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MALATTIE INFIAMMATORIE CRONICHE INTESTINALI IN ETÀ PEDIATRICA: DALLA FISIOPATOLOGIA A NUOVE STRATEGIE PER LA SCELTA E IL MONITORAGGIO DELLE TERAPIE

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PEDIATRIC-ONSET INFLAMMATORY BOWEL DISEASE: FROM PATHOPHYSIOLOGY TO NEW STRATEGIES FOR THERAPY CHOICE AND MONITORING

Abstract

Background: Anti-tumor necrosis factor monoclonal antibodies have lead to a revolution in the treatment of inflammatory bowel diseases (IBD), yet a sizable proportion of patients do not respond to therapy. There is increasing evidence suggesting that treatment failure may be associated with inadequate blood drug levels and/or the appearance of anti-infliximab antibodies (AIA). Data regarding therapeutic drug monitoring of infliximab (IFX) in children however are still incomplete.

Methods: We studied 49 pediatric (median age 14.4) IBD patients (Crohn's disease 34, ulcerative colitis 15) treated with IFX. Serum samples were collected at 6, 14, 22 and 54 weeks, before IFX infusions. IFX and AIA were measured using ELISA assays. Disease activity was determined by PUCAI or PCDAI.

Results: Clinical remission, defined as a clinical score <10, was obtained by 76.3% of patients at week 14 and by 73.9% at week 54. Median trough IFX concentration was higher in patients achieving sustained clinical remission at all time points. IFX levels during maintenance correlated also with C-reactive protein, albumin, and calprotectin. After multivariate analysis, the strongest predictor of sustained clinical remission was an IFX concentration at the end of induction > 3.11 (p-value = 3.0×10^{-5}, sensitivity 89%, specificity 80%). AIA concentrations were inversely correlated with IFX concentrations (p-value = 0.00088) and directly correlated with adverse reactions (p-value = 0.018).

Conclusions: Measurement of IFX trough levels at the end of induction therapy (week 14) allows predicting sustained long-term response in pediatric patients with IBD.

Introduction

Inflammatory bowel diseases (IBD) are immune-mediated disorders characterized by the presence of a chronic inflammatory involvement of the alimentary tract, possibly associated with extraintestinal manifestations (EIMs). IBD include Crohn's disease (CD), ulcerative colitis (UC) and inflammatory bowel disease unclassified (IBD-U). The natural history of these conditions is characterized by a chronic/relapsing progressive evolution, resulting in organ damage and severe impairment of the intestinal function, as well as impairment of the person's well-being and quality of life. Impairment of growth represents a further problem in children, both as a result of active disease and of long-term therapy with corticosteroids.

The goal of therapy of IBD is not only to induce and maintain clinical remission but also to achieve normalization of mucosal inflammation (mucosal healing), in order to normalize growth, regain quality of life, and avoid surgical complications. Apart from the risk of detrimental side effects, corticosteroid therapy does not achieve these goals in most patients.[1–3] In fact, although most patients do present a clinical response to treatment with corticosteroids, only 40% of them obtain endoscopic remission, and only 15% also histologic remission.[4] In order to overcome the shortcomings of therapy with corticosteroids, several other immunomodulatory drugs, acting more specifically on the immune system, have been introduced. Among these, monoclonal antibodies directed against tumor necrosis factor (TNF), a pivotal cytokine in the pathogenesis of IBD, were introduced in the early 90s and have led to a revolution in the treatment of these conditions, both in adult and in pediatric patients.

In the intestinal mucosa of patients with IBD, TNF is primarily secreted by macrophages, monocytes, neutrophils, T helper cells and NK cells, both as a result of stimulation by microbial antigens and other cytokines such as interferon gamma,

interleukin (IL) 2, and GM-CSF; on the other hand, TNF is inhibited by TGF-beta and IL-6.[5 6] In target cells, TNF determines the activation of signaling pathways implicated in the generation of inflammatory responses. Furthermore, it increases the proliferation of T cells, and some populations of T cells actually respond to IL-2 only in the presence of TNF.[7] Circulating TNF increases bone resorption by stimulating osteoclasts, decreases appetite and increases protein catabolism.[8] Several studies have demonstrated the presence of high levels of TNF in the blood and feces of patients with IBD, as well as an increased number of TNF-producing cells in the intestinal mucosa.[9 10] These observations, together with the fact that mice knock-out for TNF are significantly less sensitive to experimental colitis,[11] have led to identification of TNF as one of the main objectives for the development of new targeted pharmacological agents in the form of monoclonal antibodies.

Among these, infliximab, a monoclonal IgG1 immunoglobulin, was the first biological therapy approved for IBD.[12] Infliximab is a chimeric antibody, consisting of a constant human part (about 75% of the molecular weight) and a part of murine origin, representing the variable, antigen-binding portion of the molecule. Other anti-TNF antibodies followed, in particular certolizumab pegol, consisting of a pegylated antibody fragment, and two fully human antibodies, i.e. adalimumab and golimumab, which were developed in order to try to reduce the problem of immunogenicity of chimeric antibodies.

Preclinical studies have shown that treatment with anti-TNF monoclonal antibody can lead to neutralization of circulating TNF, but also to a blockade of leukocyte migration into tissues and to induction of apoptosis of T lymphocytes and activated monocytes already present in the inflamed mucosa.[13–15] Subsequent clinical trials have demonstrated that treatment with anti-TNF agents is capable to obtain clinical remission, as well as mucosal healing, in a substantial proportion of patients,[16 17] and to reduce

hospitalization and the need for surgery, thus possibly changing the natural history of these diseases.[18 19] These results have led to the idea of employing anti-TNF agents as first-line therapy in selected patients ('top down approach'), since this approach may in fact be more cost-effective than using them only after failure of conventional therapy (traditional 'step up approach'), when several anatomical and immune system changes may have already occurred that may limit the efficacy of treatment.[20–23] Treatment with anti-TNF agents, however, is associated with considerable costs and deployment of health care resources, as well as possibly with significant side effects.

Currently, patient selection for anti-TNF treatment is mainly performed on clinical grounds, identifying patients who could benefit from a more aggressive management, or those refractory to other treatments.[22 24 25] Nevertheless, it would be highly desirable to be able to select patients according to their probability of response to treatment. Furthermore, it is becoming increasingly important to be able to monitor therapy in clinical practice, mainly through the utilization of therapeutic drug monitoring (TDM) in order to adapt therapy to the single patient.

Treatment with anti-TNF agents is successful in a majority of patients with IBD, yet it can fail in a sizable proportion of patients, either because of inadequate response (primary failure: 10-40% of patients), or because of subsequent loss of response (secondary failure: up to 45% per patient-year when evaluated by both discontinuation and need for therapy intensification).[26–28] Furthermore, up to 26% of patients may experience adverse effects, possibly leading to therapy discontinuation.[29–31] Mechanisms associated with treatment failure are multiple. Apart from treatment suspension due to adverse events, they can be divided in factors affecting drug pharmacokinetics and factors related to the disease itself.

Factors affecting drug pharmacokinetics

While standard dosing regimens have been evaluated through randomized clinical trials in order to achieve the maximum efficacy in the majority of patients, subsequent analyses clearly correlated clinical response to serum drug levels.[32–34] These findings paved the way for therapy optimization through TDM and represent the rationale behind the utilization of therapy intensification schemes - either by increasing drug doses or by shortening administration intervals. Multiple studies have demonstrated, in fact, that serum drug levels are likely to increase in response to treatment intensification, and that these strategies can recapture a significant proportion of IBD patients who were failing treatment.[35–37] Drug levels, in turn, can be influenced by several factors.

Dosing regimen and adherence to treatment

Both drug dosing and administration frequency are correlated with drug trough levels. Clinical trials have demonstrated that scheduled treatment is superior to episodic or as-needed treatment.[38] Scheduled treatment is associated with higher drug blood levels, resulting in a more adequate suppression of intestinal inflammation; furthermore, episodic administration and low drug trough levels may facilitate the appearance of anti-drug antibodies (ADAs), which are associated with treatment failure.[39 40] For the same reasons, proper adherence to treatment is associated with better clinical outcomes.[41] Two recent prospective studies have shown better adherence to infliximab than to adalimumab, with no differences between CD and UC, in adult patients.[41 42] This may be due to a better patient-to-provider contact for the intravenous route of administration (infliximab) as compared to self-injections (adalimumab). A recent critical review has

also shown that patients' concerns and beliefs about medications play an important role in maintaining adherence to treatment.[43]

Antidrug antibodies

Anti-TNF monoclonal antibodies are potentially immunogenic and interaction with the host's immune system may result in the development of ADAs. Following drug administration, a polyclonal immune response occurs, resulting in multiple ADA species circulating in patients' serum, each one displaying its own binding affinity and target specificity.[44] ADAs can be heterogeneous and directed toward different immunogenic sites on the target drug. Different ADAs may have different effects: (1) neutralizing effect on TNF-binding activity; (2) non-neutralizing effect on activity, but enhanced elimination; and (3) non-neutralizing effect on activity but a sustaining effect on pharmacokinetics.[45] The clinical outcome will be determined by the prevailing ADAs; nevertheless, ADAs usually affect therapy negatively.

The chimeric antibody infliximab is structurally more immunogenic than fully human antibodies (adalimumab and golimumab), with the variable regions derived from murine sequences being the most immunogenic.[46] However, idiotypes on human anti-TNFs can also result in immunogenicity.[47] Furthermore, the higher immunogenicity of subcutaneous route of administration, being the skin an highly specialized structure for antigen processing and presentation to immune cells, may partially compromise the advantages of the fully human sequence.[48]

Low drug levels are associated with ADAs appearance,[39 40] likely because of the loss of "high zone tolerance" (i.e. immune tolerance induced by high doses of soluble protein as compared to low doses).[49] In a real-life IBD cohort, an exposure to infliximab levels below 3 mcg/mL was the strongest predictor of ADAs development, with a 4-fold

increased risk.[50] These findings support the need for TDM to reduce the risk of immunogenicity.

PEGylation is associated with reduced immunogenicity through decreased recognition of the protein by the immune system. Certolizumab pegol (CZP), the only available PEGylated anti-TNF agent, is associated with reduced likelihood of ADA formation.[51] Non-human glycosylation patterns can also affect immunogenicity, and may contribute to differences among drug batches or biosimilars formulations;[52] however, whether differences in glycosylation can actually influence clinical efficacy in patients with IBD is still unclear.

In patients with IBD, formation of ADAs may be associated with infusion reactions and loss of response to treatment through increased drug clearance.[28 53 54] When followed prospectively, most patients develop ADAs within 12 months of therapy.[55] Persistent ADAs have been defined as measurable ADAs on up to two consecutive infusions without any therapy alteration.[55] In contrast to persistent ADAs, transient ADAs can appear and disappear haphazardly at any time during treatment and usually are of little clinical significance in terms of treatment efficacy, yet they can be associated with higher risk of infusion reactions.[55 56]

The definition of what level of ADAs can be considered as a "low level" has not been clearly established, and there are no clear cut-offs defining what ADAs titers should be considered irreversible. An ADA titer > 8 mcg/mL has been suggested to be "high" (and has been associated with an odds ratio for treatment failure of 5),[57] while a titer >20 mcg/mL has been defined as "very high". Low level ADAs may be still amenable to be reverted through therapy intensification and/or the introduction of low-dose immunomodulator drugs such as methotrexate or azathioprine.[55 58] Combination therapy may also be useful for the prevention of secondary loss of response, yet the recommendations on this issue are still conflicting. Infliximab has been shown in some

studies to benefit from combination therapy, especially in the first 6-12 months, when the immunogenicity risk is higher,[59] while evidence is scarcer for other anti-TNF agents.

Increased drug clearance

Monoclonal antibodies clearance occurs mainly by endocytosis and degradation by reticuloendothelial system cells.[48] ADAs, as previously said, increase the drug catabolism and elimination. Apart from ADAs, several other factors influence drug clearance.

Neonatal Fc receptor (FcRn), also known as the Brambell receptor, is expressed on endothelial cells, and acts as a sparing agent for IgG antibodies and albumin, preventing their catabolism.[60] Systemic inflammation may increase drug catabolism by increasing reticuloendothelial activity and by saturating FcRn receptors with endogenous IgG, which may be in turn upregulated by systemic inflammatory response resulting in hypergammaglobulinemia. This increase may partly explain the association of higher baseline inflammatory status, identified by higher C-reactive protein (CRP), with treatment failure in some patients,[61] and may also suggest that patients with more severe disease may need higher drug doses. Similarly, low serum albumin has been consistently associated with higher drug clearance and worse outcomes.[50 62] Lower albumin may in fact represent a marker of low FcRn efficiency and of high fecal protein loss, therefore indicating increased drug clearance.

Besides systemic elimination, increased drug clearance may occur in target tissues through several mechanisms. Tissue inflammation may act as a "sink" for the drug, therefore decreasing its availability (there is more TNF that needs to be neutralized by greater drug doses) and increasing the formation of drug-TNF immune complexes that are more easily cleared. Actually, higher baseline TNF concentrations in colonic tissues

have been shown to be associated with a lower probability of treatment success in UC.[9] Yarur et al. also demonstrated that in inflamed tissues the ratio of TNF to anti-TNF is elevated compared to uninflamed tissue.[10] Furthermore, most patients with active endoscopic disease despite adequate serum drug levels had low levels of drug in intestinal tissues ("serum-tissue anti-TNF discordance"), thus suggesting these patients may require higher drug doses.

Factors that may explain this serum-tissue anti-TNF discordance include intestinal drug loss and intestinal drug clearance. Intestinal drug loss with feces may occur in patients with moderate-to-severe UC, likely because of increased intestinal permeability and ulcerated mucosa.[63] This may explain the possible advantage of using an intensified induction regimen in patients with severe steroid-refractory UC in which fecal drug loss may be extensive. The presence of an increased number of phagocytes in the inflamed mucosa may increase local degradation of the drug-TNF immune complexes, through an enhanced production of proteases, including matrix metalloproteinases (MMP) and human neutrophil elastase (HNE). Recently, it has been demonstrated that human MMP-3 and MMP-12, as well as homogenates from inflamed IBD mucosa are capable to cleave infliximab, adalimumab, and etanercept in vitro.[64] Similarly, HNE also degrades infliximab and adalimumab.[65] Finally, serum levels of endogenous IgG fragments cleaved by MMP3 and MMP12, were found to be higher in patients who did not respond to treatment.[64]

Factors related to the disease

Switch to non-TNF-mediated pathways

Cytokine signature changes during the natural history of IBD.[66] Interleukin (IL)-12 polarizes mucosal T cells isolated from children at first diagnosis of CD but not from late CD, into T helper type 1 with high levels of interferon (IFN)-γ.[67] In a subsequent study, TNF, IFN-γ and IL-21 have been observed to be increased in the early phases of CD; on the other hand, a Th17 pathway (dominated by IL-17A with low expression of both TNF and IFN-γ) has been documented in patients with more advanced CD.[68] CD patients may be therefore more likely to respond to anti-TNF therapy in the early phase of disease rather than in later one.[69]

Additionally, therapy with anti-TNF agents may by itself lead to a switch in the immune pathways from TNF to other cytokines. This may also lead to the onset of paradoxical adverse events.[70] Anti-TNF–induced psoriasis is associated with IL-17A- and IL-22-secreting Th17 cells. In these patients, this side effect may be treated successfully with ustekinumab, a monoclonal antibody directed against the p40 subunit of IL-12 and IL-23, able to inhibit Th1 and Th17 responses.[71] The change from TNF to other cytokines may also be corroborated by the results of clinical trials showing a greater efficacy of ustekinumab in patients with CD who had not responded to anti-TNF agents.[72]

Non-inflammatory causes of non-response

Careful selection of patients is paramount to maximize treatment efficacy. Anti-TNF should be preferentially reserved to patients with active inflammatory disease, excluding patients with other causes of persistent symptoms, including fibrostenotic

disease, abscesses, small intestinal bacterial overgrowth, bile salt malabsorption, and opportunistic infections.[73]

Cross-sectional imaging techniques, especially magnetic resonance enterography, are useful to identify patients with inflammatory disease vs. patients with fibrotic complications.[74] Similarly, abscess should be early detected by radiologic techniques. Small intestinal bacterial overgrowth is an underestimated disorder mimicking a relapse in CD patients, especially those with previous surgical procedures and a long history of CD.[75] Bile salt malabsorption is a frequently neglected condition associated with IBD, mainly with ileal CD, due to impaired conjugated bile acid reabsorption, which may lead to clinical steatorrhea and intestinal dysmotility. Among opportunistic infections in IBD, Clostridium difficile and Cytomegalovirus should be especially considered in any disease relapse.

PREDICTORS OF RESPONSE TO ANTI-TNF ANTIBODIES

Children are generally regarded to have better response to anti-TNF drugs in comparison to adults. In the REACH trial, which evaluated clinical response to infliximab in children with CD, 88.4% patients responded to infliximab and 58.9% patients achieved clinical remission after induction; at week 54, the percentages were 63.5% and 55.8% for clinical response and clinical remission, respectively.[76] These figures are higher in comparison to results from adult trials such as the ACCENT I trial.[77] However, patients in the REACH trial were also required to be on a combination therapy with an immunomodulatory drug, and the results may actually not be far from those of similarly conceived studies in adults, such as the SONIC trial, which evaluated azathioprine-infliximab combination therapy in adults with CD.[78]

On the other hand, children with very early onset IBD (i.e. patients with an IBD diagnosis before 6 years of age) have been reported to have worse response to anti-TNF therapy and shorter durability of response.[79] This may be due to a more aggressive disease in this age group, but possibly also to different drug pharmacokinetics or to a greater contribution of cytokines other than TNF in disease pathogenesis.

Disease phenotype in CD is associated with response to treatment. Luminal inflammatory disease is associated with a better response, while a stricturing phenotype has been associated with reduced response.[80] Nevertheless, some patients with stricturing phenotype may respond well, especially when an inflammatory component is also present. Pan-enteric disease has been included by ECCO/ESPGHAN guidelines among conditions for which anti-TNF therapy should be considered for primary induction, because of the increased risk of poor outcomes.[22] Other patient-related factors associated with treatment failure include previous surgery, possibly because of association with more advanced and/or fibrosing disease, and greater body weight/body mass index.[62]

In UC, severe acute colitis is associated with reduced response to treatment and risk of colectomy.[81 82] Severe colitis may be associated with lower drug levels, possibly because of greater drug clearance and loss of drug in the stools,[83] therefore therapy optimization by means of TDM, and/or higher drug dosage from the start or shorter intervals may be indicated. In fact, intensified induction regimens with infliximab (median 3 doses in 24 days) have been associated with reduced need for early colectomy in adult patients.[84]

The significance of CRP as a predictor of response to treatment may be double-edged and data are inconclusive. Increased baseline CRP in patients with CD has been associated with better response to treatment in some clinical trials,[78] but not in others;[85] furthermore, other studies, have related higher CRP with lower likelihood of response.[61]

This apparent contradiction may be due to the fact that CRP is certainly associated with an inflammatory phenotype, therefore more amenable to be treated by TNF blockade, but also possibly to a more severe and extensive disease. Furthermore, a normal CRP is not an absolute contraindication to treatment, since CRP may be normal in some patients with active disease. Overall, baseline CRP does not seem to be a useful predictor of treatment response in clinical practice. However, higher CRP is associated with greater peripheral drug clearance, as previously said,[86] therefore patients with high inflammatory load should be strictly monitored for drug levels. Fecal inflammatory markers, such as fecal calprotectin and lactoferrin, may be more sensitive than CRP in detecting active intestinal inflammation, but they have little role for response prediction. The role of positive baseline perinuclear anti-neutrophil cytoplasmic antibodies (pANCA) in predicting poor responders to infliximab has been evaluated by few studies, with variable results. A meta-analysis integrating data of 415 patients determined that pANCA positive patients had almost a two-fold lower response to anti-TNF therapy compared with pANCA negative patients;[87] Nevertheless, pANCA positivity cannot be regarded as a contraindication to treatment, yet it may be useful to identify a subset of patients at higher risk of treatment failure, possibly necessitating therapy optimization with TDM.

Therapeutic drug monitoring

TDM-guided dose optimization, based on determination of anti-TNF drug serum concentrations as well as of ADAs, has emerged as a fundamental determinant of treatment success. Indeed, there is accumulating evidence that TDM is independently associated with improved outcomes. Maintaining adequate blood levels, which are usually evaluated at pre-infusion “trough” levels, is fundamental to obtain an adequate

TNF blockade in target tissues as well as to avoid induction of ADAs. Target drug concentrations differ according to treatment phase, being usually higher and most variable during induction and then stabilizing during maintenance phase.[39 88]

Therapy intensification, either by shortening doses interval or by increasing drug doses, can recapture response in some patients with secondary loss of response, including some patients with ADAs, especially if their titers are not high.[89] TDM allows more precise guidance in case of therapeutic loss of response.[73 90] In fact, when a patient experiences loss of response that is deemed to be due to relapse of active inflammatory disease during treatment, it is crucial to identify whether drug trough levels are adequate (pharmacodynamic failure) or not. In the latter case, evaluating the presence of ADAs allows discriminating between the so-called immunogenic failure (low drug levels, high levels of ADAs) and pharmacokinetic failure (low drug levels, absent ADAs). This may guide the subsequent therapeutic strategy. In case of pharmacokinetic failure, it could be useful to escalate therapy; in immunogenic failure, it may be useful to switch to another anti-TNF agent or to add an immunomodulator, yet therapy intensification may also recapture response in some patients; finally, pharmacodynamic failure usually implies that inflammatory activity is not responsive to TNF blockade.

TDM may also allow a more cost-effective treatment.[91]

Recent studies have identified infliximab trough levels at the end of induction as the most reliable predictor of sustained clinical response. A post-hoc analysis of the ACCENT I trial evaluated the association between serum infliximab trough levels after 14 weeks with durable long-term response: optimal predictors of sustained response were week 14 trough level ≥3.5 mg/mL and a CRP decrease ≥60%.[32] These results have been replicated by several other studies, and a target range of 3-7 mcg/mL for trough levels has been identified as being related to optimal outcomes. Furthermore, TDM may be useful also in patients with stable responses to maintenance therapy. The Trough

Concentration Adapted Infliximab Treatment (TAXIT) prospective randomized controlled trial included 263 adults (178 with CD and 85 with UC) with stable responses to maintenance infliximab therapy.[92] Doses were escalated or reduced using an algorithm to reach a target trough concentration of 3–7 mcg/mL (optimization phase). Patients were then randomly assigned to receive infliximab dosing based on either clinical grounds or trough levels (maintenance phase). After therapy optimization, most patients reached trough concentrations within the therapeutic range, resulting in a higher proportion of patients in remission than before dose escalation among patients with CD, but not in those with UC. Furthermore, patients with trough levels above range were able to reduce drug dosing without compromising response, thus achieving a 28% reduction of drug costs. Finally, frequency of disease relapse was more than doubled in patients who received clinically based dosing in comparison to patients who received concentration-based dosing. In a recent real-life study on 191 patients suffering from IBD and treated with infliximab, TDM led to a change in almost one-third of decisions made, offering considerable cost savings and reducing exposure to unnecessary therapies.[93]

Also for adalimumab, several studies in patients with IBD demonstrated an inverse relationship between drug levels and disease activity. An analysis performed on the prospective CLASSIC studies, with data from 275 patients, considered association of serum adalimumab concentrations at weeks 4, 24, and 56 of therapy with clinical remission.[94 95] Median adalimumab concentrations at week 4 were significantly higher in patients who achieved clinical remission (8.10 versus 5.05 mcg/mL). No cutoff thresholds to discriminate responders could be determined.[96] A later cross-sectional study collecting samples from 118 samples from 71 patients with CD at unselected time points confirmed that high adalimumab trough levels were associated with disease remission, with a cut-off drug level of 5.85 mcg/mL yielding optimal sensitivity and

specificity (68%, 70.6%).[97] Anti-adalimumab antibodies were present in 30% of samples, and were inversely related to adalimumab drug levels. A threshold ≥3 mcg/mL for ADAs had a specificity of 98% for active disease.

In children, the number of studies correlating anti-TNF blood levels and anti-drug antibodies with response to therapy is more limited. A prospective observational cohort commencing infliximab had trough levels measured at weeks 14 and 54. Infliximab levels and CRP at week 14 were significantly associated with week 54 efficacy, suggesting that a model combining both CRP and infliximab may help to predict long-term remission.[98] These observations suggest that early dose adjustment based on drug trough levels at the end of induction may improve long-term therapeutic outcomes.[99] A recently published meta-analysis of pediatric studies has shown that patients with higher infliximab plasma levels more frequently maintained clinical response after induction and after 1 year of therapy.[100] A recent study, however, correlated infliximab levels to laboratory response (calprotectin and C-reactive protein, CRP) but not to clinical response.[101] The IMAgINE-1 study, which was a phase-3, randomized, double-blind, 52-week study, enrolled 192 patients; trough serum adalimumab (at baseline, week 2, 4, 16, 26, and 52) and anti-adalimumab antibody measurements (baseline, weeks 16, 26, and 52) were collected.[102] Higher concentrations of adalimumab were associated with greater rates of remission, even if full statistical significance was not reached in this study ($P < 0.10$), with patients not in remission having lower serum concentrations than those in clinical remission only in 60% of measurements. A prospective study is currently ongoing to examine the effect of drug level-based personalized treatment with adalimumab in children with Crohn's disease (Pediatric Crohn's Disease Adalimumab Level-based Optimization Treatment, PAILOT trial). Primary completion of this study is due by July 2018.

Recent data indicate that the concept of therapeutic range (i.e. 3-7 mcg/dL for infliximab) may be oversimplified, since different concentration targets may be associated with different outcomes. Ungar et al. reported that in a cohort of 145 patients with IBD, increasing blood levels of infliximab and adalimumab were associated with higher probability of mucosal healing.[103] According to their experience, 50% of patients reached mucosal healing when infliximab levels were above 4 mcg/ml, 85% reached mucosal healing with infliximab levels above 6 μg/ml and 90% with levels above 8 μg/ml. Importantly, no difference was observed according to IBD diagnosis (UC vs. CD). Similarly, in patients treated with adalimumab, 50% of patients reached mucosal healing when adalimumab levels were above 7.5 mcg/ml, 75% with adalimumab levels above 8 mcg/ml, and 90% with levels above 12 mcg/ml. Interestingly, similar observations have been reported for fistulizing CD by Davidov et al.,[104] and by Yarur et al., who described incremental efficacy of infliximab therapy for perianal fistula healing.[105] These data suggest that different patients may require personalized target drug levels, and that higher levels may be required in refractory patients.

The evidence on the clinical utility of tailoring therapy according to infliximab concentration has led to the idea of developing rapid tests to measure drug concentration at the point of care. Lateral flow assays have been recently made available and seem to provide analytical results comparable to ELISA assays in less than one hour.[106] The monitoring of non-trough drug levels to allow timelier dose adjustment has been also recently considered and could lead to new insights on how to monitor therapy with anti-TNF agents.[107]

Pharmacogenomic markers

Several studies have evaluated the association of genetic variants with response to anti-TNF therapy. Most studied genes include genes related to cytokines and their receptors (especially TNF), or immunoglobulin receptors. The contribution of polymorphisms of single genes is still debated, since results have been often conflicting, both in pediatric and in adult patients.[108] Possible explanations for this include the lack of statistical power among studies as well as heterogeneity in patient selection and outcome measures. Nevertheless, it may also be that contribution of single genes is difficult to ascertain because anti-TNF monoclonal antibodies probably have far more complex pharmacokinetics and pharmacodynamics than other drugs. A recent systematic review and meta-analysis has evaluated available studies including at least 100 patients with IBD.[109] According to this study, polymorphisms in FCGR3A (rs396991), TLR4 (rs5030728), TNFRSF1A (rs4149570), IFNG (rs2430561), IL6 (rs10499563) and IL1B (rs4848306) genes were significantly associated with improved response among IBD patients, while polymorphisms of TLR2 (rs3804099) and TLR9 (rs352139) variants resulted associated with reduced response. The role of polymorphisms of genes implicated in the apoptotic process have also been evaluated, in consideration of the fact that anti-TNF monoclonal antibodies exert their action also by inducing apoptosis of inflammatory cells. A composite index integrating polymorphisms of Fas, Fas ligand and caspase-9 was found to correlate with response to treatment with infliximab in patients with CD.[110] Nevertheless, the use of pharmacogenetic markers for predicting drug response has not yet widely entered clinical practice, possibly because their practical significance has not been adequately validated.

Pharmacogenomic variants involved in anti-TNF drug pharmacokinetics rather than in their pharmacodynamics may actually be more promising, in consideration of the

increasing importance attributed to drug levels in clinical outcomes. A recent retrospective single center study evaluated the effect of a variable number of tandem repeats (VNTR) polymorphism in the promoter of the neonatal Fc receptor (FCGRT gene), which is responsible for the relative stability of immunoglobulins and albumin in serum, as previously discussed. This study included a cohort of 395 infliximab-naïve IBD patients treated with infliximab and a second cohort of 139 adalimumab- naïve patients treated with adalimumab. A specific FCGRT genotype (VNTR2/VNTR3) was associated with a significantly lower infliximab and adalimumab serum concentrations compared with patients homozygous for VNTR3/VNTR3 after adjustment for immunogenicity.[111]

Multi-gene panel testing of candidate variants involved in anti-TNF pharmacokinetics and pharmacodynamics could therefore be useful to predict anti-TNF response. A recent study evaluated the predictive value of 196,524 polymorphisms in genes associated with immune function in predicting response rate and durability of response to anti-TNF therapy in CD.[112] Among these polymorphisms, 15 were found to be associated with response to treatment, including 11 IBD-risk alleles. These were combined to obtain a genetic risk score (ranging from 5 to 20), which was associated with primary nonresponse (odds ratio 2.65 per 1 unit score increase) and durable response (odds ratio 1.60 per 1 unit increase). A model combining genetic and clinical variables was superior to a model including only clinical variables. These results, however, still need to be validated in other populations.

Fecal markers

As previously discussed, loss of infliximab into stools has been associated with primary non-response in a recent study in UC patients.[63] During the first 14 days of therapy the

highest fecal concentration of infliximab was observed at day one, and correlated with low serum albumin at baseline and low serum infliximab concentration at week two. Accordingly, it seems that most loss of infliximab happens when the drug serum levels is high and mucosa is still severely ulcerated leading to protein loss. Fecal concentrations of anti-TNF drugs may therefore represent a useful marker to monitor therapy.

Another candidate fecal marker under evaluation is the intestinal microbiota. In a prospective study conducted on children with IBD, responders to anti-TNF therapy had higher baseline amounts of Bifidobacteria, *Clostridium colinum*, *Eubacterium rectale*, Clostridiales and Vibrio, and lower presence of *Streptococcus mitis* than non-responders. During the anti-TNF induction, responders increased by week 6 the microbial diversity and similarity to control microbiota much more than nonresponders.[113] This microbial signature, however, needs to be validated in larger independents cohorts.

Mucosal markers

A peculiar gene expression signature in the inflamed mucosa of IBD patients in relation to anti-TNF response is yet to be determined, and conflicting results have been reported. In fact, both a pre-treatment increased (IL-17A, IFN-γ)[114] or decreased (TNF-α, IL-1b, IL-17A, IL-6, IFN-γ)[115] expression of pro-inflammatory cytokines in the mucosa has been associated with a better response to anti-TNF therapy, therefore the possibility of mucosal cytokine profiling does not seem to be promising at this moment, possibly because the absolute values of cytokine concentrations fail to recapitulate the complexities of inflammatory profiles in inflamed IBD mucosae.[5]

Other studies evaluated TNF density at a cellular level. A study evaluating TNF-positive cells in the intestinal mucosa of 14 adult patients with UC before infliximab

treatment did not find significant differences between responders and non-responders in TNF positive cell density.[116] In the same study, however, the Authors found a significant relationship between response to therapy and TNF mRNA quantification: patients with higher TNF gene expression had a lower probability of endoscopic remission following treatment with infliximab. This relationship seems to recapitulate the relationship between a higher inflammatory status and reduced anti-TNF efficacy previously reported by other studies. As previously discussed, this may actually be due to a higher systemic drug clearance in these patients leading to lower drug levels, as well as to greater drug inactivation in the mucosa because of matrix metalloproteinases (MMP), or, finally, to greater fecal drug loss.

Confocal laser endomicroscopy is an endoscopic technique developed to obtain very high magnification and resolution images of the mucosal layer of the gastrointestinal tract during a standard endoscopic examination. In a study comprising 25 patients with CD, a fluorescent antibody targeting membrane TNF was used for detecting immune cells of the colonic mucosa during confocal laser endomicroscopy.[117] A higher number of membrane TNF positive cells was associated to a better response to adalimumab (at week 12, then sustained over a follow-up period of one year). The presence of ≥ 20 fluorescent cells had 84.6% sensitivity and 91.7% specificity for the prediction of response to adalimumab therapy. Although intriguing, the main limitation of this technique is that it is not widely available.

Experimental part

THE RELATIONSHIP BETWEEN INFLIXIMAB PHARMACOKINETICS, ANTI-INFLIXIMAB ANTIBODIES, AND CLINICAL RESPONSE IN PEDIATRIC PATIENTS WITH IBD

Aims

The main objective of our study was to assess correlation between infliximab (IFX) serum levels and clinical response to treatment in a group of 49 pediatric patients treated with the drug. In addition, the role of anti-infliximab antibodies (AIA) on IFX serum concentrations, clinical response, and adverse effects was also evaluated.

Materials and Methods

Patients and eligibility criteria

Patients with IBD treated at Institute for Maternal and Child health IRCCS "Burlo Garofolo", Trieste, Italy, and at Maggiore Hospital, Bologna, Italy, were enrolled between March, 2012 and June, 2016. The inclusion criteria were: age between 6 and 18 years, a diagnosis of active IBD, and treatment with IFX. IFX was started in case of treatment failure or intolerance to first-line therapies: mesalamine, immunosuppressants, and, in the case of CD, enteral nutrition. IFX was used also as first line treatment in selected patients, as suggested by pediatric guidelines.[22] Exclusion criteria were: presence of an ileostomy or colostomy, disease needing surgery, infectious complications (including intra-abdominal infections), fulminant UC or toxic megacolon, or contemporary presence of other noncontrolled medical conditions. Therapy with immunosuppressants was permitted; therapy with glucocorticoid was permitted if tapering was undertaken after starting treatment with IFX. The patients enrolled were all

the eligible consecutive cases at the participating centers in the time-frame of the study. The study was approved by the local ethical committees and appropriate informed consent was obtained from all patients or their parents or tutors.

Blood samples for IFX and AIA measurement were taken at the appropriate clinic visits. The therapeutic protocol included an induction phase with intravenous administration of IFX 5 mg/kg at weeks 0, 2, 6, then IFX was administered every 8 weeks during maintenance phase. In case of clinical evidence of loss of response, therapy with IFX could be escalated, either by increasing drug dose (up to 10 mg/kg/dose) or by shortening intervals between infusions. All patients were pre-medicated prior to infusion with i.v. methylprednisolone 1 mg/kg and chlorphenamine maleate 0.2 mg/kg, to reduce the risk of adverse reactions during the infusion. Patients developing anaphylactoid reactions were considered in the pharmacokinetic study up to the time when they developed the reaction.

Definition of clinical response

Clinical disease activity was assessed using Pediatric Crohn's Disease Activity Index (PCDAI) and Pediatric Ulcerative Colitis Activity Index (PUCAI) for Crohn's disease and UC patients, respectively, at the time of blood sample collection.[118 119] Disease was considered to be in remission if the disease activity index was less than 10; partial response was defined as a change of at least 15 points from baseline for CD and at least 20 points for UC.[119 120] Loss of response was considered either as clinical worsening in a patient who had previously attained clinical response/remission or as need for treatment intensification (either as increase in drug dose to 8-10 mg/kg/dose or as an increase in infusion frequency).

Measurement of IFX and AIA

IFX and AIA levels were determined by ELISA (LISA Tracker Duo IFX, Theradiag, France), on sera collected immediately available before the III, IV, V, IX infusion (weeks 6, 14, 22, 54), and, in any case before the last infusion preceding therapy discontinuation. AIA levels were measured when IFX plasma levels were less than 1.5 μg/ml. The assay results for IFX and AIA levels were obtained retrospectively.

Statistical analysis

Statistical analysis was performed using the software R (version 3.4.2). The association between IFX concentrations and therapeutic response was evaluated in a univariate analysis by generalized linear model of the gaussian family (logistic regression), using patients response to infliximab as the dependent variable and infliximab concentration as the independent variable. To identify the best predictor of IFX response, the most significant association between IFX concentrations and response at the various time-points considered was identified on the basis of the logistic regression analysis. Receiver operating characteristic (ROC) curves were then constructed for IFX concentrations, to determine the optimal cut-off to predict patients' clinical response to IFX. Sensitivity, specificity and the positive and negative predictive values (PPV, NPV, respectively) of the cut-off point were analyzed. To test the association of the identified cut-off value with demographic and clinical covariates (age, sex, IBD type, clinical laboratory parameters including C-reactive protein (CRP), albumin and calprotectin), univariate logistic regression analysis was performed, considering patients' IFX concentration below or above the cut-off point as the dependent variable and the demographic/clinical covariate as the independent variable.

Multivariate analysis was performed to test the potential confounding effect, on the association between therapeutic response and the cut-off identified for IFX concentration, by clinical and demographic covariates. This multivariate analysis was done by a logistic regression generalized linear model, using therapeutic response as the dependent variable and the cut-off for IFX concentration together with all covariates significantly associated with this cut-off in the univariate analysis, as independent variables.

An analysis on the association between post-induction infliximab concentrations and the clinical laboratory parameters was performed also by generalized linear mixed effects models, considering the clinical laboratory parameter of interest as the dependent variable and infliximab concentration as the independent variable. For the clinical laboratory parameter, normality of the distribution was evaluated by visual examination of the data histogram and by Shapiro's test and an appropriate transformation was applied to restore normality. For the association between AIA concentrations and IFX concentrations was determined by non-parametric Spearman's test.

Results

Clinical response

Forty-nine patients (CD 34, UC 15; median age 14.4, interquartile range 11.6-16.2) were enrolled. Seven patients were on concomitant immunosuppressive therapy at treatment start (azathioprine) and other 7 patients were receiving systemic corticosteroids. After induction therapy, 9 patients (18.4% total, 3 with CD and 6 with UC) did not respond to therapy, and 2 patients (4.1% total, 1 with CD and 1 with UC) discontinued IFX due to anaphylactoid reactions during induction infusions. Thirty-

eight patients (25 with CD and 13 with UC) responded to induction treatment: 8 patients presented partial response (16.3%, 5 with CD and 3 with UC) and 30 achieved clinical remission (61.2%, 25 with CD and 5 with UC). All patients with partial or complete response to treatment continued therapy with IFX after induction, and were followed till week 54. At week 54, 24 patients presented sustained clinical response (49.0% of all patients, 58.8% of those with CD and 26.7% of those with UC, p-value logistic regression = 0.056); of these 23 presented clinical remission and 1 partial response. Nine patients (18.4% of all patients, 7 CD and 2 with UC) had lost response by 54 weeks, while 5 patients (3 with CD and 2 with UC) discontinued IFX due to anaphylactoid reactions during maintenance therapy (figure 1). A total of 134 samples from 49 patients were analyzed at 6, 14, 22 and 54 weeks, for 40, 35, 33 and 26 patients, respectively.

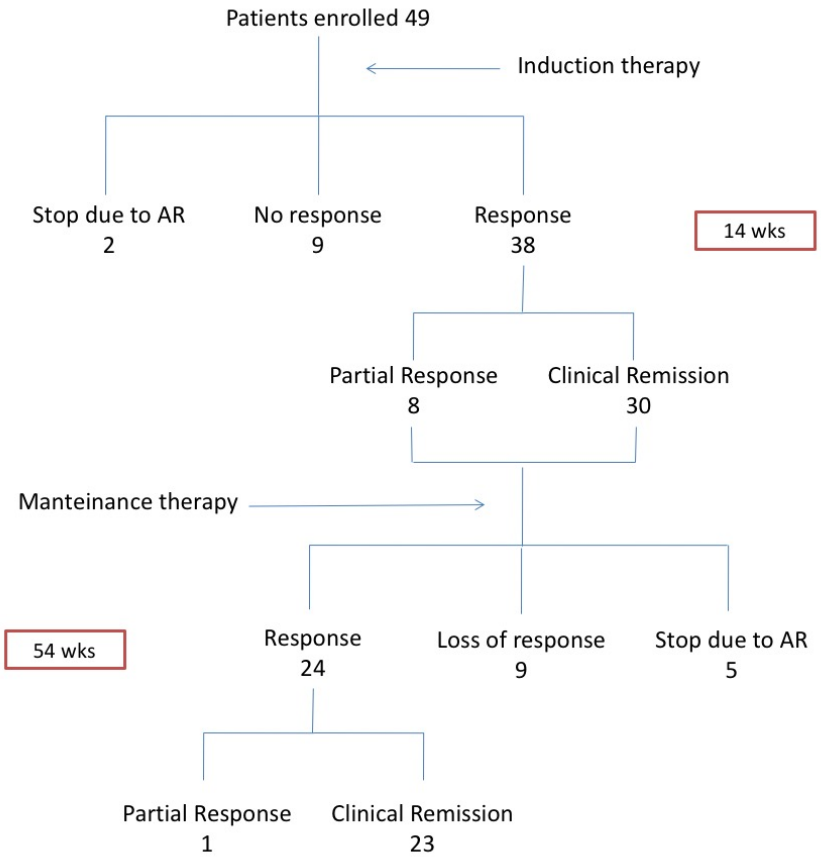

Figure 1: Clinical response to treatment with IFX.

Serum IFX levels and clinical remission after induction treatment

At third infusion (i.e. at week 6), 24 patients were in clinical remission, while 14 were not. IFX concentrations were different between these two groups (median IFX concentration 7.1 µg/ml, IQR 4.7-9.8, in patients in clinical remission vs median 9.8 µg/ml, IQR 8.4-12.6, in patients not in remission; p-value logistic regression = 0.044; Figure 2a). Also at the fourth infusion (week 14), IFX serum concentrations were significantly different (median IFX concentration 5.0 µg/ml, IQR 3.6-9.1, vs 1.0 µg/ml, IQR 0.18-2.7; p-value logistic regression = 0.00039; Figure 2b).

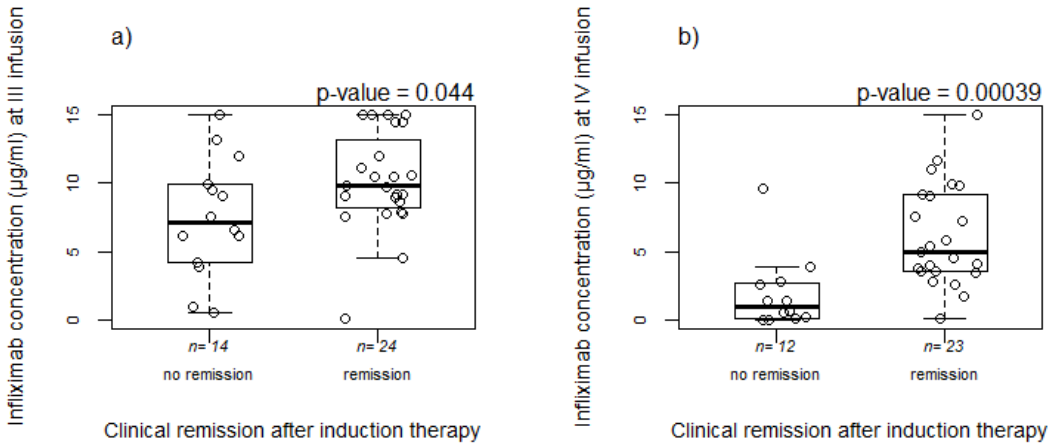

Figure 2: Boxplot comparing clinical remission at the end of induction therapy and serum IFX concentration at the III (a) and IV (b) infusion between patients according to remission status. The bold horizontal line represents the median value. Statistical significance was assessed by logistic regression analysis.

Serum IFX levels and clinical remission at 22 weeks of treatment

Considering clinical response at 22 weeks of treatment, significantly different concentrations of serum IFX were observed for samples collected before the III, IV and V infusion between patients in clinical remission and those who were not. In particular, median IFX concentrations before III, IV, and V infusion were 10.3 µg/ml (IQR 9.0-13.8), 5.0 µg/ml (IQR 3.5-9.1), and 4.4 µg/ml (IQR 2.4-8.7) in patients in clinical remission, and 7.1 µg/ml (IQR 4.7-9.8), 1.0 µg/ml (IQR 0.17-2.7), and 0.6 µg/ml (IQR 0.05-1.3), in patients not in clinical remission, respectively (p-value logistic regression < 0.01 for all comparisons; Figure 3).

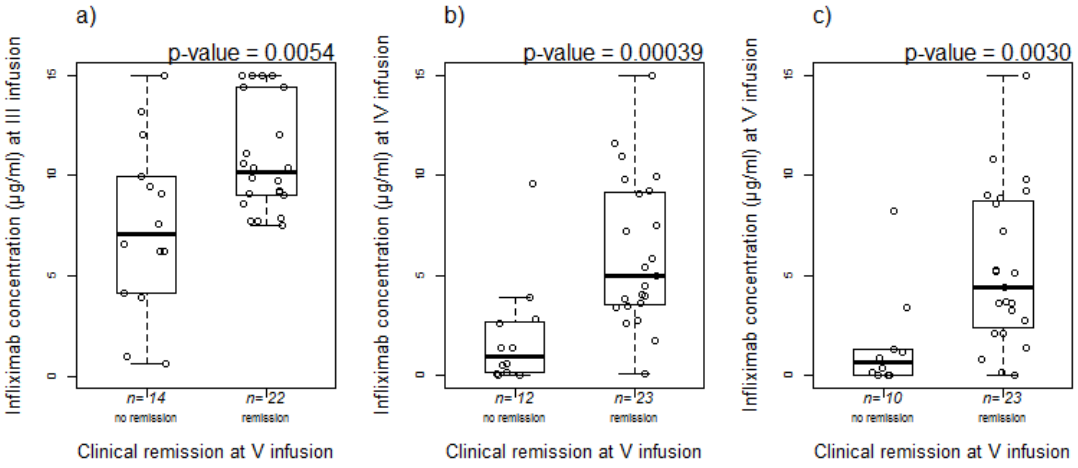

Figure 3: Boxplot comparing clinical remission at 22 weeks of treatments and serum IFX concentration at the III (a), IV (b) and V (c) infusion between patients according to remission status. The bold horizontal line represents the median value. Statistical significance was assessed by logistic regression analysis.

Serum IFX levels and clinical remission at 54 weeks of treatment

Clinical remission at 54 weeks of treatment was most significantly correlated with serum IFX levels before the IV infusion (median IFX concentration: 6.1 µg/ml, IQR 3.8-9.6 in patients in clinical remission vs 1.4 µg/ml, IQR 0.35-2.8 in patients not in clinical remission, p-value logistic regression = 0.00038, Figure 4b), which therefore emerged as the best potential predictor of sustained clinical remission. Serum IFX concentrations at the III and V infusion were also significantly associated with clinical remission at 54 weeks of treatment (median IFX concentration: at III infusion 10.4 µg/ml, IQR 9.1-14.4, vs 7.8 µg/ml, IQR 5.7-10.5, p-value logistic regression = 0.0080, Figure 4a; at V infusion 5.2 µg/ml, IQR 2.9-9.0, vs 1.0 µg/ml, IQR 0.34-1.9, p-value logistic regression = 0.0022; Figure 4c). IFX levels at IX infusion (54 weeks) were also associated with clinical remission, even though the difference was less statistically significant (median IFX concentration 3.8 µg/ml, IQR 2.7-6.0, vs 1.2 µg/ml, IQR 0.67-1.9, p-value logistic regression = 0.025, Figure 4d).

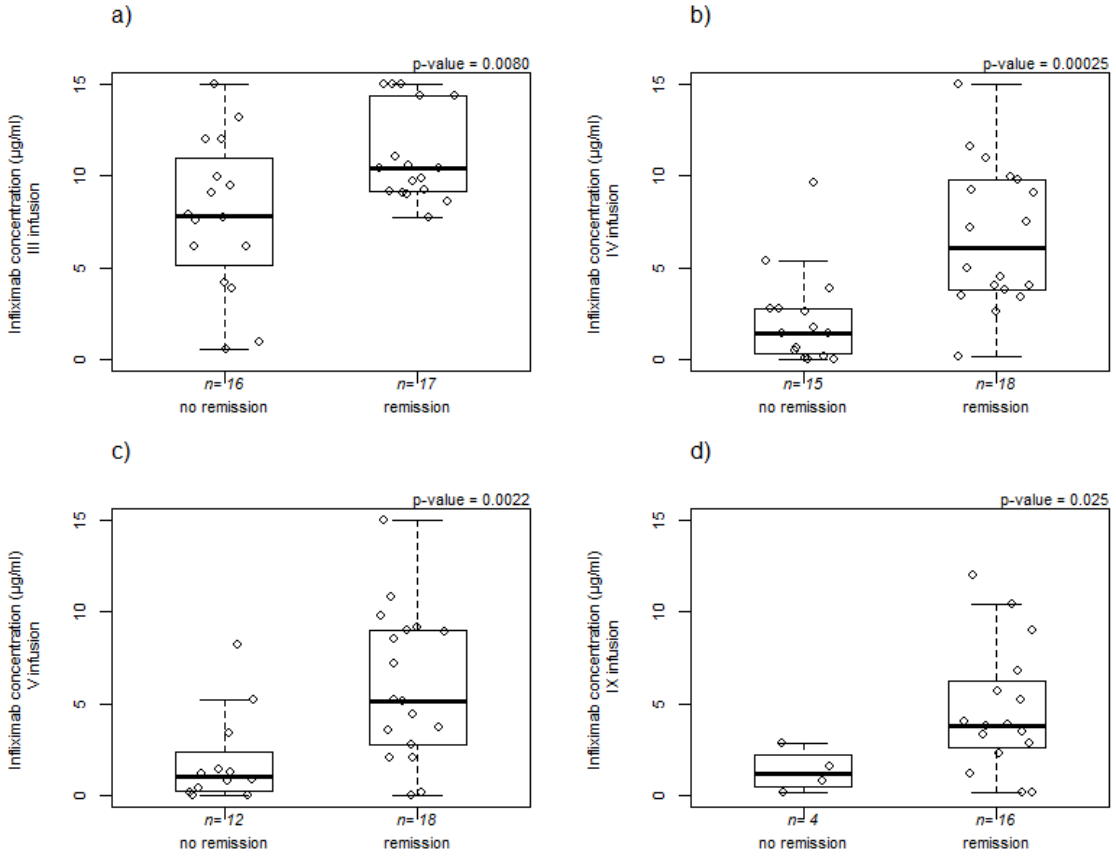

Figure 4: Boxplot comparing clinical remission at 54 weeks of treatments and serum IFX concentration at the III (a), IV (b), V (c) and IX (d) infusion between responsive and non-responsive patients. The bold horizontal line represents the median value. Statistical significance was assessed by logistic regression.

Receiver operating characteristic (ROC) curves were constructed to assign optimal cut-off values for IFX levels before IV infusion and clinical response at 54 weeks: an optimal cutoff of 3.11 µg/ml was defined. Area under the ROC curves (AUC) was 85.9% (Figure 5). The test had a sensitivity of 88.9% and a specificity of 80.0% (positive predictive value [PPV] 84.2%; negative predictive value [NPV] 85.7%) for predicting sustained remission. Logistic regression analysis confirmed patients who reached the cut-off point of 3.11 µg/ml (19 patients, 16 in sustained remission at 54w) had a higher probability of maintaining sustained remission compared to those who did not (14 patients, only 2 in sustained remission at 54w), with an odds ratio (OR) of 32.0 (95% CI 5.5 –297.8, p-value 3.0 $x10^{-5}$).

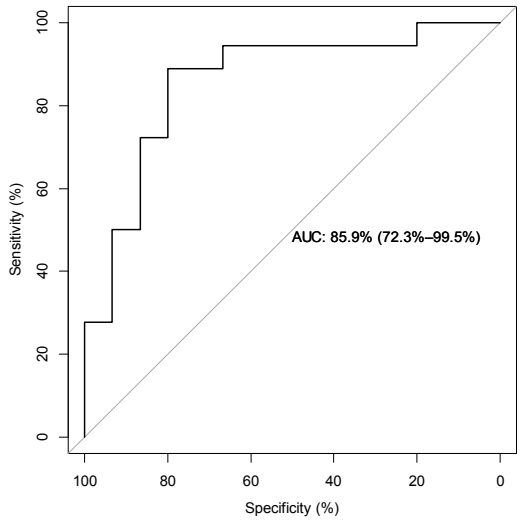

Figure 5: Areas under the ROC curves for the serum IFX quantification at the IV infusion for clinical remission at 54 weeks of treatment. ROC, receiver operating characteristic. Optimal cutoff value was 3.11 µg/ml (sensitivity 88.9%, specificity 80.0%).

Serum IFX levels and demographics, clinical and biochemichal variables

Considering demographical variables, neither sex (p-value logistic regression = 0.16) nor age (p-value logistic regression = 0.10) or IBD type (p-value logistic regression = 1.00) were significantly associated with achieving the IFX cut-off value for sustained remission (i.e. 3.11 µg/ml at the IV infusion). The cut-off value was significantly correlated with biochemichal parameters measured at IV infusion: patients not achieving the cut-off IFX concentration had significantly higher CRP and calprotectin levels and lower albumin levels (Figure 6).

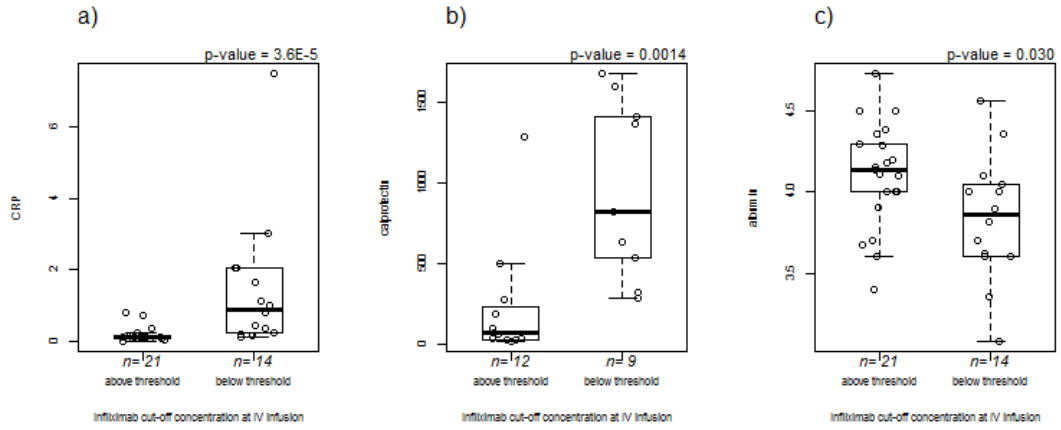

Figure 6: Level of clinically relevant laboratory parameters (a) CRP, b) calprotectin, c) albumin) in patients with a concentration of IFX at the IV infusion below or above the threshold level associated with sustained response; p-values are from Wilcoxon test.

CRP and calprotectin but not albumin at 14w also showed a significant association in a logistic regression analysis with sustained remission at 54w (Table 1). Considering all post-induction samples collected (n = 90 in 42 patients), IFX levels were significantly inversely correlated with CRP (p-linear mixed-effect model p-value=0.0008) and calprotectin (linear mixed-effect model p-value = 0.025) and directly correlated with albumin (linear mixed-effect model p-value = 0.0033) (Figure 7).

A multivariate logistic regression model was therefore performed to assess the independence of the association between CRP, calprotectin, and IFX concentration cut-off, with sustained clinical response. A model containing all variables did not converge, likely because of the missing values of calprotectin. However, a model with CRP and the IFX concentration cut-off showed a significant effect only for the IFX cut-off (adjusted logistic regression model p-value = 0.0065), which therefore was confirmed to be the most robust predictor of sustained clinical response.

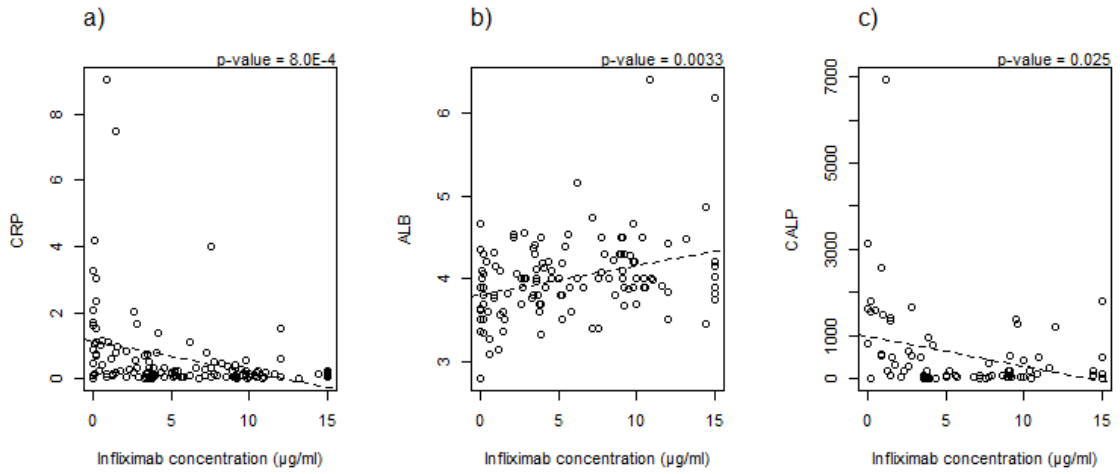

Figure 7: Concentration of IFX and relevant laboratory variables. Concentration of IFX was significantly inversely correlated with CRP and calprotectin and directly correlated with albumin (p-values and correlation lines are from linear mixed-effect models).

AIA quantification

AIA were measured in all samples that showed serum IFX levels below 1.5 µg/ml: AIA concentrations were inversely correlated with IFX trough concentration (Spearman test p-value =0.00088; Figure 8). Considering all patients samples and assuming that patients showing high serum IFX concentration were negative for AIA concentration, the correlation was even more significant (Spearman test p-value = 3.2×10−15).

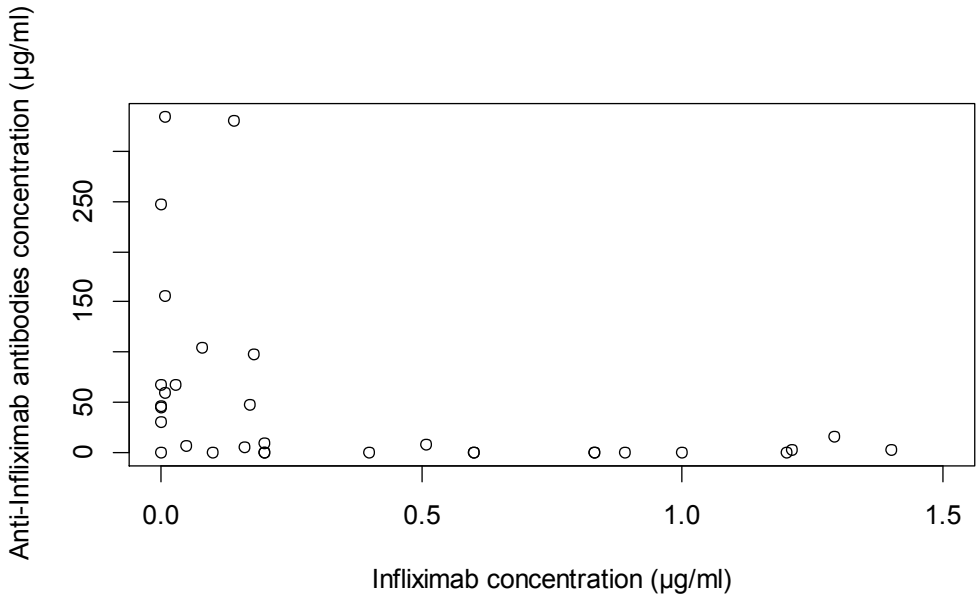

Figure 8: Scatterplot displaying IFX and AIA concentration. The correlation was assessed using Spearman's tests.

Ten patients (20.4%) resulted AIA positive, 2 at the III infusion, 3 at the IV infusion, 2 at the V infusion and 3 at the IX infusion; in all but one patient, AIA positivity persisted also at subsequent infusions (Figure 9).

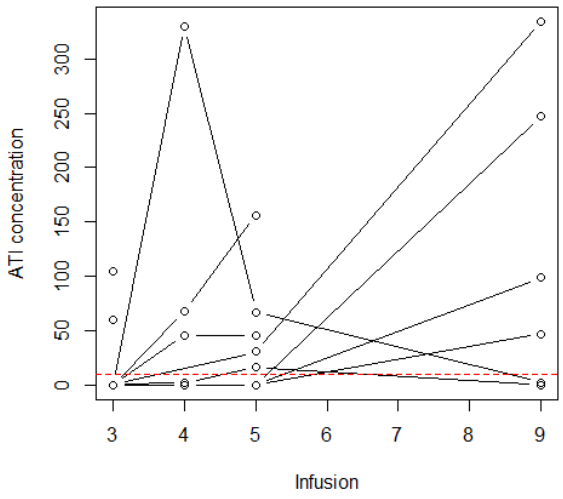

Figure 9. Trends for AIA concentrations in individual patients during the study period.

Serum AIA levels and adverse reaction

AIA levels higher than 10 ng/µl were considered positive. A statistical significant association was found between positivity to AIA and anaphylactoid reactions during treatment (logistic regression p-value = 0.018, OR 8.00, 95% CI 1.4-50.4): indeed, of the 7 patients with anaphylactoid reactions during the study, 4 were AIA positive (57%), while among the 42 showing no adverse reaction only 6 were AIA positive (14%; Figure 10).

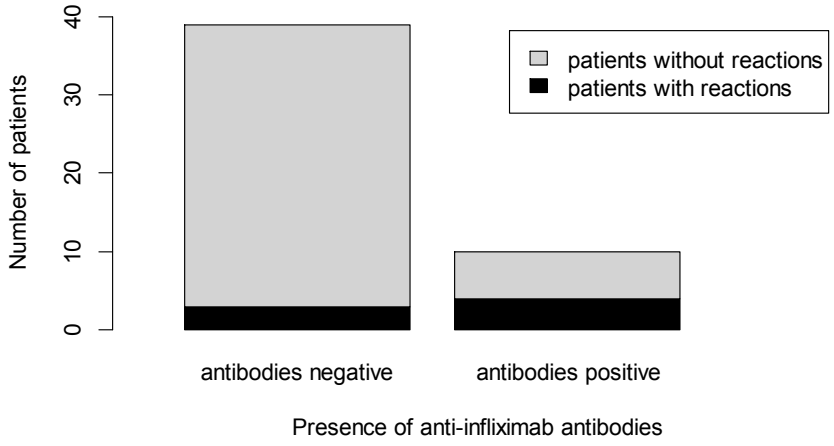

Figure 10: Anti-IFX antibodies and anaphylactoid reactions in patients.

Serum AIA levels and therapeutic response

No statistically significant association was found between positivity to AIA and clinical efficacy. Indeed, among the 24 patients showing sustained response to IFX, three (12.5%) resulted AIA positive during therapy, while among the 25 patients with unsatisfactory response, seven (28%) resulted AIA positive (logistic regression p-value = 0.19). However, considering the 5 patients with a very high AIA concentration (>100 µg/ml), a trend was observed toward worse efficacy, with just one patient (20%)

showing sustained remission, compared to 61.4% of patients non developing AIA or developing AIA at a concentration lower than 100 μg/ml (logistic regression p-value = 0.073, OR 2.5, 95% CI 0.85-130.0).

Discussion

Forty-nine percent of patients achieved sustained response within 54 weeks of therapy, while the incidence of therapeutic inefficacy was 18.4% after induction (primary failure) and the same percentage of patients lost response within 54 weeks of therapy (secondary loss of response); moreover, 14.3% of patients had to discontinue treatment because of the occurrence of adverse events (anaphylactoid reactions). Overall, therapeutic efficacy of IFX in our cohort is comparable to previous reports.

IFX trough levels were found to be significantly associated with clinical remission at all time points. Most importantly, IFX concentrations measured at the end of induction therapy (week 14) were predictive of sustained clinical remission without need for treatment intensification at 54 weeks: this is similar to what was previously reported both in adult and pediatric patients. In adult patients, Cornillie et al. reported that median week 14 trough levels in patients with and without durable sustained response to IFX 5 mg/kg were 4.0 and 1.9 μg/mL, respectively.[32] As in the present study, IFX levels at week 22 were also significantly associated with therapeutic response. Singh et al. demonstrated in pediatric patients that week 54 persistent remission was significantly associated with week 14 IFX concentration: in particular, a value of 4 mg/mL or above was predictive of sustained response.[98] The cut point identified in our study is slightly lower, even if concordant with those reported by other authors.[92 121] Interestingly, IFX concentration at the IV infusion was also predictive of response at this same infusion and at 22 weeks: this may be directly reflective of disease status at the time of sample

collection. Indeed, at the IV infusion, laboratory parameters associated with disease activity, in particular CRP, calprotectin and albumin were also significantly different between patients with IFX concentration below or above the long-term efficacy prediction threshold. CRP and calprotectin at the end of induction were also predictors of sustained IFX efficacy at week 54; however, multivariate analysis indicated that IFX concentration threshold was the best predictor, suggesting a more direct causal role for IFX efficacy. Interestingly, a strong correlation was found also between IFX trough levels and biochemical variables (CRP, calprotectin, and albumin) during maintenance (i.e. when inflammatory markers tend to reflect response to treatment).

Our data offer also the opportunity to examine causes of treatment failure in a population of pediatric patients receiving IFX. It has been suggested in fact that causes of treatment failure in patients treated with anti-TNF biologic agents can be categorized in three groups: pharmacodynamic failure (when drug levels are high and no AIA are detected); pharmacokinetic failure (when drug levels are low and no AIA are detected); and immunogenic failure (when drug levels are low and AIA are present). In addition to this, one should always consider also the possibility of inappropriate patient selection (e.g. patients with symptoms related to non-inflammatory disease complications unlikely to benefit from TNF blockade), concurrent infections (e.g. cytomegalovirus colitis), or poor adherence to treatment.[73] In our study, patients were selected at enrollment to maximize the possibility of treatment benefit, excluding patients with evidence of non-inflammatory disease. In our cohort of patients, we observed that treatment failure was mainly associated with inadequate drug levels or adverse events. Among patients failing to reach clinical remission at the end of induction, only 2 patients had IFX levels above 3 μg/ml (fig. 2b). Also at 54w, IFX levels measured at IV, V, and IX infusion were below 3 μg/ml in most patients not achieving clinical remission, with only few patients maintaining higher IFX levels (fig. 4). It seems therefore that in

children with IBD treated with IFX, inadequate blood drug levels represent the main cause of treatment failure, along with the occurrence of anaphylactoid reactions, and that so-called pharmacodynamic failures do represent a rare occurrence. It should be also noted that a minority of patients with sustained remission also had low drug levels, therefore, while failing to achieve remission seems to be associated in most cases with low drug levels, the opposite is not always true.

In our study, 20% of patients developed positivity to AIA, and among these 40% developed reactions to IFX, compared to 8% of AIA-negative patients. However, incidence of sustained IFX response was not statistically different among these two group of patients, perhaps due to the relatively low number of patients enrolled. It should be noted that AIA have been measured only in samples with low IFX concentration, and not in all samples, since the ELISA assay employed could detect AIA only in presence of low levels of AIA. This may have affected the possibility of finding a significant statistical association with treatment failure, as we may have detected also transitory AIA, which have little clinical significance. Adopting a different assay capable of detecting AIA in all conditions could possibly lead to more meaningful results.

In conclusion, our data appear in accordance with existing literature in both adult and pediatric patients, and further support the utility of measuring IFX concentration to monitor and predict therapy outcomes. IFX concentration at the end of induction therapy is confirmed as a strong predictor of sustained efficacy, and presence of AIA a determinant of reactions to IFX in children with IBD. Furthermore, our results confirm that therapeutic failure in pediatric IBD is associated in most cases with inadequate drug levels ("pharmacokinetic failure"). Greater availability of anti-TNF blood levels laboratory quantification in clinical practice is necessary to improve care of pediatric IBD patients. Point-of-care assays may allow clinicians to immediately adjust or change

treatment based on pharmacokinetic results in addition to clinical variables. Further studies should be performed to understand therapeutic strategies to improve outcomes in patients at risk of not reaching adequate IFX levels at the end of induction. These could include monitoring IFX concentration at an earlier stage than pre-infusion measurement and adopting a proactive treatment strategy, e.g. adding an immune modulatory drug to avoid low-drug level-induced immunogenicity or increasing drug levels in advance.

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