demonstrated.

In conclusion, this part of the study provides molecular and clinical evidences that GAS5 should be considered a novel pharmacogenomic biomarker useful for the personalization of GC therapy in paediatric IBD. If these preliminary data will be confirmed in a larger cohort of patients, the development of an assay based on GAS5 screening in patients' PBMCs obtained at diagnosis and treated with GCs *in vitro* could be proposed to predict clinical response, helping clinicians in the adjustment of the current protocols. Moreover, inhibition of GAS5 by a specific molecule could be considered as a strategy to restore GC response.

Emerging data have shown that GAS5 is also involved in the regulation of important mediators of tissue injury, such as MMP-2 and MMP-9, and in the process of epithelial-mesenchymal transition, factors implicated in the pathogenesis of IBD[16], but there are no data about the role of GAS5 in mediating tissue damage and maintaining gut barrier function in IBD patients. Experiments conducted on inflamed and non-inflamed colon tissues obtained from IBD patients have demonstrated that GAS5 expression was significantly decreased in inflamed mucosa of patients compared to non-inflamed sites and increased levels of MMPs gene and protein expression were observed in inflamed tissues as expected. Moreover, the in vitro experiments demonstrated that THP-1 cell line differentiated in macrophages could represent a good cellular

model to better understand the mechanism by which GAS5 could have a role in mediating gut barrier function by regulating the effect of MMPs.
These preliminary results provide new information about the functional role of GAS5 in the regulation of MMP-2 and MMP-9 in IBD patients even though further investigations are needed on a large group of IBDs patients. In order to confirm the molecular mechanism by which GAS5 regulates MMPs levels in IBD patients, RNA interference and over-expression experiments to modulate GAS5 levels and to examine its effect on the expression of MMP2 and MMP-9 in terms of transcription and activity of the proteins should be examined.

In inflamed and non-inflamed colon mucosa of IBDs patients the expression of TTP, a zinc finger protein able to interact and inhibit pro-inflammatory cytokines through the binding with ARE on mRNA sequences, was studied in the second part of my thesis. In addition, considering that phosphorylation inactivates protein activity impairing TTP ability in pro-inflammatory cytokines degradation, the role of 14-3-3 complex protein was also evaluated. Indeed the 14-3-3 complex recognizes and binds TTP only when phosphorylated. Results have shown a higher expression of the TTP and 14-3-3 proteins in the site of inflammation, demonstrating the involvement of TTP and its phosphorylation in inflamed colon biopsies. In macrophages differentiated from IBDs patients a greater endogenous expression of TTP and 14-3-3 was highlighted, demonstrating that inflammation is closely related to high levels of phosphorylated protein expression. These preliminary results, if confirmed with further experiments, could open new perspectives in the study of IBDs and in the investigation of new target therapies based on the modulation of TTP phosphorylation by phosphatases, such as the serine/ threonine phosphatase protein phosphatase 2A [59,171].

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