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XXXIII CICLO DEL DOTTORATO DI RICERCA IN SCIENZE DELLA RIPRODUZIONE E DELLO SVILUPPO

"IDENTIFICATION OF MOLECULAR MARKERS OF DISEASE AND RESPONSE TO THERAPY IN PEDIATRIC PATIENTS WITH INFLAMMATORY BOWEL DISEASES"

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INDEX

ABSTRACT

Inflammatory bowel disease (IBD) is a chronic immune-mediated condition of the gastrointestinal tract that includes Crohn's 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. In the first part of the thesis project the role of the lncRNA GAS5 was studied, reporting the possible involvement of this lncRNA in the pathogenesis of tissue damage, thorough the regulation of mediators of tissue injury, in particular MMP-2 and MMP-9. From data obtained on biopsy tissues of pediatric patients affected by IBD and from *in vitro* experiments, it is clear that GAS5 could be involved in the processes of tissue damage and in determining a chronic inflammatory state regulating the expression of MMP-2 and MMP-9, proteases that, in IBD cause serious damage to the intestinal wall. The identification of a molecular target capable of modulating the levels of these enzymes would allow not only to deepen the knowledge about the causes of an altered production of MMP-2 and MMP-9 but also to develop a targeted pharmacological strategy. Moreover by means of RNA-IP experiment it was shown that this lncRNA was able to bind and interact physically with NF-κB, a transcriptional factor involved in inflammation process. GAS5 binds NF-κB probably modulating its transcriptional activity, demonstrating the involvement of this lncRNA in inflammation and confirming its role as biomarker of inflammation.

To date, there is no curative drug therapy for IBD and the therapeutic approach is aimed at inducing remission and promoting its maintenance. Recent studies have shown that thalidomide is very effective in children with IBD, but the mechanism of action of this drug still remains ambiguous. The second part of this thesis was focused to obtain a greater knowledge of the mechanisms of action of thalidomide in patients with IBD, in order to identify not only markers of efficacy but also to investigate the molecular causes that trigger peripheral neuropathy, a serious side effect of this drug. Transcriptome analysis, performed on blood samples from IBD pediatric patients refractory to standard therapies and treated with thalidomide were performed, and 252 differentially expressed genes emerged from RNA-seq, following treatment with the drug. The hypergeometric test on gene ontology (GO) annotations, revealed a series of altered pathways implicated in the mechanism of thalidomide and its main adverse effect, including the eicosanoid and prostaglandin receptor signaling pathway and related to the G protein-coupled adenylate cyclase pathway, and also pathways important in the nervous system. To validate these transcriptomic results, PGE2 and cAMP were quantified in monocytic Thp-1 cells and Thp-1 differentiated in macrophages. Thalidomide caused a significant decrease of PGE2

production in macrophages stimulates with LPS, whereas in Thp-1 the drug treatment induced a significant increase in cAMP production. Preliminary studies conducted on the neuronal cell line sh-sy5y, in order to identify new pathways and genes involved in thalidomide induced peripheral neuropathy, revealed that this drug did not have a cytotoxic effect, nor an action at the level of mitochondrial activity. ATP quantification and analysis on intracellular calcium in sh-sy5y revealed that the drug induced an increase in ATP production and in intracellular calcium. This preliminary study provided the basis for carrying out targeted studies that will allow us to broaden our knowledge of this drug and identify response markers to promote the personalization of thalidomide treatment in pediatric patients with IBD.

RIASSUNTO

Le malattie infiammatorie croniche intestinali (MICI) sono malattie caratterizzate da una condizione cronica immuno-mediata del tratto gastrointestinale che includono il morbo di Crohn e la rettocolite ulcerosa. Le MICI che colpiscono pazienti pediatrici sono di particolare interesse poiché la loro incidenza è in aumento e, anche se vengono utilizzate strategie farmacologiche diverse, il trattamento ottimale è lungi dall'essere raggiunto. Nella prima parte del progetto di tesi è stato studiato il ruolo dell'lncRNA GAS5, mettendo in evidenza il suo possibile coinvolgimento nella patogenesi del danno tissutale, attraverso la regolazione dei mediatori di danno tissutale, in particolare la MMP-2 e MMP-9, metallo proteasi che, in queste malattie, provocano gravi danni alla parete intestinale. Dai dati ottenuti sulle biopsie tissutali di pazienti pediatrici affetti da MICI e dagli esperimenti *in vitro*, è risultato evidente che GAS5 potrebbe essere coinvolto nei processi di danno tissutale e nella determinazione di uno stato infiammatorio cronico che regola l'espressione delle MMP-2 e MMP-9. L'identificazione di un target molecolare in grado di modulare i livelli di questi enzimi consentirebbe non solo di approfondire le conoscenze sulle cause di un'alterata produzione di MMP-2 e MMP-9 ma anche di sviluppare una strategia farmacologica mirata. Inoltre grazie ad esperimenti di RNA-IP è stato dimostrato che questo lncRNA era in grado di legarsi e interagire fisicamente con NF-κB, fattore trascrizionale coinvolto nel processo infiammatorio. GAS5 si lega a NF-κB, probabilmente modulando la sua attività trascrizionale, dimostrando ancora una volta il coinvolgimento di questo lncRNA nell'infiammazione e confermando il suo ruolo come biomarcatore dell'infiammazione.

Ad oggi nelle MICI non esiste una terapia farmacologica curativa e l'approccio terapeutico è finalizzato a indurre la remissione e a favorirne il mantenimento. Recenti studi hanno dimostrato che la talidomide è molto efficace nei bambini affetti da MICI, ma il meccanismo d'azione di questo farmaco rimane ancora non del tutto chiaro. La seconda parte di questa tesi si è focalizzata ad investigare sui meccanismi d'azione della talidomide nei pazienti con MICI, al fine di identificare non solo marcatori di efficacia ma anche di indagare le cause molecolari che scatenano gli effetti collaterali noti, come la neuropatia periferica. Un'analisi del trascrittoma è stata condotta su campioni di sangue di pazienti pediatrici con MICI refrattari alle terapie standard e in trattamento con talidomide; sono emersi 252 geni differenzialmente espressi dall'analisi di RNA-seq a seguito del trattamento con il farmaco. Il test ipergeometrico basato su annotazioni di Gene Ontology (GO) ha rilevato una serie di pathway alterate implicate nel meccanismo d'azione della talidomide e nel suo principale effetto avverso, la neuropatia

periferica, tra cui la via di signaling del recettore degli ecosanoidi e delle prostaglandine e pathways associate alle proteine G accoppiate alla via dell'adenilato ciclasi, e anche pathway coinvolte nel sistema nervoso. Per validare questi risultati trascrittomici, PGE2 e cAMP sono stati quantificati in cellule monocitiche Thp-1 e Thp-1 differenziate in macrofagi. La talidomide causa una significativa diminuzione della produzione di PGE2 nei macrofagi stimolati con LPS, mentre nelle cellule Thp-1, il trattamento farmacologico induce un significativo aumento della produzione di cAMP. Studi preliminari condotti sulla linea cellulare neuronale sh-sy5y, al fine di identificare nuove pathway e geni coinvolti nella neuropatia periferica indotta dalla talidomide, hanno rivelato che questo farmaco non ha un effetto citotossico, né un'azione a livello dell'attività mitocondriale. La quantificazione di ATP e del calcio intracellulare nelle sh-sy5y ha permesso di rilevare che il farmaco induce un aumento di entrambi in seguito a trattamento. Questo studio fornisce la base per la realizzazione di studi mirati che ci consentiranno di ampliare le nostre conoscenze su questo farmaco e identificare marcatori di risposta per promuovere la personalizzazione del trattamento con talidomide di pazienti pediatrici affetti da MICI

1. INTRODUCTION

1.1 Inflammatory bowel disease

Inflammatory bowel diseases (IBD) are a group of diseases characterized by chronic inflammation of the gastrointestinal tract, with possible extraintestinal manifestations and associated immune disorders. Knowledge about the etiology of IBD still tells us that a genetic predisposition in determining the integrity of the intestinal barrier and the response of the immune system could cause an inappropriate inflammatory reaction in response to infections or other environmental factors [1]. Among IBDs, the most representative are ulcerative colitis (UC) and Crohn's disease (CD), which have some features in common, but different intestinal localization, histology, course and associated complications (Fig. 1.1).

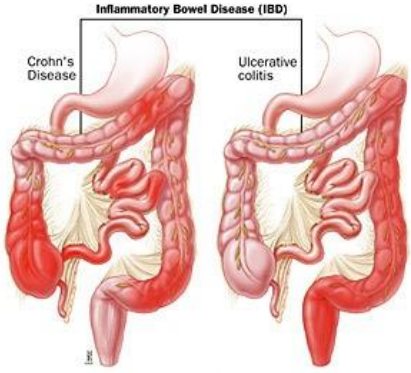


Fig. 1.1 Distribution of lesions in inflammatory bowel diseases

The incidence and prevalence of CD and UC vary considerably around the world [2] and is increasing; the diseases are often diagnosed in adolescents and young adults, with a rising incidence in the pediatric population [3]. Indeed, approximately 25% of patients with IBD are under the age of 20, 18% under the age of 10 and 4% under the age of 5 [4]. In children, negative consequences on growth, development and psychosocial function are often observed [5].

Both in CD and in UC the course of the disease is characterized by periods of activity and remission, but it is often impossible to predict the evolution of the disease as regards the duration and severity.

These diseases are distinct and clearly distinguishable on the anatomo-pathological and clinical level (Figure 1.2). CD can best be defined as a relapsing granulomatous inflammatory lesion that typically affects the terminal ileum or colon but can occur at any level of the gastrointestinal tract, from the mouth to the anus [6]. Typically, the disease is characterized by subacute or chronic, necrotizing and healing inflammation. The latter mainly affects young adults and causes ulceration of the mucosa, which induces a disproportionate connective tissue reaction and can

lead to stenosis of the intestinal lumen with the formation of multiple fistulas [7]. The clinical picture largely depends on the location of the disease and can include diarrhea, abdominal pain, fever, clinical signs of intestinal obstruction, as well as passage of blood, mucus or both [8]. UC, on the other hand, is characterized by widespread inflammatory ulcerations of the mucosa of the colon (Figure 1.2). People affected by this disease typically have bloody diarrhea, passage of pus and /or mucus, and abdominal cramps during evacuation. Severe symptoms are less common in proctitis and distal colitis [9].

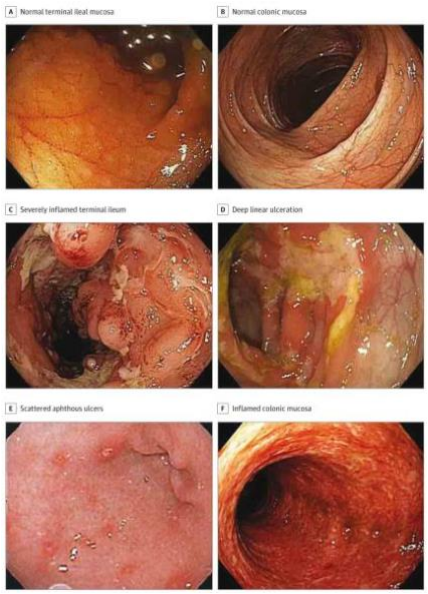


Fig 1.2 Representative endoscopic images of normal and inflamed gastrointestinal mucosa from pediatric patients with and without IBD **A**. Normal vascular pattern, villous epithelium, and lymphoid nodularity. **B**. Normal mucosa and delicate vascular network. **C**. Terminal ileum in a child with CD. **D**. Linear ulcer directly adjacent to normal colon mucosa in a young child with CD. **E**. stomach antrum of a child with CD. **F**. Inflamed colonic mucosa in an adolescent with UC.

IBD is a multifactorial disease that involves a series of interactions between genetics, environmental factors, gut microbiota and the immune response [10]. In recent years, genome-wide association studies have identified more than 200 risk loci associated with IBD [11]; many of the genes identified code for proteins involved in innate and adaptive immunity, autophagy and mucosal barrier integrity. This immune dysregulation could alter the intestinal microbial composition causing the chronic inflammation [12]. Moreover, metagenomic studies have shown that microbial diversity in IBD patients tends to decrease, due to the prevalence of species with pro-inflammatory properties over protective species. In these patients the most common bacterial strains are *E. coli*, *R. gnavus*, *Campylobacter concisus* [13], *Helicobacter pylori*, *Mycobacterium avium paratuberculosis* and *C. difficile* [14]. The tissue injury observed in IBD

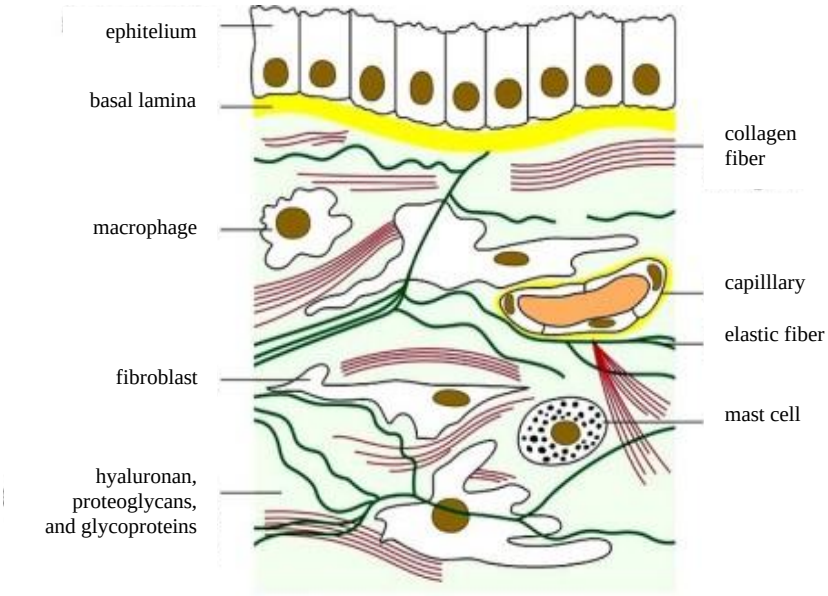
has a major role in the progression of the disease. On this perspective, an interesting role is attributed to a family of proteins called matrix metalloproteinases (MMPs).

In these diseases, an aberrant and excessive cytokine response that causes subclinical or acute mucosal inflammation is present. In particular, mucosal immune cells (macrophages, T cells and innate lymphoid cells) produce cytokines that can promote chronic inflammation of the gastrointestinal tract [15]. The cytokine TNF-α is produced both in membrane-bound and soluble form by lamina propria mononuclear cells and also by macrophages, adipocytes, fibroblasts and T cells, and is increased in IBD patients [16, 17]. TNF-α induces different pro-inflammatory effects, binding to its receptors TNFR1 and 2 and activating the transcription factor nuclear factor-κB (NF-κB); as a consequence, angiogenesis, production of metalloproteinases, activation of macrophages and effector T cells are induced [18]. Treatment of IBDs with antibodies that neutralize both soluble and membrane-bound TNF-α is highly effective, improving mucosa healing: indeed, anti TNF-α monoclonal antibodies are well established therapies in IBDs. In addition, other cytokines in addition to TNF-α have a fundamental role in controlling mucosal inflammation in IBD [14].

1.1.1 Role of the extracellular matrix in IBD

Common features of IBD are tissue damage and alteration of the intestinal architecture due to chronic inflammation. An excessive and prolonged inflammatory response occurring in the intestinal tissue is the cause of progressive changes to the structure and functioning of the intestinal tissue extracellular matrix (ECM). [19]

The ECM is a highly dynamic structure present in all tissues, which undergoes controlled remodeling. During this process, both quantitative and qualitative alterations of its components take place in order to control homeostasis and tissue architecture. Matrix components include collagen proteins: type I collagen, basement membrane collagens (type IV, VIII and X), type VI microfibrillar collagens; non-collagen proteins, such as elastin, fibronectin, laminin, thrombospondin or tenascin; proteoglycans and glycosaminoglycans, and growth factors, enzymes, including matrix metalloproteinases (MMPs). (Figure 1.3)



EXTRA CELLULAR MATRIX

The ECM is not only a scaffold for cells within the tissues, but also a dynamic component of the tissue, involved in multiple molecular pathways and processes, such as cellular proliferation, migration and adhesion. Its structure undergoes constant deposition, degradation or modification. The modifications in ECM, or so-called ECM remodeling, is caused by specific enzymes responsible for its degradation and rebuilding, such as matrix metalloproteinases (MMPs), neutrophil elastases (NE) and meprins [19].

The progression of IBD involves a disturbance to the proteolytic – anti proteolytic balance, which contributes to increased degradation of ECM components, in which MMPs take part. Aside from increased proteolytic activity, ECM remodeling during IBDs involves the creation of a new ECM, with a large increase in the synthesis of fiber-forming collagens (type I, III and V collagens). Excessive ECM formation leads to a progressive intestinal fibrosis, which in turn causes intestinal lumen narrowing. Fibrosis occurs through the activation of fibroblasts and the secretion of ECM components. The increased stiffness of the fibrotic tissue, on the other hand, contributes to further fibrogenesis. Progressive tissue damage and IBD-related complications, resulting from unbalanced and deregulated ECM remodeling, cause the disappearance of barriers between the epithelium and the endothelial tissue and contribute to the formation of fistulas, eventually resulting in intestinal perforation.

Chronic inflammation, typical of the intestinal mucosa, is also characterized by an important remodeling of the sub-epithelial connective tissue which leads to a turnover of the components of ECM. Coordinated synthesis and degradation of ECM components are critical for many

pathophysiological processes involved in inflammation, such as leukocyte invasion, epithelial migration, neo angiogenesis and wound healing process. The disturbance of the synthesis-degradation balance of the components of the ECM can lead both to the progressive destruction of the organ, as seen in the formation of ulcers, and to an excessive deposition of collagen which causes fibrosis.

1.1.2 Role of matrix metalloproteinases (MMPs) in ECM remodeling in the progression of IBD

The remodeling of ECM within the intestinal tissues, which simultaneously involves an increased degradation of ECM components and excessive intestinal fibrosis, is mediated principally by inflammatory leukocyte-derived and activated matrix metalloproteinases (MMPs). MMPs are a family of 24 zinc-dependent endopeptidases that are grouped according to their substrate into collagenase, gelatinase, stromelysins and membrane MMPs [20]. In particular, the group of gelatinases includes MMP-2 (gelatinase A) and MMP-9 (gelatinase B) [21]. Gelatinases are able to degrade the ECM and the basement membrane facilitating the migration, infiltration and cellular remodeling of tissues. These enzymes are synthesized by fibroblasts and other types of connective tissues cells, as well as by leukocytes, monocytes, macrophages and endothelial cells, and then released into the extracellular space in an inactive form (pro-MMP). The enzyme is activated by proteolytic cleavage in the pro-peptide region. The activity of metalloproteinases is precisely regulated at the transcriptional and translational level, and by endogenous inhibitors, such as α2-macroglobulin and tissue inhibitors of metalloproteinases (TIMPs) [22]. MMPs are involved in the typical lesions that characterize CD, namely transmural inflammation, fibrosis and fistula formation; while in the UC the tissue damage is limited to the superficial layers of the mucous membrane of the colon and rectum (Figure 1.4).

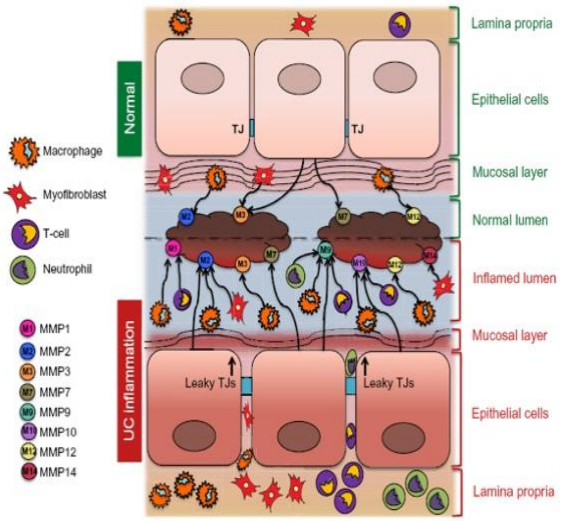


Fig. 1.4 MMPs produced by different cell types in IBD

Cytokines produced by immune cells favor the activation of these proteolytic enzymes, and furthermore their transcription is regulated by the levels of cytokines such as IL6 and TNF-α [23]. Increased levels of MMPs (MMP-1, 2, 3, 8, 9, 12 and MT1-MMP1) have been reported both in various animal models of colitis and in IBD patients [20, 21]. Many studies have shown that MMP levels are correlated with disease activity [24]. The increase of these proteases, in particular MMP-2 and MMP-9, has also been reported in biopsies, in serum and in urine samples of pediatric patients with IBD [25, 26], demonstrating that these proteases could be a useful biomarker for the evaluation of the clinical activity of IBD.

Due to their proteolytic action, these MMP2 and MMP9 proteins are able to process many cytokines and their receptors, modulating their activity during the inflammatory process [27]. For example, MMP-2 and MMP-9 are able to activate inactive TGFβ [28]. In other studies, it has been shown that MMP-9 can process the pro-inflammatory cytokine TNF-α, modulating the inflammatory process [29, 30]. MMP-9 is hyper-produced by macrophages and other inflammatory cells during colon inflammation [31]. Furthermore, it is involved in the migration process of T lymphocytes, in the process of invasion and transmigration of leukocytes, and acts as a regulator of the inflammatory process [29]. MMP-9 can also cause an increase in intestinal epithelial tight junction permeability, inducing an increase of intestinal permeability, an important pathogenic factor contributing to the development of intestinal inflammation in IBD [32]; and this event could be mediated by an activation of NF-κB signaling pathway [33]. MMP-2 in the healthy gastrointestinal tract is mainly produced by stromal cells. During inflammation it is also produced by fibroblasts, mononuclear cells, vascular endothelial cells and epithelial cells. MMP-2 maintains gut barrier function, favoring infiltration processes of leukocytes in inflamed tissues [34].

1.3 Epigenetics markers in IBD

The etiology of IBD is not well understood; however, comprehensive epidemiologic and genetic studies suggest that it is a result of complex interactions between genetics, immune dysregulation, and environmental factors, including lifestyle factors [35].

Genome wide association studies (GWAS) have been extremely successful in dissecting the genetic background and revealing pathogenetic pathways, which may lead to pathways specific therapies. To date, a high number of potential candidate genes and several possible epigenetic modifications seem to be involved in IBD pathogenesis and are still emerging, but our understanding on their functional relevance is still scarce. Recently the new technologies have

led to identify more than 240 susceptibility loci mainly in Caucasian people but also in trans-ethnic cohorts [36]. Most of these loci are shared by both CD and UC, and frequently by other immune-mediated diseases [37, 38]. However, these are common variants with small effect size that explain only a small percentage of the estimated genetic risk. In addition, there is still a lot of work to be done to understand more specifically the biological role of genetic variants and involved pathways.

Epigenetics is defined as a modification in the organism's phenotype that persists through mitosis and even meiosis without altering the underlying DNA sequence. Consequently, epigenetics is generally understood to be the study of mechanisms that control gene expression in response to environmental influences in a potentially inheritable manner [39]. Complex epigenetic states can be brought on by several converging and amplifying signals, including transcription factors, non-coding RNAs, DNA-methylation, and histone modifications. All of these processes are dynamic and reversible [40].

Epigenetic modifications have been reported in several immune mediated inflammatory diseases, cardiovascular diseases and cancers. Despite a large amount of data regarding the genetic and epigenetic involvement in IBD pathogenesis, the translational results are still poor and there is a significant gap in understanding the direct consequence of the identified genetic variants. In patients with IBD the most studied modifications have been DNA-methylation and noncoding RNAs [41].

The most widely studied epigenetic modification in mammals is DNA methylation, which comes through the covalent binding of a methyl group to the 5′ carbon of the cytosine residue, more often in the dinucleotide sequence cytosine phosphate guanine (CpG). When methylation occurs near a promoter sequence, gene expression is repressed, either because proteins bind to the methylated CpG island and initiate DNA compensation and inactivation or methylation itself blocks the DNA sequence and transcription factors are unable to bind. The conversion of cytosine to 5-methylcytosine is catalyzed by enzymes called DNA methyltransferases. The CpG sequences are present in about 1–2% of the genome and usually display a low transcriptional activity. The increase of DNA methylation may change the transcriptional activity and gene expression level, eventually impacting on diseases [42].

Several reports have been published of different mucosal methylation changes of several genes in IBD patients, with often significant differences among CD and UC. The combinations of several markers may lead to a 70–80% accuracy in differentiating IBD from controls as demonstrated by several investigators [43, 44]

1.3.1 Long non coding RNA in IBD

ncRNAs have emerged as important regulators of gene expression at both the transcriptional and post-transcriptional levels. Many ncRNAs have shown promising translatable potential for clinical applications from prediction and diagnosis to treatment and monitoring of patients.

Non-coding RNAs (ncRNAs) can be divided in two classes: the first ranges from few to 200 nucleotides and is called small non-coding RNAs and the other one, longer than 200 nucleotides to several kilobases, is called long non-coding RNA (lncRNA). Among the small ncRNA, the microRNAs (miRNAs) are the most known and studied group. miRNAs are expressed endogenously and during biogenesis they pass a multistep process that includes: transcription, nuclear maturation, export and cytoplasmic processing (Figure 1.5).

miRNAs mediate RNA silencing and gene expression at both transcriptional and post-transcriptional level, and therefore can modify several possible pathways. More specifically, in IBD they may intervene in T-cell differentiation, TH17 signaling pathway, and autophagy. There is significant evidence to demonstrate their involvement in disease onset and progression [45].

The class of lncRNAs is of emerging interest among the non-coding transcriptome. 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 recruited to the chromatin [45] (Figure 1.5).

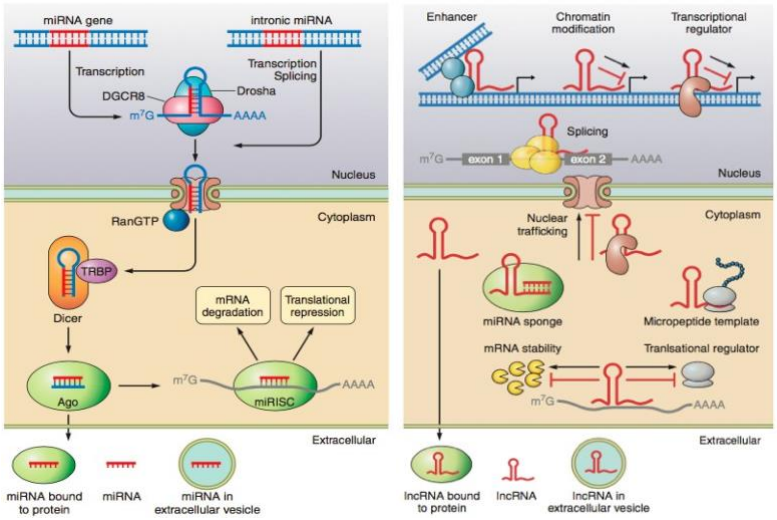


Figure 1.5 Biogenesis and function of miRNAs (on the left) and lncRNAs (on the right). [46]

The functions and mechanisms of action of most lncRNAs are not fully understood; however, based on the current findings, they exhibit diverse functional roles, including the regulation of protein-coding genes by chromatin remodeling, modulation of gene expression, regulation of protein activity, localization, and stability. In particular the ability of lncRNAs to bind protein partners makes them capable of performing numerous cellular functions. In particular, lncRNAs can act as decoy, for example by preventing the access of regulatory proteins to DNA. A known example is growth-arrest-specific transcript 5 (GAS5) lncRNA, a non-coding RNA that is over-expressed in growth arrested cells; GAS5 presents in the sequence a hairpin structure able to bind the glucocorticoid receptor (GR) blocking its ability to recognize the binding sites on the DNA of the target genes [47].

Several recent studies have investigated the association of dysregulated lncRNAs with both CD and UC using colonic biopsies or blood samples (or both) and have further highlighted their roles in disease-related processes, including the regulation of intestinal epithelial cells and inflammation [48].

For example, one of the first reports elucidating the role of lncRNAs in IBD was the study by Qiao and colleagues in which they reported that in both active and inactive disease CD patients, the lncRNA DQ786243 was upregulated compared to control subjects. [49]. Subsequent overexpression of DQ786243 in Jurkat cells has further shown that DQ786243 can regulate the function of regulatory T lymphocytes (Treg) through changes in the expression of Treg-related cAMP response element-binding protein (CREB) and fork head box P3 (Foxp3). It is established that defective Tregs play an important role in CD pathogenesis. One of the first functional studies on the role of lncRNAs in IBD identified an IFNG-AS1 lncRNA which was found to be deregulated in inflamed UC tissues and correlated with clinically validated IBD susceptibility loci [49].

lncRNAs can also cause resistance to treatment and increase the severity of IBD. In a recent study using *in vitro* cell lines and IBD patient samples, an increased level of the lncRNA GAS5 was identified after treatment with glucocorticoids (GCs). A significant increase in the level of GAS5 was shown to be the rate-limiting factor in the remission of at least 20% of IBD patients showing resistance to GC treatment [50].

1.2.2 The lncRNA GAS5

GAS5 lncRNA was first isolated in a study by Schneider and colleagues from NIH3T3 mouse cells, with the aim of identifying genes expressed during the growth arrest phase of the cell cycle [51]. The gene belongs to the family of 5'-terminal oligopyrimidine tract (5'-TOP) as,

downstream of the gene, there is an oligopyrimidine trait, which confers stability to GAS5 and protects it from degradation [52]. GAS5 is located on chromosome 1q25, is about 650 base pairs long and is composed of 11 introns and 12 exons (Figure 1.6).

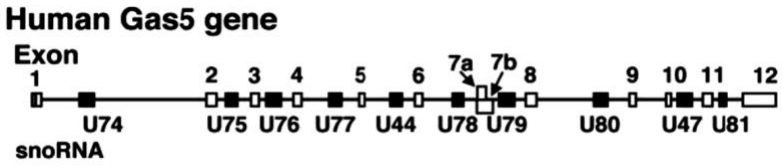


Fig. 1.6 Schematic representation of the GAS5 gene. The white boxes represent the 12 exonic sequences, the black boxes are the 10 snoRNAs

The introns of this gene express several small non-coding nucleolar RNAs (snoRNAs), while its exons contain a small non-coding ORF for a functional protein. In humans GAS5 encodes 10 C/D box snoRNAs that are involved in the 2'-O-methylation of rRNAs. The snoRNAs are transcribed by RNA polymerase II as pre-mRNA, and the functional ones derive from splicing events. From the alternative splicing of exon 7, two isoforms of GAS5 are derived, and both retain the 5 'TOP and 3' structure.

At the 3' terminal end of the GAS5 gene, there is a complementarity of about 40 base pairs with another lncRNA called GAS5 antisense RNA (GAS5-AS1), transcribed on the opposite strand. The fact that the ORF of GAS5 is very small and there are stop codons in the first exons, suggests that this transcript is subject to nonsense-mediated decay following translation [53].

There are several evidences that GAS5 can be classified as a tumor suppressor in tumors. An aberrant down-regulation of GAS5 has been revealed in breast cancer, kidney cancer, bladder cancer, colorectal cancer, prostate cancer, pancreatic cancer and kidney cancer cells [54-59]. An interaction between GAS5 and miR-21 was also identified. miR-21 was one of the first miRNAs to be described as oncomir, a cancer associated miRNA [60, 61]. This miRNA is found upregulated in various cancers such as breast, prostate, colon, liver and lung cancers. In a recent study it was found that GAS5 is a direct target of miR-21. For example, a negative correlation between GAS5 and miR-21 in breast cancer has been highlighted [62]. Furthermore, it appears that GAS5 itself can negatively regulate miR-21 through the RNA-induced silencing complex, suggesting that there is a reciprocal regulatory loop between GAS5 and miR-21 [63].

Regarding the antisense transcript of GAS5 or GAS5-AS1, Wu and collaborators in 2016 published a work which demonstrates the inverse correlation between the expression of this RNA and a greater capacity of metastasis of non-small cell lung cancer [64].

An important feature of GAS5 was discovered in 2010; Kino and collaborators have described GAS5 as repressor of the GR, influencing the GC activity as observed also in healthy donors'

peripheral blood mononuclear cells (PBMCs) and in IBD pediatric patients [47, 65, 66] (Figure 1.7).

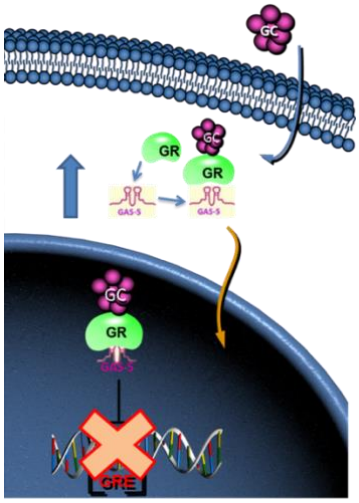


Fig. 1.7 Mechanism of action of GAS5 following the link with GR [106]

To date, there is still no curative drug therapy for IBD, and despite the introduction of highly effective biological drugs into therapy, GCs are still considered an important treatment; however, large inter-individual differences in their efficacy and side effects have been reported and the identification of individuals who are most likely to respond poorly to GCs is extremely important [67].

Kino et colleagues have observed that GAS5 is able to interact with the DNA binding domain (DBD) of the activated GR causing a block of its transcriptional activity (Figure 1.8).

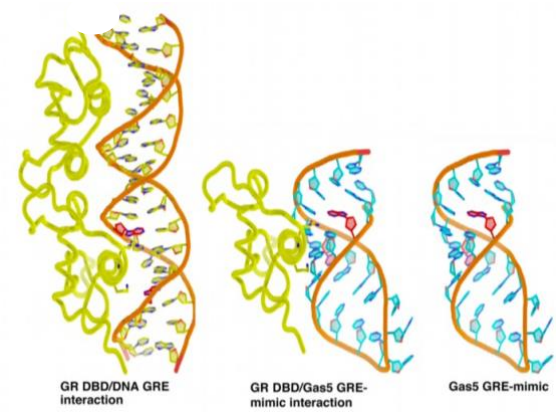


Fig 1.8 Three-dimensional structure of GR DBD/DNA GRE interaction, Gas5 GRE-mimic and its interaction model with GR DBD structure.[47]

In particular, this portion of GAS5 capable of binding the GR is defined GR-mimic as is able to simulate the glucocorticoid responsive elements (GRE) present on the GC responsive genes and also contains two sequences (GR-1 and GR-2) necessary for the formation of hydrogen bonds with the DNA biding domain (DBD) of the activated GR. When these bonds are created,

recognition of target genes with GRE sequences is inhibited, resulting in a loss of the transcription control performed by the receptor [47].

Recent studies have analyzed the possible role of the lncRNA GAS5 in the altered response to steroid treatment, in PBMC isolated from healthy subjects and from pediatric patients with IBD [65, 66, 68]. In particular, a different GAS5 expression was observed among resistant and sensitive subjects to GC: resistant subjects show an increase in GAS5 levels compared to sensitive subjects who instead undergo a downregulation of lncRNA. It has therefore been hypothesized that the failure to respond to the drug in resistant subjects is due to the fact that GAS5, by binding to the DBD domain, does not allow the receptor to perform its function. These results suggest that GAS5 may represent a potential marker of response to GCs.

The expression of the lncRNA GAS5 is down-regulated in various types of cancer and is inversely correlated with clinical pathological characteristics such as tumor size, progression of metastases [69]. Recently Chen et al. reported a new role of GAS5 as a regulator of the development of the metastatic phenotype in melanoma, proving the effects of GAS5 over-expression and silencing in *in vitro* and animal models of melanoma [70]. The authors demonstrated that GAS5 regulates the development of metastases through the modulation of MMPs, which are responsible for the degradation of ECM [71].

1.2.3 Role of lncRNA GAS5 in the regulation of NF-κB

As described previously, the function of most lncRNAs depends on their ability to interact with functional domains of signaling proteins and thus regulate signal transduction. miRNAs and lncRNAs contain modular domains through which they directly interact with NF-κB signaling proteins, thus regulating inflammatory response. [72].

Nuclear factor-κB (NF-κB) is a transcription factor that play critical roles in inflammation, immunity, cell proliferation, differentiation, and survival [73]. Various studies have demonstrated that NF-κB is a central mediator of pro-inflammatory gene induction and functions in both innate and adaptive immune cells, regulating the immune system and inflammatory processes [74, 75]. Members of NF-κB can either induce or repress gene expression by binding to specific DNA sequences in promoters of target genes. In most cell types, NF-κB complexes are retained in the cytoplasm by the actions of a family of inhibitory proteins termed inhibitors of NF-κB (IκB). The activation of the NF-κB is based on the phosphorylation of IκB, and its consequent proteaosomal degradation, by IκB kinase (IKK). The

transcriptional activity of NF-κB is regulated by two pathways: the canonical and non-canonical pathway (Figure 1.9) [76].

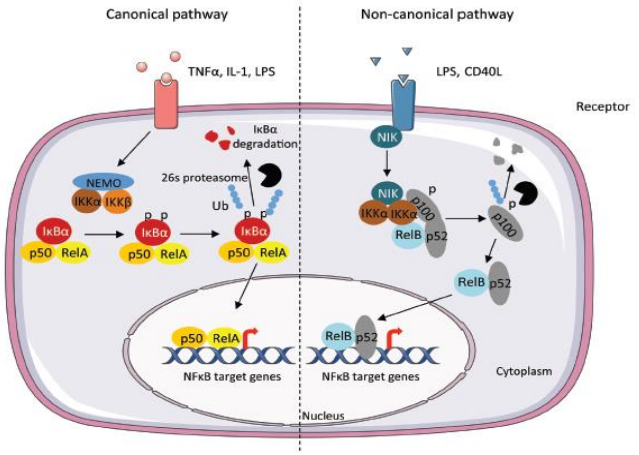


Fig 1.9 Canonical and non-canonical pathway leading to the activation of NF-κB [76]

Translocation into the nucleus and binding to target DNA sequences are important events for NF-κB to control transcription process. Genes regulated by NF-κB include those encoding IL-2, IL-6, IL-8, the IL-2 receptor, IL-12 p40 subunit, VCAM, 1-ICAM-1, TNF-α, *etc*. NF-κB has been implicated in the pathogenesis of a number of inflammatory diseases, such as rheumatoid arthritis, IBD, multiple sclerosis, atherosclerosis, systemic lupus erythematosus, type I diabetes, chronic obstructive pulmonary disease and asthma [77]. Constitutive NF-κB activation has been found in inflamed colonic tissue of IBD patients [78]. Furthermore, genetic mutations in NF-κB stimulating immune receptors, such as NOD2, and NF-κB target genes, such as IL-12 and IL-23, are associated with human IBD [79].

Also the members of the NF-κB family are associated with a regulation by ncRNAs. In 2015, Liu et al. found that the lncRNA NKILA (NF-κB interacting lncRNA) binds to NF-κB; NKILA inhibits the activation of the NF-κB signaling pathway by masking to IKK the phosphorylation site of IκB necessary for its suppression [80]. Instead, the lncRNA MALAT1 largely located in the nucleus, is directly associated with p65, preventing the p65/p50 heterodimer binding to the target promoters [81].

Recent evidence reported that also the lncRNA GAS5 was involved in the regulation of NF-κB. In particular Li et al. found that this lncRNA was down-regulated in colorectal cancer, and subsequent functional experiments revealed that knockdown of GAS5 increases IL-10 expression [82]: in particular in HCT-116 cells, GAS5 overexpression significantly decreased the level of p-NF-κB p65, and GAS5 knockdown increased the level of p-NF-κB p65 without

affecting the level of total NF-κB p65, suggesting a possible mechanisms through which GAS5 inhibits the inflammatory profile in colorectal cell lines.

In 2018 Wang and colleagues reported that in osteosarcoma cells GAS5 acts as a tumor suppressor working as "sponge" for miR-203a, and thus deactivating PI3K/AKT/GSK3β pathways and activating NF-κB signaling, reporting that the lncRNA GAS5 silencing decrease p-NF-κB p65 levels [83]. Moreover, Yue and collaborators described that in cardiomyocytes GAS5-specific siRNA decreased the protein expression of NF-κB in the nucleus, increased the cytoplasmic protein expression of NF-κB and inhibited the activity of NF-κB in cardiomyocytes, through miR-26a binding [84]. However, in a recent study of Gao and colleagues, the silencing of GAS5 in a mouse model of sepsis inactivated the NF-κB signaling pathway, suggesting that GAS5 might activate the NF-κB signaling pathway, and promote sepsis and the following myocardial depression and injury [85]. All these studies strengthen the evidence that GAS5 could be defined a modulator of inflammatory response regulating the activity of NF-κB in different cell models.

As already described above, GAS5 interacts with the activated GR, preventing its association with DNA, and consequently suppressing its activity. GAS5, through its GR-mimic portion, binds the DBD of the activated GR. Given that NF-κB is a key mediator of immune and inflammatory responses and that the GR exerts anti-inflammatory functions, the crosstalk between GR and NF-κB signaling is of particular interest [86]. The most extensively studied case has been the transrepression of NF-KB by GR. The inhibitory effect of GR is postulated to be largely due to recruitment of GR, via protein–protein interaction by the DBD domain of NF-κB or AP1 (tethering model) [87]. GR and p65 are suggested to physically interact and mutually antagonize each other's transcriptional activity [88]. In principle, multiple layers of regulation seem to be involved in the crosstalk of GR and NF-κB; however, the mechanism and extent of crosstalk is still unresolved. Furthermore, it is unknown if GAS5 has also an impact on the GR-dependent repression of NF-κB activity, but it is already known that GAS5 binds GR [47] at the same domain, of which GR could interact with NF-κB [87].

1.3 Therapeutic approaches in pediatric IBD

In face of the complex pathophysiology of IBD, a cure has not yet been found to induce complete remission of the disease. Currently, therapy is aimed at controlling the exacerbation of the disease, maintain remission and treat specific complications such as fistulas [89], rather than modifying or reversing the underlying pathogenetic mechanism. Furthermore, in some cases drug therapy fails and surgical removal of entire portions of the intestine is necessary [90].

Furthermore, people with IBD have nutritional deficiencies that require adequate assistance by identifying and correcting factors associated with malnutrition and applying oral, enteral or parenteral nutritional therapy [91].

Currently available therapies include anti-inflammatory, immunomodulatory and immunosuppressive drugs, and biological therapies against inflammatory cytokines such as TNF-α. (Figure 1.10)

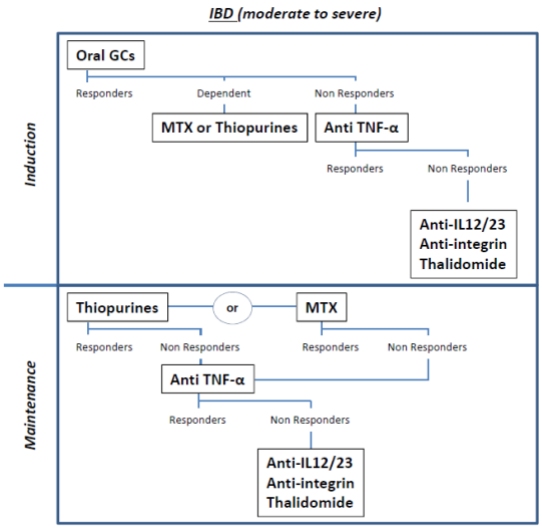


Fig 1.10 Schematic therapeutic approach in IBD (moderate to severe form)

Conventionally, a strategy involving intensification of therapy is used. Treatment with 5-aminosalicylic acid is considered appropriate for mild cases of UC, both for the induction of remission and for the prevention of relapse [92]. Glucocorticoids are largely used in IBD to induce remission in patients with moderate to severe disease, but a great inter-individual variability has 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) [93, 94].

In children, 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 [95] and no biomarkers are still available to predict the response to corticosteroids and reduce the risk of developing adverse events [96, 97].

Therapy with immunomodulators such as azathioprine (AZA), mercaptopurine (MP) and methotrexate (MTX), employed for maintaining remission is also associated with side effects and intolerance. Monoclonal antibodies against TNF-α, such as infliximab (a chimeric antibody) and adalimumab (a completely human antibody), are currently used and are highly effective; however, approximately 10-20% of patients are primary non-responders to anti-TNF-α therapy and another 20% -30% of patients lose therapeutic response over time [90]. Several other immunomodulators, such as thalidomide, can be used to treat IBD: this synthetic drug has been shown to significantly reduce inflammation associated with IBD due to its anti-TNF-α, immunomodulatory and anti-angiogenic properties [89]. Drug therapy reduces the need for surgery that involves the removal of the large intestine. While UC is treated after colon removal, CD is panenteric in nature, so the disease can still recur after surgery [98]. Therefore, it is necessary to develop a safe therapy, optimized for chronic use and more targeted for IBD, aimed at ensuring a satisfactory quality of life for the patient [90].

1.3.1 Thalidomide in IBD therapy

Despite an increasing number of treatment options, many IBD patients continue to pose a therapeutic challenge because they do not respond adequately to therapy or because they experience severe side effects following treatments. Thanks to its anti-TNF-α, immunomodulating and anti-angiogenic properties, thalidomide has been used successfully in chronic inflammatory diseases including IBD, in particular in children who do not respond to other therapies, despite the potential for adverse effects [99].

The role of thalidomide in the treatment of pediatric and adult refractory CD has been studied in small open-label studies and in a series of retrospective cases [100]. Recently, a randomized controlled clinical trial showed superiority of thalidomide over placebo for achieving clinical remission at 8 weeks of treatment and for long-term maintenance of remission in children and adolescents with refractory CD. The drug has been administered to immunomodulator/biologic resistant or intolerant patients [101].

The therapeutic effect of thalidomide in IBD is attributable to its immunomodulatory and anti-inflammatory activity through TNF-α, IL6 and IL2 inhibition [102], and also to its antiangiogenic property, reducing the expression of vascular endothelium growth factor and of fibroblasts growth factor (VEGF and FGF), involved in the process of inflammation of the mucous membranes [103].

1.3.2 Thalidomide: mechanisms of action

Thalidomide was marketed in the late 1950s as a nonaggressive and non-barbiturate sedative by the German pharmaceutical company Chemie-Grunenthal. It was sold as an over-the-counter drug with sedative, hypnotic and tranquilizing properties and was quickly found to be an effective antiemetic for treating nausea during pregnancy. However, it was introduced to the market without adequate clinical trials. Shortly after its release in 1957, reports emerged of patients developing peripheral neuropathy after taking the drug and the first reports of severe birth defects affecting various body systems due to the drug's teratogenicity appeared. In particular, the teratogenic effect of thalidomide was reported in 1961, with the first cases of embryopathy related to thalidomide [104]: damage was primarily seen to the limbs (phocomelia), face, eyes, ears, genitalia, and internal organs, including heart, kidney, and gastrointestinal tract.

Thalidomide is a synthetic derivative of glutamic acid, a natural amino acid involved in important physiological processes, including brain neurotransmission and metabolism. It is composed of two connected rings, a glutarimide ring and a phthalimide ring; the presence of a chiral carbon determines the formation of two enantiomers (R and S) which interconvert under physiological conditions forming a racemic mixture in a 1:1 ratio (Figure 1.11). One of the two enantiomers, the S-enantiomer, appears to cause the teratogenic effect [99]. However, since the drug can convert between one enantiomeric state and another, it is very difficult to create a stable form that is not teratogenic.

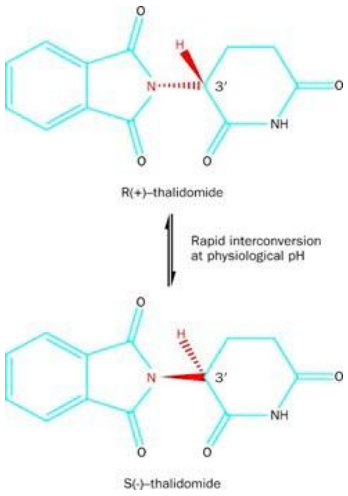


Fig 1.11. Interconversion between the two enantiomers of thalidomide

Oral absorption is effective despite being slow, variable and dose dependent. The blood peak is reached after 2-3 hours and the half-life after a single dose of 200 mg or repeated administrations, varies from 3 to 7 hours. Metabolism occurs mainly by spontaneous non-

enzymatic hydrolysis at physiological pH with the formation of over 20 metabolites. Thalidomide can still be metabolized in the liver by cytochrome p450 2C19 with the formation of molecules that could intervene in the antiangiogenic effect [99].

The immunomodulatory activity of thalidomide appears to be due to its ability to modulate the production of cytokines (Figure 1.12) [104]. Among these, there is the suppression of TNF-α, which occurs through an increase in the degradation of its mRNA [100]. The drug is able to inhibit the release of TNF-α by human monocytes stimulated *in vitro* by LPS, in a concentration-dependent manner [105]. Thalidomide also inhibits the production of IL-12 and the activation of NF-κB, as well as the induction of molecules that induce leukocyte adhesion [105]. Furthermore, it suppresses angiogenesis, probably through the inhibition of endothelial cell proliferation [106].

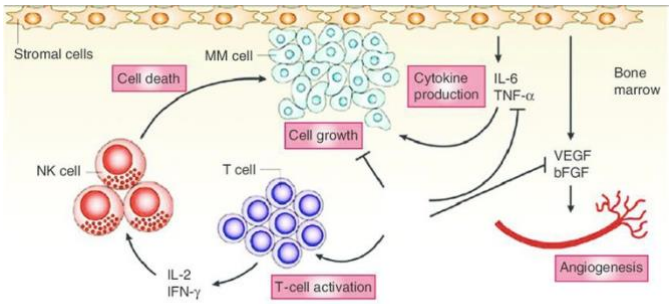


Fig 1.12 Mechanism of action of thalidomide in multiple myeloma

Angiogenesis also requires the activation of NF-κB; consequently, it has been hypothesized that thalidomide may mediate its effects through the suppression of NF-κB [106]. In addition to influencing the production of cytokines in monocytes, thalidomide is able to influence the functions of T cells, which play a central role in regulating the immune response.

In 2010, Ito et colleagues identified in cereblon (CRBN) the primary direct target of thalidomide [107] for both the anti-cancer activity and teratogenicity of thalidomide [108, 109]. CRBN forms a cullin-really interesting new gene (RING) ligase-4 (CRL4) ubiquitin ligase (E3) complex with damage-specific DNA-binding protein 1 (DDB1), Cullin 4 (Cul4), and Regulator of cullins-1 (Roc1). When thalidomide or its derivatives bind to CRBN, the protein can recognize 'neosubstrates' for subsequent ubiquitination and protein degradation. Substrate selectivity is dependent on the structure of CRBN-binding compounds, and novel compounds that target CRBN are being sought. In particular, proteolysis-targeting chimeras (PROTACs) have been developed using CRBN, and this technology is attracting the attention of researchers and of the pharmaceutical industry [110]. Some studies suggest that thalidomide exerts its teratogenic

effects by binding to CRBN, with a consequent alteration in the recognition by CRBN of the proteins to be ubiquitinated for degradation, in particular of the molecules involved in the signaling of development and embryogenesis, including signaling pathways of bone morphogenetic protein (BMP), fibroblast growth factor 8 (FGF8), spalt-like transcription factor 4 (SALL4) and p63 [100, 108, 111, 102].

In multiple myeloma it has been observed that, following the direct binding between CRBN and thalidomide, but also its derivatives lenalidomide and pomalidomide, CRBN selectively binds the transcription factors Ikaros (IKZF1, Ikaros family zinc finger protein 1) and Aiolos (IKZF3, Ikaros family zinc finger protein 3), causing rapid ubiquitination with subsequent degradation (Figure 1.13) [111].

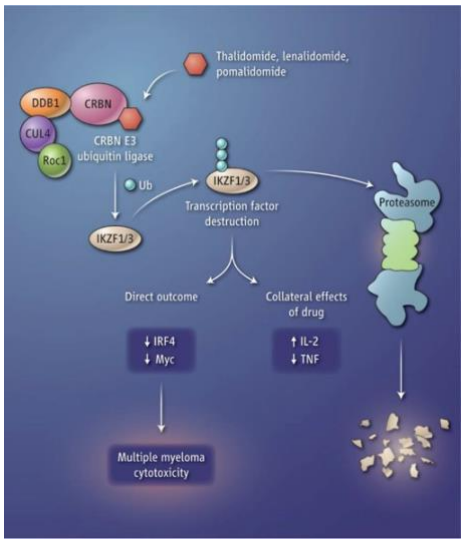


Fig 1.13 Graphic representation of the mechanism of action of thalidomide in multiple myeloma [105]

Ikaros and Aiolos are transcriptional regulators of B and T cell development. Under normal conditions Ikaros suppresses the expression of the gene encoding IL-2 in T cells, but on the contrary stimulates the expression of the interferon regulatory factor 4 (IRF4), a transcription factor that increases in infections; it is therefore deduced that thalidomide simultaneously causes both an activation of the immune system, since the degradation of Ikaros is followed by an increase in the production of IL-2 by T cells with consequent stimulation of immune responses, and a degradation of the function of B cells , as a result of reduced IRF4 expression [111].

Recently many other studies have reported that CRBN, by interacting with thalidomide or its derivatives, lenalidomide and pomalidomide, alters the expression of other proteins and transcriptional factors, inducing ubiquitination and degradation: casein kinase I (CK1α) [112], G1 to S phase transition 1/eukaryotic release factor 1 (GSPT1) [113], AMP-activated protein kinase (AMPK) [114] and promyelocytic leukemia zinc finger (PLZF) [115].

1.3.3 Peripheral neuropathy induced by thalidomide

In addition to the well-known teratogenicity of this drug, peripheral neuropathy is one of most frequent adverse events of thalidomide and is a common cause of treatment discontinuation.

At a pathologic level, thalidomide-induced peripheral neuropathy (TiPN) tends to manifest with axonal, length-dependent, sensory injuries. Neuropathy often presents as painful paresthesia or numbness [116]; this adverse event is reported as reversible in most patients after drug discontinuation [117]. The most common electrophysiological alterations are a reduction or absence of the nerve action potential that may occur before the outcome of the symptoms. Some risk factors are known, including: previous neuropathy, age, previous chemotherapy, vitamin B12 deficiency and folate deficiency. The frequency of TiPN varies in the current literature depending on the age of the patients, the disease, the drug doses, concomitant treatments and the duration of follow-up [118].

TiPN occurs in 25–75% of patients, with dose-dependent prevalence and severity [119]. I pediatric IBD patients in treatment with thalidomide TiPN was found in 72.5% of patients, but the prevalence depended on the treatment length [120]. Some recent studies have also shown that the development of this side effect is closely related to the dose of thalidomide [121].

The pathophysiology of TiPN remains not fully understood. To date three main hypotheses have arisen to explain thalidomide neurotoxicity. First, as thalidomide has antiangiogenic activities, this process is proposed to be responsible for the secondary ischemia and hypoxia of nerve fibers, followed by an irreversible damage of sensory neurons [122]. Second, it is suggested that thalidomide reduces neural cell survival by downregulation of TNF-α, triggering the inhibition of NF-κB and accelerating neuronal cell death via neurotrophins and their receptors dysregulation [123]. Finally, it has been hypothesized that thalidomide metabolites can cause reactive oxidative species (ROS) damage to DNA, leading to neuronal damage [124]. ROS-dependent mechanism has been confirmed in platinum-based neurotoxicity development, but not in TiPN. In 2011 Johnson and collaborators published a paper in which some genetic factors were studied as predisposing to the development of peripheral neuropathy in cancer patients suffering from multiple myeloma treated with thalidomide [125]. In this study, the identification of a series of single nucleotide polymorphisms (SNPs) in ABCA1 (*rs363717),* ICAM1 (*rs1799969*), PPARD (*rs2076169),* SERPINB2 (*rs6103*), and SLC12A6 (*rs7164902*) which were associated with TiPN were described. Although information on TiPN mostly derives from studies on adults with multiple myeloma, and little is known about the risk factors and the course of TiPN in IBD patient, a recent study conducted on pediatric IBD patients reports that SNPs in ICAM1 *(rs1799969)* and *S*ERPINB2 *(rs6103)* reduce the risk of neuronal damage and favour

TiPN resolution [119], proving that polymorphisms in genes involved in neuronal inflammation may be protective. Understanding the underlying mechanisms of TiPN could be a useful tool to prevent the onset of this complication and to delay or treat the most severe symptoms.

2. AIM OF THE PROJECT

IBDs are a group of diseases characterized by chronic inflammation of the gastrointestinal tract that promotes progressive destruction and pathological remodeling of the tissues of the gastrointestinal system. The exact causes of these pathologies remain unknown but studies have shown the importance of complex multi-factor interactions [1]. During the activity phase of disease, CD4 + T lymphocytes are capable of releasing inflammatory cytokines, which activate other inflammation cells. These pro-inflammatory factors also favor the activation of proteolytic enzymes including MMPs, which play an important role in the ECM remodeling [22]. Among the MMPs, of particular interest is the group of gelatinases which includes MMP-2 and MMP-9 [21]. In IBD, increased levels of both MMP-2 and MMP-9 have been reported, and the high concentration of these two proteases suggests that inflammatory mediators may be involved in their regulation [120]. It has recently been shown that the expression of these two gelatinases in melanoma cells is inversely correlated with the expression levels of the GAS5 lncRNA [70]. Recent discoveries on lncRNAs as possible regulators of gene expression led us to investigate the potential role of the lncRNA GAS5 in mediating tissue damage and maintaining intestinal barrier function in pediatric patients with IBD and *in vitro*. In particular the first aim of the present investigation was to increase the knowledge about the possible regulation mechanism of GAS5 on MMP-2 and MMP-9 expression and activity in IBD patients, by a comparative analysis of gene expression profile of these genes between inflamed and non-inflamed colon tissue obtained from IBD pediatric patients at diagnosis. Moreover, the role of GAS5 in regulating the expression of these two MMPs was investigated on Thp-1 cell line. Considering the important role of NF-κB as regulator of the enzymes responsible for ECM remodeling in the digestive tract and the constitutive NF-κB activation in inflamed colonic tissue of IBD patients, the potential role of GAS5 in the modulation of NF-κB activity was also studied; an analysis of the interaction of P65, (a subunit NF-κB complex), and GAS5 through the RNA immunoprecipitation assay in Hela cells was performed.

To date, a curative drug therapy for IBDs does not exist and the therapeutic approach is directed to the treatment and control of inflammation. A significant portion of patients treated with standard therapies does not respond or experience adverse effects that can be serious. These patients are therefore treated with other drugs, in some cases very expensive and with important side effects. A multicenter randomized placebo-controlled study conducted by IRCCS Burlo Garofolo has shown that thalidomide is very effective in children with refractory Crohn's disease [127]. Despite numerous attempts to clarify how thalidomide acts, the exact mechanisms still remain ambiguous and controversial. The second purpose of this thesis work was to obtain a

greater knowledge of the mechanisms of action of thalidomide in patients with IBD, in order to identify not only markers of efficacy but also to investigate the molecular causes that trigger the important side effect, peripheral neuropathy. To achieve this goal, blood samples from pediatric patients with IBD refractory to standard therapies and then treated with thalidomide were collected starting from 2016 and used for the study of transcriptome by next generation sequencing (NGS). To validate the RNA-seq results, *in vitro* experiments were conducted on Thp-1 cell line, a monocytic cellular line, and on sh-sy5y, a neuronal cell model, trying to investigate the molecular mechanism of peripheral neuropathy induced by this drug.

3. MATERIALS AND METHODS

3.1 Clinical samples

Samples for MMPs and GAS5 correletion study

Twenty-five children with IBD (mean age at enrolment 12.5 years, 12 UC and 13 CD) were enrolled at diagnosis in this study, at the Gastroenterology department of IRCCS Burlo Garofolo in Trieste. For each patient, during a colonoscopy, two biopsies (inflamed and non-inflamed) were collected. Both endoscopic and histologic evaluations were performed for all patients enrolled in the study.

Histologic appearance of biopsies was described using a predefined histologic inflammatory score and an architectural abnormalities score, developed for a previous study [99]. Both inflammatory and architectural score were described with a range from 0 (absence of inflammatory activity) to 5 (maximal inflammatory activity) calculated considering the active inflammation (range from 0 to 3), presence of crypt abscesses (range from 0 to 1), and erosions and ulcerations. Architectural abnormalities comprised glandular crypts alteration (range from 0 to 3); basal plasmacytosis (range from 0 to 1), and epithelioid granulomas (range from 0 to 1). The study was submitted for approval by the local Bioethics Committees, and patients were enrolled only after having been adequately informed and having signed informed consent, personally, when possible, or from parents or guardians.

Samples for transcriptomic thalidomide study

The analysis carried out in this part of the project was part of a multicentre study that involved the pediatric gastroenterology departments of the "Burlo Garofolo" hospitals in Trieste and "Meyer" in Florence. Starting from 2016, 10 IBD pediatric patients (pre-treatment, PRE) treated with thalidomide were enrolled and blood samples were taken for pharmacogenomic analyzes. An additional blood sample was drawn after 3 months of treatment with thalidomide 1.5-2.5 mg/kg/day (POST). Clinical activity, inclusive of clinical and inflammatory markers evaluation, was assessed by PCDAI and PUCAI scores.

3.2 Isolation of PBMCs from whole blood

Blood mononuclear fraction cells were isolated from EDTA anticoagulated peripheral blood from pediatric IBD patients included in the study. Cells were isolated following centrifugation (600 x g, 40 minutes at 15 ° C) by density gradient, using Ficoll PaqueTM Plus (GE Healthcare, density: 1.077g / mL).

The PBMCs, stratified in the Ficoll-plasma interface, were collected using a Pasteur pipette and subsequently washed with PBS by centrifuging at 600 x g for 10 minutes at 15 ° C. The cells,

after last wash, were resuspended in 2 ml of PBS and counted by the Trypan Blue exclusion method. Finally, about 10 x10^6 cells were resuspended in 1 ml of Trizol and stored at - 80 ° C until the time of RNA extraction.

3.3 RNA extraction from PBMCs

The total RNAs were extracted using the "PureLink RNA Mini Kit" (Thermo Fisher Scientific), according to the instructions given by the company. The RNA samples were then quantified with the NanoDrop 2000 Spectrophotometer.

3.4 mRNA-sequencing and mapping

The RNA samples were shipped to the IGA sequencing center in Udine for libraries preparation using the Ilumina TruSeq kit and subsequent paired-end sequencing on the Illumina Hiseq 2000 NGS platform. In order to remove non-informative sequences, raw reads were trimmed with the "Trim reads" tool from CLC Genomics WorкBench v12.0 (**https://digitalinsights.qiagen.com/**) as follows:

- Quality trim = Yes
- Quality limit = 0.05
- Ambiguous trim = Yes
- Ambiguous limit = 0
- Automatic read-through adapter trimming = Yes
- Remove 5' terminal nucleotides = Yes
- Number of 5' terminal nucleotides = 20
- Remove 3' terminal nucleotides = No
- Discard short reads = Yes
- Minimum number of nucleotides in reads = 30
- Discard long reads = No
- Save discarded sequences = No
- Save broken pairs = No
- Create report = Yes

Trimmed reads were mapped on annotated human genomic DNA (Ensembl release 68) using the RNA-Seq Analysis tool included in CLC Genomics WorкBench v.12.0, obtaining expression data for each of the 53,816 annotated genes. The mapping was carried out separately for each sample. The number of reads mapped for each gene was normalized to TPM and the

normalized counts were used for differential expression analysis between post-treatment (POST) vs pre-treatment patient samples (PRE).

3.4.1 Differential Gene Expression analysis on CLC Genomics WorкBench

The statistical analysis relating to the differential expression was conducted using the Differential Expression for RNA-Seq tool of CLC Genomics WorкBench v12.0 which implements a generalized linear model. The Differentially Expressed Genes (DEGs) were selected considering a FDR-corrected p-value (FDR) < 0.05 the proportion Fold Change (FC) > 2 (which characterizes the up-regulated transcripts) and < -2 (which characterizes the down-regulated genes). The differential analysis was performed starting from the gene-expression read tracks (GE) obtained from the mapping with the “Differential Expression for RNA-Seq” tool of the workbench. The “pre-treatment” samples were used as a "control".

3.4.2 Enrichment test (Gene Ontology)

The differential analysis was integrated with a functional analysis, implemented by the hypergeometric test on the Gene Ontology annotations. Gene Ontology (GO) is a bioinformatics project designed with the aim of creating a universal standard terminology for the annotation of genes. The classification concerns three areas: cellular component, molecular function and biological process. The purpose of the analysis was to verify the presence of enriched categories of Gene Ontology, determining whether the number of genes observed among the DEGs for each GO category was significantly greater than the number of genes expected for the same category. The intent of this study was to identify the biological processes and molecular functions that are perturbed by the treatment.

3.4.3 Ingenuity Pathway Analysis

Ingenuity Pathway Analysis (IPA) (Qiagen) is a tool capable of generating networks of biological interest that help to understand how genes of interest are related to each other, showing the significant interactions between them based on the IPA Knowledge Base. The latter is a database constantly updated that organizes biological and functional interactions based on relationships between proteins, genes, cells, tissues, drugs and diseases, limited to humans, mice and rats. These reports are constantly edited and include details and references to the original

publications, facilitating the formulation of functional biological hypotheses starting from data derived from large-scale genomic, transcriptomic and proteomic experiments.
Starting from a list of DEGs associated with a fold change and a p-value, IPA can also:

- identify key regulators upstream of the DEGs that can explain their pattern of expression ("upstream regulator" analysis);
- predict downstream effects on biological and pathological processes;
- build interactive models of experimental systems.

3.5 Cell cultures

The following immortalized cell lines were used in this study:

- Thp-1
- HeLa
- Sh-sy5y

The Thp-1 (ATCC, TIB-202) is a monocyte cell line growing in suspension, isolated from a pediatric patient with human acute monocytic leukemia; this cell line was grown in Roswell Park Memorial Institute (RPMI) 1640 medium (EuroClone®). The HeLa (ATCC, CCL-2) is a human cervical carcinoma cell line, and was grown in Dulbecco's modified Eagle's medium (DMEM, EuroClone®); lastly, sh-sy5y (catalog number 94030304, Sigma-Aldrich) is a human neuroblastoma cell line, with an epithelial-like cell phenotype, and maintained in Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (Sigma-Aldrich), supplemented with non-essential amino acid (10 mM for each amino acid; Sigma-Aldrich).
All media were supplemented with 10% (v/v) fetal calf serum (FBS, Sigma-Aldrich), 1% (v/v) L-glutamine 20nM, 1% (v/v) penicillin 10,000 UI/mL and streptomycin 10 mg/mL (EuroClone®). Cell cultures were maintained according to standard procedures in a humidified incubator at 37 °C with 5% CO_2, and cell passage was performed twice a week.

3.5.1 Cells differentiation

To differentiate macrophages from the immortalized cell line Thp-1, after seeding in completed medium, cells were stimulated with phorbol 12-myristate 13-acetate (PMA, Life Technologies) 5 ng / ml and left in the incubator for 48 hours to induce the differentiation of monocytes into macrophages. To induce differentiation of Sh-sy5y cell line into a neuron-like phenotype cell with neurite outgrowth and branches, one day after plating, cells were incubated in Neurobasal medium (Gibco), supplemented with B-27 supplement (Gibco) and l-glutamine (EuroClone®).

Dibutyryl cyclic AMP (DbcAMP) (1mM, Sigma-Aldrich) was added to the medium for 3 days. After 3 days, cells were differentiated in a neuronal phenotype cell.

3.5.2 Cell lines stimulation

For the *in vitro* study and analysis of GAS5, MMP2 and MMP9 expression, Thp-1 were treated with different pro-inflammatory stimuli, in order to study the effects of the inflammatory response on the expression of the examined genes. After seeding, Thp-1 were stimulated with phorbol 12-myristate 13-acetate (PMA, Life Technologies) 5 ng / ml and left in the incubator for 48 hours to induce the differentiation of monocytes into macrophages. After 48 hours, the culture medium was aspirated and washed with sterile PBS; subsequently the Thp-1, differentiated and not, were stimulated with lipopolysaccharide (LPS, Life Technologies) at a concentration of 1 μg/ml for 3 hours.

3.6 Cytotoxicity Assay

MTT (3,4-dimethylthiazole-2,5-diphenyltetrazolium bromide) (Sigma- Aldrich) assay test was used to quantify the possible cytotoxic effect of thalidomide in sh-sy5 cells either un- and differentiated. Cells were treated with different concentrations (50, 100, 200, 400 μM) of thalidomide and at different times of incubation. 4 hours before treatment expires, cells were incubated with MTT and finally levels of MTT/formazan were determined by measuring the absorbance. The plate is read at the double wavelength of 540 nm and 630 nm (the absorption wavelength of the formazan salt corresponds to 570 nm).

3.7 RNA extraction from biopsies and cell lines

Total RNA was extracted using TRIzol® reagent (Thermo Scientific) from Thp-1, HeLa, Sh-sy5y 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 °C for 10 minutes. Then, the RNA concentration and purity were calculated by Nano Drop instrument (NanoDrop 2000, EuroClone®).

3.8 Quantitative real-time PCR

mRNA expression levels of different genes studied were evaluated by real-time RT-PCR TaqMan® analysis and Syber Green 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 1 µg of total RNA per 20 µL of reaction containing 10 µL of 2x RT Buffer, 1 µL of 20x RT Enzyme Mix. The thermal protocol (Applied Biosystems 2720 Thermofisher Scientific) provides a first incubation of samples to start the reaction at 37 °C for 60 minutes and a stop of the reaction by heating to 95 °C for 5 minutes and a final hold step at 4 °C. The cDNA obtained is ready for real-time PCR analysis.
For quantifying the expression level of the genes analyzed in this thesis, TaqMan® Gene Expression Assay (Applied Biosystem) and the SensiFASt SYBR Lo-ROX Kit (Meridian-Bioscience) were used.
RT-PCR TaqMan® analysis 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 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. TaqMan gene expression assays used are summarized in Table 3:

PROBE	CODE	COMPANY
18S	Hs99999901_s1	Thermo Fisher
GAPDH	Hs99999905_m1	Thermo Fisher
GAS5	Hs03464472_m1	Thermo Fisher

RPLP0	Hs99999902_m1	Thermo Fisher
MMP-9	Hs00957562_m1	Thermo Fisher
MMP-2	Hs01548727_m1	Thermo Fisher

Table 3: TaqMan® gene expression assays 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, Gapdh and ß-Actin were used, while for biopsies the RPLP0, that codes for a ribosomal protein of 60S subunit, was employed. The expression levels of the selected transcripts were determined using the Livak method for relative expression (RE) and relative expression of ΔΔCt [129]. The results are provided as the mean and standard error (SE) of three replicates.
SYBR Green is a fluorescent dye, able to bind double-stranded DNA molecules by intercalating to determine the amount of DNA amplified. Predesigned (KiCqStart® SYBR® Green Primers, Sigma-Aldrich) primers were used table 4. Beta-actin was used as normalizer and expression levels were reported as 2-ΔCt, using the Livak method [129].

GENE	CODE	COMPANY
CACNG6	FH1_CACNG6	SIGMA
ADAMTS2	FH1_ADAMTS2	SIGMA
SLC1A3	FH1_SLC1A3	SIGMA
ACTIN B	FH1_ACTB	SIGMA
NRCAM	FH1_NRCAM	SIGMA
GABRE	FH1_GABRE	SIGMA

Table 4. Predesigned (KiCqStart® SYBR® Green Primers, Sigma-Aldrich)

3.9 Gene transfection and over-expression

To verify the role of GAS5 in modulating the expression of MMP-2 and MMP-9, a gene overexpression experiment was performed, transfecting the cells with a plasmid vector in which the gene encoding GAS5 was cloned. The vector used is a pCDNA3.1 +, a commercial plasmid. The transfection was carried out on the Thp-1 cell line with the pCDNA3.1 + GAS5 expression vector using the Lipofectamine 2000 reagent (Invitrogen), following the protocol suggested by the manufacturer. In parallel, the empty plasmid vector pcDNA3.1 + was used as a control. Lipofectamine 2000 DNA Transfection Reagent (Invitrogen) is a reagent composed of cationic lipids that form liposomes with a positively charged surface that allows the association of DNA and mediates interaction with the plasma membrane.

3.10 Protein isolation

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

1) to study the expression of MMP2 and MMP9 in IBD patients, colon biopsies were collected, frozen and isubsequently 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 lysis buffer, composed of Tris- HCl 10 mM pH 7.4, EDTA 100 mM, NaCl 100 mM, SDS 0.1%, with protease inhibitor cocktail 1x (all from Sigma-Aldrich) 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 contains the whole protein lysate.

2) to quantify the effect of thalidomide on Sh-sy5y cell line differentiation, cells were collected and washed twice with cold PBS. To lyse the membrane 100 µL of a lysis buffer with protease inhibitor cocktail 1x (Life technologies) 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.

3) for RNA-immunoprecipitation experiments, Hela cells after being seeded and cultured for 2 days were collected and washed twice with cold PBS. To lyse the membrane, 600 µL of RIP buffer, composed with 20 mM HEPES PH 7.7 (Euroclone®), 150 mM NaCl, 0.5 mM EDTA, 1 mM DTT, 10% Glycerol, 0.1% TritonX with protease inhibitor cocktail (all from Sigma-Aldrich) was used. Lysate were incubated for 30 minutes in ice and then centrifugated at 10,000 x g for 20 minutes at 4 °C to remove cell debris. The supernatant contains the whole protein lysate. Protein lysates were quantified with Bradford assay for the Western blot assay and for RNA-Immunoprecipitation. Proteins bind to Coomassie dye under acidic conditions resulting in its change of color from brown (cationic form) to blue (anionic form). Standards (BSA at concentrations of 1,5–0.125 µg/µL and 0 µg/µL) and samples are placed in a 96-well flat-plate with Bradford reagent (Sigma-Aldrich) in ratio 1:50. After 15 minutes of incubation at room temperature in the dark, absorbance is read at 590 nm by a spectrophotometer (Automated Microplate Reader EL311, BIO-TEK® Instruments). A calibration curve is obtained, and the protein content of the sample is calculated from its absorbance value.

3.11 RNA-Immunoprecipitation

RNA Immunoprecipitation is an antibody-based technique used to map in vivo RNA-protein interactions and RNA modifications. The RNA binding protein (RBP) of interest is

immunoprecipitated together with its associated RNA to identify bound transcripts (mRNAs, non-coding RNAs) and transcripts can be detected by real-time PCR, microarrays, or sequencing. Hela cell line was used to study the role of GAS5 in modulating the activity of the trascriptor factor NF-κB through a RNA-IP experiment.

Protein lysates, obtained as described previously, were incubated with 50 µl of Sepharose G Beats (1:1) (Sigma-Aldrich) for 20' at 4 °C with gentle rotation for sample pre-clearing, which is an optional step designed to remove proteins and ligands that bind non-specifically to the beaded support. After, beads were pelleted at 1000 RPM for 30'' and the supernatant was incubated over night at 4 °C with gentle rotation with 300 µl of RIP buffer, 5 µg/mL heparin and with primary antibody (1:200, anti-P65, Santa Cruz Biotechnologies). As negative control, protein lysate pre cleared was incubated with anti IgG (1µg/mL, Sigma-Aldrich). 20 µl of Sepharose G Beats were added and incubated for 4 hours at 4 °C with gentle rotation. At the end samples were divided in two aliquots and then centrifugated at 1000 RPM for 30 sec. For the first aliquot, used for RNA isolation, the supernatant was removed and beads were resuspended in 500 µl of RNA wash buffer (HGEN buffer with DOC 0.2% and Urea 5mM), incubated 10' at 4 °C with gentle rotation, then centrifugated for at 5000g for 30'', and repeated for a total of 4 washes. Beads were resuspended in 1 ml of TRIzol® reagent (Thermo Scientific). Co-precipitated RNAs were isolated and RT-PCR for GAS5. The second aliquot was used for protein isolated by the beads. Beads were resuspended in 500 µl of Wash buffer (PBS 0.2% TritonX100, all from Sigma-Aldrich), then centrifugated for at 1000 RPM for 1', and the centrifugation repeated for a total of 4 washes. Beads were then resuspended in Loading Buffer 4X (Thermofisher) and protein (P65) was detected by Western blot analysis.

3.12 Immunoblot assay

Immunoblot assay or western blot is an analytical technique used to detect and quantify specific proteins in a biological sample. Samples are prepared as follows: 20 µg of proteins are added with 5 µL of LDS Loading buffer 1x (Thermo Fisher) to a final volume of 20 µL with RIPA buffer under non-reducing conditions. Each sample is then loaded on Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) 10% (precast Bolt 10%, Bis-Tris Plus Gels, Thermo Fisher) which permits to separate proteins based on their molecular weight since it breaks tertiary and secondary protein structures and charges amino acids negatively. Gel is also loaded with 5 µL of protein marker (Thermo Fisher) that separates in colored bands depending on the molecular weight in order to follow protein run. The gel is inserted in an electrophoretic chamber filled with Running Buffer 1x (Thermo Fisher) that is connected to electrodes on which

a voltage of 200 V is applied for about 50 minutes. Gel is then put in a Power Blotter (Thermo Fisher) placed on a nitrocellulose membrane (Thermo Fisher) previously wetted with Transfer Buffer 1x (Thermo Fisher). A constant electric current of 1.3 A (25 V) is applied for 10 minutes. After transfer on nitrocellulose, the membrane is cut using markers as a guide to separate proteins of interest from the others. Afterwards, each sample is incubated with 5% milk solution in T-TBS (0.012 % p/V Trizma® base (Sigma); 0.009 % p/V Sodium chloride (Sigma); 0.001% Tween 20 (Sigma)) for 1 hours at 4 °C on a rocking platform. This phase is needed to bind all non-specific binding sites on the membrane. After blocking, incubation with the following primary antibodies at 4 °C is performed:

Antibody	**Host**	**Final dilution**	**Company**
anti MMP9	**rabbit**	**1:1000**	Sigma Aldrich
anti MMP2	rabbit	1:1000	Sigma Aldrich
anti p65	mouse	1:500	Santa Cruz Biotechnologies
anti β tubulin III	rabbit	1:1000	Cell Signaling
Anti- HSP90	rabbit	1:1000	Santa Cruz Biotechnologies
anti- β Actin	mouse	1:3000	Abcam

Table 4: Primary antibodies used for Western blot.

Anti-mouse (Cell Signaling Technologies) and anti-rabbit HRP conjugated (OriGene technologies and Sigma-Aldrich) secondary antibodies were incubated for 1 hour at 4 °C. After each step of antibody incubation, membranes are washed with T-TBS, four times for 5 minutes and are ready to be developed. In order to develop the membranes, LiteABLOT Turbo Chemioluminescent Substrate (EuroClone®) is used. The kit is composed of Peroxidase Buffer and Luminol: HRP enzyme in presence of hydrogen peroxide catalyzes the oxidation of luminol, resulting in a fluorescent emission. The solution is spread on the membrane and incubated for 5 minutes. The fluorescence produced by luminol is impressed on the photographic plate (Sigma-Aldrich) placed on the membrane for 20 minutes in a cassette. The plate is then developed with Developer solution (Thermo Fisher), washed with water and stained with Fixer solution (Thermo Fisher). Images were analyzed with ImageJ program and data were normalized on β-actin.

3.13 Enzyme-linked immunosorbent assays (ELISA)

In this study three ELISA assay were performed for the quantification of MMPs, PGE2 and cAMP on supernatants of Thp-1 cells.

- For MMPs quantification, supernatants were collected from Thp-1cells and run on a Bio-Plex 200 System (Bio-Rad Laboratories); the results were calculated using Bio-Plex Manager 6.0 software (Bio-Rad Laboratories).
- For PGE2 quantification, supernatants were collected form Thp-1cells and the quantifications were performed using Prostagland E2 Elisa kit- monoclonal (Cayman Chemical) and the plate were measured at the GloMax -Multi Detection System (Promega).
- For cAMP quantification, supernatants were collected form Thp-1cells and quantified by means of cyclic AMP Elisa kit (Cayman Chemical) and the plate were measured at the GloMax -Multi Detection System (Promega).

3.14 Adenosine triphosphate (ATP) determination

Sh-sy5y cells were seeded in 96 well plates and after 24 hours were stimulated for 6 and 72h with thalidomide (50-200 μm). After drug incubation, cells were lysed using 100 μl of phosphate buffer saline (PBS) with 0.02% of TritonX100 (Sigma-Aldrich). Adenosine triphosphate (ATP) was measured using the ATP determination kit (Invitrogen, Thermo Fisher Scientific) following manufacturer instruction in 10 μl of the lysates and the luminescence was read at the GloMax-Multi Detection System (Promega) and expressed as ATP concentration using the standard provided by the kit. ATP level was normalized on protein concentration, quantified with Bradford assay (Sigma Aldrich).

3.15 Mitochondrial membrane potential and reactive oxygen species measurement

To study the possible effect of thalidomide treatment on mitochondrial membrane potential (MMP) and on ROS production, sh-sy5y cells were seeded in 96 well plates and after 24 hours cells were stimulated for 6 and 72 h with thalidomide (50-400 μm). After drug incubations, MMP and ROS were measured. For MMP, JC-1 probe (Sigma-Aldrich) was used, loading cells with probe (10 μm) for 30 minutes at 37 °C, and then the fluorescence was read immediately. JC-1 is a cationic carbocyanine dye that accumulates in mitochondria. The dye exists as a monomer at low concentrations and yields green fluorescence, similar to fluorescein. At higher concentrations, the dye forms J-aggregates that exhibit a broad excitation spectrum and an emission maximum at ~590 nm. The red/green fluorescence intensity ratio can be used as a measurement of MMP: a decrement indicates a mitochondrial depolarization, an increment a

hyperpolarization. The results were expressed as green (525 nm) to red (590 nm) fluorescence intensity ratio; normalized on untreated cells.

For ROS quantification, CellROX Green Reagent (Invitrogen, Thermo Fisher Scientific), a cell-permeant dye, was added to each well at a concentration of 10 μmol/L. After 30 minutes of incubation, fluorescence was measured at the GloMax -Multi Detection System (Promega). The dye is weakly fluorescent when in a reduced state and exhibits bright green photostable fluorescence upon oxidation, with absorption/emission maxima of 485/520 nm. ROS levels were normalized on protein concentration, quantified with Bradford assay (Sigma Aldrich).

3.16 Intracellular calcium quantification

To evaluate and study an effect of thalidomide on intracellular calcium levels, Fluo-4, acetoxymethyl ester, a cell-permeant dye (F10472, Thermo Fisher Scientific) was used to test the effect of this drug on intracellular calcium. Sh-sy5y, treated with thalidomide at different concentration (50 - 400 μM) for 72 hours, were loaded with the dye (10 μM) for 30 minutes at 37 °C and then the fluorescence was analyzed with Cytation5 (Biotek). Fluorescence was read by excitation at 485 nm, and emission at 520 nm, measuring every 0.2 s. After 60 s, an automated dispenser was used to distribute thapsigargin, a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca2+ ATPase (SERCA), (10 μL, 10 μM, T9033; Sigma Aldrich) in each well and followed by continuous fluorescence monitoring. Fluorescence reads were then normalized on baseline (means of first 20 s readings). Data were reported as maximum normalized fluorescence after thapsigargin stimulation. Untreated cells were used as control.

3.17 Immunofluorescence imaging analysis

In order to study the effect of the thalidomide on cell differentiation mechanisms and to evaluate morphological cellular parameters, sh-sy5y were differentiated with dbcAMP for three days, and simultaneously treated every 24 hour for 3 days with thalidomide (100-400 μM), followed by fixation with 4% paraformaldehyde-PBS for 20 minutes, then washed, and finally permeabilized with PBS 0.2% TritonX100 (PBS-T). Blocking was performed in 10% normal goat serum (NGS) in PBS with 0.2% TritonX100 (PBS-T). Cells were then incubated overnight at 4 °C with anti β -Tubulin III antibody (1:1000, Cell Signaling Technologies) in PBS-T 1.5% NGS. Cells were then incubated for 1 hour with the anti-rabbit Alexa Fluor 568 secondary antibody (1:1000, Thermo-Fisher Scientific). A mounting medium containing 40,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich) was used to seal the cells. Fluorescent pictures were

acquired using Cytation5 (Biotek). Cellprofiler software was used to analyzed immunofluorescence images [130]. Images stitched from 4 different fields from each well were subjected to analysis using a custom-made pipeline; in brief, DAPI and β -Tubulin III channels were separated and the first used to define the single objects in each field, followed by recognition of the same objects in β -Tubulin III channel.

Secondly, these objects were reduced to lines and the number of branches for each cell were automatically counted; in abscissa are the numbers of branch per cells, subdivided in "bins", while in ordinate the frequency of each bin, i.e. the number of cells having that exact number of branches divided the frequency of each bin.

3.18 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 7.00. $P < 0.05$ was considered statistically significant.

t-test, one and two-way ANOVA with post-tests were used for the analysis of gene and protein expression, for ELISA assays, for measurement of intracellular ATP, calcium quantification analysis, quantification of ROS, MMP and for MTT assay in sh-sy5y cells.

For the analysis of correlation between GAS5 and MMPs, and between GAS5 and scores of the disease, Spearman test and linear models were used. Wilcoxon signed rank test (paired test) was used for gene expression analysis in colon biopsies of IBD patients.

4. RESULTS

-part 1

4.1 IBD patients' characteristics for MMPs study

In order to compare the expression levels of the lncRNA GAS5, MMP-2 and MMP-9 between inflamed and non-inflamed intestinal tissues, 25 pediatric patients with IBD were enrolled at diagnosis, before starting drug treatment. The baseline characteristics of patients enrolled and histologic findings of their biopsies are shown in Table 1.

IBD patient's characteristics	
Patients (n)	25
Age (mean, range)	12.5, 6.2–18
Male (%)	13 (52%)
CD (%) UC (%)	13 (52%) 12 (48%)
Clinical score: PCDAI (mean, range) PUCAI (mean, range)	 30.6, 7.5–55 30.1, 10–75
Histologic findings: Inflammation score	Score (% of patients) 0 (0%) 1 (4%) 2 (24%) 3 (36%) 4 (28%) 5 (8%)
Architectural score	Score (% of patients) 0 (0%) 1 (8%) 2 (28%) 3 (52%) 4 (12%)

Table 1. Characteristics and histologic scores of the patients.

4.1.1. Quantification of MMP-2 and MMP-9 gene expression levels in colon biopsies of pediatric patients with IBD

Gene expression analysis of MMP2 and MMP9 in colon biopsies of IBD patients, as reported in Fig. 4.2, clearly confirms that levels of the two metalloproteinases are significantly higher in inflamed than in non-inflamed tissues, confirming what has already been reported in the literature.

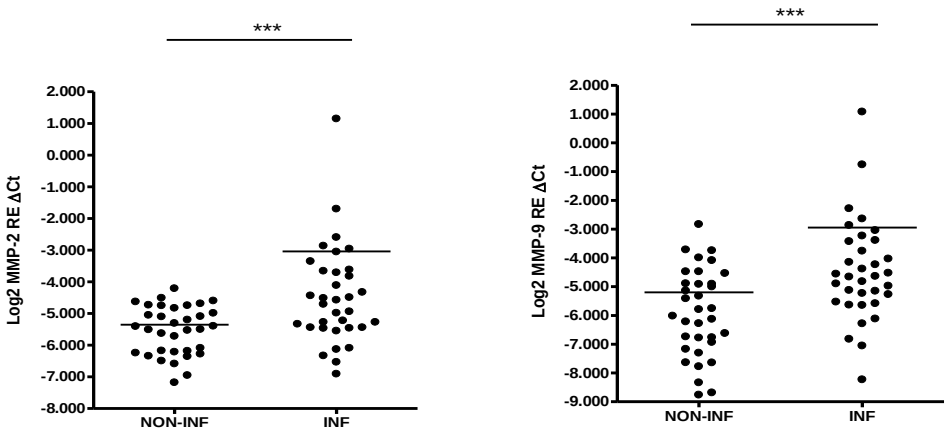


Fig. 4.1.1 Quantification of MMP-2 and MMP-9 expression levels in inflamed (INF) and non-inflamed (NON-INF) intestinal tissues of IBD patients. MMP2 and MMP9 expression were calculated with respect to the housekeeping RPLP0 gene. Expression reported in Log2 of MMP-2 and MMP-9 relative expression (RE, values are expressed as $2^{-\Delta Ct}$). Statistical analyses with Wilcoxon signed rank test, p = 0.0008 (MMP2), p = 0.0002 (MMP9).

4.1.2. Quantification of MMP-2 and MMP-9 protein expression levels in colon biopsies of pediatric patients with IBD

Immunoblotting analysis of the MMP9 and MMP2 proteins was also performed on inflamed and non-inflamed biopsies of three additional patients (mean age at enrolment 13.8 years, two UC and one CD, two males and one female).

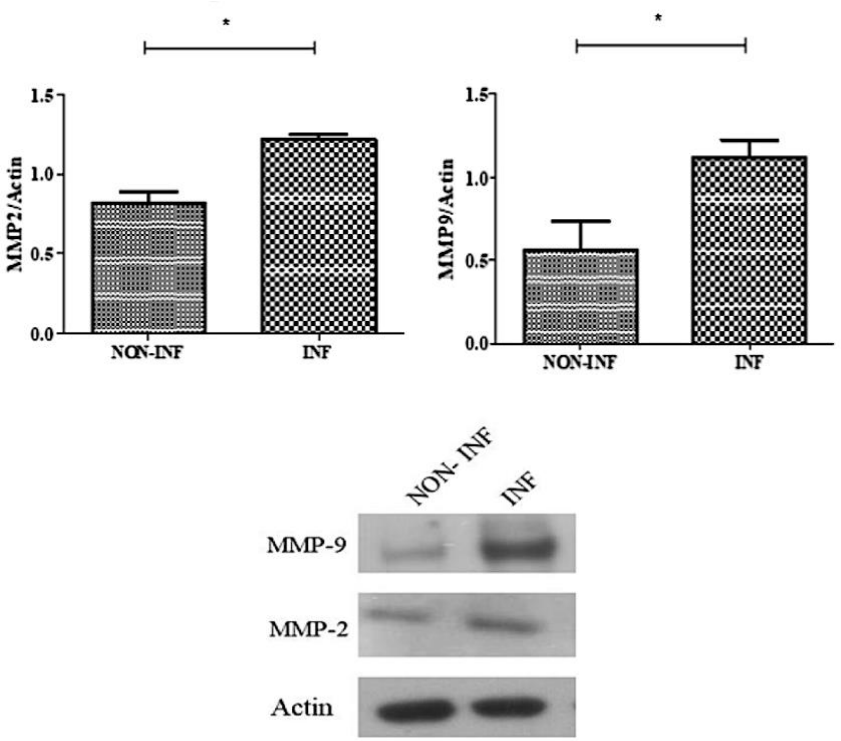


Figure 4.1.2. MMP2 (74 kDa) and MMP9 (92 kDa) protein expression in colon biopsies of IBD pediatric patients analyzed by western blot. Protein lysates were obtained from inflamed (INF) and non-inflamed (NON-INF) tissues. Beta-actin (42kDa) was used as internal

loading control. Representative western blot image of one biopsy reflecting MMP2, MMP9 and actin protein levels. Paired t test * $p < 0.05$.

An increase in the expression of both proteins in inflamed compared to non-inflamed ones was observed (Figure 4.1.2).

4.1.3. Quantification of GAS5 gene expression levels in colon biopsies of pediatric patients with IBD

Gene expression analysis, as shown in Fig. 4.1.3, demonstrates that GAS5 levels are significantly lower in inflamed than in non-inflamed tissues.

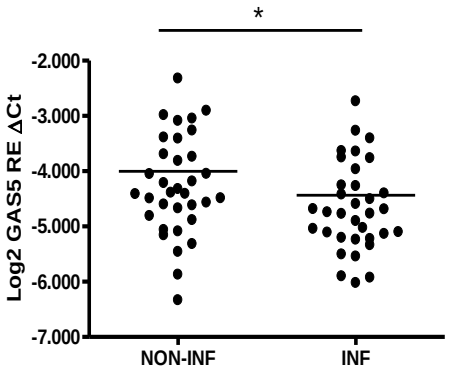


Fig. 4.1.3. Quantification of expression levels of GAS5 in inflamed (INF) and non-inflamed intestinal tissues (NON-INF) of IBD pediatric patients. GAS5 expression was calculated with respect to the housekeeping RPLP0 gene. Expression reported in Log2 of GAS5 relative expression (RE, values are expressed as $2^{-\Delta Ct}$).
Statistical analyses with pair *t test* * $p < 0.05$.

The results obtained suggest that the expression of GAS5 may be reduced in the presence of pro-inflammatory stimuli that are mainly released by macrophages, monocytes and lymphocytes infiltrated in the inflammation site.

4.1.4 Correlation between GAS5 and inflammation and clinical scores

To analyze the possible effect of GAS5 as biomarker of disease and inflammation, an analysis of correlation was performed between GAS5 and the inflammation and clinical scores.

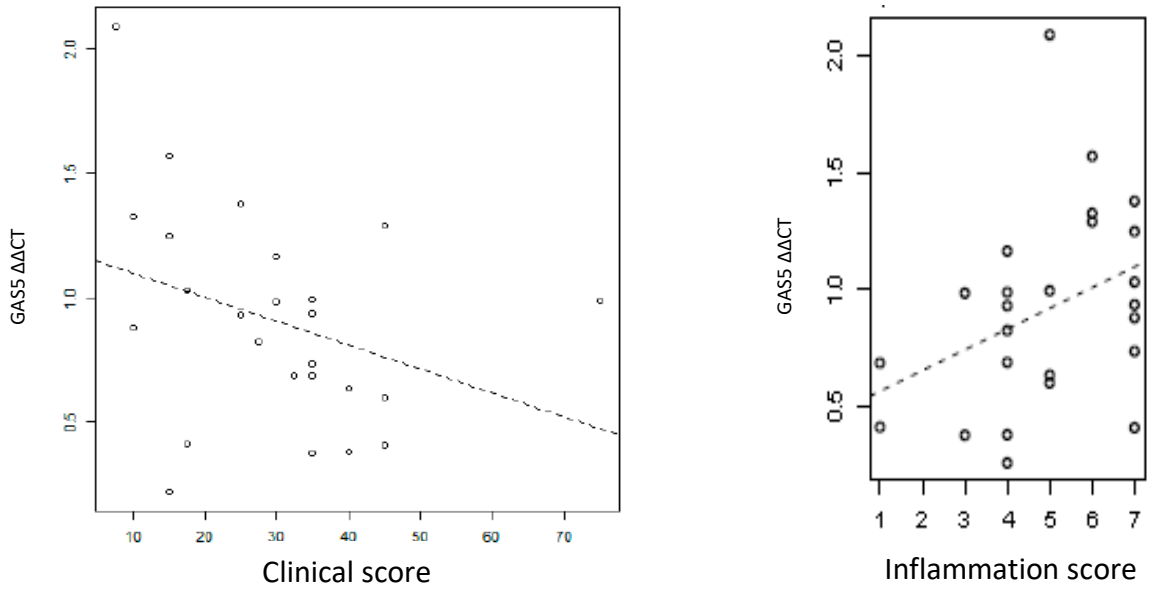


Fig. 4.1.4 Correlation between GAS5 expression (ΔΔCT) and the inflammation and clinical scores. Statistical analyses with Spearman's rank test, inflammation score: $p > 0.05$; clinical score: $p = 0.048$.

No significant association was found with the inflammation and architectural parameters, described in Table 1 (Figure 4.1.4) However, a significant negative correlation between GAS5 levels and the clinical scores PUCAI and PCDAI has emerged.

4.2 Analysis of gene expression levels in Thp-1 cell line

To analyze the expression of lncRNA GAS5 and of MMP-2 and MMP-9 *in vitro*, the human monocyte Thp1-1 line was treated with different pro-inflammatory stimuli, in order to study the effects of the inflammatory response on the expression of the examined genes, known to be involved in IBD. It is clear that this model is not exhaustive of all aspects of the pathology under study, but literature data show that they are able to reproduce the inflammatory phenotype [131].

4.2.1 Quantification of GAS5, MMP-2 and MMP-9 gene expression levels in response to LPS and PMA in Thp-1

Among the cell types that characterize inflamed intestinal tissue and considering that remodeling of the ECM and cell surface by MMPs is one of the most important function of monocytes and macrophages, the role of GAS5 in the regulation of MMPs expression was studied in the Thp-1 monocyte cell line. In particular, *in vitro* experiments at different stages of differentiation, from monocyte to macrophages, stimulated with a proinflammatory factor (LPS), were conducted (Fig. 4.2.1).

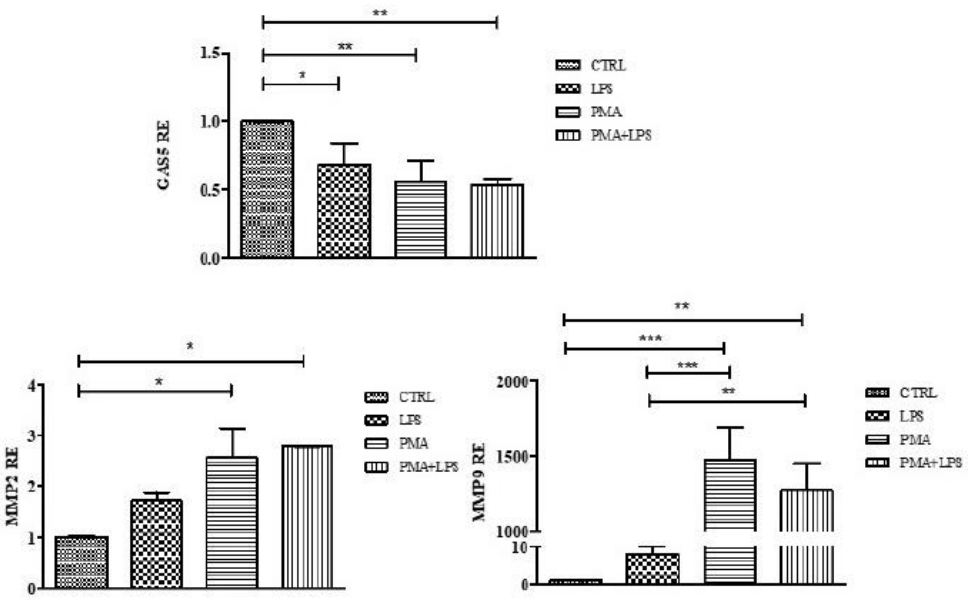


Fig. 4.2.1 Quantification of gene expression levels of GAS5, MMP-2 and MMP-9 on Thp-1 (monocytes) in response to LPS and PMA, used to differentiate monocytes into macrophages. Gene expression were calculated with respect to the housekeeping 18s gene. Expression reported as relative expression (RE, values are expressed as $2^{-\Delta Ct}$). One-way ANOVA: GAS5 p = 0.048; MMP-2: p = 0.0071; MMP-9 p< 0,0001; Bonferroni's post-test = * p<0.05, * * p<0.01, *** p<0.001.

As observed for inflamed colon biopsies of patients, the endogenous levels of GAS5 decrease in the presence of a pro-inflammatory stimulus, highlighting how these factors can alter its expression; this phenomenon is particularly evident in macrophages. As regards MMP-2, following treatment with LPS, in monocytes we observe a slight unsignificant increase in the gene's expression. On the other hand, the differentiation of monocytes into macrophages induces a significant increase in MMP-2 levels which do not tend to increase further in the presence of LPS. For MMP-9, as for MMP-2, in monocytes we observe a slight increase in response to LPS. In macrophages, on the other hand, there is a high increase which tends to not increase in presence of LPS.

4.2.2 Evaluation of MMP-2 and MMP-9 gene expression in cell transfected with the pcDNA3.1-GAS5 Plasmid

Following the transient transfection with pcDNA3.1-GAS5 plasmid and with the vector without the insert, the efficiency of overexpression of the lncRNA both in monocytes (Thp-1) and macrophages (Thp-1 +PMA) was assessed with Real Time-PCR (Fig 4.2.2).

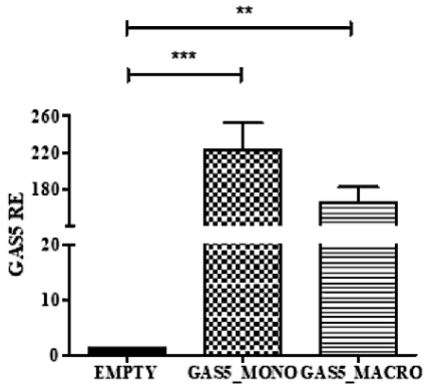


Figure 4.2.2. Evaluation of GAS5 levels after transfection with pcDNA-GAS5 plasmid in monocytes (GAS5_MONO) and macrophages (GAS5_MACRO). GAS5 expression was normalized using GAPDH gene and relative expression (RE) values are expressed as $2^{\wedge-\Delta Ct}$. One-way ANOVA: GAS5 p = 0.0005, Bonferroni's post-test ** p < 0.01, *** p < 0.001.

Gene expression levels of MMP-2 and -9 were evaluated in transfected monocytes and macrophages stimulated or not with LPS (Fig 4.2.3).

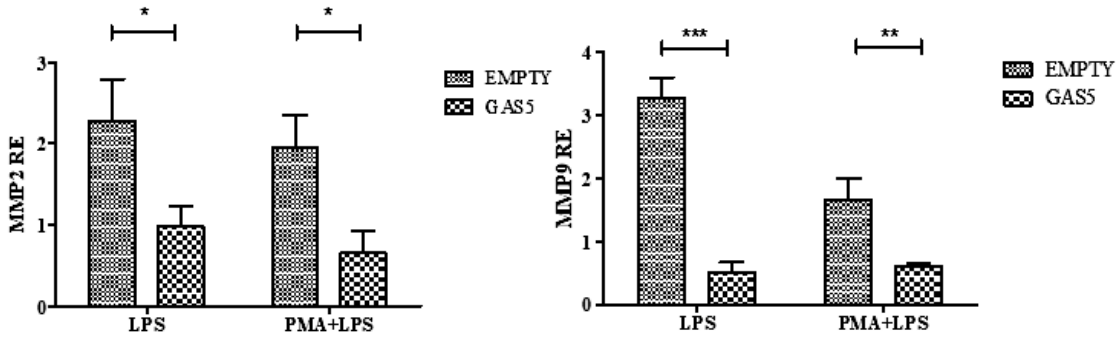


Figure 4.2.3 Levels of MMP-2 and MMP-9 after overexpression of GAS5 (GAS5) in monocyte stimulated with LPS (LPS) and macrophages stimulated with LPS (PMA + LPS) and in controls (EMPTY). MMPs expression were normalized using GAPDH gene and relative expression (RE) values are expressed as $2^{-\Delta\Delta Ct}$. Two-way ANOVA: EMPTY vs. GAS5 MMP-2 p = 0.002, MMP-9 p < 0.0001 Bonferroni's post-test
* p < 0.05, ** p < 0.01, *** p < 0.001.

Data reported in figure 4.2.3 show a significant downregulation in LPS-stimulated monocytes and macrophages compared with the respective control transfected with the empty vector, demonstrating the involvement of the lncRNA in the regulation of gene expression of the two MMPs.

4.2.3 Quantification of MMP-2 and MMP-9 proteins released by Thp-1 cells overexpressing GAS5

To confirm the role of GAS5 in regulating the expression of MMP-2 and MMP-9, their levels in the supernatant of both LPS-stimulated monocytes and PMA-differentiated macrophages overexpressing GAS5 were evaluated by enzyme-linked immunosorbent assay (ELISA) test.

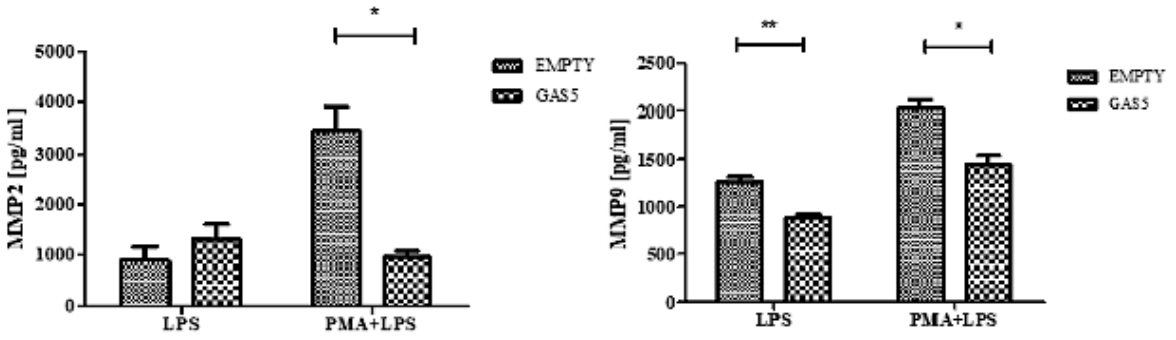


Figure 4.2.4 Evaluation of levels of MMP-2 and MMP-9 after overexpression of GAS5 (GAS5) in monocyte stimulated with LPS (LPS) and macrophages stimulated with LPS (PMA + LPS) and controls (EMPTY). Two-way ANOVA: EMPTY vs. GAS5 MMP-2 $p = 0.02$, MMP-9 $p < 0.0001$ Bonferroni's post-test * $p < 0.05$, ** $p < 0.01$.

As reported in Figure 4.2.4, MMP-9 was less expressed in GAS5-transfected monocytes with respect to the control; no difference was detected measuring MMP-2 levels. GAS5 overexpression significantly downregulated both MMP proteins in macrophages stimulated with LPS compared to the cells transfected with the empty vector, supporting the role of GAS5 in modulating their expression.

4.3 Role of GAS5 on modulation of NF-κB activity: RNA-IP assay result

The potential role of GAS5 in the modulation of NF-κB activity was also studied. After electrophoretic mobility shift assay (EMSA) results previously obtained in collaboration with prof Pavlovic' team, which highlighted an effect of GAS5 in increasing NF-kB DNA biding activity, the potential interaction between the lncRNA and the NF-κB subunit P65, through a RNA-IP experiment on Hela cells was studied.

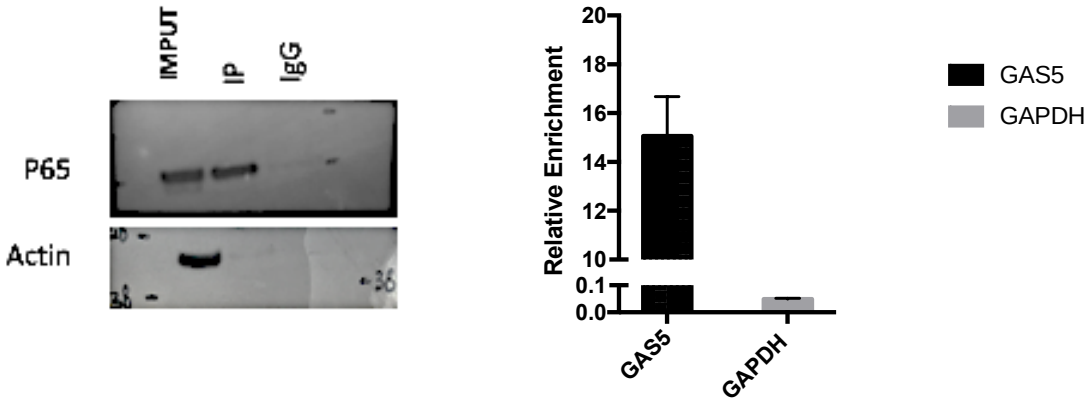


Fig 4.3 RNA -IP experiment. The protein-mRNA immune-complex with either anti-P65 or the control anti-IgG complexes were processed for immunoblotting and for Real Time-PCR. First lane in the blot corresponding to total lysate (IMPUT) was not subjected to immunoprecipitation. Actin was used as control for IP specificity. The relative enrichment, reported in the graph, was calculated by the ratio between IP mRNA levels and IMPUT mRNA, and normalized to negative control (anti-IgG). Data was from one representative experiment.

The result reported in figure 4.3 shows that the immunoprecipitation of P65 was obtained, and the IgG background control (IgG) showed lack of immunoprecipitation, confirming IP specificity. The Real Time-PCR result clearly shows that GAS5 is bound to P65. The housekeeping gene GAPDH was used to confirm the specific enrichment of the assay.

5. DISCUSSION

- part 1

In IBD pediatric patients, genetic and environmental factors are responsible for the alteration in epithelial barrier; the activity of pro-inflammatory elements released from macrophages, T cells, and innate lymphoid cells is also important [132]. MMPs have a key role in this context. In addition to play a central role in ECM turnover, they activate or degrade a variety of other substrates including chemokines, cytokines, growth agents, and junctional proteins [133]. Several studies have shown that MMP-2 and MMP-9 are highly expressed in inflamed colonic mucosa, serum, urine, and fecal samples of IBD patients [134-136]. However, the molecular mechanism by which the levels of these two enzymes can be modulated during the inflammatory process still remains unclear. Recently, it has been suggested that lncRNAs may play an important role in the pathophysiology of IBD, and many researches have therefore covered this topic. Forty-seven lncRNAs dysregulated in IBD have been identified, and it has been suggested that they might hold a crucial role in the regulation of inflammatory pathways [35]. This part of the thesis project is focused on the study of the role of the lncRNA GAS5 in the regulation of MMP-2 and the MMP-9. In particular, the expression of GAS5, MMP-2, and MMP-9 were evaluated in intestinal biopsies of 25 pediatric patients with CD and UC, not in drug treatment. The results show that the expression of the lncRNA GAS5 in patients with IBD is lower in inflamed tissues compared to the adjacent normal part, while the expression levels of MMP2 and MMP-9 increase in inflamed biopsies [137]. The results obtained in this cohort of patients confirm what has already been reported in the literature in patients with IBD [26, 138]. The role of MMP-2 in IBD seems to be related to the infiltration process of leukocytes in the inflamed tissue and with the maintenance of the epithelial barrier function, while MMP-9 has been associated with an enhanced inflammation because of the activation of growth factors and pro-inflammatory cytokines [139].

As reported in the literature, GAS5 seems to play a role in various inflammatory and autoimmune diseases in which significantly reduced GAS5 levels in immune cells were shown [140]. Previous reports have already shown that GAS5 is involved in the regulation of thestwo MMPs, indeed Chen et al. have shown that the expression of MMP-2 and MMP-9 were inversely correlated with the levels of the lncRNA GAS5 in melanoma cells [70]. Overexpression of GAS5 reduced the levels of the proteins, whereas the knockdown increased their expression [70]. The potential role of GAS5 in the regulation of MMPs was described also in IBD patients [136]. Interestingly, data obtained from this cohort of patients highlighted a lack of correlation between GAS5 and inflammation and architectural parameters, whereas a negative correlation between GAS5 levels and the clinical scores PUCAI and PCDAI was observed. Clinical scores were significantly higher in patients with active disease while a score of <10 is consistent with

inactive disease [28]. Inflammation and architectural parameters of biopsies and clinical indexes are not always perfectly correlated because some symptoms are not related only to the mucosal inflammation, as previously described [141]. Therefore, GAS5 levels could be a specific molecular marker useful to better predict disease activity and stratify the severity of the disease. Significant correlations between two other lncRNAs in biopsies of CD patients, LINC01272 and HNF4A-AS1, and more severe mucosal injury have been already found, but no correlation with clinical disease activities has been reported [142].

Considering that the remodeling of the ECM and cell surface by MMPs is one of the most important function of monocytes and macrophages, to investigate the role of GAS5 in the regulation of MMP expression, the Thp-1 monocyte cell line was used. In particular, *in vitro* experiments at different stages of differentiation of cells, from monocyte to macrophages, stimulated with LPS were conducted. The results confirmed the trend observed in patients. The stimulation with LPS induced a down regulation of GAS5 in both monocytes and macrophages, while an increase of MMPs was observed. This data highlight that, in response to immune activation, GAS5 is transcriptionally repressed but the mechanism involved needs to be further investigated. Moreover, GAS5 seems to have a protective role during the induction of inflammation: its upregulation repressed the MMPs, acting as an anti-inflammatory agent. Actually, only one paper describing GAS5 expression after LPS-induced inflammation was published: the authors found that GAS5 was downregulated in ATDC5 chondrocyte cells and its overexpression alleviated LPS-induced injury [143]. Moreover, the expression levels of TNF-α, interleukin (IL)-1*b*, IL-6, and IL-8 were all significantly lowered by GAS5 overexpression. The mechanism proposed by the authors is that GAS5 could modulate LPS-induced inflammatory damage through regulation of Krüppel-like factor 2 (KLF2) expression and inhibition of NF-κB pathway [144].

The effects of GAS5-overexpression on the production of MMPs in response to LPS in terms of gene and protein expression were also studied. Overexpression of GAS5 in monocytes and macrophages stimulated with LPS led to a reduction of MMPs at RNA and protein level, supporting the role of GAS5 in modulating their expression, probably at the transcriptional level. It would be interesting to evaluate in our cellular model whether the reduced expression of GAS5 could induce an increase of MMP-2 and MMP-9 expression, as already reported in other cells [70].

The function of most lncRNAs depends on their ability to interact with functional domains of signaling proteins and thus regulate signal transduction. miRNAs and lncRNAs contain modular domains through which they directly interact with NF-κB signaling proteins thus regulating inflammatory response [72]. NF-κB is a central mediator of pro-inflammatory gene induction

and functions in both innate and adaptive immune cells, regulating the immune system and inflammatory processes; moreover, it is a known regulator of genes responsible for ECM remodeling in the digestive tract [75]. NF-κB has been implicated in the pathogenesis of a number of inflammatory diseases including IBD [78] and polymorphisms and mutations in NFKB1 gene have been associated with IBD [79, 145, 146]. Recent evidences reported that GAS5 was involved in the regulation of NF-κB [82-84]. But to date, it is unknown if GAS5 has an impact on NF-κB DNA biding. In previous experiments performed in collaboration with the University of Belgrade, the effect of overexpressed GAS5 on NF-κB activity in Hela cells was analysed through an EMSA assay.

The effect of overexpression of GAS5 on NF-κB activity suggested that this lncRNA increased the binding of NF-κB to DNA in a time-dependent fashion. These results suggest that GAS5 could have a role in NF-κB signalling pathway, stimulating the transcription factor DNA binding activity, an effect induced by various intra and extra cellular stimuli including cytokines like TNF-α. Indeed, the RNA-IP result highlighted the ability of GAS5 to bind NF-κB, as reported in literature for other lncRNA. Liu et al. described that the lncRNA NKILA (NF-κB interacting lncRNA) binding to NF-κB, inhibits the activation of the NF-κB signaling pathway by masking to IKK the phosphorylation site of IκB necessary for its suppression [80]. Instead, the lncRNA MALAT1 largely located in the nucleus, is directly associated with p65, preventing the p65/p50 heterodimer binding to the target promoters [81].

This data will be substantiated in other RNA -IP experiments, also treating HeLa cells overexpressing GAS5 with GC or a pro-inflammatory stimulus, as TNF-α.

6. RESULTS

-part 2

6.1 Patients' characteristics for thalidomide study

Ten IBD pediatric patients (mean age at enrolment 13.1 years, 6 CD, 6 males) refractory to previous pharmacological therapies and with active disease were enrolled for this study. Before starting thalidomide treatment, patients had already undergone other therapeutic attempts with conventional medications for the treatment of IBD. These were found to be unable to induce remission or to maintain it in the long term. The efficacy of thalidomide was assessed after 3 months of treatment. Patients in whom the treatment led to an improvement, evidenced by a reduction of at least 10 points in the PCDAI or the PUCAI scores at the end of the 3-months cycle were defined sensitive (PS). Patients in whom the 3-month course of thalidomide could not control disease, which remained active on the basis of clinical scores, were defined as resistant and therefore were not included in the study.

6.2 RNA-sequencing

RNA extracted from the PBMCs of 10 patients, isolated from whole blood before (PRE) and after 3 months of treatment (POST) with thalidomide, was sent to the Institute of applied genomics (IGA) in Udine for the preparation of the libraries and the sequencing of the transcriptome (RNA-seq). The analysis of the transcriptome, performed on an Illumina platform, made it possible to identify the expression profiles of the mRNAs for each patient, at two experimental times. The comparison between the expression profiles of the post-treatment and the respective pre-treatment samples allowed to highlight the variations in terms of gene expression associated with the treatment (Figure 4.4.1).

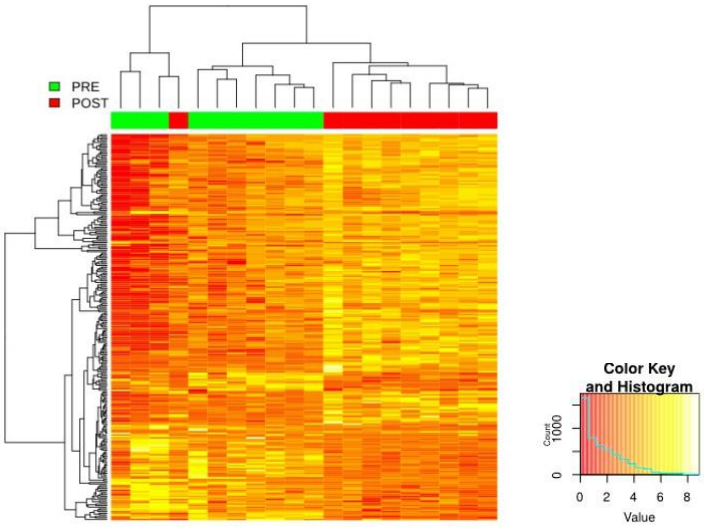


Figure 4.4.1 Heatmap obtained from the count of the mRNA reads differentially expressed in each patient, before (PRE) and after treatment (POST) with thalidomide.

In particular, the RNA-seq analysis identified 251 differentially expressed genes, of which 76 were downregulated and 175 up regulated following thalidomide treatment (Table 2).

GENES UP REGULATED			GENES DOWN REGULATED		
GENE ID	**FC**	**FDR p-value**	**GENE ID**	**FC**	**FDR p-value**
CLC	8.850	0	HAPLN3	-7.2	0
SLC28A3	6.890	0	C11orf95	-6.5	4.78E-12
CLEC5A	3.354	2.99E-12	DUSP8	-5.8	8.96E-12
IGFBP2	19.883	3.35E-09	ADAMTS2	-108.0	3.93E-11
TMEM63C	3.945	5.20E-09	CELSR2	-82.2	3.73E-10
ALDH2	2.539	1.49E-08	MPP2	-8.0	3.28E-06
CMKLR1	3.733	1.98E-08	SH3PXD2B	-4.2	7.63E-06
ZNF692	2.715	2.33E-08	SLC38A11	-2.9	1.92E-05
SIGLEC8	23.116	4.92E-08	TK2	-2.2	2.20E-05
RXFP2	3.446	9.61E-08	SH3RF3	-3.1	2.80E-05
GATA2	3.249	9.61E-08	KCNH3	-3.1	3.61E-05
LAMB2	4.004	2.40E-07	ADAMTS17	-3.0	4.70E-05
PRRG1	5.035	1.09E-06	PMEPA1	-2.8	6.91E-05
DSC2	3.100	1.81E-06	ZDHHC23	-2.4	7.31E-05
UNC13B	3.657	2.17E-06	GNG7	-2.2	7.38E-05
ZNF366	3.363	2.17E-06	BEAN1	-3.3	9.59E-05
HP	3.062	2.84E-06	C17orf107	-20.9	1.38E-04
CHI3L1	6.237	3.13E-06	EBF1	-2.5	1.79E-04
HDC	2.961	4.18E-06	COBLL1	-2.4	1.98E-04
HOPX	3.381	4.70E-06	NRCAM	-3.2	2.04E-04
CPA3	4.109	6.19E-06	HIP1R	-2.1	4.40E-04
SIGLEC1	2.644	7.39E-06	INHBA	-5.9	5.02E-04
HRH4	3.477	8.35E-06	ABHD6	-2.6	5.17E-04
SPTSSB	4.311	1.21E-05	PTPRS	-2.8	5.39E-04
AKAP12	3.416	1.53E-05	SEMA3F	-7.8	8.63E-04
CD209	3.730	1.89E-05	CAMK2A	-17.6	1.18E-03
LDB2	3.439	2.04E-05	MSR1	-2.5	1.30E-03
NCAM1	2.863	2.27E-05	C7orf55 LUC7L2	-17.1	1.34E-03
CES1	2.638	2.27E-05	RPS6KL1	-3.0	1.38E-03
CACNG6	5.954	3.24E-05	TNFRSF13C	-2.4	2.03E-03
PRSS33	10.534	3.39E-05	LARGE	-2.3	2.25E-03
C1orf115	3.035	3.39E-05	ACAN	-9.9	2.42E-03
STEAP3	2.598	3.67E-05	NETO1	-3.2	2.42E-03
RBMS2	2.089	3.71E-05	LZTS3	-2.4	2.55E-03
PRSS23	3.480	3.94E-05	SLC22A23	-2.3	3.17E-03
TRIM71	4.467	6.91E-05	MRAS	-2.8	3.34E-03
CLIC2	2.620	6.95E-05	FBLN2	-2.9	4.76E-03
TPBG	9.113	7.31E-05	BAIAP3	-2.4	5.26E-03
JPH4	3.960	7.84E-05	SDC2	-4.7	5.47E-03
CD36	3.100	7.84E-05	EML6	-2.1	6.58E-03
PPFIA4	2.870	8.38E-05	FMO5	-58.7	6.97E-03
ASB9	5.915	9.59E-05	FN1	-2.5	7.58E-03
RAB7B	6.243	1.10E-04	NBL1	-3.1	7.97E-03
SLCO4C1	2.014	1.29E-04	LOC101927509	-4.7	8.49E-03
PTGER3	3.554	1.32E-04	SDK2	-2.1	8.49E-03
CLEC10A	3.132	1.80E-04	RBM11	-2.4	8.61E-03
CYSLTR2	2.667	2.60E-04	PLEKHG1	-2.1	8.92E-03
KIR2DL4	4.392	2.78E-04	LOC107983947	-49.7	9.13E-03
STON2	2.761	2.81E-04	DIRAS1	-2.9	9.34E-03

CCR3	2.760	3.00E-04	UGT8	-3.0	0.012
COLGALT2	3.003	3.14E-04	ITGA3	-2.9	0.012
UGT2B11	3.498	3.83E-04	ARSI	-11.4	0.014
GABRE	11.043	4.09E-04	EPB41L5	-2.1	0.015
NRG1	2.735	4.09E-04	PEG10	-2.5	0.015
FCER1A	2.688	4.09E-04	PLD5	-4.6	0.015
MARCO	2.655	4.09E-04	SYNGR3	-3.1	0.015
DTHD1	3.021	4.32E-04	COL4A3	-2.0	0.017
EOMES	2.771	4.66E-04	FGF9	-4.2	0.017
MARVELD1	2.670	5.02E-04	TMEM119	-3.6	0.018
RETN	3.793	5.14E-04	KIRREL2	-5.7	0.020
FAT4	3.607	5.35E-04	NAV3	-7.3	0.020
ZMAT4	3.148	6.29E-04	SEC14L2	-2.1	0.020
KLRF1	2.514	6.32E-04	L1CAM	-5.3	0.021
FADS2	2.425	6.32E-04	SH3BP4	-2.0	0.021
FXYD6-FXYD2	3.209	6.42E-04	PALD1	-2.6	0.024
CDK15	10.183	6.49E-04	CXCL16	-2.1	0.028
PVR	2.080	6.76E-04	CHD7	-2.0	0.029
TDRD6	2.975	6.96E-04	DENND2C	-2.9	0.030
CD163	2.209	8.58E-04	LOC105379752	-9.9	0.030
CPM	2.006	8.69E-04	MYL9	-2.3	0.032
GZMA	2.562	1.08E-03	LGMN	-2.2	0.033
VIT	5.415	1.10E-03	PHLDB1	-3.5	0.037
MMP14	2.498	1.14E-03	SLC35D3	-9.9	0.041
KCTD15	2.242	1.28E-03	LRFN3	-3.6	0.045
KLRC1	2.594	1.40E-03	SGCE	-4.0	0.047
CABP1	6.277	1.53E-03	RGPD5	-2.4	0.049
KLRD1	2.597	1.62E-03			
VSIG10	2.916	1.67E-03			
OLIG1	2.415	1.82E-03			
RGS9	2.443	1.83E-03			
IL5RA	3.240	1.89E-03			
HFE	2.203	2.11E-03			
HECW2	3.020	2.42E-03			
GLP1R	3.756	2.45E-03			
TFEC	2.091	2.45E-03			
EGR1	3.106	2.82E-03			
DLEU7	2.211	2.88E-03			
RAB44	2.070	3.14E-03			
B3GNT3	5.294	3.43E-03			
WDR49	2.594	3.43E-03			
PRSS41	5.838	3.54E-03			
SERPINH1	2.771	3.87E-03			
KLRC3	2.210	3.87E-03			
CYP2S1	2.331	4.06E-03			
ADRB2	2.306	4.06E-03			
PGA3	9.735	4.32E-03			
DBNDD2	2.043	4.37E-03			
GZMB	2.350	4.45E-03			
PTGFR	4.380	4.58E-03			
HSPA1A	2.187	4.76E-03			
HOXB3	2.311	5.33E-03			
HNMT	2.074	5.33E-03			
PTGDR2	3.453	5.35E-03			
GPBAR1	2.149	5.41E-03			
LGI2	2.936	5.60E-03			
CX3CR1	3.180	6.80E-03			

MS4A6A	2.119	7.04E-03
SLC1A3	2.131	7.05E-03
MPZL2	2.930	7.61E-03
B3GAT1	2.285	7.90E-03
ADARB2	2.510	7.97E-03
GCSAML	2.218	8.01E-03
LGALS12	2.402	8.75E-03
SIRPB2	2.080	8.92E-03
TRIM36	2.549	9.87E-03
ADGRG5	2.350	0.010
GPR141	2.067	0.010
SLAMF8	2.673	0.010
PLA2G7	2.302	0.011
FCRL6	2.575	0.011
MLC1	2.030	0.011
ADORA3	3.624	0.011
ACVRL1	2.870	0.013
STAC	2.983	0.013
KIAA1522	2.043	0.013
IKZF2	2.071	0.014
MARC2	2.727	0.014
XCL2	2.709	0.014
ZNF365	2.311	0.014
GNLY	2.543	0.014
SOX13	2.266	0.014
TPM2	2.242	0.015
NEDD4	2.069	0.016
PTMS	2.077	0.017
DPYSL2	2.012	0.017
ANGPT4	2.324	0.018
DHCR24	2.023	0.018
HOXA9	2.709	0.018
KLRC2	2.231	0.019
NDFIP2	2.282	0.019
IDO1	3.924	0.020
FGFBP2	2.211	0.020
TMEM45A	3.981	0.020
WNT5B	2.770	0.021
SPON2	2.121	0.021
ME3	2.025	0.021
ADGRD1	2.316	0.022
TRIM49D1	5.245	0.022
LIMCH1	4.149	0.023
FLJ44635	2.047	0.023
TNIP3	2.437	0.024
PTGDR	2.597	0.024
KL	2.595	0.025
CLEC11A	2.110	0.027
MCEMP1	2.093	0.028
KIR3DL1	2.712	0.028
SPN	2.159	0.028
PRKCDBP	3.713	0.029
MPP3	2.455	0.030
SPR	2.707	0.030
CACNG8	3377	0.031
SH2D1B	2.028	0.031
RNF207	2.522	0.033

FZD4	2.807	0.034
TLR3	2.892	0.036
COL13A1	2.313	0.037
CYYR1	2.535	0.037
MS4A2	2.542	0.040
FAM124B	2.219	0.040
TIGIT	2.083	0.046
GZMK	2.094	0.046
ERICH3	4.128	0.047
S100A12	2.163	0.048
TPPP3	2.210	0.048
GATA1	2.716	0.048

Table 2. Genes up- and downregulated after treatment.
FC: fold change; FDR**:** false discovery rate.

6.2.1 Hypergeometric test and Ingenuity Pathway Analysis (IPA)

In order to give an interpretation of the biological effects caused by the treatment with thalidomide, an integrative approach, the analysis with a hypergeometric test, was used looking for the altered GO (Gene Ontology) terms in the subset of transcripts differentially expressed after treatment with thalidomide. The molecular functions, biological processes and cellular compartments most significantly influenced were thus identified (Table 3).

Molecular function (GO ID)	**FDR p-value**
Eicosanoid receptor activity (GO:004953)	7,51E-03
Carbohydrate binding (GO:0030246)	1,27E-02
Molecular transducer activity (GO:0060089)	1,98E-02
Transmembrane signaling receptor activity (GO:0004888)	2,13E-02
Signaling receptor activity (GO:0038023)	2,13E-02
Prostaglandin receptor activity (GO:0004955)	2,16E-02
Prostanoid receptor activity (GO:0004954)	2,34E-02
Biological process (GO ID)	**FDR p-value**
Defense response (GO:0006952)	2,28E-06
Cell adhesion (GO:0007155)	6,21E-06
Inflammatory response (GO:0006954)	2,54E-04
Regulation of immune system process (GO:0002682)	1,71E-03
Regulation of cell migration (GO:0030334)	3,31E-03
Regulation of cell motility (GO:2000145)	4,18E-03

Immune system process (GO:0002376)	6,08E-03
Synapse organization (GO:0050808)	9,53E-03
Positive regulation of cellular process (GO:0048522)	9,89E-03
Adenylate cyclase-modulating G protein-coupled receptor signaling pathway (GO:0007188)	9,96E-03
Regulation of transporter activity (GO:0032409)	1,27E-02
G protein-coupled receptor signaling pathway, coupled to cyclic nucleotide second messenger (GO:0007187)	3,29E-02
Cellular components (GO ID)	**FDR p-value**
Plasma membrane (GO:0005886)	8,75E-07
Extracellular matrix (GO:0031012)	8,40E-05
Collagen-containing extracellular matrix (GO:0062023)	8,74E-04
Postsynaptic density membrane (GO:0098839)	3,19E-03
Synaptic membrane (GO:0097060)	7,49E-03
Catalytic complex (GO:1902494)	8,18E-03
Postsynaptic membrane (GO:0045211)	1,26E-02
Endocytic vesicle membrane (GO:0030666)	1,78E-02
Glutamatergic synapse (GO:0098978)	5,07E-02
Integral component of postsynaptic density membrane (GO:0099061)	5,25E-02

Table 3. Summary of GO categories identified as significantly affected by hypergeometric annotation testing in PBMCs of IBD patients treated with thalidomide.

Data related to the differentially expressed genes that emerged from the POST vs PRE comparison were further analyzed by exploiting IPA database to understand the most significant biological interactions. The study identified a number of canonical pathways predicted as "altered" (Table 4).

Ingenuity Canonical Pathways	**-log (p-value)**
Natural killer cell signaling	4.77
Crosstalk between dendritic cells and natural killer cells	3.35
Eicosanoid signaling	3.04
Phagosome formation	3.03
Axonal guidance signaling	2.86
Neuroprotective role of THOP1 in Alzheimer's disease	2.67

Nicotine degradation II	2.24
Gαs Ssignaling	2.18

Table 4. Most significant canonical pathways identified as altered by IPA analysis

6.3 In vitro studies for mRNA sequencing data validation

To assess and validate the analysis of the transcriptome of IBD patients treated with thalidomide, in vitro experiments were performed on Thp-1 cell line, in particular to confirm some pathways that had emerged as altered from the hypergeometric test and IPA analysis.

6.3.1 Evaluation of the effect of thalidomide on the eicosanoid signaling: PGE2 quantification in Thp-1 cells

To demonstrate a possible involvement of the prostaglandin's pathway in the mechanism of action of thalidomide, PGE2 production was quantified in Thp-1 cell line and Thp-1 differentiated, as described previously, after treatment with thalidomide and LPS.

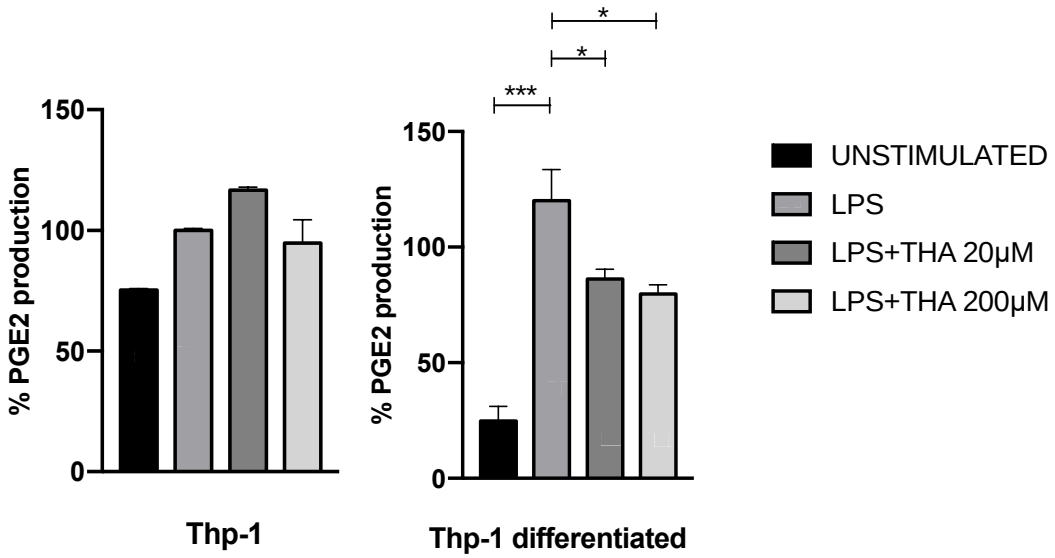


Fig. 4.5.1 PGE2 production. Evaluation of levels of PGE2 production in Thp-1 and Thp-1differentiated in macrophages, non stimulated (UNSTIMULATED) or stimulated with LPS (LPS) after 72 h of treatment with 20 μM (LPS +THA 20 μM) and 200 μM (LPS +THA 200 μM) thalidomide. The graphs represent the percentages of PGE2 production normalized on stimulated cells (LPS). One-way ANOVA: Thp1: $p>0.05$; Thp-1 differentiated $p=0.0001$. Tukey's multiple comparison *$p < 0.05$; ***$p<0.001$.

As shown in ig.4.5.1, thalidomide reduced the production of PGE2 after co-stimulation with LPS in Thp-1 differentiated in macrophages, whereas results obtained in monocytic Thp-1 cells

were not significant; in monocytes LPS did not cause an increase in PGE2 in comparison to unstimulated cells.

6.3.2 Evaluation of the effect of thalidomide on the adenylate cyclase-modulating G protein-coupled receptor signaling pathway: cAMP quantification in Thp-1 cells

To study the possible alteration by thalidomide in cAMP related signaling pathways, the production of cAMP was quantified in Thp-1 cells and in cells differentiated in macrophages, both treated with thalidomide and LPS.

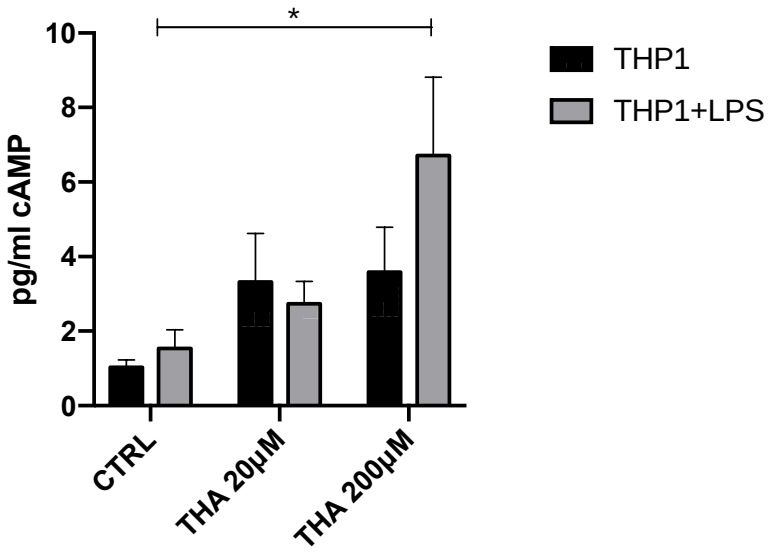


Fig. 4.5.2 Evaluation of levels of cAMP in Thp-1 stimulated or not with LPS after 72 h of treatment with 20 and 200 µm thalidomide (THA). Two-way ANOVA: MONO: p=0,01; Tukey's multiple comparison * $p < 0.05$

The results (Fig. 4.5.2) indicate that, in monocytes, thalidomide induces a significant increase in cAMP production, in particular in cells co-treated with LPS and 200 µM of thalidomide. Data obtained in macrophages are not shown because very low levels of cAMP were detected, out of the detection range of the ELISA kit used.

6.4 Neuro-related genes from sequencing analysis data

RNA-seq analysis identified 252 genes differentially expressed before and after thalidomide treatment (Table 2). The hypergeometric test (Table 2) revealed that some genes were found to belong to GO categories linked to functions and components of the nervous system. In particular, the most interesting GO categories emerged are: "synapse organization", "synaptic

membrane", "postsynaptic membrane", "glutamatergic synapse". From IPA analyses (Table 3), "Axonal Guidance Signaling pathway" resulted as one of the most altered pathways. The most relevant peripheral neuropathy-related genes are described in table 4.

GENE	Gene Title	FC	FDR p-value
ADAMTS2	A disintegrin and metalloproteinase with thrombospondin motifs 2	**-108**	**3.93E-11**
LAMB2	Laminin subunit beta-2	**4**	**2.40E-07**
MPP2	MAGUK p55 subfamily member 2	**-8**	**3.28E-06**
UNC13B	Protein unc-13 homolog B	**3,6**	**2.17E-06**
GNG7	Guanine nucleotide-binding protein G(I)/G(S)/G(O) subunit gamma-7	**-2.2**	**7.38E-05**
ADAMTS17	A disintegrin and metalloproteinase with thrombospondin motifs 17	**-3**	**4.70E-05**
CACNG6	Voltage-dependent calcium channel gamma-6 subunit	**5.9**	**3.24E-05**
NCAM1	Neural Cell Adhesion Molecule 1	**2.86**	**2.27E-05**
SEMA3F	Semaphorin-3F	**-7.8**	**8.63E-04**
PTPRS	Receptor-type tyrosine-protein phosphatase S	**-2.8**	**5.39E-04**
ABHD6	Abhydrolase Domain Containing 6	**-2.6**	**5.17E-04**
HIP1R	Huntingtin-interacting protein 1-related protein	**-2.1**	**4.40E-04**
GABRE	Gamma-aminobutyric acid receptor subunit epsilon	**11.04**	**4.09E-04**
NRG1	Pro-neuregulin-1, membrane-bound isoform	**2.7**	**4.09E-04**
NRCAM	Neuronal cell adhesion molecule	**3.2**	**2.04E-04**
SDK2	Protein sidekick-2	**-2.2**	**8.49E-03**
SLC1A3	Solute Carrier Family 1 Member 3	**2.31**	**7.05E-03**
CX3XR1	CX3C chemokine receptor 1	**3.2**	**6.80E-03**
SDC2	Syndecan-2	**-4.7**	**5.47E-03**
MRAS	Muscle RAS Oncogene Homolog	**-2.8**	**3.34E-03**
ACAN	Aggrecan core protein	**-9.9**	**2.42E-03**
NETO1	Neuropilin and tolloid-like protein 1	**-3.2**	**2.42E-03**
RGS9	Regulator of G-protein signaling 9	**2.4**	**1.83E-03**
CABP1	Calcium-binding protein 1	**6.27**	**1.53E-03**
CAMK2A	Calcium/calmodulin-dependent protein kinase type II subunit alpha	**-17.6**	**1.18E-03**
MMP14	Matrix metalloproteinase-14	**2.5**	**1.14E-03**

Table 4: List of neuro-related genes emerged from sequencing analysis

6.5 In vitro studies for validation of neuro-related genes and pathways altered from sequencing analysis data

To investigate the possible mechanism of action of thalidomide to induce peripheral neuropathy in IBD patients, sh-sy5y cells were used to study the effect of this drug on an in vitro cellular model with a neuronal phenotype.

6.5.1 Cytotoxicity of thalidomide in sh-sy5y cells and sh-sy5y cells differentiated

The cytotoxicity of thalidomide in vitro was evaluated both in sh-sy5y cells and in sh-sy5y differentiated, as described previously. The drug concentrations tested were 50, 100, 200 and 400 μM. For sh-sy5y cells, the exposure to the drug was performed using two different approaches: in the first case the drug was added only once followed by a 72 hours incubation, in the second case the drug was added every 24 hours for 3 days.

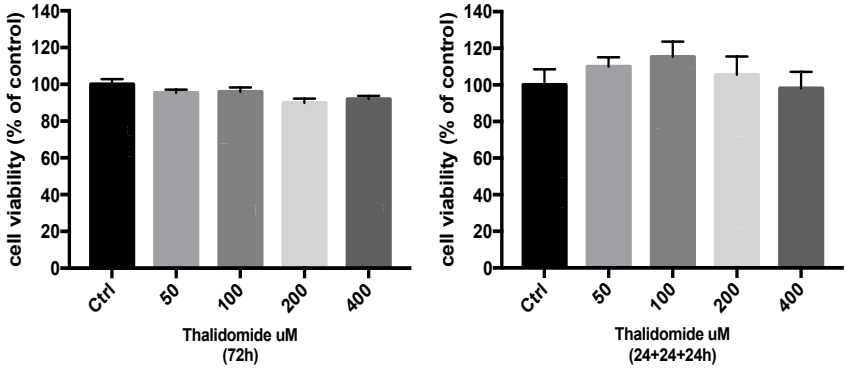


Figure 6.1 Evaluation of the cytotoxic effect of thalidomide using the MTT assay in sh-sy5y cells. Cells were treated once with thalidomide for 72 h or by adding the drug every 24 hours for 3 days (24 h + 24 h + 24 h). The percentages were calculated on the untreated cells (Ctrl). One-way ANOVA: $p > 0.05$. The data are reported as means ± SE of three independent experiments performed in quadruplicate.

The data reported in figure 6.1 show that thalidomide does not cause changes in viability in the cell models tested at all concentrations, in both experimental conditions.

To study thalidomide cytotoxic effect on differentiated sh-sy5y cells, the exposure to the drug was performed using two different ways: in the first case the drug was added after cell differentiation, and followed by a 72 hours drug incubation, in the second case the drug was added every 24 hours for 3 days, simultaneously to the addition of the stimulus to induce differentiation.

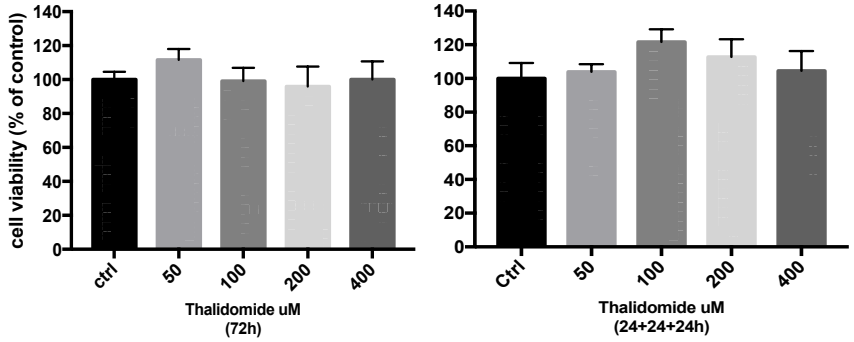


Figure 6.2 Evaluation of the cytotoxic effect in sh-sy5y differentiated with dbcAMP (1mM) using the MTT assay. The cells were treated with thalidomide for 72 hours and every 24 hours during the differentiation process (24h+24h+24 h). The percentages were calculated on the untreated cells (Ctrl). One-way ANOVA: $p > 0.05$. The data are reported as means ± SE of three independent experiments performed in triplicate.

Also, in this case, data (Fig 6.2) show that this drug does not cause changes in viability at all concentrations, suggesting another mechanism for its neurotoxic effect.

6.5.2 Mitochondrial membrane potential (MMP) and ROS quantification in sh-sy5y cells

To assess the effect of thalidomide on mitochondrial activity, MMP and ROS were measured in sh-sy5y cells treated for 6 and 72 hours with the drug. MMP was evaluated with JC-1, a membrane-permeant cationic probe exhibiting potential-dependent accumulation in the mitochondria.

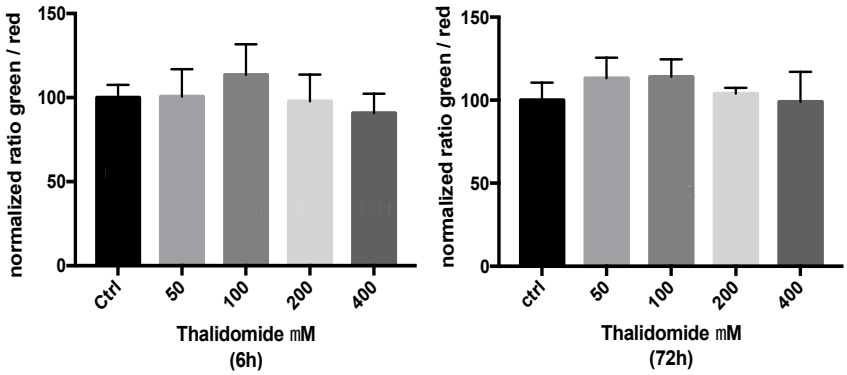


Figure 6.3 Effect of thalidomide on mitochondrial membrane potential (MMP) in sh-sy5y cell line using JC-1 dye after treatment with thalidomide (50- 400µM) for 6 and 72 hours. The pictures represent the ratios of JC-1 monomeric form to JC-1 aggregates (green/red fluorescence) for untreated (Ctrl) and treated cells, normalized on untreated cells. Statistical analyses with one-way ANOVA: $p > 0.05$. The data are reported as means ± SE of three independent experiments performed in quadruplicate.

As shown in Figure 6.3, thalidomide treatment for 6 or 72 hours resulted in no significant changes in MMP at all concentration tested.

The intracellular ROS levels were assayed by using CellRox dye as a fluorescence probe. As for MMP, no significant alteration in ROS production in this cell line was observed after treatments (Fig 6.4).

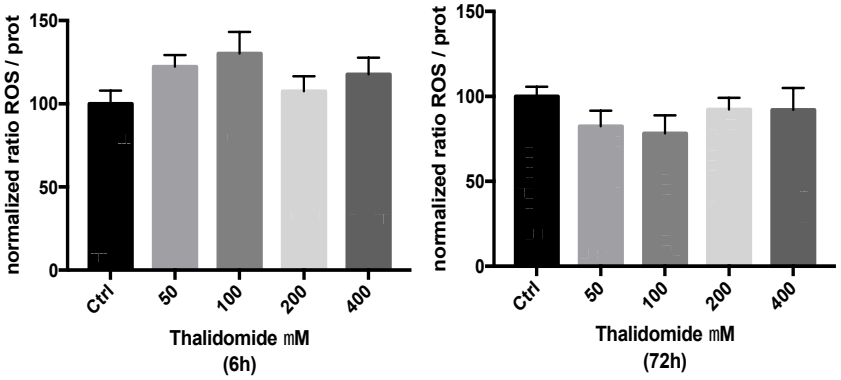


Figure 6.4 Quantification of ROS production in SH-SY5Y cell line after treatment with thalidomide (50-400µM) for 6 and 72 hours. The ratio between ROS and protein concentration was normalized on untreated cells (Ctrl). Statistical analyses with one-way ANOVA: p >0.05. The data are reported as means ± SE of three independent experiments performed in quadruplicate.

6.5.3 Adenosine triphosphate (ATP) quantification in sh-sy5y cell line

To analyze the possible effect of thalidomide treatment on ATP synthesis in sh-sy5y cells, ATP was then evaluated. As for MMP and ROS quantification, cells were incubated with thalidomide (50-400 µm) for 6 and 72 hours.

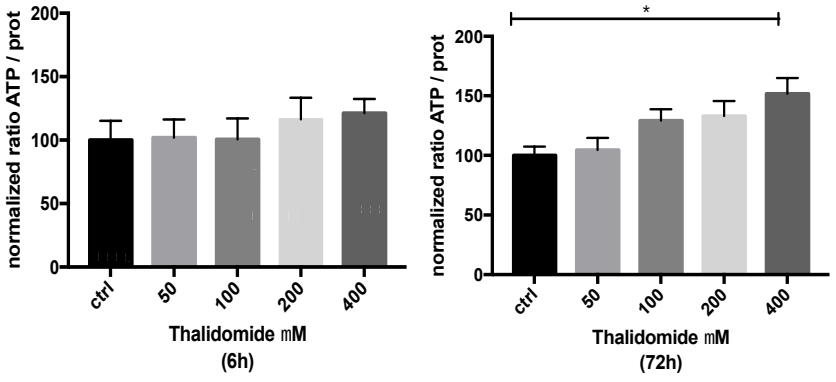


Figure 6.5 Quantification of ATP levels after treatment with thalidomide (50-400 µm) for 6 and 72 hours. One-way ANOVA: 72 h: p-value =0.023, Bonferroni post-test: 6 h: p-value>0.05; 72h: *p <0.05. The data are reported as means ± SE of four independent experiments performed in quadruplicate.

Data reported in figure 6.5 show that thalidomide induced a significant, although moderate increase in ATP production after the 72 hours treatment. For the short treatment, there was a similar but unsignificant trend.

6.5.4 Intracellular calcium quantification in sh-sy5y cell line

To monitor the changes in intracellular calcium, in particular the effect of the drug on calcium release from ER, the site of intracellular calcium storage, Fluo-4 AM was used as fluorescent probe. To quantify the release of calcium from ER, thapsigargin, a non-competitive inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA), was added in each well after 60 seconds from the start of fluorescence measuring, and followed by continuous fluorescence readings.

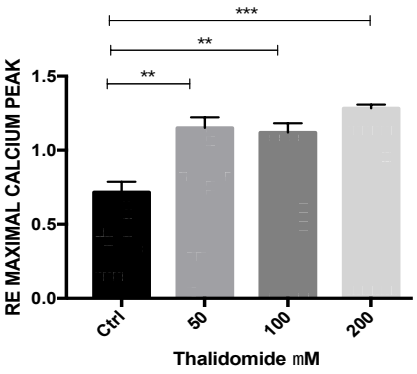


Figure 6.6 Quantification of intracellular calcium. Relative expression of calcium peaks in sh-sy5y cells treated with thalidomide (50-200 µM) for 72 hours. Maximal fluorescence levels were normalized on baseline readings (means of first 20 s readings). Untreated cells were used as controls. One-way ANOVA: $p<0.001$. Bonferroni post-test **$p<0.01$, ***$p<0.001$. The data are reported as means ± SE of three independent experiments performed in quadruplicate.

As reported in figure 6.6, a significant change in intracellular calcium levels upon thapsigargin stimulation was evident, with a marked increase of Ca^{2+} peaks at all thalidomide concentration used, suggesting an increase of calcium release from the ER induced by the drug.

6.5.5 Quantification of neuro-related gene expression levels in sh-sy5y cells

For the study and analysis of the expression of neuro-related genes in vitro, sh-sy5y cells were treated with thalidomide for 72 hours, in order to study the effects of the drug on the expression of the examined genes in a neuronal phenotype cell line. The expression levels of 6 neuro-related genes, selected as most relevant from the gene list previously described in table 4, were assessed

by RT-PCR. Genes selected were: ADAMTS2, CABP1, CACNG6, GABRE, NRCAM and SLC1A3.

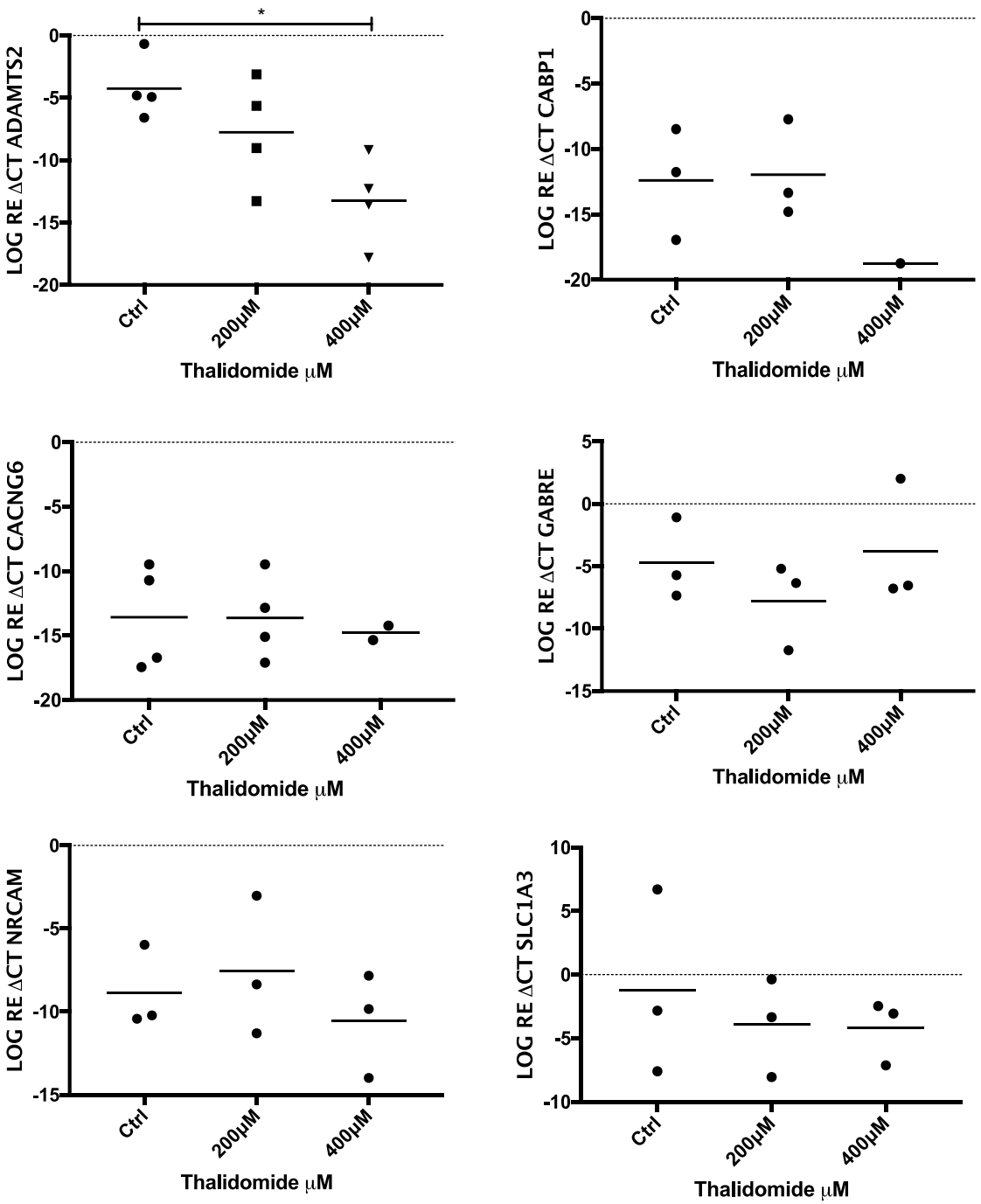


Figure 6.7 Quantification of gene expression levels in sh-sy5y treated with thalidomide. Gene expression was calculated with respect to the housekeeping beta-actin gene. Expression reported in Log2 of RE ΔCt. One-way ANOVA: ADAMTS2 p=0.019; Bonferroni's post-test * $p < 0.05$. One-way ANOVA: other genes $p>0.05$.

As reported in figure 6.7, thalidomide treatment significantly downregulated the expression of ADAMTS2 gene. Regarding the expression of the other genes, no significant changes following

treatment with thalidomide was observed, probably due to the high variability of gene expression through the experiments.

6.5.6 Differentiation marker assessment in sh-sy5y cells differentiated and immunofluorescence imaging analysis

To test whether this drug exerts an inhibitory effect on cell differentiation and on axonal outgrowth, expression of ß tubulin III was assessed both in immunoblot experiments and microscopic analysis of cells differentiated and treated with thalidomide."

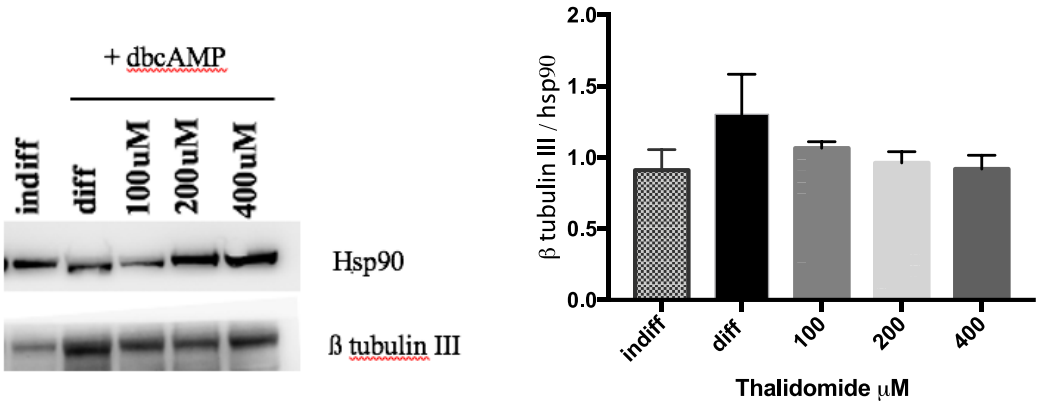


Figure 6.8. Beta tubulin III (55 kDa) protein expression in sh-sy5y cells analyzed by western blot. Protein lysates were obtained from sh-sy5y cells (indiff) and from cells differentiated (diff) with dbcAMP in co-treatment with thalidomide (100-400 μM). HSP90) was used as internal control. Representative western blot image of one of three replicates of the experiments reflecting protein levels.
Statistical analysis: One-way ANOVA: $p>0.05$.

Immunoblot results show that thalidomide treatment during the process of differentiation of this cell line seems to reduce the expression of ß tubulin III, but data were not significant.

To analyze and study the possible impact of thalidomide on neuritogenesis and differentiation, immunofluorescence images were collected in sh-sy5y cell differentiated and treated with thalidomide during the process of differentiation.

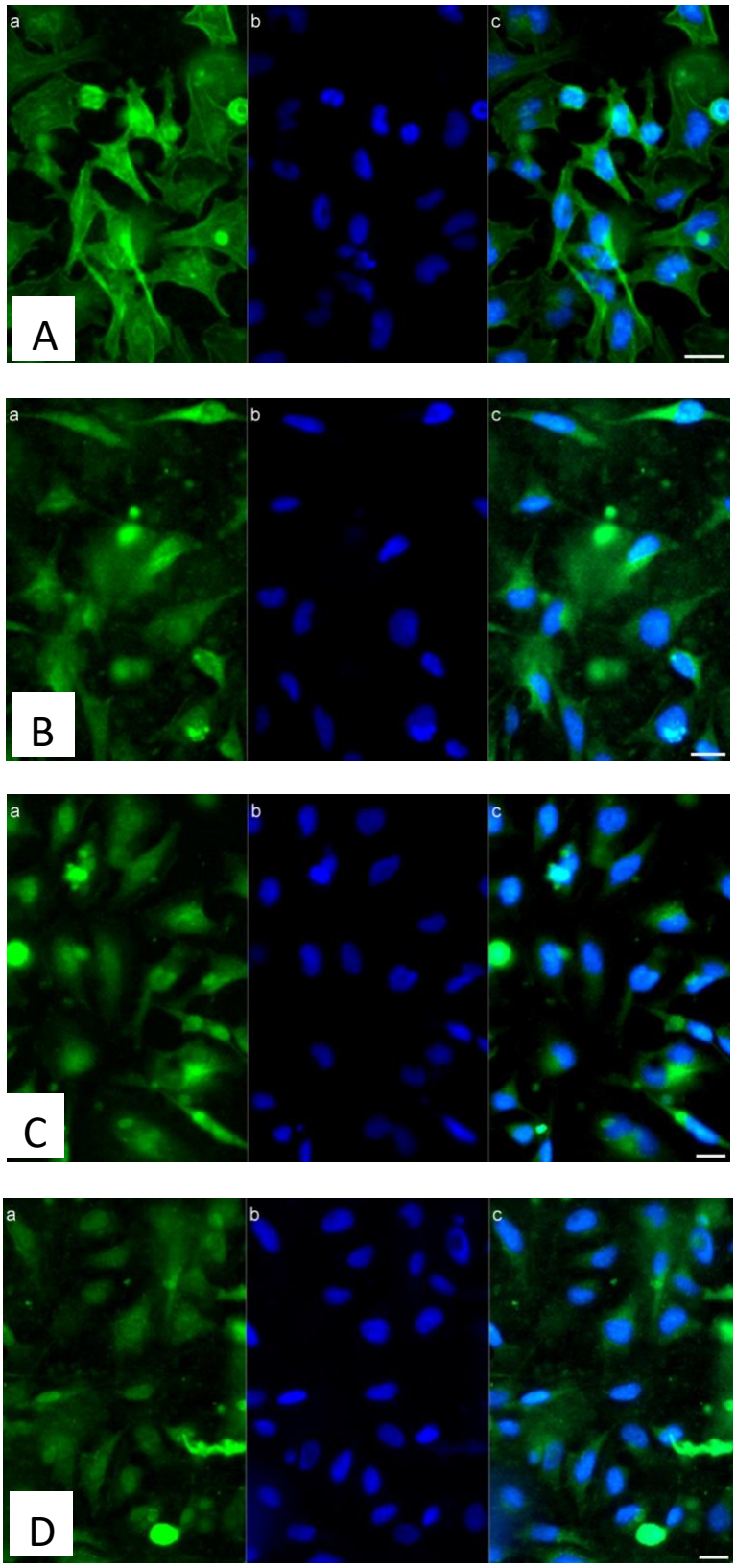


Figure 6.9 Representative images of immunohistochemistry of sh-y5y differentiated and treated with thalidomide. A) Differentiated cells; B) Differentiated cells treated with 100µM of thalidomide; C) Differentiated cells treated with 200µM; D) Differentiated cells treated with 400µM. In each panel (a) shows ß -tubulin staining while (b) DAPI and (c) both channels. Scale bar is 20 mM.

In figure 6.9 representative immunohistochemistry images of sh-sh5y differentiated and treated with thalidomide (100-400 µM) are shown. Panel A shows differentiated untreated sh-sy5y cells. It possible to observe an elongated, neuron-like morphology. Panel 2, 3 and 4 illustrates the effects of increasing doses of thalidomide, in which the signal of ß tubulin III seems to decrease.On the basis of immunohistochemistry results, a morphological analysis of cells was performed.

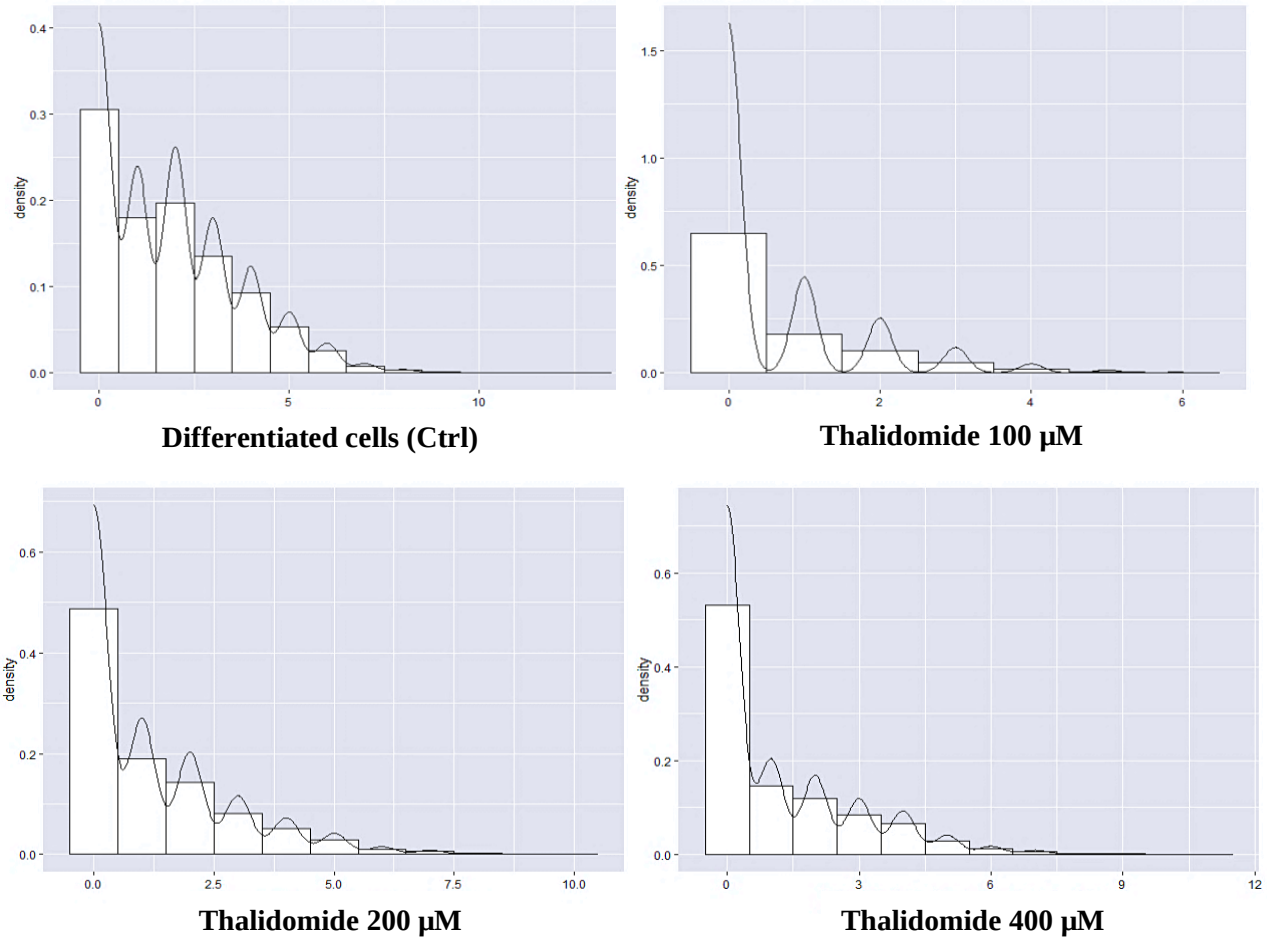


Fig 6.10 Morphological analysis of differentiated sh-sy5y cells and treated with thalidomide (100-400 µM). Graph represents the numbers of branch per cells, respect the cellular density. A dedicated software (CellProfiler) was used [130]. Representative analysis of one experiment.

As reported in figure 6.10 the number of cellular branches seems to be reduced after thalidomide treatment. In differentiated cells there were more branches per cells in comparison to the treated cells, in which the density of the number of branches per cell was very low.

7. DISCUSSION

-part 2

IBDs are a group of pathologies with highly variable characteristics; the cause of these diseases has been not yet fully understood but a multifactorial alteration has been hypothesized which includes genetic predisposition, impaired immune response and other external factors. The quality of life is strongly compromised in patients with IBD, particularly in pediatric patients, due to the chronic nature of the disease which involves frequent hospitalizations and aggressive therapies, with a significant risk of side effects. To date, a curative drug therapy for these diseases does not exist and the therapeutic approach is directed to the treatment and control of inflammation. A recent study has shown that thalidomide is very effective in children and adolescents with Crohn's disease and ulcerative colitis [127]. The molecular mechanisms underlying the immunomodulatory effects of thalidomide in IBD patients are unclear and published data derive mainly from studies conducted on patients with other diseases. In order to broaden the knowledge on the mechanisms of action of thalidomide, blood samples from pediatric patients with IBD refractory to standard therapies and treated with thalidomide were collected starting from 2016 and used for the study of transcriptome by NGS. The 10 patients included in this study were responders to thalidomide, a response that was defined on the basis of clinical scores at 3 months of treatment. The RNA extracted from PBMCs isolated before treatment and after 3 months of therapy was analyzed and 252 genes differentially expressed following treatment were identified. In order to give an interpretation of this biological effect, an integrative approach was used, the analysis with hypergeometric test, in search of the altered Gene Ontology (GO) terms in the subset of differentially expressed transcripts.

Among the most altered biological processes identified by the hypergeometric test, pathways related to the immunomodulatory effect of thalidomide emerged, as defense response, cell adhesion, inflammatory response, regulation of immune system process, regulation of cell migration, regulation of cell motility and immune system process. These biological processes have been already described in the literature as the principal pathways altered from thalidomide [147, 149].

Among the molecular functions most significantly affected were the activity of eicosanoid receptors and prostaglandins. Eicosanoids are derivatives of arachidonic acid, a fatty acid which is released from membrane phospholipids by enzymes belonging to the class of phospholipases A2 (PLA2). Through the action of the enzyme prostaglandin G/H synthase (PHS) prostaglandin G2 and subsequently H2 (PGH2) originates, which in turn is transformed by specific tissue isomerases in the various prostanoids, involved in many physiological functions, including the protection of the gastric mucosa, and in inflammatory processes. Prostaglandins (PGs) signal in an autocrine and/or paracrine manner through their distinct G protein–coupled receptors.

There is an evidence of the possible bioactivation of teratogenic compounds, including thalidomide metabolites, by PHS which would lead to the generation of reactive oxygen species (ROS) that could be involved in the teratogenic effect of thalidomide [150, 151]. In particular, the relevance of this mechanism has been evaluated in pregnant rabbits treated with thalidomide in co-treatment or not with acetylsalicylic acid, an inhibitor of PHS [151]. Treatment with acetylsalicylic acid was found to be embryo-protective, resulting in reduction in anomalies of fetal limbs caused by thalidomide.

Among the genes differentially expressed and belonging to the GO terms significantly altered following treatment with thalidomide, both an enzyme belonging to phospholipases A2 (PLA2G7) and 4 receptors for prostaglandins (PTGDR, PTGDR2, PTGER3, PTGFR) are upregulated, suggesting a potential involvement of this pathway in the mechanism of action of thalidomide. One of the hypotheses considered is that thalidomide, due to the activation process by PHS and the production of PGs, can trigger a positive feedback circuit that, interfering with PGs synthesis by PHS, could regulate the expression of prostaglandins receptors and therefore of the downstream signaling pathways, as described in endometrial adenocarcinoma cells [152]. To demonstrate the possible involvement of these pathways and genes differentially express in the mechanism of action of thalidomide, PGE2 production was quantified in Thp-1 cell line. In macrophages stimulated with a pro inflammatory stimulus, thalidomide treatment inhibited the secretion of PGE2 in a dose-dependent manner. PGE2 is present in most tissues at biologically functional nanomolar levels, and its levels are increased in sites of inflammation [153]. It is a critical molecule that regulates the activation, maturation, migration, and cytokine secretion of several immune cells, particularly those involved in innate immunity such as macrophages. Reduced levels of PGs were reported in mucosal tissue from UC patients after treatment with sulfasalazine, sulfapyridine, and 5-aminosalicylic acid by 34, 32, and 62%, respectively [154]. Thalidomide may reduce levels of PGE2, through the modulation of PHS activity, as described in previous works [152, 155], proving its therapeutic effect in IBD patients. In addition, the modulating G protein-coupled receptor (GPCR) pathway emerged from the enrichment analysis as one of the most significantly altered. Many drugs act on GPCRs and produce their effects by increasing or decreasing the activity of adenylate cyclase, thereby modulating the production of cyclic adenosine monophosphate (cAMP). cAMP is a potent regulator of innate and adaptive immune cell functions [156]. To demonstrate the possible alteration by thalidomide in cAMP related signaling pathways, the production of cAMP was quantified in Thp-1 cells. Thalidomide led to a significant increase in cAMP levels in monocytes stimulated with LPS. It is known that an increment in cAMP levels leads to a consequent

activation of phospholipase C (PKA). This enzyme regulates the production of inflammatory mediators involved in the inflammatory process of IBD through the phosphorylation of the transcription factor cAMP response element binding protein (CREB), which inhibits the expression of NF- κB [157]. It will therefore be important to investigate whether in these cells, after treatment with thalidomide, an activation and phosphorylation of CREB, is induced.

Moreover, thalidomide could induce an alteration of the activity of adenylate cyclase, or an inhibition of phosphodiesterase (PDE), the enzymes that convert cAMP to AMP, causing an increase in cAMP. This mechanism is reminiscent of that of apremilast, sildenafil and derivatives, and analogues of thalidomide [158]. Munoz-Perez et colleagues in their work described the increase in cAMP levels in the rat uterus caused by two thalidomide analogs, acting as PDE inhibitors [159]. In the future, further experiments will be carried out to confirm this data.

In addition to the pathways and genes analyzed related to the immunomodulatory mechanism of thalidomide, the hypergeometric test revealed some genes belonging to GO categories linked to functions and components of the nervous system. "Axonal Guidance Signaling pathway" resulted also as one of the most altered pathways. Peripheral neuropathy is one of the most frequent adverse events of thalidomide and is a common cause of treatment discontinuation [119]. Looking to all genes belonging to these categories, the main mechanisms that seem to emerge were changes in intracellular calcium levels, alteration in glutamate signaling, impaired activation of glial cells, and alteration in axonal guidance signaling-related genes.

In order to better define a pathophysiological mechanism of this complication in patients treated with thalidomide and to validate, in a neuronal cell model, genes and pathways emerged from transcriptome analysis, sh-sy5y cells were used to study the effect of this drug in vitro. The cytotoxicity data on undifferentiated sh-sy5y and cells differentiated in a more neuronal phenotype cell indicated that thalidomide did not induce a negative effect on cell viability in sh-sy5y cells following a continuous treatment of 72 hours or repetitive treatments every 24 hours. Moreover, the same result was obtained in differentiated cells, treated at the end of differentiation process for 24 hours or during this process for 3 days. These results were in agreement with some paper already published in other cellular models, in which thalidomide does not appear cytotoxic on leukocytes and monocytes exposed to the drug for 18 hours [160]. A contrasting finding is that reported in a study conducted on induced pluripotent stem cells; after a single treatment for 96 hours no reduction in cell viability was observed, whereas the change of medium with drug every 48 hours induced a significant reduction in cell viability

[161]. These MTT data demonstrated that thalidomide is not cytotoxic in these cellular models, suggesting another mechanism of action in the neurotoxicity.

In the literature, the mechanisms by which drugs as chemotherapeutics lead to a peripheral neuropathy has been largely described and involves principally oxidative stress, alteration in ion channel activity, neuroinflammation, and mitochondrial damage [162]. Therefore, mitochondrial function in terms of MMP, ROS production, ATP synthesis and intracellular calcium levels were measured in sh-sh5y cells treated with the drug. In line with results obtained from cytotoxicity analysis, thalidomide treatment didn't cause an alteration in MMP or ROS production.

Thalidomide caused a significant increase in ATP production following a 72 hours of cell treatment. This alteration in intracellular ATP levels could be indicative of the activation of multiple signaling pathways. One of the hypotheses, is that this ATP increment could be linked to an alteration of the ubiquitination process, an ATP-dependent mechanism, caused by the biding of thalidomide with its ligand CRBN, which affects the ubiquitination and degradation process of some proteins [109]. Moreover in literature, it was reported that the concentration of ATP involves changes in the specificity of the protein substrates of the proteasome allowing the cell to rapidly regulate the activity of the proteasome under stress conditions [163]. The alteration of ubiquitination proteasomal activity, due to the binding of thalidomide with CRBN, could induce an increase in ATP levels as a feedback loop mechanism to maintain proper protein degradation.

Intracellular calcium (Ca^{2+}) was measured in sh-sy5y cells treated with thalidomide for 72 hours. The results demonstrated a significant increase of calcium release from RE after thapsigargin stimulation, an inhibitor of sarco(endo) plasmic reticulum Ca^{2+} ATP-ase (SERCA), underlying an effect of thalidomide on calcium homeostasis. In the literature, the effect of thalidomide on intracellular calcium levels is currently poorly studied, in particular in neuronal cell models. Abnormal neuronal calcium homeostasis has been implicated in numerous diseases of the nervous system, including peripheral neuropathies induced by chemotherapy [164]. A dysregulation of calcium homeostasis and signaling pathways, inducing apoptotic changes in peripheral nerves, was described for oxaliplatin and cisplatin, as a consequence of an increase ROS production [165]. The authors described that an increase of intracellular Ca^{2+}concentration may result in calpain, a potent protease, activation which leads to unregulated proteolysis, directly triggering axon degeneration [166]. Moreover a dysregulation of intracellular Ca^{2+} was observed in a peripheral neuropathy model caused by paclitaxel, causing the release of Ca^{2+} from mitochondria, and from the ER [165]. In this work, authors hypothesized that a taxol

binding protein, neuronal Ca^{2+}sensor 1 (NCS-1), interacts directly with IP3R, causing release of Ca^{2+} and consequent cytosolic Ca^{2+}alterations. Future experiment will performed to understand and investigate this Ca^{2+} increment after thalidomide treatment in this cells.

From IPA analysis, Axonal Guidance Signaling pathway emerged as one of the most altered pathways. Axon guidance, also called axon pathfinding, is a subfield of neuronal development, concerning the process by which neurons send out axons to reach their correct targets. Axons need to be properly guided to their targets to form synaptic connections, and this requires interactions between extracellular and transmembrane ligands and their cell surface receptors. [167]. The mechanisms of axonal guidance signaling were not only related to developmental mechanisms, but they have also been associated with neuronal regeneration mechanisms [168]. To date, there are few studies in literature that analyze the alteration of axonal guidance pathways linked to peripheral neuropathy, and they were principally focused on the mechanism of axonal degeneration [168]. In the analysis of expression levels of genes associated with the nervous system and pathways, ADAMTS2 gene emerged as significantly altered from thalidomide treatment. This gene was down regulated in sh-sh5y cells after drug treatment, confirming the trend emerged from patients. ADAMTS2 (ADAM Metallopeptidase With Thrombospondin Type 1 Motif 2) is a secreted metalloproteinase which excises the N-propeptide of the fibrillar procollagens types I-III and type V [169]. This gene seems to have a role also in the signaling of Netrin, which is one of the axonal chemoattractant cues, altering the expression on plasma membrane of its receptor [170]. In a recent study Ruso-Julve et al. investigated the mechanisms implicated in the regulation of gene expression of ADAMTS2 in schizophrenia and psychotic disorders, reporting that the expression of this gene was activated by a dopaminergic signaling, which in turn activated the downstream signaling of cAMP/CREB pathway [171]. The other genes analyzed seems to be not altered following thalidomide treatment in this in vitro model. It will therefore be important to modify the time of treatment of cells, and then to identify other cellular models to validate the expression of these genes.

In order to evaluated the possible effects in the mechanisms of neuronal differentiation and neurite formation (neuritogenesis), linking to the axonal guidance pathways, preliminary studies were conducted on sh-sy5y differentiated, analyzing the expression of β-tubulin III, which has been considered a neuron-specific marker molecule of differentiation [172]. The immunoblot result indicated that the thalidomide treatment during the process of differentiation seems not to significantly influence the expression of this protein. Future experiments will be necessary to confirm this result. In the literature, recent studies have demonstrated the effect of some chemotherapy drugs, for example cisplatin, on neurite growth and differentiation, leading to

neuron degeneration [173]. Preliminary data obtained from immunohistochemistry images analysis allowed us to assume that thalidomide could influence the mechanisms of neurite growth, confirming data emerged from IPA analysis, in which Axonal Guidance pathway was reported as one of the most altered pathways from RNA-seq analysis. Therefore, thalidomide treatment could modulate basal mechanisms of neurites development, causing a damage on peripheral neurons, altering for example the structure of cytoskeleton, fundamental for axonal transport and neurotransmission [174]. However, these data and hypotheses will be confirmed in future experiments on this in vitro model.

8. CONCLUSIONS

In conclusion, the first part of this study demonstrated:

- data obtained on biopsy tissues of IBD pediatric patients demonstrates that the levels of MMP-2 and MMP-9, are significantly higher in the inflamed sites than in the non-inflamed ones while the levels of the lncRNA GAS5 are significantly lower, suggesting that GAS5 may play a role in the pathogenesis process of tissue damage, regulating MMPs, and therefore in determining a chronic inflammatory state;
- in vitro experiments on Thp-1 cells confirmed the trend observed in patients for the three genes: the stimulation with a pro inflammatory stimulus, LPS, promoted a downregulation of GAS5 and an increase of the two MMPs;
- GAS5 overexpression experiments showed that higher levels of the lncRNA lead to a decrease of both enzymes, in terms of gene and protein expression, confirm the role of GAS5 in the modulation of the expression of the two proteases;
- RNA-IP experiment highlighted that this lncRNA was able to bind and interact physically with NF-κB probably modulating its transcriptional activity, demonstrating the involvement of this lncRNA in inflammation and confirming its role as biomarker of inflammation.

The second part of this thesis project demonstrated:

- transcriptome analyses performed on blood samples from 10 IBD pediatric patients refractory to standard therapies and treated with thalidomide, revealed 252 differentially expressed genes emerged following treatment with thalidomide;
- the hypergeometric test on gene ontology (GO) annotations and IPA analysis, revealed a series of altered pathways implicated in the mechanism of action of thalidomide and also pathways related to the nervous system, suggesting new genetic determinants involved in peripheral neuropathy induced by thalidomide;
- to validate transcriptomic results, preliminary in vitro experiments were conducted on Thp-1 cells, showing that thalidomide could be involved in prostaglandin signaling pathways and G protein-coupled adenylate cyclase pathway;
- studies on sh-sy5y cell line reported that thalidomide did not seem to have a cytotoxic effect, nor an action on mitochondrial functions and activity, but an increase in ATP levels and intracellular calcium were observed;
- preliminary data obtained on differentiated sh-sy5y cells, suggest a potential role of this drug on neuronal differentiation mechanism, including neurite formation.

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