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

"BIOSENSOR DEVELOPMENT AND GENOMIC ANALYSIS FOR THE STUDY OF THE INFLIXIMAB RESPONSE IN PEDIATRIC INFLAMMATORY BOWEL DISEASE PATIENTS."

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INDEX

ABSTRACT

Pharmacological therapy of inflammatory bowel disease (IBD), and in particular the use of antibodies against tumor necrosis factor alpha (TNFα), such as infliximab and adalimumab, has led to a revolution in the treatment of IBD thanks to their capability to induce and maintain clinical remission. Treatment with anti-TNFα agents is successful in a majority of patients with IBD, yet it can fail in a proportion of patients leading to loss of response during the induction phase (10-20%) or over time (up to 45%) and to the development of adverse drug reactions (20%). There is increasing evidence suggesting that treatment failure may be associated with inadequate blood drug levels, the appearance of anti-drug antibodies and/or the presence of genetic variants. Studying the mechanism underlying this inter-individual variability becomes an important research goal to improve clinical practice.

In this context, this thesis has demonstrated:

- ✓ the utility of point of care as reliable option for real-time therapeutic drug monitoring of infliximab in children, showing a good agreement with traditional ELISA assays;
- ✓ encouraging preliminary results of AFM methodology for infliximab monitoring in sera of pediatric IBD patients. A good correlation was found between signal height variation and infliximab concentration and this technique can be exploited in the future, thanks to its multiplexing capability, to dose infliximab and anti-drug antibodies at the same time;
- ✓ the use of therapeutic drug monitoring to predict the efficacy of anti–TNFα agents in order to optimize treatment and minimize side effects. Early treatment modification can avoid complications: higher adalimumab levels during early treatment obtained from non–trough level serum samples predict long-term remission in children with IBD;
- ✓ the role of *FCGR3A* SNP in pediatric IBD patients: this SNP seems to affect infliximab response and influence anti-drug antibodies production susceptibility; these data support the utility of genotyping candidate genes to predict infliximab response in children with IBD, resulting in more cost-effective and safe therapies;
- ✓ the validity of Jurkat CD4+ T cells in vitro model for the study infliximab mechanism of action;

- ✓ the role of CD69 as marker of infliximab response.

RIASSUNTO

La terapia farmacologica delle malattie infiammatorie croniche intestinali (MICI), ed in particolare l'uso di anticorpi monoclonali contro il fattore di necrosi tumorale alfa (TNFα), come ad esempio l'infliximab e l'adalimumab, ha completamente rivoluzionato il trattamento delle MICI, grazie alla loro capacità di indurre e mantenere la remissione clinica. Il trattamento con agenti anti-TNFα ha successo nella maggior parte dei pazienti affetti da MICI, ma una percentuale di pazienti va incontro ad una perdita di risposta durante l'induzione (10-20%) o nelle fasi più tardive del trattamento (fino al 45%) e allo sviluppo di reazioni avverse al farmaco (20%). Vi sono prove crescenti che suggeriscono che il fallimento del trattamento possa essere associato a livelli di farmaco nel sangue inadeguati, alla comparsa di anticorpi anti-farmaco e/o alla presenza di varianti genetiche. Lo studio dei meccanismi alla base di questa variabilità interindividuale diventa quindi un obiettivo importante nella pratica clinica, con lo scopo di migliorare gli *outcomes* clinici e ottenere una terapia personalizzata, *ad hoc* per il paziente.

In questo contesto, questa tesi ha dimostrato:

- ✓ l'utilità del *point of care* come alternativa ai saggi ELISA, per il monitoraggio terapeutico dell'infliximab nei pazienti pediatrici affetti da MICI;
- ✓ incoraggianti risultati preliminari nell'utilizzo dell'AFM come metodica innovativa per il monitoraggio dell'infliximab nei sieri di pazienti pediatrici con MICI. È stata infatti dimostrata una buona correlazione tra la variazione di altezza e la concentrazione di infliximab permettendo così di sfruttare in futuro questa tecnica, grazie alla sua capacità di analisi *multiplexing*, per dosare contemporaneamente infliximab e anticorpi anti-farmaco;
- ✓ una correlazione tra livelli più elevati di adalimumab durante l'induzione e la risposta clinica a lungo termine nei pazienti pediatrici affetti da MICI;
- ✓ il ruolo del polimorfismo nel gene *FCGR3A* (rs396991) nei pazienti pediatrici con MICI: questo polimorfismo sembra influenzare la risposta all'infliximab e la suscettibilità alla produzione di anticorpi anti-farmaco. Questi dati supportano l'utilità della genotipizzazione di geni candidati per predire la risposta all'infliximab, riducendo i costi delle terapie ed aumentando l'efficacia del trattamento;

- ✓ la validità del modello in vitro di linfociti T CD4+ (Jurkat) per lo studio del meccanismo d'azione dell’infliximab;
- ✓ il ruolo del CD69 come marker di risposta all’infliximab.

1. INTRODUCTION

1.1 Inflammatory bowel disease

Inflammatory bowel disease (IBD) are complex disorders characterized by a chronic inflammation of the gastrointestinal tract and include Crohn's disease (CD) and ulcerative colitis (UC). These disorders have a progressive evolution, resulting in organ damage and heterogeneous manifestations [1]. Clinical features are similar between children and adults but pediatric onset disease is often characterized by a more aggressive course, impairment of growth and delayed puberty [2]. In less than 20% of IBD patients it is difficult to distinguish between CD and UC and to make a correct diagnosis, so the term indeterminate colitis (IC) is used. IC is characterized by features typical for both CD and UC but, despite the similarities with the two main IBD diseases, IC presents a different clinical course and prognosis. Patients diagnosed with IC are usually children and present extensive ulcers involving the colon [3].

From the 21st century, IBD has become a global disease with increasing incidence in newly industrialised countries (Africa, Asia and South America). However, the highest incidence (0.3%) has been reported in Europe and North America [4]. The incidence of IBD in the pediatric age has been increasing and approximately 25% of patients develop the disease before the age of 20 years. Among children, in 4% of cases the disease starts before the age of 5 years and in 18% before the age of 10 years, with the peak incidence in adolescence. In addition, pediatric onset disease is often characterized by aggressive course, with extensive intestinal involvement and rapid clinical progression [2].

Clinically, CD and UC present similar symptoms (diarrhea, hematochezia, and abdominal pain), whereas the location and depth of inflammation can be different. Extraintestinal manifestations (EIMs) are common in CD patients and can affect the dermatologic, musculoskeletal, hepatic, ocular or hematologic systems. Other organs and systems, such as kidneys, pancreas, lungs and venous system are less commonly affected [5].

CD may involve the whole gastrointestinal tract and the inflammation extends through the entire thickness of the bowel wall from the mucosa to the serosa (transmural inflammation). CD can progress from an initially mild to moderate and severe penetrating condition with several relapses and complications such as fistulae and intra-abdominal or perianal abscess formation [6]. On the contrary, UC is characterized by a continuous mucosal inflammation extending from the rectum to the more proximal colon. UC results in diffuse friability and superficial erosions on the colonic wall associated with bleeding [7] [Figure 1].

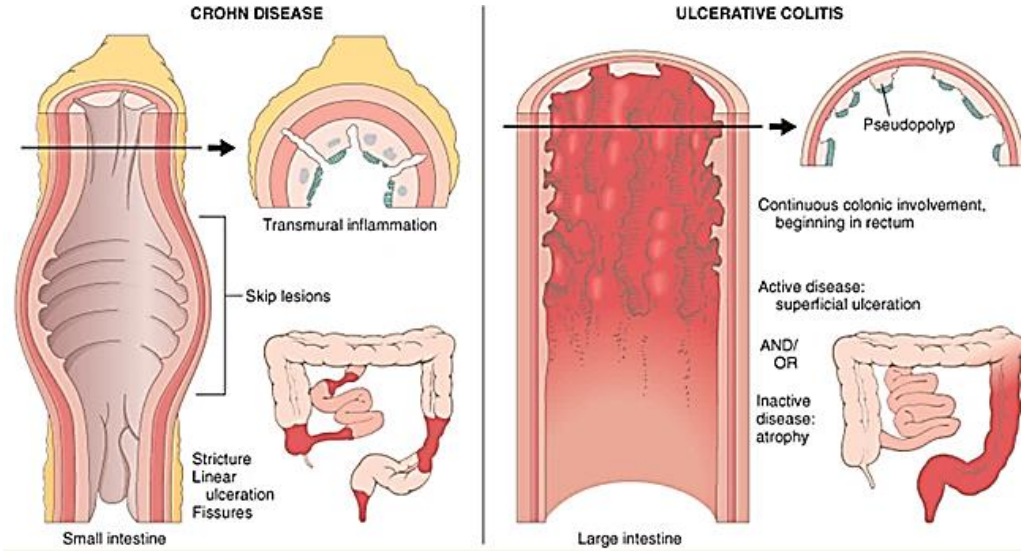

Figure 1: *Comparison between Crohn disease and ulcerative colitis [https://www.hopkinsmedicine.org/]*

Endoscopy and colonoscopy are currently used for the differential diagnosis of CD and UC; however, these are invasive and expensive test, which require considerable patient preparation. There are also the non-invasive routine laboratory investigations that currently include blood testing for C-reactive protein, albumin, transaminases and erythrocyte sedimentation rate in addition to fecal testing for calprotectin and lactoferrin. These investigations permit only to identify a systemic inflammation and are complementary to the invasive tests used for IBD discrimination [8].

Despite the increasing evidences, the mechanisms underlying the IBD pathogenesis are not fully understood. However, recent studies indicated an involvement of various factors such as genetic susceptibility, external environment, and intestinal dysbiosis, contributing together to the development of the disease [9].

1.2 Immune system and IBD

A combination of genetic and environmental factors has a crucial role in the pathogenesis of IBD, leading to an alteration of the epithelial barrier. The immunopathogenic mechanism in IBD is thought to be due to the initial activation of the innate immune system causing a non-specific response, followed by the upregulation and maintenance of this inflammation by activation of the adaptive immune system, involving various mucosal immune cells, such as macrophages, T cells and innate lymphoid cells that can promote chronic inflammation of the gastrointestinal tract [10].

Recent studies have demonstrated that the proportions of circulating activated T-helper (Th) CD4+ and cytotoxic CD8+ lymphocytes are increased in patients with IBD and are related to plasma concentration of pro-inflammatory cytokines [11]. CD69, a membrane receptor transiently expressed on activated lymphocytes at inflammatory sites is increased during inflammation, inducing several signalling cascades, including nuclear factor-kappa B (NF-κB) pathway, that enhances the release of pro-inflammatory cytokines that induce and maintain the expression of this activator marker [12].

Cytokines drive intestinal inflammation inducing intestinal stenosis, rectal bleeding, abscess and fistula formation [13]. The CD4+ T cells have been identified as important drivers of inflammation in IBD. Upon recognition of their antigen presented by antigen presenting cells (APCs), naïve CD4 + T cells can differentiate into several distinct Th cell types: Th1, described as important cell subset in the pathogenesis of CD, Th2, reported to be more involved in mediating UC inflammation, Th17 and inducible regulatory T (iTreg) cells. These T cell subsets can be characterized by their specific transcription factor (T-bet/Stat4, GATA-3/Stat5, RORγt/Stat3 and Foxp3/Stat5 for Th1, Th2, Th17 and iTreg respectively) and their cytokine profiles (IFN, IL6 and TNFα for Th1, IL4, IL5 and IL13 for Th2, IL17 for Th17 and IL10 for iTreg) [14] [Figure 2].

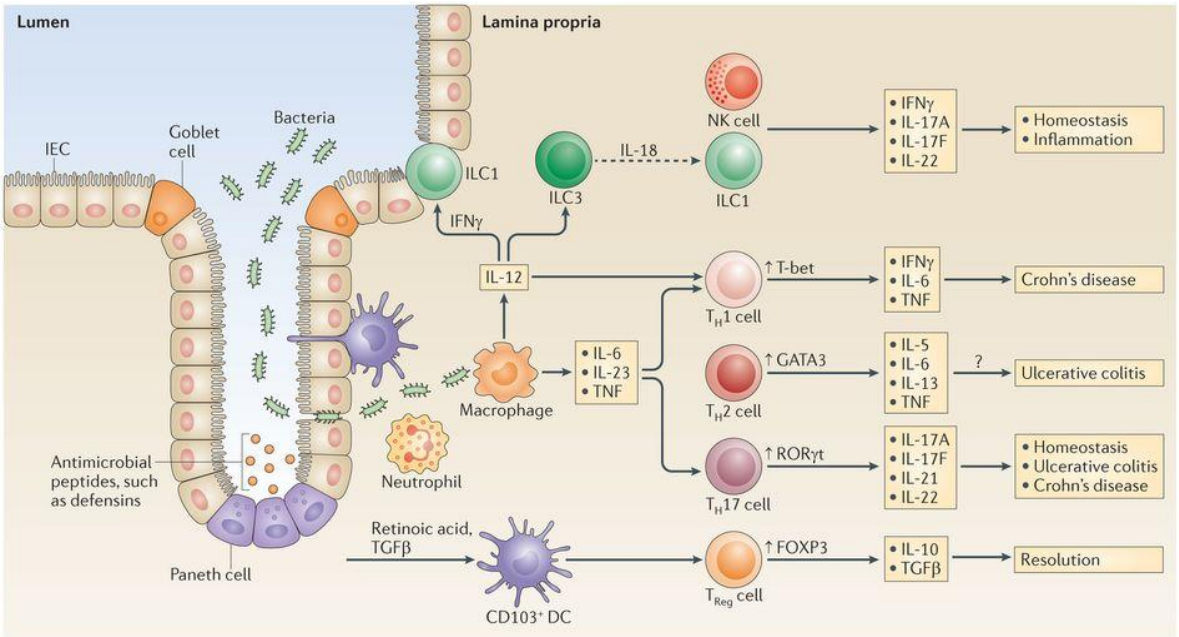

Figure 2: *Cytokines in IBD pathogenesis [15]*

A pivotal component of Th1 mediated colitis is TNFα. TNFα has been proposed as a central player in inflammatory cell activation and recruitment and is suggested to play a critical role in the development of IBD. This pro-inflammatory cytokine is produced as a 26

kDa transmembrane protein precursor (mTNFα). Cleavage of mTNFα by TNFα-converting enzyme (TACE) results in release of a 17 kDa soluble form (sTNFα). The mTNFα and sTNFα are biologically active and preferably interact with TNF receptor (TNFR)1 and TNFR2 respectively. TNFR1 is ubiquitously expressed on several different cell types, whereas the expression of TNFR2 is highly regulated by different stimuli and more restricted in certain cell types (immune, neuronal or endothelial cells). TNFR1 pathway involves caspase (CASP)-dependent death signalling and pro-survival NF-κB activation while TNFR2 activates exclusively pro-inflammatory and pro-survival signalling pathways [16, 17]. Several studies have demonstrated that TNFα is increased in the blood and stool of IBD patients, showing a large number of TNFα-producing cells in the intestinal mucosa [18, 19]. These findings have identified the TNFα as pharmacological targets for IBD treatment.

1.2.1 The role of CD69 in IBD

CD69 is a disulphide-linked homodimer protein composed by two glycosylated subunits. Each subunit presents an extracellular C-type lectin domain (CTLD) linked to a transmembrane region followed by a short cytoplasmic tail [20]. The *CD69* gene is located in the natural killer (NK) gene complex, in chromosome 12. The sequence contains a TATA element with a number of putative binding sites for inducible transcription factors such as NF-κB and activation protein-1(AP-1), involved in the control of *CD69* gene expression [20]. CD69 is commonly used as marker of activated cells and is expressed on infiltrated leukocytes at inflammatory sites in several chronic inflammatory disorders such as rheumatoid arthritis and systemic lupus erythematosus [21, 22]. The cytoplasmic tail of CD69 is able to activate different intracellular signals through Janus kinase (JAK)-2 and 3, which then activate the transcriptional factor STAT3 and 5 permitting to balance the T cell differentiation towards regulatory T cells or Th profile, underling the important role of CD69 in immune regulation. Furthermore, the JAK/STAT signalling pathway regulates the production of interferons and interleukins such as IL-2, IL-6 and TNFα, key drivers of inflammatory process.

The role of CD69 remains ambivalent because it is induced by pro-inflammatory molecules but upregulation of CD69 leads to a decreased migration of activated CD4+ T cells to the intestine and to an increased regulatory response which attenuates inflammation [23].

1.3 Therapeutic approaches in IBD

Consensus guidelines for IBD management suggest the "step-up" approach with initial immunosuppressive treatment (corticosteroid and steroid) before starting anti-TNFα agents for inducing remission and controlling inflammation. In particular, the use of 5-aminosalicylate compounds, followed by steroids and then an immunomodulator such as 6-mercaptopurine (6-MP), azathioprine (AZA) or methotrexate (MTX) are adopted in those individuals with rapid disease recurrence once steroids are withdrawn [24, 25].

While the immunomodulators are generally not effective in inducing remission in IBD, thiopurines (6-MP and AZA) have proven to be effective for the maintenance of response and remission in IBD patients; as well as the acid folic analogue MTX [26]. However, the several side effects and inability of long-term remission phase maintenance created a need for the novel treatment strategies.

Several studies concerning the role of cytokines in intestinal inflammation have identified key drivers of disease such as TNFα, IL12, IL23 and the α4β7 integrin [27]. The introduction of biologic antibodies able to target these cytokines have provided therapeutic benefits to IBD patients, achieving a good clinical response and mucosal healing [28, 29]. For this reason, in recent years there has been a shift towards the "top-down" approach, starting with the use of monoclonal antibodies, especially in severe disease phenotypes and this appears to be a better solution for the maintenance of remission [30, 31] [Figure 3].

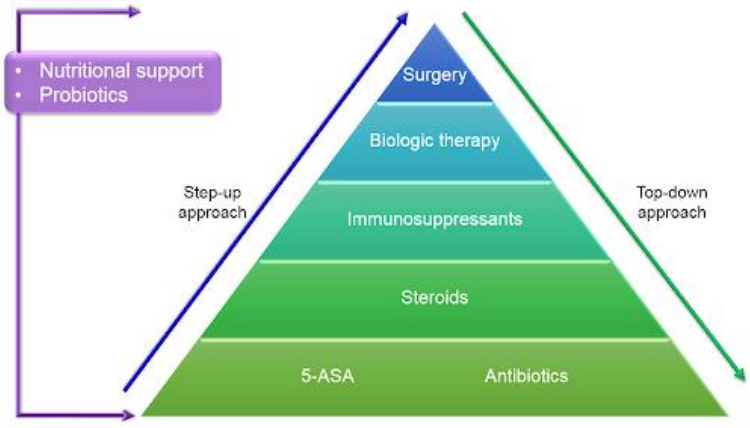

Figure 3: *Step-up versus Top-down approach in IBD [32].*

1.3.1 Anti-TNFα therapy

The advent of biological therapies, in particular the anti-TNFα agents, has changed the management of IBD in particular in patients with severe disease activity and refractoriness to standard therapies. These therapies have improved quality of life, decreased hospitalization and reduced side effects caused by the use of corticosteroids and immunomodulators [33].

Biologicals that are currently approved to treat IBD are: infliximab, adalimumab, golimumab and certolizumab-pegol. However, in pediatric IBD patients, infliximab and adalimumab are the only biologicals approved by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) [34].

All anti-TNFα agents are monoclonal antibodies (mAbs) of the immunoglobulin G1 (IgG1) type and consist of a F(ab') fragment and a Fc-portion. The former is able to form a complex with TNFα and the latter activates Fc-receptor mediated complement-dependent cytotoxicity (CDC) and antibody-dependent cell cytotoxicity (ADCC). These mechanisms limit cellular functions, such as cell activation, immune regulation and cytokine and chemokine production [29].

Infliximab is a chimeric mAb with the variable regions of a mouse anti-human TNFα mAb fused with the constant region of a human IgG1 and was the first biological therapy approved for IBD. Infliximab has a half-life of approximately 14 days, is administered intravenously at 5 mg/kg given in a 0, 2, and 6 weeks induction regimen followed by a maintenance regimen with infusions every 8 weeks.

Adalimumab is a fully humanised monoclonal immunoglobulin G1, effective in inducing and maintaining remission in children with CD and is used off-label as second-line therapy in UC. Adalimumab is given subcutaneously on days 1 and 15 at 160 mg and 80 mg for body weight ≥40 kg, or 80 mg and 40 mg in case of body weight less than 40 kg for induction, followed by every other week maintenance dose of 40 or 20 mg.

Certolizumab pegol comprises the Fab portion of the antibody conjugated to polyethylene glycol, and in some countries is used off-label for pediatric IBD treatment. It has been shown to be effective in reducing symptoms of moderately to severely active CD in adult patients who not response to conventional therapy. Golimumab is a fully human mAb and

acts blocking soluble and transmembrane TNFα. This agent is approved for treatment of moderate to severe UC in adults but in children is only available off-label [35].

1.3.2 Anti-TNFα mechanism of action

Although anti-TNFα antibodies are approved for the therapy of IBD, their mechanism of action is still not completely understood. Initially, a direct neutralization of pro-inflammatory TNFα cytokine by these agents was suggested, leading to the suppression of inflammation [36].

All anti-TNFα agents have the same target, but the affinity for TNFα has been reported not to be the same. Moreover, it has been described that up to three infliximab molecules can bind to each TNFα homotrimer. In contrast, etanercept is able to bind only one TNFα trimer resulting in a non-complete block of sites. Probably this is the reason why etanercept failed to show any clinical efficacy and is therefore not approved for IBD treatment [37].

In recent year several new mechanisms of action of anti-TNFα agents were studied [Figure 4]. Reverse signalling through mTNFα was induced by infliximab through the activation of JNK/p53 signalling pathway and the up-regulation of Bax, Bak, IL-10, and p21. The cytoplasmic domain of mTNFα contains serine residues that are essential for apoptosis and cell cycle arrest [38]. Apoptosis is a fundamental mechanism of inflammation resolution and can be induced by the Fc region of anti-TNFα antibodies involving the complement or NK cells.

ADCC is a mechanism used by anti-TNFα agents, characterized by the recognition of the Fc domain by the Fc receptor of effector immune cells, such as NKs. The NK cells then release cytotoxic proteins such as perforins and granzymes that induce the lysis of the target cell. The binding of antibodies to a target cell can result also in complement activation leading to the formation of a membrane attacking complex, inducing a pore within the cell and inducing cell death. Infliximab and adalimumab are more able to induce Fc-mediated apoptosis than other biologics. However, as certolizumab pegol is not able to induce ADCC, but is effective in CD, it has been suggested that Fc-mediated apoptosis is not a central mechanism of action of anti-TNFα agents [37].

Another mechanism of action has been identified: anti-TNFα agents neutralize the binding of mTNFα to TNFR2 on T cells and block TNFR2 signalling pathways and in particular TRAF2 and NF-κB activation. As a consequence of anti-TNFα therapy, a significant

increase of active caspase-3 and an increase in the pro-apoptotic Bax/Bcl-2 ratio could be detected, leading to the apoptosis of TNFR2 expressing cells [37].

Although biologics revolutionized the treatment of IBD, more than a third of patients are primary non responders and a variable proportion of patients (up to 50%) loses the response over time (secondary failure). This variability is related to several factors, including drug pharmacokinetic effects [33, 39].

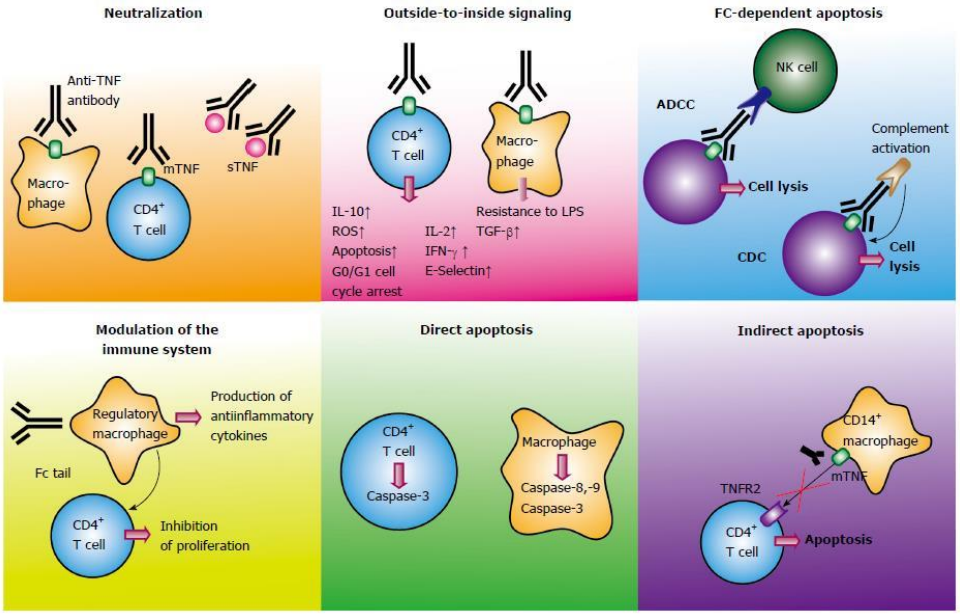

***Figure 4**: Anti-TNF agents' mechanism of action [37].*

1.4 Therapeutic drug monitoring: proactive vs reactive

Dose optimization based on therapeutic drug monitoring (TDM) during induction might result in better long-term clinical outcomes avoiding side effects and treatment failure. TDM is an important part of personalized medicine wherein drug dosing is adjusted based on serum drug concentration. In fact, in case of loss of response it is important to assess whether drug trough levels are acceptable or not. In the latter case, the pharmacokinetic failure, the evaluation of the presence of anti-drug antibodies may guide the subsequent therapeutic strategy. [40].

In recent years two TDM strategies have been described: the reactive and the proactive TDM. Reactive TDM concerns measuring infliximab concentration in patients with treatment failure despite ongoing previously successful infliximab therapy and guides

interventions based on drug intensification, change to another TNFα inhibitor, or switching out of a biologic drug class [41]. On the contrary, proactive TDM permits to modify anti-TNFα drug dosage based on serum drug concentration rather than waiting for the patient's symptoms. This strategy is used for patient in clinical remission in order to avoid or minimize the risk of future treatment failure.

Numerous studies and post hoc analyses have shown that higher drug concentrations are associated with favourable short-term and long-term outcomes in adults and children with IBD [42-46]. Moreover, there are increasing evidences in literature that support the utility of proactive strategy over the reactive one.

In the Trough Level Adapted Infliximab Treatment (TAXIT) trial, the infliximab dose was optimized using an algorithm to reach a target trough concentration of the drug (3-7 μg/mL) and then randomized to receive either a clinically based or a concentration- and anti-drug antibodies-based dosing of infliximab. The major benefit was related to the initial dose optimization, resulting in a higher proportion of CD patients in remission, in safe reduction of the dose and in substantial drug cost savings [47, 48].

In the drug concentration versus symptom-driven dose adaptation of infliximab in patients with active Crohn's disease (TAILORIX) trial, anti-TNFα naïve patients were randomized between infliximab dose adaptations based on TDM, symptoms, and biomarkers or based on clinical symptoms. No benefit was observed in infliximab dosing based on TDM, clinical symptoms and biomarkers compared to treatment intensification based on clinical symptoms alone [49].

The Pediatric Crohns Disease Adalimumab-Level-Based Optimization Treatment (PAILOT) study was the first randomized controlled trial that showed better CD outcome in children treated with adalimumab undergoing proactive TDM versus reactive TDM. The proactive monitoring of adalimumab trough concentrations and adjustment of doses and intervals resulted in significantly higher rates of clinical remission than reactive monitoring [50].

The evidence of the clinical utility of adapting therapy on the basis of drug levels has led to the need of developing new analytical assays to quantity drug levels and to provide results quickly. Furthermore, dose adjustment based on non-trough drug levels has been also recently considered for adalimumab treatment and could lead to new insights on how to monitor therapy [51]. Nevertheless, before TDM can be widely applied in clinical practice,

there are several points to clarify: when to use TDM, how to interpret the results and define the optimal drug concentration thresholds.

1.5 Immunogenity of anti-TNFα therapy

The formation of anti-drug antibodies occurs after drug administration, as a polyclonal immune response characterized by multiple types of anti-drug antibodies circulating in patients' serum. Anti-drug antibodies can be heterogeneous and each one displays its binding affinity and target specificity showing different effects: neutralizing effect on TNFα-binding activity or enhanced elimination rate of the drug. Detection of these antibodies is dependent on the sensitivity of analytical assays, which are generally optimized to detect IgG isotypes [52].

Different drug-specific epitopes can be identified: all anti-TNFα agents, except etanercept, contain complementary determining regions (CDR). These hypervariable loops form the TNFα binding region, which contain a unique amino acid sequence. This antigen-binding site forms the immunogenic region targeted by anti-drug antibodies [53]. Anti-drug antibodies production can affect drug efficacy and safety and is considered one of the reasons why a patient can lose response.

Infliximab is more immunogenic in comparison to the other anti-TNFα drugs, likely because of the variable regions derived from murine sequences. In patients treated with infliximab, anti-drug antibodies have been reported in 8-60% of cases; for adalimumab, a fully human antibody, the risk of immunogenicity is up to 38%. Anti-drug antibodies production impact both on drug pharmacokinetics and pharmacodynamics by enhancing the drug clearance [54]. Anti-drug antibodies production is associated with worse treatment response, not only for the high risk of hypersensitivity reactions, but also for a lower bioavailability caused by higher anti-TNFα clearance. In fact, the generation of anti-drug antibodies would facilitate the opsonization and phagocytosis of anti-TNFα agents, reducing their efficacy and leading to treatment failure.

In some patients, anti-drug antibodies production is transient, suggesting a mechanism of immune tolerance. Immune tolerance refers to the exhaustion of the immune response to a particular immunogenic antigen preventing an excessive response. In the case of anti-TNFα treatment, this event can occur as a decrease of anti-drug antibodies titer over time. Besides their ability to affect pharmacokinetics and contribute to clinical inefficacy, the presence of

anti-drug antibodies is also linked to adverse events. Infusion reactions are the most common adverse event described and include symptoms like fever, pruritus, bronchospasms, or cardiovascular collapse within the first day after drug administration.

For infliximab, the reported incidence rate of infusion-related reactions varies between 4–15%. Infusion reactions can range from mild, characterized by rash, pruritus, and dyspnea to severe. In the latter, anaphylactic-like reactions such as hypotension and respiratory distress are described. These symptoms could suggest an involvement of IgE antibodies but their role in infliximab infusion reaction is controversial. IgE antibodies were generally found in very low levels, and all IgE positive patients were also IgG positive and for this reason the IgG isotypes seem to be more important than IgE [55].

Factors that seem to contribute to the incidence of adverse events are size and shape of immune complexes. The majority of the complexes are dimers, but in some cases a multimerization of antibodies can occur activating, via their Fc portion, the complement cascade [56].

Several treatment strategies to overcome immunogenicity are already being applied in clinical practice. The combination of biologic therapies with immunosuppressive agents has been proposed to reduce the development of anti-drug antibodies: the use of concomitant methotrexate is associated with less anti-drug antibodies toward adalimumab and infliximab [57, 58]. A plausible explanation could be related to the capability of MTX to suppress early B- and T-cell responses, leading to modulation of the immune response. Another mechanism could be related to the role of MTX in reducing FcγR levels, which might lead to a lower clearance of anti-TNFα agents [59]. Observational studies performed in patients with IBD showed that concomitant use of AZA and glucocorticoids reduced the incidence of anti-drug antibodies, however the efficacy of these drugs in reducing immunogenicity are still controversial [60].

Other options to avoid treatment failure could include the dose adjustment of the TNFα inhibitor. In this context, TDM could help to improve decision clinical for patients with IBD.

1.6 Analytical assays in anti-TNFα TDM

Several assays, such as the radioimmunoassay (RIA), the homogeneous mobility shift assay (HMSA) and the cell reporter gene assay (RGA), have been set up to measure the

concentrations of anti-TNFα drugs and anti-drug antibodies, however the enzyme-linked immunosorbent assay (ELISA) is probably the most common test used for TDM in clinical practice. The inability to detect anti-drug antibodies in the presence of drug, was an important limitation of the first ELISA assay and for this reason, recently, a new ELISA test (Immundiagnostik, Germany) has been introduced allowing a determination of ADAs even in the presence of anti-TNFα agents. RIA is similar to ELISA in terms of sensitivity and specificity but the use of a radioactive agent, iodine 125, the higher costs, and the prolonged incubation time needed, limit its practical use. HMSA and RGA have been subsequently designed to overcome these limitations. HMSA is characterized by a homogeneous liquid-phase condition where the antibody and antigen-binding reactions take place, enhancing detection of all immunoglobulin isotypes and all subclasses of IgG.

Thanks to high-pressure liquid chromatography and size exclusion chromatography, HMSA permits to dissociate drug-anti-drug antibody complexes and to quantify them independently. RGA permits to obtain quantitative measurements shortly and economically. Recently other assays have been introduced in order to achieve a rapid readout of the results: the fiber-optic surface plasmon resonance (FO-SPR) and lateral flow (LF) assays. FO-SPR studies the interaction between two molecules: one immobilized on a chip and the other flowing through a microfluidic system over surface. The major advantage is that it can determine anti-TNFα drug and anti-drug antibody concentrations simultaneously and rapidly and without the use of radio-labelled molecules.

The Quantum Blue (Bühlmann Laboratories, Schönenbuch, Switzerland) was the first rapid test introduced for TDM and later, another lateral flow assay was developed: the RIDAQuick (R-Biopharm, Darmstadt, Germany). These tests are based on the same principle and consist in a small device able to analyse the test membrane spotted with the patient serum, returning a quantitative value of signal intensity (Figure 5).

According to the needs of the clinicians, the use of these techniques could be useful for proactive strategies permitting to have an immediate result in order to change clinical decision [39].

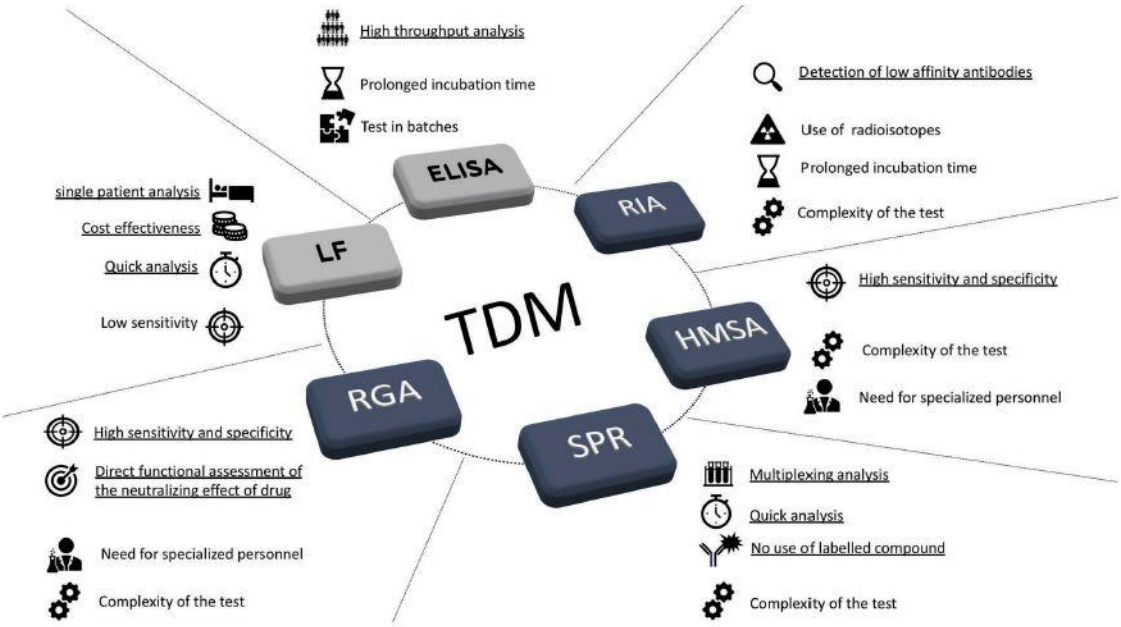

Figure 5*: Schematic representation of the different methods used for therapeutic drug monitoring (TDM). The advantages are underlined. In light grey the solid phase immunoassays (ELISA and LF), in dark grey the other ones: (RIA, HMSA, RGA and SPR assays) [39].*

1.6.1 Alternative tool in TDM: the atomic force microscopy

Current in vitro techniques cannot accurately identify small differences in concentration in samples containing few molecules. Nanotechnology overcomes these limitations with the possibility of measuring nanomolar protein concentrations in a small volume. In fact, in the recent years, the development of diagnostic devices based on diverse physical principles for the highly sensitive, non-invasive, and fast detection of biomarkers have changed the analytical practice [61].

Atomic force microscopy (AFM) is a scanning probe technique that combines a nano scale resolution and a higher imaging capacity and is considered a promising method for the analysis of biological samples. The instrument consists of a cantilever with a conductive material tip at its extremity: when a sample is scanned, van der Waals forces are created between the surface and the tip, and the deflection of the latter is recorded by a detector [Figure 6].

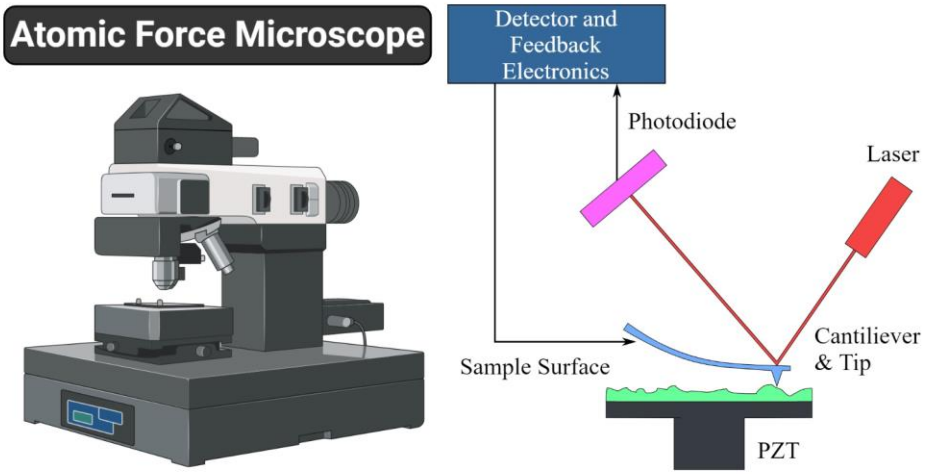

Figure 6: *Atomic force microscopy approach [62].*

AFM permits to control density and orientation of protein layers on gold surfaces for optimal biorecognition through nanolithography techniques, and simultaneously the measurement of binding interactions in a label-free way. The assay is based on molecular manipulation to create functional spots of surface immobilized binders and differential topography measurements [63].

AFM approach consists in the confinement of high-affinity antigen-binding molecules on a surface by means of AFM nanografting of single-stranded DNA (ssDNA) and DNA-directed immobilization (DDI) of conjugated binders. Surface-bound ssDNA can be used to create a ligand-binding assays. Thanks to nanografting technique and Watson-Crick base pairing, a ssDNA nanoarrays can be created on the surface [64].

In the last decades nanotechnology have developed a strong impact in medicine. A variety of sensors based on different nanostructured materials and techniques have increased research interest due to their potential for being developed to point-of-care type of device [65]. Miniaturization in particular is the key for the creation of point of care devices capable of testing low volume of biological sample. Menotta et colleagues showed, for the first time, the use of AFM to quantify the amount of a drug, tacrolimus, in blood samples [66]. Compared with the gold standard ELISA, multiplexing nano-arrays have several advantages: the high-throughput nature, efficiency in terms of time and cost and the ability to measure one antigen in the context of multiple others.

1.7 Pharmacogenetic of anti-TNFα therapy

The efficacy of anti-TNFα drugs is characterized by a high interpatient variability that depends on several factors, such as the severity and localization of the disease, environmental factors and habits. However, an important role in this variability should be ascribed to genetic factors. Increasing evidences suggest that these differences in drug response and anti-drug antibodies production could be caused by genetic variability and several studies considering therapeutic response and drug efficacy have been performed evaluating single nucleotide polymorphisms (SNP) in genes involved in immune processes and inflammation [17].

The contribution of polymorphisms of single genes is still debated both in pediatric and in adult patients [67]. However, the contribution of single genes is difficult to ascertain because of the complex pharmacokinetics and pharmacodynamics of anti-TNFα agents. Most studied genes include genes related to immune processes, inflammation, autophagy and apoptosis.

Considering the central role that TNFα and IL6 play in the pathogenesis of IBD [27, 68], it is possible that different production of these cytokines may affect the response to therapy. Many variants in the untranslated region of the human *IL6* and in *TNFα* gene have been described and seem to alter transcriptional process, decreasing IL6 level and increasing TNFα production, respectively [69, 70]. One of the most studied SNPs is the *TNFα* promoter polymorphism -308 G>A that has previously been associated with susceptibility to a range of autoimmune disorders, among which IBD and rheumatoid arthritis.

Allele A confers a major transcriptional activation and increases the level of TNFα production comparing to the common allele G. The AA and GA genotypes were correlated with non-response to anti-TNFα treatment; furthermore, the combination of TNF -308 A allele and the CC genotype in the SNP rs763110 of the *FAS* ligand (*FASL*) gene gives an additive effect conferring to patients a nearly five-fold higher odd of being non-responders [69]. Other predictors of good response for patients treated with infliximab are the genetic variation involved in NF-κB signalling pathway: rs4149570 in *TNFR1*, rs976881, rs1061622 and rs652625 in *TNFR2* and rs2430561 in interferon gamma (*IFNG*) [69].

Polymorphisms in other cytokine genes could influence the predisposition to IBD and therapeutic response. IL6 signalling may participate in disease perpetuation by mediating the resistance of lamina propria T cells against apoptosis via STAT-3 signalling, causing

chronic inflammation. It has been shown that the neutralization of TNFα results in the suppression of various proinflammatory cytokines, including IL6, and interrupts a positive feedback loop of TNFα production. Moreover, IL6 promotes Th17 cell lineage and function and inhibits regulatory T cells leading to the breakdown of immunological tolerance, polyclonal B-cell activation and autoantibody production [71].

Fujino et al. have shown that serum levels of IL-17 are increased in patients with IBD and suggested that this cytokine might be associated with altered inflammatory responses [72]. Caucasian patients with the genetic variant rs10889677 in the 3'-untraslated region (UTR) of the *IL-23R* gene, enhancing IL-23R mRNA levels and protein production, had an higher probability to respond to infliximab therapy [73]. Bank et colleagues demonstrated a correlation with several SNPs in Toll Like Receptor (TLR)-related genes and good response to anti-TNFα. In fact, the rs1816702CT/TT and rs3804099TC/CC genotypes in *TLR2*, conferred an increased expression of receptor, leading to a decreased expression of TNFα, IL-1β and IL-6. In addition *TLR9* rs187084TC genotype induced a decreased expression of TLR9 and a decrease of NF-κB signalling pathway activation, leading to a good anti-TNFα response while the rs4696480TT and rs11938228CA/AA genotypes in *TLR2*, the rs1554973TC/CC in *TLR4* and rs352139AA in *TLR9* were associated with non-response [74, 75].

As discussed before, the production of anti-drug antibodies against anti-TNFα drugs is a crucial cause of loss of response. Several studies have evaluated the association between genetic variants in proteins important for antibodies degradation, such as Fc-gamma receptors (FcγRs) and the monoclonal antibodies efficacy. The main clearance mechanism for anti-TNFα drugs is through the reticulo-endothelial system (ERS) which depends on two receptors: the Brambell's receptor (FcRn) that is expressed by endothelial ERS cells and increases IgG half-life; and the FCGR expressed by macrophages, NK cells and neutrophils, that induce the degradation of IgG-FcγR complexes in the endolysosomes of these innate immune cells [76]. Interestly, the presentation of IgG antigens on these cells through the class II major histocompatibility complex (MHC) increases the probability of anti-drug IgG antibodies production by activated plasma cells [76].

For these reasons, polymorphisms in these genes could affect the clinical response to anti-TNFα agents and anti-drug antibodies production. The rs396991 is a non-synonymous polymorphism in the receptor for the Fc portion of IgG (FCGR)3A (c.818A>C, F158V, rs396991). rs396991 A encodes for a protein with phenylalanine in position 158 and is

identified as F allele that binds with low affinity IgG1 and IgG3, while the rs396991 C encodes the variant valine (V) that has a high-binding affinity. Recently, it was reported that *FCGR3A* 158V/V genotype was associated with increased infliximab elimination and risk of relapse after infliximab discontinuation in CD patients [58]. However, other authors failed to demonstrate this associations [77, 78]. Romero-Cara et al., in a recent meta-analysis, could not find an association of the *FCGR3A* high affinity V variant allele with better response to TNFα inhibitors in patients with arthritis; in addition, they confirmed that patients carrying the *IL6* SNP (-174 G>C, rs1800795) C variant allele, a promoter polymorphism that increases the level of this pro-inflammatory cytokine, were poor responders to anti-TNFα treatment [76, 79].

Sazonovs et al. demonstrated that the genetic variation rs2097432 *HLA-DQA1*05*, carried by approximately 40% of Europeans, significantly increased immunogenicity during anti-TNFα treatment and reduced the bioavailability of the drug, despite the use of concomitant immunomodulator [80].

The role of SNPs in genes involved in apoptotic process has also been evaluated, given the mechanism of action of anti-TNFα drugs that are implicated in apoptosis process of inflammatory cells. Association with the anti-TNFα response was found for the Fas cell surface death receptor (FAS), a member of the tumor necrosis factor receptor family containing a death domain and playing a central role in programmed cell death. The FASL triggers apoptosis, contributing to T and NK ability to kill target cells. One intronic polymorphism, rs7896789, was identified as possibly associated with a lower response to anti-TNFα agents, however, its clinical implications remain unknown [81].

Autophagy Related 16 Like 1 (ATG16L1) plays an important role in autophagy and is able to suppress inflammatory cytokines, regulating mitochondrial antiviral signalling (MAVS)-dependent type I IFN production and Nucleotide-Binding Oligomerization Domain (NOD)-1 and NOD-2-driven inflammatory cytokine response. Genetic variations in this gene could influence the clinical response in IBD patients: a strong association with rs10210302 SNP and adalimumab response was observed in CD Slovenian patients: T allele confers low levels of C reactive protein leading to a better adalimumab response compared to the CC genotype [82]. This correlation was not confirmed by Netz and colleagues: in CD Caucasian patients, the rs10210302 and rs2241880 SNPs, linked to reduced autophagy, were not associated with anti-TNFα response [69]

2. AIM OF THE RESEARCH

Pharmacological therapy of inflammatory bowel disease (IBD), and in particular the use of antibodies against tumor necrosis factor alpha (TNFα), such as infliximab and adalimumab, has led to a revolution in the treatment of IBD thanks to their capability to induce and maintain clinical remission.

Nevertheless, a sizable proportion of patients' loss response: 10-20% during induction phase of treatment (primary failure) and up to 45% had a secondary loss of response. There is increasing evidence suggesting that treatment failure may be associated with inadequate blood drug levels and/or the production of anti-drug antibodies. Studying the mechanism underlying this inter-individual variability becomes an important research goal to improve clinical practice. The main purposes were:

- to compare two point-of-care (POC) devices for quantification of serum infliximab concentration with two validated ELISA assays in children with IBD;
- to set-up an innovative method for the measurement of infliximab concentrations in pediatric IBD patients using atomic force microscopy (AFM) technique;
- to assess the predictivity of adalimumab concentrations at the end of induction and early during maintenance for long term response;
- to study the role of pharmacogenetics in infliximab response in a cohort of pediatric IBD patients;
- to evaluate the role of CD69 in infliximab mechanism of action and as possible marker of infliximab response on Jurkat cells, an activated immortalized T cell line and on PBMC of healthy donors.

The results of this research could be useful for studying anti-TNFα mechanisms of action and to achieve a personalized therapy and may influence the choice of anti-TNFα treatment in pediatric IBD patients.

3. MATERIAL AND METHODS

3.1 Patients

3.1.1 Infliximab patients' cohort

Patients with IBD treated with infliximab were enrolled at the Pediatric Gastroenterology Unit of the Institute for Maternal and Child Health IRCCS Burlo Garofolo, in Trieste and the Pediatric Unit of Ca' Foncello Hospital in Treviso.

Children were treated according to a therapeutic protocol consisting of intravenous administration of infliximab at 5 mg/kg in 2 hours infusions at weeks 0, 2, 6 (induction phase), followed by a maintenance phase in which infusions were performed every 8 weeks. The inclusion criteria were age between 7 and 18 years, previous diagnosis of IBD and treatment with infliximab. Infliximab was started as first line therapy or in case of treatment failure or intolerance to first-line therapies. The exclusion criteria were: patients with ileostomy or colostomy, disease needing surgery or contemporary presence of other non-controlled pathologies. For each patient clinical efficacy was assessed, using Pediatric Crohn's Disease Activity Index (PCDAI) and Pediatric Ulcerative Colitis Activity Index (PUCAI) for CD and UC patients, respectively, at the end of induction and during maintenance phase (at 22 and 52 weeks). Disease was considered in remission if indexes were ≤10. Local Ethic Committee approval and appropriate informed consent were obtained from all patients' parents or caregivers.

3.1.2 Adalimumab patients' cohort

Patients with IBD treated with adalimumab were enrolled at the Pediatric Gastroenterology Unit of the Institute for Maternal and Child Health IRCCS Burlo Garofolo, in Trieste, at the Pediatric Unit of Ca' Foncello Hospital in Treviso and at the Pediatric Gastroenterology Unit, of Maggiore Hospital in Bologna. Adalimumab was started in case of treatment failure or intolerance to standard therapies (steroids, exclusive enteral nutrition, immunomodulators) or infliximab. Children were treated according to a therapeutic protocol consisting of subcutaneous administration of adalimumab on day one at 160 mg and 80 mg, and on day 15 at 80 mg and 40 mg, for bodyweight ≥40 kg or <40 kg, respectively (induction phase), followed by every other week maintenance at 40 mg or 20 mg for body weight ≥40 kg or <40 kg, respectively (maintenance phase). Concomitant

therapy with immunomodulators was permitted. Exclusion criteria were the same considered in infliximab patients' cohort; and the disease was considered in remission if PCDAI/PUCAI indexes were ≤10. Local Ethic Committee approval and appropriate informed consent were obtained from all patients' parents or caregivers.

3.2 Determination of serum infliximab concentration by point-of-care devices in the infliximab patients'cohort

Serum samples were collected immediately before infusion (trough level), both during the induction and maintenance phases of infliximab treatment. For the pharmacokinetic analysis, samples were stored at -20 °C and were analysed retrospectively, according to the manufacturer, with 2 different ELISA assays (Lisa Tracker-Duo® Infliximab and Promonitor assay) and 2 POC devices (Quantum Blue and RidaQuick), retrospectively. Anti-drug antibodies were measured by Promonitor ELISA assay when infliximab plasma levels were ≤ 1.5 μg/ml. Patients with anti-drug antibodies levels higher than 10 units per milliliter (AU/ml) were considered positive.

3.3 The innovative atomic force microscopy method for serum infliximab measurement in healthy donors and pediatric IBD patients

Serum samples of healthy donors and serum samples of pediatric IBD patients in treatment with infliximab were collected immediately before infusion, stored at -20 °C and analysed retrospectively to check the compatibility of the AFM system with the serum.

The AFM is based on the measurement of signal variation of a nanostructured gold surface covered with a self-assembled monolayer (SAM) of alkanethiols. In order to avoid unspecific protein adsorption, alkanethiols were terminated with oligoethylene glycol (TOEG). This system is composed by proteins able to recognise the target present in solution (infliximab), and works as described:

1) DNA nanostructures (ssDNA) are fabricated through nanografting, an AFM-based nanolithography technique, inside a TOEG-SAM carpet;
2) A new single stranded DNA (ssDNA) sequence complementary to the nanografted one (ssDNA), is immobilized on the nanospot via DNA-DNA interaction. This process is known as DNA-Directed Immobilization (DDI).

3) The final structures (the conjugate) composed by double-stranded DNA linked to TNFα on the top, is now ready to recognize the infliximab present in solution (Figure 7).

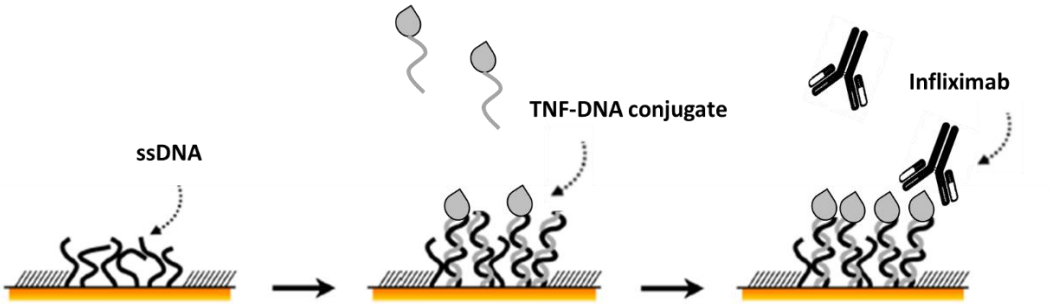

Figure 7: *Schematic representation of AFM.*

To fine-tune the AFM technique, several concentrations of infliximab (20, 30, 40 and 50 nM) were initially tested in Tris EDTA (TE) buffer 1M NaCl. Afterwards, sera of healthy donors treated with known concentrations of infliximab within the therapeutic range (10, 20, 30, 40 and 50 nM) and the sera of pediatric IBD patients treated with infliximab whose concentration has already been determined by ELISA gold standard assay, were used to check the compatibility of the AFM system with the serum. The sample was diluted 1:100 to avoid the formation of non-specific bonds and EDTA was used at a concentration of 10 mM. The addition of EDTA, a chelator of Mg^{2+} ions, allows to maintain the integrity of the patches created during the nanografting phase, minimizing the action of nucleases on nanographed DNA.

All AFM experiments were performed with a XE-100 Park Instruments. Thiol modified ssDNA (SH-cF9: SH- (CH2) 6-5'-CTTATCGCTTTATGACCGGACC-3' from Biomers GmbH Ulm, Germany) were immobilized inside the TOEG6 SAM by serial AFM-based nanografting technique: an AFM tip with sufficient rigidity (MicroMasch NSC 19/no Al, spring constant 0.6 Nm^{-1}) was operated at high load (set point/force ~100 nN) on areas of 2 x 2 μm in order to locally displace the TOEG6 SAMs and to facilitate the exchange with the thiolated ssDNA biomolecules (5 μM in TE buffer 1 M NaCl) present in the liquid cell. All the AFM images were taken at a frequency of 1 Hz and result from 256 x 256 lines. The images were recorded in gentle contact at minimum force values and the topography analysis was determined using the Xei software.

3.4 Pharmacokinetic analysis in the adalimumab patients' cohort

Serum samples for adalimumab levels measurement were collected during routine visits between adalimumab administrations and therefore not necessarily at trough, both during the induction (week 4 ± 4) and maintenance phases (week 22 ± 4, week 52 ± 4, week 82 ± 4). Samples were stored at -20 °C and were analysed retrospectively. All patients with complete clinical response (disease activity score ≤ 10) to treatment after induction continued therapy with adalimumab and were followed until week 82.

3.5 DNA extraction and pharmacogenetic analysis in the infliximab patients' cohort

Total DNA was isolated from infliximab patients' peripheral blood using a commercial kit (Sigma-Aldrich, Milan, Italy) according to the manufacturer's protocol. The DNA concentration and purity were calculated by Nano Drop instrument (NanoDrop 2000, EuroClone, Milan, Italy). TaqMan® SNP genotyping assays (Applied Biosystems, USA), polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) and droplet digital-PCR (ddPCR) were used to characterize the SNPs of interest (*FCGR3A* rs396991, *IL6* rs1800795 and *TNFα* rs1800629, respectively).

The *FCGR3A* rs396991 was genotyped with TaqMan Designed SNP Genotyping Assays (Sequence_VIC/FAM:TCTGAAGACACATTTTTACTCCCAA[C/A]AAGCCCCCTGCA GAAGTAGGAGCCG) using the CFX96 real-time system-C1000 Thermal Cycler (Bio-Rad Laboratories). The thermal cycling conditions for TaqMan assays were as follows: 5 min at 95 °C followed by 50 cycles at 95 °C for 15 s and 60 °C for 60 s. Through the software unit (Sequence Detection System) the 3 different populations' genotypes (wt, hz and mut) were discriminated. The analysis of the *IL6* rs1800795 and *TNFα* rs1800629 promoter polymorphisms were determined by PCR- RFLP [83]. Briefly, for *IL6* rs1800795 (sequence forward and reverse primers: 5′-GACTTCAGCTTTACTCTTGT-3′ and 5′-CTGATTGGAAACCTTATTAAG-3′) and TNFα rs1800629 (sequence forward and reverse primers: 5′-GAGCAAAGGTTTTGAGCCCAT-3′ and 5′-GGGACACACAAGCATCAAG-3′) the PCR products obtained by amplification of gDNA, were digested with NlaIII (New England Biolabs) and NcoI (New England Biolabs) and run on a 3.5% SYBR™ Safe-stained agarose gel, respectively. To confirm the data obtained with PCR-RFLP, the analysis of these polymorphisms was determined also by

ddPCR Bio-Rad's QX200 system (Bio-Rad Laboratory, Hercules, California, USA). PCR was performed in a Veriti PCR instrument (Thermo Fisher) according to manufacturer instructions. After thermal cycling, plates were read in the QX200 Droplet Reader. QuantaSoft software was used for analysis (Bio-Rad Laboratory, Hercules, California, USA).

3.6 Cell line

Jurkat cells are a CD4+ T cell line, cultured in RPMI 1640 (Euroclone) with the addition of:

- ❖ 10 % of Fetal Bovine Serum (FBS) (Sigma);
- ❖ 1 % of L-glutamine 200 mM (Sigma);
- ❖ 1 % of penicillin 10.000 UI/mL and streptomycin 10.000 μg/mL (Sigma).

Cells are grown in T25 flasks and passages are made twice a week. Cells are counted using the Trypan blue exclusion test which allows to discriminate between alive cells and dead cells, both necrotic and apoptotic, whose membrane is permeable to the dye and thus appear blue in contrast to alive cells that appear white. The cell suspension is diluted 1:10 in a Trypan blue: PBS 1:1 solution and cell count is made using a Burker chamber. The following formula is applied in order to count alive cells in 1 mL of suspension:

$$Cells/mL = mean\ cell\ number * diluition\ factor * 10^4$$

After each passage, cells are seeded at $1.5*10^6$ cells in T25 and are kept in an incubator with humidity control at 37 °C with 5 % CO2.

3.7 Sample preparation and Bradford assay

Protein samples were suspended in RIPA buffer (1 mM EDTA; 150 mM NaCl; 50 mM Tris HCl; sodium deoxycholate 0.5%; NP-40 1%; protease inhibitor cocktail 1x, all from Sigma), sonicated for about 30 seconds in ice to lyse cell membranes and centrifuged at 10,000 x g at 4 °C for 10 minutes. The supernatant is withdrawn and the pellet quantified with Bradford assay for the Western blot assay. Proteins bind to Coomassie dye under acidic conditions resulting in its change of colour from brown (cationic form) to blue

(anionic form). Standards (BSA at concentrations of 2 – 1 – 0.5 – 0.25 and 0 μg/μL) and samples are placed in a 96-well flat-plate with Bradford reagent (Sigma) in ratio 1:50 (5 μL of samples and 250 μL of Bradford reagent). 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.

3.8 Western blot analysis

Western blot or immunoblot assay 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 coloured 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 7 minutes. To verify if the gel is completely transferred on the membrane, Ponceau red is used. This solution binds proteins unselectively and imparts them a bright red colour. It is washed away using distilled water. 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 (Sigma); 0.009 % p/V NaCl (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: anti-p65 1:500 (Santa Cruz Biotechnologies, Dallas, TX USA, Cat. N. sc-372), anti-IKB 1:13000 (Abcam, Cambridge UK, Cat. N. ab32518) and anti-Actin 1:3000 (Abcam, Cambridge UK, Cat. N. ab8227). Anti-mouse (Cell Signalling, Danvers, MA USA, Cat. N.

7076S) and anti-rabbit HRP conjugated (OriGene technologies, Rockville, MD USA, Cat. N. TA13002) 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 Chemiluminescent Substrate (Euroclone) is used. The kit is composed of Peroxidase Buffer and Luminol: HRP enzyme in presence of hydrogen peroxide catalyses 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) placed on the membrane for 20 minutes in a cassette. The plate is then developed with Developer solution (1:5 in water, Thermo Fisher), washed with water and stained with Fixer solution (1:5 in water, Thermo Fisher). Images were analyzed with ImageJ program and data were normalized on β-actin.

3.9 Total RNA extraction

Total RNA of Jurkat cells was extracted using the TRIzol® reagent (Thermo Scientific). TRIzol® reagent is a monophasic solution of phenol and guanidine isothiocyanate that maintains the integrity of RNA due to the highly effective inhibition of RNase activity. The samples were incubated with 1 mL of TRIzol® for 5 minutes at room temperature to dissociate the nucleoprotein complex. Chloroform (0.2 mL; Sigma-Aldrich) was added and after 3 minutes of incubation at room temperature, a centrifugation at 12,000 × g for 15 minutes at 4 °C was performed. The homogenate is allowed to separate into a clear upper aqueous layer (containing RNA), and interphase and red lower organic layers (containing the DNA and proteins). The upper phase was then transferred into a new RNase-free tube to proceed with the RNA isolation. RNA is precipitated from the aqueous layer with 500 μL of 100% isopropanol (Sigma-Aldrich) and washed with 1 mL of 75% ethanol (Sigma-Aldrich). The precipitated RNA pellet was resuspended in 20 μL RNase-free water (Gibco-Life Technologies) and incubated in a water bath at 55–60 °C for 15 minutes. Then, the RNA concentration and purity were evaluated by a Nano Drop instrument (NanoDrop 2000, EuroClone®).

3.10 Reverse transcription

Reverse transcription (RT) is a process that converts RNA to single-stranded complementary DNA (cDNA). The RT was performed using the High Capacity RNA to-

cDNA Kit (Applied Biosystem) with up to 1 μg of total RNA per 20 μL of reaction containing 10 μL of 2 x RT Buffer, 1 μL of 20x RT Enzyme Mix. The thermal protocol (Applied Biosystems 2720 Thermofischer 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.

3.11 Real-time PCR

For quantifying the expression level of the genes analyzed in this thesis, the KiCqStart® SYBR® Green qPCR ReadyMix™ kit (Sigma-Aldrich) was used. SYBR Green is a fluorescent dye, able to bind double-stranded DNA molecules by intercalating between the DNA bases. The fluorescence can be measured at the end of each amplification cycle to determine the amount of DNA amplified. Predesigned (KiCqStart® SYBR® Green Primers, Sigma-Aldrich) primers were used and their sequences are reported in table 1. The thermal cycler was the CFX96 real-time system-C1000 (Bio-Rad Laboratories). Beta-actin was used as normalizer and expression levels were reported as 2-ΔCt. The results are provided as the mean and standard error of three replicates.

Table 1. Real-time PCR analysis of tumor necrosis factor receptor gene expression.

GENE	PRIMER	SEQUENCE 5'->3'	T (°C) MELTING
B-Actin	Forward	CGCCGCCAGCTCACCATG	86.5
	Reverse	CACGATGGAGGGGAAGACGC	
TNFR1	Forward	CTGCCTCAGCTGCTCCAAA	62.3
	Reverse	CGGTCCACTGTGCAAGAAGAG	
TNFR2	Forward	CTCCAACACGACTTCATCCA	62
	Reverse	CTCCAACACGACTTCATCCA	

Abbreviation: TNFR, tumor necrosis factor receptor

3.12 PBMC isolation

Ficoll-Paque products are a ready-to-use density gradient media for isolating peripheral blood mononuclear cells (PBMCs) from healthy donor in high yield and purity, using a simple and rapid centrifugation procedure based on the method developed by Bøyum [84].

Differential migration of cells during centrifugation results in the formation of layers containing different cell types:

• The bottom layer contains erythrocytes, which have been aggregated by Ficoll and therefore sedimented completely;

• the layer immediately above the erythrocytes contains mostly granulocytes;

• At the interface between the plasma and the Ficoll-Paque layer, mononuclear cells are found together with other particles (e.g., platelets) with low density.

PBMCs were recovered using Pasteur pipettes and washed twice in PBS. The PBMCs are resuspended in 1 mL of medium and the count was performed after a dilution 1:1000 in a Trypan blue: PBS 1:1 solution.

3.13 Flow cytometry analysis: CD69 expression on Jurkat cells and PBMC

Flow cytometry was used to identify the ratio of CD69 positive cell subpopulations present on Jurkat cell and on CD4+ and CD8+ T cells isolated from PBMCs of healthy donors. Staining cells for surface markers was performed using specific antibodies directly conjugated to a fluorochrome. Different fluorochromes emit light when hit by lasers of particular wavelengths. Stained cells in suspension are led through different lasers one by one and when hit by the laser light, one detector measures the forward scatter (FSC) which is a measure of cell size, and another detector measures the side scatter (SSC) which is the granularity of the cells. Thus, data generated for each cell includes cell size, granularity and fluorescent labelling.

Flow cytometry analysis were performed on a BD FACSC flow cytometer (BD Biosciences) as the following protocol:

- 250.000 Jurkat cells were cultured in complete RPMI 1640, in 12-well plate and activated with PMA/ionomycin (eBioscience) for 4 hours;
- after activation, cells were centrifugated to remove the stimulus and treated with increased concentration of infliximab (0-1-10-100 µg/mL) overnight;
- at the end of infliximab treatment, Jurkat cells were washed and resuspended in 250 µL of PBS and incubated with 1 µL of CD69 APC antibody, for 20 minutes in the dark;
- after incubation, Jurkat cells were washed to eliminate the unbound antibody and were than fixed with PBS plus 1% paraformaldehyde for the flow cytometry

analysis. The expression of CD69 was evaluated in terms of mean fluorescent intensity (MFI).

The same protocol was used for the evaluation of CD69 MFI in PBMCs, but the following step were modified:

- 250.000 PBMCs were cultured in RPMI 1640, in 12-well plate, with the addition of 10% of human serum (to avoid unspecific activation), and activated with PMA/ionomycin (eBioscience) for 8 hours and then treated with the same concentration of infliximab overnight;
- after infliximab treatment PBMCs were washed and resuspended in 250 µL of PBS and incubated with 1 µL of CD69 APC antibody, 2 µL of CD3 VioBlue, 2 µL of CD4 PE and 5 µL of CD8 PerCP for 20 minutes in the dark;
- The CD69 MFI was evaluated both in CD4+ and CD8+ T cell populations.

3.14 Statistical analysis

Statistical analysis was performed using the R software (version 3.4.2). $P < 0.05$ was considered statistically significant.

In the pharmacokinetic analysis of infliximab, intraclass Correlation Coefficient (ICC) and Bland-Altman plots were assessed for quantitative comparison between POC assays, Promonitor ELISA assay and Lisa-Tracker ELISA assay, conventionally assumed as reference. Weighted kappa statistics were determined after stratification of results by therapeutic interval (<3 µg/mL, ≥3 – <7 µg/mL and ≥7µg/mL) and were used for qualitative comparison.

For AFM analysis, linear regression function was used to assess the correlation between infliximab concentration and signal variation (height variations). Graph-Pad Prism version 6.00 (GraphPad, La Jolla, CA, USA) was used for the one-way ANOVA statistical analysis.

In the pharmacokinetic analysis of adalimumab, the association between adalimumab concentrations and the therapeutic response was evaluated by linear mixed effect (LME) models, which allow to account for repeated measures, using patient's response to adalimumab as the dependent variable and adalimumab serum levels as the independent variable. To find the optimal drug levels for predicting efficacy, the association between

adalimumab concentrations and response at the various time points was identified using logistic regression analysis. The potential confounding effect of adalimumab dose was tested by considering the difference in adalimumab weight normalised dose at different time-points by LME. Receiver operating characteristic (ROC) curves were then constructed for adalimumab concentrations, to determine the optimal cut-off to predict clinical response. Sensitivity, specificity and positive and negative predictive values of the cut-off point were analysed. An analysis of the association between adalimumab concentrations considering all time points and the clinical and biochemical parameters (clinical scores, CRP and fecal calprotectin) was carried out by non-parametric Spearman's test. The association between anti-drug antibodies and drug concentrations was determined by Spearman's test. Continuous data are presented as medians with interquartile ranges (IQRs) and categorical data are presented as absolute numbers and percentages.

For the pharmacogenetic analysis, the association between clinical response and anti-drug antibodies production was evaluated by generalized linear models of the binomial family (logistic regression), using clinical response or anti-drug antibodies production as the dependent variable and SNPs, clinical (type of disease) demographic (age and gender) characteristics of population and concomitant treatment as the independent variables. The correlation between infliximab levels and the three SNP was assessed by the non-parametric Kruskal-Wallis test. Multivariate analysis was done by a logistic regression model, using clinical response as the dependent variable and all covariates significantly associated with this variable, as independent.

Flow cytometry, RealTime and western blot analysis were performed using Graph-Pad Prism version 6.00 (GraphPad, La Jolla, CA, USA). Two-way and one-way ANOVA with Bonferroni post-test and t test were used for the analysis of gene and protein expression.

4. RESULTS

4.1 Determination of serum infliximab concentration by point-of-care devices

Thirty-two serum samples were obtained from 19 pediatric IBD patients. The demographic characteristics of the population are summarised in Table 2.

Table 2. Demographic and clinical characteristics of the population enrolled.

	Overall (n= 19)	**Crohn's Disease (n= 15)**	**Ulcerative colitis (n= 4)**
Age - years (IQR)	15.5 [13.6-16.9]	13.13 [10.8-15.7]	15.33 [13.1-16.1]
Disease duration - months (IQR)	38.64 [24.1-54.3]	44.60 [30.4-62.2]	21.6 [18.7-29.9]
Gender - n (%)			
Female	9 (47.4)	7 (46.7)	2 (50)
Male	10 (52.6)	8 (53.3)	2 (50)

Abbreviations: IQR, interquartile range; PCDAI, Pediatric Crohn's disease activity index; PUCAI, Ulcerative Colitis Activity index.

The drug trough levels were determined with two commercial ELISA assays (Promonitor and Lisa-tracker) and 2 point of care (POC) assays: the quantum Blue (POC IFX/QB) and Ridaquick (POC IFX/RQ). The results of the POC assays showed good agreement with both ELISA assays in the quantitative analysis (ICC: 0.82 and 0.87 for POC IFX/QB and POC IFX/RQ, respectively). A good association was also found between the two ELISA assay used (ICC: 0.87) (Figure 8).

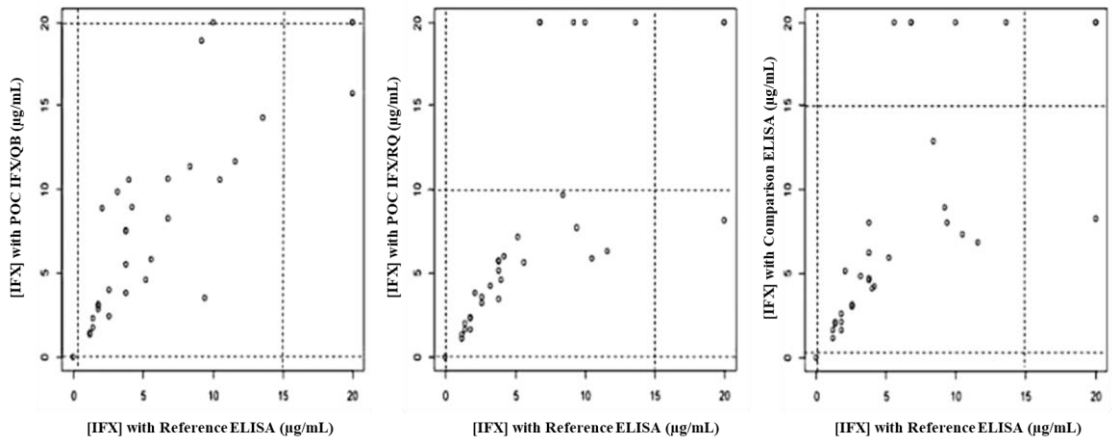

***Figure 8**: Plots representing infliximab (IFX) concentrations evaluated with reference ELISA assay and point of care (POC) IFX/Quantum Blue (QB), POC IFX/RidaQuick (RQ) and comparison ELISA assay. Dashed lines represent the lower and the upper detection limits of the tests.*

The Bland-Altman plots showed a good dispersion between the reference ELISA assay and the POC devices: a better result was achieved for lower infliximab concentration (Figure 9).

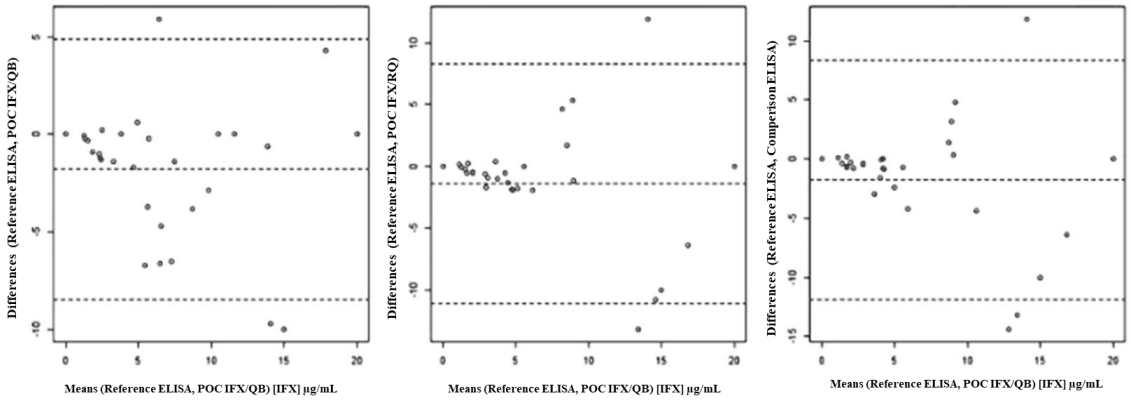

Figure 9: *Bland-Altman plots comparing the reference ELISA assay with POC IFX/QB, POC IFX/RQ and comparison ELISA assay.*

As reported in Table 3, a good agreement was shown between pairs of assays, stratifying the results into three therapeutic intervals (<3 µg/mL, ≥3 - <7 µg/mL and ≥7µg/mL). A total agreement with ELISA was obtained when the result of the POC assay was < 3 µg/mL (7/7 and 8/8 cases for POC IFX/QB and POC IFX/RQ, respectively); for POC results between 3 and 7 µg/mL agreement was present with the reference ELISA in 4/8 (50%) cases (POC IFX/QB) and 8/13 (62%) cases (POC IFX/RQ); finally, when POC results were > 7 µg/mL, agreement with reference ELISA was present in 9/17 (53%) and 8/11 (73%) cases for POC IFX/QB and POC IFX/RQ, respectively. Sensitivity, specificity, and test predictive values were then calculated for the identification of low drug levels (i.e. <3 µg/mL). For the POC IFX/QB, sensitivity was 63.6% (95% CI 31.6-87.6%), specificity 100% (95% CI 81-100%), positive predictive value 100% (95% CI 56-100%), and negative predictive value 84% (95% CI 63-95%). For POC IFX/RQ, sensitivity was 72.7% (95% CI 39.3-92.7%), specificity 100% (95% CI 81-100%), positive predictive value 100% (95% CI 59.7-100%), and negative predictive value 87.5% (95% CI 66.5-96.7%). Notably, sensitivity, specificity, and predictive values of the second ELISA assay (Promonitor), as compared to the reference ELISA, were identical to those of the POC IFX/RQ.

Table 3. Comparison of infliximab concentrations obtained by ELISA assays and POC.

		Reference ELISA (Lisa-Tracker)			
		IFX < 3µg/mL	**3 ≥ IFX < 7 µg/mL**	**IFX ≥ 7 µg/mL**	**Weighted Kappa (K) statistics**
POC IFX/QB	IFX < 3µg/mL	7	0	0	K= 0.67 $p = 3.5e^{-05}$
	3 ≥ IFX < 7 µg/mL	3	4	1	
	IFX ≥ 7 µg/mL	1	7	9	
POC IFX/RQ	IFX < 3µg/mL	8	0	0	K= 0.80 $p = 4.3e^{-06}$
	3 ≥ IFX < 7 µg/mL	3	8	2	
	IFX ≥ 7 µg/mL	0	3	8	
Comparison ELISA (Promonitor)	IFX < 3µg/mL	8	0	0	K= 0.81 $p = 2.4e^{-06}$
	3 ≥ IFX < 7 µg/mL	3	7	1	
	IFX ≥ 7 µg/mL	0	4	9	

Abbreviations: IFX, infliximab; POC, point of care; K, Kappa statistics; QB, Quantum Blue, RQ, RidaQuick.

4.2 Measurement of serum infliximab concentration by atomic force microscopy

The sera of 6 healthy donors and 6 pediatric IBD patients in treatment with infliximab were analysed by AFM. The demographic characteristics of the IBD patients are summarised in Table 4.

Table 4. Demographic and clinical characteristics of the IBD population enrolled.

	Overall (n= 6)	**Crohn's Disease (n= 3)**	**Ulcerative colitis (n= 3)**
Age - years (IQR)	14.88 [12.7-16.1]	13.13 [10.8-15.7]	15.33 [13.1-16.1]
Disease duration - months (IQR)	39.4 [25.2-51.6]	43.18 [28.4-56.6]	22.6 [17.3-29.5]
Disease activity index at inclusion – n (IQR)			
PCDAI		14.2 [7 - 19]	-
PUCAI		-	15.1[8-21.3]
Gender - n (%)			
Female	3 (50)	1 (33.3)	2 (66.7)
Male	3 (50)	2 (66.7)	1(33.3)

Abbreviations: IQR, interquartile range; PCDAI, Pediatric Crohn's disease activity index; PUCAI, Ulcerative Colitis Activity index.

To quantify infliximab by AFM, a functionalized gold structure was constructed. In particular the gold surface is covered by self-assembled monolayer (SAM) composed by top oligo ethylene glycol (TOEG). Single stranded thiolated DNA (cF9-SH) is immobilized

on surface using nanografting, an AFM-based nanolithography technique. cF9_TNFα conjugate is subsequently immobilized on nanospots through DNA-DNA interactions. The resulting structure is capable of capturing the target (infliximab) in a specific manner. The system was initially fine-tuned by testing infliximab concentration (10-20-30-40-50 nM) in buffer Tris EDTA (TE) 1M NaCl pH 8, and then in healthy donor serum (Figure 10). The preliminary results have shown a good correlation between height variation and drug concentration obtained in TE buffer and control serum (linear regression: R^2=0.9638 and p-value= 0.0030; R^2=0.9275 and p-value=0.002 respectively).

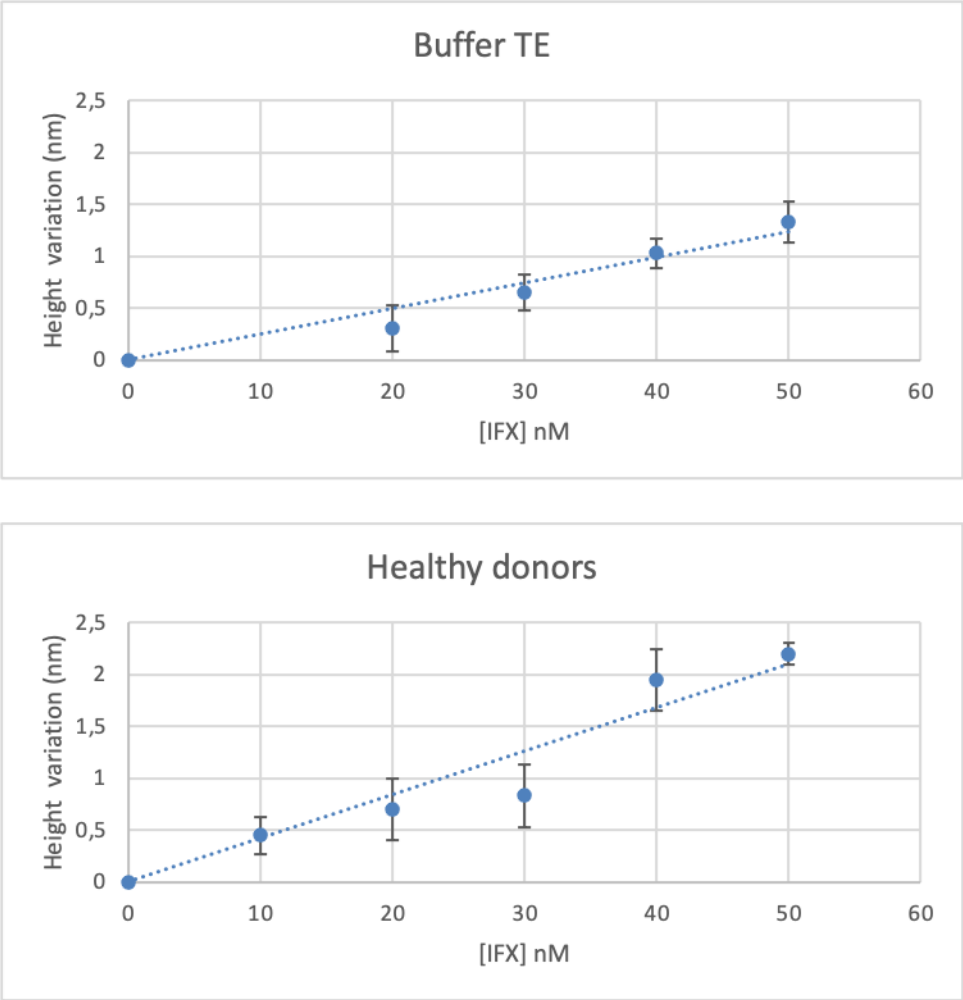

Figure 10: *Calibration obtained in Buffer Tris EDTA (TE) 1M NaCl pH 8 and healthy donor sera.*

The sera of 6 pediatric IBD patient were previously analysed by the gold standard ELISA. The data obtained were then used to correlate infliximab concentration and height variation measured by AFM. The preliminary results have shown a good correlation between height variation and drug concentration (R^2=0.98) (Figure 11).

However, a higher height variation was observed in pediatric patients compare to healthy donor and buffer TE samples.

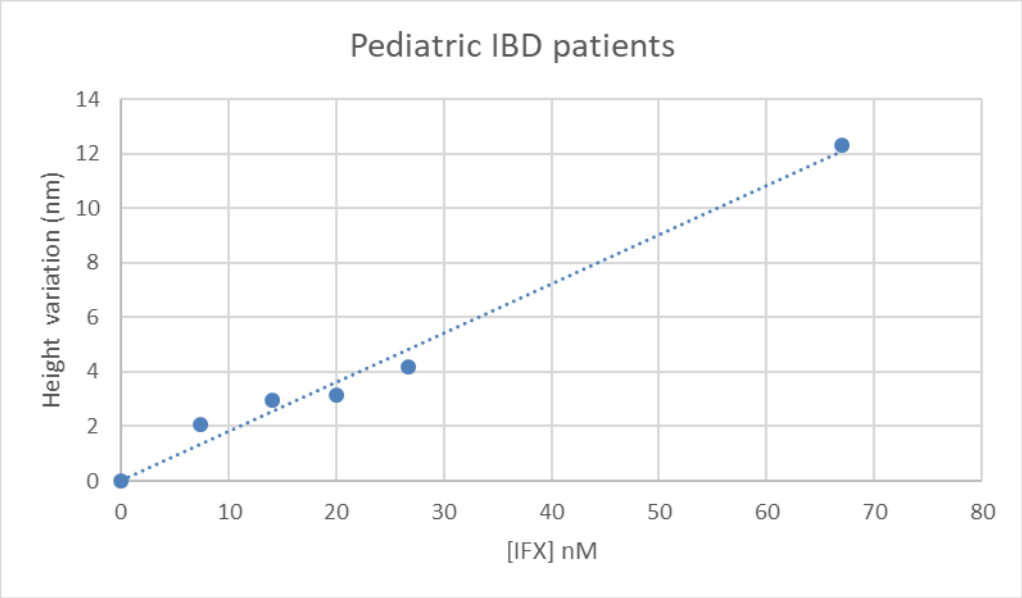

Figure 3: *Calibration line obtained in six pediatric patients' sera.*

4.3 Correlation between adalimumab levels at the end of induction and early during maintenance for long term response and anti-drug antibodies production.

4.3.1 IBD patients' cohort

Thirty-two pediatric patients with IBD were enrolled. The demographic characteristics of the population are summarised in Table 5. Twenty-eight patients (87.5%) had stopped infliximab before adalimumab, and 4 patients (12.5%) were anti-TNFα naive. Infliximab was stopped because of adverse events in 13 (46.4%) patients, loss of response due to anti-infliximab antibodies in 8 (28.6%) patients and loss of response during induction phase in 7 (25%) patients.

At adalimumab start, 8 (28.6 %) patients were in remission and had biochemical alterations (elevated CRP and fecal calprotectin levels); however the pre-adalimumab therapy score was not associated with response or adalimumab concentration at later timepoints (pre-adalimumab therapy score vs clinical response at 4, 22, 52 and 82 weeks: $p = 0.47$, $p = 0.11$, $p = 0.56$ and $p = 0.67$ respectively, pre-adalimumab therapy score vs adalimumab concentration at 4 and 22 weeks: $p = 0.88$ and $p = 0.86$ respectively).

Table 5. Demographic and clinical characteristics of the population enrolled.

	Overall (n= 32)	**Crohn's Disease (n= 24)**	**Ulcerative colitis (n= 8)**
Age - years (IQR)	14.88 [12.1-16.7]	14.72 [11.2-16.3]	16.46 [14.3-17.3]
Disease duration - months (IQR)	41.73 [23.3-67.4]	46.30 [31.2-68.8]	23.3 [19.9-30.9]
Gender - n (%)			
Female	12 (37.5)	7 (29.2)	5 (62.5)
Male	20 (62.5)	17 (70.8)	3 (37.5)
Disease activity index at inclusion – n (IQR)			
PCDAI		15 [8 - 20]	-
PUCAI		-	15 [8–20]
Concomitant therapy – n (%)			
Steroids	6 (18.8)	5 (20.8)	1 (12.5)
Methotrexate	2 (6.3)	2 (8.3)	0
Azathioprine	1 (3.1)	1 (4.2)	0
Thalidomide	2 (6.3)	2 (8.3)	0

Abbreviations: IQR, interquartile range; PCDAI, Pediatric Crohn's disease activity index; PUCAI, Ulcerative Colitis Activity index.

Sixteen, 15, 14 and 12 patients were in remission at week 4, 22, 52 and 82, respectively. All patients were steroid-free, and three patients stopped immunomodulators within six months. Five (15.6%) patients lost the initial clinical response: 2 recovered spontaneously while 3 shortened intervals between injections without the need for additional treatments, including steroids. Three (9.4%) patients were lost at follow up before week 52, and 7 (21.9%) patients before week 82. One (3.1%) patient discontinued treatment because of an adverse event (anaphylactoid reaction), which however occurred after 52 weeks of therapy, allowing his inclusion in the study. No statistical association between clinical response and demographic (age, gender) and clinical (type of IBD) characteristics of the population was found after logistic regression analysis ($p > 0.05$).

4.3.2 Correlation between adalimumab concentration, disease activity and clinical and biochemical variables.

Serum adalimumab levels at 4 weeks were correlated with clinical remission during maintenance. Higher serum adalimumab levels at week 4 were found for patients in clinical remission compared to patients with clinically active disease at week 22 (22.72 µg/ml IQR: 8.4 µg/ml vs 8 µg/ml IQR: 5.08 µg/ml, $p = 0.0029$), at week 52 (22 µg/ml IQR: 8.4 µg/ml

vs 8 µg/ml IQR: 4.66 µg/ml, p = 0.003) and at week 82 (22.72 µg/ml IQR: 9.39 µg/ml vs 8 µg/ml IQR: 4.5 µg/ml, p = 0.003) (Figure 12). The same trend was observed also considering shorter timeframes in the induction interval and long-term response (Figure 13).

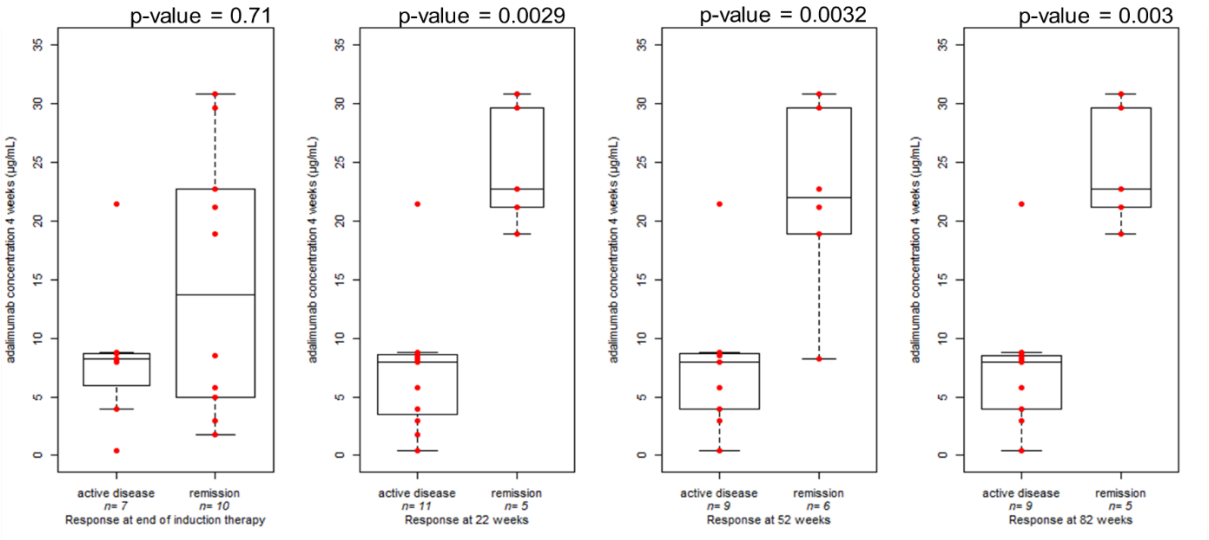

Figure 12: *Boxplot comparing clinical disease activity at the end of induction (17 measurements in 13 patients), at 22 (16 measurements in 12 patients), 52 (15 measurements in 11 patients) and 82 weeks (14 measurements in 10 patients) and serum adalimumab concentration during induction therapy (4 weeks). The bold horizontal line represents the median value. P-values are from linear mixed effect model (LME), accounting for repeated measures.*

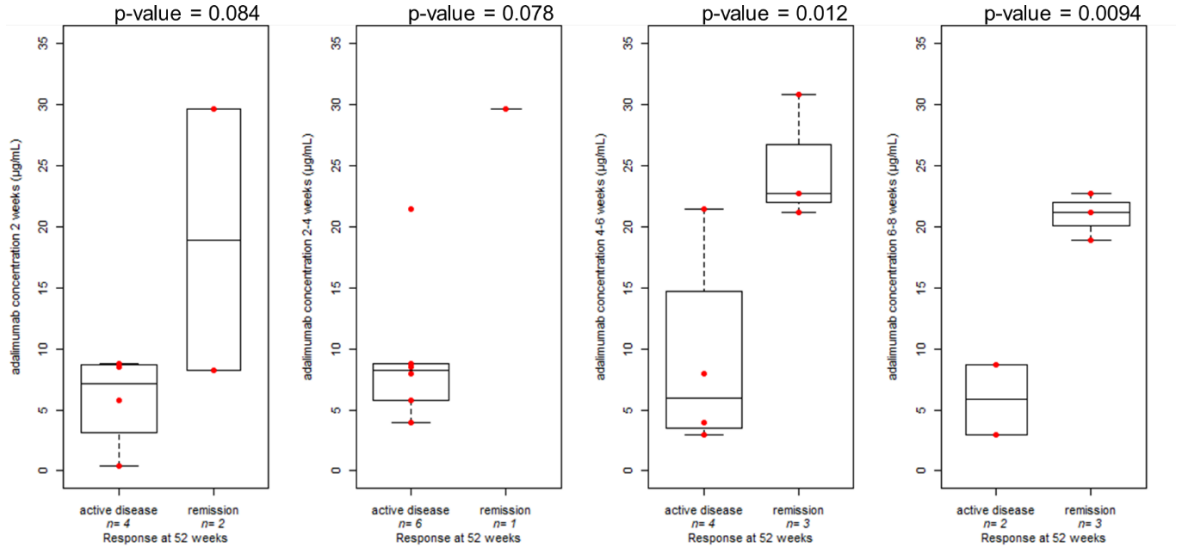

Figure 13: *Correlation between long term response and adalimumab levels considering shorter timeframes in the 2-8 weeks interval.*

However, median adalimumab levels measured at week 4 did not differ between patients in clinical remission and patients with clinically active dis-ease at the end of induction (13.7 µg/ml IQR: 17.13 vs 8.26 µg/ml IQR: 2.73 µg/ml, p = 0.71) and the same trend was observed considering median adalimumab levels at week 22 and the clinical response at

weeks 22 (7.04 µg/ml IQR: 9 µg/ml vs 7.14 µg/ml IQR: 10.34 µg/ml, p = 0.85). Patients in remission at weeks 52 and 82 had higher adalimumab levels at week 22 compared to patients with clinically active disease (12.44 µg/ml IQR: 9.62 µg/ml vs 6.38 µg/ml IQR: 3.82 µg/ml, p = 0.09; and 10.8 µg/ml IQR: 9.72 µg/ml vs 5.4 µg/ml IQR: 5.09 µg/ml, p = 0.016, respectively) (Figure 14).

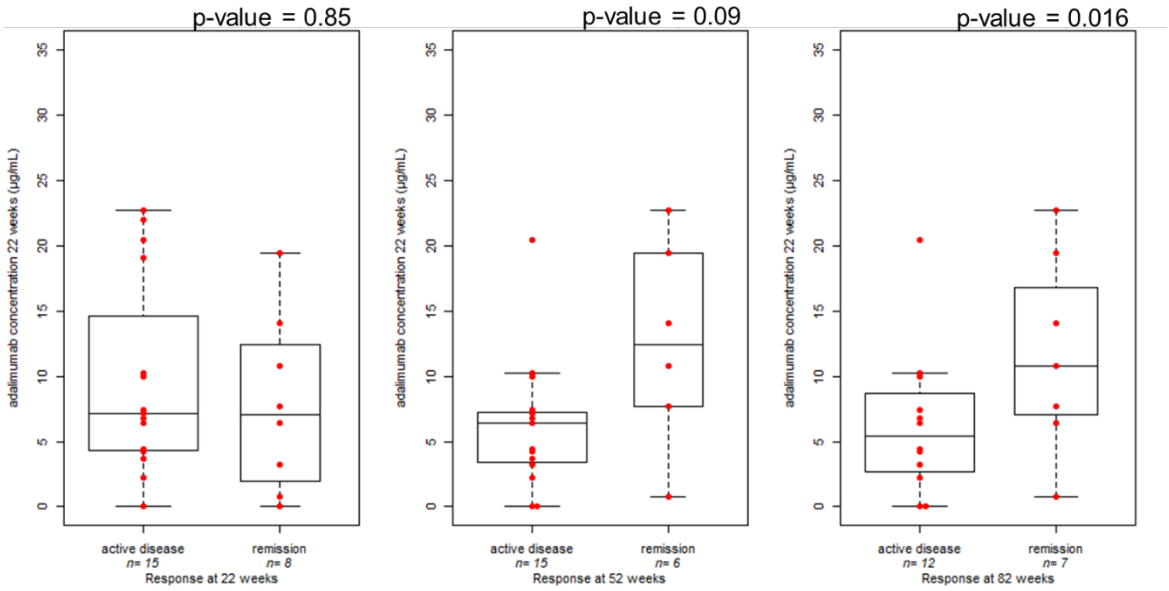

Figure 14: *Boxplot comparing clinical disease activity at 22 (23 measurements in 19 patients), 52 (21 measurements in 18 patients) and 82 (19 measurements in 16 patients) weeks of treatment and serum adalimumab concentration during week 22. The bold horizontal line rep-resents the median value. P-values are from linear mixed effect model (LME), accounting for repeated measures.*

No significant difference in adalimumab dose, normalised on patients' weight was observed at week 4, while at week 22, patients with clinically active disease were treated with higher adalimumab weight normalised doses (Figure 15).

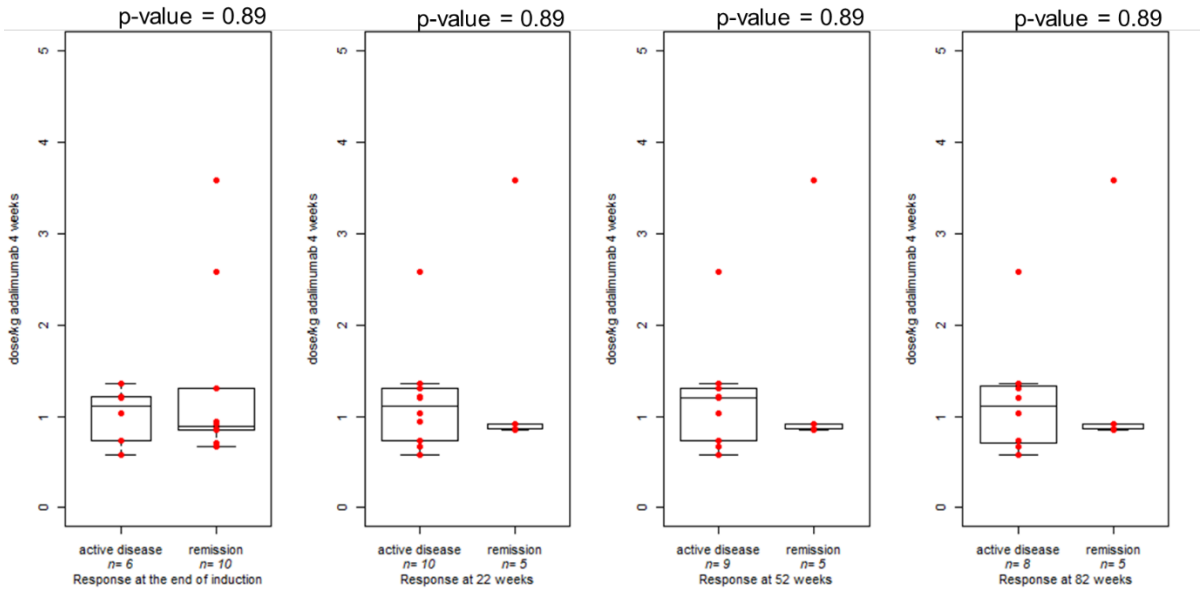

Figure 15: *Correlation between clinical response and adalimumab levels after dose adjustment normalised on patients' weight.*

ROC curves were constructed to assign optimal cut-off values for adalimumab levels at the end of induction therapy and after early maintenance phase to predict clinical response at 52 and 82 weeks (Figure 16). Optimal cut-off values were 13.85 µg/ml and 7.54 µg/ml at week 4 and 22, respectively, to predict clinical response at week 52. The cut-off values of 13.85 µg/ml and 10.51 µg/ml were obtained at weeks 4 and 22 respectively, to predict clinical response at week 82. Logistic regression analysis confirmed that patients who reached the cut-off point of 13.85 µg/mL at the end of induction (6 patients, 5 in sustained remission both at weeks 52 and 82) had a higher probability of maintaining remission at week 52 and week 82 compared to those who did not (9 patients, only 1 in sustained remission at week 52; 8 patients, none in remission at week 82), with an odds ratio (OR) of 40.0 (95% confidence interval (CI) 2.0 – 794.3, p = 0.015) and OR of 62.33 (95% CI 2.13 – 1822.7, p = 0.016) respectively at weeks 52 and 82.

Patients above the cut-off point of 7.54 µg/mL during week 22 (8 patients, 5 reached remission at week 52) had a higher probability of maintaining remission at week 52 compared to those who did not (13 patients, only 1 in sustained remission at week 52) with an OR of 20 (95% CI 1.65 – 241.7, p = 0.018). Patients with the cut-off point of 10.51 ug/mL during week 22 reached remission at 82 weeks (5 patients, 4 in sustained remission); compared to those who did not (14 patients, only 3 in sustained remission) with an OR of 14.66 (95% CI 1.16 – 185.24, p = 0.037).

The area under the ROC curve (AUC) was 88.9% at week 4 and 78.9% at week 22, considering the response at week 52, and 95.6% and 73.8% respectively at weeks 4 and 22, considering the response at week 82. The test had a sensitivity of 83.3% and specificity of 88.8% considering week 4 (positive predictive value, PPV 83.3%; negative predictive value, NPV 88.8%) and a sensitivity of 83.3% and specificity of 80% at week 22 (PPV 62.5%; NPV 92.3%) for predicting sustained remission at week 52. A sensitivity of 100% and a specificity of 88.9% were obtained considering week 4 (PPV 83.3%; NPV 100%) and a sensitivity of 57.14% and a specificity of 91.66% considering week 22 (PPV 80%; NPV 78.57%) for predicting sustained remission at week 82.

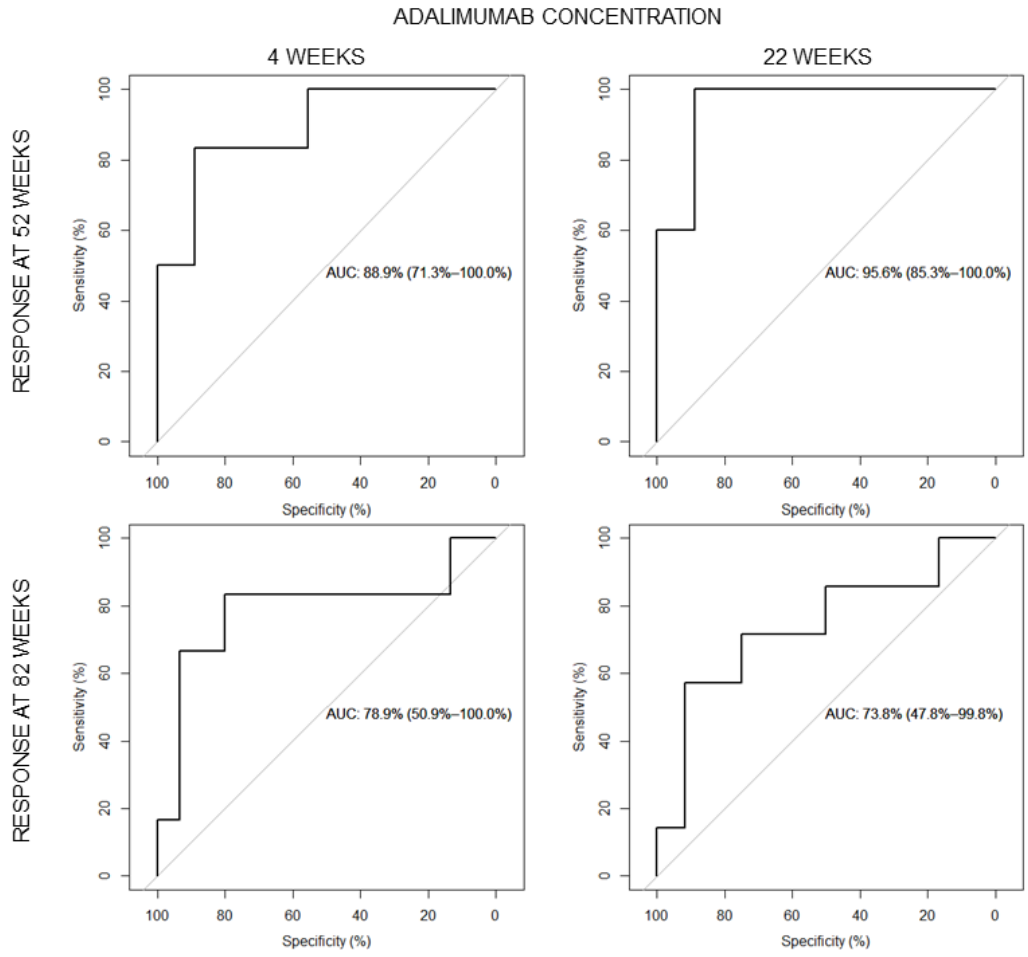

Figure 16: *Areas under the ROC curves for the serum adalimumab quantification at the end of induction and week 22 for clinical disease activity at 52 (above) and 82 weeks (below).*

Sex, age and IBD type were not significantly associated with adalimumab concentration. The cause of infliximab failure and the concomitant treatment with steroids or immuno-modulators did not affect adalimumab levels ($p = 0.97$). A statistically inverse correlation ($p < 0.05$, Spearman's test) between serum adalimumab levels and clinical laboratory

variables, including disease activity score, CRP and fecal calprotectin was observed (Figure 17).

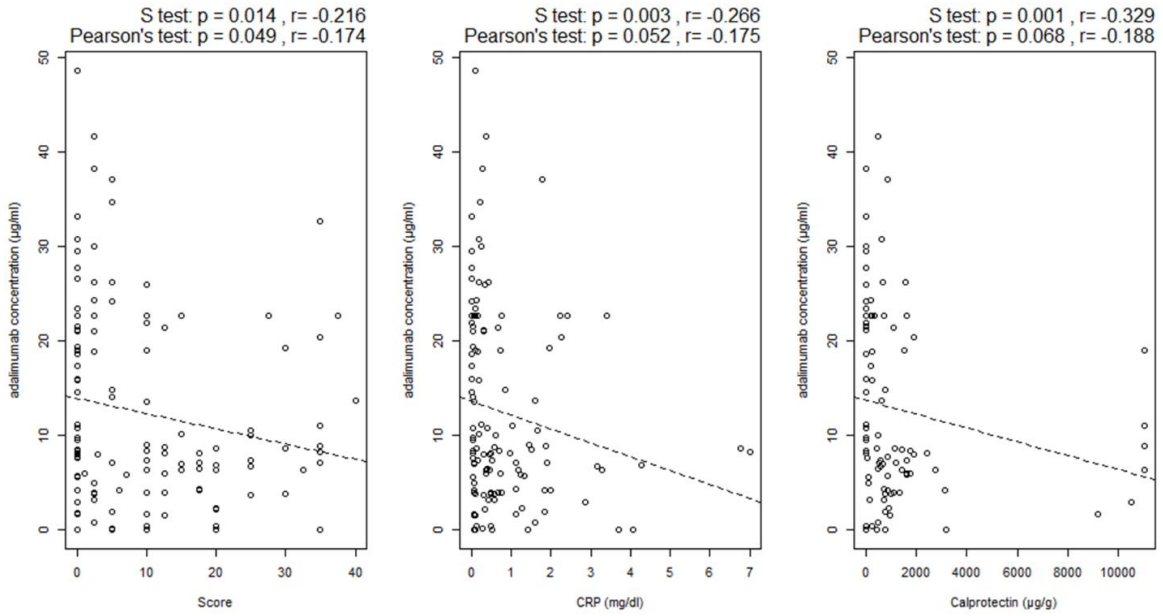

Figure 17: *Concentration of adalimumab was significantly inversely correlated with the clinical score (129 measurements in 32 patients), CRP (124 measurements in 32 patients) and fecal calprotectin (95 measurements in 27 patients).*

The adalimumab cut-off value for sustained remission (i.e. 13.85 µg/ml at the end of induction) significantly correlated with fecal calprotectin measured during the induction: patients not achieving the cut-off for adalimumab concentrations had significantly higher fecal calprotectin compared to others, and the same trend was observed for CRP (Figure 18).

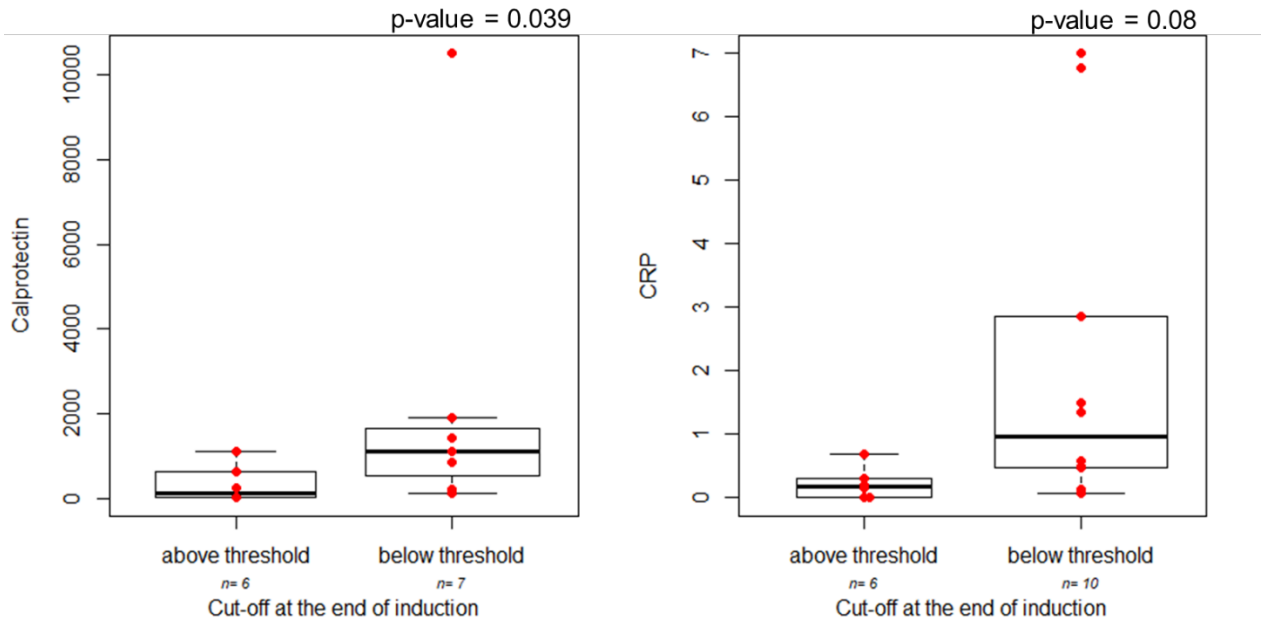

Figure 4: *Levels of CRP and calprotectin in patients with a concentration of adalimumab below or above the threshold level associated with sustained response.*

4.3.3 Correlation between serum adalimumab levels and anti-drug antibodies

The concentrations of anti-drug antibodies were measured in 9 samples from 6 patients (18.8%) that showed serum adalimumab levels below 1.5 µg/ml. Only 4 samples from 2 patients (6.3%) resulted positive to anti-drug antibodies. None had previously developed anti-infliximab antibodies. The concentrations of anti-drug antibodies were inversely correlated with adalimumab levels, but the correlation was marginally significant (Spearman test, p = 0.073) (Figure 19).

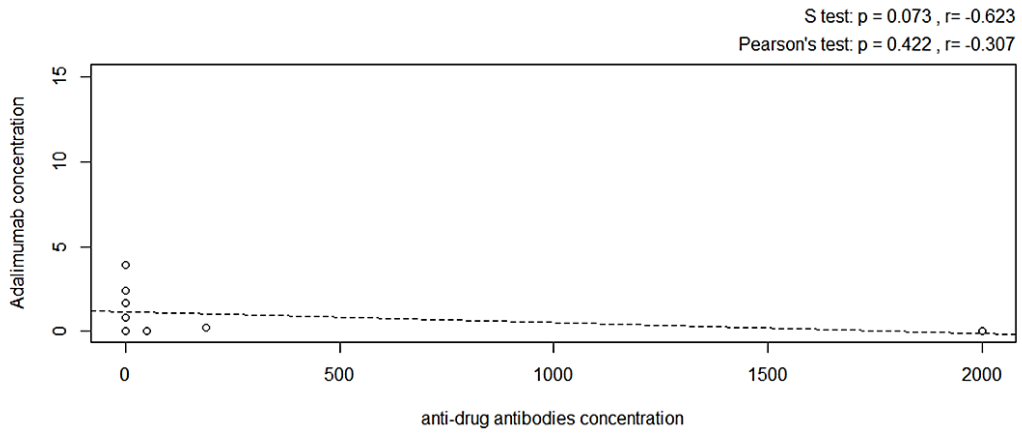

Figure 19: *Concentration of adalimumab was significantly inversely correlated with the anti-drug antibodies production.*

4.4 Correlation between *FCGR3A*, *IL6* and *TNFα* genetic variants and infliximab clinical response, pharmacokinetics and anti-drug antibodies production in pediatric IBD patients.

4.4.1 IBD patients' cohort

Seventy-six children in treatment with infliximab were enrolled by the Gastroenterology Unit of IRCCS Burlo Garofolo Hospital in Trieste and the Pediatric Unit of Ca' Foncello Hospital in Treviso. The demographic characteristics of the population are summarized in Table 6.

Table 6. Demographic and clinical characteristics of the population enrolled.

	Overall (n= 76)
Age - years (IQR)	14.7 [12.3-16.3]
Disease duration - months (IQR)	40.3 [27.1-60.2]
Disease type - n (%)	
CD	50 (65.8)
UC	26 (34.2)
Gender - n (%)	
Female	37 (48.7)
Male	39 (51.3)
Primary failure – n (%)	24 (31.6)
Secondary failure – n (%):	
At 22 weeks	31 (48.4)
At 52 weeks	30 (48.4)
ADA production	13 (56.5)
Concomitant therapy – n (%)	
Steroids	5 (12.8)
Azathioprine+ steroid	29 (74.4)
Azathioprine	4 (10.2)
Methotrexate	1 (2.6)

Abbreviations: IQR, interquartile range; CD, Crohn disease; UC, ulcerative colitis; ADA, anti-drug antibodies; n, number.

Considering demographical variables, neither gender nor age or IBD type were significantly associated with infliximab clinical response at the end of induction ($p = 0.18$, $p = 0.1$ and $p = 0.8$ respectively), at 22 weeks ($p = 0.12$, $p = 0.96$ and $p = 0.47$ respectively) and with anti-drug antibody production ($p = 0.57$, $p = 0.6$ and $p = 0.5$ respectively) after univariate logistic regression analysis. A significant association between gender and clinical response at 52 weeks was found with an increased therapy failure in girls compared to

males (p = 0.035). However, no association was found with age and IBD type (p=0.6 and p=0.2 respectively). Evaluating the possible confounding effect of concomitant treatment (antimetabolite drugs and steroids) on infliximab response, no association was found with the response at the end of induction (p = 0.62), at 22 weeks (p = 0.31), at 52 weeks (p = 0.2) and with anti-drug antibodies production (p = 0.83).

4.4.2 Association between SNP variations and clinical response

The effect of the *FCGR3A* SNP on infliximab response and anti-drug antibodies production was assessed. The genotype distribution of the population is summarized in Table 7. Genotype frequencies were in Hardy–Weinberg equilibrium (HWE, p > 0.05).

Table 7. *FCGR3A* SNP and genotype distribution of pediatric IBD patients

GENE	SNP	POSITION	IBD PEDIATRIC PATIENTS (N=76)			
			wt (%)	hz (%)	mut (%)	HWE
FCGR3A	rs396991	chr1:161544752; 559 A>G (F158V)	33 (43.4)	29 (38.2)	14 (18.4)	0.1

Abbreviations: HWE, Hardy–Weinberg equilibrium; wt, wild type; hz, heterozygote; mut, mutated.

A significant association between the *FCGR3A* SNP and clinical response at the end of induction (p = 0.004, odds ratio (OR) FF vs VF genotype = 6.76, CI = 1.89-24.19; OR FF vs VV genotype = 5.43, CI = 1.22-24.07), at 22 weeks (p = 0.001, OR FF vs VF genotype = 8.9, CI = 2.52-31.41; OR FF vs VV genotype = 4.4, CI = 1.05-18.35) and at 52 weeks (p = 0.001, OR FF vs VF genotype = 6.6 , CI = 1.91-23.17; OR FF vs VV genotype = 2.5, CI = 0.61-10.11) was observed (Figure 20).

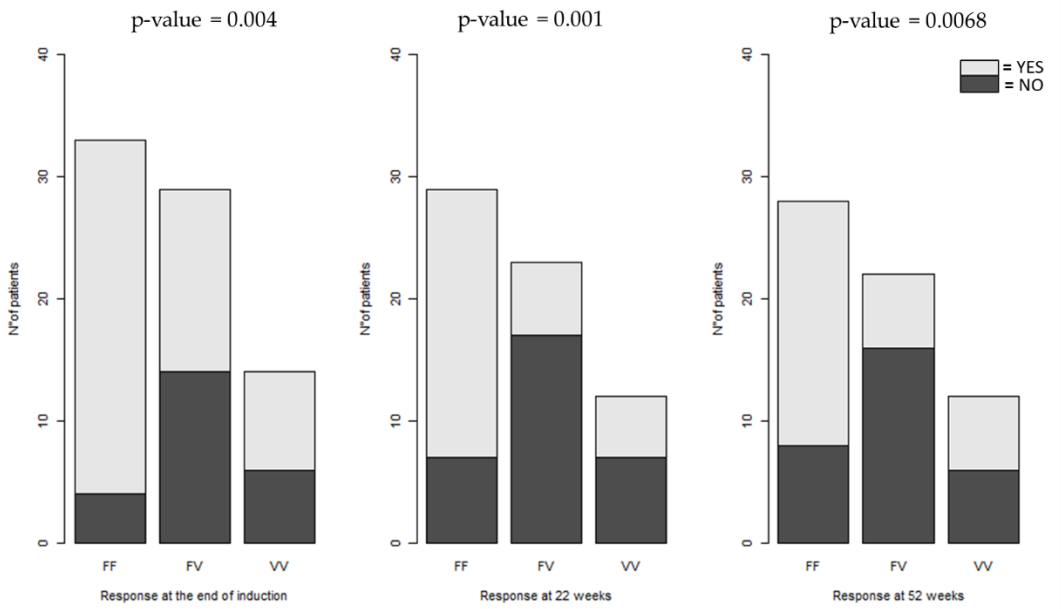

Figure 20: *Correlation between FCGR3A SNP, clinical response at the end of induction and during maintenance (at 22 and 52 weeks). Logistic regression model was used for statistical analysis.*

No significant association was found between *IL6* SNP (genotype distribution in Table 8) and clinical response at the end of induction (p = 0.8, OR GG vs GC genotype = 1.14, CI = 0.38-3.39; OR GG vs CC genotype = 1.56, CI = 0.39-6.24), at 22 weeks (p = 0.8, OR GG vs GC genotype = 0.85, CI = 0.28-2.55; OR GG vs CC genotype = 0.6, CI = 0.13-2.79) and at 52 weeks (p = 0.8, OR GG vs GC genotype = 1, CI = 0.3-3.05; OR GG vs CC genotype = 0.66, CI = 0.14-3.1) (Figure 21).

Table 8. *IL6* SNP and genotype distribution of pediatric IBD patients

GENE	SNP	POSITION	IBD PEDIATRIC PATIENTS (N=76)			
			wt (%)	hz (%)	mut (%)	HWE
IL6	rs1800795	chr7:22727026; -174 G>C	28 (36.8)	35 (46.1)	13 (17.1)	0.8

Abbreviations: HWE, Hardy–Weinberg equilibrium; wt, wild type; hz, heterozygote; mut, mutated.

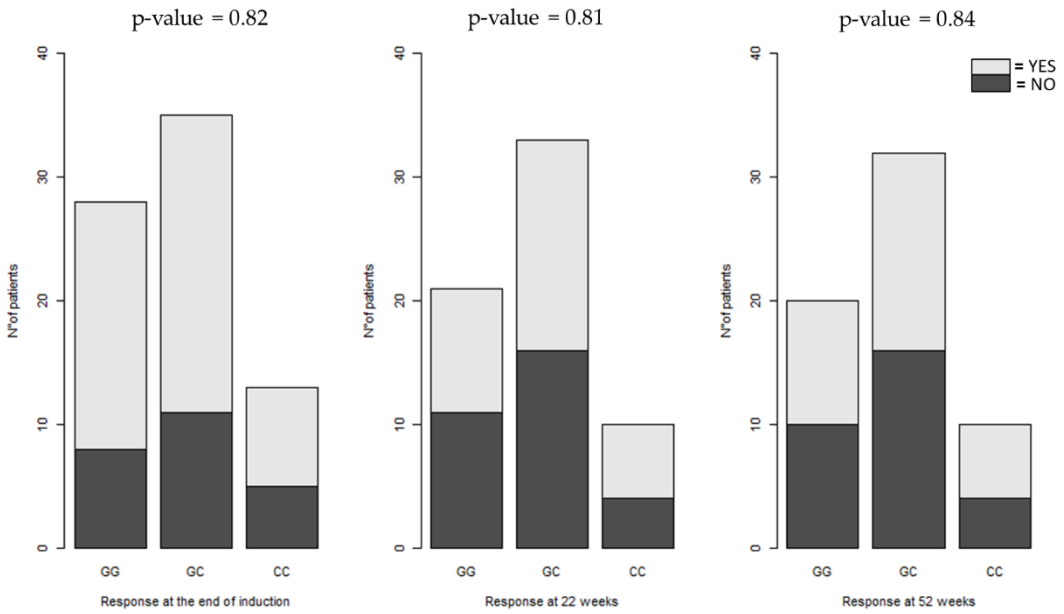

Figure 21: *Correlation between IL6 SNP, clinical response at the end of induction, and during maintenance (at 22 and 52 weeks) and ADA production. Logistic regression model was used for statistical analysis.*

The same trend was found between the *TNFα* SNP (genotype distribution in Table 9) and clinical response at the end of induction (p = 0.8, OR GG vs AG genotype = 1.13, CI = 0.34-3.81; OR GG vs AA genotype = 2.27, CI = 0.13-38.5), at 22 weeks (p = 0.7, OR GG vs AG genotype = 1.75, CI = 0.44-6.9; OR GG vs AA genotype = 1.16, CI = 0.07-19.67), at 52 weeks (p = 0.7, OR GG vs AG genotype = 1.94, CI = 0.42-9; OR GG vs AA genotype = 1.2, CI = 0.07-19.7) (Figure 22).

Table 9. *TNFα* SNP and genotype distribution of pediatric IBD patients

GENE	SNP	POSITION	IBD PEDIATRIC PATIENTS (N=76)			
			wt (%)	hz (%)	mut (%)	HWE
TNFα	rs1800629	chr6:31575254; -308 G>A	59 (77.6)	15 (19.8)	2 (2.6)	0.3

Abbreviations: HWE, Hardy–Weinberg equilibrium; wt, wild type; hz, heterozygote; mut, mutated.

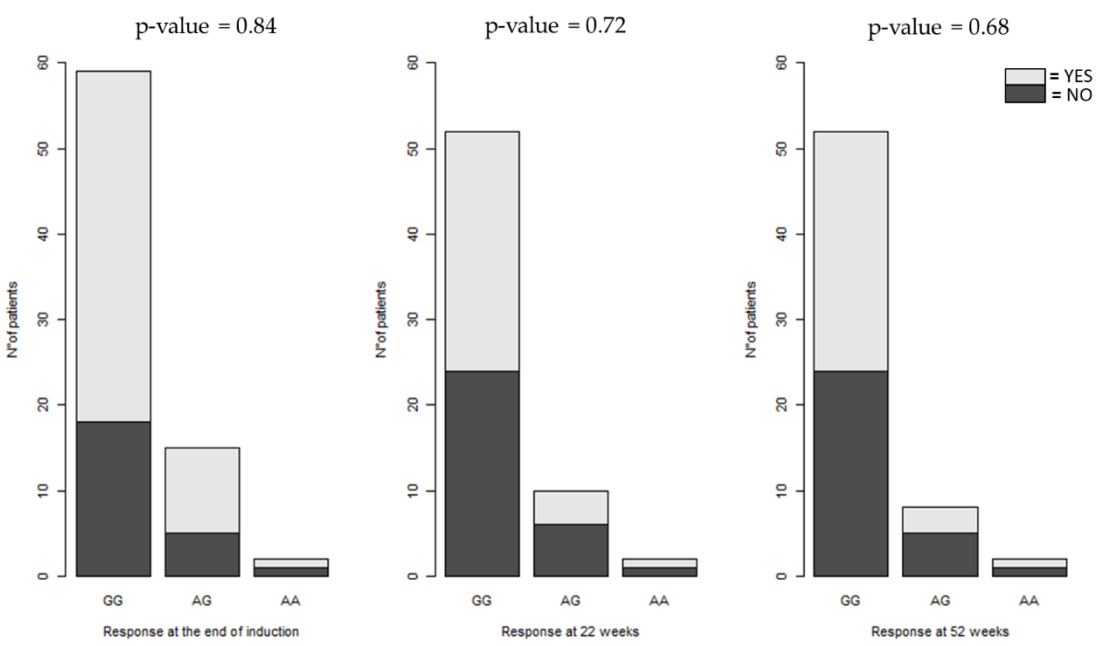

Figure 22: *Correlation between TNFα SNP, clinical response at the end of induction, and during maintenance (at 22 and 52 weeks) and ADA production. Logistic regression model was used for statistical analysis.*

4.4.3 Association between serum infliximab levels, anti-drug antibodies production and *FCGR3A*, *IL6* and *TNFα* polymorphisms.

The correlation between infliximab pharmacokinetic and *FCGR3A*, *IL6* and *TNFα* polymorphisms was evaluated in a subgroup of patients, based on the availability of patient's sera (N=56). The data have shown a significant association between the *FCGR3A* variant and median infliximab levels measured during maintenance phase: patients with the FF genotype had higher infliximab levels compared to patient with the variant allele V (6.8 µg/ml, IQR:9.2; vs 1.23 µg/ml, IQR:1.3; vs 2.6 µg/ml, IQR:3; p = 0.04 for FF, VF and VV respectively). No significant association was found between serum infliximab concentration and the IL6 (6 µg/ml, IQR:5.9; vs 2.18 µg/ml, IQR:9.17; vs 4µg/ml, IQR:8.6; p = 0.22 for GG, GC and CC respectively) and *TNFα* (4.2 µg/ml, IQR:8.8; vs 2.08 µg/ml, IQR:10.2; vs 4.1 µg/ml, IQR:0; p = NA for GG, AG and AA respectively) genotypes (Figure 23).

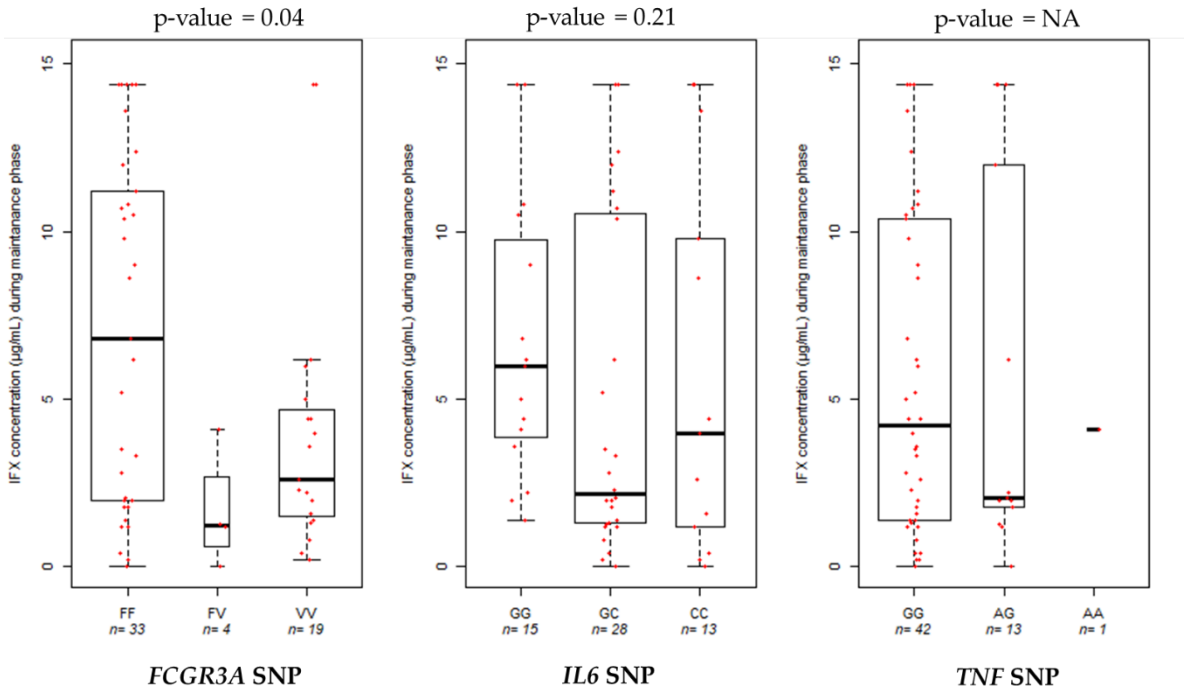

Figure 23: *Boxplot comparing serum infliximab (IFX) concentration and TNFα genotypes. The bold horizontal line represents the median value. Statistical significance was assessed by Kruscal Wallis test.*

A significant association was found between *FCGR3A* SNP and anti-drug antibodies production (p = 0.024). In particular, patients with the variant allele V had a higher probability to produce ADA (OR FF vs VF genotype = 14, CI = 1.54-127.2; OR FF vs VV genotype = 10.5, CI = 0.66-165.1). No association was found between ADA production and *IL6* (p = 0.1, OR GG vs GC genotype = 0.9, CI = 0.11-6.7; OR GG vs CC genotype = 0.079, CI = 0.003-2.24) and *TNF* SNP (p = 0.5, OR GG vs AG genotype = 0.77, CI = 0.11-5.1; OR GG vs AA genotype = 2.3, CI =0.083-66.88) (Figure 24).

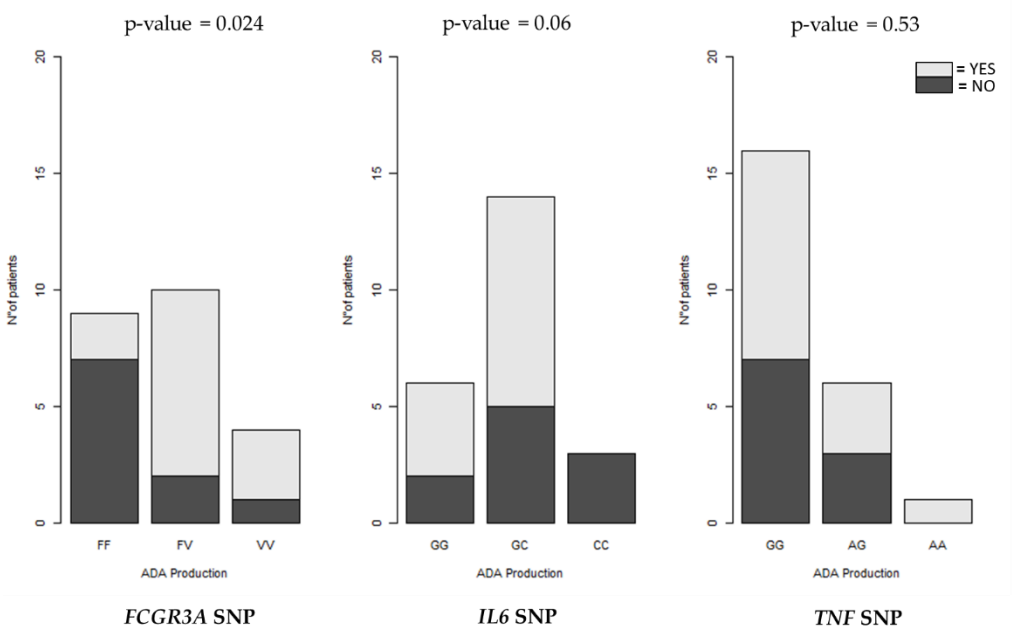

Figure 24: *Boxplot comparing the production of anti-drug antibodies (ADA), measured in patient with infliximab levels below 1.5 µg/mL, and FCGR3A, IL6 and TNF genotypes. The bold horizontal line represents the median value. Statistical significance was assessed by LME.*

4.4.4 Multivariate analysis

Multivariate analysis was performed to test the potential confounding effect, on the association between therapeutic response to infliximab, genetic variants (*FCGR3A, IL6* and *TNFα* SNPs) and demographic covariates. This multivariate analysis was done by a logistic regression generalized linear model, using therapeutic response as the dependent variable and genetic variants with all covariates significantly associated with these SNPs in the univariate analysis (only the gender), as independent variables. Multivariate analysis confirmed that the *FCGR3A* SNP was associated with clinical response at the end of induction, at 22, at 52 weeks of treatment (p= 0.013, p=0.001 and p=0.014) and with anti-drug antibodies production (p = 0.0003). Furthermore, also the female gender was associated with the loss of long-term response at 52 weeks (p = 0.045).

4.5 An in vitro model for the study of infliximab mechanism of action and the role of CD69 as marker of infliximab response

4.5.1 Evaluation of CD69 expression in Jurkat cells after infliximab treatment

To evaluate the possible role of CD69 as marker of infliximab response, Jurkat cells are activated or not with PMA/ionomycin (0.05/0.96 µg/mL) for 4 hours and treated with different concentration of infliximab (0-1-10 and 100 µg/mL) overnight. The expression of CD69, in term of mean fluorescence intensity (MFI), was evaluated by flow cytometry analysis. As reported in Figure 25, CD69 MFI decreased from 100 to 91.7 ± 3.61, 87.4 ± 6 and 73.1 ± 0.98 % after treatment with infliximab 0, 1, 10 and 100 µg/mL respectively ($p \leq 0.0001$) (Figure 25).

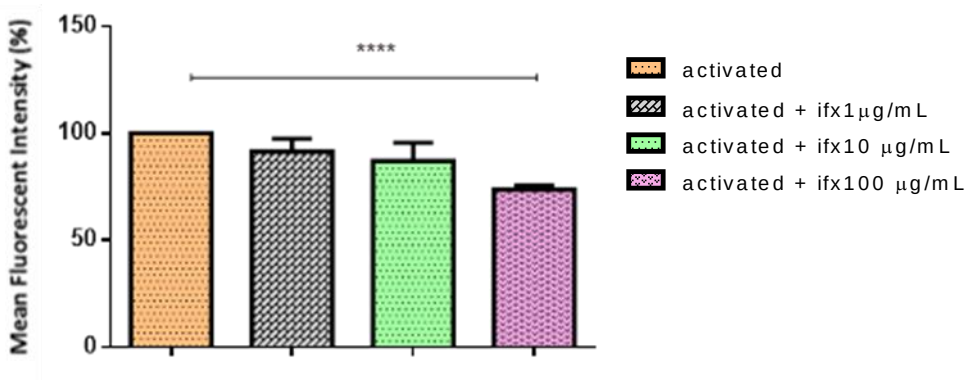

Figure 25: *Cytofluorimetric analysis reveals that CD69 cell surface expression decreases from 100 to 91.7 ± 3.61, 87.4 ± 6 and 73.1 ± 0.98 % in activated Jurkat cells treated with 0 (control), 1, 10 and 100 µg/mL of infliximab (IFX) overnight, respectively (Two-way ANOVA: p-value < 0.0001; Bonferroni's multiple comparisons test: *= p < 0.05; ** = p < 0.01; ***= p < 0.001; **** = p < 0.0001.*

4.5.2 Evaluation of TNFR1 and TNFR2 expression in Jurkat cells after infliximab treatment

To characterize the Jurkat T cell model and to investigate the mechanism by which CD69 MFI decreased after infliximab treatment, a real-time PCR was performed to evaluate the expression of TNFR1 and TNFR2 (Figure 26). Unstimulated Jurkat cells express only the TNFR1 receptor and not the TNFR2. After activation with PMA/ionomycin for 4h, the expression of TNFR1 increased significantly but not TNFR2 (One-way ANOVA, $p < 0.0001$) (Figure 26) suggesting that the signalling involved in Jurkat infliximab response is driven by TNFR1.

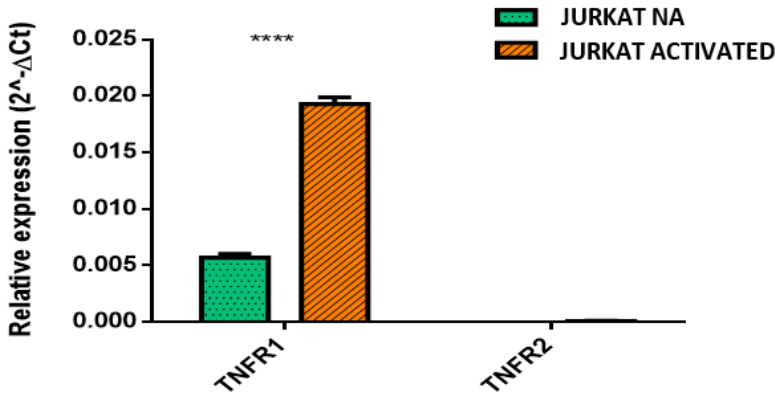

Figure 26: *Relative expression (RE) of TNFR1 and TNFR2 in Jurkat cells activated and not (NA) with PMA/iono: cells express more TNFR1 than TNFR2 (One-way Anova: p-value < 0.001). Gene expression values were normalized using beta actin as reference gene. The data are reported as means ± standard error (SE) of three technical replicates.*

4.5.3 The relationship between NF-κB, its inhibitor IkB, and CD69

The protein expression of p65 and its inhibitor IkB in Jurkat cells activated and not with PMA/ionomycin after treatment with infliximab 100 µg/mL overnight was assessed by western blot. As shown in figure 15, a significant difference among the groups analyzed was observed ($p < 0.0001$); in particular, the results demonstrated a significant decrease of IkB protein level in activated cells compared to the control (fold change = 0.6 ± 0.03). The treatment of activated Jurkat cells with infliximab 100 µg/mL led to an increase of IκB protein expression in comparison to untreated cells (fold change = 1.24 ± 0.1). No significant differences were observed considering p65 protein expression (fold change = 1.06 ± 0.07) (Figure 27).

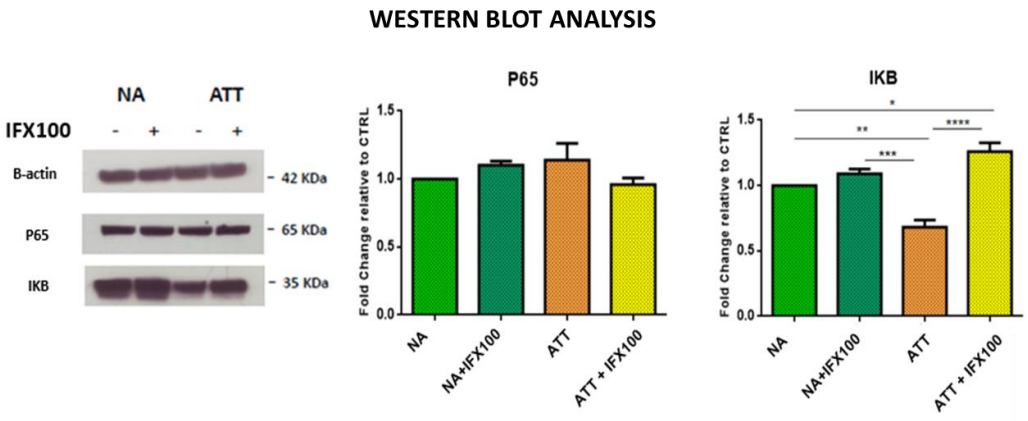

Figure 27: *Protein levels of P65 and IkB in control (NA) and activated Jurkat cells, treated and not with infliximab (IFX) 100 µg/mL. Activated cells have a lower IKB protein expression than ctrl (fold change = 0.6 ± 0.03). The treatment of activated Jurkat cells with infliximab 100 µg/mL led to an increase of IκB protein expression in comparison to untreated cells (fold change = 1.24 ± 0.1). Two-way ANOVA p < 0.0001, Bonferroni's multiple comparisons test: *= p < 0.05; ** = p < 0.01; ***= p < 0.001; **** = p < 0.0001.*

4.5.4 CD69 expression in PBMC of healthy donors after infliximab treatment

Preliminary data were obtained on CD4+ and CD8+ T cells, labelled with anti-CD4 and anti-CD8 human antibodies (Figure 28), from PBMCs of 3 healthy donors.

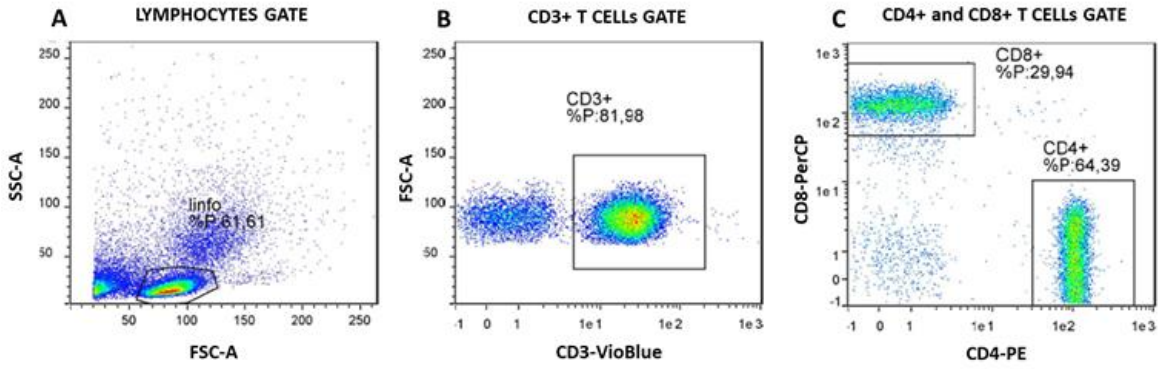

Figure 28: *Representative cytograms of T-cell subpopulations. In A the gate of the lymphocytes; in B the T-lymphocytes gated CD3+ and in C the T-lymphocytes gated CD4+ vs CD8+.*

In the CD4+ cells, CD69 MFI decreased from 100 to 90, 72.5 and 61.2% and in the CD8+ cells CD69 MFI decreased from 100 to 87.2, 69.2 and 48.5% after treatment with infliximab 0, 1, 10 and 100 µg/mL, respectively (Figure 29).

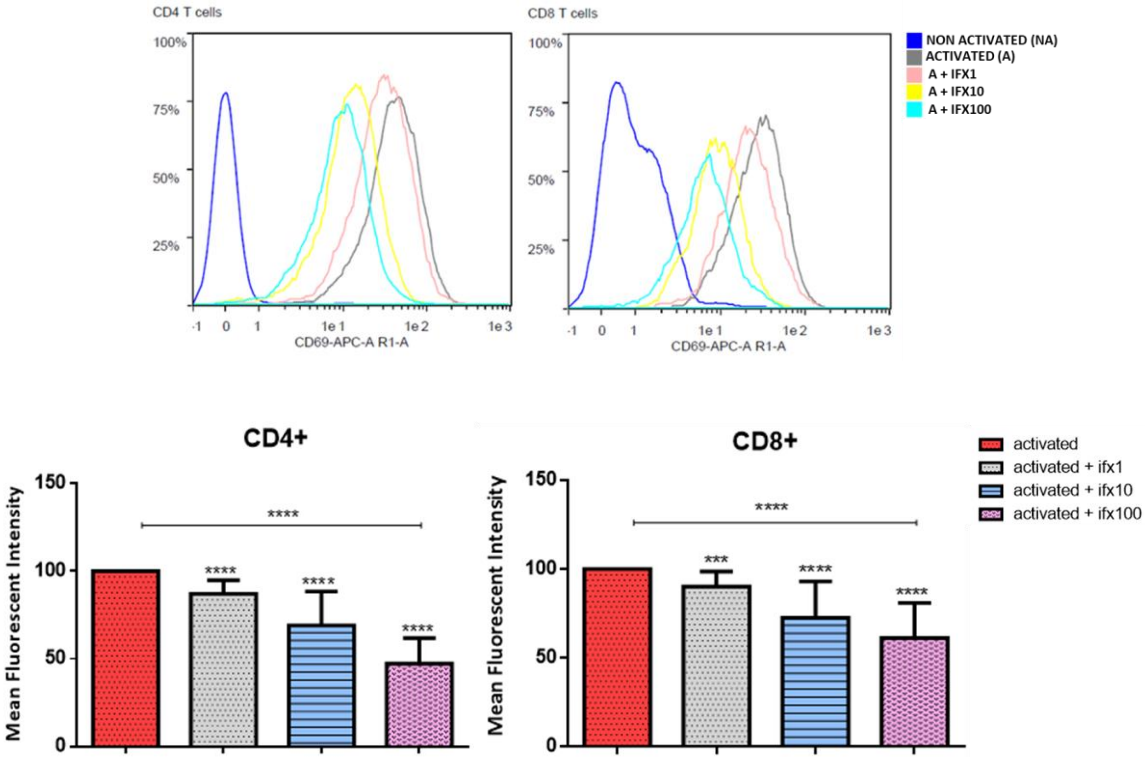

Figure 29*: Cytofluorimetric analysis reveals that CD69 cell surface expression decreases from 100 to 73.5, 30.9 and 23.3% and from 100 to 72.3, 31.3 and 22.4% after treatment with infliximab (IFX) 0, 1, 10 and 100 µg/mL overnight, respectively. in CD4+ and CD8+ T-cells isolated from PBMCs of healthy donors (Two-way ANOVA: p-value < 0.0001; Bonferroni's multiple comparisons test: *= p < 0.05; ** = p < 0.01; ***= p < 0.001; **** = p < 0.0001.*

Considering the CD8+ cell gate (30% of the T-lymphocytes), the percentage of CD69+ CD8+ cells decreased after infliximab treatment from 100 to 98.5, 93 and 89 %. No change was observed in the percentage of CD69+CD4+ selected cells after infliximab treatment.

5. DISCUSSION

In the first part of the study, we show that infliximab trough levels can be assessed with good accuracy with two POC assays in children with IBD. Limited studies have compared ELISA test and POC infliximab assays, only in adult patients showing a good accuracy and a reliable alternative to ELISA gold standard technique [85, 86] .A good agreement was observed between the POC assays and ELISA gold standard technique. In particular, the Bland-Altman plots showed a good dispersion between the reference ELISA assay and the POC devices, mostly at lower concentrations, which are the most interesting testing range from a clinical point of view. In this context, POC devices could be a good option for infliximab quantification in clinical practice especially when an immediate result is needed. The agreement was not perfect, and the POC IFX/QB seemed to overestimate the concentration of infliximab compared to the other assays. POC assays could improve the possibility of proactive dose adjustment in clinical practice. In the TAXIT and TAILORIX trials, the clinical utility of proactive strategy to dose infliximab on the basis of TDM was tested, however no significant differences in terms of clinical remission were observed in comparison with the gold standard strategy. In this context the use of ELISA assays could delay the dose adjustment at the next infusion leading to a lack of improvement in clinical outcomes. A recent study assessed the utility of proactive adjustment of infliximab therapy during induction phase in children and young adults. In the absence of concomitant immunosuppression, proactive TDM at week 10 may maintain higher infliximab concentrations during maintenance as established in a retrospective cohort of 83 IBD children and adolescents [87]. The data in our pediatric IBD cohort, suggest that POC assays can represent a possible alternative for TDM of infliximab in children with IBD, in particular when an immediate result is necessary to adjust the therapy and to achieve clinical remission. The use of POC is very useful in term of cost effectiveness, in particular when a limited number of samples have to be analysed. However, this rapid assay had very good positive predictive values for the detection of low drug levels but suboptimal sensitivity for high levels. In recent year the introduction of biosensors based on different nanostructured materials and techniques, such as the atomic force microscopy, in the rapid quantification of drugs, has been proposed for therapeutic drug monitoring.

AFM technique provides a real-time three-dimensional image under physiological conditions, with high imaging resolution and sensitivity. Accurate determination of cell stiffness, the determination of adhesion properties of cells, nanoparticle interaction with cells and drug quantification are possible application of AFM technique [28, 88].

On this basis, we have used AFM technique as an innovative tool to quantify infliximab in buffer TE, healthy donors and pediatric IBD patients' sera. A good agreement was observed between signal variation and drug concentration both in buffer and in control sera. The same trend was maintained in IBD patients' sera, but a higher signal variation was observed compared to healthy donors. These results could be explained by the fact that analysis in serum is complex because of the presence of high-abundance proteins, high level of salt and other interfered compounds that make difficult the detection of target analytes [89]. Probably patients' sera have different a protein profile with an increased expression of some proteins involved in inflammation and these characteristics could affect the AFM analysis. For this reason, the use of AFM as innovative tool for TDM is under investigation and requires further analysis and validations.

TDM of anti-TNFα agents is an important strategy to maximize the response rates in children with IBD. Differences in clinical response are common and several studies have considered the possibility that these differences are caused by pharmacokinetic and/or genetic factors.

In this context, our study showed that higher adalimumab levels during early treatment obtained from non–trough level serum samples predict long-term remission in children with IBD and that the cut-off value of 13.95 and 7.54 µg/ml at week 4 and 22 respectively, emerged as a predictor of remission at week 52 and 82. These findings are in line with studies in adult patients which showed that clinical remission and mucosal healing after 52 weeks of treatment could be predicted from adalimumab trough levels above 5 µg/mL measured at week 26 [90-92]. Similarly, it has been recently reported that in children with CD that have clinically responded to the first two doses of adalimumab, maintaining trough levels above 5 µg/mL starting on week 4 with a proactive strategy, is associated with a remission rate of 82% at week 72 [50]. It could be hypothesised that adalimumab dose could be intensified using drug concentrations measured at a definite time point (end of induction) to improve long-term efficacy. In this context, Rinawi and collegues have found that higher induction dose in early phase of therapy correlates positively with anti-drug antibodies trough levels at week 4 and 8 (22.5 and 12.5 µg/mL, respectively) leading to clinical remission at week 24, in pediatric IBD patients with CD [93]. Our results demonstrate an inverse correlation between drug levels and biochemical variables, in particular CRP and fecal calprotectin and the absence of a significant effect on adalimumab concentration of concomitant therapy with immunosuppressants, as previously reported

[57]. Anti-drug antibodies developed in 6.3% of patients, and a marginal inverse correlation was found between drug and antibody concentration. A previous study conducted by our team demonstrated that 20% of patients were positive to anti-infliximab antibodies. Therefore, in this cohort adalimumab appears less immunogenic than infliximab (6% vs 20% of patients have developed anti-drug antibodies) [33] probably because of the humanisation level of the antibody.

In addition to pharmacokinetic factors, also genetic interindividual variability could affect the clinical response to anti-TNFα agents. Pharmacogenomic variants involved in anti-TNFα drug pharmacokinetics may hence be promising, in consideration of the increasing importance attributed to drug levels in clinical outcomes.

Our results have shown a significant association between the *FCGR3A* SNP and loss of response at the end of induction and during maintenance phase. This is in line with several studies that have demonstrated the association of this functional polymorphism and clinical response to biological therapy in chronic inflammatory disorders [94, 95]. However, other studies gave different results, maybe because clinical response is influenced by several factors such as the pharmacokinetics and pharmacodynamics, but also clinical features, concomitant treatments and patient's medication [77]. The association between *FCGR3A* SNP and the response to infliximab remains controversial and little is known about the mechanism through which the polymorphism affects drug response. A functional polymorphism in *FCGR3A* gene, the rs396991 (F158V), has been reported to influence human IgG1 binding and ADCC activity [96]. Ternant and colleagues were the first to demonstrate the influence of this SNP on pharmacokinetics of infliximab in CD patients: the higher binding affinity of the receptor to IgG in patients carrying the V variant alleles induces a higher infliximab elimination rate probably due to an increase in NK cells recruitment and increased ADCC activity. These findings suggest that infliximab may be cleared faster in V/V patients than in F-carriers, inducing underexposure to infliximab and relapse [58]. Our results confirm that patients with variant V allele have lower levels of infliximab and worse clinical response compared to F/F patients. These findings underline the importance of ADCC in infliximab clinical efficacy, and a relevant role of this SNP in the prediction of infliximab response.

The production of antibodies against anti-TNFα agents is an important cause of loss of response in patients with IBD [76]. Anti-drug antibodies production is associated with worse treatment response, not only for high risk of hypersensitivity reactions, but also for a

lower bioavailability caused by higher anti-TNFα clearance. In fact, the generation of anti-drug antibodies would facilitate the opsonization and phagocytosis of anti-TNFα agents reducing their efficacy, leading to treatment failure. It is known that FCGRs are involved in the degradation of IgG complexes, and for this reason polymorphisms in *FCGR3A* gene could be involved in anti-drug antibodies production. Our results showed that pediatric patients with the variant allele V have a higher probability to produce anti-drug antibodies and these findings are in line with previous studies in adult IBD patients, treated with infliximab and adalimumab [76]. Because of the increasing binding capacity of Fc gamma receptor in patients with variant allele V, it can be supposed that an increased ADCC activity could induce the release of antibody fragment leading to autoimmunity events, anti-drug antibodies generation and treatment resistance. [58].

Considering the central role that TNFα and IL6 play in the pathogenesis of IBD, it is possible that different production of these cytokines may affect the response to therapy. Many variants in untranslated region of the human *IL6* and in *TNFα* gene have also been described and seem to alter the transcriptional process, decreasing IL6 level and increasing TNFα production [69, 97]. IL6 signalling may participate in disease perpetuation by mediating the resistance of lamina propria T cells against apoptosis via STAT-3 signalling, causing chronic inflammation [97]. It has been shown that the neutralization of the TNFα results in the suppression of various proinflammatory cytokines, including IL6 and interrupts a positive feedback loop of TNFα production [98]. Moreover, IL6 promotes Th17 cell lineage and function and inhibits regulatory T cells leading to the breakdown of immunological tolerance, polyclonal B-cell activation and auto-antibody production [99]. For this reason, lower levels of this pro-inflammatory cytokine may reduce the risk of immunogenicity and anti-drug antibodies production. A meta-analysis of 9 studies concludes that rs1800629 A allele carriers respond less to TNFα inhibitors when used in the treatment of rheumatoid arthritis; probably due to higher TNFα levels [100]. The same association was also observed in adult patients with CD [101]. Patients with the variant TNFα allele might have higher production of TNFα in the intestine and thereby show a reduced therapeutic response to infliximab. Despite in literature there are works that support the association of this SNPs with the clinical response to TNFα inhibitors, we have not found an association between *IL6* and *TNFα* SNPs, infliximab response and infliximab levels probably because our infliximab patients' cohort is too small. For this reason, further analysis on a larger cohort of IBD patients will be needed in the future to define the role of these SNPs in the response to anti-TNFα agents.

The innate and adaptive immune system responses are altered in IBD and the effect of TNF-blockers on T-lymphocyte subsets is largely unknown. CD69 has been for decades used as a simple marker of activated lymphocytes without knowing any concrete role this receptor could play in the regulation of anti-TNFα response and immune system balance. The flow cytometry analysis has demonstrated that the CD69 MFI expression decreased proportionally after infliximab treatment in Jurkat CD4+ T cells and in order to assess which is the mechanism involved in this reduction we have evaluated the expression of TNFR1 and TNFR2, the receptors through which TNFα exerts its action.

Jurkat CD4+ T cells resulted to have a TNFR1 increased expression compared to TNFR2. TNFR1 can trigger multiple signal transduction pathway to induce proliferation and inflammation, among this NF-κB signalling [102]. TNFR1 has also a cytoplasmic death domain that can activate the caspase-8 and caspase-3–dependent apoptosis involved in anti-inflammatory response. A same action could be played by TNFR2 that through a "reverse signalling" in TNF-producing cells, triggers apoptosis and/or proinflammatory cytokine suppression [103]. Since after cytofluorimetric analysis we have not seen cell death we have excluded apoptosis signalling and we have hypothesized the involvement of NF-κB signalling pathway in the mechanism of CD69 reduction and infliximab response.

When the transcriptional factors NF-κB and AP1 are induced by proinflammatory stimuli and mitogenic signals, such as phorbol esters and ionomycin, the expression of CD69 increases [104]. Specifically, the synergy of ionomycin with phorbol esters in triggering T cell activation enhances activation of protein kinase C that promotes survival, proliferative (via AP-1) and NF-κB signalling pathways, leading to release of pro-inflammatory cytokines (among which TNFα and IL-2), that up-regulate and maintain the expression of the T cell activation marker, CD69 [12].

Therefore, when T cells are treated with infliximab, probably the inhibition of TNFα and, consequently, the increase of the inhibitor of NF-κB, IκB, induce a reduction of NF-κB activity that negatively regulate the expression of CD69, making it a possible marker of infliximab response (Figure 30).

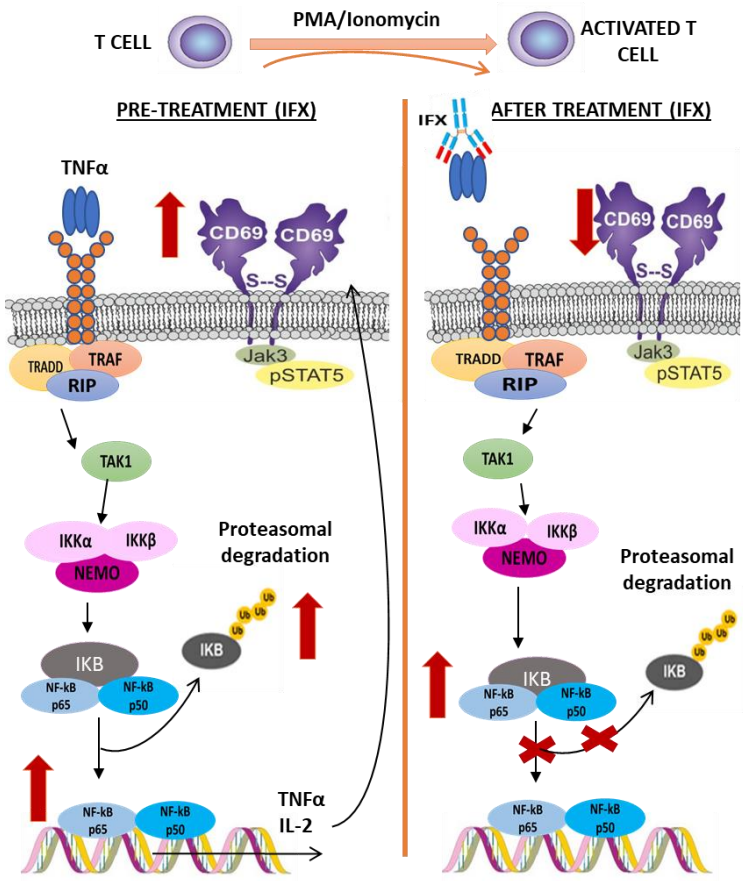

Figure 30: *Schematic representation of the pathways involved before and after infliximab action.*

Abbreviations: Tumor necrosis factor (TNF); tumor necrosis factor receptor type 1 (TNFR1); TNFR1-associated death domain (TRADD); TNFR-associated factor (TRAF); receptor-interacting protein (RIP); transforming growth factor b-activated kinase 1 (TAK1); nuclear factor kappa B (NFkB); NFkB essential modulator (NEMO); inhibitor of nuclear factor kappa-B kinase (IKK); inhibitor of nuclear factor kappa-B (IkB); ubiquitin (Ub) interleukin-2 (IL-2); janus kinase (JAK); signal transducer and activator of transcription (STAT); infliximab (IFX).

To demonstrate the possible role of CD69 as marker of infliximab response, the expression of CD69 was evaluated ex-vivo, on PBMCs of healthy donors, a cellular model that could be used in pediatric IBD patients. PBMCs were activated with PMA/ionomycin and then treated overnight with different concentration of the drug. As observed in the Jurkat model, the CD69 decreased after infliximab treatment, and in particular the expression of this activation marker decreased both in CD4+ and in CD8+ T cell subpopulations. Several papers reported in the literature a dysregulated intestinal T cell response in patients with IBD, showing increased percentages of activated CD4+ and CD8+ T cells [105].

The impact of TNF-blocking agents on the composition of the adaptive immune system is largely unexplored. Dublic and colleagues have observed a decrease of CD8+CD69+ T-cells in CD patients in remission, in treatment with anti-TNFα therapy [106]. Our results are in line with these observations and demonstrate that infliximab is able to reduce T cell activation as measured by CD69 MFI and induce a suppression of CD8+CD69+ cells but

not of CD4+CD69+. Probably the CD4+ T cells, not reduced by infliximab treatment, are able to induce the formation of regulatory T cells (CD4+CD25+FOXP3+) that positively modulate the immune system response.

6. CONCLUSION

In conclusion, this study demonstrates:

- the utility of point of care assays as reliable option for real-time therapeutic drug monitoring in children treated with infliximab, showing a good agreement with traditional ELISA assays;

- encouraging preliminary results of AFM methodology for infliximab monitoring in sera of pediatric IBD patients. A good correlation was found between signal variation and infliximab concentration and this technique can be exploited in the future, thanks to its multiplexing capability, to dose infliximab and anti-drug antibodies at the same time;

- the use of therapeutic drug monitoring to predict the efficacy of anti–TNFα agents in order to optimize treatment and minimize side effects. Early treatment modification can avoid complications: higher adalimumab levels during early treatment obtained from non–trough level serum samples predict long-term remission in children with IBD;

- the role of *FCGR3A* SNP in pediatric IBD patients: this SNP seems to affect infliximab response and influence anti-drug antibodies production. These data support the utility of genotyping candidate genes to predict infliximab response in children with IBD, resulting in more cost-effective and safe therapies;

- the validity of Jurkat CD4+ T cells in vitro model for the study of CD69 and infliximab mechanism of action. Furthermore, a decrease of CD4+ and CD8+CD69+ T cells was observed in PBMCs obtained by healthy donor, indicating a possible role of CD69 as marker of infliximab response.

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