THERAPEUTIC DRUG MONITORING – AN EMERGENT APPROACH IN INFLAMMATORY BOWEL DISEASE

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Céptico como os cépticos, crente como os crentes.
A metade que avança é crente, a metade que confirma é céptica.
Mas o cientista perfeito é também jardineiro:
acredita que a beleza é conhecimento.

Gonçalo M. Tavares

Ao meu Avô

À minha Mãe

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List of Abbreviations

ADA – Anti-Drug Antibodies

ADCC - Antibody-Dependent Cellular Cytotoxicity

ADL - Adalimumab

AHLC - Anti-Human Lambda Chain

APC - Antigen-Presenting Cells

ATG16L1 - Autophagy related 16 like 1

CD – Crohn's Disease

CDC - Complement-Dependent Cytotoxicity

CRP – C-Reactive Protein

DDW – Digestive Disease Week

ECCO - European Crohn´s and Colitis Organisation

ELISA - Enzyme-Linked Immunosorbent Assays

EMA - European Medicines Agency

FC – Faecal Calprotectin

FcRn - Brambell receptor

FDA - American Food and Drug Administration

HMSA - Homogeneous Mobility Shift Assay

HPLC - High-Pressure Liquid Chromatography

HRP - Horseradish Peroxidase

IBD – Inflammatory Bowel Disease

ICC - Intraclass Correlation

IFX - Infliximab

IgG – Immunoglobulin G

IL – Interleukin

KAPPA - Cohen's kappa coefficient

LPS - Lipopolysaccharides

MHC II - Major Histocompatibility Complex Class II

mTNF - membrane-bound TNF

NLR - NOD-like receptors

NOD - nucleotide-binding oligomerization domain-like

NSAID – Non-Steroids Anti-Inflammatories Drugs

PD – Pharmacodynamic

PK - Pharmacokinetic

QB – Quantum Blue

RA - Rheumatoid Arthritis

RGA - Reporter Gene Assay

RIA - Radioimmunoassay

ROC - Receiver Operating Characteristic

SFPE - Semi-Fluid Phase Enzyme Immunoassay

sTNF - soluble-bound TNF

TDM – Therapeutic Drug Monitoring

TGF-β - Transforming growth factor beta

Th - T helper

TL – Trough Levels

TLR - Toll-Like Receptors

TNF-α – Tumour Necrosis Factor α

TNFR1 - Tumour Necrosis Factor Receptor 1

UC – Ulcerative Colitis

UEGW- United European Gastroenterology

USA – United States of America

Abstract

The discovery of anti-TNF-α drugs revolutionized the treatment of inflammatory bowel disease (IBD). However, a considerable portion of patients don't respond to treatment or lose response over time. Loss of response is frequently managed empirically. Therapeutic Drug Monitoring (TDM) is an emergent alternative strategy in the management of IBD patients. Measuring drug levels and anti-drug antibodies (ADA) levels may help clinicians to adjust therapy on an individual patient basis. A correct use of TDM in clinical practice relies on the knowledge of pharmacokinetic (PK) properties that influence treatment response and the accurate use of assays for the detection of drug and ADAs.

The overall aim of this thesis was to understand the impact of methodological and PK factors on TDM approach in IBD patients under anti-TNF-α antibodies.

We have assessed the impact of different assays on results and consequent biases on TDM interpretation. This work demonstrated that the presence of IFX influences significantly the detection of ADA and that this impact is assay dependent. Our data also showed that the amount of ADA modulates the impact of IFX in all assays. Furthermore, serum samples double positive (IFX+/anti-IFX+) and double negative (IFX-/anti-IFX-) lead to higher disagreement between assays, indicating the need for careful decision making in these situations. Our work was the first to show that, a recently developed methodology, the rapid test, could substitute old methodologies (ELISAs) in TDM, revolutionizing the way physicians use TDM in their clinical practice. The rapid test, as well as ELISA assays, were tested for evaluation of drug levels of biosimilars of IFX, CT-P13 and SB2. This work exposed that mostly all assays showed a good performance with exception of ELISA Sanquin for CT-P13.

In this work TDM was used to assessed pharmacokinetics properties in ulcerative colitis (UC) patients treated with anti-TNF-α agents. Our data demonstrated that IFX trough levels correlated with endoscopic remission and low degree of inflammation in UC patients indicating that TDM may be used to discriminate PK and

pharmacodynamic reasons for therapy failure. Nevertheless, in clinically-stable UC patients ADA presence was the main factor influencing PK of IFX. Our study revealed a strong advantage to include TDM (namely, determination of ADA) alongside with faecal calprotectin (FC) assessment in clinically-stable UC patients.

Overall, presented data supports that TDM-based strategy should be recommended in scenarios of a) loss of response guiding physicians to discriminate among PK and PD reasons for therapy failure; and b) predict loss of response combining TDM with biomarkers assessment, namely, FC, in stable patients. Moreover, TDM management must be constantly integrated with the recognition of methodological related biases and with individual clinical evaluation. Both drug and ADAs must be assessed by the same assay throughout time. If not possible, physicians must be aware of potential methodological biases in results interpretation.

Resumo

A descoberta dos anticorpos anti-TNF-α revolucionou o tratamento da doença inflamatória intestinal (DII). No entanto, uma parte considerável dos doentes não responde ao tratamento ou perde a resposta ao longo do tempo. A perda de resposta é frequentemente gerida de forma empírica. A monitorização terapêutica de fármacos (MTF) é uma estratégia emergente no tratamento de doentes com DII. A avaliação dos níveis de fármaco e dos níveis de anticorpos anti-fármaco (AAF) pode ajudar os médicos a ajustar a terapêutica de forma individualizada. O uso adequado da MTF depende do conhecimento das propriedades farmacocinéticos que influenciam a perda de resposta e do uso correto dos métodos para a deteção dos níveis de fármaco e AAF.

O objetivo geral desta tese foi compreender o impacto dos fatores metodológicos e farmacocinéticos na abordagem com MTF em doentes com DII tratados com anticorpos anti-TNF-α.

Avaliámos o impacto de diferentes ensaios nos resultados e consequentes vieses na interpretação da MTF. Este trabalho demonstrou que a presença de fármaco influencia significativamente a deteção de AAF e este impacto varia consoante a metodologia. Os nossos resultados também demonstraram que a quantidade de AAF modula o impacto da presença de fármaco em todos os ensaios. Além disso, nas amostras de soro duplamente positivas (IFX^+/anti-IFX^+) e duplamente negativas (IFX^-/anti-IFX^-) observou-se uma discordância significativa entre os ensaios, indicando a necessidade de um cuidado adicional na aplicação da MTF nessas situações. O nosso trabalho inclui o primeiro estudo a demonstrar que a metodologia recentemente desenvolvida, o teste rápido, pode substituir as metodologias antigas (ELISAs) na MTF, revolucionando a forma como os médicos usam a MTF na sua prática clínica. O teste rápido, bem como teste ELISA, foram também testados para avaliação dos níveis de fármacos dos biossimilares do IFX: o CT-P13 e o SB2. Este trabalho mostrou que a maioria dos

ensaios apresenta um bom desempenho, com exceção do ELISA Sanquin para o CT-P13.

Neste trabalho a MTF foi utilizada para avaliar as propriedades farmacocinéticas em doentes com colite ulcerosa tratados com agentes anti- TNF-α. Os nossos resultados demonstraram que os níveis mínimos de IFX se correlacionam com remissão endoscópica e baixo grau de inflamação em doentes com colite ulcerosa, indicando que a MTF pode ser usada para discriminar as razões de perda de resposta. Já em doentes com colite ulcerosa, mas clinicamente estáveis, a presença de AAF mostrou ser o fator principal a influenciar a farmacocinética do IFX. Desta forma, existe uma grande vantagem em incluir a MTF (em particular, a determinação de AAF) juntamente com a avaliação de calprotectina fecal (CF) em doentes com colite ulcerosa clinicamente estáveis.

Este trabalho mostra que a estratégia baseada na MTF deverá ser recomendada em cenários de a) perda de resposta, permitindo aos médicos discriminar entre razões de farmacocinética e farmacodinâmica para o insucesso do tratamento; e b) em doentes estáveis para predizer a perda de resposta, desde que combinada com avaliação de biomarcadores, nomeadamente a CF. Além disso, a utilização da MTF deve ser sempre integrada com a identificação de possíveis vieses metodológicos e a avaliação clínica do doente. Os níveis de fármaco e AAF devem ser avaliados pelo mesmo ensaio ao longo do tempo. Se não for possível, os médicos devem estar cientes dos possíveis vieses metodológicos na interpretação dos resultados.

I. INTRODUCTION

1. Inflammatory Bowel Disease

Inflammatory Bowel Disease (IBD) is a group of inflammatory disorders of the gastrointestinal tract, including two major diseases: Crohn's Disease (CD) and Ulcerative Colitis (UC). Both conditions are idiopathic, chronic and relapsing, and although there are some overlap features, they are separate conditions with distinct pathologic and clinical features [1-3].

Crohn's Disease presents a transmural (all layers from mucosal to serosa) inflammation that can affect any part of the gastrointestinal tract, from the mouth to the anus, but typically affects gut segments as the ileum, colon or both [4]. Though, CD presents normally an inflammatory phenotype at time of diagnosis it evolves frequently to a more aggressive phenotype with strictures, fistulas, or abscesses [4, 5].

Ulcerative Colitis is characterised by inflammation limited only to mucosal surface and affects colon and the rectum. The rectum is usually the first segment impacted and inflammation either remains localized or extends proximally through the entire colon [6, 7].

1.1. Epidemiology

Traditionally IBD was considered a western disease affecting mainly Caucasians across Europe, North America and Australia. The incidence and the prevalence of UC and CD seems to be stabilizing in these regions, while in the Middle East and Asia an increase has been reported [8, 9]. Table 1 shows the highest annual incident and prevalence rate reported for IBD [1]. Western incidence and prevalence is still higher than the rest of the word. Portugal is between the highest and the lowest prevalence of European countries, showing an IBD prevalence of 146 patients per 100 000 persons in 2007 and an annual incidence around 15 per 100 000 person-years[10]. Urban areas and higher social economic classes present a higher prevalence of CD than rural areas and lower social economic classes. IBD is an emerging global disease present in societies at all levels of socioeconomic development [1, 8, 9, 11].

Table 1. Highest incident and prevalence rate reported for IBD (data from World Gastroenterology Organization, reported in 2015 [1]).

	Highest annual incidence (per 100,000 person-year)		Highest prevalence (per 100,000 person)	
	UC	CD	UC	CD
Europe	24.3	12.7	505	322
Asia/Middle East	6.3	5.0	114	29
North America	19.2	20.2	249	319
Australasia	11.2	17.4	145	155

1.2. Pathophysiology of IBD

The ethology of IBD is unknown, nonetheless it is believed to arise in genetically susceptible individuals. Bowel inflammation in these individuals seems to be triggered by uncontrolled immune response after interaction between commensal gut flora and an unknown environmental agent or modifier [1, 12, 13].

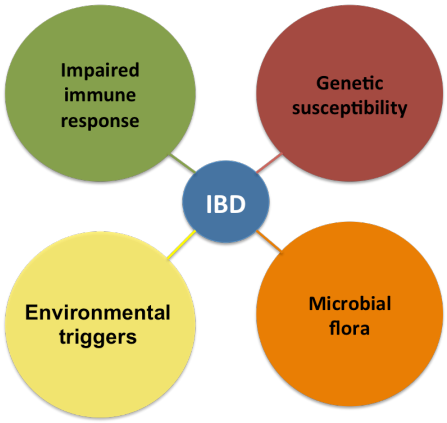


Figure 1 – Factors contributing to the onset of Inflammatory Bowel Disease.

1.2.1. Impaired immune response

The initiating causes are unclear. It is believed that the primary mechanism of inflammation in IBD is immune mediated, after barrier failure to enteric flora. T-cells

and macrophages activation leads to secretion pro-inflammatory cytokines (IL-1, IL-6, IL-8, IL-12, IL-17, IFN-γ, IL23, IL13, IL5 and TNF-α) and downregulatory cytokines (IL-10, and TGF-β) in the lamina propria [9, 14, 15]. The inability to ensure an equilibrium between both pro- and anti-inflammatory cytokines may generate chronic inflammation. Indeed, the onset of the chemokine gradient leads to recruitment of leukocytes to the site of inflammation, transmigration of neutrophils, and consequent tissue damage by metalloproteases, cytokines, growth factors, reactive substances and other mediators. These leads to amplification of the inflammatory response and disruption of the epithelial barrier which itself causes additional ingress of enteric flora and their products (Figure 2) [11, 12, 14].

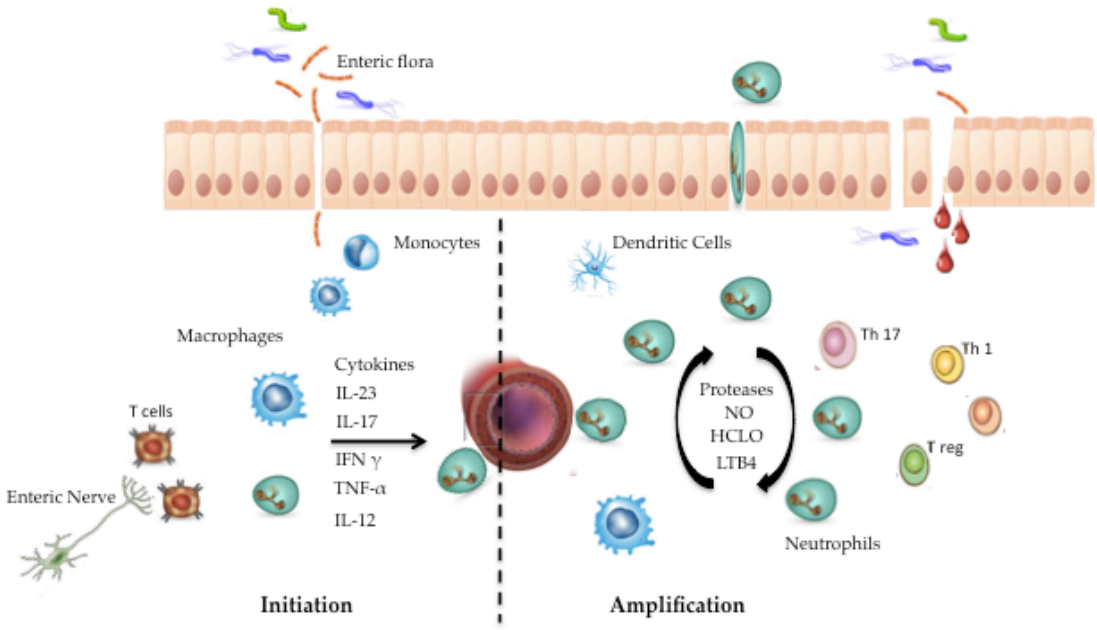


Figure 2 – Immune mechanisms involved in the pathogenesis of IBD (scheme was adapted from Rampton, 2016 [11]).

1.2.2. Genetic susceptibility

Both UC and CD present genetic susceptibility in about 8-15% and 12-15% of the cases, respectively [4, 7, 9]. The risk is particularly high in first-degree relatives. The lifetime risk of developing UC and CD is around 1-6 and 2-5% among non-Jewish populations and 4-5 and 7-8% among Jewish populations, respectively [16]. African Americans and Asian descents are linked to a lower risk of CD [17, 18]. A meta-analysis of genome-

wide association scans (GWAS) of large cohort IBD patients showed 163 disease-related genetic loci. Of the 163 loci, 110 were associated with both IBD conditions, 23 were associated only to UC and 30 only to CD [19]. Most of these genetic differences are implicated in several disease mechanisms such as mucosal barrier function or immune regulation. Homozygous NOD2 gene mutation is associated to a forty-fold increased risk for CD [11, 17, 20]. NOD2 polymorphism confer CD predisposition by altering immunorecognition of the constituents of bacterial flora and by modifying activation of nuclear transcription factor, NF-κB [11, 20]. Mutation of autophagy related genes (ATG16L1) result in increase of intracellular bacterial load [21]. Polymorphism of the gene encoding interleukin-23 receptor (IL-23R) can lead to an upregulation of interleukin (IL23) resulting in generation of Th17 effector cells, increasing interleukin 17 (IL-17) and consequently augmenting inflammation [22]. Nevertheless, no single susceptibility gene is sufficient to lead to disease. Gene-environmental interactions may be required for full development of the disease.

1.2.3. Environmental triggers

Several environmental factors have been attributed to IBD onset. Western diet has been associated to the pathogenesis of IBD. Indeed, high intake of fats, sugar and animal protein in meat and fish has been linked to an increased risk of IBD whereas high fruit and fibre intake have been seen to be protective [23]. Smoking showed to be a major risk for the onset of CD and for the disease presentation. Instead, for UC seems to have a protective role [24]. Pharmacological agents can also act as risk factors for IBD. Non-steroids anti-inflammatory drugs (NSAIDs), antibiotics, oral contraceptives and hormone replacement therapy have been described as possible risk factors [25-28]. Hygiene may also explain increasing IBD prevalence in newly developed countries since rigorous sanitary conditions may decrease early exposure to selected pathogens, reducing microbial diversity and augmenting risk factors for disease development [12].

1.2.4. Microbial flora

The role of gut microbial flora in the onset and pathogenesis of IBD has been reported in several studies [29, 30]. Gut microbiota is fundamental in the maturation and maintenance of normal mucosal immunity. It acts as a metabolic organ being an important barrier to the colonisation of pathogens and contributing to the production of short chain acids and vitamins.

While some microbial products have anti-inflammatory properties, others promote inflammation in susceptible individuals. In healthy individuals, pro-inflammatory toll-like receptors (TLR) and NOD-like receptors (NLR) pathways, are blocked by inhibitory molecules such as IL-10 and TGF-β [30]. When this homeostasis is disturbed, the state of immune tolerance is threatened and may result in gut inflammation.

1.3. Pharmacologic therapy

Pharmacological therapy for IBD is traditionally managed by a "step-up" strategy. Corticosteroids are usually initiated at the time of diagnosis or at disease flares [1, 31]. For UC, aminosalicylates are added to corticosteroids at mild to moderate active disease and alone for maintenance of remission [32, 33]. For CD, conventional immunosuppressive agents such as methotrexate and thiopurines (azathiopurine and mercaptopurine) are added to corticosteroids dependents or when flares are recurrent [5, 34]. If disease is still active or thiopurine intolerant, treatment with biological agents are usually the next step. Several biologic agents are available: antagonists to tumour necrosis factor α (anti-TNF-α) and antagonist to α4β7 integrin, for both UC and CD, and antagonist to p40 subunit of IL12 and IL23 only for CD [1, 5, 31-34].

Although, IBD pharmacologic therapy is developing quickly, with many new therapeutic agents under research that are likely to alter therapeutic approaches deeply in the next decade.

1.4. Antagonists to TNF-α

Tumour necrosis factor-α (TNF-α) is a proinflammatory cytokine that plays a central role on the inflammatory cascade that leads to chronic inflammation in IBD. TNF-α, produced mainly by activated macrophages and lymphocytes, promotes apoptosis and inflammatory response through activation of Nf-κB pathway [35]. Anti-TNF drugs are antibodies, produced by genetic engineering, that block TNF-α, thus supressing the inflammatory process mediate by this cytokine [36]. Several studies suggest a more complex function of TNF-α antagonists beyond simple neutralization. The putative mechanisms of action of anti-TNF-α are described in Table 2 [37]. Antagonists of TNF-α neutralize directly soluble and membrane form of TNF-α, but also modulate immune system facilitating inflammation resolution. These agents are also able of reverse signalling, induce apoptosis and induce both antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity.

Several anti-TNF-α inhibitors were approved for the treatment of IBD: infliximab (IFX), adalimumab (ADL), certolizumab pegol (for CD in the USA only) and golimumab (for UC).

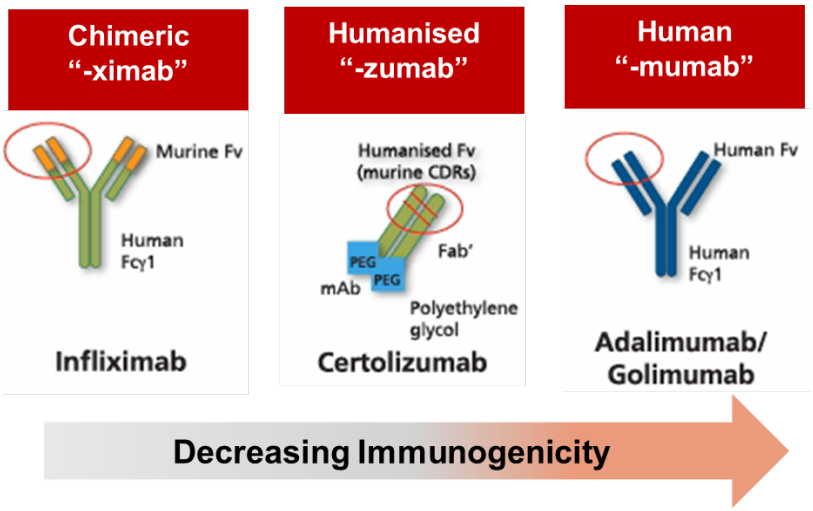
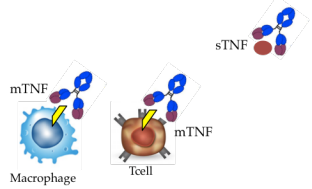
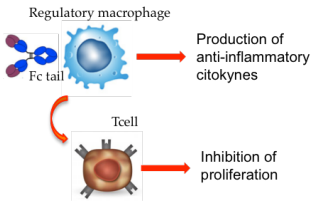
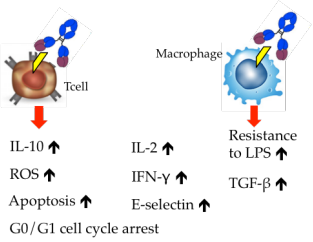
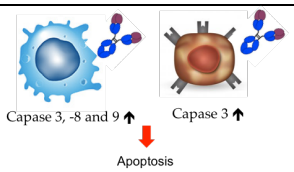
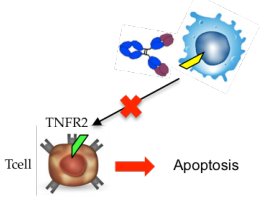
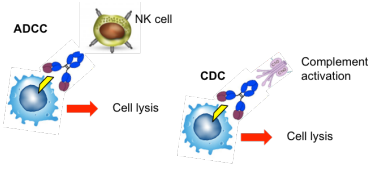


Figure 3 – Anti-TNFs antibodies approved for IBD therapeutic and potential impact of molecular structure on immunogenicity.

Table 2 – Mechanism of action of anti-TNF-α [37].

Mechanism of action of anti-TNF-α	Description	
TNF-α neutralization	TNF-α antagonists bind both soluble TNF-α (sTNF-α) and membrane-bound form of TNF (m TNF-α). Affinity of anti-TNF antibodies to sTNF has been reported higher than mTNF-α for some anti-TNFs antibodies. However, neutralization of mTNF seems to be more important than sTNF in IBD therapy.	
Modulation of the immune system	Anti-TNFs antagonists reduce proinflammatory T cells subsets and induce regulatory macrophages facilitating inflammation resolution.	
Reverse signalling or Outside-to-inside signalling	mTNF acts not only as a ligand but also as a receptor. Reverse signalling presents resistance to bacterial LPS; enhanced secretion of IL-2 and IFN-γ; upregulation of E-selectin in T-cells; induced expression of IL-10; activation of ROS accumulation and increased apoptosis induced G0/G1 cell cycle arrest.	
Direct Apoptosis of inflammatory immune cells	Anti-TNFs (namely IFX) induced direct apoptosis of monocytes and Tcells in a caspase-dependent way.	
Indirect Apoptosis of inflammatory immune cells	Indirect apoptosis may occur if anti-TNF binds to mTNF preventing TNFR2 activation by mTNF. TNFR2 activation leads to NfkB induction followed by IL-6 production, and subsequent T cell resistance to apoptosis.	
Antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)	ADCC is a mechanism of action of antibodies that possess Fc domain. After binding of anti-TNF to mTNF, Fc domain is recognised by NK cells that release cytotoxic proteins that subsequently result in lysis of the target cell (cytolysis). Binding of antibodies to target cells may also activate complement system (CDC).	

Infliximab was the first protein-based therapy against TNF-α available in the market for IBD treatment. Infliximab is a chimeric monoclonal IgG1 antibody against TNF-α. It presents 25% murine variable region and 75% human constant region and binds both to the soluble TNF-α (in its monomeric and trimeric form) and membrane-bound TNF (mTNF-α) [38]. Adalimumab and Golimumab are fully human IgG1 antibodies against both sTNF-α and mTNF-α. Both have been described as less immunogenic than infliximab due to full human origin [39, 40]. Certolizumab pegol is a pegylated humanized monoclonal Fab-fragment against both forms of TNF-α [39] (Figure 3).

1.5. Biosimilars

Therapeutic proteins (or biologics) have transformed the management of IBD patients and other immunoinflammatory diseases. These drugs account for almost 20% of total pharmaceutical sales market [41]. Recently, some important therapeutic antibodies, such as infliximab and adalimumab, have expired their patents and several pharmaceutical companies developed new similar products called biosimilars. The U.S. Food and Drug Administration (FDA) describes a biosimilar as a biological product that has been tested to be highly similar to the reference biological product, and with no significant clinical differences related to safety, purity and potency, despite minor differences in clinically inactive components [42].

Remsima® and Inflectra® are the first biosimilars produced based on the original infliximab- Remicade® (CT-P13) and have been approved by regulatory agencies for use (2013 – European Medicines Agency (EMA); 2016 – United States Federal Drug Administration (FDA)). A second infliximab biosimilar has been produced – Flixabi® (SB2) and approved by regulatory agencies for use (2016 – EMA; 2017 – FDA) [43].

A biosimilar cannot been seen as an exact copy of its originator. These compounds are derived from living organisms resulting in minor differences inherent of such a complex production. There are important differences in the degree of fucosylation, mannosylation and galactosylation, notwithstanding these differences have no impact

on the clinical properties [44]. It is uncertain if antibodies to the original drug may cross-react to the biosimilars. With the growth of biosimilars in IBD patient's management due to its lower price, one might expect the need to use TDM also in IBD patients under biosimilars. The role of TDM for biosimilars is therefore of growing interest.

1.6. Calprotectin – a inflammation biomarker

Secondary loss of response is typically referred when a patient responds to treatment during induction period but loses response to treatment during maintenance. Additional definitions have been suggested for loss of response or therapy failure: starting a new immunomodulator or biological drug, switching immunomodulator, regime intensification of current biological (increasing dose or decreasing interval), switching biological or need to undergo bowel-related surgery. Usually response and remission are determined using clinical symptoms indices: Mayo score for UC and Harvey Bradshaw index for CD. Higher scores are associated with disease activity [45]. Disease activity measurement based only in clinical symptoms may be ambiguous and clinicians often use other tools to support diagnosis. A combination of clinical features, endoscopy, histology, radiology and laboratory assessments are often required. Some of these exams are invasive (endoscopy, histology) and expensive. Laboratory exams, such as blood and stool analyses, are non-invasive, cheaper and faster than other procedures, alleviating patients from physical and financial burden [46, 47].

Calprotectin is a calcium and zinc-binding protein present mainly in neutrophil granulocytes but can also be found on the membrane of monocytes, macrophages and epithelial cells, in lower concentrations. Calprotectin has anti-microbial and anti-inflammatory properties and represents around 60 % of the protein content on the neutrophil's cytosol [48, 49]. In the presence of inflammation in the gastrointestinal tract, neutrophils migrate from the circulation to the affected mucosa. Active inflammation leads to changes in the epithelial barrier resulting in impaired permeability and neutrophils leakage to the lumen. During this process, calprotectin

is released to the lumen and consequently to the feces [49]. Fecal calprotectin (FC) has been considered a highly sensitive biomarker of intestinal inflammation. Studies have shown a high correlation between calprotectin concentration in the feces and intestinal inflammation [50]. This biomarker is stable in feces for 7 days, at room temperature and was reported to be distributed homogenously in feces [49].

2. Therapeutic Drug Monitoring

Infliximab and adalimumab have been approved for the treatment of moderate to severe CD, when conventional therapy (corticosteroids and/or immunosuppressant drugs) failed to respond [1, 5, 34], and for UC as a rescue therapy in corticosteroid-refractory severe disease [1, 32]. Both agents induce sustainable clinical response in IBD. Anti-TNF agents have been described to reduce hospitalizations and surgery rates in patients with IBD [51]. Nevertheless, about 20-30% patients fail to respond favourably in induction period (primary non responders) and up to 40% of patients sooner or later experience treatment failure (secondary non responders) [52, 53]. The loss of response may be related to immunogenicity (development of antibodies against TNF-α antagonists), individual differences in bioavailability, pharmacokinetics factors or other factors that increase drug clearance [53]. Before switching to another therapeutic class optimization of dose regimen and combination therapy might help these patients.

2.1. Clinic-based approach to drug optimization

When treatment with anti-TNF-α fails, clinicians need to act promptly to ensure treatment salvage. There is a risk of irreversible tissue damage since long-term periods with uncontrolled disease may lead to disease progression [54, 55]. Clinic-based approach relies in patients' symptoms and a "trial and error" strategy. Guidelines suggest handling non responders with a stepwise approach until a response is achieved. Firstly, with regime intensification of current anti-TNF-α (increasing dose or decreasing interval). Secondly, switching to other anti-TNF-α; and finally changing to

another class of drugs. Concomitant treatment with conventional immunosuppressive agents, co-treatment with corticosteroids and surgery is also suggested [31, 56, 57]. Unsuccessful trials lead not only to a negative impact on patients' clinical status, but also to significant financial burden. In IBD, TNF-α antagonists represent the highest healthcare cost being higher than surgery and medical hospitalisation costs [58, 59]. Chronic diseases such as CD and UC also have a significant impact in indirect disease related costs. Treatment failure has immediate impact on patients due to diminished productivity and leave use increase; and on their extended family due to the potential need to use leave to care for the IBD patient [58].

2.2. Therapeutic Drug Monitoring reactive approach to drug optimization

A tailored approach to drug optimization may be an alternative to a clinical-based strategy. Rather than handling patients as equal, applying a standardized treatment to all patients who lose response, one can adjust treatment according to individual characteristics. The complexity of clinical and disease characteristics and the involvement of numerous genes, cytokines and metabolites in drug response result that a given drug can be effective in a group of individuals and ineffective in others. It is also reported that some individuals experience adverse effects while others are not affected [60].

The use of therapeutic drug monitoring, by measuring drug and anti-drug antibodies in the blood, may help obtaining the highest therapeutic benefit with the lowest adverse effect. It can also aid to identify the underlying cause to therapy failure, which may include pharmacokinetic and pharmacodynamic factors and immunogenicity.

2.3. Pharmacokinetics and Pharmacodynamics of anti-TNF-α drugs

The mechanism of action of anti-TNF-α drugs was previously summarized in Table 2. Lack of response to anti-TNF-α drugs may be related with other significant inflammatory routes independent of TNF-α pathway [61]. Pharmacodynamics of anti-

TNF-α drugs seems to be influenced by patient´s individual variability. Indeed, the efficacy of anti-TNF-α drugs differs between individuals presenting comparable drug levels [62]. It has been reported that even with high circulating levels of anti-TNF-α drugs, several patients do not respond to treatment (primary non-responders) or lose response to treatment over time (secondary non-responders) [52, 53]. In such scenario, other mechanism of action rather than TNF-α must be present.

Pharmacokinetics (PK) of monoclonal antibodies is different from PK of conventional small drugs. As anti-TNF-α drugs are large molecules, administration is often intravenous (e.g. Infliximab) or either subcutaneous or intramuscular (e.g. adalimumab, certolizumab). Intravenous route allows the administration of a large volume of drug resulting in less variability in drug exposure, due to immediate central distribution, and less immunogenicity. Extravascular administration uses smaller injection volumes and distribution seems to occur by lymphatic drainage resulting in individual variability of absorbed dose. Subcutaneous administration is often more immunogenic [63-66].

Due to large size and hydrophilic nature of antibodies, distribution occurs mainly within the central compartment (peripheral blood) with low tissue penetration, and therefore the volume of distribution is small (≈ 0.1 L/Kg) [64, 65]. The transport into the cells may likely occur via pinocytosis (fluid-phase endocytosis) in endothelial cells, or via receptor-mediated endocytosis through Fcγ receptors present in immune cells membranes. At a lesser degree, transport may occur via convective transport across paracellular pores in the vascular endothelium [66-68].

The large size of anti-TNF-α antibodies (≈150 KDa) prevents renal elimination. Only small fragments of antibody (e.g. Fab portion) are filtered by the kidney and the majority of the filtered protein is reabsorbed by proximal tubular cells. Bile excretion represents a minor elimination pathway of anti-TNF-α, since only small quantities of antibodies are found in bile [68]. Thus, the elimination route of monoclonal antibodies seems to occur by intracellular proteolytic catabolism and comprise 3 main pathways:

a) pinocytosis with lysosomal degradation (fluid-phase endocytosis); b) degradation of internalised antibodies by receptor-mediated endocytosis process; and c) degradation of internalized antibody-antigen complex (target mediated endocytosis) [65, 66, 68].

Binding of Fab or Fc domain to cell surface receptors, triggers the internalization of the antibody into a vesicle and subsequent intracellular catabolism by proteases [66]. When the complementarity-determining region of the Fab fragments of the antibody bind to specific target in cell surface the endocytosis and the elimination is called target-mediated endocytosis (Figure 4b). The expression of the target, the dose of antibody and the affinity to the target influence the rate of elimination [66]. On the other hand, when Fc domain bind to Fcγ receptors expressed in immune cells the endocytosis and elimination is called receptor-mediated endocytosis (Figure 4a).

Fluid-phase endocytosis is an unspecific pathway of internalization into cells. After endosome formation, degradation may occur. In an acidic environment (pH 6.0) and the presence of Brambell receptor, FcRn, the antibody may be protected from degradation. When Fc domain binds to FcRn no degradation occurs in the endosome and IgG antibody is released again to the plasma and/or interstitial space [65]. This process not only contributes to IgG antibody homeostasis but also aids IgG antibody transport trough cells (Figure 4c).

Absorption, distribution and elimination of anti-TNF-α drugs are affected by several variables related to the disease, to the patient, to the target antigen and to the structure of the drug. Several studies have tried to identify these variables. Buurman et al, showed that sex influences clearance of IFX (being 35% higher in males than in females) [69]. Body weight index was described to increase clearance as well. This increase seems to be mediated by a higher inflammatory load in obese patients [70, 71]. Nevertheless, low weight patients show lower IFX trough levels probably because clearance is not linearly correlated to weight in these patients, but dosing is weight-based [72]. Low albumin was reported to be associated to increased clearance. Low albumin and high CRP levels are markers of high inflammatory burden [70, 73].

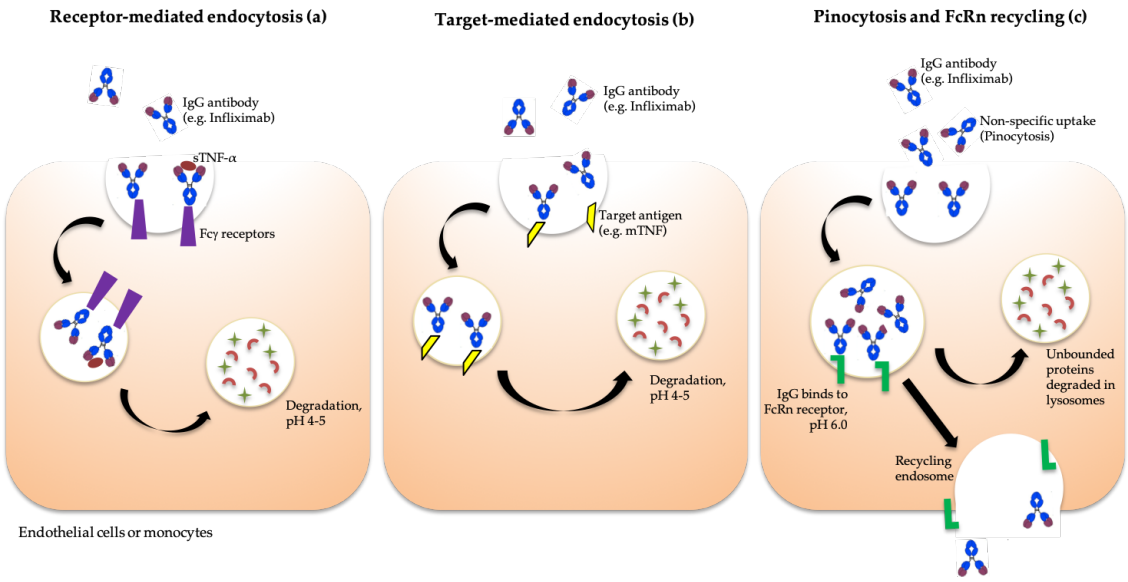


Figure 4 – Main elimination pathways of IgG antibodies drugs.

The degree of inflammation is also a factor of enhanced clearance. Firstly, because of increase IgG catabolism by reticuloendothelial system, secondly, because high levels of TNF-α will consume faster the anti-TNF-α drug, and thirdly because during active intestinal inflammation there is a rise of intestinal permeability and consequent fecal loss of anti-TNF-α drug [65, 74]. The individual variability of Fcγ receptors and/or FcRn receptors may also influence PK of anti- TNF-α antibodies, due to the importance of this receptors in anti-TNF-α elimination [75, 76]. Additionally, the formation of antibodies to the drug was reported to impact clearance of anti-TNF-α drug and consequently impact response to treatment [77].

2.4. Anti-TNF-α antibodies (ADAs)

Treatment with anti-TNF-α drugs may provoke an immunogenic response leading to development of endogenous antibodies against the foreigner protein (immunogenicity). Formation of ADAs may affect treatment safety due to hypersensitivity reactions and reduce therapeutic efficacy of the drug and, in some cases, therapy failure [65, 77].

Antibody formation is triggered by the interaction of three main cells: antigen-

presenting cells (APCs); T helper (Th) cells and B cells. Briefly, the therapeutic drug is recognized and engulfed by APC, through pinocytosis or receptor mediated endocytosis. Inside the cell, in the lysosomes, therapeutic drug is then degraded to small peptides. These peptides bind to the major histocompatibility complex class II (MHC II) which displays them at the surface of APC. Th cells will then recognize, through T cells receptors (TCRs), the complex MCH-antigen. In the presence of co-stimulatory molecules, such as CD80 and CD86, provided by APC, Th cell is fully activated. Without co-stimulation with CD80 and CD86 molecules, Th cells are not activated. When fully activated, Th cells divide and generate a set of pro-inflammatory cytokines that recruit naïve B cells. IgM and IgD receptors of naïve B cells bind to the epitope of MCH-antigen-TCR complex and this interaction induces B-cells differentiation to memory B cells and production of antibody-secreting plasma cells [65, 78, 79].

Several factors may elicit the generation of endogenous antibodies. Route, dose and frequency of administration can contribute to immunogenicity. Indeed, subcutaneous administration is more immunogenic than intravenous administration and a single infusion was described in the ACCENT I study to be more immunogenic than maintenance regime every eight weeks (ADAs incidence: <9% for maintenance vs 28% for episodically) [80]. Individuals with higher level of tolerance to a foreigner protein, prolonged treatments may eventually invoke the formation of endogenous antibodies. Ungar et al, showed that 90% of sustained ADAs formation occurs during the first year and the first detection of ADAs happened on average 4.5 months after treatment initiation [81].

The nature of the anti-TNF-α drug also influences the degree of immunogenicity. In fact, immunogenicity decreases with the level of humanization of the antibody (fully-human IgG < humanized IgG < chimeric IgG < murine IgG (Figure3) [65]).

Size, structural complexity and protein sequence variation may affect as well. Therapeutic antibodies are produced in cell lines of mammals (human and non-human), plants, bacteria, yeast and virus. During production, minor differences in

post-translational modification, such as glycosylation, may impact the immunogenicity. An immune response may be stimulated by the glycan structure itself or its presence may affect protein structure in a way that the protein becomes immunogenic [79, 82]. Process or product-related compounds such as impurities or additives may also impact immunogenicity. Even small amounts of impurities are able to invoke an immune response [83].

The amount of aggregates formed is directly correlated with the level of immunogenicity. Despite the developments in drug production over the last years, aggregates are one of the highest concerns of regulatory agencies, since their incidence may act as a strong activator of immune response of innate defense system [84]. Protein aggregation may occur in several steps of drug production, storage, transport and drug administration.

Patients characteristics might also influence immunogenicity. Genetic differences, the type of disease and the immune status of the patient can influence the capacity to invoke a significant immune reaction. A higher immune reaction may be expected in patients with a highly active immune system. Bartelds et al. showed that rheumatoid arthritis (RA) patients positive for antibodies to adalimumab presented higher disease activity and C-reactive protein levels at baseline [85].

Concomitant treatment of anti-TNF-α and immunomodulators in IBD patients was described to lower incidence of ADAs. The SONIC trial showed prolonged response to treatment in patients with concomitant therapy when compared with monotherapy [86]. The combination therapy had showed to eliminate existing ADAs and regain clinical response [87].

2.5. Methodologies for measuring anti-TNF-α drugs and antibodies

Awareness of the potential value of TDM has led to the development of different methodologies for assessing levels of anti-TNF-α and antibodies to anti-TNF-α. Numerous assays are available in the market, such as binding assays based on immunochemical processes, including solid or liquid phase immunoassays. The

available methodologies have different limitations and may yield different results. Therapeutic drug monitoring interpretation may be impacted by this potential bias.

2.5.1. Anti-TNF-α drugs levels assays

Levels of anti-TNF-α drug may be determined by distinctive assays. Figure 5 depicts three different methodologies.

Enzyme-linked immunosorbent assays (ELISAs) are the most commonly used assays [88]. ELISA requires costly equipment, highly trained analysts, sample batching and is time-consuming (~8 hours). Figure 5 a) and b) represent the principle of the assay. The TNF-α (5a) or an antibody to anti-TNF-α (5b) drug is adsorbed passively onto the wells of a microtiter plate, by incubating for a few hours. After rigorous washing to remove unbound excess of the antigen, a blocking buffer (often, 1% bovine serum albumin (BSA)) is added to the plate to block nonspecific binding sites. Diluted serum samples are then added to the plate and anti-TNF-α present in the samples will bind to the antigen immobilized in the plate (TNF-α (5a) or an antibody to anti-TNF-α (5b)). After an hour of incubation for total binding, plate is washed again to remove unbound material. For detection, an antibody (anti-Fc domain or anti-TNF-α antibody) linked to an enzyme (horseradish peroxidase) is added to the plate. After, incubation period, the plate is washed again and a substrate to the enzyme (tetramethylbenzidine) is added. In the presence of the substrate a colorimetric product is formed, and the reaction is stopped with an acidic solution. The absorbance of the colorimetric product is measure at a specific wavelength [89].

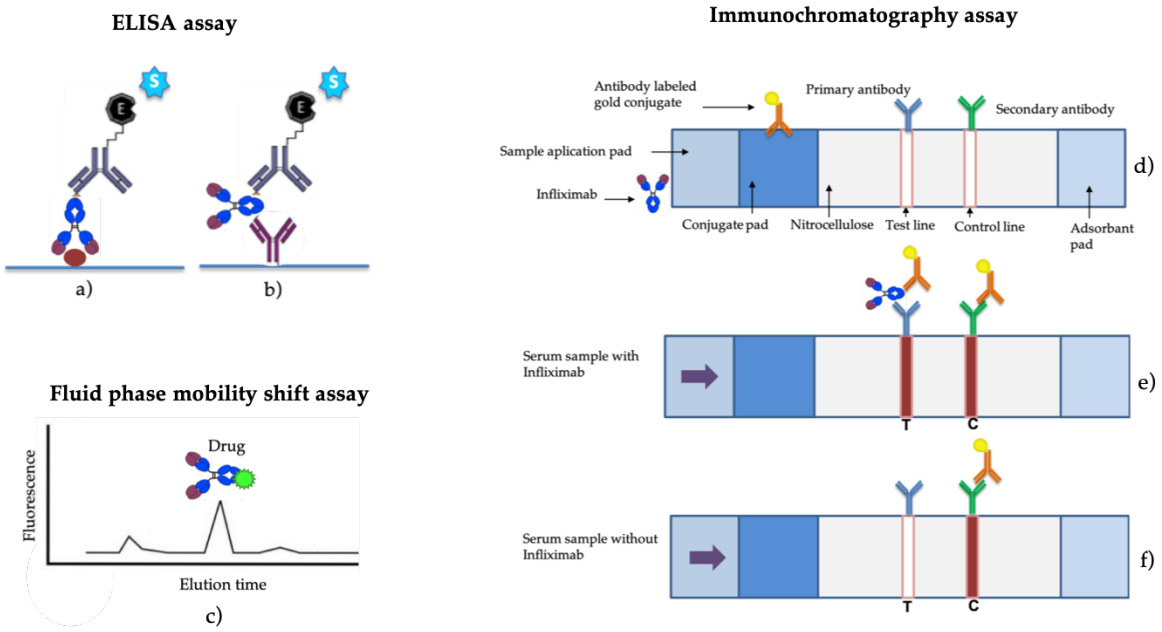


Figure 5 – ELISA and immunochromatography assays for drug assessment.

The fluid phase homogeneous mobility shift assay (HMSA) is a high-pressure liquid chromatography (HPLC) base mobility assay. A fluorescent-labelled-TNF-α complex incubation with the serum is performed before injection in the chromatographic column. The molecular size separation in the chromatographic column allows the identification of free drug with a fluorescence detector (5c) [90].

Recently, a new rapid test was launched in the market. This new assay relays in the lateral-flow based assay technology which allows results in only 15 minutes. Figure 5 d), e) and f) details the principle of this methodology [91].

Two anti-drug antibodies are used in this assay. One is immobilized in a chromatographic membrane (primary antibody), at the test line, the other, labeled with a colloidal gold conjugate, is impregned between the application pad and the chromatographic membrane. There is a third antibody (secondary antibody) that is use as test control (Figure 5 d)).

Once a sample with anti-TNF-α drug is added to the cassette, the sample migrates through the membrane, binding first to the antibody labelled to colloidal gold (conjugate). This immunocomplex drug-conjugate continues through the chromatographic stream moving to the test and control zones. When the complex

drug-conjugate finds the primary antibody, they bind, and a positive reaction is seen by the red line in the test zone. Then, the excess of free conjugate binds to the secondary antibody and a red line is formed in the control zone. This line assures that the test is a valid test (Figure 5e)).

In a non-drug sample, the conjugate (antibody labelled to colloidal gold) is carried by the chromatographic stream and binds only to the secondary antibody in the control zone and no colour is seen in the test line (Figure 5f)).

The quantification of the drug is determined by an equipment which is able to scan the chromatographic membrane, by focusing light and measuring the reflected light attenuation by the surface of the membrane in the test line and control line.

Still, more assays are available for the anti-TNF-α antibody levels determination. Less used because of the complexity of the assay and safety concerns (handling of radioactive material) are the radioimmunoassay's (RIA). It uses the same principle of ELISA, but in a liquid-phase assay and with a radio-label antibody for detection step.

2.5.2. Antibodies to anti-TNF-α levels drugs assays

Assessing the levels of ADA in IBD patients under anti-TNF-α therapy is a challenging task. There is no gold standard for ADA levels determination. It is important to acknowledge that assay heterogenicity may decrease TDM accuracy, and consequently lead to erroneous clinical decisions. One of the main pitfalls of ADA assays is the drug presence in the serum sample, as the drug is an antibody itself [92]. Bridging ELISA assay is one of the most common commercial assays for ADA levels evaluation (Figure 6). This methodology has some drawbacks resulting in false positives and false negatives. False positives usually result from cross-binding of drug Fc-fragments with rheumatoid factors, activated complements and anti-allotypic antibodies present in the serum (Figure 6a)). The drug antibody pre-adsorbed to the solid phase uses ADA to 'bridge' with the enzyme-labelled drug antibody used for the detection phase. When other components, as rheumatoid factor, are able to mimic this 'bridge', false positives may arise (Figure 6b)). Drug presence may cause false negative

tests because ADA is already bound to the drug and cannot bind to the drug antibody pre-adsorbed in the plate, being lost in the washing steps (Figure 6c)). Additionally, if ADA are of the IgG4 type, that is functionally monovalent, it binds to the adsorbed antibody but is unable to bind to the enzyme-labelled drug antibody (Figure 6d)) [93, 94].

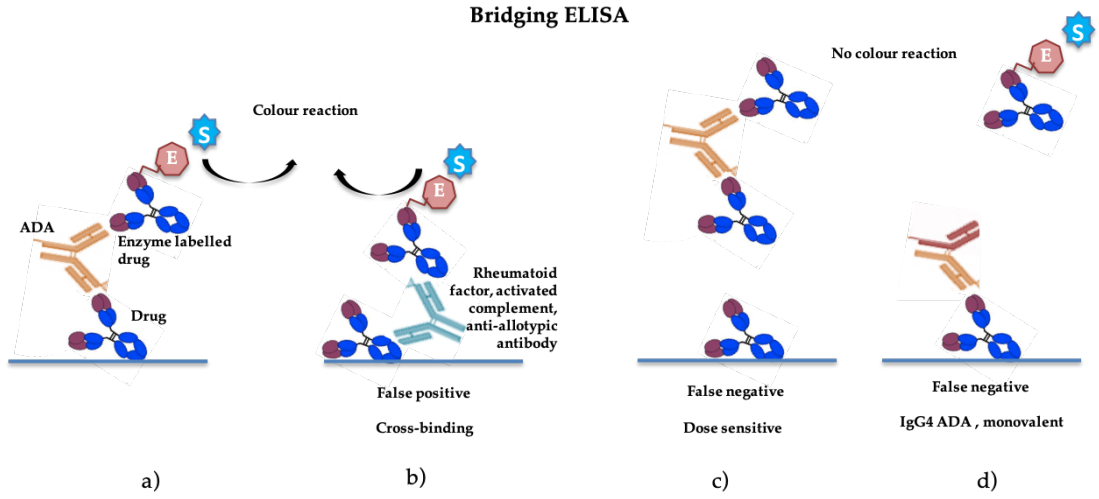


Figure 6 – Bridging ELISA for ADA levels assessment

To overcome these limitations other methodologies were developed. The anti-human lambda chain assay (AHLC) is an ELISA assay that uses a λ light chain antibody labelled to enzyme for detection step (Figure 7a)) [89]. The anti-TNF-α antibodies are IgG-κ light chains. On the other hand, the endogenous ADAs comprise both IgG-k and -λ light chains. Using a λ light chain antibody for detection step is likely to minimize drug interference. There are also some limitations to this assay. False negative tests may arise when detecting anti-idiotypic ADAs, since the TNF-α pre-adsorbed to the plate binds to idiotypes in the anti-TNF-α antibody precluding anti-idiotypic antibodies binding (Figure 7b)). Drug presence may also hamper ADAs detection with AHLC assay (Figure 7c)) [93].

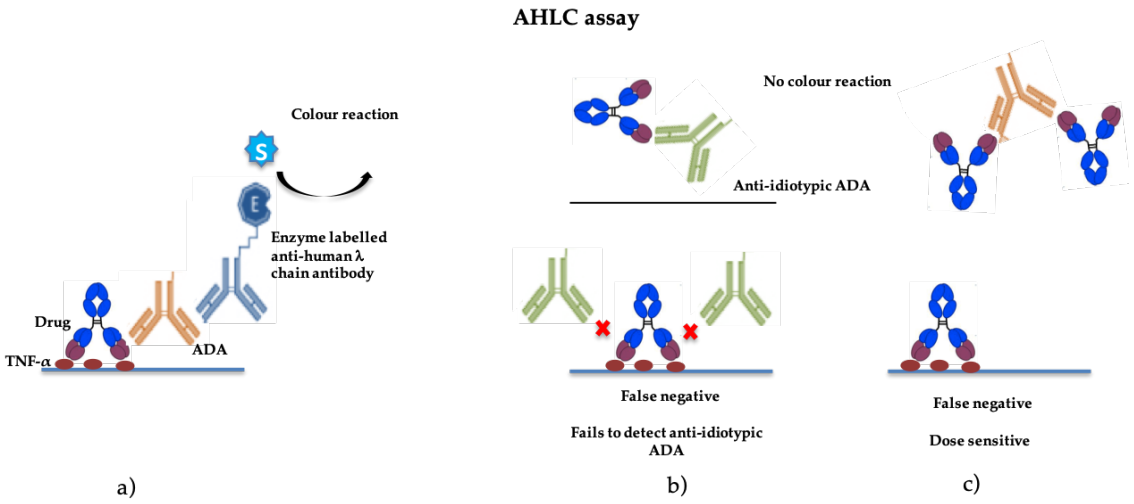


Figure 7 – AHLC ELISA for ADA levels assessment

The semi-fluid phase enzyme immunoassay (SFPE) is an ELISA assay that uses an initial acid buffer treatment to dissociate the drug-ADA complex and two drug-conjugates: a biotin labelled drug conjugate to immobilize ADA to the plate (pre-treated with streptavidin), and a horseradish peroxidase (HRP) labelled drug conjugate for detection step. After immune complex dissociation at low pH, both drug-conjugates bind to ADA and colour is formed in the presence of a substrate (Figure 8 a)). However, when ADA binds to double biotin-labelled drug conjugate (Figure 8 b)) or to double HRP- labelled drug conjugate (Figure 8c)) false negatives may occur.

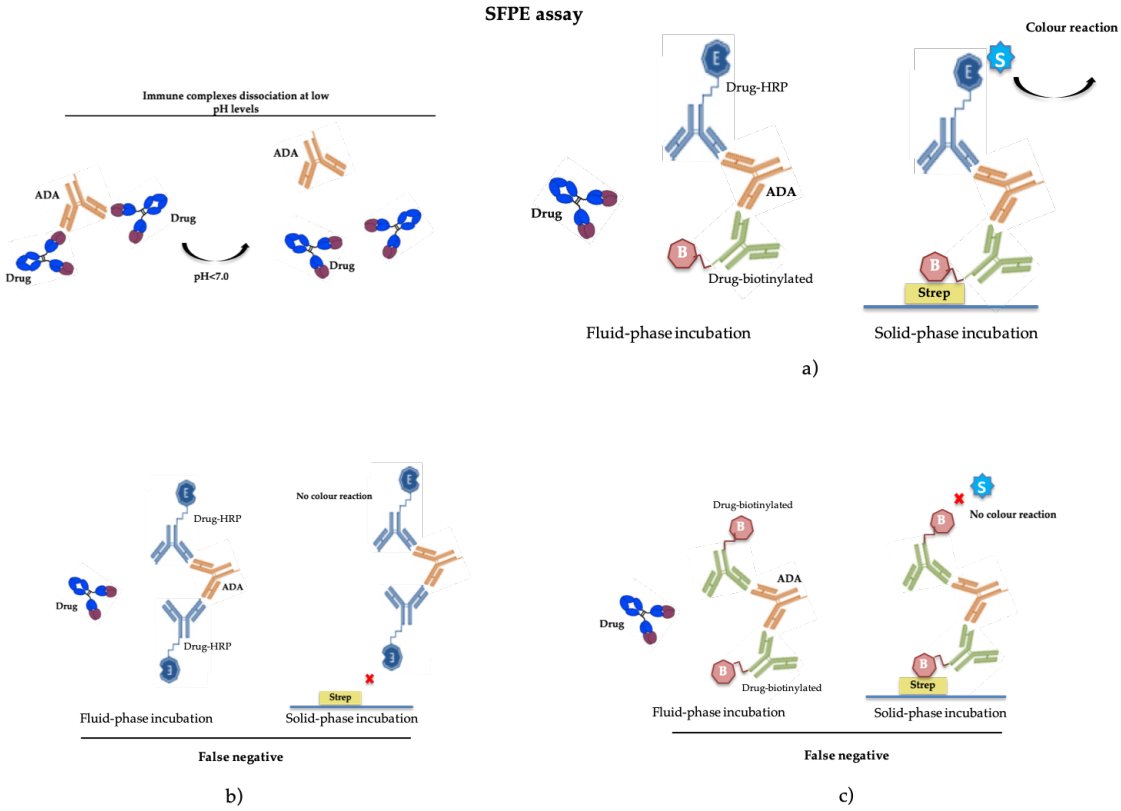


Figure 8 – SPFE assay for ADA levels assessment

Though, it should be notice, that solid-phase methodologies may be hampered by artifacts, namely neoepitope formation due to aggregates formation in plastic surfaces and epitope masking resulting in false-positives and false-negatives results, respectively [89].

Liquid-phase methodologies are less artificial reproducing better *in-vivo* conditions. Fluid phase HMSA was described previously for anti-TNF-α antibody detection (Figure 5). For ADAs detection an extra step is added: an acidic treatment step to dissociate the drug-ADA complex, previous to incubation with the fluorescent-labelled drug. Chromatographic separation in the column allows to detect ADAs that bind to the fluorescent-labelled drug [90].

Another fluid-phase assay, RIA, is used for ADA detection. Serum with ADA is incubated with radio-labelled drug, which after centrifugation is detected in a gamma-

counter apparatus. As mentioned in Section 2.5.1, handling radioactive material arises safety concerns which turns this assay less attractive.

For quantifying neutralizing ADAs, a cell-based assay was developed. The cell-based reporter gene assay (RGA) measures only levels of neutralizing ADAs. Briefly, human recombinant TNF-α is added to cell culture (earlier, transfected with reporter-gene construct encoding the enzyme firefly luciferase). The cytokine binds to its receptor, tumour necrosis factor receptor 1 (TNFR1), and intracellular signalling is then initiated through activation of Nf-κB. Transcription factor migrates to the nucleus where it activates transcription of reporter-gene. Cells are then lysed and by addition of a luciferase-catalysed substrate, light emission is quantified. When drug is added to TNF-α, the cytokine is neutralized, and no light emission is observed. On the other hand, if TNF-α is previously incubated with serum with ADAs, the drug is blocked by neutralizing-ADAs. TNF-α is then free to induce intracellular signalling and light emission is perceived [93].

II. AIM

The aim of this investigation and presented in this thesis was to understand the impact of methodological and pharmacokinetic factors on TDM approach in IBD patients under anti-TNF-α antibodies.

For this purpose, 3 objectives were defined and separated by chapters as follow:

Chapter 1 – Systematic Review on IFX and ADL drug monitoring

- Evaluate the evidence available in this field through a systematic review on IFX and ADL drug monitoring (study I).

Chapter 2 – Influence of methodology on drug levels and anti-drug antibodies

- Understand the impact of different assays on results and consequent biases on TDM interpretation (study II-V);
- Evaluate performance of IFX-originator-optimized assay in the assessment of IFX-biosimilars levels (study IV and V).

Chapter 3 – Pharmacokinetic factors of anti-TNF-α antibodies and clinical targets

- Pharmacokinetic factors of anti-TNF-α antibodies and clinical targets and clarify TDM and biomarkers role to identify reasons to therapy failure (study VI and VII).

III. CHAPTER 1

Systematic Review on Infliximab and Adalimumab Drug Monitoring

Study I - "A Systematic Review on Infliximab and Adalimumab Drug Monitoring: Levels, Clinical Outcomes and Assays"

Inflamm Bowel Dis. 2016 Sep;22(9):2289-301

A Systematic Review on Infliximab and Adalimumab Drug Monitoring: Levels, Clinical Outcomes and Assays

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Background: Immunogenicity to therapeutic proteins has been linked to loss of response by a large percentage of patients taking anti–tumor necrosis factor-alpha agents. Drug monitoring can be extremely useful, allowing physicians to adjust the therapeutic scheme individually. This article aims to systematically review the published data with respect to cutoff levels of infliximab (IFX) and adalimumab (ADA) and relate them to the methodology adopted for quantification of IFX and ADA levels and clinical outcomes.

Methods: The PubMed database was searched to identify studies focusing on the association between IFX or ADA cutoff levels and clinical outcomes in patients with inflammatory bowel disease.

Results: Of the 1654 articles initially selected by queries, 20 were included. A receiver operating characteristic curve analysis was performed to identify cutoff levels of IFX or ADA that correlated with a clinical outcome, but only 6 studies performed the same analysis for antidrug antibody levels. Cutoff levels were different between studies. The methodology chosen for level quantifications, clinical outcomes, and sample size and characteristics were also different. Nevertheless, measurement of drug levels should be performed during maintenance, and with loss of response, with persistent high levels of C-reactive protein, and when mucosal lesions are still present. In these scenarios, drug and antidrug levels were correlated with clinical outcomes.

Conclusions: Concerning drug levels monitoring any methodology is adequate. With respect to antidrug antibody levels, it will be necessary to define a gold standard method or to establish different cutoff levels for different methodologies.

(Inflamm Bowel Dis 2016;22:2289–2301)

Key Words: anti-infliximab antibodies, clinical outcomes, infliximab trough levels, therapeutic drug monitoring

Infliximab (IFX) and adalimumab (ADA) are antitumor necrosis factor-alpha (TNFα) agents that have changed the clinical course of many autoimmune diseases such as inflammatory bowel disease (IBD), psoriasis, and rheumatoid arthritis. These agents have been successfully used in the past decades to treat patients with IBD, even in those who were refractory to conventional therapy.[1–5] Introduction of these agents to the drug market allowed physicians to aim for more than clinical remission, as these new drugs were proven to induce endoscopic remission and mucosa healing in patients with either Crohn's disease (CD) or ulcerative colitis (UC).[6–8] Despite this, up to 70% of patients lose responsiveness over time.[9] Many mechanisms may be involved in the loss of response, but immunogenicity to the antibody itself is so far the best studied.[10] The presence of antibodies to IFX (ATIs) in patients' serum was associated with a 3-fold higher risk of loss of response than in patients who did not have ATIs in their serum.[9] Although ADA is a fully human monoclonal antibody drug, immunogenicity to this drug has already been described and a negative correlation between the presence of antibodies to ADA (ATA) and ADA trough levels (TLs) was demonstrated.[11] However, the influence of ADA levels in clinical and endoscopic remission is not well established yet.

When patients lose response to anti-TNFα agents, their physicians have roughly 4 options: (1) dose escalation, (2) addition of an immunomodulator, (3) change to another class of drugs, or (4) change to another anti-TNF agent.[12–17] Currently, physicians have to empirically decide since measurement of drug and antidrug antibody levels is not yet used in daily practice. Many authors have highlighted the importance of knowing drug and antidrug antibody levels to better adjust the therapeutic scheme.

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Nonetheless, most authors emphasize the need to find a valid assay, especially to measure antidrug antibodies and to set cutoff levels to help in decision-making.[18–20] The aim of this article was to systematically review the published data with respect to IFX and ADA levels, the methodology applied, and the relationship with clinical outcomes.

MATERIALS AND METHODS

A systematic review focusing on the association between IFX, ADA TL, ATIs, ATAs, and clinical outcomes in patients with IBD was performed.

Search Strategy

A literature search was performed, through July 2015, using the PubMed database with the following keywords and Medical Subject Headings (MeSH) terms: "{(adalimumab[All fields]) OR (infliximab[All fields])} AND {(inflammatory bowel disease [MeSH Terms]) OR (inflammatory bowel diseases[MeSH Terms]) OR (crohn's disease[MeSH Terms]) OR (colitis, ulcerative[MeSH Terms]) OR (crohn disease[MeSH Terms])} AND ([clinical response] OR [clinical remission] OR [disease activity] OR [clinical outcomes])." Considering this is a hot topic, we decided, on December 2015, to perform an additional literature search on abstracts presented on 3 reference congresses. The European Crohn's and Colitis Organisation (ECCO) Website was searched for all published abstracts related with this topic, using the terms "infliximab ifx" and "adalimumab ada"; The United European Gastroenterology Week (UEGW) Website was searched for abstracts from the last United European Gastroenterology week in Barcelona; The Digestive Disease Week (DDW) Website was searched for abstracts from the past 5 years, using the terms "infliximab levels" and "adalimumab levels" in title, abstract, or keywords.

Eligibility Criteria

The inclusion criteria were: (1) articles studying the association between IFX or ADA cutoff levels and clinical outcomes in patients with IBD and (2) articles written in English.

We excluded studies that (1) were systematic reviews, (2) used another anti-TNF-α agent rather than IFX or ADA, (3) enrolled patients with other diseases rather than IBD (psoriasis, rheumatoid arthritis), (4) only assessed the relationship between IFX or ADA TL and clinical outcomes but did not perform a receiver operating characteristic (ROC) curve analysis, or (5) did not present the specificity and sensitivity values of the ROC curve analysis. This last criterion was defined so that we could infer the accuracy of the cutoff value (i.e., a cutoff value with a sensitivity and/or specificity of 50% would be no better at identifying true positives than flipping a coin). It was not applied to abstracts found on ECCO, UEGW, or DDW databases.

Study Selection and Data Collection Process

Studies were screened and selected by 2 reviewers. First, all titles and abstracts were read and the inclusion and exclusion criteria were applied. Second, the articles considered for inclusion after selection by title/abstract reading were read fully and the inclusion and exclusion criteria were applied again. The data collected from each study were: the type of study and location, number of patients enrolled, and the type of IBD, definitions of clinical outcomes, antidrug antibodies incidence, type of assay used to measure IFX/ADA and ATIs/ATAs serum levels, and the results from the ROC curve analysis (cutoff levels and specificity and sensitivity values), except for the studies obtained in ECCO, UEGW, or DDW databases. In these studies, we have only had access to the abstract. A quality assessment was performed using a qualitative classification of the risk of bias. We used a 4-item classification based on the Meta-analysis of Observational Studies in Epidemiology checklist.[21] The items were chosen based on the factors that can incorporate bias, i.e., inclusion and exclusion criteria, justification of the cohort (eligibility criteria, sources and methods of selecting participants, and the methods used to describe follow-up), the type of disease (if they pointed out whether the patients included had CD or UC), and the assay used to measure drug and antidrug antibody levels (Fig. 1).

RESULTS

Search and Study Selection

A total of 1237 articles were identified with our query (Fig. 2). Of these, 1160 were excluded by title and/or abstract alone, mainly because they did not study the association between IFX or ADA TL and clinical outcomes. Therefore, 77 articles were considered for full text analysis and after that 13 were included in our systematic review (Fig. 2). Two additional articles were included after searching those related to the 13 articles selected by query.[22,23] From the search on ECCO, UEGW, and DDW abstract databases, 417 abstracts were found but only 5 were included, according to the inclusion criteria previously defined (Fig. 3).

Description of Studies

Of the 20 studies included, all but one[24] were conducted in adult patients. One study[25] only involved patients with UC, 11 studies[6,11,22,23,26–33] only encompassed patients with CD and 7 studies pertained to patients with either UC or CD.[8,24,34–38] Fifteen of the 19 studies involved IFX maintenance therapy[6,8,22,24–28,30–35,37] (Table 1), whereas the other 4 involved ADA maintenance therapy (Table 2).[11,23,29,36] One study encompassed patients from both regimens, IFX and ADA maintenance therapy.[38] Seven studies did not report information about the incidence of ATIs,[25,29,31–33,38,39] and only 6 performed an ROC curve analysis to find a cutoff value for ATI[22,32,34,35,37] or ATA[11] levels.

In 6 studies, the clinical outcome was "clinical remission"[8,11,22,24,28,36] usually assessed by the Harvey-Bradshaw Index–Mayo score and/or C-reactive protein (CRP) levels. In 4 studies, the outcome was "loss of response,"[26,32,35,37] defined as an initial good clinical response to IFX induction treatment followed by a loss of clinical response to IFX during maintenance treatment

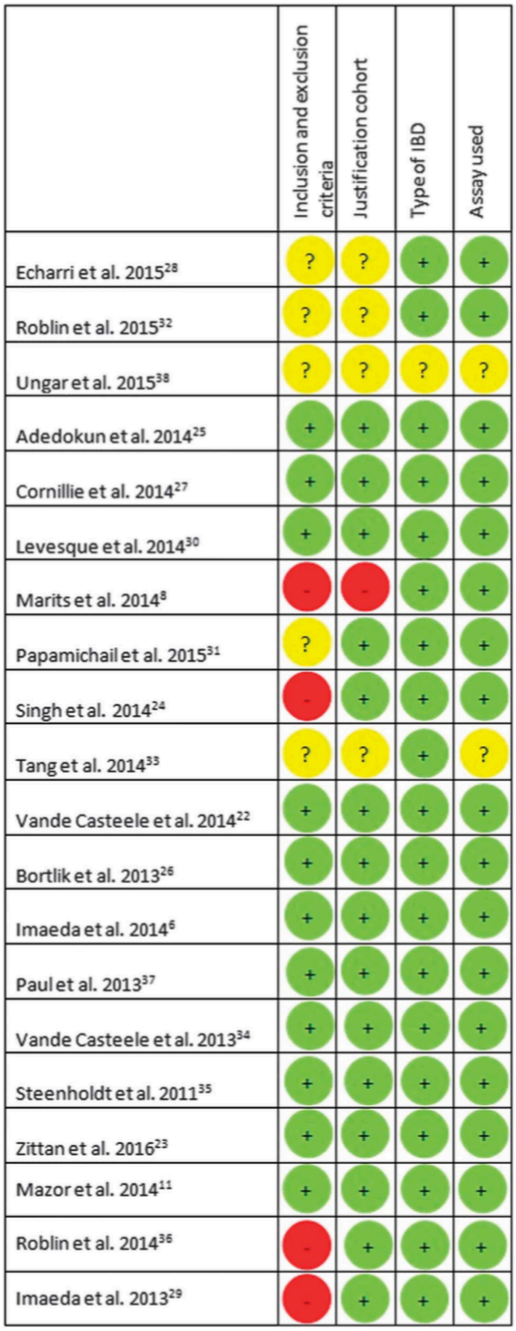

FIGURE 1. Summary of risk of bias.

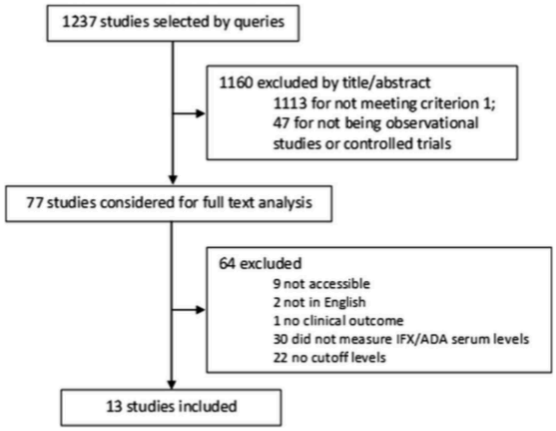

FIGURE 2. Data collection process.

leading to discontinuation of the drug. For Adedokun et al,[25] the endpoint was the "clinical response" defined as a decrease from the baseline in the total Mayo score of ≥3 points and at least 30%, and a decrease in the subscore for rectal bleeding of ≥1 or an absolute subscore for rectal bleeding of 0 or 1. For Levesque et al,[30] there were 2 endpoints which were an "increase in CD activity index ≥70" and an "increase in CRP ≥ 5 mg/L." Imaeda et al[6] defined 2 endpoints for IFX, including "mucosa healing," meaning an endoscopic score of 0 or 1, and "CRP ≤ 0.3 mg/L," whereas for ADA,[29] they only used "CRP ≤0.3 mg/L." Four more studies defined "mucosa healing" as the endpoint of interest.[23,31,33,38] Cornillie et al[27] defined clinical outcome as a "sustained response at week 54," which was expressed as clinical remission based on the relevant disease activity index at week 54, in the absence of any dose intensification during IFX maintenance therapy. Paul et al[37] also defined 2 endpoints: "loss of response" and "absence of clinical remission." Vande Casteele et al[34] described 3 endpoints which were "ATI formation," "IFX discontinuation," and "unsuccessful intervention." The intervention (change in therapy) was considered successful if, at the second infusion after the intervention, the symptoms had disappeared and CRP, if elevated before the intervention, had

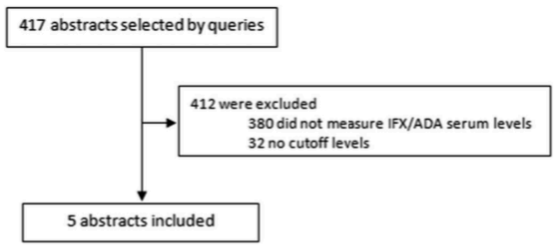

FIGURE 3. Data collection process.

TABLE 1. IFX Trough Levels and Antidrug Antibodies Cutoff, Methodology and Clinical Outcomes

Authors	Study Design	Population	Regimen	Country	Time Point
Echarri et al[28]	—	36 Adults with CD	IFX	Spain	W0, W6, W14, W30
Roblin et al[32]	Prospective cohort	119 Adults with CD	IFX	France	Trough level
Ungar et al[38]	Retrospective cross-sectional	78 Adults with IBD	IFX	Israel	No data
Adedokun et al, 2014[25]	Observational (post-hoc ACT1-2)	454 Adults with UC	IFX; induction regimen followed by maintenance therapy	globally	W8 W30 W54
Cornillie et al[27]	Observational (analyses of ACCENT I)	573 Adults with CD	IFX; induction regimen followed by maintenance therapy	North America, Europe, Israel	W14
Levesque et al[30]	Prospective cohort	327 Adults with CD	IFX; maintenance therapy	Canada	W8
Marits et al[8]	Retrospective	63 Adults with CD, 15 adults with UC, 1 adult with U-IBD	IFX	Sweden	Just before next infusion
Papamichail et al, 2015[31]	Retrospective	101 adults with CD	IFX	Belgium	W 0, 2, 6, 14
Singh et al[24]	Prospective cohort	58 pediatric patients (<21 years) with CD and UC	IFX; induction regimen followed by maintenance therapy	USA	W14
Tang et al[33]	No	15 adults with CD	IFX	China	No data
Vande Casteele et al[22]	Observational	483 adults with CD	IFX; maintenance therapy	Belgium Canada	No data
Bortlik et al[26]	Retrospective	84 adults with CD	IFX	Czech Republic	W14–22
Imaeda et al, 2014[6]	Prospective cohort	65 adults with CD	IFX; maintenance therapy	Japan	Just before next infusion
Paul et al[37]	Prospective cohort	103 adults with IBD	IFX; maintenance therapy	France	Just before next infusion
Vande Casteele et al[34]	Retrospective	64 adults with CD, 26 adults with UC	IFX	Belgium	Just before next infusion
Steenholdt et al[35]	Retrospective	85 adults with CD, 21 adults with UC	IFX	Denmark	Just before next infusion

	Drug			Antidrug antibodies				
Authors	Method	Cutoff, μg/mL	Spec/Sens, %	Method	Incidence, n (%)	Cutoff	Spec/Sens, %	Endpoint
Echarri et al[28]	ELISA	>3 (w6)	No	ELISA	No (26)	No	No	Good response and sustained remission
Roblin et al[32]	ELISA (commercial kit, Theradiag)	<2	No	ELISA	No	>20 ng/mL	No	Loss of response
Ungar et al[38]	No	>5	85/—	No	No	No	No	Mucosa healing

TABLE 1 (*Continued*)

	Drug			Antidrug antibodies				
Authors	Method	Cutoff, μg/mL	Spec/Sens, %	Method	Incidence, n (%)	Cutoff	Spec/Sens, %	Endpoint
Adedokun et al, 2014[25]	Classic ELISA	>41 (w8) >3.7 (w30) >1.7 (w54)	62/63 (w8) 71/65 (w30) 64/89 (w54)	Bridging ELISA	No	No	No	Clinical response
Cornillie et al[27]	Classic ELISA	≥3.5	78/64	Bridging ELISA	ATI+ = 2 (9)	No	No	Sustained response at w54
Levesque et al[30]	HMSA (commercial kit, Prometheus Laboratories)	≤2.8–4.6 (a) ≤2.7–2.8 (b)	68/61 (a) 74/64 (b)	HMSA (commercial kit)	ATI+ = 57 (18)	No	No	(a) Increased CDAI ≥70; (b) Increased CRP ≥5 mg/L
Marits et al[8]	Classic ELISA	>4.1 (CD)	44/87	Inhibition ELISA	ATI+ = 22 (79); ATI transient = 4 (18)	No	No	Remission (HBI-Mayo and CRP)
Papamichail et al, 2015[31]	ELISA	>22.5 (w2) >12.8 (w6)	No	No	No	No	No	Short-term mucosa healing
Singh et al[24]	Classic ELISA and HMSA (Prometheus Laboratories)	≥5 ≥7	85/50 100/33	Bridging ELISA and HMSA (Prometheus Laboratories)	No (10 at w14), no (26 at w54)	No	No	Week 54 persistent remission
Tang et al[33]	No	>4.87	77/88	No	No	No	No	Mucosa healing
Vande Casteele et al[22]	HMSA (commercial kit)	>2.79	77.6/52.5	HMSA (commercial kit)	23.7% (IFX−/ATI− = 6.5%; IFX +/ATI− = 69.8%; IFX−/ ATI+ = 16.4%; IFX+/ATI+ = 7.3%)	< 3.15 U/mL	87.4/38.0	Remission (CRP ≤ 5 mg/L)
Bortlik et al[26]	Classic ELISA (Q-INFLIXI, Matriks Biotek)	<3	62/70	Bridging ELISA (commercial kit)	ATI+ = 14 (17), ATI− = 24 (28), ATI inconclusive = 46 (55)	No	No	Loss of response at 1 year using IFX
Imaeda et al, 2014[6]	Classic ELISA	>4 (a) >0.6 (b)	70/71 (a) 62/73 (b)	ELISA (+acid dissociation and immunoaffinity crhomatography)	No	No	No	(a) Mucosa healing (b) CRP < 0.3 mg/L
Paul et al[37]	ELISA (commercial kit, Theradiag)	<2 (a)	82.3/76	Bridging ELISA (commercial kit)	ATI+ = 34 (32.8), CD = 25.4%, UC = 41.5%,[a] ATI+ = 65.3%[b]	>200 ng/mL (b)	93.5/22.0	(a) Absence of clinical remission (b) Loss of response.

TABLE 1 (*Continued*)

Authors	Drug			Antidrug antibodies				Endpoint
	Method	Cutoff, μg/mL	Spec/Sens, %	Method	Incidence, n (%)	Cutoff	Spec/Sens, %	
Vande Casteele et al[34]	Classic ELISA	<13 (w6) (a) <2.2 (w14) (a) <2.2 (w14) (c)	81/72 94/79 74/82	HMSA	ATI+ = 53 (59), Transient ATI = 15 (28), Sustained ATI = 38 (72)	>9.1 U/mL (b)	82/65	(a) ATI formation (b) Unsuccessful Intervention (c) IFX discontinuation.
Steenholdt et al[35]	Fluid-phase RIA	<0.5 (CD) <0.8 (UC)	85/86 (CD) 100/75 (UC)	Fluid-phase RIA	ATI+ = 35 (33.3)	≥10 U/mL (CD and UC)	90/81 (CD) 100/80 (UC)	Loss of response

[a]Cutoff for ATI positivity >10 ng/mL.
[b]Cutoff for ATI positivity >5 ng/mL.
CDAI, CD activity index; HBI, Harvey-Bradshaw index; sens, sensitivity; spec, specificity; U-IBD, unclassified inflammatory bowel disease.

decreased by >50% than the value at the time of loss of clinical response. Quality assessment was limited in those cases to which we only had access to the abstract.[28,31–33,38] Taking into consideration the other studies, all but 4 had suitable inclusion and exclusion criteria,[8,24,29,36] and all papers indicated the type of IBD and the assay used to measured drug and antidrug antibody levels.

Assays Used to Measure Drug and Antidrug Antibody Levels

One aspect that should be taken into consideration when analyzing drug TL and antidrug antibody levels is the assay used to measure them. All but 3 of the included works measured IFX or ADA TL using classic enzyme-linked immunosorbent assay (ELISA).[6,8,11,24–29,31,32,34,36,37] Zittan et al,[23] Levesque et al,[30] and Vande Casteele et al[22] used a homogeneous mobility shift assay (HMSA), whereas Steenholdt et al[35] used a fluid-phase radioimmunoassay (fluid-phase RIA). Singh et al[24] tested 2 methodologies, the classic ELISA and HMSA.

Regarding antidrug antibody measurements, 7 studies used bridging ELISA, either via home-made assays or commercial kits.[25–28,32,36,37] Mazor et al[11] applied an adaptation of the anti-human lambda chain-based ELISA. In 2 studies by Imaeda et al, ATI[6] and ATA[29] levels were also measured with ELISA, but samples were previously treated with acid in order to dissociate immune complexes. Other methods were used, namely HMSA,[23] fluid-phase RIA,[35] and inhibition ELISA.[8] Singh et al[24] tested 2 methodologies, bridging ELISA and HMSA. Figure 4 displays all methodologies used.

Infliximab Levels

By Week of Measurement

Of the 16 IFX studies, 7 specified the time point measurement[24–27,30]: one measured drug levels at week 2,[31] 2 at week 6,[28,31] 2 at week 8,[25,30] 3 at week 14,[24,26,27] 1 at week 22,[26] and 1[25] also measured IFX levels at weeks 30 and 54. Others only indicated that measurements were made before each infusion, thus representing drug TL.[6,8,11,29,34,35,37]

In Papamichaiel et al,[31] 2 cutoff levels were proposed (Table 1), both correlating with short-term mucosa healing, but after multiple logistic regression analysis, only IFX levels >12.8 μg/mL at week 6 were retained as an independent factor to predict short-term mucosa healing (OR: 3.6, $P = 0.004$). Echarri et al[28] presented a largely different cutoff level for the same time point. They suggest that IFX levels >3 μg/mL at week 6 had a positive-predictive value for "good response and sustained remission" of >90%. Adedokun et al[25] showed that IFX levels >41 μg/mL at week 8 correlated with clinical response with a specificity of 62% and a sensitivity of 63% (Table 1). The median serum IFX concentration was significantly higher at week 8 in patients with clinical response or mucosal healing during induction than those not achieving these endpoints. Levesque et al[30] found a different cutoff: a mean IFX trough concentration <3 μg/mL at week 8 was

TABLE 2. ADA Trough Levels and Antidrug Antibodies Cutoff, Methodology and Clinical Outcomes

Authors	Study Design	Population	Regimen	Country	Time point	Drug		
						Method	Cutoff, μg/mL	Spec/Sens, %
Zittan et al, 2016[23]	Observational	60 Adults with CD	ADA	Canada	—	HMSA (commercial kit, Prometheus Laboratories)	8.14	76.0/91.4
Ungar et al[38]	Retrospective, cross-sectional	67 Adults with IBD	ADA	Israel	—	—	>7.1	85/—
Mazor et al[11]	Observational, cross-sectional	71 Adults with CD	ADA	Israel	Just before next infusion	Classic ELISA	>5.85 (a)	70.6/68
Roblin et al[36]	Observational cross-sectional	40 Adults with IBD	ADA; maintenance therapy	France	W22	ELISA (commercial kit, Theradiag)	<4.9 (a) >4.85 (b)	85/66 67/81
Imaeda et al, 2014[29]	Prospective cohort	40 Adults with CD	ADA; maintenance therapy	Japan	Just before next infusion	Classic ELISA	>5.9	92/67

Authors	Antidrug antibodies				Endpoint
	Method	Incidence, n (%)	Cutoff	Spec/Sens, %	
Zittan et al, 2016[23]	HMSA (commercial kit, Prometheus Laboratories)	No (30.9)[a]	—	—	Mucosa healing
Ungar et al[38]	—	—	—	—	Mucosa healing
Mazor et al[11]	(adapted) Antihuman lambda chain-based ELISA	No (30.5 samples)[b] no (12.7 samples)[c]	≥ 3 μg/mL (b)	98/20.6	(a) Remission; (b) Active disease
Roblin et al[36]	Bridging ELISA (commercial kit)	9 (22.5)	No	No	(a) Absence of mucosa healing (b) Clinical remission
Imaeda et al, 2014[29]	ELISA (+acid dissociation)	35 (23)	No	No	CRP ≤ 0.3 mg/dL

[a]Cutoff for ATA positivity >1 U/mL.
[b]Cutoff for ATA positivity >1.5 μg/mL-eq.
[c]Cutoff for ATA positivity >3 μg/mL-eq.

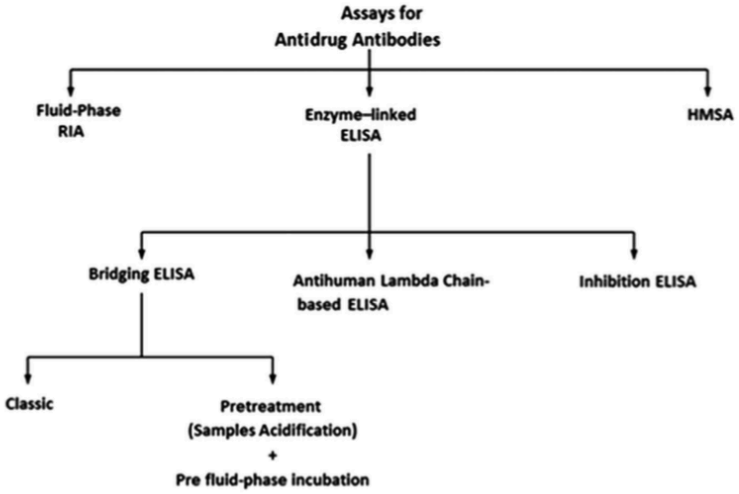

FIGURE 4. Methodologies for antidrug antibodies quantification.

significantly associated with a ≥70-point increase in the mean total CD activity index score between infusions ($P < 0.001$).

In measurements performed at weeks 14 and/or 22, cutoff values varied from <3[26] to ≥7 μg/mL[24] (Table 1). Patients with TL >3 μg/mL at weeks 14 and/or 22 had an approximately 66% lower likelihood to lose their response to IFX than those with subtherapeutic levels.[26] These findings are similar to data from the post-hoc analysis of the ACCENT I trial (A Crohn's Disease Clinical Trial Evaluating Infliximab in a New Long-term Treatment Regimen I),[27] which found that an IFX level >3.5 μg/mL at week 14 was a good predictor of sustained response at week 54. Patients with sustained response to scheduled maintenance IFX at 5 mg/kg had higher median IFX TL than those who lost response during the 54 weeks follow-up (4.0 versus 1.9 μg/mL, $P = 0.0331$). Adedokun et al[25] also measured IFX levels at weeks 30 and 54 and the levels, related with clinical response, were 3.7 and 1.7 μg/mL, respectively (Table 1). They suggested that more weight should be given to the threshold estimate at week 30 (3.7 μg/mL) because it was most representative of the steady-state trough concentration for both Active Ulcerative Colitis Trial studies. Therefore, patients with IFX TL >3.7 μg/mL at week 30 are more than twice as likely to have clinical response than patients with IFX TL <3.7 μg/mL.

Mucosa Healing

Imaeda et al[6] found that IFX TL >4 μg/mL was a good predictor of mucosa healing (Table 1). The authors also showed that the deterioration of the endoscopic findings was significantly associated with lower IFX TL. Two abstracts reported similar cutoff levels.[33,38] In both of them, IFX levels were significantly higher in the mucosal healing group than in patients with active disease (4.3 versus 1.7 μg/mL, $P = 0.0002$[38]).

Loss of Response

Steenholdt et al[35] showed that IFX TL <0.5 in CD and <0.8 in UC were good predictors of loss of response. IFX TL were significantly higher in both patients with CD and patients with UC who had maintained response to IFX compared with those who had lost response (median 2.8 μg/mL versus median 0 μg/mL, for CD; and 3.8 μg/mL versus 0 μg/mL for UC). Higher levels were identified by Bortlik et al[26] (<3 μg/mL) and Roblin et al[32] (<2 μg/mL).

Biomarkers

Imaeda et al[6] showed that IFX levels >0.6 μg/mL could predict normalized CRP levels (<3 mg/dL) with good sensitivity and specificity (Table 1). C-reactive protein levels were significantly higher in the nonmucosal healing group than in the mucosal healing group (0.09 versus 1.32 mg/dL). Levesque et al[30] showed that IFX concentrations <2.7 to 2.8 μg/mL predicted serum CRP levels >5 mg/L. Therefore, they suggested that a mean IFX trough concentration <3 μg/mL at week 8 was significantly associated with a higher probability for serum CRP concentrations >5 mg/L at that time point. In a study by Vande Casteele et al,[22] an IFX TL >2.79 μg/mL was considered to be a good predictor of CRP <5 mg/L, meaning that patients with IFX levels <2.79 μg/mL in a "current" sample were at higher risk of not achieving remission, defined as CRP <5 mg/L.

Adalimumab Levels

ADA information is sparse. Imaeda et al[29] evaluated 40 adults with CD and performed an ROC curve analysis to identify threshold levels of ADA that could predict normalized CRP levels (i.e., CRP ≤ 3 mg/dL). ADA levels >5.9 μg/mL predicted normalized CRP with high specificity (Table 2). Mazor et al[11] and

Roblin et al[36] conducted cross-sectional studies in patients taking ADA maintenance therapy; Mazor et al[11] enrolled patients with CD and Roblin et al[36] enrolled patients with CD or UC. In the study by Mazor et al, ADA TL >5.85 μg/mL predicted remission with a specificity and sensitivity of 70.6% and 68.0%, respectively. Roblin et al[36] showed that ADA serum concentrations <4.9 μg/mL predicted an absence of mucosa healing. The median ADA TL was significantly higher in cases of mucosa healing (6.5 versus 4.2 μg/mL in those without mucosa healing; $P < 0.005$). Moreover, serum levels higher than 4.85 μg/mL predicted clinical remission, defined as CD activity index <150 points or total Mayo score <3 (Table 2). Higher ADA TL were found in the work by Zittan et al (14.7 μg/mL in the mucosa healing group, versus 3.4 μg/mL in the non-MH group, $P = 6.25 \times 10^{-5}$).[23] Furthermore, Zittan et al suggested that ADA TL <8.14 μg/mL predicted MH with high sensitivity (Table 2). In the work by Ungar et al,[38] ADA levels >7.1 μg/mL identified patients with mucosa healing with 85% specificity. He also found that the association between higher levels of ADA and increased rate of mucosa healing reached a plateau at 12 μg/mL.

Incidence of ATIs and ATAs

Antidrug antibodies are described as the main cause of loss of response to biologic drugs over time. However, the incidence of antidrug antibodies varies significantly between studies. Taking into consideration those included in this systematic review, the ATI incidence varied from 9%[27] to 63.5%[37] (Table 1). In Bortlik et al,[26] 17% of the patients had ATIs but 55% were considered inconclusive. Marits et al[8] reported 22 out of 28 patients with ATIs, wherein 18% of them were ATI transient, meaning that patients presented with ATIs in their serum which at some point disappeared. The same was reported by Vande Casteele et al,[34] where 15 of the 53 patients considered with ATIs were transient (Table 1). Vande Casteele et al[22] reported an ATI incidence of 23.7%, and the authors were able to distinguish 4 groups of patients based on ATI and IFX status (Table 1). Paul et al[37] showed a global incidence of ATIs of 32.8%, considering a cutoff for ATIs of 10 ng/mL; with a cutoff of 5 ng/mL, the incidence was 63.5%. In the pediatric setting, 10% of the patients had ATIs in their blood at week 14, but the incidence increased to 16% at week 54.[24]

Although ADA is a fully human antibody, some patients develop ATAs. Imaeda et al[29] described a 23% incidence of ATAs. Roblin et al[36] found a similar value (22.5%). In the cross-sectional study from Mazor et al,[11] 12.7% of the samples had ATA levels ≥3 μg/mL; when a cutoff of ≥1.5 μg/mL was established, the incidence rose to 30.5% (Table 2). Zittan et al[23] described an ATA incidence of 30.9%, using a cutoff of >1 U/mL.

Cutoff Levels of ATIs and ATAs

Only 5 studies[11,22,34,35,37] performed an ROC curve analysis to identify threshold levels for antidrug antibodies. Steenholdt et al[35] reported that ATI levels, measured with fluid-phase RIA, >10 U/mL in patients with CD predicted "loss of clinical response" with a specificity of 90% and sensitivity of 81%. In the subgroup of patients with UC, the specificity was higher (Table 1). ATI were significantly lower in both patients with CD and patients with UC who had maintained response to IFX compared with those who had lost response (median 0 U/mL versus median 35 U/mL for CD, and median 0 U/mL versus median 85 U/mL for UC).

Paul et al[37] also performed an ROC curve analysis using "loss of response" as the target clinical outcome. The authors suggested that ATI levels >200 ng/mL, assessed by the ELISA assay, predicted loss of response with a high specificity but with a low sensitivity (Table 1). A combined analysis was also performed on patients with CD with IFX levels <2 μg/mL and ATI levels <200 ng/mL. The ATIs predicted clinical remission with a high specificity and sensitivity (Table 1); patients with UC showed higher specificity (100%) but lower sensitivity (70%). The same analysis using "mucosa healing" as the clinical outcome was also supplied (Table 1). An ROC curve analysis for a threshold >9.1 U/mL at the time of loss of response predicted an "unsuccessful intervention" with a specificity of 82% and a sensitivity of 65%.[34] Therefore, patients having ATI TL >9.1 U/mL at the time of loss of response had a likelihood ratio of 3.6 for an unsuccessful intervention. It was also reported that patients with ATI levels <3.15 U/mL had a higher probability of being in remission.[21]

With regard to ADA, Mazor et al[11] suggested that a cutoff level ≥3 μg/mL, when using an adapted anti-human lambda chained-based ELISA assay, predicted active disease with high specificity but low sensitivity (Table 2). The authors showed a negative correlation between ADA drug levels and ATA levels and found that for patients with ATA levels ≥3 μg/mL-eq, the maximal ADA level was only 0.5 μg/mL.

DISCUSSION

The importance of measuring drug levels and antidrug antibody levels to adjust therapy is undisputable. The major hindrance to its implementation in daily clinical practice is the lack of a universally valid assay and the absence of a cutoff level clearly related with a clinical outcome. One cannot easily compare results from different studies, as they use distinct assays that have different limitations and lower limits of quantification.

Regarding the measurement of IFX levels, classic ELISA is the methodology most frequently used, but other methods are available, such as HMSA and fluid-phase RIA. Studies[40–42] that have compared performance of different methods to measure drug levels have concluded the same; there is a good qualitative correlation between different assays (e.g., IFX detection rates of 76% with ELISA and 82% with RIA[42]). Furthermore, in some cases, there is a good quantitative correlation (e.g., ELISA and RIA, $R^2 = 0.98$, $P = 0.001$[40]; ELISA and RIA, Pearson $r = 0.91$, $P < 0.0001$[41]) but not a perfect agreement on drug concentrations (e.g., maximum difference of 1.41 μg/mL between ELISA and RIA[40]), and this emphasizes the importance of

establishing different cutoff levels according to the methodology used. The threshold levels assessed by ROC curve analysis were quite different between the studies. This can be due to (1) different methodology (even using the same principle, such as bridging ELISA, home-made ELISA, and commercial kits), (2) different study design and sample characteristics, and/or (3) different endpoints. This heterogeneity justifies the obstacle to perform a meta-analysis. A systematic review and meta-analysis was recently published on this topic and suggested a cutoff level of 2 μg/mL to predict remission (RR = 2.9, 95% confidence interval, 1.8–4.7, $P < 0.001$), but there was a high statistical heterogeneity ($I^2 = 88\%$).[43] However, TL were always associated with a better clinical endpoint: clinical remission, mucosa healing, normalized CRP, or loss of response. Our review emphasizes the importance of measuring drug levels during maintenance therapy as well as in cases of loss of response, cases with persistent high levels of CRP, and when mucosal lesions are still present. In the induction phase, the only study reported did not show any advantage of measuring IFX at 2 weeks because this corresponded to the loading period and it was not possible to differentiate responders from nonresponders. However, at weeks 8, 14, and 30, the different studies found significant differences between responders and nonresponders, and one of these time periods should be chosen by clinicians for strategic therapeutic decisions, namely increasing drug dose or addition of 1 immunomodulatory drug. Active Ulcerative Colitis Trial subanalysis suggested week 30 is ideal and argued that this time corresponds to the steady state of the drug. Two studies (TAILORIX[44] and TAXIT[45]) have concluded that in maintenance phase, concentration-based dose adjustment was not superior to dose adjustment based on symptoms alone. However, TAXIT trial also showed that patients in the "clinically based dosing" group had more flares during the course of treatment than those in the "concentration-based dosing" group.

Overall, there is evidence for determining drug levels in weeks 6, 14, 22, 30, and 54. During maintenance, therapeutic drug monitoring should be considered in case of loss of response, mucosal ulceration, and elevated biomarkers, such as CRP and fecal calprotectin (Fig. 5).

Figure 6 shows how therapeutic drug monitoring may be used to highlight factors influencing loss of response. Two branches are schematized: for patients with loss of response and high levels of drug (pink branche) and for patients with loss of response and low drug levels (green branche). Pharmacodynamic, pharmacokinetic, and immunogenicity factors may be identified and help clinicians to handle therapeutic decisions.

All methodologies available (ELISA, HMSA, fluid-phase RIA) seem qualitatively equivalent, so either one can be used to monitor drug levels. However, the clinician should take into consideration that there are disagreements on IFX concentration between assays, therefore for each patient, drug levels should be always measured with the same assay. Concerning antidrug antibody levels, the variability among methods is more significant. Enzyme-linked immunosorbent assay (ELISA) is the methodology most frequently used; however, not all ELISAs use the same principle. A bridging ELISA, or double antigen ELISA, uses the drug, in this case, IFX or ADA as the captured antigen and as the detection antibody. Consequently, this method is susceptible to several limitations, namely false-positive results, caused by rheumatoid factors or activated complement fragments that cross-bind the drug's fragment crystallizable region. False-negative results are due to the assay's inability to detect monovalent immunoglobulin G4 (IgG4) and antidrug antibodies in the presence of the drug. This method was used by 6 of the studies included in this systematic review.[24–27,36,37] Since this assay has no sensitivity to detect antidrug antibodies in the presence of the drug, some of the studies did not measure antidrug antibodies if there were drug levels in the serum and considered those samples

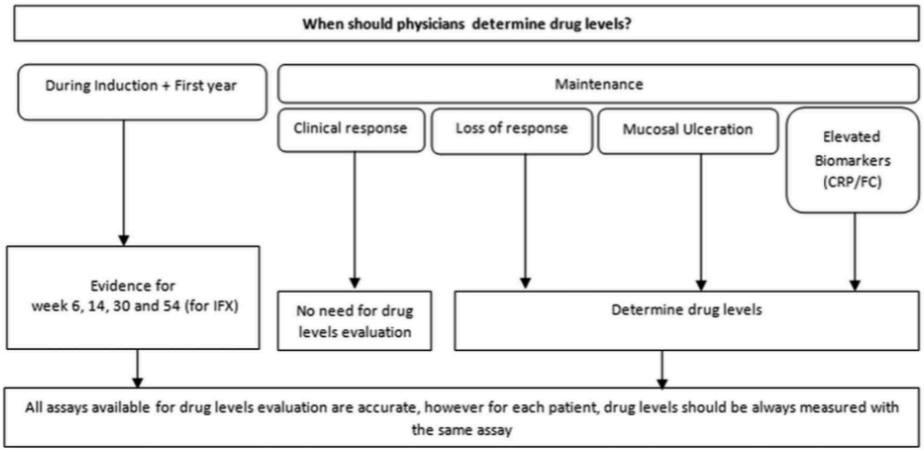

FIGURE 5. Time points for drug level determination.

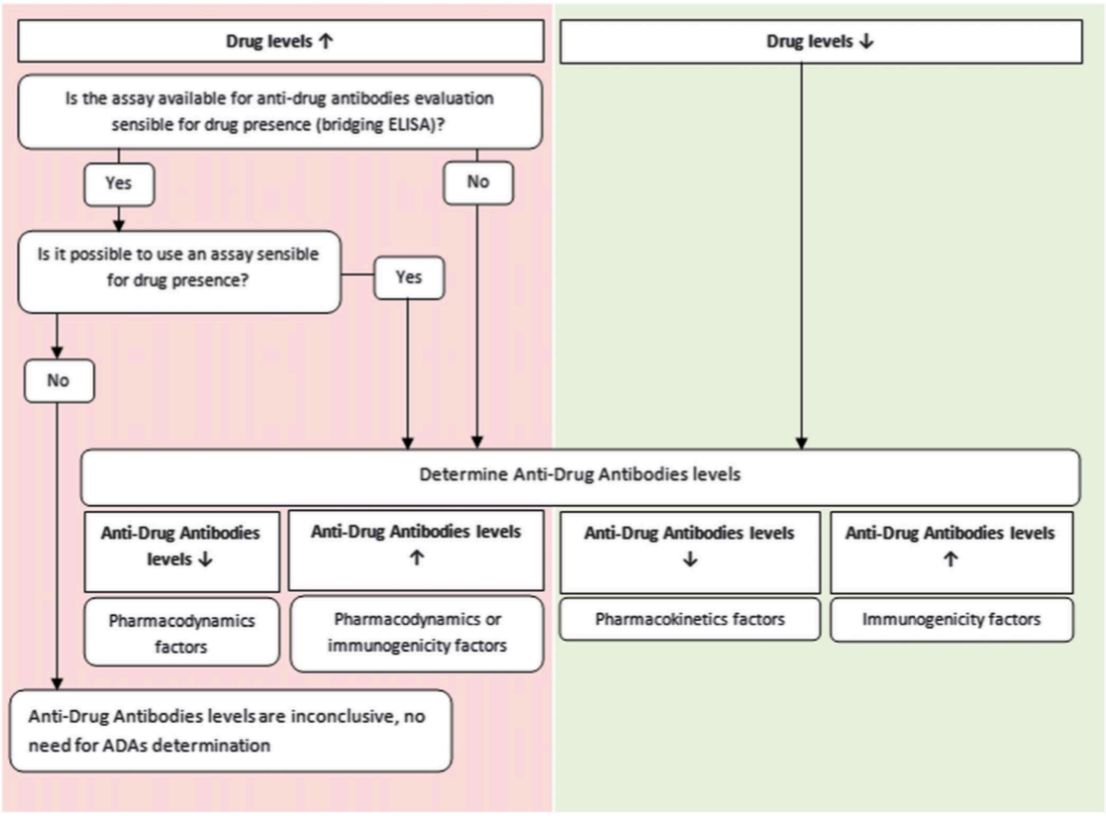

FIGURE 6. Therapeutic drug monitoring brought to light the factors influencing the loss of response.

as "ATI inconclusive." Comillie et al[27] considered samples that had IFX levels >0.1 μg/mL as "ATI inconclusive," whereas Bortlik et al[26] only considered samples that had IFX levels >3 μg/mL as "ATI inconclusive." Given that half of the patients in clinical trials had the drug in their serum, the use of a bridging ELISA for anti-IFX detection may lead to serious bias. This must be taken into consideration when one tries to draw conclusions about the therapeutic importance of ATIs using bridging methodology.[35]

We should also keep in mind that study populations and study designs were different. Some included only patients with CD or UC, whereas others comprised both types of patients; some were prospective cohorts while others were cross-sectional studies or post-hoc analyses of controlled trials. These differences can explain why the incidence of antidrug antibodies was so varied between them, even when using the same assay. For example, both studies from Cornillie et al[27] and Paul et al[37] used a bridging ELISA to measure antidrug antibodies but the incidence of ATI positivity was 9% and 32.8%, respectively. This could be explained by the fact that the first study was a post-hoc analysis of the ACCENT I trial that enrolled 573 adult patients with CD, whereas the second was a prospective cohort with 103 adults with CD or UC.

Kopylov et al[46] developed a different ELISA method, anti-human lambda chain-based ELISA, to overcome the false-negative results associated with the presence of the drug. The authors took advantage of the fact that antidrug antibodies have a lambda light chain, whereas the drug has a kappa light chain, and they used an anti-lambda antibody as the detection antibody, ensuring that they were only measuring antidrug antibodies. Mazor et al[11] adapted this method to measure antibodies to ADA. Those authors described an incidence for ATA positivity of 30.5%, which showed the sensitivity of anti-lambda chain ELISA and its low rate of drug interference. However, in serum with high levels of a drug, even anti-lambda chain ELISA is not able to completely overcome drug interference.[47] Anti-lambda chain ELISA is also unable to detect anti-idiotypic antibodies, i.e., antibodies that recognize functional binding epitopes.[47]

One way of overcoming drug interference is to perform a prior acidic dissociation. Imaeda et al pretreated samples with

acid in both the IFX study and ADA study.[6,29] In a previous work, the authors showed the ability of this new method to detect ATIs in samples containing detectable levels of IFX, which proved to be more accurate than the bridging ELISA.[48] From a total of 58 samples, the methodology by Imaeda et al could detect an additional 14 positive samples, of which, by the bridging ELISA, 8 had been considered negative and 6 "inconclusive."

Three studies[24,30,34] used the HMSA to measure ATIs, an alternative assay to ELISA. The HMSA uses size exclusion high-performance liquid chromatography.[47] Although HMSA requires expensive equipment, the authors of those studies state many advantages, including the ability to overcome many potential artifacts encountered in the solid-phase ELISA, the ability to detect high and low affinity antibodies (low affinity antibodies may not be detected by ELISA due to multiple washing steps), the detection of all immunoglobulin isotypes and all IgG subclasses (including IgG4), and the fact that it is not affected by substances present in serum.[49] However, a different ATI incidence was reported by the 3 studies, which can be explained by differences in the study population and sample size (Table 1).

Another assay is able to bridge the gaps of the ELISA methodology. In fluid-phase radioimmunoassay (RIA), used by Steenholdt et al,[35] a radio-labeled antibody to detect and quantify the amount of antidrug antibodies is applied. It has proved to be more sensitive than ELISA, as it is able to detect antidrug antibodies in the presence of the drug and IgG4 isotype.[40,41] Moreover, fluid-phase RIA overcomes matrix effects encountered in solid-phase assays due to epitope masking via protein aggregation. The major limitation of RIA is the need for advanced laboratory facilities.[47]

Therefore, the differences in methodology, study design, and sample size and characteristics may also explain why the 4 studies with IFX[22,34,35,37] that performed an ROC curve analysis in order to find a cutoff level of antidrug antibodies related with a clinical outcome found different threshold levels. It is also not easy to compare the thresholds between studies because they used different units (U/mL; μg/mL; ng/mL) and defined different endpoints. A serious limitation of all of the studies was the inability to show whether or not antidrug antibodies were neutralizing.

It is important to address whether or not antidrug antibodies are functional, because we know that antidrug antibody detection in serum does not always correlate with loss of clinical response.[47,50] Moreover, sometimes the presence of antidrug antibodies may actually increase the half-life of the drug; if 1 or 2, but not more, antidrug antibodies bind to the drug, the complex will bind to Neonatal fragment crystallizable receptor and will escape elimination.[51] A study comparing different methodologies (ELISA, EIA, RGA, RIA) to measure antidrug antibody levels has been published and concluded that the ability to detect anti-ATIs is comparable with respect to basic analytical properties. ELISA and RIA showed a good correlation ($R^2 = 0.73$, $P = 0.03$), but the agreement was not so good, with a mean titer difference of −2400 (−5000 to 200), which can be partially explained by the inability of bridging ELISA to detect IgG4 antidrug antibodies. The authors suggest that clinicians should choose an assay where assessments take place in fluid phase and where all anti-IFX IgG isotypes are quantified.[40]

CONCLUSION

Currently, there is no doubt that drug levels correlate with clinical and endoscopic outcomes, and this knowledge is the basis of drug monitoring. Nevertheless, it can only be widely used in clinical practice when there is a consensus on the thresholds of drug and antidrug antibody levels that correlate with a specific clinical outcome, including either clinical remission or loss of response. Concerning drug level monitoring, any methodology is adequate but the data published by now is insufficient to come up with a cutoff level. With respect to antidrug antibody levels, assays have significantly different sensitivity, therefore it will be necessary to define a gold standard method or to establish different cutoff levels for different methodologies.

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IV. CHAPTER 2

Influence of Methodology on drug levels and anti-drug antibodies

Study II - "Detection of anti-infliximab antibodies is impacted by antibody titer, infliximab level and IgG4 antibodies: a systematic comparison of three different assays"

Therap Adv Gastroenterol. 2016 Nov;9(6):781-794

Study III - "Proactive therapeutic drug monitoring of infliximab: a comparative study of a new point-of-care quantitative test with two established ELISA assays"

Aliment Pharmacol Ther. 2016 Oct;44(7):684-92

Study IV - "Therapeutic drug monitoring of CT-P13: a comparison of four different immunoassays"

Therap Adv Gastroenterol. 2017 Sep;10(9):661-671

Study V - "The performance of Remicade®-optimized quantification assays in the assessment of Flixabi® levels"

Therap Adv Gastroenterol. 2018 Sep 23;11:1756284818796956

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Detection of anti-infliximab antibodies is impacted by antibody titer, infliximab level and IgG4 antibodies: a systematic comparison of three different assays

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***Abstract*:**
Background: There is scant information on the accuracy of different assays used to measure anti-infliximab antibodies (ADAs), especially in the presence of detectable infliximab (IFX). We thus aimed to evaluate and compare three different assays for the detection of IFX and ADAs and to clarify the impact of the presence of circulating IFX on the accuracy of the ADA assays.
Methods: Blood samples from 79 ulcerative colitis (UC) patients treated with infliximab were assessed for IFX levels and ADAs using three different assays: an in-house assay and two commercial kits, Immundiagnostik and Theradiag. Sera samples with ADAs and undetectable levels of IFX were spiked with exogenous IFX and analyzed for ADAs.
Results: The three assays showed 81–96% agreement for the measured IFX level. However, the in-house assay and Immundiagnostik assays detected ADAs in 34 out of 79 samples, whereas Theradiag only detected ADAs in 24 samples. Samples negative for ADAs with Theradiag, but ADA-positive in both the in-house and Immundiagnostik assays, were positive for IFX or IgG4 ADAs. In spiking experiments, a low concentration of exogenous IFX (5 μg/ml) hampered ADA detection with Theradiag in sera samples with ADA levels of between 3 and 10 μg/ml. In the Immundiagnostik assay detection interference was only observed at concentrations of exogenous IFX higher than 30 μg/ml. However, in samples with high levels of ADAs (>25 μg/ml) interference was only observed at IFX concentrations higher than 100 μg/ml in all three assays. Binary (IFX/ADA) stratification of the results showed that IFX+/ADA- and IFX-/ADAs+ were less influenced by the assay results than the double-positive (IFX+/ADAs+) and double-negative (IFX-/ADAs-) combination.
Conclusions: All three methodologies are equally suitable for measuring IFX levels. However, erroneous therapeutic decisions may occur when patients show double-negative (IFX-/ADAs-) or double-positive (IFX+/ADAs+) status, since agreement between assays is significantly lower in these circumstances.

Keywords: anti-infliximab antibodies, anti-Infliximab antibody methodologies, infliximab trough levels, therapeutic drug monitoring

Introduction

Infliximab (IFX) is a chimeric (human–murine) monoclonal IgG1 anti-tumor necrosis factor alpha (TNF-α) antibody used in the treatment of inflammatory bowel disease (IBD) [Bendtzen, 2013]. Although IFX has profoundly improved the treatment of inflammatory diseases, not all patients respond to induction therapy, and up to 50% of patients experience the loss of clinical response over time (secondary loss of response) [Yanai and

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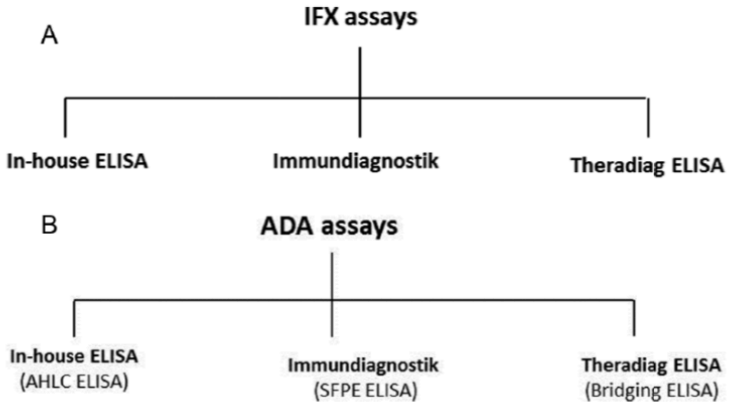

Figure 1. (A) Infliximab assays. (B) Anti-infliximab antibodies assays.

Hanauer, 2011; Steenholdt *et al.* 2013]. When managing loss of response, clinicians may empirically intensify treatment with the existing drug (increase dosage and/or increase frequency), switch to another TNF-α antagonist or switch to a totally different class of drug. This empirical approach has disadvantages: risk of irreversible tissue damage while the physician searches for an effective new drug, and significant economic consequences of unsuccessful trial and errors [Bendtzen and Svenson, 2011; Steenholdt *et al.* 2014a]. A more astute strategy is probably to use therapeutic drug monitoring (TDM), which enables clinicians to identify patients in whom a medication or change in medication is likely to be effective [Roblin *et al.* 2014; Steenholdt *et al.* 2014b; Yanai *et al.* 2015]. Indeed, a rational evidence-based and tailored therapy according to individual needs may reduce delays in effective treatment [Bendtzen, 2013; Steenholdt *et al.* 2014b].

Awareness of the value of TDM has led to the development of different techniques for assessing levels of infliximab and anti-infliximab antibodies (ADA) in patients, but these different methodologies have distinctive limitations and may yield different results. This potential bias may have a significant impact on TDM results and interpretation. There is still little information allowing us to compare different assays, in particular in relation to ADAs detection, which is likely to be subject to interference by detectable levels of IFX [Casteele *et al.* 2012; Kopylov *et al.* 2012; Steenholdt *et al.* 2013].

In order to incorporate therapeutic drug monitoring into clinical practice it is pertinent to recognize the potential for assay heterogeneity and accuracy. Therefore, the objective of this study was to evaluate and compare three different methodologies used to detect IFX and ADA and to clarify the importance of detectable IFX levels when measuring ADA levels namely on the accuracy of ADA assays.

Methods

Patients and sera

Trough blood samples were collected from 79 IFX treated ulcerative colitis (UC) patients. Blood samples were centrifuged, and the serum collected and stored at –80°C. This was a multicenter, open-label, single-arm trial. Study participants were recruited from ten IBD centers in Portugal. The trial was conducted in accordance with the Declaration of Helsinki and Ethical Principles of Good Clinical Practice and was approved by the local Ethics Committees. All participants gave their written informed consent.

Evaluation of IFX levels

IFX levels were evaluated using a sandwich enzyme-linked immunosorbent assay (ELISA) from three different sources (Figure 1A): one in-house ELISA and two commercial ELISA kits. The upper limit of the measurement for the three assays was calculated as the highest concentration of the standard curve × sample dilution factor used.

IFX levels were evaluated using the in-house ELISA as previously described by Baert and

colleagues with some modifications [Baert *et al.* 2003; Ben-Horin *et al.* 2011]. Briefly, 100 ml of 1:100 diluted serum was added to a plate precoated with 750 ng/ml TNFα (Peprotech, Rocky Hill, NJ, USA) and incubated for 60 min. Following washing, horseradish peroxidase (HRP) labelled goat anti-human Fc fragment antibody (MP Biomedicals, Solon, OH, USA) at a concentration of 0.62 μg/ml was added for 60 min. After washing, a 3-min reaction was performed with tetramethylbenzidine (TMB) substrate and stopped with H_2SO_4, 2 M. The results were read at 450/540 nm using a Power Wave 340 (Biotec Instruments). The infliximab concentration was quantified using a standard curve constructed using exogenous infliximab (Schering Plough, NJ, USA). The lower limit of quantification was 0.1 μg/ml.

IFX levels were also evaluated in parallel using the commercial TNFα-Blocker ELISA (Immundiagnostik AG, Germany) and Lisa-Tracker Premium Infliximab ELISA (Theradiag, France) kits according to the instructions provided by the manufacturer. The lower limit of quantification was 0.5 and 0.1 μg/ml, for Immundiagnostik and Theradiag, respectively.

Evaluation of ADA levels

Antibodies to infliximab were evaluated using three distinct methodologies (Figure 1B), one in-house assay [anti-human lambda chain assay (AHLC)], and two commercially available kits [a semi-quantitative assay, TNFα- Blocker ADA (Immundiagnostik AG, Germany); and a quantitative assay, Lisa-Tracker Premium Infliximab ELISA (Theradiag, France)]. The upper limit of the measurement for the three assays was calculated as the highest concentration of the standard curve × sample dilution factor used.

In-house AHLC. ADA levels were determined in all samples using an in-house ELISA as previously described by Ben-Horin and colleagues [Ben-Horin *et al.* 2011]. The AHLC is a sandwich ELISA that uses anti-human lambda chain conjugate antibody in the detection step, taking advantage of the exclusively kappa chain composition of IFX [Kopylov *et al.* 2012]. Briefly, IFX was added to a plate precoated with TNFα (Peprotech, Rocky Hill, NJ, USA). After Diluted serum was added and incubated for 60 min at room temperature. Goat anti-human lambda chain HRP-labeled antibody (Serotec, Oxford, UK) was added and incubated for 60 min, at room temperature. After a 6-min reaction with TMB the reaction was stopped with H_2SO_4. Absorbances were read at 450/540 nm and the results are expressed as μg/ml-equivalent (μg/ml-e) after normalization against results obtained using a standard curve of goat anti-human F(ab')2 fragment antibody (MP Biomedicals). For the purpose of brevity, the results are thereafter expressed as μg/ml, rather than μg/ml-e. The lower limit of quantification was 1.2 μg/ml.

Immundiagnostik semi-fluid phase enzyme immunoassay. ADA levels were determined in a semi-fluid phase enzyme immunoassay (SFPE) using a commercial kit (TNFα-Blocker ADA) purchased from Immundiagnostik (Germany) according to the instructions provided by the manufacturer. The SFPE uses an initial acid buffer treatment to dissociate the IFX–ADA immune complexes and two IFX conjugates with different conjugates. Acidified samples are incubated with IFX–biotin conjugate which immobilizes ADAs on the plate, and IFX–peroxidase conjugate is used for detection [Imaeda *et al.* 2012].

Theradiag bridging ELISA. ADA levels were determined in a bridging ELISA (BE) performed using a commercial kit (Lisa-Tracker Premium Infliximab ELISA) purchased from Theradiag (France) according to the instructions provided by the manufacturer. The BE uses a double-antigen bridge: ADAs create a bridge between IFX immobilized on the plate and IFX enzyme-linked conjugate. A total of 4 out of 79 samples determined with Theradiag were higher than the upper limit of the kit: in these cases the upper limit was considered as the result. The lower limit of quantification was 10 ng/ml.

Exogenous IFX in ADA-positive sera samples

Exogenous IFX (Schering Plough, NJ, USA) was used to spike ADA-positive sera samples that contained undetectable levels of IFX.

Evaluation of exogenous IFX incubation time. The incubation time of exogenous IFX in sera was evaluated. Sera samples with ADAs and undetectable levels of IFX were incubated with several concentrations of IFX (5, 10, 15, 30, 100 and 300 μg/ml) for different lengths of time (0, 0.5, 3, 6, 12 and 24 h). Levels of ADAs were then determined using the AHLC assay as described above.

Evaluation of stratified ADA levels (four groups) with exogenous IFX spiking using AHLC methodology. Four groups with undetectable IFX (<0.1 µg/ml) were stratified according to ADA-positive concentration as previously measured by AHLC assay: group A (ADAs within 1.7–3 µg/ml); group B (ADAs within 3–10 µg/ml); group C (ADAs within 10–25 µg/ml); group D (ADAs >25 µg/ml). Sera were preincubated with several concentrations of exogenous IFX (5, 10, 30, 100 and 300 µg/ml) for 30 min, at room temperature. Levels of ADAs were then determined using the in-house AHLC ELISA as described above.

Evaluation of ADA levels with exogenous IFX spiking in two groups of sera samples using three different methodologies. Two groups of ADA-positive sera with undetectable IFX (<0.1 µg/ml) were selected from sera previously measured by AHLC assay: high levels of ADAs (>25 µg/ml) and intermediate levels of ADAs (>3 µg/ml and <10 µg/ml). Sera were preincubated with several concentrations of exogenous IFX (5, 10, 15, 30, 100 and 300 µg/ml) for 30 min, at room temperature. Levels of ADAs were then determined using the three different methodologies: in-house AHLC, Immundiagnostik SFPE and Theradiag BE as described above.

Measurement of ADA IgG4 in the sera

Sera samples containing ADAs were evaluated for ADA IgG4. ADA IgG4 levels were determined using an ELISA developed and described by Bendtzen and colleagues and adapted for IgG4 detection [Bendtzen and Svenson, 2011; Steenholdt, 2013]. Briefly, diluted serum was added and incubated for 60 min at room temperature to a plate precoated with anti-IgG4 antibody (Serotec, Oxford, UK). Plates were then washed and HRP-labeled infliximab was added for 60 min, at room temperature. A 5-min reaction with TMB substrate was performed and stopped with 2 M H_2SO_4. Absorbances were read at 450/540 nm and the results were expressed as µg/ml-e after normalization against results obtained using a standard curve of goat anti-human F(ab')2 fragment antibody (MP Biomedicals).

Statistical analysis

The intraclass correlation coefficient (ICC) was used to determine the quantitative agreement between IFX levels evaluated by the three assays. ICC within 0.4 and 0.75 was considered moderate, lower than 0.4 poor and higher than 0.75 excellent. Agreement between assays was also assessed using Bland–Altman analysis, in which the mean differences and limits of agreement are descriptive. The KAPPA coefficients of 0–0.20 were considered to indicate slight, 0.21–0.40 fair, 0.41–0.60 moderate, 0.61–0.80 substantial and 0.81–1 almost perfect agreement [Landis and Koch, 1977]. The mean difference (bias) and its 95% confidence interval (CI) were computed. Limits of agreement (LOAs) were defined as mean difference ± 2 standard deviations of difference (LOA = bias ± SDD). Ninety-five per cent of differences are expected to lie between LOAs.

The quantitative agreement between assays for ADA could not be assessed because data were reported using different and arbitrary units (AU/ml).

Kappa coefficient and percentage agreement were therefore used to determine the qualitative agreement between IFX levels, ADAs and status (IFX+/ADAs-; IFX+/ADAs+; IFX-/ADAs-; IFX-/ADAs-). Correlations between assays were determined only for IFX levels using linear correlation analysis expressed as Pearson's correlation coefficient (Pearson's *r*).

Results

IFX levels

A total of 79 sera samples were evaluated for IFX levels using three types of ELISA. Samples were collected from multicentric cohort of UC patients, composed of 44% men and with a median [interquartile range (IQR)] age of 35 (23–48) years. All patients were in the maintenance phase (after 14 weeks). A total of 35% of the patients were in IFX monotherapy and the remain cohort in concomitant therapy [38% azathioprine (AZA); 12% 5-aminosalicylic acid (5-ASA); 7% AZA + 5ASA; 1% methotrexate; 1% corticosteroids; 2% AZA + 5-ASA + corticosteroids; 1% AZA + corticosteroids + antibiotics].

Quantitative agreement was calculated using the ICC and mean differences (Table 1). Comparisons showed that ICC varied from a moderate agreement of 0.694 (0.618–0.805) between Immundiagnostik and Theradiag up to an excellent agreement of 0.957 (0.933–0.972) between the in-house ELISA and Immundiagnostik kit.

Table 1. Intraclass correlation (ICC) and mean differences between different assays.

	ICC	95% CI	Differences	
			Mean	95% CI
IFX				
In-house ELISA/ Theradiag ELISA	0.762	0.627–0.848	3.99	2.63–5.36
In-house ELISA/ Immundiagnostik ELISA	0.957	0.933–0.972	–0.43	–1.31 to 0.44
Immundiagnostik ELISA / Theradiag ELISA	0.694	0.618–0.805	4.43	2.76–6.10

CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; IFX, infliximab.

Mean differences (bias) confirmed the ICC evaluation. Moreover, a Bland and Altman plot analysis (graphics not shown) indicate that the significant bias between in-house and Theradiag is a result of dispersion of values along all of infliximab concentration, even for minor concentrations. Regarding in-house and Immundiagnostik, bias is more significant for concentrations higher than 15 µg/ml.

The Pearson correlation between different assays was also calculated: 0.84 ($p<0.001$) for in-house *versus* Theradiag; 0.79 ($p < 0.001$) for Theradiag *versus* Immundiagnostik and 0.92 ($p<0.001$) for in-house *versus* Immundiagnostik.

In order to quantify the qualitative agreement of the different methods, two different cutoffs were applied to the measured concentrations: one used the limit of detection determined by the manufacturers for each assay as the cutoff, conveying an analytical perspective; and the other used clinically relevant cutoffs, as defined in the literature [Kopylov *et al.* 2012; Ungar *et al.* 2015].

Regarding the analytical approach, IFX was considered positive when above 0.1 µg/ml using the in-house and Theradiag methods. For the Immundiagnostik kit, positivity was considered for IFX concentrations above 0.5 µg/ml. The in-house ELISA technique detected trough levels in 61 of 79 samples, a detection rate similar to that verified using the Immundiagnostik method (78%). Using Theradiag the IFX trough levels were detected in fewer sera (71%). The accuracy and KAPPA coefficients for these methods using the analytical cutoff are depicted in Table 2(A). In-house and Theradiag ELISA showed a moderate agreement (0.509), whereas the in-house/Immundiagnostik and Immundiagnostik/Theradiag had a substantial agreement (0.69 and 0.726, respectively). Moreover, a good accuracy was found among the three assays (81–88%).

Considering the clinical approach, IFX was defined as positive for concentrations above 1 µg/ml. The cutoff was settled taking in consideration cutoffs reported previously in the literature [Kopylov *et al.* 2012; Ungar *et al.* 2015].

The in-house ELISA detected positive IFX trough levels in 48 of the 79 samples (61%), while the Immundiagnostik kit did so in 45 of the 79 samples (57%) and the Theradiag kit did so in 49 of the 79 samples (62%). The data regarding IFX trough levels obtained using the different assays is shown in Table 2(B).

The accuracy and KAPPA coefficient between the three different assays were calculated (Table 2B). All assays showed excellent agreement regarding IFX levels, with the KAPPA coefficient ranging from 0.835 (0.709–0.960) between Immundiagnostik and Theradiag results, to 0.922 (0.836–1.000) between the in-house assay and Immundiagnostik results. A high level of accuracy was found between the three assays, with the in-house and Immundiagnostik assays showing the highest accuracy (96%).

ADA levels

The ICC and mean difference for ADAs could not be evaluated because the different assays used different units of measurement (µg/ml for in-house assay and Theradiag and AU/ml for Immundiagnostik).

From an analytical point of view, ADAs positivity was defined as levels above 1.2 µg/ml for in-house method, 0.01 µg/ml for the Theradiag kit and 10 AU/ml for Immundiagnostik kit. All techniques have identified the same number of ADAs-positive samples (46%). Accuracy and KAPPA coefficient were calculated and are depicted in Table 3(A): a good accuracy was found for the

Table 2. Agreement between different assays regarding levels of IFX: (A) analytical approach and (B) clinical approach.

	Agreement	
	Accuracy	KAPPA [95% CI]
(A) IFX (>0.1 µg/ml in-house and Theradiag; 0.5 µg/ml Immundiagnostik)		
In-house ELISA/ Theradiag ELISA	81%	0.509 [0.295–0.722]
In-house ELISA/ Immundiagnostik ELISA	87%	0.697 [0.526–0.868]
Immundiagnostik ELISA / Theradiag ELISA	88%	0.726 [0.559–0.893]
(B) IFX (>1 µg/ml)		
In-house ELISA/ Theradiag ELISA	92%	0.840 [0.716–0.963]
In-house ELISA/ Immundiagnostik ELISA	96%	0.922 [0.836–1.000]
Immundiagnostik ELISA / Theradiag ELISA	92%	0.835 [0.709–0.960]

CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; IFX, infliximab.

Table 3. Agreement between different assays regarding levels of ADAs: (A) analytical approach and (B) clinical approach.

	Agreement	
	Accuracy	KAPPA [95% CI]
(A) ADAs (>1.2 µg/ml for in-house and >0.01 µg/ml Theradiag; >10 AU for Immundiagnostik)		
In-house/ Theradiag	85%	0.695 [0.539–0.851]
In-house/ Immundiagnostik	82%	0.643 [0.472–0.814]
Theradiag / Immundiagnostik	82%	0.635 [0.463–0.807]
(B) ADAs (>1.7 µg/ml for in-house and Theradiag; >10 AU/ml for Immundiagnostik)		
In-house/ Theradiag	81%	0.602 [0.435–0.769]
In-house/ Immundiagnostik	85%	0.692 [0.531–0.852]
Theradiag / Immundiagnostik	83%	0.653 [0.492–0.814]

AU, arbitrary units; CI, confidence interval; ELISA, enzyme-linked immunosorbent assay; IFX, infliximab.

three assays (82–85%), whereas KAPPA coefficient shown a moderate agreement.

A clinical approach to the ADAs positivity is shown in Table 3(B). Samples were considered ADA-positive when levels exceeded 1.7 µg/ml for the in-house (AHLC) as applied by Kopylov and colleagues [Kopylov *et al.* 2012]. For Theradiag the same cutoff was applied. For the Immundiagnostik (SFPE) assay ADA-positive were considered when >10 AU (cutoff defined by the manufacturer for positivity). The in-house and Immundiagnostik assays detected ADAs in 34 out of 79 samples (43%), while Theradiag detected ADAs in 24 out of 79 samples (30%). Although the in-house and Immundiagnostik assays detected the same number of samples with ADAs not all samples matched. In fact, ADAs were confirmed by both the in-house and Immundiagnostik assay in 28 out of the 34 samples (82%). Regarding the comparative performance of Theradiag with the in-house and Immundiagnostik assays, of the 24 samples with ADAs identified by Theradiag, 21 (87.5%) were also positive in both the in-house and Immundiagnostik assays. Nevertheless, the in-house and Immundiagnostik assays considered positive 13 and 12 samples, respectively, that were negative with Theradiag.

Moderate agreement was found between the in-house and Theradiag assays, with a KAPPA coefficient of 0.602 (0.435–0.769) while the in-house and Immundiagnostik assays showed good agreement with a KAPPA coefficient of 0.692 (0.531–0.852). Accuracy ranged from 81% to 85% among the different pairs of assays (Table 3B).

IFX and ADA status

Figure 2 depicts the levels of IFX and ADAs evaluated by all assays. The vertical dotted line separates samples positive for IFX from samples negative for IFX. The horizontal dotted line separates samples positive for ADAs from samples negative for ADAs. Both analytical and clinical perspectives are represented in Figure 2. From the analytical point of view, 71–77% of total samples are included in IFX+ group, and 45% in anti-IFX+. On the other hand, from a clinical perspective all the three assays identified 25% of samples positive for ADAs and IFX-negative.

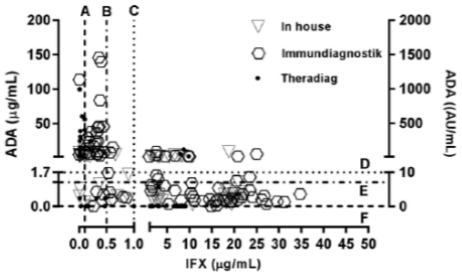

Figure 2. Levels of infliximab (IFX) and anti-infliximab antibodies (ADAs) determined by all methodologies. Dotted lines represent cutoffs for analytical approach (A, B, D, E and F) and clinical approach (C and D).
Cutoffs for IFX levels: (A) 0.1 µg/ml (in-house and Theradiag); (B) 0.5 µg/ml (Immundiagnostik); (C) 1 µg/ml (all assays).
Cutoffs for ADAs levels: (D) 1.7 µg/ml (in-house and Theradiag) and 10 AU/ml (Immundiagnostik); (E) 1.2 µg/ml (in-house); (F) 0.01 µg/ml (Theradiag).

However, the group of samples that had a double-positive status for both IFX and ADAs were identified almost exclusively by the in-house and Immundiagnostik assays. Theradiag detected only three samples that were positive for ADAs in the presence of the drug. However, these three samples were considered IFX-negative by the other two assays.

Thereafter, we focused on the double status of IFX and ADAs. As the IFX/ADAs group definition depends directly on the cutoffs applied, the two different approaches (analytical and clinical) were taken into consideration. Tables 4 and 5 show the agreement between assays for different combinations of IFX/ADA status from an analytical and clinical perspective, respectively.

IFX+/ADAs-. The analytical approach has shown that 50% of samples had an IFX+/ADAs-status (Table 4). Agreement between assays was considered good (85%).

The clinical perspective exhibited a strong agreement ranging between 75.6 and 100% (Table 5). The poorest agreement between assays was observed when using Theradiag as the reference assay and Immundiagnostik as the confirmatory assay (75.6%).

IFX+/ADAs+. The double-positive samples obtained using the cutoffs given by the analytical perspective is shown in Table 4. The agreement was low (31.3–50%). The lowest agreement rate occurred for Theradiag and Immundiagnostik (considering Theradiag as the standard assay).

In contrast, for the double-positive status (IFX+/ADAs+) defined using the clinical approach, there was a significant discrepancy between Theradiag and the in-house and Immundiagnostik assays. The in-house assay detected a total of 11 double-positive sera and Immundiagnostik a total of 10 IFX+/ADAs+. Theradiag was able to detect three double-positive samples but neither were in agreement with the other assays. In fact, in those three samples, both the in-house and Immundiagnostik assay detected ADAs whereas IFX was considered undetectable.

IFX-/ADAs-. Using the analytical perspective (Table 4) only two or three double-negative samples were detected. Furthermore, Theradiag and the other two assays never agreed. The in-house and Immundiagnostik kit agreed in one out of three samples.

The detection of samples with double-negative status (IFX-/ADAs-) by clinical approach showed similar levels of agreement between pairs of assays (Table 5). When considering the in-house and Immundiagnostik assay there was 62.5% agreement, but when considering Theradiag as the reference assay, there was a lower level of agreement between methodologies (50–55.6%).

IFX-/ADAs+. From an analytical perspective, Immundiagnostik and Theradiag had a good agreement (75–90%) concerning IFX-/ADAs+. A very good agreement was also obtained for this status between the in-house and Immundiagnostik assays when considering in-house the standard test.

From the clinical point of view, IFX-/ADAs+ status showed good agreement (more than 84%) between the in-house and Immundiagnostik assays. The in-house and Theradiag assays showed a lower level of agreement, although it was still considered good at 90.5% and 79.2%, respectively.

Exogenous IFX in ADA-positive samples

To evaluate the effect of IFX on in-house, Immundiagnostik and Theradiag assessment of ADAs in sera, an experiment was designed that involved spiking with exogenous IFX. The influence of exogenous IFX incubation time was assessed previously and no differences were found

Table 4. Agreement between the three assays for all combinations of IFX/ADAs status (analytical approach).

			Agreement	
			Accuracy	KAPPA [95% CI]
In-house assay *versus* Theradiag assay[a]		Theradiag assay *versus* In-house assay[b]		
IFX + ADA– 85% (*n* = 40)	IFX + ADA + 38.1% (*n* = 21)	IFX + ADA – 85% (*n* = 40)	IFX + ANTI + 50% (*n* = 16)	68%	0.502 [0.355–0.649]
IFX - ADA – 0% (*n* = 2)	IFX – ADA + 75.0% (*n* = 16)	IFX - ADA – 0% (*n* = 2)	IFX – ADA+ 57.1% (*n* = 21)		
In-house assay *versus* Immundiagnostik[c]		Immundiagnostik assay *versus* In-house assay[d]			
IFX + ADA – 85.0% (*n* = 40)	IFX + ADA + 35.0% (*n* = 20)	IFX + ADA – 85.0% (*n* = 40)	IFX + ADA + 58.3% (*n* = 12)	72%	0.566 [0.425–0.707]
IFX - ADA – 33.3% (*n* = 3)	IFX – ADA + 93.8% (*n* = 16)	IFX - ADA – 33.3% (*n* = 3)	IFX – ADA + 62.5% (*n* = 24)		
Immundiagnostik assay *versus* Theradiag assay[e]		*Theradiag assay versus* Immundiagnostik[f]			
IFX + ADA – 84.6% (*n* = 39)	IFX + ADA + 41.7% (*n* = 12)	IFX + ADA – 82.5% (*n* = 40)	IFX + ADA + 31.3% (*n* = 16)	77%	0.562 [0.415–0.707]
IFX - ADA – 0.0% (*n* = 2)	IFX – ADA + 75.0% (*n* = 24)	IFX - ADA – 0.0% (*n* = 1)	IFX – ADA + 90.0% (*n* = 20)		

[a]In-house assay was considered the reference assay and it was evaluated whether Theradiag confirmed the double status result.
[b]Theradiag assay was considered the reference assay and it was evaluated whether in-house confirmed the double status result.
[c]In-house assay was considered the reference assay and it was evaluated whether Immundiagnostik confirmed the double status result.
[d]Immundiagnostik assay was considered the reference assay and it was evaluated whether in-house confirmed the double status result.
[e]Immundiagnostik assay was considered the reference assay and it was evaluated whether Theradiag confirmed the double status result.
[f]Theradiag assay was considered the reference assay and it was evaluated whether Immundiagnostik confirmed the double status result.
CI, confidence interval.

(data not shown). An incubation time of 30 min was selected for further experiments.

Evaluation of ADA levels with exogenous IFX spiking in four groups of sera samples. To evaluate whether different intrinsic concentrations of ADAs are important when assessing the influence of exogenous IFX, we used stratified concentrations of ADA levels in sera (four different groups as evaluated previously: 1.7–3, 3–10, 10–25 and >25 µg/ml) and spiked them with exogenous IFX. The evaluation was performed using the in-house assay only. The results are presented in Figure 3. In-house assay lost the capability to detect levels of antibodies in sera samples with ADA levels between 1.7 and 3 µg/ml at concentrations of IFX over 5 µg/ml. The influence of exogenous IFX was also evident in the 3–10 µg/ml group, but loss of antibody detection capacity was only observed beyond 100 µg/ml IFX, which generally exceeds the therapeutic concentrations of infliximab *in vivo* even during induction phase [Adedokun *et al.* 2014]. A decreased ADA detection capacity was observed in the two upper groups (10–25 and >25 µg/ml) in a concentration-dependent manner, but in both groups the in-house assay was able to detect antibodies even with 300 µg/ml of exogenous IFX. Nevertheless, a significant reduction of detection capacity of 75% and 61% was observed in the 10–25 µg/ml and >25 µg/ml groups, respectively.

Evaluation of ADA levels with exogenous IFX spiking in two groups: high and intermediate levels of ADAs. Taking into account the above-mentioned results, we decided to explore the performance of

Table 5. Agreement between the three assays for all combinations of IFX/ADAs status (clinical approach).

				Agreement	
				Accuracy	KAPPA [95% CI]
In-house assay *versus* Theradiag assay[a]		Theradiag assay *versus* In-house assay[b]			
IFX + ADA– 100% (*n* = 35)	IFX + ADA + 0% (*n* = 11)	IFX + ADA – 77.8% (*n* = 45)	IFX + ANTI + 0% (*n* = 3)	76%	0.620 [0.489–0.751]
IFX - ADA – 62.5% (*n* = 8)	IFX – ADA + 79.2% (*n* = 24)	IFX - ADA – 55.6% (*n* = 9)	IFX – ADA+ 90.5% (*n* = 21)		
In-house assay *versus* Immundiagnostik[c]		Immundiagnostik assay *versus* In-house assay[d]			
IFX + ADA – 83.8% (*n* = 37)	IFX + ADA + 54.5% (*n* = 11)	IFX + ADA – 88.6% (*n* = 35)	IFX + ADA + 60% (*n* = 10)	81%	0.716 [0.591–0.841]
IFX - ADA – 62.5% (*n* = 8)	IFX – ADA + 95.7% (*n* = 23)	IFX - ADA – 62.5% (*n* = 8)	IFX – ADA + 84.6% (*n* = 26)		
Immundiagnostik assay *versus* Theradiag assay[e]		Theradiag assay *versus* Immundiagnostik[f]			
IFX + ADA – 100% (*n* = 34)	IFX + ADA + 0% (*n* = 10)	IFX + ADA – 75.6% (*n* = 45)	IFX + ADA + 0% (*n* = 3)	76%	0.625 [0.491–0.758]
IFX - ADA – 66.7% (*n* = 6)	IFX – ADA + 76.9% (*n* = 26)	IFX - ADA – 50% (*n* = 8)	IFX – ADA + 100% (*n* = 20)		

[a]In-house assay was considered the reference assay and it was evaluated whether Theradiag confirmed the double status result.
[b]Theradiag assay was considered the reference assay and it was evaluated whether in-house confirmed the double status result.
[c]In-house assay was considered the reference assay and it was evaluated whether Immundiagnostik confirmed the double status result.
[d]Immundiagnostik assay was considered the reference assay and it was evaluated whether in-house confirmed the double status result.
[e]Immundiagnostik assay was considered the reference assay and it was evaluated whether Theradiag confirmed the double status result.
[f]Theradiag assay was considered the reference assay and it was evaluated whether Immundiagnostik confirmed the double status result.
CI, confidence interval.

the three assays under the interference of exogenous IFX. The two most representative groups were used: samples with ADAs within 3–10 µg/ml (intermediate) and those with >25 µg/ml (high level). Figures 4 and 5 present the results of the groups spiked with exogenous IFX at different concentrations (0, 5, 10, 15, 30, 100 and 300 µg/ml). Table 6 shows the half maximal inhibitory concentrations (IC_{50}).

Figure 4 (high levels of ADAs) illustrates that exogenous IFX only interfered with ADA detection at concentrations higher than 100 µg/ml. Spiking 100 µg/ml of exogenous IFX in sera samples with high levels of ADA resulted in a decline in ADA detection of 87%, 46% and 16% in the Theradiag, in-house and Immundiagnostik assays, respectively. Nevertheless, in-house showed a higher IC_{50} than Theradiag, at 143.6 µg/ml *versus* 74.9 µg/ml, respectively, indicating that it was less affected than Theradiag by the presence of IFX. In the Immundiagnostik assay the influence of exogenous IFX showed a lower decline in ADA detection. In fact, 300 µg/ml decreased ADA detection by 17% (the IC_{50} was therefore impossible to determine), while in in-house and Theradiag assays, ADA detection decreased by 75% and 93%, respectively. However, all methodologies were able to detect ADAs, in sera originally positive for high levels of ADAs, even with 300 µg/ml of exogenous IFX.

Figure 5 shows exogenous IFX interference in sera samples with intermediate levels of ADAs. The presence of exogenous IFX decreased ADA detection in Theradiag and in-house with the

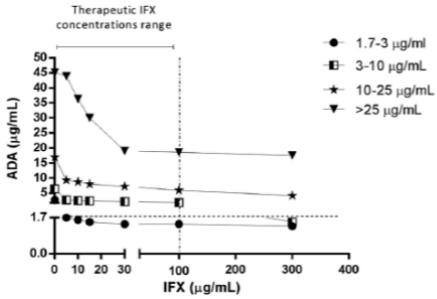

Figure 3. Stratified anti-infliximab antibodies (ADA) levels in the presence of exogenous infliximab (IFX), evaluated by in-house assay. Therapeutic IFX concentrations range was considered between 0 and 100 µg/ml [Adedokun *et al.* 2014].

lower concentration (5 µg/ml) of exogenous IFX. For Theradiag this decrease led to the inability to detect ADAs and consequently it was impossible to determine the IC_{50}. In-house showed an IC_{50} of 0.88 µg/ml while Immundiagnostik showed a higher value of 140.1 µg/ml. At 300 µg/ml the interference was stronger in Theradiag (97%) while in in-house detection decreased by 74%. In-house failed to detect ADAs at IFX concentrations of 300 µg/ml, which are above the therapeutic levels achieved *in vivo*. Above 30 µg/ml IFX the Immundiagnostik assay was influenced by an additive concentration effect but was able to detect ADAs even at the higher concentration (300 µg/ml) of exogenous IFX (total decrease of 60%).

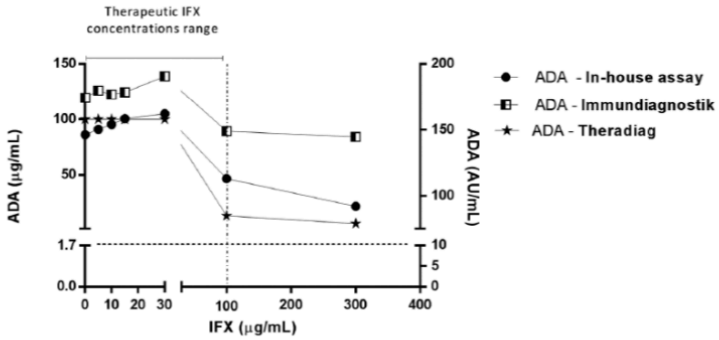

Figure 4. High anti-infliximab antibodies (ADAs) levels in the presence of exogenous infliximab (IFX) evaluated by in-house, Immundiagnostik and Theradiag assay. Therapeutic IFX concentrations range was considered between 0 and 100 µg/ml [Adedokun *et al.* 2014].

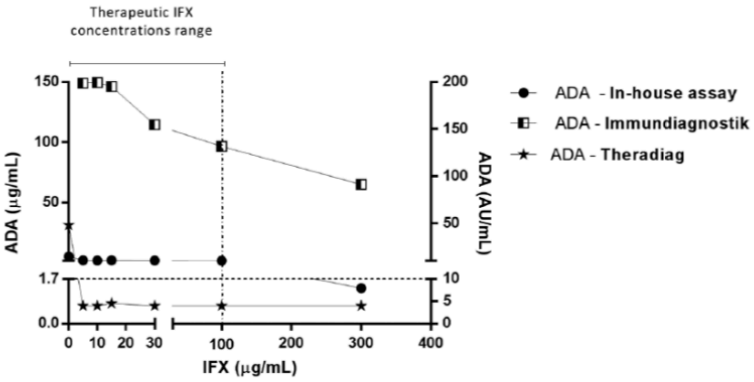

Figure 5. Intermediate anti-infliximab antibodies (ADAs) levels in the presence of exogenous infliximab (IFX) evaluated by in-house, Immundiagnostik and Theradiag assay. Therapeutic IFX concentrations range was considered between 0 and 100 µg/ml [Adedokun *et al.* 2014].

Table 6. Infliximab (IFX) inhibition effect on ADAs at intermediate and high levels in different assays.

	IC_{50} (µg/ml)	
	Intermediate levels 3 µg/ml > ADA <10 µg/ml	High levels ADA >25 µg/ml
In-house (AHLC)	0.88	143.60
Immundiagnostik (SPFE)	140.10	NA
Theradiag (BE)	NA	74.89

IC_{50}, half maximal inhibitory concentration; NA, IC_{50} value not determined because either 50% inhibition fell outside the highest concentration or because inhibition was 100% above the lowest concentration of IFX; ADA, anti-infliximab antibodies; AHLC, anti-human lambda chain; SPFE, semi-fluid phase enzyme; BE, bridging enzyme-linked immunosorbent assay.

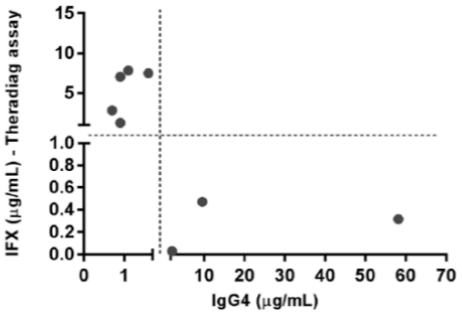

Figure 6. Levels of IFX and IgG4 in samples that were ADA-negative in Theradiag and ADA-positive in both the in-house and Immundiagnostik assays.

IgG4 ADAs

In order to evaluate the influence of IgG4 ADAs, samples positive for ADAs were tested for the presence of IgG4. IgG4 ADAs were found in 54.8% of the ADA-positive samples. Figure 6 shows the levels of IFX and IgG4 in samples that were ADA-negative with Theradiag but ADA-positive for both the in-house and Immundiagnostik assays. Of the eight samples negative for antibodies with Theradiag, five presented positive levels of IFX and three presented IgG4 ADAs.

Discussion

Several methodologies are available for use in drug monitoring. However, different methodologies return different results. There is still little information comparing different assays, in particular for ADA detection, which is susceptible to interference in the presence of detectable levels of IFX.

The aim of this study was to evaluate and compare three different assays for detecting IFX and ADAs and to clarify the importance of the presence of detectable levels of IFX on the accuracy of the ADA assays.

Cutoffs for a qualitative evaluation of the assays are difficult to establish, particularly for ADAs assays. In fact, ADAs results are expressed in different units by the different assays. In addition, Immundiagnostik defines a cutoff for positivity that is different from the limit of detection and not suggested by all commercial assays. The manufacturers' instruction for Theradiag do not contain any suggestion for a cutoff for ADAs positivity besides the limit of detection of 0.01 µg/ml. Van Schouwenburg and colleagues have shown that BE assays (Theradiag methodology) performance is depend not only of the amount of antibody in the serum but also of the affinity of ADAs to the assay [Van Schouwenburg *et al.* 2016]. This indicates a possible source of intervariability of the assay, namely for values near the limit of detection. In fact, our results show that with Theradiag 70% of the values considered to be positive, with analytical approach, are between 0.01 and 0.04 µg/ml. This might be a reflection of the intervariability of the assay, rather than a faithful quantification of the presence of antibodies. Taking this into consideration, we have decided to perform two different approaches in the qualitative analyses of the results: an analytical one, in which cutoffs were defined by the manufacturers' instructions; and a clinical one, in which the cutoffs were defined according to the literature.

From an analytical perspective, IFX assays showed a substantial agreement and a good accuracy, although the in-house and Theradiag methods had only a moderate agreement. On the other hand, upon applying a cutoff of 1 µg/ml, the behavior of the three assays is very similar.

This suggests that using the limit of detection as a cutoff may decrease the quality agreement between assays.

The behavior of the three assays in terms of IFX determination was very similar. In fact, an accuracy of over 92% for IFX was observed when analyzing the agreement between the three assays. An agreement of 100% (data not shown) was observed between the in-house and Immundiagnostik assays for IFX-negative samples, while the Theradiag assay identified four IFX-positive samples that the other two assays returned as negative. Casteele and colleagues also evaluated three IFX assays based on the sandwich ELISA principle and found good correlation between them [Casteele *et al.* 2012]. The authors used the same commercial kit as used here (Theradiag). In fact, Theradiag returned 11 positive samples that were negative according to the other two assays, showing that this assay can detect nonspecific binding. Overall, high titers in one assay were also high in the other two assays. Nevertheless, the Immundiagnostik and in-house assays were able to detect higher levels of IFX in sera samples, which may indicate the use of a more specific anti-human IgG antibody in the detection step in these assays than in Theradiag. The agreement between the three assays seemed to be more qualitative than quantitative. The three methodologies use the same ELISA sandwich principle, but there are some idiosyncratic differences that can explain the small discrepancies in quantitative evaluation: different detection limits and test sensitivity; reagent stability; different calibration standards and curve adjustment; and different sample dilution, manipulation and processing (e.g. different incubation times). Nevertheless, from a clinical point of view, the information given by the three assays (presence of positive/negative levels; low/therapeutic/high levels of the drug) showed acceptable interassay agreement. However, clinicians should be aware that changing drug level assays during patient follow up could induce errors in the interpretation of results and subsequent therapeutic strategies.

ADAs were evaluated using three methodologies: in-house AHLC, Immundiagnostik SFPE and Theradiag BE. These three assays have different methodological principles. The in-house AHLC is a sandwich ELISA that uses anti-human lambda chain conjugate antibody in the detection step, benefitting from IFX kappa chain exclusive composition [Kopylov *et al.* 2012]. The Theradiag BE uses a double-antigen bridge: ADAs create a bridge between IFX immobilized on the plate and IFX enzyme-linked conjugate. The Immundiagnostik SFPE uses an initial acid buffer treatment to dissociate the IFX–ADA immune complexes and two IFX conjugates with different types of conjugates. Acidified samples are incubated with an IFX–biotin conjugate, which immobilizes ADAs to the plate, whilst an IFX–peroxidase conjugate is used for detection.

A total of 79 samples of sera were compared using the three assays. The analytical approach showed the same moderate agreement for the three assays than the clinical approach. However, and from the analytical perspective, Theradiag was able to detect 37 positive ADAs, whereas from a clinical perspective only 24 patients were considered to be positive for ADAs. Conversely, the in-house assay detected almost the same number of ADA-positive patients with the two approaches (34 for analytical *versus* 37 for clinical). The results obtained using a clinical perspective with the Theradiag kit suggest a high rate of false-negative outcomes. This high prevalence of false-negative outcomes with Theradiag was reported previously by other authors [Imaeda *et al.* 2012; Steenholdt *et al.* 2013]. Kopylov and colleagues reported that BE was unable to detect ADAs in the presence of IFX [Kopylov *et al.* 2012]. Our results showed that 22 out of 24 ADA-positive samples were IFX-negative. Only three patients showed a double-positive status with Theradiag, however neither was confirmed as IFX-positive in the in-house or Immundiagnostik assay. These samples were probably false positive for IFX. In fact, the disagreement of the in-house and Immundiagnostik assay with Theradiag for double-positive status (IFX+/ADAs+) was 100%. Since Theradiag uses labeled IFX as the detection antibody, the presence of IFX may compete with the detection of the ADAs. On the other hand, the BE is unable to detect monovalent IgG4 ADAs, contributing to the false-negative results. Our results were consistent with these two statements and showed that samples positive for ADAs with the in-house and Immundiagnostik assays, but negative with Theradiag, one of two situations occurred: (i) sera samples were IFX-positive; or (ii) ADAs were IgG4 antibodies. The inability to detect antibodies in the presence of the drug is important as two recent studies have shown that patients with IFX levels >3 μg/ml but with positive ADA have significantly higher levels of C-reactive protein (CRP) and less mucosal

healing [Casteele *et al.* 2015; Yanai *et al.* 2015], indicating a reduced control of inflammation mediated by these antibodies even when drug levels are adequate.

In order to understand the influence of drug presence on ADA detection, we designed an experiment using IFX-negative sera that were incubated with different concentrations of exogenous IFX. The in-house assay was slightly affected by 5 µg/ml exogenous infliximab but was able to detect antibodies up to 100 µg/ml of IFX. Kopylov and colleagues described the same drug concentration dependency for the AHLC assay [Kopylov *et al.* 2012]. However, our results permit us to define which concentration of IFX decrease the assay capacity to determine ADAs. The Immundiagnostik assay showed the best behavior in the presence of exogenous IFX. Interference was observed above 100 µg/ml of exogenous IFX but the assay was able to detect ADAs even at the higher concentration of 300 µg/ml of IFX. However, the results obtained in sera with high levels of ADAs were surprising and to the best of the authors' knowledge have not been reported previously. Even the Theradiag BE, which is widely described as being unable to detect antibodies in the presence of the drug, was able to identify ADA-positive samples in the presence of high levels of exogenous IFX. The results indicated that assays are not only limited by the levels of drug in the sera but also by the concentration of ADAs. These phenomena could be explained as follows: (i) in Theradiag, ADAs bind to all free drug in serum but there is still enough ADAs to bind to the plated IFX and bridge it to the conjugated one; (ii) in Immundiagnostik high levels of ADAs are most likely to diminish free IFX interference.

In summary, for IFX drug level determination, the three methodologies are equivalent, however the agreement between them seems to be more qualitative than quantitative. Regarding ADAs, despite being equally effective, the three assays show significant differences. Disagreement increased when samples had a double-positive or double-negative status, probably related to the specific limitations of each assay. The choice of cutoff to discriminate positive *versus* negative also enhances disagreement. This led us to define two different approaches with different cutoffs and present both evaluations. Clinical approach seems to highlight inabilities of the assays. In fact, Theradiag showed inability to detect positive ADA levels in samples with IFX (<5 µg/ml) or a high percentage of IgG4 ADAs. IgG4 ADAs are monovalent and unable to create a bridge with the labeled IFX used in detection step. In contrast, both the in-house assay and the Immundiagnostik assay are able to detect ADAs in the presence of IFX up to levels of >100 µg/ml which conform with the therapeutic concentrations observed in patients receiving IFX. Unexpectedly, in samples with high levels of ADAs (>25 µg/ml), the presence of IFX was not as important and even Theradiag was able to detect antibodies. Nevertheless, Immundiagnostik was least affected by IFX, followed by the in-house assay. In conclusion, our results indicated that assays are not only limited by the levels of drug in the sera but also by the concentration of ADAs.

Clinicians must be aware when optimizing treatment that binary (IFX/ADAs) stratification of results as positive and negative can differ according to the assay used. There is a strong agreement between assays for IFX+/ADAs- and IFX-/ADAs+ status. The choice of assay will probably have little influence on therapeutic decisions to change the class of drug (IFX+/ADA-) or change anti-TNFα antibody (IFX-/ADAs+). However, erroneous therapeutic decisions may occur when patients show double-negative or double-positive status, since agreement between assays is significantly lower in these circumstances. Treatment intensification (IFX-/ADAs-) and change of drug class or concomitant use of immunomodulators (IFX+/ADAs+) should take into account the fact that the results are assay dependent.

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data analysis, interpretation and drafting of the manuscript, and revised it critically for important intellectual content. All authors read and approved the final version of the manuscript.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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AP&T Alimentary Pharmacology and Therapeutics

Proactive therapeutic drug monitoring of infliximab: a comparative study of a new point-of-care quantitative test with two established ELISA assays

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SUMMARY

Background
Therapeutic drug monitoring is a powerful strategy known to improve the clinical outcomes and to optimise the healthcare resources in the treatment of autoimmune diseases. Currently, most of the methods commercially available for the quantification of infliximab (IFX) are ELISA-based, with a turnaround time of approximately 8 h, and delaying the target dosage adjustment to the following infusion.

Aim
To validate the first point-of-care IFX quantification device available in the market – the Quantum Blue Infliximab assay (Buhlmann, Schonenbuch, Switzerland) – by comparing it with two well-established methods.

Methods
The three methods were used to assay the IFX concentration of spiked samples and of the serum of 299 inflammatory bowel diseases (IBD) patients undergoing IFX therapy.

Results
The point-of-care assay had an average IFX recovery of 92%, being the most precise among the tested methods. The Intraclass Correlation Coefficients of the point-of-care IFX assay vs. the two ELISA-based established methods were 0.889 and 0.939. Moreover, the accuracy of the point-of-care IFX compared with each of the two reference methods was 77% and 83%, and the kappa statistics revealed a substantial agreement (0.648 and 0.738).

Conclusions
The Quantum Blue IFX assay can successfully replace the commonly used ELISA-based IFX quantification kits. This point-of-care IFX assay is able to deliver the results within 15 min makes it ideal for an immediate target concentration adjusted dosing. Moreover, it is a user-friendly desktop device that does not require specific laboratory facilities or highly specialised personnel.

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INTRODUCTION

Infliximab (IFX), the first anti-tumour necrosis factor alpha (TNFα) to be approved for the treatment of inflammatory bowel disorders, is a chimeric monoclonal IgG1 composed by a murine variable region (25%) and a constant human region (75%).[1–3] Upon binding to TNF, IFX elicits a number of mechanisms that reduce and control the inflammatory response, including the down-regulation of local and systemic pro-inflammatory cytokines, the induction of T-cell apoptosis, and the reduction in leucocytes and lymphocytes migration towards the inflammatory focus.[2, 3]

However, and in spite of the efficacy demonstrated by IFX in the treatment of several autoimmune inflammatory disorders, certain patients do not respond or have a limited response to IFX therapy. In the specific case of inflammatory bowel disorders (IBD), 10–30% of the patients do not respond to IFX induction (primary non-responders), whereas an annual rate of 13% of the initial responders tend to stop due to loss of response.[4–6] It is nowadays increasingly acknowledged that low trough levels of serum IFX can explain the lack of response to therapy. Although the cut-off levels are still debatable, it has been clearly established in the literature that IFX nonresponders have significantly lower serum trough levels of the drug.[4, 7] Moreover, several published studies demonstrate a clear positive correlation between IFX serum levels and rates of endoscopic improvement and remission, whereas undetectable IFX levels place patients at an increased risk of colectomy.[8–11] Overall, IFX serum trough levels between 3 and 7 μg/mL are commonly accepted by physicians and researchers as being the optimal therapeutic window during the maintenance phase.[6, 12–14]

The interindividual variation in IFX serum levels in IBD patients is known to be the result of multiple factors: whereas the immunogenicity of the drug and the consequent formation of antibodies to IFX is widely studied, other factors, such as body mass index, albumin serum concentration, gender, smoking and disease activity/duration are known to impact the pharmacokinetics and pharmacodynamics of IFX, therefore, having a role in the drug availability on the patients' serum.[4, 15, 16] Despite the underlying reasons for this variability, monitoring of serum IFX concentrations and of the formation of anti-drug antibodies during therapy (Therapeutic Drug Monitoring, TDM) is a powerful tool to aid physicians in the therapeutic decision-making process in the case of loss or of suboptimal response.[12–14, 17] Moreover, TDM may also support IFX de-escalation in case of supratherapeutic serum concentrations, enhancing the cost-effectiveness of the therapeutic process and avoiding unnecessary side effects.[12–14, 18]

As TDM relies on an efficient and accurate quantification of serum IFX levels and anti-drug antibodies, several methods have been developed, validated and made commercially available for use in hospitals and reference laboratories. Some of them are able to measure both IFX and antibodies to IFX, whereas others are specific for one of these quantifications. Most of the available methods rely on an Enzyme-Linked Immunosorbent Assay (ELISA) technique, whereas alternative ones, based on, for instance, immunoaffinity magnetic purification coupled with high-temperature reversed-phase liquid chromatography, fluid-phase radioimmunoassays (RIA), reporter gene assays and liquid chromatography linked with mass spectrometry are becoming increasingly acknowledged.[19–25]

This study aimed to evaluate the performance of the first point-of-care IFX-quantification assay available in the market by comparing it to two established ELISA methods, using spiked samples and a large and wide-ranged set of clinical samples.

MATERIAL AND METHODS

Samples and patients

Spiked samples of known IFX concentrations (0.5, 1, 1.5, 3, 5, 7, 10, 15, 20, 30 and 40 μg/mL) were generated by diluting the appropriate amount of exogenous IFX (Schering Plough, New Jersey, USA) into a pool of serum from control donors (after signing consent forms). Serum samples for IFX quantification were prospectively and consecutively obtained from a multicentric cohort of IBD out-patients in the maintenance phase of IFX therapy during routine consultations and immediately before the IFX infusion. The Ethics Committees of all involved institutions approved this study, and all patients were required to sign a written informed consent prior to their participation. Blood samples were collected, centrifuged and serum samples were kept at −80 °C until being processed.

IFX-quantification assays

The assay being tested was the Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Buhlmann, Schönenbuch, Switzerland) – hereafter referred to as point-of-care QB – and the sample quantification was carried out strictly respecting the manufacturers' instructions in an open-label fashion. A chip card, provided with each

test kit, supplied the point-of-care QB reader with the test information and calibration curve for each specific cartridge lot. Briefly, serum samples were diluted 1:20 and a 70 μL aliquot was loaded into the port of the test cartridge. After a 15 min reaction, the cartridge was read and the results were shown on the point-of-care QB reader display. According to the manufacturer, this kit has the following analytical characteristics: the limit of detection is 0.15 μg/mL, and the lower and upper limits of quantification are 0.4 μg/mL and 20 μg/mL respectively.

The point-of-care QB was compared with two well-established ELISA-based IFX-quantification tests: the Level Infliximab M2920 kit (Sanquin, Amsterdam, the Netherlands) – hereafter referred to as Sanquin – and an 'in house' validated procedure. The Sanquin was performed according to the manufacturers' instructions, whereas the 'in house' procedure has been carried out as described elsewhere.[26–29] Briefly, regarding the 'in house' procedure, serum samples were diluted (1:100) and added to a plate pre-coated with TNFα (Peprotech, Rocky Hill, NJ, USA). After 60 min of incubation and an appropriate number of washes, a horseradish peroxidase (HRP)-labelled goat anti-human Fc fragment antibody (MP Biomedicals, Solon, OH, USA) was added and the plate was incubated for 60 min. Afterwards, tetramethylbenzidine (Millipore, MA, USA) substrate was added, and the reaction was stopped 3 min later with 2M H_2SO_4. Finally, the samples' absorbance was read at 450/540 nm, and the IFX was quantified by interpolating the absorbance values in a standard curve built with known concentrations of exogenous IFX (Schering Plough, NJ, USA). According to the manufacturer, the Sanquin kit has the following analytical characteristics: the lower and upper limits of quantification are 0.08 μg/mL (1:200) and 47 μg/mL (1:2000) respectively. For the 'in house' procedure, the upper limit of quantification was calculated as the highest concentration of the standard curve × the sample dilution factor used.

Statistical analyses

Continuous variables were described using the median and interquartile range (IQR) or the average and the standard deviation (when appropriate). The level of statistical significance was set at 0.05. For statistical purposes, the results of all patients' samples for which the concentration measured by the point-of-care QB was below or above the limits of quantification (0.4 μg/mL and 20 μg/mL, respectively) were considered to be those same limits. The accuracy and kappa statistics were computed based on a pre-established therapeutic window of IFX. Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY, USA) and GraphPad Prism version 6.0 for Windows (GraphPad Software, La Jolla California USA) were used for the statistical analysis and plots' design.

RESULTS

The point-of-care QB assay and the two reference methods were used to measure the IFX concentrations in known IFX-spiked samples to compare their intravariability and recovery rates (Figure 1). The point-of-care QB assay had the smaller intravariability and the better (i.e. closer to real concentrations) recovery rates. In fact, the average recovery rate of the point-of-care QB assay was 92% (ranging from 68% to 108%), compared to 151% (ranging from 57% to 225%) and 114% (ranging from 79% to 160%) for the 'in house' and Sanquin methods respectively.

Moreover, IFX was quantified in a total of 299 serum samples using the three different tested assays. These samples were collected from the same number of IBD patients, a multicentric cohort with a median (IQR) age of 34 (24–45) years and composed of 50% males. All these patients were in the maintenance phase of IFX therapy. The correlation between the IFX levels obtained with each of the three assays is shown in Figure 2. All correlations are significant, with the highest correlation coefficient being found for the Sanquin and the point-of-care QB assay (0.952). Considering the correction made for samples which results were above the limit of detection with the point-of-care QB, these samples were

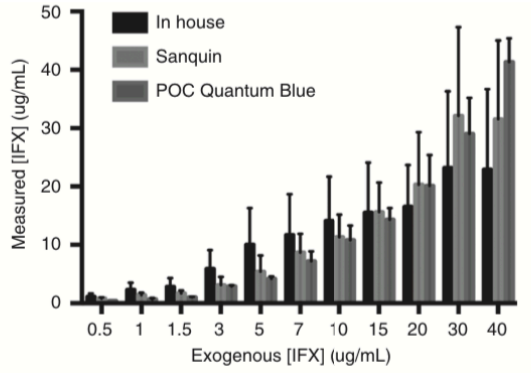

Figure 1 | IFX quantification of exogenously spiked IFX samples of known concentrations. The bars indicate the mean concentration obtained with each assay in the different concentrations and the error bars refer to the standard deviation.

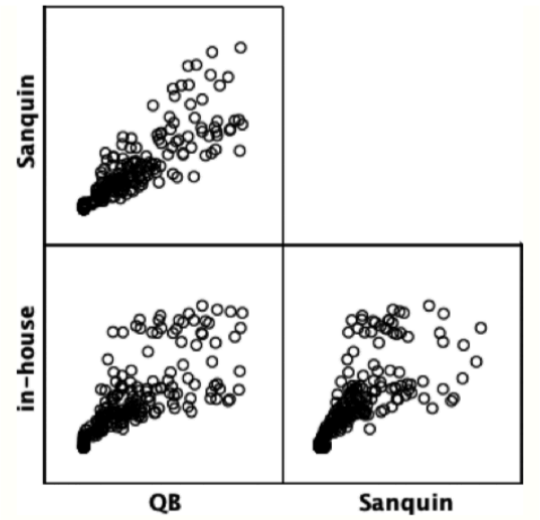

Figure 2 | Dispersion matrix of the IFX quantification in the 299 patients' samples considering the three used methods (μg/mL). The Spearman correlation coefficients are the following: 0.919 for the 'in-house' method vs. the Sanquin kit ($P < 0.001$); 0.913 for the 'in-house' method vs. the point-of-care Quantum Blue kit ($P < 0.001$); and 0.952 for the Sanquin kit vs. the point-of-care QB kit ($P < 0.001$).

excluded and a new correlation analysis was made (Table S1). The pattern of correlations and their significance are similar to those using the entire set of samples.

Quantitative analysis

The Intraclass Correlation Coefficients (ICC) for the paired-comparisons of the IFX-quantification assays used in this study are listed in Table 1. The highest ICC was found when comparing the Sanquin and point-of-care QB assays (0.939). Interestingly, the highest mean difference was also found between these two assays (0.92), and the 95% IC did not include 0, showing that the concentrations measured by the Sanquin kit were consistently higher than those measured by the point-of-care QB. Moreover, these analyses were repeated excluding all samples that had a result higher than 20 μg/mL with the point-of-care QB kit, and the results were similar (Table S2). The only difference was the highest mean difference being found for the 'in house'-QB comparison, and its 95% CI also excluding 0.

The Bland–Altman plots show a greater dispersion in the comparison of the 'in house' with the point-of-care QB assay (Figure 3). It should be noticed that this dispersion is mainly localised in the 10–15 μg/mL range of concentrations, whereas the dispersion in the comparison of the Sanquin with the point-of-care QB assay seems to increase gradually with the increase in the measured concentrations. These analyses were repeated excluding all samples that had a result higher than 20 μg/mL with the point-of-care QB kit and the results were similar (Figure S1).

Table 1 | Intraclass correlation coefficients (ICC) and differences found upon comparing the different IFX-quantification assays

	ICC		Differences	
	ICC	95% CI	Average	95% CI
In house point-of-care QB	0.889	0.861–0.911	0.37	−0.07–0.81
Sanquin point-of-care QB	0.939	0.924–0.952	0.92	0.54–1.30

Qualitative analysis

For the qualitative analyses, the results for each method were stratified according to a commonly accepted IFX therapeutic window – lower than 3 μg/mL, 3–7 μg/mL and higher than 7 μg/mL. The agreement, that is, the percentage of patients that were classified in the same group using the point-of-care QB and one of the reference assays – was then analysed (Table 2). The accuracies between the point-of-care QB and the 'in house' and Sanquin assays were high (77% and 83% respectively). The kappa statistic revealed that the point-of-care QB assay had a substantial agreement with both reference assays, according to the kappa's level of agreement published by Landis and Koch.[30]

DISCUSSION

This study aimed to evaluate the performance of a new point-of-care assay to determine serum IFX concentrations that has been recently launched in the market. To do so, this new test was used to quantify IFX in spiked samples and in the serum of 299 IBD patients under IFX maintenance therapy, and the results were compared with those obtained using two well-established ELISA methods (the Sanquin and an 'in house' assay). The results were very promising, as the new point-of-care QB assay revealed an excellent performance when measuring the spiked samples with known IFX concentrations. In fact, the point-of-care QB assay performed better than

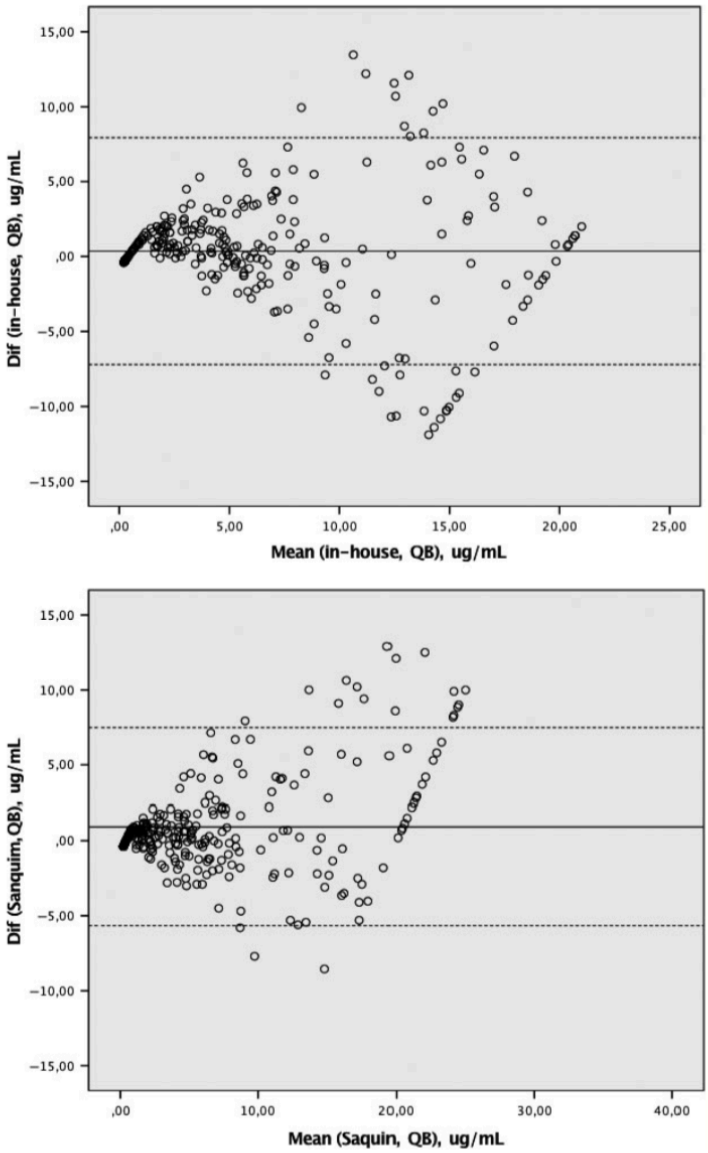

Figure 3 | Bland–Altman plots comparing the point-of-care Quantum Blue with the *in house* and Sanquin assays.

Table 2 | Qualitative comparison between the point-of-care Quantum Blue and the reference assays

	QB < 3 μg/mL *n* (%)	3 μg/mL < QB > 7 μg/mL *n* (%)	QB ≥ 7 μg/mL *n* (%)	Accuracy	Kappa (95% CI)
In house <3 μg/mL	100 (75.8)	1 (1.4)	0 (0.0)	77%	0.648 (0.577–0.719)
3 μg/mL ≤ in house > 7 μg/mL	31 (23.5)	48 (66.7)	14 (14.7)		
In house ≥ 7 μg/mL	1 (0.8)	23 (31.9)	81 (85.3)		
Sanquin < 3 μg/mL	119 (90.2)	4 (5.6)	0 (0.0)	83%	0.738 (0.673–0.803)
3 μg/mL ≤ sanquin > 7 μg/mL	12 (9.1)	48 (66.7)	14 (14.7)		
Sanquin ≥ 7 μg/mL	1 (0.8)	20 (27.8)	81 (85.3)		

the two reference methods both in terms of intravariability and recovery rates. Regarding the quantitative analysis of the results obtained from the patients' serum, both reference methods had a significant correlation with the point-of-care QB, and the ICCs were consistently high (0.889 and 0.939). Interestingly, although the Sanquin assay was the closest to the point-of-care QB, it also had a bias of 0.92, revealing that the former kit measures consistently higher values than the latter. This result finds support at the concentrations obtained from the spiked samples, in which in all but the 40 μg/mL sample higher values were obtained when using Sanquin instead of point-of-care QB. Moreover, this IFX overestimation of the Sanquin assay has been noticed elsewhere, where the average percentage of IFX recovery for this kit was shown to be 139%.[21] The differences found upon comparing the different methods, as well as those between measured and nominal values, are noteworthy. However, one should keep in mind that these differences are inherent to the nature of IFX itself and that of the ELISA method. In fact, IFX is a monoclonal antibody, and therefore, affinity variation is an issue in ELISA assays.[31] Moreover, being a solid-phase capture assay, ELISA techniques may present some problems in the anti-TNF-antibody ligation. Steenholdt *et al.* have recently published a revision in which the technical biases of measuring IFX are well described and documented.[32]

Interestingly, the Bland–Altman plots of the patient's measurements locate the differences between the point-of-care QB and the other two assays in different ranges of concentrations. In fact, and concerning the 'in house' assay, most differences that fall out of the 95% CI are located in the 10–15 μg/mL range of concentrations, whereas concerning the Sanquin assay the differences increase as the IFX concentrations raise.

The qualitative analysis of the results is more important in this context, as the placement of a patient within a certain range of IFX concentrations will be reflected in the clinical decisions made if TDM is applied. In this regard, the samples were stratified according to one possible IFX therapeutic window for the maintenance phase of IFX treatment: lower than 3 μg/mL, within the range of 3–7 μg/mL, and higher than 7 μg/mL. The groups formed by the point-of-care QB and each of the reference assays based on this particular criteria were rather similar: indeed, the kappa statistics indicate a substantial agreement between them. From a clinical point of view, the quantification of IFX coupled with the assessment of the anti-drug antibodies is a powerful tool to guide the physicians' therapeutic decisions: using this particular IFX therapeutic window, a patient with IFX levels lower than 3 μg/mL should escalate the dosage (either increasing the amount of IFX in the infusions or decreasing the interval between them) or switch to a different intra- or interclass drug – depending on the presence and concentration of antibodies to IFX; a patient with IFX levels within the range of 3–7 μg/mL does not need dosage adaptation; and a patient with IFX levels higher than 7 μg/mL can safely de-escalate the dosage.[6, 12–14] According to our results, the clinical decision based on the IFX quantification only (but prone to further adjustments should the concentration of antibodies to IFX be known) would be of a similar nature in 77% of the patients – using the point-of-care QB instead of the 'in house' assay – or in 83% of the patients – using the point-of-care QB instead of the Sanquin assay. Conversely, 24% and 10% of the patients considered to have an IFX concentration below the optimal therapeutic window when evaluated by the point-of-care QB were actually within or above this window when their serum was assayed with the 'in house' or Sanquin methods respectively. Moreover, approximately 33% of the patients considered within the therapeutic window by the point-of-care QB assay were actually above or below it according to the tests used as reference, whereas 15% of the patients placed by the QB point-of-care test above the therapeutic window were considered to be within that window when assayed by the Sanquin and 'in house' methods. Although these percentages may seem noteworthy, it is important to highlight that the decisions made following a TDM approach must be integrated in the patients' clinical context. As so, it is likely that considering the presence of symptomatology and other disease markers would dilute these differences, particularly in the cases where patients are placed in different groups using two different assays, but the measured IFX concentrations are actually borderline the therapeutic window. Moreover, the 3–7 μg/mL therapeutic window used in our study is a commonly used reference, but different thresholds have been established for different methods by different authors. Our results are only indicative of an overall qualitative agreement during IFX maintenance therapy, but the specific percentages would obviously vary should a different therapeutic interval be used. Further studies are needed to integrate the clinical status and disease progression of the patients with the performance of the point-of-care QB assay in a TDM context, as well as to define specific IFX cut-offs that should be applied with this method in the different therapeutic phases.

The clinical advantage of using a point-of-care assay for the monitoring of IFX (and other variables) in the IBD patients is rather evident: the turnaround time of the point-of-care QB assay is 15 min, compared to the approximately 8 hours taken by a common ELISA-based kit. This allows the physician to optimise the IFX treatment immediately when using the point-of-care QB, as opposed to optimise the treatment in the following infusion (usually 6–8 weeks later) when using one of the commercially available ELISA-based kits. The benefits of optimising the IFX therapy in IBD and other inflammatory diseases' treatment are amply acknowledged in the literature. In fact, adjusting the IFX doses (either escalating or de-escalating) and the infusion intervals has been proved to be a clinically powerful tool and a cost-effective strategy.[12–14, 16–18, 33] For those reasons, a serious effort of the medical and research community has been recently applied to the development of novel point-of-care assays concerning the IBD patients monitoring.[34–37] However, most of these tests are focused on disease activity. To the best of our knowledge, there are only a couple of pilot studies describing other lateral flow based assays for the on-site monitoring of serum IFX levels.[38, 39] The Quantum Blue® Infliximab: Quantitative Lateral Flow Assay from Buhlmann assay is the first test already developed and launched in the market for this purpose, and our analysis is the first extensive evaluation of its performance by comparing it with other two already established methods.

Another important advantage of this point-of-care QB assay is its user friendliness: this desktop device may be operated by any nurse, physician or researcher, as opposed to the current commercially available ELISA-based kits and other IFX-quantification methods, which require highly trained personnel and specific laboratory facilities. Moreover, the ELISA-based assays should be run in batches for the sake of cost-effectiveness, which, depending on the dimension of the IBD population being followed in each care centre, may represent a further delay. The point-of-care QB test, on the other hand, is designed to be individually used for each patient sample. As a limitation, whereas ELISA-based methods are usually able to measure antibodies to IFX in addition to IFX itself, the point-of-care QB assay, for the time being, measures only the IFX concentration.

As the main aim of this study was to assess whether the point-of-care QB could effortlessly replace the time-consuming and difficult to apply ELISA-based kits, we focused our efforts in the comparison between the different assays, and we have not taken into consideration the analytical performance characteristics of the point-of-care QB kit. These have, however, been thoroughly analysed by the manufacturers, have been reported elsewhere,[40] and are clearly indicated in the instructions booklet that accompanies the kit. In this respect, it is worth noticing that the point-of-care QB limits of quantification are 0.4–20 μg/mL. The lower and upper limits of detection may hold a disadvantage when compared to the ELISA-based kits or other IFX-quantification methods. Although the point-of-care QB assay was shown to perform rather well in concentrations above its upper limit after adjusting the dilution factor (Figure 1), we were unable to do so in the patients' samples and we have chosen to strictly respect the indications available in the kit's booklet. As so, and for statistical purposes, we have considered all patients' samples that were below the limit of detection to have an IFX concentration of 0.4 μg/mL, and all patients' samples that were above the limit of detection to have an IFX concentration of 20 μg/mL. This strategy had an obvious impact in the quantitative analyses of the patients' samples, although the overall results remained very satisfactory. Moreover, the quantitative analyses were repeated excluding all values that had a result higher than 20 μg/mL with the point-of-care QB assay, and the results were shown to be similar (Tables S1, S2 and Figure S1). Finally, and as the limits of detection are rather far apart the IFX therapeutic window (3–7 μg/mL), they should have no clinical impact in the physician assessment and decision-making process.

This study involved spiked samples and a large number of serum samples, which were obtained from a multicentric and heterogeneous cohort and are therefore a faithful representation of the reality. Notwithstanding, there were a couple of limitations that should be noticed and taken into account: the IFX quantification was always made by the same researcher, which hampers the assessment of the interassay variability; moreover, the presence of antibodies to IFX and other compounds that may differentially interfere with the IFX quantification in each kit was unaccounted for.

On the basis of this study, we may conclude that the point-of-care Quantum Blue is a reliable alternative to the time-consuming ELISAs, allowing the fast and accurate assessment of IFX levels, which in turn contributes towards a proactive and cost-effective therapeutic managing of IBD patients. In the era of the personalised medicine, the fast implementation of tailored therapeutic solutions is important for the patients' quality of life and the healthcare resources optimisation.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Bland–Altman plots excluding samples >20 μg/mL according to the point-of-care QB assay.

Table S1. Spearman correlation coefficients excluding samples >20 μg/mL according to the point-of-care QB assay.

Table S2. Intraclass Correlation Coefficients and differences found upon comparing the different IFX-quantification assays excluding samples >20 μg/mL (according to the point-of-care QB assay).

AUTHORSHIP

Guarantor of the article: Fernando Magro.

Author contributions: J Afonso: study concept and design; acquisition,interpretation, analyses of data and drafting of the manuscript. CC Dias: Statistical analyses. F Magro: Study concept and design; acquisition, interpretation and analyses of data; drafting of the manuscript; study supervision and critical revision of the manuscript for important intellectual content. All the other authors: patients' enrolment and samples collection.

All authors approved the final version of the manuscript.

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Therapeutic drug monitoring of CT-P13: a comparison of four different immunoassays

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Abstract

Background: The commercialization of CT-P13, an infliximab (IFX) biosimilar, has the potential to decrease health-related costs and enhance access to biological therapies. This study aimed to address the accuracy and inter-assay agreement of the CT-P13 quantification using four different assays initially developed to assess IFX.

Methods: The four different methods, one in-house method and three commercially available kits, were used to quantify exogenously-spiked samples and the sera from 185 inflammatory bowel disease (IBD) patients on CT-P13 therapy.

Results: The quantification of the spiked samples unveiled a consistent and accurate behaviour of three of the tested methods, with average percentage recoveries of 90%, 102% and 109%. Results from the clinical samples demonstrated that these three assays were also highly correlated, both concerning Spearman's rank coefficients (range 0.890–0.947) and intraclass correlation coefficients (range 0.907–0.935). There were a few systematic deviations among them, but their impact in the clinical stratification of the patients using different cut-offs was minimal, particularly when these cut-offs were in the 3–4 μg/ml range, for which the strength of agreement (as assessed by the Kappa statistics that ranged from 0.732 to 0.902) was substantial to almost perfect.

Conclusions: Our results indicate that three of the tested IFX quantification methods can be used to accurately quantify CT-P13 without any adjustments.

Keywords: CT-P13, therapeutic drug monitoring, drug quantification

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Introduction

The knowledge of the pivotal role played by the cytokine tumour necrosis factor (TNF)α in chronic immune-mediated inflammatory diseases brought along new therapeutic perspectives and expectations. The development of monoclonal antibodies (mAbs) targeting TNFα (anti-TNFα) has dramatically changed the natural evolution of these diseases. Particularly in the case of inflammatory bowel diseases (IBDs), of which the two main types are Crohn's disease (CD) and ulcerative colitis (UC), the introduction of anti-TNFα as a therapeutic strategy has led to steroid sparing, mucosal healing, decreased rates of surgery and hospitalization, and an overall improvement in health-related quality of life, both in the short and long term.[1–3] However, the high costs of these biological agents carry a heavy economic burden to health care institutions and patients, and end up restraining their utilization. With the advent of patent expiration dates, the development and market release of biosimilars (i.e. products that are similar, though not identical, to an originator biological drug) have the potential to considerably reduce healthcare costs, while improving and generalizing early access to these innovative therapeutic strategies.

CT-P13, commercialized under the brand names Remsima® (Celltrion, South Korea) and Inflectra® (Hospira, USA) is a biosimilar of infliximab (IFX; Remicade®, Merck Sharp &

Dohme, Ireland). CT-P13 was the world's first biosimilar mAb to be approved by the regulatory agencies, in 2013 by the European Medicines Agency and in 2016 by the United States Food and Drug Administration, and can now be used for all adult and paediatric indications of the originator drug, including rheumatoid arthritis (RA), ankylosing spondylitis (AS), psoriatic arthritis (PsA), psoriasis, CD and UC.[4,5] Being a biosimilar, CT-P13 has the same formulation and a similar physicochemical profile as its originator IFX.[6,7] Moreover, CT-P13's pharmacodynamic properties resemble those of IFX, having an equivalent effectiveness and being generally well tolerated.[6,7] The biological and therapeutic equivalence of CT-P13 was originally demonstrated in two keystone studies using AS and RA patient cohorts.[8,9] Although the extrapolation of these results were sufficient to grant CT-P13 indication to treat IBD, multiple postmarketing and observational trials have confirmed the validity of this extrapolation and consistently reported the absence of significant differences between IFX and CT-P13 in terms of efficacy, safety and immunogenicity when used to treat CD and UC patients.[10–12]

Despite IFX's effectiveness in many IBD patients, a considerable number of them fail to respond to this drug either in the induction phase (primary nonresponse) or in the maintenance phase (secondary loss of response). Studies performed during the last decade have consistently associated this lack of effectiveness to a low concentration of IFX, usually assessed immediately before the following infusion (trough levels of IFX).[2,13–22] A great effort has been made in the attempt to establish therapeutic cut-offs for IFX, (i.e. fixed concentrations below which one can predict lack of response with an appreciable certainty), and therefore adjust the IFX dosing accordingly.[2,13,17–23] In this context, several algorithms and dashboards are being developed to facilitate, automatize and standardise the physician's decision-making process, therefore optimizing IFX therapy.[24–28] This tailored therapeutic management relies heavily on an accurate assessment of IFX levels in a patient's serum, known as therapeutic drug monitoring (TDM) and several methods, usually based on enzyme-linked immunosorbent assays (ELISAs), have been developed and are currently available in the market, allowing an easy, efficient and relatively fast quantification of IFX. Overall, TDM-based algorithms have been proved to be a cost-efficient approach, maximizing benefit while reducing toxicity risks.

One can rationally expect that the relationship seen between the serum levels and the clinical, histological and endoscopic outcomes for IFX is maintained with its biosimilar CT-P13.[29] However, and to smooth and assure a safety transition, one must guarantee that the assays used to measure IFX in the patient's serum are equally efficient and accurate to measure CT-P13. This study aims to address the efficacy, accuracy and inter-assay agreement of CT-P13 quantification using four different assays that were initially developed to assess the originator IFX from patient's serum.

Materials and methods

Spiked samples of known CT-P13 concentrations (0.5, 1, 1.5, 3, 5, 7, 10, 15, 20, 30 and 40 µg/ml) were generated by diluting the appropriate amount of exogenous CT-P13 (Remsima®, Celltrion, South Korea) into a pool of serum from control donors. Clinical samples were prospectively and consecutively obtained from 185 IBD patients that were on CT-P13 therapy (Remsima®; Celltrion, South Korea), followed in four different university and community hospitals. All IBD patients over18 years old and on Remsima® were invited to participate in the study.

This study was approved by the ethic committee of all hospitals involved and by the Portuguese Data Protection Authority. All patients and control donors enrolled signed an informed written consent.

IFX quantification assays

A total of four different assays were used to quantify CT-P13 from the patient's serum: one in-house assay and three commercially available kits. The in-house method is an ELISA commonly used in our laboratory and was carried out as previously described by Ben-Horin and colleagues[20,30–34] Briefly, serum samples were diluted (1:100) and added to a plate pre-coated with TNFα (Peprotech, Rocky Hill, NJ, USA). After 60 min of incubation and an appropriate number of washes, a horseradish peroxidase (HRP)-labelled goat anti-human Fc fragment antibody (MP Biomedicals, Solon, OH, USA)

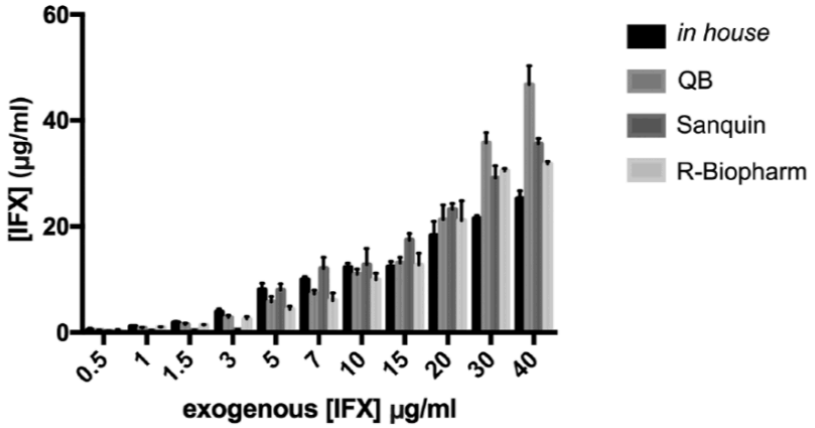

Figure 1. CT-P13 quantification of exogenously-spiked samples (µg/ml). The bars indicate the mean concentration obtained with each assay in the different concentrations, and the error bars refer to the standard deviation.
IFX, infliximab; QB, Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Bühlmann, Schönenbuch, Switzerland).

was added and the plate was incubated for 60 min. Afterwards, tetramethylbenzidine (Millipore, MA, USA) substrate was added, and the reaction was stopped 3 min later with 2M H_2SO_4. Finally, the sample's absorbance was read at 450/540 nm, and the CT-P13 was quantified by interpolating the absorbance values in a standard curve built with known concentrations of exogenous CT-P13 (Remsima®; Celltrion, South Korea). The three commercially available kits used were the following: Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Bühlmann, Schönenbuch, Switzerland), hereafter referred to as QB; Level Infliximab M2920 kit (Sanquin, Amsterdam, Netherlands), hereafter referred to as Sanquin; and RIDASCREEN® IFX Monitoring (R-Biopharm AG, Darmstadt, Germany), hereafter referred to as R-Biopharm. All these kits were used strictly following manufacturer's instructions. The lower and upper limits of quantification are 0.4 µg/ml and 20 µg/ml for the QB assay, and 0.08 µg/ml (1:200) and 37.5 µg /ml (1:1500) for the Sanquin assay, respectively: whenever the results obtained in the clinical samples were below or above these limits of quantification, they were considered to be at those limits. The R-Biopharm kit manufacturer provides no information on the quantification limits. For the in-house procedure, the upper limit of quantification was calculated as the highest concentration of the standard curve × the sample dilution factor used.

Statistical analysis

Categorical variables were described through absolute (n) and relative (%) frequencies and continuous variables were described as mean and standard deviation, median, percentiles, and minimum/maximum values when appropriate. All the reported p-values were two-sided, and p-values <0.05 were considered to be statistically significant. The accuracy and Kappa statistics were computed based on a pre-established therapeutic window of IFX. All data were arranged, processed and analysed with SPSS® v.20.0 data (Statistical Package for Social Sciences, IBM Corp., Armonk, NY). Graphs were computed with Prism 7® (GraphPad Software, Inc., CA).

Results

Quantitative analysis

The four tested methods were used to quantify CT-P13 from 11 spiked samples varying from 0.5 to 40 µg/ml (Figure 1). As shown in the error bars on Figure 1, the intra-assay variation was roughly the same for all methods: the standard deviations varied from 0.07 to 2.66 for the in-house method, 0.05–3.56 for the QB assay, 0.02–3.03 for the Sanquin assay, and 0.11–3.67 for the R-Biopharm assay. On the other hand, the average recovery was 109% (range 63–162%) for the in-house method, 102% (range 80–119%) for the QB, 91% (range 17–172%) for the Sanquin and 90% (range

Table 1. Cohort characterization.

	n	%
Sex, *n* (%)		
Male	71	38%
Female	114	62%
Disease, *n* (%)		
CD	148	80%
UC	37	20%
Age at diagnosis, median (IQR)	41	28–50
Years of follow-up, median (IQR)	14	11–16
5-ASA, *n* (%)	20	15%
AZA, *n* (%)	69	51%
Corticosteroids, *n* (%)	11	8%
Methotrexate, *n* (%)	2	1%
Corticodependency, *n* (%)	38	64%
CT-P13 therapeutic phase, *n* (%)		
induction	69	37.5%
maintenance	115	62.5%

5-ASA, 5-aminosalicylic acid; CD, Crohn's disease; IQR, interquartile range; UC, ulcerative colitis.

79–106%) for the R-Biopharm. Overall, the QB kit had the best average recovery, whereas the R-Biopharm had the least dispersion of percentage recoveries across all tested concentrations.

The four assays were also used to quantify CT-P13 levels in the serum of 185 IBD patients, with a median age of 41 and of which 62% were women. Most of these patients had CD (80%) while 20% of them had UC, and their median [interquartile range (IQR)] follow-up time was 14 years (11–16). Concerning concomitant medications, 15% of these patients were on 5-aminosalicylic acid (5-ASA), 51% were on azathioprine (AZA), 8% were on corticosteroids, and 1% was on methotrexate. As for the CT-P13 therapeutic phase, 37.5% of all patients were in the induction, whereas 62.5% were in the maintenance phase (Table 1).

The median (IQR) CT-P13 concentration obtained from the clinical samples was the highest when using the R-Biopharm kit [6.00 µg/ml (0.40–12.50)], and the lowest when using the Sanquin kit [2.60 µg/ml (0.90–12.10)]. The in-house method and QB assays measured medians were, respectively, 3.40 µg/ml (0.30–10.80) and 4.20 µg/ml (0.40–11.50). These results were significantly correlated among themselves, as shown in Table 2 and Supplementary Figure 1, with the maximum Spearman's rank correlation coefficient being obtained for the pair QB and R-Biopharm (0.947). The intraclass correlation coefficient (ICC) was also computed for each pair of assays, once again showing a good correlation between the different methods (Table 3). The highest ICC was found for the pair QB and R-Biopharm, the only pair for which no consistent systematic difference could be encountered. As for the rest of the comparisons, the in-house method results were consistently lower than those obtained with the Sanquin, QB and R-Biopharm kits (by an average of 2.05, 1.27 and 0.83 µg/ml, respectively), whereas the Sanquin results were consistently higher than those obtained with the QB and R-Biopharm assays (by an average of 0.78 and 1.22 µg/ml, respectively). Blant–Altman plots (Supplementary Figure 2) reveal that the dispersion between the values measured by each pairs of methods tends to increase as the CT-P13 concentration raises. This dispersion is particularly narrow for the pairs in-house-QB, in-house-R-Biopharm and QB-R-Biopharm, but broadens up whenever Sanquin is included in the comparison.

To test whether the ICC between the different methods was dependent on the therapeutic phase (and, therefore, on the range of IFX values being assessed), samples were stratified according to the patient's therapeutic phase (induction *versus* maintenance) and ICCs were independently calculated for each of these groups (Supplementary Table 1). The results show that the induction ICCs were quite similar to the maintenance ICCs for each pair of methods, most of the times, the 95% confidence intervals (CIs) were at least partially overlapping. On the other hand, the average differences seem to be larger in the induction phase.

Qualitative analysis

To analyse these results from a clinical perspective, CT-P13 levels were stratified according to a predefined and commonly accepted IFX therapeutic window: lower than 3 µg/ml (subtherapeutic levels), between 3 and 7 µg/ml (therapeutic levels) and >7 µg/ml (supra-therapeutic levels). The patients' distribution by these categories

Table 2. Spearman's rank correlations.

		In-house	Sanquin	QB
Sanquin	Correlation coefficient	0.785		
	Sig. (two-tailed)	<0.0010		
	N	185		
QB	Correlation coefficient	0.901	0.875	
	Sig. (two-tailed)	<0.0010	<0.0010	
	N	185	185	
R-Biopharm	Correlation coefficient	0.890	0.824	0.947
	Sig. (two-tailed)	<0.0010	<0.0010	<0.0010
	N	185	185	185

QB, Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Bühlmann, Schönenbuch, Switzerland); sig, significance.

Table 3. Intraclass correlation coefficient (ICC).

	ICC		Difference	
	ICC	**CI 95%**	**Average**	**CI 95%**
In-house-Sanquin	0.760	0.6800–0.821	−2.05	−3.15 to −0.96
In-house-QB	0.907	0.876–0.930	−1.27	−2.85 to −0.70
In-house-R-Biopharm	0.912	0.883–0.935	−0.83	−1.32 to −0.33
Sanquin-QB	0.904	0.872–0.928	0.78	0.00–1.56
Sanquin-R-Biopharm	0.773	0.696–0.830	1.22	0.16–2.28
QB-R-Biopharm	0.935	0.913–0.951	0.44	−0.3–0.92

CI, confidence interval; ICC, intraclass correlation coefficient; QB, Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Bühlmann, Schönenbuch, Switzerland).

after quantification by each assay is depicted in Table 4. The accuracy was higher for the pair QB and R-Biopharm (88%), with an almost perfect strength of agreement according to the Kappa interpretation of Landis and Koch[35] (Kappa = 0.874). All the other pairs had a substantial agreement, with the exception of the Sanquin-in-house comparison, that displayed only a moderate agreement (Kappa = 0.597).

This qualitative analysis using the 3–7 µg/ml therapeutic interval was further stratified into samples taken from patients in the induction or in the maintenance phase (Supplementary Table 2). The results show that the concordance between each pair of methods is similar using samples from different phases, as the Kappa 95% CI were, most of the times, at least partially overlapping.

The agreement analysis was further extended to admit other cut-offs and to identify the cut-off with the highest agreement (Table 5). The highest Kappa values (0.901 and 0.902) were obtained for the QB-R-Biopharm comparison at cut-offs of 3 and 4, respectively. Interestingly, whereas agreement tends to decrease with the increase in the cut-off value, such tendency appears to be absent or inverted when the comparisons involve Sanquin.

Discussion

The expiration of biological patents and commercialization of biosimilar drugs holds the potential of lowering health-related costs and generalizing access to these therapies. However, and particularly in the case of IFX, a long path has been made towards a target-concentration adjusted

Table 4. Qualitative comparison between the CT-P13 quantification assays using the therapeutic interval 3–7 µg/ml.

		In-house				
		<3 (*n*)	**3–7 (*n*)**	**⩾7 (*n*)**	**Accuracy**	**Kappa**
Sanquin	**<3 (*n*)**	81	14	4	75%	0.597 (0.496–0.698)
	3–7 (*n*)	2	11	12		
	⩾7 (*n*)	1	13	47		
QB	**<3 (*n*)**	78	4	0	80%	0.776 (0.177–0.840)
	3–7 (*n*)	5	14	9		
	⩾7 (*n*)	1	20	54		
R-Biopharm	**<3 (*n*)**	72	3	0	77%	0.752 (0.685–0.819)
	3–7 (*n*)	10	10	3		
	⩾7 (*n*)	2	25	60		
		Sanquin				
		<3 (*n*)	**3–7 (*n*)**	**⩾7 (*n*)**	**Accuracy**	**Kappa**
QB	**<3 (*n*)**	79	3	0	80%	0.671 (0.577–0.766)
	3–7 (*n*)	18	9	1		
	⩾7 (*n*)	2	13	60		
R-Biopharm	**<3 (*n*)**	75	0	0	77%	0.622 (0.522–0.721)
	3–7 (*n*)	15	7	1		
	⩾7 (*n*)	9	18	61		
		QB				
		<3 (*n*)	**3–7 (*n*)**	**⩾7 (*n*)**	**Accuracy**	**Kappa**
R-Biopharm	**<3 (*n*)**	74	1	0	88%	0.874 (0.824–0.922)
	3–7 (*n*)	8	14	1		
	⩾7 (*n*)	0	13	74		

QB, Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Bühlmann, Schönenbuch, Switzerland).

dosing, which has been proved to be both clinically- and cost-effective. With the arrival of CT-P13, the usefulness and applicability of the relationships previously explored between drug levels and clinical outcomes is limited by the lack of knowledge on whether the assays optimized to quantify the originator IFX are equally efficient and accurate for the quantification of CT-P13. In fact, and despite having the same amino-acid sequence, originator IFX and CT-P13 may have post-translational differences as a consequence of their complex manufacturing processes. In this study, we have explored and compared the performance of four IFX quantification assays, an in-house method and three commercially available kits, Sanquin, QB and R-Biopharm, in the quantification of CT-P13. The three assays: in-house, Sanquin and R-Biopharm, are traditional ELISA-based methods, and therefore have a turnaround time of approximately 8 h. The QB assay has the added advantage of being a rapid assay with a turnaround time of 15 min, allowing an immediate adjustment of the drug dosage, as opposed to delaying this adjustment to the following infusion, which commonly happens with all ELISA-based assays.

The results show that, upon measuring CT-P13 levels from exogenously-spiked samples, all methods have an acceptable performance as assessed

Table 5. Kappa (SE) between the different methods using different cut-offs.

Cut-off	3 µg/ml	4 µg/ml	5 µg/ml	6 µg/ml	7 µg/ml	8 µg/ml	9 µg/ml	10 µg/ml
In-house								
Sanquin	0.774 (0.046)	0.748 (0.049)	0.6961 (0.054)	0.660 (0.058)	0.636 (0.060)	0.6161 (0.063)	0.582 (0.066)	0.615 (0.065)
QB	0.891 (0.034)	0.806 (0.043)	0.738 (0.050)	0.696 (0.053)	0.655 (0.057)	0.630 (0.060)	0.624 (0.062)	0.601 (0.064)
R-Biopharm	0.835 (0.041)	0.732 (0.049)	0.753 (0.047)	0.687 (0.051)	0.699 (0.053)	0.636 (0.057)	0.629 (0.058)	0.660 (0.058)
Sanquin								
QB	0.753 (0.047)	0.711 (0.050)	0.711 (0.059)	0.774 (0.047)	0.815 (0.044)	0.8198 (0.044)	0.823 (0.045)	0.817 (0.047)
R-Biopharm	0.744 (0.047)	0.684 (0.050)	0.684 (0.050)	0.676 (0.052)	0.691 (0.052)	0.702 (0.052)	0.721 (0.051)	0.742 (0.052)
QB								
R-Biopharm	0.901 (0.032)	0.902 (0.032)	0.860 (0.037)	0.849 (0.038)	0.847 (0.039)	0.832 (0.041)	0.771 (0.048)	0.747 (0.051)

QB, Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Bühlmann, Schönenbuch, Switzerland); SE, standard error.

by their average recovery percentage. However, and looking more closely at the individual results, one can see that the Sanquin assay has a wide variation of recovery, measuring consistently low values for concentrations below 5 µg/ml. In fact, the percentage recovery for this kit concerning samples with 0.5, 1, 1.5 and 3 µg/ml is 44%, 35%, 28% and 17%, respectively. Notwithstanding, and for concentrations between 5–20 µg/ml, the Sanquin kit tends to overestimate CT-P13 levels, reaching recovery percentages as high as 160 and 172% for 5 and 7 µg/ml, respectively. Its average recovery for this drug shows a general underestimation (91%), whereas when used with IFX Sanquin has been shown to overestimate the true amount of product in the samples.[30,36]

The unstable behaviour of Sanquin is once again noticeable upon measuring clinical samples: whereas it measures the lowest median concentration (2.60 µg/ml), it also measures consistently higher values than the other methods, by an average difference of 2.05, 0.78 and 1.22 units to the results obtained with the in-house, QB and R-Biopharm assays, respectively. Not surprisingly, it also has the lowest correlation coefficients when compared with the other methods.

The results from the quantification of clinical samples obtained from all the other tested methods (in-house, QB and R-Biopharm assays) show a strong correlation punctuated by minor systematic differences. This pattern of inter-assay relationships has been commonly observed in other methodological comparisons between two or more IFX quantification assays, ELISA or not ELISA-based: whereas a comparable and stable accuracy usually results in moderate to high correlation values, systematic differences occur, likely due to the utilization of different antibodies with varying IFX affinities.[36–42] The highest correlation in this study was observed for the QB-R-Biopharm pair. Moreover, the correlations were fairly similar between samples taken from patients in the induction and in the maintenance phase of the CT-P13 therapy. The larger average differences generally seen in the induction phase are likely the reflection of the higher CT-P13 levels present in these samples: in accordance to the Bland–Altman plots, the dispersion between kits is larger in higher CT-P13 levels.

Perhaps more importantly than the quantitative comparison of the results obtained, one must look

at the consequences in terms of clinical decisions. The utilization of algorithms and dashboards to interpret the results obtained from TDM and optimize anti-TNFα therapy is becoming popular.[24–28] Therefore, one must verify how much does the systematic deviations observed in these assays impact the subsequent clinical decisions. To do so, we have stratified the results according to a popular and commonly accepted therapeutic window, which includes levels from 3 to 7 μg/ml: concentrations below this interval are considered to be infra-therapeutic, whereas concentrations above this interval are considered to be supra-therapeutic. By comparing the stratification obtained using the results from the different assays, one can see that the agreement is usually substantial, being lower for the Sanquin-in-house comparison and almost perfect for the R-Biopharm-QB pair. Moreover, the agreement seemed to be fairly similar when samples were stratified according to each patient's therapeutic phase.

As the 3–7 μg/ml therapeutic window is a generalized one, and cut-offs must always be outcome, assay and disease-specific, we have also measured the agreement between the assays using different cut-offs (from 3–10 μg/ml). The results are substantial to almost perfect in most of the cases, being once again higher for the R-Biopharm-QB comparison. However, when Sanquin is involved, results tend to be weaker. Besides, whereas the Kappa usually lowers as the cut-off increases, likely the result of a higher dispersion of measurements in higher concentrations, the opposite tends to happen when Sanquin is compared with QB and R-Biopharm. This likely reflects the poor recovery and overall poor behaviour of the Sanquin kit when measuring low concentrations.

The rationale for the need to validate these assays with CT-P13 and the reason for the differences encountered, particularly conspicuous in the case of Sanquin, relies on the complex nature of biological drugs and consequently of their biosimilar molecules. Although sharing the same amino-acid sequence, the originator IFX and CT-P13 may have a number of differences in post-translational features (e.g. glycosylation, sulfurylation, phosphorylation and side-chain additions or subtractions) due to disparities in the cell line used, growth conditions and purification processes, as well as in storage and transport.[5,7,11] The pharmacokinetics, pharmacodynamics, immunogenicity and safety studies performed so far show no influence of those differences in these features. However, they may influence capture antibody affinity, particularly through epitope disposition and exposure, impacting the results of the quantification assays. Our results are in line with those previously obtained by Schulze and colleagues, Malíckova and colleagues and Gils and colleagues, showing that antibodies and assays initially developed to quantify IFX can be accurately used to measure CT-P13,[43–45] although previous validation is absolutely required to identify less favourable cases.

This study has a few strengths that ought to be underlined: the spiked concentrations include a wide range of values, allowing one to observe the assay behaviour both at low and high CT-P13 concentrations; and a high number of patient's sera was assessed, assuring a representative real-world sampling. However, there are a couple of limitations that should also be acknowledged: the presence of antibodies to CT-P13 (or other inhibitory molecules) in the clinical samples was unknown; and the coefficient of variation within the same assay used in different moments or by different researchers was unaccounted for.

This study addresses the validity of using four different IFX-optimized assays to quantify the IFX biosimilar CT-P13. Overall, the results are very promising and show that three out of four methods can be accurately used to measure CT-P13: an in-house method previously described by Ben-Horin and colleagues[32] and the R-Biopharm and QB kits that are commercially available. The QB kit has the added advantage of being a bedside point-of-care solution, releasing results within 15 min of sampling, and therefore allowing an immediate adjustment of CT-P13 dosing.

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content. JA: drafting of the manuscript; CT-P13 assays; analysis and interpretation of data. CCD: statistical analysis. All the other authors: recruitment of patients and collection of samples. All the authors read and approved the final version of the manuscript.

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Conflict of interest statement

FM served as speaker and received honoraria from Merck Sharp & Dohme, Abbvie, Vifor, Falk, Laboratorios Vitoria, Ferring, Hospira and Biogen.

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The performance of Remicade®-optimized quantification assays in the assessment of Flixabi® levels

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Abstract

Background: The advent of Remicade® biosimilars, Remsima®, Inflectra® and, more recently, Flixabi®, has brought along the potential to decrease the costs associated with this therapy, therefore increasing its access to a larger group of patients. However, and in order to assure a soft transition, one must make sure the assays and algorithms previously developed and optimized for Remicade perform equally well with its biosimilars. This study aimed to: (a) validate the utilization of Remicade-optimized therapeutic drug monitoring assays for the quantification of Flixabi; and (b) determine the existence of Remicade, Remsima and Flixabi cross-immunogenicity.

Methods: Healthy donors' sera spiked with Remicade, Remsima and Flixabi were quantified using three different Remicade-quantification assays, and the reactivity of anti-Remicade and anti-Remsima sera to Remicade and to its biosimilars was assessed.

Results: The results show that all tested Remicade-infliximab-optimized assays measure Flixabi as accurately as they measure Remicade and Remsima: the intraclass correlation coefficients between theoretical and measured concentrations varied from 0.920 to 0.990. Moreover, the interassay agreement values for the same compounds were high (intraclass correlation coefficients varied from 0.936 to 0.995). Finally, the anti-Remicade and anti-Remsima sera reacted to the different drugs in a similar fashion.

Conclusions: The tested assays can be used to monitor Flixabi levels. Moreover, Remicade, Remsima and Flixabi were shown to have a high cross-immunogenicity, which supports their high similarity but prevents their switching in nonresponders with antidrug antibodies.

Keywords: biosimilars, Flixabi®, Remicade®, therapeutic drug monitoring

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Introduction

Inflammatory bowel diseases (IBD) are a group of immunity-driven conditions characterized by the presence of flares intertwined with remission periods. These conditions include Crohn's disease (CD) and ulcerative colitis (UC), and are thought to arise from a complex interplay involving environmental and immunological factors on a susceptible genetic background. Tumour necrosis factor α (TNFα) is a key cytokine that plays a major role in IBD pathophysiology.[1] The development of anti-TNFα monoclonal antibodies has therefore revolutionized the therapeutic approach and natural progression of IBD: the utilization of these biological therapies led to decreased rates of steroid utilization, surgery and hospitalization, increased rates of clinical remission and mucosal healing, and an overall improvement in the health-related quality of life of IBD patients.[2–4] Four different anti-TNFα agents are currently being used for the treatment of IBD, of which infliximab (name brand Remicade®, Remicade is manufactured by Merck Sharp and Dohme, Ireland) was the first to be approved (Remicade will be used throughout

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this article when referring to the original infliximab drug). Remicade is a chimeric monoclonal immunoglobulin G1 (IgG1) anti-TNFα antibody composed of a murine variable region (25%) and a constant human region (75%). Its multiple mechanisms of action include the reduction of lymphocyte and leucocyte migration to sites of inflammation, the downregulation of pro-inflammatory cytokines, and the induction of TNFα-producing cell apoptosis, among others.[4]

Notwithstanding their pivotal role in the treatment of IBD and other autoimmune diseases, biologic therapies are substantially expensive. In fact, they are currently the main drivers of cost in IBD units.[5] For that reason, biosimilars are an attractive alternative: these molecules are highly similar (though not identical) to their reference products in structural, functional, biological and clinical terms. With an expedited regulatory process, biosimilars have the potential to reduce the cost of biological therapies by 25–40%, hence increasing their availability.[5] Despite some controversy linked to the regulatory process, mostly concerning the extrapolation of clinical indications,[6] two Remicade biosimilars have been approved both in Europe and in the USA.

Remsima® (Celltrion, Incheon, South Korea) and Inflectra® (Hospira, Illinois, USA) are the brand names of CT-P13, the first Remicade biosimilar approved by the European Medicine Agency (EMA) in September 2013 and by the US Food and Drug Administration (FDA) in April 2016. Flixabi® (Samsung Bioepsis, South Korea) is the brand name of SB2, which was the second Remicade biosimilar that received marketing authorization from the EMA (in May 2016) and from the FDA (in April 2017). Given the biosimilar expedited regulatory process, Remsima, Inflectra and Flixabi were approved for all the therapeutic indications of their originator drug, including CD and UC. Remsima is the only Remicade biosimilar for which real-world observational data concerning IBD therapy are already available: so far, these studies are promising, as they show no significant differences between Remsima and Remicade in what concerns efficacy, safety and immunogenicity.[7,8]

There have been several attempts to optimize Remicade therapy in IBD patients. It is now commonly accepted that the rates of response and remission increase when a drug concentration-guided individualized therapy is followed.[3,9,10] Given their overall similarity to Remicade®, one can rationally expect that this pharmacokinetic/pharmacodynamic relationship also occurs with the biosimilars Remsima and Flixabi.[11] The process of adjusting the drug dosage and the infusions' interval in order to achieve a particular therapeutic window, within which the drug has its maximum efficacy with the minimum associated toxicity, is dependent on an accurate and systematic assessment of drug levels, named therapeutic drug monitoring (TDM). Multiple systems, mostly enzyme-linked immunosorbent assay (ELISA)-based, have been developed and are now available to monitor patients' Remicade levels throughout time. However, to safely employ TDM to tailor treatment in Flixabi- and Remsima-treated patients, one must determine whereas the systems developed and optimized to quantify Remicade are equally accurate in the quantification of its biosimilars.

Our group has previously demonstrated that a number of Remicade quantification methods can be safely applied to quantify Remsima.[12] This study was meant to extend those analyses in order to include the recently-approved Flixabi. Shortly, our aim was to assess the efficacy, accuracy and interassay agreement of three Remicade quantification assays in the monitoring of Flixabi levels. Additionally, we have also tested the cross-reactivity of antidrug antibodies (ADAs) anti-Remicade and anti-Remsima with Remicade, Remsima and Flixabi.

Material and methods

Spiked samples and quantification assays

Spiked samples of known Remicade, Remsima and Flixabi concentrations were generated by diluting the appropriate amount of each drug (Remicade, Remsima, Flixabi) into a pool of sera extracted from control donors. Each spiked concentration was repeated between six and nine times and analysed in duplicate. Samples were then quantified using one in-house assay and two commercially available kits: the Quantum Blue® infliximab: quantitative lateral flow assay (Buhlmann, Schönenbuch, Switzerland), hereafter referred to as Buhlmann; and the RIDASCREEN® IFX monitoring (R-Biopharm AG, Darmstadt, Germany), hereafter referred to as R-Biopharm.

The in-house method was an ELISA assay commonly used in our laboratory and was carried out as previously described by Ben-Horin and colleagues.[13–18] Briefly, serum samples were diluted and added to a plate precoated with TNFα (Peprotech, Rocky Hill, NJ, USA). After 60 min of incubation and an appropriate number of washes, a horseradish peroxidase (HRP)-labelled goat antihuman fragment-crystallizable fragment antibody (MP Biomedicals, Solon, OH, USA) was added and the plate was incubated for 60 min. Afterwards, tetramethylbenzidine (Millipore, MA, USA) substrate was added, and the reaction was stopped 3 min later with 2 mol/l H_2SO_4. Finally, the samples' absorbance was read at 450/540 nm, and the Remicade was quantified by interpolating the absorbance values in a standard curve built with known concentrations of exogenous Remicade. The upper limit of quantification was calculated as the highest concentration of the standard curve multiplied by the sample dilution factor used.

Concerning the Buhlmann assay, a chip card containing the test information and calibration curve for each specific cartridge lot was supplied with each test kit. Briefly, serum samples were diluted 1:20 and an 80 µl aliquot was loaded into the port of the test cartridge. After a 15 min reaction, the cartridge was read and the results were shown on the point-of-care Buhlmann reader display. The lower and upper limits of quantification were 0.4 and 20 µg/ml, respectively.

Concerning the R-Biopharm method, the samples were diluted and added to the assay plate. After 60 min of incubation at 37°C and several washes, a conjugate was added to the plate and incubated for 30 min at the same temperature. Afterwards, the substrate was added and the reaction was interrupted 10 min later by adding the stop reagent. The sample absorbance was read at 450/620 nm. The manufacturer provided no information on the limits of quantification.

Whenever the results obtained were below or above the limits of quantification indicated for the in-house and Buhlmann methods, they were rounded to match those limits.

Antidrug antibodies' cross-reactivity

Serum samples from IBD patients being treated with Remicade or Remsima were extracted immediately before an infusion. The presence of ADAs was determined routinely in these patients, and 74 serum samples were included in the study. Only samples positive for anti-Remicade or anti-Remsima antibodies were used. The presence of cross-reactivity between Remicade and its biosimilars was determined using an in-house procedure previously described by Ben-Horin and colleagues.[13–18] Briefly, Remicade, Remsima or Flixabi were added to a plate precoated with TNFα. Afterwards, a diluted sample of serum (anti-Remicade or anti-Remsima) was added to the plate and incubated for 60 min at room temperature. Goat antihuman lambda chain HRP-labelled antibody (Serotec, Oxford, UK) was then added, followed by another room temperature 60 min-incubation. Finally, TMB (3,3',5,5'-tetramethybenzidine, Merckmillipore, USA) was added and allowed to react for 6 min, after which the reaction was stopped with H_2SO_4. Absorbances were read at 450/540 nm, and the results were obtained upon interpolation in a standard curve of goat antihuman F(ab')2 fragment antibody (MP Biomedicals) and expressed as µg/ml-equivalent (for the purpose of brevity, the results are hereafter expressed as µg/ml). The lower limit of quantification was 1.2 µg/ml.

This study was approved by the ethics committees of all hospitals involved and by the Portuguese Data Protection Authority. All patients and control donors enrolled have signed an informed written consent giving permission for blood sample collection for medical research.

Statistical analysis

Continuous variables were described using median, interquartile range, minimum and maximum values. The association between theoretical/measured concentrations, methods and the antidrug reactivity of Remicade and its biosimilars was assessed by calculating the intraclass correlation coefficient (ICC) and the corresponding 95% confidence intervals. Moreover, Bland and Altman plots were used to compare the different techniques. The level of statistical significance was set at 0.05. All statistical analyses were performed using the Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY, USA), whereas graphs were designed using Prism 7®.

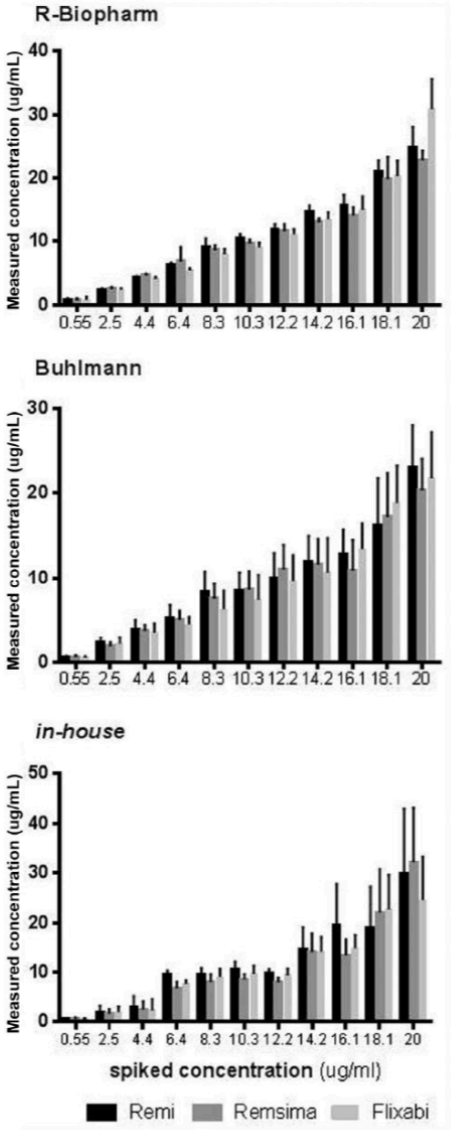

Figure 1. Remicade, Remsima and Flixabi-spiked samples measured by R-Biopharm, Buhlmann and the in-house assays.
Buhlmann, Quantum Blue® infliximab: quantitative lateral flow assay; R-Biopharm, RIDASCREEN® IFX monitoring.

Results

Drug quantification assays

The spiked samples of Remicade, Remsima and Flixabi were quantified using the three assays referred to in the material and methods section (Figure 1). The results show that these assays measure similar amounts of each compound at any given concentration, with the standard deviations (SDs) being larger for the Buhlmann method. Accordingly, the mean intra-assay coefficient of variation was 6.4%, 3.4% and 11.7% for the in-house, R-Biopharm and Buhlmann assays, respectively. The average recovery rates of each drug were higher with the R-Biopharm assay (105%, 102%, and 105% for Remicade, Remsima and Flixabi, respectively) when compared with the Buhlmann (91%, 87%, and 86%, respectively) and the in-house methods (105%, 97%, and 99%, respectively).

Table 1 shows the intraclass ICCs and the average differences between the theoretical and the measured concentrations obtained using the different methods. The most accurate assay to quantify Remicade and Remsima is the R-Biopharm (with ICCs of 0.986 and 0.990, respectively), whereas the most accurate method to quantify Flixabi is the Buhlmann (with an ICC of 0.983). Still, all ICCs are rather high (above 0.920) and therefore all methods seem to accurately measure the different drugs. The R-Biopharm and the in-house methods have a negative average difference between theoretical and measured concentrations, which means that both methods tend to overestimate the drugs' concentrations, whereas the opposite is observed for Buhlmann. The 95% confidence interval (CI) of the average difference in Remsima and Flixabi quantified with Buhlmann is positive and excludes 0, which means that, in these cases, the underestimation is consistently observed throughout the entire range of tested concentrations.

The ICCs between the different assays are shown in Table 2. Values tend to be high (the minimum is 0.936), which means that similar concentrations are obtained for each compound using different assays. R-Biopharm is particularly close to Buhlmann in what comes to Remicade and Remsima, whereas Buhlmann is particularly close to the in-house method in what comes to Flixabi. Overall, the Buhlmann assay yields values consistently lower than those obtained with R-Biopharm for all three drugs; on the other hand, the in-house method yields values consistently higher than those obtained with Buhlmann in what concerns Remicade and Flixabi. Moreover, the Bland–Altman plots suggest that the differences between

Table 1. Intraclass correlation coefficient between the theoretical and measured concentrations.

	ICC		Difference		
	ICC	CI 95%	Average	CI 95%	
R-Biopharm					
Spiked concentrations: Remicade	0.986	0.949–0.996	−0.72	−1.82	0.38
Spiked concentrations: Remsima	0.990	0.964–0.997	−0.10	−0.98	0.77
Spiked concentrations: Flixabi	0.945	0.796–0.985	−0.69	−3.05	1.68
Buhlmann					
Spiked concentrations: Remicade	0.982	0.932–0.995	0.94	−0.23	2.11
Spiked concentrations: Remsima	0.985	0.945–0.996	1.33	0.31	2.35
Spiked concentrations: Flixabi	0.983	0.938–0.996	1.28	0.14	2.41
In house					
Spiked concentrations: Remicade	0.951	0.818–0.987	−1.31	−3.54	0.92
Spiked concentrations: Remsima	0.920	0.702–0.978	−0.46	−3.42	2.50
Spiked concentrations: Flixabi	0.972	0.896–0.992	−0.39	−1.99	1.22

Buhlmann, Quantum Blue® infliximab: quantitative lateral flow assay; CI, confidence interval; ICC, intraclass correlation coefficient; R-Biopharm, RIDASCREEN® IFX monitoring.

the methods increase for higher concentrations but rarely exceed the ±1.96 SD interval (Supplementary Figure 1).

Cross-immunogenicity

In order to determine the presence of cross-immunogenicity, the three drugs were tested with anti-Remicade and anti-Remsima sera extracted from IBD patients (Figure 2). The results show that the amount of antisera that reacted to Remicade, Remsima and Flixabi was similar ($p = 0.293$ for the anti-Remicade, and $p = 0.538$ for the anti-Remsima). In fact, the ICCs between the different drugs' reaction to anti-Remicade and anti-Remsima sera were close to 1.0 (Table 3).

Discussion

TDM is increasingly considered as a key step to optimize anti-TNFα treatment in IBD patients. Therefore, the advent of Remicade biosimilars carries along the necessity of validating the utilization of Remicade-quantifying assays, which were optimized for Remicade, with these somehow modified compounds. This study addressed the performance of three different Remicade-optimized quantification procedures, already validated to be used with Remsima, in the assessment of Flixabi concentrations. Moreover, we have addressed the presence of cross-immunogenicity between Remicade, Remsima and Flixabi. The Buhlmann assay tested in this study is particularly suitable for a clinical environment as the results are available within 15 min of placing the sample into the cartridge test, which allows an immediate adjustment of the drug dosage. In fact, when a traditional ELISA method is used, the dosage adjustment (if needed) is usually postponed to the next infusion, as the results take approximately 8 h.

The three assays used, R-Biopharm, Buhlmann and the in-house method, seem to be almost equally accurate in what concerns the quantification of Remicade and of its biosimilars. In fact, R-Biopharm and Buhlmann are slightly more accurate when measuring Remsima than when measuring its originator Remicade; as for

Table 2. Intraclass correlation coefficient between the different methods.

	ICC		Difference		
	ICC	CI 95%	Average	CI 95%	
Remicade					
R-Biopharm–Buhlmann	0.990	0.961–0.997	1.66	0.70	2.63
R-Biopharm–in house	0.978	0.918–0.994	−0.59	−2.20	1.02
Buhlmann–in house	0.968	0.881–0.991	−2.25	−4.08	−0.43
Remsima					
R-Biopharm–Buhlmann	0.995	0.980–0.999	1.44	0.79	2.08
R-Biopharm–in house	0.957	0.839–0.988	−0.35	−2.61	1.90
Buhlmann–in house	0.936	0.761–0.983	−1.79	−4.42	0.84
Flixabi					
R-Biopharm–Buhlmann	0.974	0.905–0.993	1.96	0.29	3.63
R-Biopharm–in house	0.979	0.922–0.994	0.30	−1.32	1.92
Buhlmann–in house	0.986	0.946–0.996	−1.66	−2.85	−0.48

Buhlmann, Quantum Blue® infliximab: quantitative lateral flow assay; CI, confidence interval; ICC, intraclass correlation coefficient; R-Biopharm, RIDASCREEN® IFX monitoring.

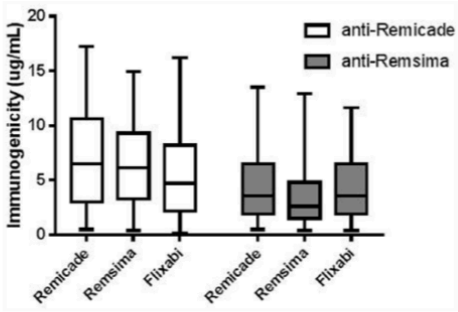

Figure 2. Reactivity of Remicade, Remsima and Flixabi to anti-Remicade and anti-Remsima sera.

the in-house method, measured values are closer to the theoretical concentrations in the case of Flixabi. Moreover, the values obtained when measuring each drug with the different quantification assays are rather similar, and the differences encountered tend to be larger when the drugs' concentrations are above the critical values considered to be in the therapeutic window, and therefore should have no effect in the clinical practice.[3,17,19] Overall, Buhlmann slightly underestimates Remicade, Remsima and Flixabi when compared with R-Biopharm, whereas the in-house method slightly overestimates Remicade and Flixabi when compared with Buhlmann. These results consolidate what has been previously published in the literature concerning Remsima, that is, Remicade-optimized methods perform equally well when measuring biosimilars' levels.[12,20–22] One can see only slight differences that are mostly likely the result of the small modifications in the biosimilars' structure, which can be attributed to dissimilarities in the compounds' biological synthesis (different cell lines or growth media, for instance), storage and transport.[8,23,24]

Immunogenicity is a key issue in Remicade and other anti-TNFα therapies: the formation of ADAs may directly or indirectly lower or even prevent the drug's action.[3] Cross-immunogenicity, that is, the abitility of ADAs to react against compounds other than the one that stimulated their appearance, is of utmost important from a clinical point of view. In fact, when an anti-TNFα therapy fails due to the presence of ADAs, one must

Table 3. Intraclass correlation coefficient between the antidrug reactivity of Remicade and its biosimilars.

	ICC		Difference		
	ICC	CI 95%	Average	CI 95%	
Anti-Remicade serum					
Flixabi–Remsima	0.988	0.977–0.994	−0.83	−1.13	−0.53
Flixabi–Remicade	0.992	0.984–0.996	−1.49	−1.77	−1.22
Remicade–Remsima	0.986	0.972–0.993	0.66	0.31	1.01
Anti-Remsima serum					
Flixabi–Remsima	0.989	0.978–0.994	0.29	0.07	0.52
Flixabi–Remicade	0.987	0.975–0.993	−0.36	−0.61	−0.11
Remicade–Remsima	0.993	0.986–0.996	0.65	0.46	0.84

CI, confidence interval; ICC, intraclass correlation coefficient.

consider the absence of cross-immunogenicity as a criterion for choosing a second anti-TNFα agent. Our results reveal that Remicade, Remsima and Flixabi react to a similar extent to anti-Remicade and anti-Remsima sera. These results come in line with what has been previously published regarding the cross-immunogenicity of Remsima and its originator.[6,22,25]

This study has a couple of limitations that we hereafter acknowledge: the results are based on *in vitro*-spiked samples only (no clinical samples were used); the *in vitro* samples were obtained spiking healthy donor sera (instead of sera extracted from IBD patients naïve to Remicade) and the cross-immunogenicity assays neither included an anti-Flixabi serum nor an anti-TNFα other than Remicade as a control serum.

This study is, to our knowledge, the first to demonstrate that Remicade-optimized quantification methods can be used to measure Flixabi levels, while consolidating the previously published results concerning Remsima in this context. In fact, our results suggest that either R-Biopharm, Buhlmann and the described in-house method can be used to measure Remicade biosimilars Remsima and Flixabi in an accurate fashion. Moreover, we have demonstrated the existence of cross-immunogenicity between Remicade, Remsima and Flixabi. This not only reinforces the similarity among these drugs, but also has some clinical implications: according to our results, a patient medicated with Remicade or Remsima whose therapy fails due to the presence of ADAs would not benefit from switching to Remicade, Remsima or Flixabi.

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Guarantor of the article: Fernando Magro.

FM: Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; study supervision; critical revision of the manuscript for important intellectual content. JA and CR: quantification and cross-immunogenicity assays; analysis and interpretation of data. CCD: statistical analysis. All the other authors: recruitment of patients and collection of samples.

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Conflict of interest statement

FM served as speaker and received honoraria from Merck Sharp and Dohme, Abbvie, Vifor, Falk, Laboratorios Vitoria, Ferring, Hospira and Biogen. IR served as a speaker/consultant for Merck Sharp and Dohme, Abbvie, Falk, Ferring, Hospira, Janssen and Takeda.

Supplemental Material

Supplemental material for this article is available online.

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V. CHAPTER 3

Pharmacokinetic Factors of Anti-TNF-α Antibodies and Clinical targets

Study VI - "Calprotectin and the Magnitude of Antibodies to Infliximab in Clinically-stable Ulcerative Colitis Patients are More Relevant Than Infliximab Trough Levels and Pharmacokinetics for Therapeutic Escalation"

EBioMedicine. 2017 Jul;21:123-130

Study VII - "Clinical performance of an infliximab rapid quantification assay"

Therap Adv Gastroenterol. 2017 Sep;10(9):651-660

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Research Paper

Calprotectin and the Magnitude of Antibodies to Infliximab in Clinically-stable Ulcerative Colitis Patients are More Relevant Than Infliximab Trough Levels and Pharmacokinetics for Therapeutic Escalation

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ABSTRACT

Although infliximab (IFX) is an efficient therapy for ulcerative colitis (UC) patients, a considerably high rate of therapeutic failures still occurs. This study aimed at a better understanding of IFX pharmacokinetics and pharmacodynamics among clinically-asymptomatic UC patients. This was a multicentric and prospective study involving 65 UC patients in the maintenance phase of IFX therapy. There were no significant differences between patients with positive and negative clinical, endoscopic and histological outcomes concerning their IFX trough levels (TLs), area under the IFX concentration *vs.* time curve (AUC), clearance and antibodies to infliximab (ATI) levels. However, the need to undergo therapeutic escalation later in disease development was significantly associated with higher ATI levels (2.62 μg/mL *vs.* 1.15 μg/mL, $p = 0.028$). Moreover, and after adjusting for disease severity, the HR (hazard ratio) for therapeutic escalation was significantly decreased for patients with an ATI concentration below 3 μg/mL (HR = 0.119, $p = 0.010$), and increased for patients with fecal calprotectin (FC) level above 250 μg/g (HR = 9.309, $p = 0.018$). In clinically-stable UC patients, IFX pharmacokinetic features cannot predict therapeutic response on a short-term basis. However, high levels of ATIs or FC may be indicative of a future therapeutic escalation.

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1. Introduction

The knowledge of the crucial role played by the tumor necrosis factor α (TNFα) on the pathophysiology of auto-immune inflammatory disorders, such as inflammatory bowel diseases (IBD), led to the development of a class of biological drugs that target this cytokine. Infliximab (IFX) was the first anti-TNFα approved for the treatment of IBD (Danese et al., 2015). Since its introduction, IBD patients experienced an improvement in their quality of life, a decrease on the number of bowel-related surgeries and hospitalizations, and an increase in steroid-free remission and mucosal healing rates (Gecse et al., 2016; Strik et al., 2016). Notwithstanding, and despite the therapeutic success of these biological drugs, some patients fail to respond to anti-TNFα in the induction period (primary non-responders), whereas others initially benefit from the treatment but eventually loose response (secondary non-responders) (Mould et al., 2016). Immunogenicity, *i.e.*, the development of anti-drug antibodies, is an unavoidable drawback of biological treatments and a possible explanation for the lack or loss of response. Antibodies to infliximab (ATIs) can directly neutralize the IFX effects by interfering with the TNFα-binding domain, or can affect the drug's clearance rate by forming immune complexes with IFX, thereby promoting its removal from the circulating system (Gecse et al., 2016).

Therapeutic drug monitoring (TDM)-based dosing is an interesting and efficient strategy to overcome IFX lack or loss of response. In order to establish an accurate algorithm to support the decision-making process on a TDM approach, many studies have attempted to elucidate IFX pharmacokinetics and to define therapeutic thresholds for IFX exposure (often using serum trough levels [TLs] as a proxy) and for ATI levels that can guide dose adjustments (Strik et al., 2016; Moore et al., 2016; Williet et al., 2016; Vande Casteele et al., 2015; Warman et al., 2015; Paul et al., 2013; Cornillie et al., 2014).

In parallel with drug monitoring, disease monitoring through non-invasive biomarkers plays an important role in IBD patients, as it allows an assessment of the inflammatory burden without the risks involved in colonoscopy-related procedures. Calprotectin constitutes up to 60% of the cytosolic protein content in granulocytes, and its presence in feces reflects the migration of neutrophils through the inflamed bowel wall to the mucosa (Gisbert and McNicholl, 2009). Recent evidences suggest that fecal calprotectin (FC) levels can be used to discriminate organic from functional disease, to assess disease activity and response to therapy, and to predict relapses (Benítez and García-Sánchez, 2015).

This study aimed to explore IFX pharmacodynamics and to assess the utility of monitoring drug and FC levels among a specific population of ulcerative colitis (UC) patients: those that are asymptomatic and considered to be in remission according to the Montreal classification. The main goal of this study was thus to define how useful – from a clinical point of view – is the monitoring drug, anti-drug antibodies and disease biomarker's levels in clinically-stable patients.

2. Material and Methods

2.1. Patients

UC patients in the maintenance phase of IFX therapy - 5 mg/kg infusions every six or eight weeks - were prospectively and consecutively recruited from 10 different hospitals. Only patients older than 18 years, with at least 14 weeks of IFX treatment and in remission according to the Montreal classification (at baseline and at least in the immediately previous consultation) were invited to participate. Moreover, all patients were in their regimens (6 or 8 weeks-interval infusions) for at least three infusions, to ensure stability. The decision of enrolling these patients in biological therapy had been done previously by the attending physician, following an inadequate response to AZA (azathioprine) or 6-MP (6-mercaptopurine) after a period of treatment equal or superior to three months, intolerance to these agents, or a severe acute relapse. Patients in the 6-weeks infusion interval had been initially allocated to the 8-weeks regimen, but were empirically placed in the shorter interval due to loss of response (LOR). Previously defined concomitant medication was maintained (dose and regimen) throughout the entire study. Exclusion criteria included patients with proctitis only; history of malignancy in the previous five years, opportunistic infections or demyelinating diseases; existence of adenomatous polyps or known viral infections; pregnancy and breastfeeding; and use of topical treatment (5-ASA or steroids) during the study period or in the previous month.

This study was approved by the ethic committee of all hospitals involved and by the Portuguese Data Protection Authority (Comissão Nacional de Protecção de Dados). All patients enrolled did so voluntarily and after signing a written informed consent. The national coordinator of the Portuguese IBD group (GEDII – Grupo de Estudo de Doenças Inflamatórias Intestinais) monitored the study.

2.2. Study Design

This was a multicentric and prospective observational study. All patients were closely monitored for six or eight weeks after an IFX infusion. Demographic and baseline characteristics were collected before the infusion (T = 0), whereas histological, endoscopic and clinical outcomes were assessed immediately before the following infusion (T = 42 or 56 days). IFX and ATIs were quantified 2 h and 14 days after the initial infusion, as well as immediately before the following one (T = 42 or 56 days). The different assessments and their timings are depicted in Fig. 1.

2.2.1. IFX and ATI Quantification

The levels of IFX were quantified using an *in-house* ELISA assay, as previously described by Ben-Horin et al. (Ben-Horin et al., 2011). The presence and amount of ATIs were assessed using the anti-human lambda chain assay (AHLC), an *in-house* ELISA procedure also described by Ben-Horin et al. (Ben-Horin et al., 2011). The ATI concentrations are expressed in μg/mL-equivalent, hereafter referred to as μg/mL for the purpose of brevity. The concentration of IFX at each time point was used to construct a concentration *vs.* time curve. The area under the curve (AUC) was calculated for each individual using the Linear Up/Log Down Trapezoidal method, whereas clearance was computed as the total IFX dose per patient divided by the correspondent AUC.

2.2.2. Endoscopic Activity

Endoscopic activity was evaluated using Ulcerative Colitis Endoscopic Index of Severity (UCEIS) (Travis et al., 2012), and the presence of macroscopic lesions was assessed with the Mayo endoscopic sub-score (Schroeder et al., 1987). Patients were considered to be in endoscopic remission whenever UCEIS was below 2, whereas mucosal healing was defined as a Mayo endoscopic sub-score either equal to 0 or lower than 2.

2.2.3. Histological Activity

To assess the presence of histological inflammation, an average of two samples per localization was collected from the sigmoid and rectum. Histological activity was evaluated following the Geboes score (Geboes et al., 2000), and histological remission was defined as a Geboes index lower than 3.1. All samples were the subject of a central reading by two independent pathologists blinded to the patients' disease status and endoscopic results. Disagreements between pathologists were resolved by a review including a third pathologist (K. Geboes) and using a multiheaded microscope, defining the final score.

2.2.4. Clinical Remission

Clinical remission was evaluated according to the Global Mayo score. Patients were considered to be in clinical remission if their global Mayo score was below or equal to 2 and no individual sub-score was above 1.

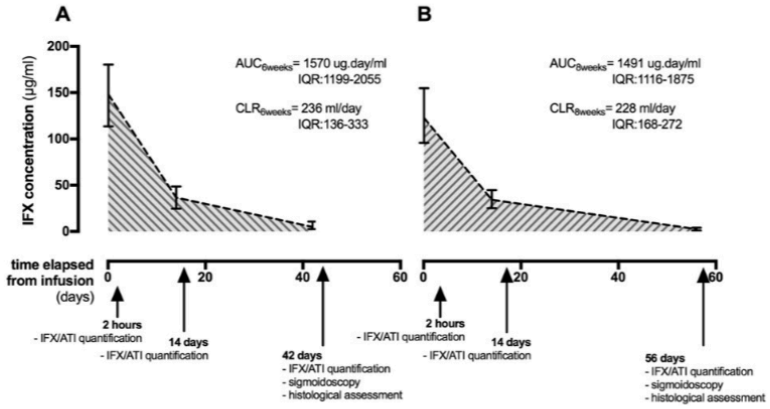

Fig. 1. IFX levels variation throughout time in patients on the 6-weeks (A) or the 8-weeks (B) schedule. The different assessments made during this study and their timing is indicated in the time bar.

2.2.5. FC Quantification

Stool samples were collected and kept at 4 °C (for a maximum of 48 h) until shipment to the central laboratory (Department of Pharmacology and Therapeutics, Faculty of Medicine of University of Porto). FC was extracted from stools within a maximum of seven days after collection using the 'Fecal sample preparation kit' (Roche Diagnostics, Germany) according to the instructions provided by the manufacturer and stored at −80 °C until quantification. FC samples were quantified using a fluoroenzyme immunoassay (EliA Calprotectin®, Thermo Fisher Scientific, Germany) according with manufacturers' instructions.

2.2.6. Assessment of Therapeutic Escalation

The files of all patients included in this study were later assessed in order to evaluate how many required therapeutic escalation. Therapeutic escalation was defined as has been previously suggested (Kalla et al., 2016), and included the presence of at least one of the following events: starting a new immunomodulator or biological drug; switching immunomodulator; increasing biological dosage or shortening infusion interval; switching biological drug due to LOR; need to undergo bowel-related surgery.

2.3. Statistical Analysis

Categorical variables were described through absolute (n) and relative (%) frequencies and continuous variables were described as mean and standard deviation, median, interquartile range (IQR), and minimum/maximum values, whenever appropriate. When testing hypothesis concerning continuous variables, nonparametric Kruskall Wallis tests were used as appropriate, taking into account normality assumptions and the number of groups compared. In order to have a more thorough understanding of the factors associated with clearance, univariate and multivariate logistic regression modelling were used. The time elapsed from assessment to therapeutic escalation was evaluated using survival analysis. To determine the factors associated to therapeutic escalation, Cox regression was used. The cumulative probabilities of event-free survival were estimated with the Kaplan-Meier method using log-rank and Breslow tests. The reported p values were two-sided, and p values below 0.05 were considered to be statistically significant. The cut-offs used to stratify the outcomes concerning IFX trough levels, clearance, and ATI levels were chosen based on the literature (Afonso et al., 2016; Vande Casteele et al., 2015). All data was arranged, processed and analyzed with SPSS® v.20.0 data (Statistical Package for Social Sciences), whereas graphs were designed using Prism 6.

3. Results

3.1. Characterization of the Cohort

The cohort analyzed in this study included 65 UC patients in remission being treated with 5 mg/kg IFX every six weeks (n = 21, 32.3%) or every eight weeks (n = 44, 67.7%) (Table 1). Overall, most patients were female (56.9%) and had never smoked (68.9%). The location of the disease was distributed as follows: 50.8% of the patients had left-side and 49.2% patients had extensive colitis. Concerning concomitant therapies, 67.7% of the patients were or had been on AZA, whereas 10.8% were or had been taking steroids. There were no significant differences between the baseline characteristics of the patients doing the 6-weeks' and the 8-weeks' regimen (data not shown).

3.2. Pharmacokinetics

Patients were followed during one IFX infusion cycle, and the assessments made throughout time are illustrated in Fig. 1. The IFX trough levels (TLs) were significantly higher in the patients enrolled in the 6-weeks' regimen when compared to those in the 8-weeks' one: median $TL_{6\ weeks}$ = 5.00 µg/mL, IQR: 2.68–9.60 *vs.* median $TL_{8\ weeks}$ = 2.43 µg/mL, IQR: 0.91–3.70, p = 0.006. However, there was no significant difference between the two regimens concerning the ATI concentration (median $ATI_{6\ weeks}$ = 1.15 µg/mL, IQR: 0.88–2.48 *vs.* median $ATI_{8\ weeks}$ = 1.51 µg/mL, IQR: 0.80–2.18, p = 0.592),

Table 1
Cohort characterization.

	n	%
Gender		
Male	28	43.1
Female	37	56.9
Smoking status		
Never smoked	42	68.9
Former smoker	15	24.6
Smoker	4	6.6
Location of disease		
Left-side colitis	33	50.8
Extensive colitis	32	49.2
Extra-intestinal manifestations	16	26.2
Azathioprine	44	67.7
Azathioprine intolerant	10	16.1
Steroids	7	10.8
Corticodependent	39	60.9
Corticoresistent	12	18.5

the AUC ($p = 0.768$) or the clearance ($p = 0.941$). The concentration of ATIs at the 6th/8th week was inversely correlated with IFX levels 14 days after the infusion (Spearman correlation coefficient = −0.295, $p = 0.022$) and IFX TLs (Spearman correlation coefficient = −0.480, $p < 0.001$).

To address the importance and interaction between ATIs and IFX-TLs concerning clearance and AUC, these parameters were analyzed in patients stratified according to their status (positive or negative) regarding clinical cut-offs of IFX TLs (3 μg/mL) and ATI concentration at the 6th/8th week (1.7 μg/mL) (Afonso et al., 2016; Vande Casteele et al., 2015) (Table 2). Both clearance and AUC of the 8 weeks-regimen patients varied in a significant fashion according to the ATI/IFX-defined patient group. These parameters were clearly associated with the presence of ATIs, as ATI positive patients had a higher clearance and consequently a lower AUC. Concerning only ATI-negative patients, those that were positive for IFX trough levels had a lower clearance and a higher AUC.

As expected, clearance and ATI levels were correlated in a significant fashion (Spearman's coefficient: 0.391, $p = 0.005$). A multiple regression analysis was made using clearance as the dependent variable and considering patients' height, weight, albumin, UCEIS (as a proxy for inflammatory burden) and ATI concentration. The multivariate model is depicted in Table 3 and shows that ATI concentration is the only independent predictor of clearance in these patients. Moreover, when UCEIS was replaced by either the endoscopic Mayo score (stratified by 0 *vs.* ≥1 or ≤1 *vs.* >1) or by the Geboes index (stratified by <3.1 *vs.* ≥3.1), the results were similar (Supplementary Tables 1, 2 and 3).

3.3. Pharmacodynamics

The patients' outcomes after the infusion cycle were evaluated in an inclusive way, including the Mayo Global score assessment, the presence of endoscopic activity and histological inflammation, and the FC levels (Table 4). Most patients (71.5%) had a global Mayo score equal to or below 2, and 70.8% were considered to be in clinical remission (defined as global Mayo score below or equal to 2 and no individual subscore above 1). Endoscopic activity according to the UCEIS was absent in 76.2% of the patients, whereas 60.3% and 82.5% did not exhibit macroscopic lesions when the Mayo endoscopic score threshold was set at 0 and 1, respectively. Histological inflammation was present in 31.3% of the patients, and 22.2 and 11.1% were above the FC threshold when that was set at 150 and 250 μg/g, respectively. There were no significant differences between the outcomes of the patients under the 6-weeks' and the 8-weeks' regimen (data not shown).

To test whether the IFX pharmacokinetic features were related to patients' response in the cohort under study, patients were stratified according their outcomes, and IFX TLs (Supplementary Table 4), ATIs (Supplementary Table 5), AUC (Supplementary Tables 6 and 7) and clearance (Supplementary Table 8) were compared between positive and negative outcomes. However, there were no significant differences to report.

Table 3
Regression analyses of the clearance rate (mL/day).

Variables	OR	95% CI	p-Value
Height (m)	413.589	−127.938; 955.115	0.130
Weight (kg)	1.028	−1.483; 3.539	0.411
Albumin	0.758	−6.114; 7.630	0.824
[ATI] μg/mL	**12.210**	**2.381; 22.040**	**0.016**
UCEIS			
≤1	Ref		
>1	0.849	−66.549; 68.247	0.980

All variables were included using the "enter" method; $R^2 = 0.293$; OR-Odds Ratio 95% CI – 95% confidence interval.

3.4. Therapeutic Escalation

A total of 60 patients were re-evaluated to detect whether a therapeutic escalation was required later in their follow-up (five patients of the initial cohort were lost to follow up). Overall, 10 patients escalated, and the time spent from initial assessment to escalation was, in median, 15.00 months (IQR: 8.00–20.00). To test whether the IFX pharmacokinetic features assessed previously were related to patients' escalation, values of IFX TLs, ATIs, AUC and clearance were compared between patients with or without the need to escalate their therapy (Fig. 2). Patients are undistinguishable based on IFX TLs, clearance and AUC. However, there is a clear trend for higher ATIs among patients who later require therapeutic escalation.

Moreover, a Kaplan-Meyer analysis showed that patients with ATIs levels above 1.7 μg/mL (Fig. 3A) and above 3 μg/mL (Fig. 3B) escalate faster than those with lower levels, although only the 3 μg/mL cut-off has statistical significance. This analysis was expanded in order to include the biomarker FC, and the results show that patients with higher levels of FC also escalate faster than their counterparts (Fig. 3C and D), although significant results are only present for the 250 μg/g cut-off. Furthermore, the escalation was also faster when any of these conditions (or both) were present (*i.e.*, ATI above 3 μg/mL or FC above 250 μg/g), as compared to those patients whom had both values below the cut-offs (Fig. 3E). Finally, this faster escalation is unrelated to the disease severity from an histological and endoscopic perspective (Supplementary Fig. 1). In fact, a Cox regression considering all these parameters shows that only ATI and FC levels are significant for therapeutic escalation: whereas an FC level above 250 μg/g has an HR (hazard ratio) of escalating of 9.309, an ATI level below 3 μg/mL has an HR of 0.119 (Table 5). These values are maintained irrespective of whether the endoscopic Mayo score cut-off was placed at 1 or 2.

4. Discussion

The success of IFX in the treatment of many UC patients, materialized in a decrease of the number of surgeries and hospitalizations and an increase in these patients' quality of life, is overshadowed by the no-

Table 2
Median and IQRs for clearance rates and AUC values stratified by ATI and IFX trough levels.

[ATI] cut-off = 1.7 [IFX] cut-off = 3	ATI− IFX−	ATI− IFX+	ATI+ IFX−	ATI+ IFX+	p-Value[a]
Clearance (mL/day)	228 [212.5–280.0]	164 [120–236]	323.5 [252–360]	228.5 [168–327.5]	<**0.001**
N	8	23	14	4	
AUC 6/6 weeks (μg·day/mL)	1388 [1206–1570]	1789 [1328–2389]	826 [468–1199]	1448 [1186–2055]	0.071
N	2	11	3	3	
AUC 8/8 weeks (μg·day/mL)	1532 [1310–1617]	1821 [1603–2302]	1090 [991–1479]	1426 [1426–1426]	**0.004**
N	6	14	12	1	

[a] Kruskall Wallis test.

Table 4
Outcomes at 6/8 weeks post-infusion.

	n	%
Global mayo score		
1	36	55.4
2	10	16.1
3	6	9.7
4	2	3.2
5	5	4.8
6	2	3.2
7	1	1.6
8	1	1.6
11	1	1.6
Remission = no	19	29.2
Remission = yes	46	70.8
Endoscopic mayo score		
0	38	60.3
≥1	25	39.7
≤1	52	82.5
>1	11	17.5
UCEIS		
≤1	48	76.2
>1	15	23.8
Histology (Geboes score)		
<3.1	44	68.8
≥3.1	20	31.3
FC (at 6/8 weeks) (μg/g)		
<150	49	77.8
≥150	14	22.2
<250	56	88.9
≥250	7	11.1

ticeable number of treatment failures. In order to address this issue, IFX pharmacokinetics, pharmacodynamics and disease outcomes – including the biomarker FC – were closely monitored during one IFX infusion cycle in a population of clinically-stable UC patients.

The values of IFX TLs, AUC and clearance reported in this study were within the range of those previously described in different studies and clinical trials (Fasanmade et al., 2009; Brandse et al., 2016; Paserchia, 1999; Anon, n.d.). Interestingly, the different IFX regimens had similar AUCs and clearance values, but could be distinguished based on their IFX TLs, which were significantly higher in the shorter regimen. Shortening the infusion interval is a commonly used strategy to intensify IFX therapy, shown to be superior or, at least, equivalent to the increase of IFX dosage in LOR (Katz et al., 2012; St Clair et al., 2002). As in the ATTRACT study, our results show that a shorter interval is associated with higher IFX TLs (St Clair et al., 2002). Moreover, this increase in IFX TLs - even without a concomitant increase in the IFX AUC - was sufficient for patients who suffered a LOR in the 8-weeks regimen regain response to IFX. In fact, their outcomes were similar to those experienced by the patients that remained in the 8-weeks' regimen.

Interestingly, ATIs levels were the only significant factor affecting IFX clearance in this cohort. One can hypothesize that variables that affect clearance in more severely ill patients (such as albumin, height, weight and inflammatory burden) are not significant in the population addressed in this study, which was constituted by patients in a stable condition. Therefore, the presence of ATIs seems to be the main factor affecting IFX availability, although these results worth a confirmation on a larger population.

The analysis of the cohort outcomes and disease indicators show that, despite being classified as in remission according to the Montreal classification, a considerable proportion of patients still has endoscopic lesions and a relatively high inflammatory burden. Interestingly, neither IFX TLs, AUC, clearance nor ATI concentrations were able to differentiate patients with positive and negative outcomes. IFX TLs are considered to be particularly useful for this: in fact, a search through the literature shows that IFX TLs are many times used to monitor this drug on a therapeutic scenario, and different authors have found significant differences between IFX TLs in responders and non-responders, many times using cut-off values close to the one used in this study (3 μg/mL), as described by Silva-Ferreira et al. and references included (Silva-Ferreira et al., 2016). Our analysis, however, suggest that these differences are absent or undiscernible when assessing clinically-stable and asymptomatic patients.

We have then analyzed whether the pharmacokinetic profile of these patients could be used to evaluate their long-term risk of requiring therapeutic escalation. Interestingly, there was a clear and significant trend for patients with higher ATI levels to need therapeutic escalation later on their lives. Moreover, a cut-off of 3 μg/mL could be statistically associated with the requirement of therapeutic escalation.

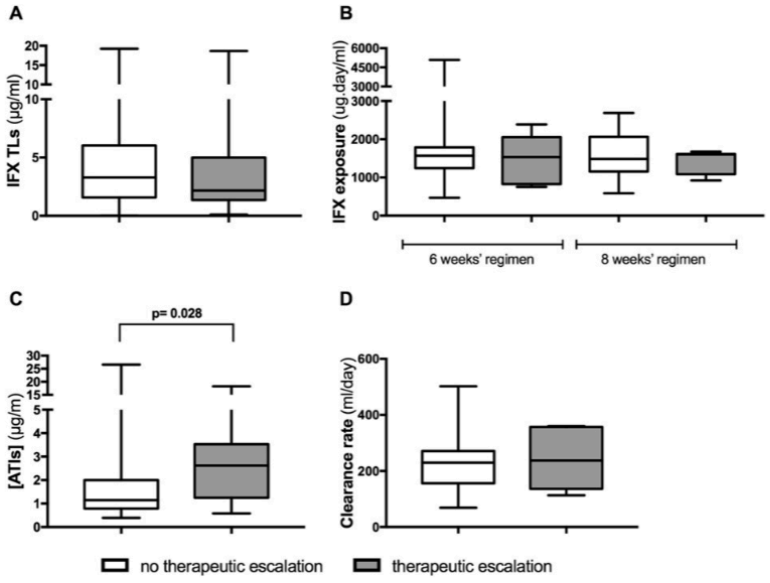

Fig. 2. Relationship between therapeutic escalation episodes and the IFX TLs (A), IFX AUC (B), ATI levels (C) and ATI clearance (D).

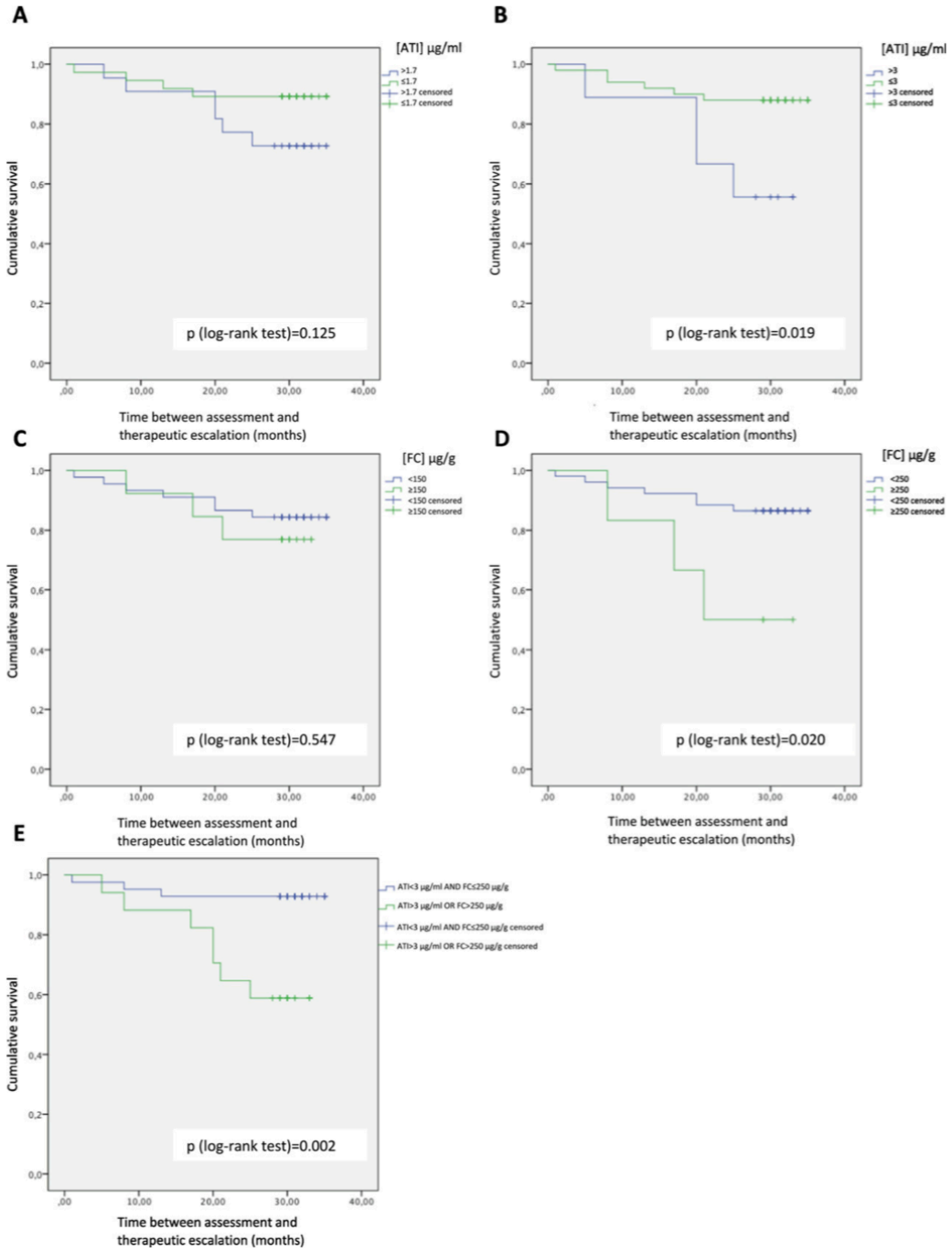

Fig. 3. Kaplan-Meyer survival curves for time to therapeutic escalation of: ATI levels using a cut-off of 1.7 µg/ml (A) or 3 µg/ml (B); FC levels using a cut-off of 150 µg/g (C) or 250 µg/g (D); ATI levels above 3 µg/ml or FC levels above 250 µg/g (E).

These results concur with the data published previously by Edlund et al., who have shown that the presence of ATIs in Crohn's disease (CD) patients, irrespective of their concentration, eventually leads to a drop in IFX levels to values below a critically minimum concentration (Edlund et al., 2016). Moreover, Ungar et al. have shown that ATI development often precedes the onset of a clinical flare (Ungar et al., 2014).

Additionally, a similar analysis including the FC levels has shown that values above 250 µg/g are also significantly associated with the

Table 5
Multi-variate Cox regression to therapeutic escalation.

	p-Value	HR	95% CI	
IFX TLs	0.771	1.021	0.887	1.176
FC (ref: <250 μg/g)	0.018	9.309	1.455	59.561
ATIs (ref: >3 μg/mL)	0.010	0.119	0.024	0.594
Geboes index (ref: <3.1)	0.602	0.648	0.127	3.301
Mayo endoscopic score (ref: 0)	0.851	1.151	0.265	5.008
IFX TLs	0.774	1.020	0.889	1.171
FC (ref: <250 μg/g)	0.019	9.036	1.445	56.511
ATIs (ref: >3 μg/mL)	0.009	0.119	0.024	0.592
Geboes index (ref: <3.1)	0.575	0.619	0.116	3.310
Mayo endoscopic score (ref: ≤1)	0.772	1.318	0.204	8.530

HR- Hazard Ratio; 95% CI-95% confidence interval.

requirement of therapeutic escalation. Such a relationship has been suggested before by Burri et al., who claimed that changes of FC levels between measurements were related to therapeutic escalation (Burri et al., 2015). From a different angle but supporting the same core idea, Papamichael et al. have recently shown that the risk of relapse after IFX de-escalation in CD patients in composite deep remission is relatively low when FC levels are maintained within the normal range (Papamichael et al., 2016). Moreover, the results of a meta-analysis including six different studies suggest that FC is useful to predict relapses in quiescent UC and CD patients (Mao et al., 2012).

The simultaneous analysis of ATI and FC levels shows that the therapeutic escalation is associated to high values of either these variables. Importantly, their impact in the need of a future therapeutic escalation is independent of the disease severity, as is shown by the fact that neither histological score nor endoscopic lesions are significant variables in this context. A combination of a biomarker and ATIs levels to predict disease development has been shown before: in fact, C-reactive proteins levels combined with IFX-TLs and ATI stability were shown to predict LOR in IBD patients (Roblin et al., 2015). Our results, together with the literature, suggest that high levels of ATIs and FC found in otherwise stable UC patients may indicate a future disease flare and its consequent therapeutic escalation. These findings have some important clinical implications: TDM on stable patients is useful if ATI levels are included and should be performed alongside with FC determination: the presence of elevated ATIs of FC levels – even in the absence of clinical symptoms – should alert the physician to act in order to prevent future therapeutic escalations.

This study has several strengths that should be noticed, namely its prospective design with a systematic and multidimensional evaluation of the therapeutic response: endoscopic, histological and clinical data was retrieved, in parallel with the quantification of a biomarker. Nevertheless, there were also a few limitations that should be taken into consideration: the inclusion of a single infusion cycle and the fact that we have not taken into account the amount of IFX lost through the feces.

In short, this study explores the IFX pharmacokinetics and the utility of drug and disease monitoring among UC patients in remission. Our findings show that, in these patients, IFX clearance is mainly related to the presence of ATIs. Moreover, and irrespective of the IFX regimen, IFX TLs, AUC, clearance and ATI concentration are unable to differentiate patients according to their outcome. Conversely, high ATI levels are significantly associated with the long-term need to undergo therapeutic escalation, as are FC levels above 250 μg/g. Therefore, the usefulness of TDM in clinically-stable UC patients relies on the possibility of avoiding future disease progression that can be predicted based on the ATI levels. Moreover, the monitoring of FC should also be carried out in these patients, as this biomarker is also increased in patients that eventually need to undergo a therapeutic escalation.

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Conflict of Interests

FM served as speaker and received honoraria from Merck Sharp & Dohme, Abbvie, Vifor, Falk, Laboratorios Vitoria, Ferring, Hospira and Biogen.

Author Contributions

FM: Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; study supervision; critical revision of the manuscript for important intellectual content. JA: IFX, anti-IFX antibodies and fecal calprotectin assays; analysis and interpretation of data. JL: histological analysis. CCD: statistical analysis. AF: pharmacokinetic calculations. KG: supervisor of the histological analysis; critical revision of the manuscript for important intellectual content. FC: responsible for the histological analysis; critical revision of the manuscript for important intellectual content. All the other authors: recruitmen of patients and collection of samples.

All authors read and approved the final version of the manuscript.

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Clinical performance of an infliximab rapid quantification assay

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Abstract

Background: Therapeutic drug monitoring (TDM)-based algorithms can be used to guide infliximab (IFX) adjustments in inflammatory bowel disease (IBD) patients. This study aimed to explore a rapid IFX-quantification test from a clinical perspective.

Methods: This manuscript describes a prospective cohort study involving 110 ulcerative colitis (UC) patients on the maintenance phase of IFX. IFX trough levels were quantified using a rapid quantification assay and a commonly-used reference kit.

Results: Irrespective of the assay used to measure IFX, its through levels were statistically different between patients with and without endoscopic remission (Mayo endoscopic score = 0), as well as between patients stratified by their faecal calprotectin (FC) levels. Despite the fact that the two methods correlated well with each other [Spearman's rank correlation coefficient = 0.843, $p < 0.001$; intraclass correlation coefficients = 0.857, 95% confidence interval (CI): 0.791–0.903], there was a discernible systematic variation; values obtained with the reference kit were on average 2.62 units higher than those obtained with the rapid assay. Notwithstanding, 3 µg/ml was shown to be an acceptable cut-off to assess endoscopic status and inflammatory burden levels using both assays. The percentage of patients that had a positive outcome when the IFX concentration measured by the rapid assay ranked above 3 µg/ml was 88% both for a Mayo endoscopic score $\leqslant 1$ and for an FC concentration <250 µg/g.

Conclusions: Based on this study, we concluded that using the rapid IFX assessment system with a 3 µg/ml threshold is a reliable alternative to the time-consuming enzyme-linked immunosorbent assays in patients on the maintenance phase of IFX.

Keywords: infliximab, ulcerative colitis, therapeutic window

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Introduction

The introduction of anti-tumour necrosis factor (TNF)α monoclonal antibodies as therapeutic agents in auto-inflammatory disorders has revolutionized the medical management strategies of these diseases and the health-related quality of life of patients. In the case of inflammatory bowel diseases [IBDs, which include Crohn's disease (CD) and ulcerative colitis (UC)], the use of anti-TNFα agents has led to a decrease in hospitalization rates, risk of surgery and health-related costs.[1]

However, and despite the anti-TNFα success in the treatment of many IBD patients, some of them do not respond to the drug during the induction phase, whereas others experience a loss of response later during treatment.[2] Accumulating evidence from the literature suggests that the outcomes of CD and UC patients on infliximab (IFX) are strongly related

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with the levels of the drug found in the organism.[3–10] From a physician's perspective, understanding the reasons that lead to unresponsiveness is key to delineate future therapeutic strategies, which can include a dose intensification, a switch to another anti-TNFα agent, or adding immunosuppressive drugs or steroids. In this context, the precise and accurate measurement of the circulating drug levels, known as therapeutic drug monitoring (TDM), has a key role. Several TDM-based algorithms and dashboards are being developed to assist the physician in the therapeutic decision-making process.[2,11–13] Moreover, TDM may also be useful to identify cases with supra-therapeutic drug levels (which can be de-escalated to prevent the appearance of adverse effects), and has been proven as a cost-effective strategy when compared with the traditional empirical-based adjustment of drug dosage.[14,15]

Given the importance of TDM in patients on IFX, one can easily find a number of different commercial kits that can measure the concentration of this agent from the patient's serum, most of them relying on an enzyme-linked immunosorbent assay (ELISA) approach. However, these kits have a turnaround time of approximately 8 h, delaying the IFX dose adjustment to the following infusion (usually 6–8 weeks later). A rapid IFX-quantification system, which allows a fast (15 min) assessment of IFX from a patient's serum, has been recently launched in the market by the Bühlmann® company (Schönenbuch, Switzerland). Not only does this system allows an immediate adjustment of the IFX dosage, but it also has the advantage of being a user-friendly desktop device, which can be easily operated by any nurse, technician or physician without the requirement of specific laboratory facilities.

We have recently validated the utilisation of the Bühlmann® rapid assay in a laboratorial context, and concluded that this kit constitutes a reliable and fast alternative to the traditional ELISA kits.[16] In this study we aimed to take a step further and to assess the clinical sensitivity and specificity of this rapid assay, by addressing the existence and interpretability of IFX cut-off values able to guide the therapeutic decision-making process.

Material and methods

Cohort

UC patients on the maintenance phase of IFX therapy were prospectively and consecutively recruited from 10 different university and community hospitals. Only patients older than 18 years and with at least 14 weeks of IFX treatment were invited to participate. Exclusion criteria included history of malignancy in the previous 5 years, opportunistic infections or demyelinating diseases; existence of adenomatous polyps or known viral infections; and pregnancy and breastfeeding.

This study was approved by the ethic committee of all hospitals involved and by the Portuguese Data Protection Authority. All patients enrolled signed an informed written consent.

IFX-quantification assays

A total of 110 samples collected from the same number of patients were assayed to determine their serum trough IFX levels using two different commercial kits: Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Bühlmann, Schönenbuch, Switzerland), hereafter referred to as QB, and Level Infliximab M2920 kit (Sanquin, Amsterdam, the Netherlands), hereafter referred to as Sanquin. Both kits were used strictly following manufacturers' instructions. The lower and upper limits of quantification were 0.4 µg/ml and 20 µg/ml for the QB assay, and 0.08 µg/ml (1:200) and 25 µg/ml (1:1500) for the Sanquin assay, respectively. Whenever the results obtained were below or above these limits of quantification, they were considered to be at those same limits. Sanquin was chosen as the reference test as it is a widely used kit in both laboratorial and clinical contexts. All measurements were carried out by the same researcher.

Assessment of disease outcomes

Disease status, including clinical evaluation, endoscopic and histological activity, and quantification of faecal calprotectin (FC), was assessed at the same time as the IFX concentration (i.e. immediately before an IFX infusion).

Clinical evaluation. Clinical remission was evaluated according to the global Mayo score. Patients were considered to be in clinical remission if their global Mayo score was ⩽2 and no individual subscore was above 1.

Endoscopic evaluation. Endoscopic activity was evaluated using the Mayo endoscopic subscore:[17] mucosal healing was defined as a Mayo endoscopic subscore equal to 0 or ⩽1.

Histological evaluation. The presence of histological inflammation was evaluated through the analysis of an average of two biopsy samples from the sigmoid and the rectum. Samples were classified following the Geboes score,[18] and histological remission was defined as a Geboes index ⩽3.0. All samples were the subject of a central reading by two independent pathologists blinded to the patients' disease status and endoscopic results. Disagreements between pathologists were resolved by a review including a third pathologist (K. Geboes) and using a multiheaded microscope, defining the final score.

Quantification of faecal calprotectin. Stool samples were collected and kept at 4°C (for a maximum of 48 h) until shipment to the central laboratory (Department of Pharmacology and Therapeutics, Faculty of Medicine of University of Porto, Portugal). FC was extracted from stools within a maximum of 7 days after collection using the 'faecal sample preparation kit' (Roche Diagnostics, Germany) according to the instructions provided by the manufacturer, and stored at −80°C until quantification. FC concentration in each sample was determined using the QB kit according to the manufacturer's instructions.

Statistical analysis

Categorical variables were described through absolute (*n*) and relative (%) frequencies and continuous variables were described as mean and standard deviation, median, percentiles, and minimum/ maximum values when appropriate. All the reported *p*-values were two-sided, and *p*-values <0.05 were considered to be statistically significant. The ability of the measured IFX concentrations to assess the various disease outcomes was evaluated by plotting Receiver Operating Characteristic curves and computing the Area Under the Curve. All data were arranged, processed and analysed with SPSS® v.20.0 data (Statistical Package for Social Sciences, IBM Corp., Armonk, NY). Graphs were computed with Prism 7 (GraphPad Software, Inc., CA, USA).

Results

Characterization of the cohort and disease outcomes

The main baseline characteristics of this study's cohort are depicted in Table 1. Females constituted 55% of the entire population, and only 5% of all patients were current smokers. A minority of patients (2%) had a proctitis diagnosis, whereas 49% of them had left-side colitis and an equal percentage had extensive colitis. Overall 22% of the patients were azathioprine (AZA) intolerant, whereas 59% and 23% were classified as corticodependent and corticoresistant, respectively. At the time of study inclusion, 61% and 9% of all patients were on AZA and steroids, respectively.

Table 1. Cohort characterization.

	n	%
Sex		
Male	50	45%
Female	60	55%
Smoking status		
Never smoked	74	71%
Former smoker	25	24%
Smoker	5	5%
Location of disease		
Proctitis	2	2%
Left-side colitis	50	49%
Extensive colitis	51	49%
Azathioprine	66	61%
Azathioprine intolerant	23	22%
Steroids	10	9%
Corticodependent	65	59%
Corticoresistant	25	23%
Montreal classification		
Clinical remission	86	80%
Mild UC	16	15%
Moderate UC	5	5%
Severe UC	0	0%

UC, ulcerative colitis.

The disease outcomes addressed during this study are listed in Table 2. Regarding clinical evaluation, the majority of patients (72%) had a global Mayo score ⩽2, and 69% of the entire population were considered to be in clinical remission (i.e. had a global Mayo score ⩽2 and no individual subscore >1). Moreover, 58% or 81% of all patients were considered to be in mucosal healing (endoscopic Mayo score = 0 or ⩽1, respectively). Regarding FC levels, 66% of the population were below the threshold of 250 µg/g. Finally, the overall median [interquartile range (IQR)] of the IFX trough levels was 6.59 µg/ml (3.03–14.66) using the Sanquin kit, and 5.25 µg/ml (1.70–9.58) using the rapid QB assay.

Analytical comparison between the two different IFX-quantification methods

IFX through levels measured by the Sanquin and QB levels were highly correlated [Spearman's rank correlation coefficient = 0.843, $p < 0.001$; intraclass correlation coefficient (ICC) = 0.857, 95% CI: 0.791–0.903], as shown in Supplementary Figure 1. However, the mean difference and its CI show that the concentrations obtained with the Sanquin kit were, on average, higher than those obtained with the QB (average difference = 2.62 µg/ml, 95% CI: 1.64–3.60). Finally, the Bland–Altman plot shows that the difference between values measured with both kits increases with the increase in IFX concentrations, but is close to 0 for concentrations below 5 µg/ml (Supplementary Figure 2).

Association between IFX trough levels and outcomes

The medians of serum trough IFX concentrations detected with each method for contrasting disease outcomes (concerning clinical remission, endoscopic Mayo score and FC levels) are represented in Figure 1. The results show that IFX trough levels were higher in patients who had positive outcomes irrespective of the assay used, and these results were significant for endoscopic remission (using endoscopic Mayo score = 0 as the criterion for remission) and FC.

We then applied different IFX cut-offs (from 1–10) to the results obtained from each kit, and assessed their ability to predict patient outcomes. The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), accuracy and Kappa for each case are depicted in Supplementary Table 1. A positive test was defined as having an IFX level below the cut-off, whereas the disease status was defined as having a negative outcome (not being in clinical remission, having an endoscopic Mayo score >0 or >1, or having an FC level >250 µg/g). NPV represents the percentage of patients who have a positive outcome (no disease) among those who have an IFX above the defined cut-off (negative test result), whereas PPV represents the percentage of patients who have a negative outcome (disease) among those that have an IFX below the defined cut-off (positive test result).

Perceptively, the performance values vary widely with the cut-off chosen and the outcome evaluated, but are considerably similar for both kits when the conditions mentioned are kept stable (i.e. same cut-off and outcome). Figure 2 represents the accuracy (i.e. the sum of true positives and negatives) of the results obtained with either QB or Sanquin in terms of clinical status, endoscopic score and FC level using different cut-offs. The results show that Sanquin and QB have a very similar variation of the accuracy along the different cut-offs. Overall, a value of 3 µg/ml seems to be an acceptable cut-off for QB, although lower values could be considered in a few situations.

NPV has an important role in this context, as it represents the percentage of patients who have an IFX concentration above the cut-off and would not benefit from a drug adjustment. And in fact, 74, 62, 83 and 86% of patients with an IFX trough level >3 µg/ml measured by the Sanquin

Table 2. Disease outcomes.

	n	%
Global Mayo score (*n*, %)		
1	60	57%
2	16	15%
3	8	7%
4	3	3%
5	6	6%
6	3	3%
7	4	4%
8	3	3%
9	1	1%
11	2	2%
Clinical remission = no	34	31%
Clinical remission = yes	76	69%
Endoscopic Mayo score (*n*, %)		
0	63	58%
⩾1	45	42%
⩽1	87	81%
>1	21	19%
FC (µg/g) (*n*, %) QB		
<250	59	66%
⩾250	31	34%
IFX, (median, IQR)		
Sanquin	6.59	3.03–14.66
Quantum Blue	5.25	1.70–9.58

FC, faecal calprotectin; IFX, infliximab; QB, Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Bühlmann, Schönenbuch, Switzerland).

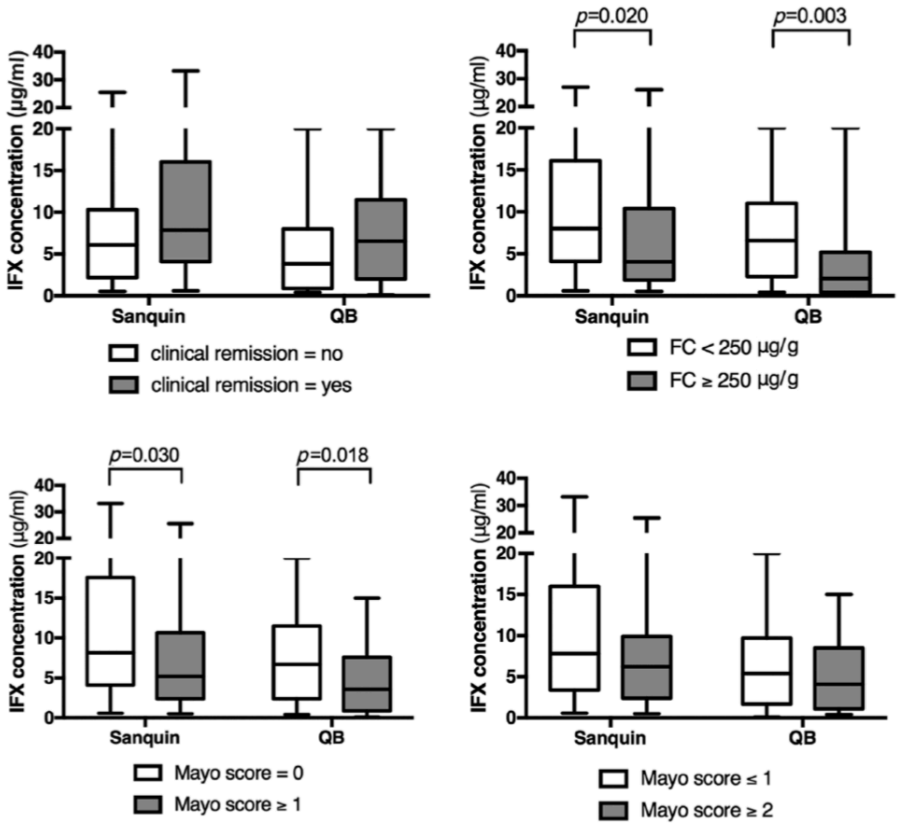

Figure 1. IFX concentrations quantified using the different methods and stratified by disease outcomes.
IFX, infliximab; QB, Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Bühlmann, Schönenbuch, Switzerland); Sanquin, Level Infliximab M2920 kit (Sanquin, Amsterdam, the Netherlands).

kit are in clinical remission, have a Mayo endoscopic score of 0, have a Mayo endoscopic score ⩽1, and have an FC level <250 µg/g, respectively, whereas these values are 74, 65, 88 and 88% for the QB kit.

When adjusting the IFX cut-off to evaluate clinical status to 1 (with Sanquin) or 2 (with QB), the percentage of patients that test above these values and are, indeed, in clinical remission, is 71% and 73%, respectively. This shows that although accuracy can be higher, the NPV is slightly smaller for these lower cut-offs. The same thing occurs when one addresses endoscopic remission (using endoscopic Mayo score ⩽1 as the remission criterion) using IFX cut-offs <3 µg/ml: the accuracy is higher, but the NPV is lower. On the other hand, the PPV (percentage of patients that are below the IFX cut-off and could benefit from an IFX dose adjustment) are consistently lower than the NPVs, and for a cut-off of 3 µg/ml vary from 23–50% with the Sanquin kit, and from 23–55% with the rapid QB kit.

To test whether the IFX values measured by these kits could also be used to assess deep remission, the Geboes index was considered as a criterion to establish histological remission, and the sensitivity, specificity, PPV, NPV, accuracy and Kappa for each cut-off concerning the occurrence of

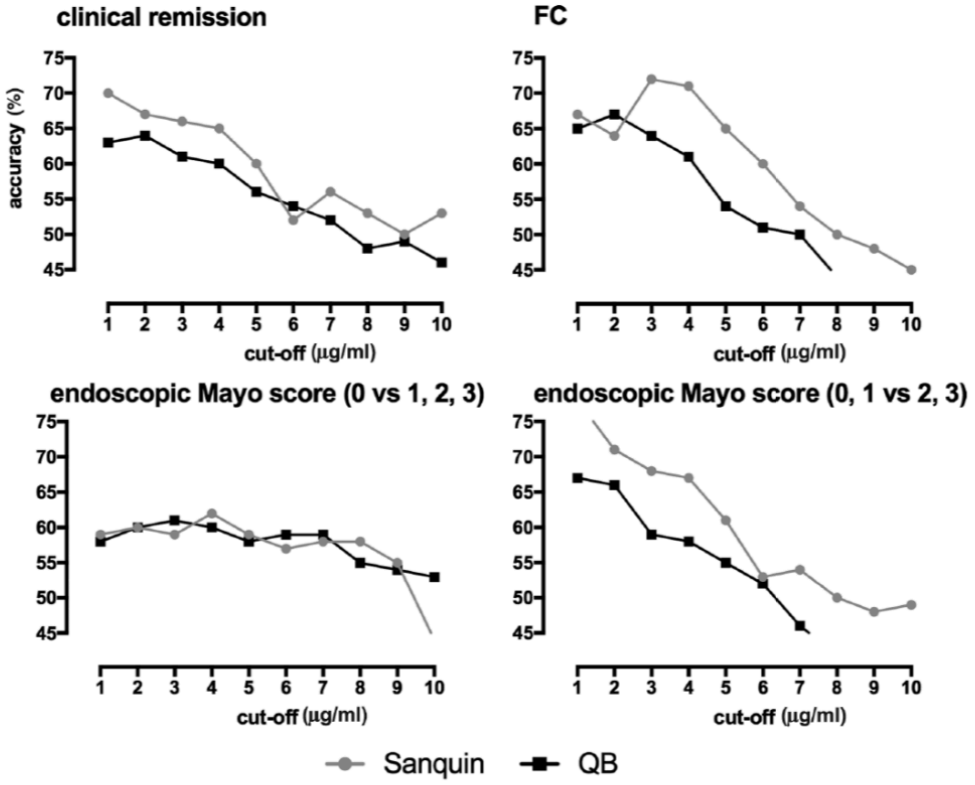

Figure 2. Accuracy values of the different cut-off values (only those between 45–75% represented).
FC, faecal calprotectin; QB, Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Bühlmann, Schönenbuch, Switzerland); Sanquin, Level Infliximab M2920 kit (Sanquin, Amsterdam, the Netherlands); vs, versus.

deep remission (with or without the histological criterion) are depicted in Supplementary Table 2. Although a cut-off of ⩽3 µg/ml seems to be acceptable to assess histological remission irrespective of the kit used, the identification of one specific cut-off in what concerns deep remission is hampered by the overall stability of accuracy across the different cut-offs.

Qualitative comparison between the two different IFX-quantification methods

A qualitative comparison of the assays for a cut-off of 3 µg/ml is depicted in Table 3, and shows an accuracy of 88% and a Kappa (standard error of the mean) of 0.718 (0.070). In fact, the distribution of patients according to a 3 µg/ml cut-off is rather similar between both methods, with only 13 patients (12.0%) being placed differently (they have IFX levels <3 µg/ml when using the rapid QB test, but above that cut-off when using the Sanquin test).

Discussion

Several commercial kits and different protocols have been optimized for an accurate determination of IFX levels from patient serum, but the recent development of a rapid IFX assessment test holds the promise of revolutionizing the TDM-based therapeutic algorithms, by allowing an immediate adjustment of the IFX dosage (as opposed to delaying this intervention to the following infusion cycle). This study aimed to assess the clinical sensitivity and specificity of this rapid assay, by using it to measure samples from 110 patients, fully characterized regarding their clinical, endoscopic and inflammatory burden status.

Table 3. Qualitative comparison between Sanquin and QB assays.

			QB		Total
			⩾3 µg/ml	<3 µg/ml	
Sanquin	**⩾3 µg/ml**	*n*	68	13	81
		%	84.0%	16.0%	100.0%
	<3 µg/ml	*n*	0	26	26
		%	0.0%	100.0%	100.0%
Total			68	39	107

QB, Quantum Blue® Infliximab: Quantitative Lateral Flow Assay (Bühlmann, Schönenbuch, Switzerland); Sanquin, Level Infliximab M2920 kit (Sanquin, Amsterdam, the Netherlands).

The overall results and the clinical stratification obtained using different cut-offs were compared with those obtained using an already validated and widely used IFX-quantification kit (Sanquin).

The results reported here show that although the concentrations obtained by the different methods are strongly correlated, there is a systematic variation: the concentrations measured by the Sanquin kit were, on average, 2.62 units higher than those measured by the rapid QB test, which is consistent with the median IFX values obtained with each method for the entire population. The Sanquin kit's bias towards measuring higher values when compared with other kits has been noticed before.[16,19] Overall, other methodological comparisons involving two or more IFX-quantification assays show that, most of the times, the assays compare quite well against each other (even when they are not ELISA-based), but systematic deviations are rather common and are likely to result from the fact that different assays use different antibodies with varying IFX affinities.[19–25]

The association of IFX serum levels with disease outcomes or inflammatory markers such as clinical response, clinical remission, mucosal healing, endoscopic improvement and C-reactive protein levels have been often reported.[3,5,6,8–10,26] Accordingly, our results show that IFX trough levels were significantly lower when patients had an endoscopic Mayo score ⩾1 or an FC concentration ⩾250 µg/g. A similar pattern was found for clinical remission and for an endoscopic Mayo score >0 (i.e. patients who have a negative outcome had lower IFX trough levels), although in this case the results were not significant. This might be due to the small size of the cohort, or to the fact that the patients analysed were very stable, most of them (80%) in clinical remission according to the Montreal classification and with over 14 weeks of IFX therapy (primary nonresponders were excluded).

Given the systematic differences encountered in the quantification, one would expect the two different methods to have different clinical cut-offs. However, that is not the case: 3 µg/ml is an acceptable threshold for both assays particularly in what concerns assessment of endoscopic status and inflammatory burden (measured by the FC levels). Regarding clinical status, although 3 µg/ml may be a satisfactory cut-off, values of 1 and 2 µg/ml can be considered for the Sanquin and QB assays, respectively. These cut-offs have a marginally better accuracy and smaller NPV when compared with 3 µg/ml. The same holds true for IFX cut-offs of 1 and 2 µg/ml when addressing endoscopic activity using an endoscopic Mayo score ⩽1 as criterion for remission: the accuracy raises and the NPV drops when compared with those of a cut-off of 3 µg/ml. Concerning deep remission, however, the different cut-offs seem to behave similarly and it is not easy to choose a single value. This is likely related to the fact that deep remission is a composite endpoint, and therefore reflects the different behaviours of its components.

The lack of impact of the systematic bias observed in the optimal clinical cut-off is easily explained by observing the Bland–Altman plot: in fact, this plot shows that the differences encountered in the values measured by both methods are particularly close to 0 for IFX levels <5 µg/ml. In other words, at levels as low as those considered for the clinical threshold, the assays seem to behave in a similar fashion. This is supported by the comparative analysis of the assay's results, which shows that for a threshold of 3 µg/ml, 88% of the patients fall equally above or below the cut-off irrespective of the method used.

In practical terms, a clinical cut-off should help a physician decide whether a patient may benefit from an IFX dose adjustment. A cut-off of 3 μg/ml has considerably high NPVs, which means that it can exclude patients from benefiting of an IFX dose adjustment with a considerable degree of certainty. Conversely, the PPVs are rather low, which means that having an IFX trough concentration below the defined cut-off does not necessarily imply having clinical activity, endoscopic lesions or a high inflammatory burden. In other words, not all patients with IFX levels below the cut-off will benefit from a dose intensification, and such a decision must be contextualized with other indicators (such as symptomatology, presence of antibodies to IFX and biomarkers).

The 3 μg/ml (or closer) cut-off has been often referred to in the literature,[3,26,27] but so have lower[8,27–29] and higher ones,[7,8,10,27] showing that cut-offs are deeply related to the method used and outcome being assessed, and studies such as these are absolutely necessary to validate thresholds and explore their interpretability. One word of caution should be added: our results were derived from a UC patient cohort, and are therefore only applicable in the context of UC. In fact, the literature shows several instances in which the parallel analysis of CD and UC patients yields different cut-offs or different behaviours of the same cut-off.[8,27,28]

This study has several strengths that should be noticed, namely its prospective design with a systematic and inclusive evaluation of the therapeutic response; and the fact that all quantifications were performed by the same researcher, and therefore the user can be excluded as a source of variation. On the other hand, and as a limitation, one should point out that the sample size was relatively small, although similar to that used in analogous studies;[5,6,8,20,28] and that the occurrence and amount of anti-IFX in the clinical samples was not taken into consideration.

In conclusion, we have explored the applicability of IFX trough level cut-offs using a recently launched rapid QB test and comparing it with a widely used ELISA kit. Overall, both assays have a good quantitative and qualitative agreement, and a cut-off of 3 μg/ml seems to be appropriate, namely when one is assessing the endoscopic status (using an endoscopic Mayo score = 0 as the criterion for remission) or the inflammatory burden. Different cut-offs can be considered for specific situations, and this ultimately depends on whether the user wants to optimize the accuracy or the NPV of the results.

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Author contributions were as follows: FM: Study concept and design; acquisition of data; analysis and interpretation of data; drafting of the manuscript; study supervision; critical revision of the manuscript for important intellectual content. JA: IFX and faecal calprotectin assays; analysis and interpretation of data. JL, KG and FC: histological analysis. CCD: statistical analysis. All the other authors: recruitment of patients and collection of samples. All authors read and approved the final version of the manuscript.

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Conflict of interest statement

FM served as speaker and received honoraria from Merck Sharp & Dohme (NJ, USA), Abbvie (IL, USA), Vifor (Glattbrugg, Switzerland), Falk (USA), Laboratorios Vitoria (Amadora, Portugal), Ferring (Saint-Prex, Switzerland), Hospira (IL, USA) and Biogen (MA, USA).

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VI. GENERAL DISCUSSION

The discovery of anti-TNF-α agents and the recognised success in the treatment of IBD patients, allowed physicians to aim for more than merely clinical remission, with the control of symptoms. Induce endoscopic remission and mucosa healing are now the new goals of IBD treatment. Despite the success of these agents, some patients do not respond to induction treatment and others lose response over time. Several mechanisms may be related with loss of response, but immunogenicity to the drug itself seems to have an important role. Antibodies to TNF-α are large proteins synthesised by living organisms administrated in considerable doses, and consequently these agents have the ability to invoke the formation of antibodies to the drug. The level of the drug available to block TNF-α and modulate immune response may also impact loss of response. Measuring drug and anti-drug antibodies levels in patients under anti-TNF-α antagonists may help to understand the underlying causes of loss of response.

1. Chapter 1 – Systematic Review on IFX and ADL drug monitoring

A systematic review on this subject was the first step of this research. Studies that presented the following characteristics were identified: a) cut-off levels of both IFX and ADL TL and antibodies to the drug; b) correlation with clinical outcome; and c) detailed information of the assays used. Of the 1237 studies identified through queries only 15 were selected for subsequent analysis. An additional 5 were added for subsequent analysis after searching abstracts from major IBD conferences (ECCO, UEGW and DDW). This work revealed that the cut-offs assessed by ROC curve analysis were significantly different between studies. For drug levels, Papamichaiel et al [95] suggest TL> 12,8 μg/mL to predict short-term mucosa healing; Echarri et al [96] propose TL> 3 μg/mL for sustained remission; and Adedokun et al [97] recommend TL> 41 μg/mL for clinical response (all at week 6/8). Different methodologies may have distinct impacts on results but different studies design, different sample characteristics and different endpoints may also explain discrepancies of threshold levels. In fact, these differences regarding study design and endpoints did not allowed us to perform a meta-analysis.

To our knowledge only one more review and meta-analysis was performed for this topic [98]. The authors included 20 studies and suggested a cut-off of 2 μg/mL for TL to predict remission but a substantial to considerable heterogeneity was obtained (I^2=88%) which compromised this meta-analysis. Nevertheless, Moore et al[98] also concluded that higher levels of TL were associated with a better clinical outcome.

The measurement of anti-drug antibodies also showed significant variability between assays. ELISA methodologies used presented different theoretical principles. As an example, studies using bridging ELISA, considered all samples in the presence of the drug to be inconclusive for the evaluation of antibodies to the drug. Since half of the patients in clinical trials had drug in the serum, the use of bridging ELISA for ADAs may have led to considerable bias. This technique was used in 6 of the 20 studies included in this systematic review.

For ADAs, only 5 studies performed a ROC curve analysis to propose a cut-off and all studies proposed different threshold levels. Steenholdt et al [99], suggested a cut-off of >10 U/mL, while Paul et al [100], proposed >200 ng/mL to predict loss of response. Beyond the already described hindrances for drug cut-offs, it is noted that different assays for ADAs assessment present different units (U/mL; μg/mL; ng/mL) which makes comparation between studies challenging. Furthermore, different assays present different limitations: bridging ELISA present false-negatives (sensible for drug presence) and false-positives (presence of rheumatoid factors or activated complements fragments); and anti-lambda chain ELISA only detects non-functional antibodies. Notwithstanding, all studies reported that lower levels of ADAs were correlated with sustained response to treatment. Nanda et al [101] published a meta-analysis on the impact of ADAs to clinical outcome in IBD patients and concluded that patients positive for ADAs have a threefold higher increased risk to therapy failure compared to those ADAs negative.

Overall, this systematic review showed evidence for determining drug levels in induction period (week 6), throughout the first year (14, 22, 30 and 54) and in maintenance period after the first year in case of loss of response, mucosal ulceration,

and elevated biomarker (CRP and calprotectin). In study I a diagram depicting TDM power to highlight factors influencing loss of response has been proposed. Patients with loss of response and high levels of drug may lose response due to pharmacodynamic factors and/or immunogenicity (when ADAs are also high). Indeed, drug ineffectiveness may be explained by non-TNF-α driven disease or the presence of anti-drug antibodies. For patients with low levels of drug, loss of response may be influence by pharmacokinetic factors (when no ADAs are detected) or due to immunogenicity (when ADAs are present). Insufficient drug bioavailability due to non-immune mediated mechanisms (associated with high inflammatory burden) or due to increase clearance by anti-drug antibodies (mainly neutralizing ADAs) may explain loss of response.

As shown with this systematic review, methodology appears to have a significant impact in the results and consequently in TDM interpretation. Moreover, pharmacokinetics factors and immunogenicity seem to be involved in the augment of intestinal inflammation and consequent therapy failure.

2. Chapter 2 – Influence of Methodology on drug levels and anti-drug antibodies

Awareness of TDM potential led to the development of assays for both drug levels and anti-drug antibodies assessment. As notice in Chapter 1 different studies use distinctive assays which pose a challenge to compare results and define thresholds. Only a few studies have compared different assays. This second chapter was aimed to further the understanding of the impact of different assays on results and consequent biases on TDM interpretation.

2.1. Assays for drug assessment

2.1.1. ELISA assays for drug assessment

In study II, a comparison between three ELISA assays for evaluation of drug levels in IFX treated UC patients was performed. All three assays presented an overall good

correlation between assays. However, Theradiag had an inferior behaviour compared with the other two. Theradiag showed only moderate agreement when compared with both *in-house* and Immundiagnostik ELISAs (ICC=0.694 and 0.762, respectively). IFX-negative samples, *in-house* and Immundiagnostik agreed 100% while Theradiag returned four IFX-positive samples that the other two identified as negative. Casteele et a l[102], also identified similar phenomenon in their work. Theradiag assay identified 11 positive samples that were considered negative by other ELISA assays. All these false positive samples presented high levels of ADAs. Non-specific binding or/and the use of non-specific secondary antibody could explain these results. The use of a more specific IgG antibody in the detection step by some ELISAs was confirmed by study II: *in-house* and Immundiagnostik detected higher levels of IFX than Theradiag. It is important to note that although ELISAs formats are based on the same sandwich principle there are inherent differences to each assay. Different capture and detection antibodies are used. Different assays present different detection limits and test sensitivity. Different assays use different reagents (blocking, washing and diluent buffers) and different calibration standards. Manipulation and processing steps (distinctive incubation times and temperature requirements) vary from assay to assay. Additionally, sample dilution factor differs between ELISAs and usually diluted samples are less influenced by matrix effects. Matrix effects can occur due to the presence of endogenous components (phospholipids and carbohydrates) and endogenous metabolites (bilirubin) or due to interaction between the analyte and the matrix, such as covalent binding to plasma proteins [103]. Solid-phase assays are also more prone to mask epitopes that are naturally present *in vivo* or, on the other hand, to present novel epitopes not present *in vivo*. This solid-phase hindrance may vary from assay to assay and even between-days of analyse [104]. Fluid-phase assays, such as RIA and HMSA, mimic better *in vivo* conditions as epitopes are free to bind to capture and detection antibodies. These assays are more prone to measure functional bioactive drug concentration. HMSA allows to distinguish free drug from drug-ADAs complexes, due to chromatographic separation of samples compounds. Bodini et al [105], compared HMSA and ELISA assay and

reported that HMSA presented consistently higher ADL TL than ELISA, which may indicate a higher sensitivity of HMSA assay. As HMSA includes an acid dissociation step, this assay measures both circulating drug and drug entirely neutralized by ADAs. Important to note that RIA and HMSA demand specialised human resources and facilities, and RIA requires the use of radioisotopes.

2.1.2. Rapid tests for drug assessment

A lateral flow immunochromatographic assay for drug TL evaluation was developed and launched recently. This method is considered a rapid assay with a result turnover of only 15 minutes. Optimizing treatment with TDM approach implies that clinicians have access to drug and anti-drug level results on the shortest time span. The use of a rapid test allows clinicians to optimize infliximab treatment immediately as opposed to optimize treatment in the following infusion (6-8 weeks). Indeed, rapid test assays returns results in only 15 minutes, while an ELISA assays takes approximately 8 hours. Moreover, ELISA-assays require around 40 samples to be performed, highly trained professionals and specific laboratory facilities. Rapid test may be performed by medical professionals and no special facilities are required. The use of a rapid test rather than an ELISA assay contributes to a fast and more personalized treatment of the patient avoiding months of delay of successful treatment. Study III was the first study comparing two ELISA assays and the newly launched rapid assay Quantum Blue (QB) from Buhlmann, Switzerland. Infliximab concentration of 299 samples from IBD patients receiving IFX in maintenance phase and IFX-spiked samples were analysed by the three assays. The rapid test was the most precise among the methods tested, with a recovery of 92% and the smallest intra-variability. The intraclass correlation coefficient (ICC) for the paired comparisons revealed the highest ICC for the pair QB rapid test – Sanquin ELISA (ICC: 0.939). Nevertheless, Sanquin measurements were consistently higher than QB rapid test (mean differences: 0.92 μg/mL, 95% CI: 0.54-1.30).

This was also observed in study IV and VII, where the mean differences and interval of confidence (CI) showed that the concentrations obtained with Sanquin assay were, on

average, higher. Systematic overestimation of Sanquin's assay was also reported by Ruiz-Argüello [106]. The rise in the magnitude of IFX concentration may be explain by non-specific binding. Non-specific binding was more pronounced in study IV, where assays for originator infliximab were tested for the assessment of levels of CT-P13 and an unstable behaviour of Sanquin was reported. Sanquin ELISA underestimated CT-P13 levels consistently below 5 μg/mL and overestimated for concentrations between 5-20 μg/mL. For the concentrations below 5 μg/mL poor recovery rates were obtained (between 17-44%). In this study, Sanquin showed also the lowest correlation coefficients when compared with the other methods. Sanquin ELISA presents acceptable results for infliximab originator assessment but according to our work is not recommendable for use with infliximab biosimilar, CT-P13.

For better evaluation of drug assays behavior in the clinical decisions made when TDM is applied, qualitative analyses were performed in study III and study IV. Patients results were placed between a certain range of IFX concentration (<3μg/mL; [3-7μg/mL]; >7μg/mL). Drug levels between 3-7μg/mL have been suggested by authors of the TAXIT study [107] as an optimal therapeutic window during the maintenance phase. Although an overall agreement was identified between the rapid test and ELISA assays (study III and IV), some disagreement occurred - 31,9% of samples considered higher than 7 μg/mL by the *in-house* were considered between 3-7 μg/mL by the rapid test (study III). Consequently, it is important to note that TDM approach must be integrated in patient's clinical context, considering the presence of symptomatology and other disease markers to help with the decision process.

Studies II-V showed that although assays for drug assessment present an overall good agreement there is a noteworthy drug concentration variability between assays. Clinicians may be aware of this variability when measuring drug levels. The same assay should be used to measure drug levels for the same patient every time a measurement is necessary.

2.2. Assays for anti-drug antibodies assessment

Assessment of ADAs is usually more complex than drug levels evaluation due to the presence of the drug itself. In study II three different ELISAs formats were compared: an *in-house* anti-human lambda chain assay (AHLC), a semi-fluid phase enzyme immunoassay (SFPE) from Immundiagnostik and a bridging ELISA from Theradiag. Overall comparison showed a moderate to substantial agreement between assays (KAPPA: 0.602-0.692) but Theradiag only detected ADAs in 24 samples (30%) while both *in-house* and Immundiagnostik detected ADAs in 34 samples (43%). Theradiag ELISA uses a double-antigen bridge for ADAs detection. False negatives may arise due to the presence of the drug. Theradiag uses labelled IFX as detection antibody, the presence of the drug may compete with the detection of ADAs. Theradiag ELISA is also unable to detect monovalent IgG4 ADAs. Of the 8 samples ADAs-negative by Theradiag and positive with both *in-house* and Immundiagnostik, 5 were positive for IFX and 3 presented IgG4 ADAs. Contradictorily, Theradiag presented ADAs-positive in the presence of the drug. Though, those 3 samples were considered IFX negative by the other two assays, suggesting that these samples were probably false positive for IFX, due to non-specific binding. The high incidence of false-negatives results by Theradiag was reported by other authors. Kopylov et al[92] described that bridging ELISA format was unable to detect ADAs in the presence of the drug. Our study confirmed the inability of Theradiag to detect positive ADAs in samples with levels of IFX higher than 5 μg/mL and established the concentrations at which IFX decrease the capacity of the assay to detect ADAs. *In-house* and Immundiagnostik were able to detect ADAs in the presence of IFX up to levels of 100 μg/mL, which correspond to the therapeutic concentrations of patients under IFX. Still, Immundiagnostik was the assay less influenced by the presence of drug in the sera. This may be explained by the initial acid buffer treatment this assay uses to dissociate IFX-ADAs immune complexes.

Surprisingly, our study revealed that for high concentrations of ADAs (>25 μg/mL) the presence of drug was less significant and even Theradiag was able to detect antibodies.

Our work demonstrate that assays are not only limited by the levels of drug in the sera but also by the concentration of ADAs.

2.3.Clinical impact of methodologies biases

Low levels of the drug and/or presence of ADAs have been associated with loss of clinical response. Depending on the binary status (drug levels/ADAs levels) of sera from patients under anti-TNF-α different clinical decisions are taken. In study II the impact on clinical decisions was evaluated when different assays are used to defined binary status of sera of patients. A total (100%) disagreement was obtained when comparing Theradiag with Immundiagnostik or with *in-house* for double positive status (IFX +/ADAs+). The result for double negative status (IFX-/ADAs-) was very similar, showing an agreement of only 50% and 55.6% when comparing Theradiag with Immundiagnostik and *in-house*, respectively. Erroneous therapeutic decisions may occur when patients show double-positive or double negative status. Treatment intensification in a double negative scenario and change of drug class and concomitant use of immunomodulators in a double positive scenario should take into account that these results are assay dependent. Nevertheless, for IFX -/ADAs + or IFX+/ADAs- a strong agreement was observed (75.6-100%) and probably the choice of the assay will have little influence on therapeutic decisions to change class of drug (IFX+/ADAs-) or change anti-TNF-α antibody (IFX-/ADAs+). In study I, a diagram was proposed to distinguish factors influencing loss of response using a binary status. These new findings show us that careful must be taken when interpreting TDM results, regarding the double positive and negative status.

The inability of some assays to determine ADAs in the presence of the drug may hindrance clinical interpretation of TDM. Some authors support the idea that there is no advantage to determine ADAs in the presence of the drug, claiming that ADAs most significant effect is mediated through their impact on drug clearance [79]. However, Casteele et al [108], reported that patients with IFX>3 μg/mL and ADAs positive have

significant higher levels of C-reactive protein (CRP) and less mucosal healing indicating a reduced control of inflammation mediated by the presence of ADAs. Authors suggest that ADAs neutralize drug activity through direct binding to drug epitope. Kopylov et al [92] indicate that the subgroup of patients with double positive status may indicate a growing immune response to IFX resulting in future low drug TL and loss of response. Double positive status may also occur due to the presence of transient antibodies. Around 30% of patients present ADAs that disappear at subsequent reassessment during IFX maintenance [81, 109]. These transient antibodies have been described to have little clinical significance since they are not correlated with loss of response. Casteele et al [109] suggested that some patients treated with anti-TNF-α, that initially produce ADAs, afterward develop tolerance toward the drug. Tolerance is mediated trough an activation of the regulatory immune response. Regulatory T-cells are activated through recognition of regulatory T-cells epitopes on the Fc part of anti-TNF-α. However, antibodies may be also considered transient due to false negatives at the time of reassessment. As showed in study II assessment of ADAs is largely dependent of the presence of the drug and the levels of the ADAs itself. Variations on drug levels and ADAs at time of sampling may impact methodology ability to detect ADAs. Still, ADAs transiency have been reported by several studies, reinforcing the idea that in some cases ADAs are indeed present in circulation only transitorily. This may impact interpretation of TDM and lead to misinterpretations and therefore integrate TDM with clinical context and biomarkers is essential. One should also be aware that transient ADAs were described to appear in lower levels than persistent antibodies. Besides, persistent antibodies are usually detectable within the first year while transient ADAs may develop after years of anti-TNF-α therapy [81].

2.4.Assays for biosimilars

Biosimilars to infliximab, CT-P13 and SB2 have recently been introduced in the treatment of IBD patients. The use of TDM to dose adjusting and to facilitate physician

decision process is not an issue only to patients under originator infliximab but also to those treated with biosimilars. All assays for drug and anti-drug antibodies evaluation, available in the market, were developed for the originator anti-TNF-α antibody. It is important to guarantee that the assays used to measure originator IFX are accurate to measure biosimilars of IFX. Notwithstanding having the same amino-acid sequence, originator IFX and biosimilars may have post-translational differences (for instance, glycosylation). Study IV and V compared methodologies developed for originator IFX measurements and their performance was evaluated to determine levels of CT-P13 and SB2. Commercial ELISA kits, an *in-house* ELISA kit and a rapid test were tested. Samples of IBD patients under CT-P13 and serum samples spiked with known concentrations of CT-P13 and SB2 were evaluated. Both CT-P13 and SB2 had an overall good recovery rate for all kits although some systematic differences were observed particularly in one assay. These studies showed that for CT-P13 evaluation, an unstable behaviour was observed with Sanquin assay. This assay presented the lowest median concentration (2.6 μg/mL) but consistently higher values than the other methods. As already mention above in this discussion, our results show that it is not recommended to use this assay for CT-P13 measurement. The other assays presented high ICC and good accuracies with minor systematic differences with no implication on the overall performance of the assays. These differences are likely to occur due to the use of different antibodies with varying IFX affinities, mainly through epitope disposition and exposure.

These studies also evaluated the agreement between assays applying the TAXIT proposed therapeutic window (3-7 μg/mL and eight other possible cut-offs (3, 4, 5, 6, 7, 8, 9 and 10 μg/mL) and results presented good agreement. Kappa tend to decrease as the cut-offs decrease likely in result of a higher dispersion of measurements in higher concentrations. This dispersion in higher concentrations (> 15 μg/mL) was observed in Blandt-Altman plots throughout all assays and both originator IFX and biosimilars (study III-V).

In study V, we have used donor's samples spiked with known concentrations of SB2 but also of CT-P13 and of originator IFX. No clinical samples of patients under SB2 therapy were available since this drug was not commercialized in Portugal at the time of the study. R-Biopharm assay and QB rapid test were slightly more accurate when measuring CT-P13 than the originator infliximab. *In-house* was more accurate in the case of SB2. Regardless the minor differences between assays that are also observed in this study, probably due to dissimilarities in the compounds manufacturing (e.g. different cell lines), storage and transport, both ELISA R-Biopharm and *in-house*, and the QB rapid test can be used to monitor the biosimilars SB2 and CT-P13.

Anti-TNF-α antibodies may invoke immunogenicity due to minor differences in manufacturing process [110]. Biosimilars are highly similar to its biologic originator but small differences in clinically inactive components have been described [111]. Lee et al[44] showed that SB2 had lower percentage of charged glycan than IFX while CT-P13 had higher percentage of galactosylated glycan than the other two drugs. Nevertheless, CT-P13 was proved to invoke immunogenicity in the same proportion of patients that IFX [112]. Instead, SB2 showed higher rates of ADA than IFX in clinical equivalent studies [110]. Little is known about bioequivalence of CT-P13 and SB2 regarding cross-immunogenicity to IFX and potential immunogenic adverse events. Cross-immunogenicity between IFX, CT-P13 and SB2 have been assessed. In study V, sera IFX-ADAs-positive and CT-P13-ADAs-positive were tested against IFX, CT-P13 and SB2. Results showed ICC between different drugs' reaction to anti-IFX and anti-CT-P13 sera was close to 1.0 (0.986-0.993) showing high cross-immunogenicity. Ben-Horin showed the same high cross-reactivity between IFX ADAs and CT-P13 [113]. These results reinforce the similarity among these drugs and the idea that these drugs probably share a common epitope. Patients medicated with IFX or CT-P13 and that developed ADAs, presenting adverse events or therapy failure will not benefit from switching to IFX, CT-P13 or SB2.

3. Chapter 3 –Pharmacokinetic factors of anti-TNF-α antibodies and clinical targets

This chapter aimed to address PK factors of anti-TNF-α antibodies and clinical targets and clarify TDM and Biomarker role to identify reasons for therapy failure.

Study VII explored the association between the serum TL and clinical targets such as clinical remission, endoscopic remission and degree of inflammation. This study included 110 UC patients on maintenance phase of IFX therapy. Clinical, endoscopic evaluation and calprotectin quantification was assessed at the same time as IFX TL concentration. The results showed that IFX TL were higher in patients who had positive outcomes. Moreover, a significative association was found between higher levels of IFX TL and endoscopic remission (Mayo score <1) and faecal calprotectin (FC<250μg/g). Association between levels of the drug and clinical outcomes have been described in some studies, as shown in Chapter 1, mostly relating higher levels of the drug with a better response to treatment.

In study VI, the correlation between drug levels and the clinical targets (clinical, endoscopic and histologic outcome) in UC patients that were asymptomatic, i.e., that were clinically stable was also investigated. Sixty-five UC patients under IFX therapy in maintenance phase (6-8 weeks-interval infusions) were included in this study. Patients were followed during one IFX infusion cycle. Those patients included in the 6-week regimen had also started with an 8-week regimen but were empirically changed to a shorter interval due to loss of response. Different regimens presented similar AUC and clearance values but IFX TL values were significantly higher in the 6-week regimen ($TL_{6\text{-}weeks}$=5.00 μg/mL vs $TL_{8\text{-}weeks}$=2.43 μg/mL, p=0.006). Infliximab TL increase allowed 6-weeks patients to regain response to treatment. For ADAs levels no significant difference was found between regimens, however the presence of ADAs was correlated with a higher clearance. This was observed when patients were grouped by IFX TL vs ADAs

status (positive/negative). ADAs was the only independent predictor affecting clearance. Important to stress that this population was clinically stable at the time of study inclusion which may explain why other variables, such as albumin, height, weight and inflammatory burden appear not to affect significantly clearance. Indeed, body weight was described to increase clearance in the presence of a higher inflammatory burden possibly due to contribution of mesenteric adipose tissue as source of TNF-α production [72]. Albumin also has showed to correlate to clearance in previous studies when systemic inflammation is present [114]. It is not clear if albumin is merely a marker of inflammatory activity or if it affects PK of anti-TNF-α antibodies by increasing the number of FcRn receptors at high albumin levels. Loss of albumin may also occur through faecal loss due to intestinal permeability augmented during active inflammation [72]. A high inflammatory burden was also associated to increase clearance. Systemic inflammation leads to an increase in IgG catabolism clearance through Fcγ receptors, internalization and proteolytic degradation. When intestinal inflammation rises, as mention above, leads to an increase in intestinal permeability and consequent loss of anti-TNF-α antibodies through faeces.

In this study patients were clinically stable at the beginning of the study and only 17.5% presented macroscopic lesions at the end of the infusion cycle. This study revealed that in clinically-stable UC patients ADAs are the main factor influencing PK of anti-TNF-α antibodies.

The overall low disease activity presented by this population may also be a reason for IFX TL, ADAs, AUC and clearance were not able to discriminate patients with positive and negative outcome.

This population was also evaluated for their long-term risk of requiring therapeutic escalation. Patients with higher ADAs (cut-off of 3 μg/mL) were more prone to the need of escalating therapy later in disease development -. Additionally, faecal calprotectin of 250 μg/g was also correlated with the need to undergo therapeutic escalation. When a patient simultaneously present ADAs>3 μg/mL and FC>250 μg/g the risk of a future

therapeutic escalation increases. This study shows that the presence of ADAs eventually leads to a drop in IFX levels below a minimum concentration and consequent loss of response.

Interestingly, the results show that faecal calprotectin is able to supervise a potential loss of response and is more useful to predict the need for future escalation then disease severity presented by endoscopic or histologic scores. Noteworthy, no significantly association was found between low levels of IFX and the need to escalate therapy, showing that even in the presence of adequate drug levels some patients need to escalate therapy. The presence of ADAs was described by Ungar et al[81] to precede the onset of clinical flare, suggesting that a lag time may occur between the presence of immunogenicity, IFX TL decrease and the resurgence of tissue inflammation. Likewise, local tissue inflammation in the presence of therapeutic serum levels of anti-TNF-α antibodies was previously described [115]. Authors suggest that high levels of TNF-α in local inflamed tissue may act as a sink for anti- TNF-α antibodies.

Serum TL and ADAs assessment in IBD patients under anti-TNF-α therapy may have an important role guiding physicians through therapeutic management. Higher TL have been associated with better clinical response whereas higher ADAs with inferior response to treatment. The TAXIT study was the first study to compare proactive concentration-based dosing with clinically-based dosing and showed little benefit for TDM. Nevertheless, concentration-based dosing was associated with fewer flares during treatment [107]. Steenholdt, et al[116] showed that using an algorithm TDM-based for therapeutic adjustment rather than clinically-based approach in patients with secondary loss of response significantly reduces treatment related costs with the same clinical efficacy. The American Gastroenterological Association recently published guidelines for TDM in IBD recommending reactive TDM to guide treatment changes [117], but claim a gap in knowledge to use TDM in quiescent disease. The pharmacokinetic study (study VI) showed that IFX TL and ADAs levels were not able to differentiate clinical, endoscopic and histologic outcome of a clinically-stable population but showed a

significant contribute of ADAs assessment to predict risk of therapeutic escalation. The results indicate a strong advantage to include TDM (determination of ADAs) on stable patients alongside with FC assessment. Indeed, even in the absence of clinical symptoms, the presence of both elevated ADAs and FC levels should alert the physician to act in order to prevent future therapeutic escalation. Robin et al also showed that a combination of CRP levels, IFX TL and persistent ADAs may help to predict loss of response in IBD patients [73]. Furthermore, the CALM study points to the importance of biomarkers as predictors of treatment response and considers FC a potential target to avoid disease progression and development of serious complications. FC was described to predict relapses in quiescent UC and DC patients [118] which stresses the need to use FC determination as a complement to TDM approach. In Chapter 1 it was highlighted the need to assess drug levels in different points of anti-TNF-α therapy, considering ADAs assessment as supplementary evaluation to discriminate PK and PD factors of loss of response. These new findings show the importance of assessing ADAs when FC rises even in clinically stable patients.

Conversely, study VII showed that some patients may also benefit of drug TL determination since TL were associated disease activity. This study comprises almost the double of patients of study VI and included non-clinically and clinically stable UC patients. In study VI, all patients were in clinical remission according to Montreal classification, at inclusion time, while in study VII, twenty percent showed mild to moderate disease activity. For study VII, our results show that IFX TL were higher in patients who had a positive outcome independently of the assay used. As seen in chapter 1 and 2, cut-offs vary between studies and different methodologies may lead to different cut-offs. Study VI aimed to evaluate the clinical sensitivity and specificity of two different methodologies, an ELISA and a rapid test, by proposing IFX cut-off values able to guide the therapeutic decision-making process. A cut-off of 3 μg/mL, independently of the assay used, was considerate the best cut-off to discriminate endoscopic remission and low degree of inflammation in UC patients. A cut-off of 3 μg/mL presented significantly higher NPVs and rather low PPVs. A high NPV means that having an IFX

TL above 3 μg/mL can exclude patients from benefiting of an IFX dose adjustment with a certain degree of certainty. On contrary, low PPV indicates that patients with IFX TL below 3 μg/mL do not necessarily present clinical activity, endoscopic lesions or high inflammatory burden and may not necessarily benefit from a dose intensification. Careful must be taken when applying cut-offs alone to take therapeutic options. Therapeutic drug monitoring in IBD must be always contextualized with other markers such as symptomatology, presence of ADAs and biomarkers.

VII. CONCLUSIONS

Therapeutic Drug Monitoring-based strategy should be recommended in scenarios of a) loss of response guiding physicians to discriminate among PK and PD reasons for therapy failure; and b) predict loss of response combining TDM with biomarkers assessment, namely, calprotectin, in stable patients. TDM management must be constantly integrated with identification of methodological related biases and with individual clinical evaluation. Physicians should be aware of assays limitations. Both drug and ADAs must be assessed by the same assay throughout time. If the use of the same assay is not possible, physicians should be informed of potential methodological biases in results interpretation.

Major findings of this thesis are summarized as follows:

- Methodology has a significant impact in TDM, especially TNF-α antibodies assays:
  - Impact of IFX on levels of anti-IFX antibodies is different for different assays. The amount of anti-IFX antibodies can also influence the impact of IFX;
  - Serum samples double positive (IFX^+/anti-IFX^+) and double negative (IFX^-/anti-IFX^-) lead to higher disagreement between assays;
- New methodologies (rapid tests) can safely substitute old methodologies (ELISAs) in TDM;
- Not all Infliximab originator-optimized quantification assays can be used to measure IFX-biosimilars in an accurate fashion:
  - ELISA Sanquin can be used to assess IFX originator levels, but we don't recommend it for IFX biosimilar CT-P13;
- There is cross-immunogenicity between Infliximab originator and both IFX biosimilars, CT-P13 and SB2;
- Antibodies to the drug is the main factor influencing pharmacokinetic of IFX in clinically-stable UC patients:
  - IFX TL, AUC and clearance are less important than antibodies to IFX or the degree of inflammation (calprotectin) for therapeutic escalation in clinically-

stable UC patients;

- There is a strong advantage to include TDM (determination of ADAs) on stable patients alongside with FC assessment in clinically-stable UC patients;

- IFX TL correlate with endoscopic remission and low degree of inflammation in UC patients indicating that TDM may be used to discriminate PK and PD reasons for therapy failure:
  - A cut-off of 3 $\mu g/mL$ seems to be appropriate for endoscopic remission and low degree of inflammation, using both rapid test and ELISA assay, in UC patients on maintenance phase of IFX.

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VII. ANNEX

Study III - Proactive therapeutic drug monitoring of infliximab: a comparative study of a new point-of-care quantitative test with two established ELISA assays

Supplementary Table 1. Spearman correlation coefficients excluding samples >20 µg/mL according to the point-of-care QB assay.

		in-house	Sanquin	QB
in-house	Correlation coefficient	1.000	.919**	.907**
	P-value	-	.000	.000
	N	299	299	276
Sanquin	Correlation coefficient	.919**	1.000	.942**
	P-value	.000	-	.000
	N	299	299	276
QB	Correlation Coefficient	.907**	.942**	1.000
	Sig. (2-tailed)	.000	.000	-
	N	276	276	276

Study IV - Therapeutic drug monitoring of CT-P13: a comparison of four different immunoassays

Supplementary Table 1: Intraclass correlation coefficient stratified by therapeutic phase (induction* vs maintenance).

	ICC		Differences		
	ICC	IC 95%	average	IC 95%	
***in house*-Sanquin**					
Induction	0.660	0.397-0.808	-7.70	-10.56	-4.84
Maintenance	0.607	0.431-0.728	.08	-.96	1.12
***in house*-QB**					
Induction	0.857	0.747-0.919	-3.30	-4.71	-1.89
Maintenance	0.846	0.777-0.893	-.60	-1.24	.05
***In house*-r-biopharm**					
Induction	0.842	0.719-0.911	-.12	-1.35	1.10
Maintenance	0.877	0.822-0.915	-1.33	-1.91	-.74
Sanquin-QB					
Induction	0.842	0.720-0.911	4.40	2.24	6.56
Maintenance	0.860	0.797-0.903	-.68	-1.38	.03
Sanquin-r-biopharm					
Induction	0.622	0.330-0.787	7.58	4.74	10.41
Maintenance	0.791	0.698-0.856	-1.41	-2.25	-.57
QB-r-biopharm					
Induction	0.897	0.818-0.942	3.18	2.08	4.28
Maintenance	0.930	0.898-0.951	-.73	-1.20	-.26

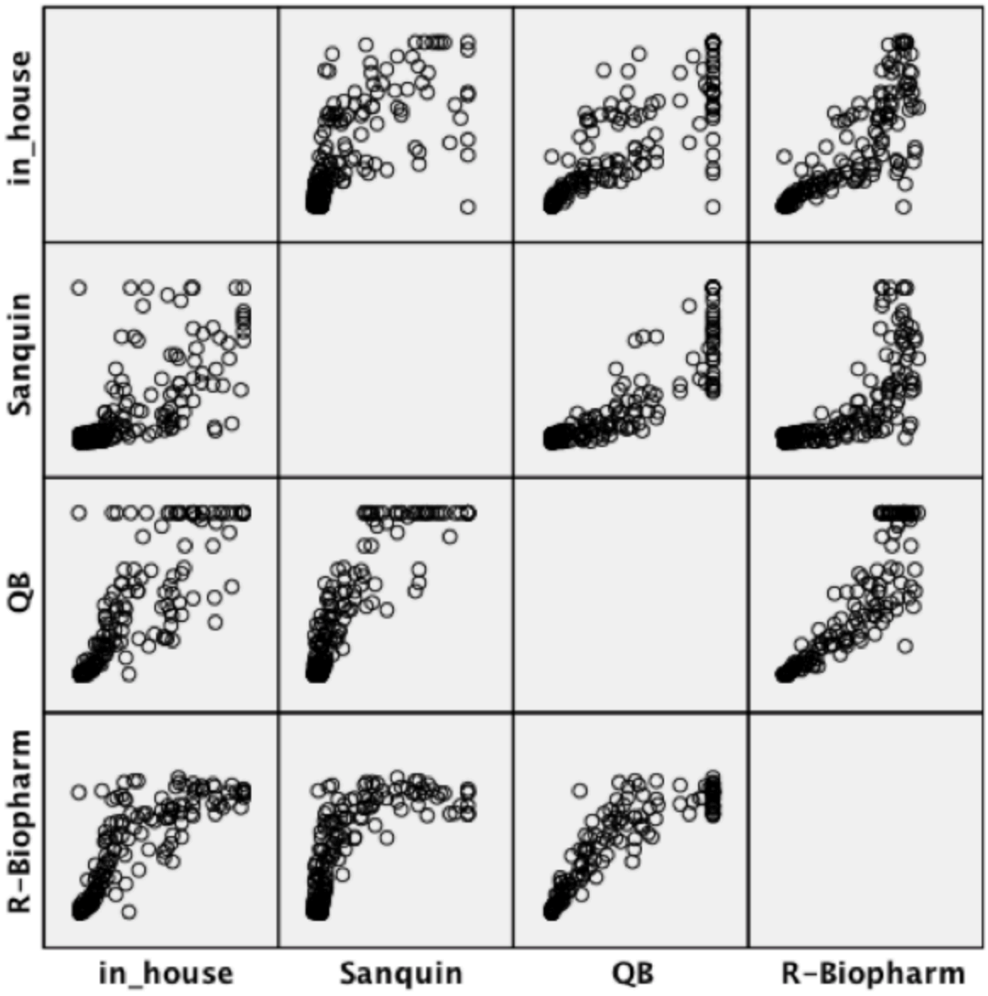
* samples taken before the first IFX infusion were excluded from this analysis.

Supplementary Table 2: Qualitative comparison between the CT-P13 quantification assays using the therapeutic interval 3-7 (i.e., <3, [3-7[, and ≥7) and stratified by therapeutic phase (induction* vs maintenance).

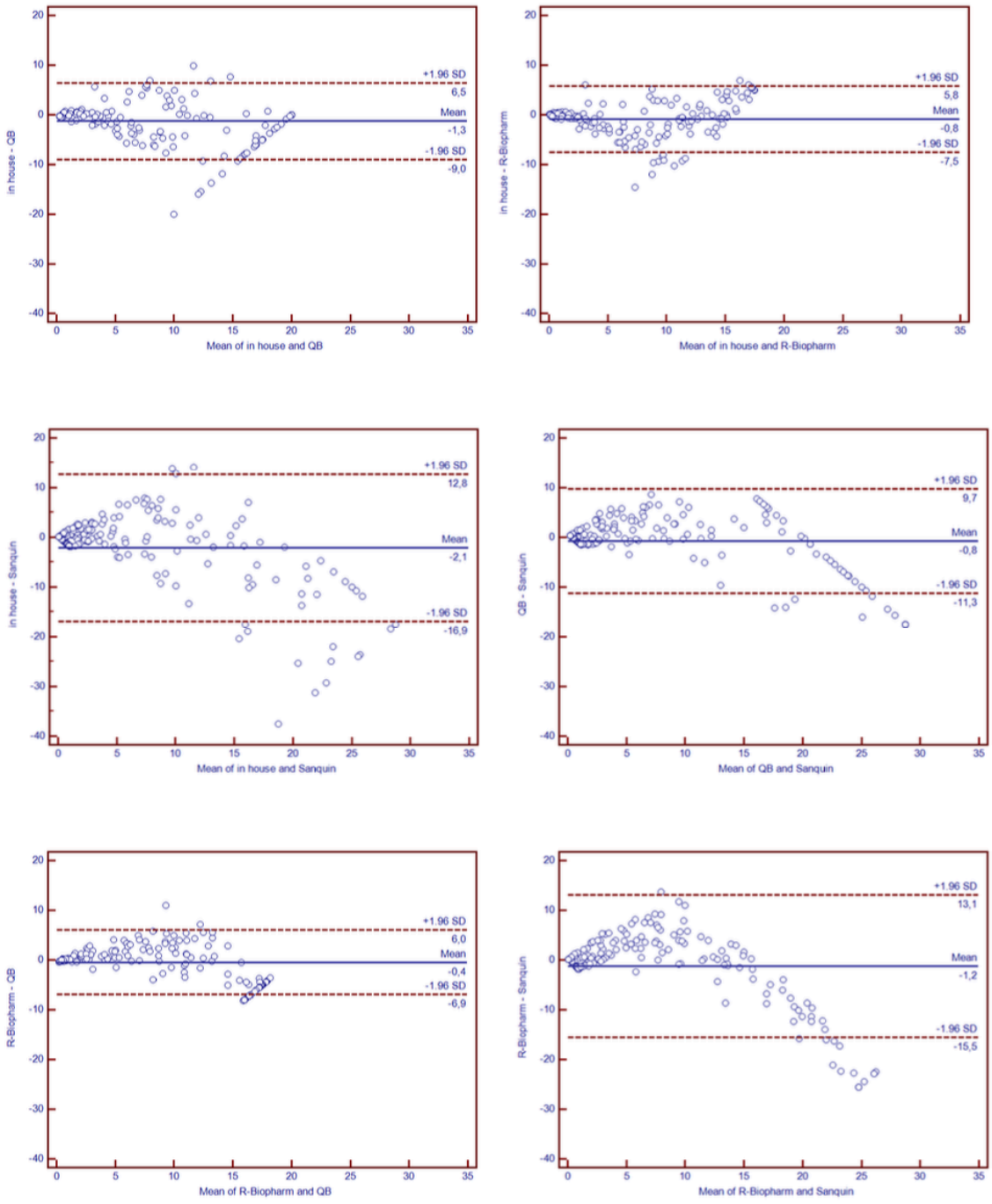
	Accuracy	Kappa [CI 95%]
		in house
Sanquin	**75%**	**0.597 [0.496-0.698]**
Induction	84%	0.670 [0.441-0.899]
Maintenance	68%	0.578 [0.468-0.688]
QB	**80%**	**0.776 [0.177-0.840]**
Induction	89%	0.785 [0.587-0.982]
Maintenance	71%	0.676 [0.585-0.767]
r-biopharm	**77%**	**0.752 [0.685-0.819]**
Induction	91%	0.817 [0.630-1.000]
Maintenance	67%	0.639 [0.545-0.733]
		Sanquin
QB	**80%**	**0.671 [0.577-0.766]**
Induction	92%	0.857 [0.725-0.989]
Maintenance	71%	0.653 [0.548-0.758]
r-biopharm	**77%**	**0.622 [0.522-0.721]**
Induction	92%	0.818 [0.637-0.999]
Maintenance	66%	0.570 [0.461-0.679]
		QB
r-biopharm	**88%**	**0.874 [0.824-0.922]**
Induction	98%	0.959 [0.878-1.000]
Maintenance	81%	0.799 [0.723-0.876]

* samples taken before the first IFX infusion were excluded from this analysis.

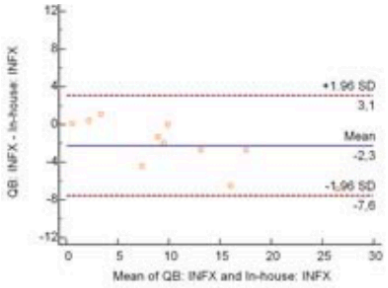
Supplementary Fig. 1

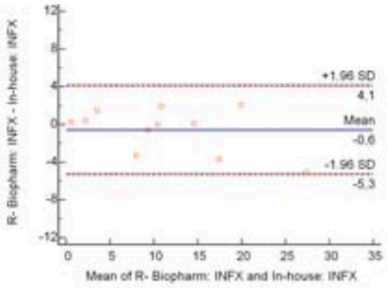


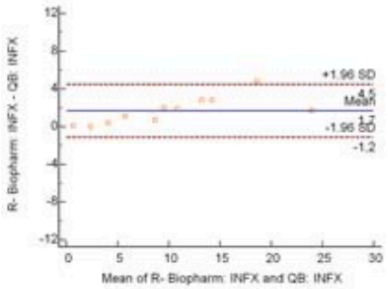
Supplementary Fig. 2

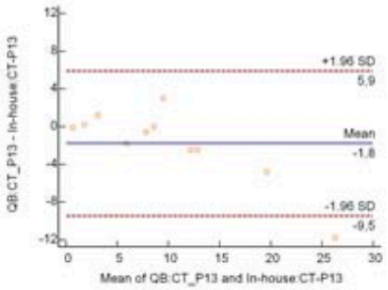


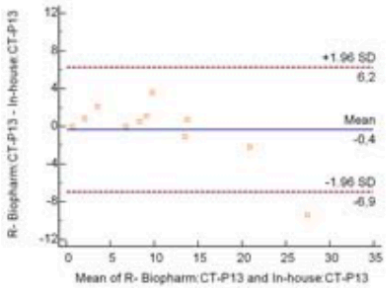
Study V - The performance of Remicade®-optimized quantification assays in the assessment of Flixabi® levels

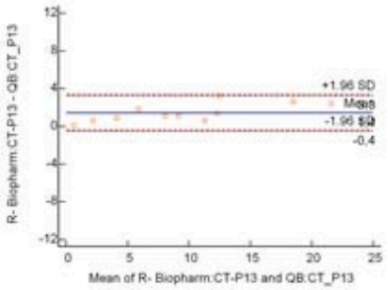


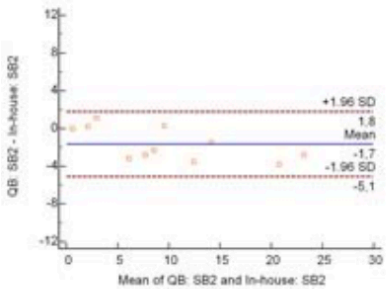


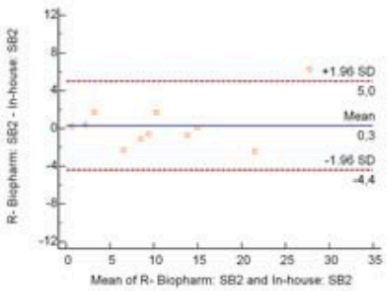


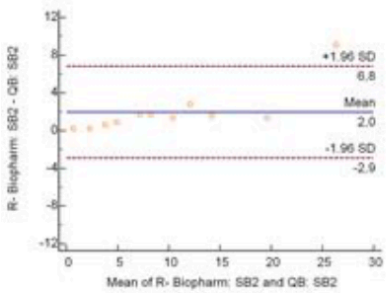




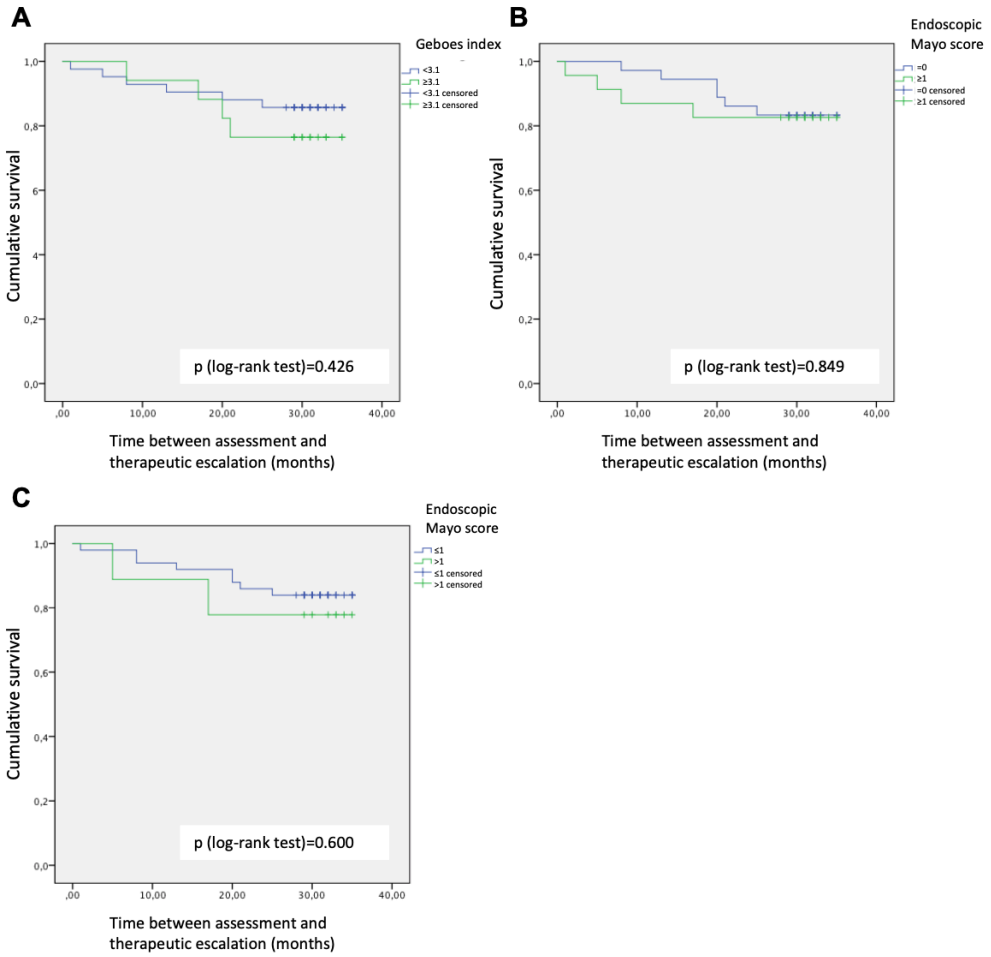








Study VI - Calprotectin and the Magnitude of Antibodies to Infliximab in Clinically-stable Ulcerative Colitis Patients are More Relevant Than Infliximab Trough Levels and Pharmacokinetics for Therapeutic Escalation



Supplementary Fig.1 Kaplan-Meyer survival curves for time to therapeutic escalation for histological inflammation (A) and endoscopic lesions (B and C).

Supplementary Table 1

		Multivariable OR[1] [95%CI]	
Height (m)	391.617	-147.717;930.951	0.149
Weight (kg)	1.063	-1.391;3.516	0.385
Albumin	0.645	-6.157;7.447	0.848
[ATI] µg/ml	**12.196**	**2.417;21.974**	**0.016**
Endoscopic Mayo			
0	Ref		
≥1	17.349	-44.075;78.774	0.570

[1]All variables were included using the "enter" method; R^2=0.300

Supplementary Table 2

		Multivariable OR[1] [95%CI]	
Height (m)	409.089	-131.029;949.208	0.133
Weight (kg)	1.035	-1.431;3.501	0.400
Albumin	0.711	-6.146;7.569	0.834
[ATI] µg/ml	**12.086**	**2.131;22.042**	**0.019**
Endoscopic Mayo			
≤1	Ref		
>1	6.379	-75.039;87.796	0.874

[1]All variables were included using the "enter" method; R^2=0.294

Supplementary Table 3

		Multivariable OR[1] [95%CI]	
Height (m)	419.753	-144.415;983.920	0.140
Weight (kg)	1.003	-1.539;3.545	0.428
Albumin	1.023	-6.312;8.357	0.778
[ATI] µg/ml	**12.452**	**1.432;23.472**	**0.028**
Geboes index			
<3.1	Ref		
≥3.1	-2.910	-83.981;78.161	0.942

[1]All variables were included using the "enter" method; R^2=0.294

Supplementary Table 4: IFX TLs

		P25	Median	P75	n	p-value
Global Mayo score	**no remission**	1.82	3.19	4.94	18	0.914
	remission	1.28	2.91	7.16	44	
Endoscopic Mayo score	**0**	1.28	3.20	7.85	36	0.786
	≥1	1.94	3.11	5.00	24	
Endoscopic Mayo score	**≤1**	1.36	3.03	6.37	49	0.901
	>1	2.19	3.18	4.94	11	
UCEIS	**remission (≤1)**	1.19	2.99	6.37	45	0.533
	activity (>1)	2.19	3.21	5.72	15	
Histology (Geboes score)	**<3.1**	1.46	3.01	6.37	42	0.815
	≥3.1	.77	3.03	5.72	19	
FC (µg/g)	**<150**	1.19	2.91	5.05	46	0.286
	≥150	2.37	3.33	5.72	14	
FC (µg/g)	**<250**	1.36	3.03	5.05	53	0.636
	≥250	1.91	2.56	8.87	7	

Supplementary Table 5: ATIs

		P25	Median	P75	n	p-value
Global Mayo score	**no remission**	.79	1.05	3.12	19	0.797
	remission	.84	1.45	2.05	45	
Endoscopic Mayo score	**0**	.84	1.53	2.05	37	0.600
	≥1	.79	1.05	2.94	25	
Endoscopic Mayo score	**≤1**	.83	1.32	2.18	51	0.706
	>1	.70	1.05	3.50	11	
UCEIS	**remission (≤1)**	.83	1.51	2.48	47	0.253
	activity (>1)	.72	.93	2.88	15	
Histology (Geboes score)	**<3.1**	.84	1.32	2.03	43	0.953
	≥3.1	.72	1.10	3.33	20	
FC (µg/g)	**<150**	.88	1.52	2.82	48	0.072
	≥150	.64	.99	1.25	14	
FC (µg/g)	**<250**	.83	1.44	2.76	55	0.794
	≥250	.72	1.15	2.48	7	

Supplementary Table 6: AUC (6 weeks' regimen)

		P25	Median	P75	n	p-value
Global Mayo score	**no remission**	1243.00	1328.00	1789.00	5	0.517
	remission	1199.00	1645.00	2150.00	14	
Endoscopic Mayo score	**0**	1186.00	1724.50	2150.00	10	0.683
	≥1	1243.00	1448.00	1789.00	9	
Endoscopic Mayo score	**≤1**	1199.00	1570.00	2150.00	15	0.617
	>1	855.50	1516.00	1828.50	4	
UCEIS	**remission (≤1)**	1199.00	1570.00	2055.00	13	>0.999
	activity (>1)	1243.00	1558.50	1868.00	6	
Histology (Geboes score)	**<3.1**	1327.00	1724.50	2269.50	12	0.108
	≥3.1	826.00	1243.00	1789.00	7	
FC (µg/g)	**<150**	1006.00	1649.50	2102.50	12	0.461
	≥150	1206.00	1243.00	1328.00	5	
FC (µg/g)	**<250**	1186.00	1448.00	2055.00	15	0.881
	≥250	1199.00	1494.00	1789.00	2	

Supplementary Table 7: AUC (8 weeks' regimen)

		P25	Median	P75	n	p-value
Global Mayo score	**no remission**	1198.00	1514.50	1631.00	10	0.557
	remission	1097.00	1491.00	2091.00	23	
Endoscopic Mayo score	**0**	1203.50	1546.00	1899.50	20	0.371
	≥1	1102.00	1315.50	1746.00	12	
Endoscopic Mayo score	**≤1**	1116.00	1546.00	2032.00	26	0.176
	>1	1088.00	1312.00	1603.00	6	
UCEIS	**remission (≤1)**	1203.50	1616.00	2061.50	24	0.074
	activity (>1)	1102.00	1201.50	1514.50	8	
Histology (Geboes score)	**<3.1**	1116.00	1601.00	2032.00	25	0.538
	≥3.1	1097.00	1205.00	1617.00	7	
FC (µg/g)	**<150**	1097.00	1484.50	1875.00	26	0.597
	≥150	1198.00	1617.00	2302.00	7	
FC (µg/g)	**<250**	1116.00	1478.00	1875.00	29	0.473
	≥250	1352.50	1692.00	2203.50	4	

Supplementary Table 8: Clearance

		P25	Mediana	P75	n	p-value
Global Mayo score	**no remission**	224.00	230.00	248.00	13	0.838
	remission	143.00	230.50	313.50	36	
Endoscopic Mayo score	**0**	150.00	232.00	308.00	29	0.768
	≥1	164.00	230.00	296.00	19	
Endoscopic Mayo score	**≤1**	152.50	230.50	302.00	40	0.635
	>1	225.50	237.00	291.00	8	
UCEIS	**remission (≤1)**	152.50	230.50	313.50	36	0.886
	activity (>1)	217.00	234.00	251.00	12	
Histology (Geboes score)	**<3.1**	152.50	220.00	302.00	36	0.439
	≥3.1	225.50	241.00	303.00	12	
FC (µg/g)	**<150**	156.00	229.00	296.00	37	0.731
	≥150	205.00	238.00	357.00	11	
FC (µg/g)	**<250**	156.00	231.00	296.00	42	0.867
	≥250	205.00	234.00	357.00	6	

Study VII - Clinical performance of an infliximab rapid quantification assay

Supplementary Table 1: Performance of each IFX quantification method in the detection of the outcomes using different IFX cut-offs.

	cut-off=1											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no clinical remission						Mayo endoscopic score=>0					
Sq	9	96	50	71	70		7	95	50	59	59	0.024/0.053
QB	27	80	38	70	63		27	80	50	60	58	0.075/0.089
	Mayo endoscopic score≤1						FC (QB)>250					
Sq	5	94	17	81	78		13	97	67	68	67	0.116/0.079
QB	24	78	21	81	67	0.0147/0.098	37	80	48	71	65	0.173/0.108
	cut-off=2											
	no clinical remission						Mayo endoscopic score>=0					
Sq	21	87	41	72	67		21	87	53	61	60	0.085/0.081
QB	38	76	42	73	64		38	77	55	63	60	0.155/0.094
	Mayo endoscopic score≥1						FC (QB)>250					
Sq	15	84	18	81	71		26	85	47	68	64	0.115/0.102
QB	38	73	26	83	66	0.094/0.099	47	78	52	74	67	0.253/0.107
	cut-off=3											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no clinical remission						Mayo endoscopic score>=0					
Sq	33	80	42	74	66		30	79	50	62	59	0.095/0.091
QB	47	68	40	74	61		49	71	55	65	61	0.197/0.096
	Mayo endoscopic score≥1						FC (QB)>250					
Sq	30	77	23	83	68	0.063/0.100	39	79	27	86	72	0.149/0.105
QB	43	64	23	88	59	0.048/0.090	56	66	25	88	64	0.144/0.088
	cut-off=4											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no clinical remission						Mayo endoscopic score>=0					
Sq	39	76	42	74	65		39	78	55	65	62	0.172/0.094
QB	50	65	40	74	60		51	67	54	65	60	0.184/0.096
	Mayo endoscopic score≥1						FC (QB)>250					
Sq	40	74	26	84	67	0.112/0.099	50	75	29	88	71	0.196/0.100
QB	48	61	23	83	58	0.063/0.087	56	63	23	87	61	0.116/0.084
	cut-off=5											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no clinical remission						Mayo endoscopic score>=0					
Sq	45	66	37	74	60		45	68	50	64	59	0.139/0.096
QB	59	55	38	75	56		60	57	51	66	58	0.170/0.095
	Mayo endoscopic score≥1						FC (QB)>250					
Sq	45	64	23	84	61	0.068/0.089	61	66	27	89	65	0.182/0.087
QB	62	53	25	85	55	0.094/0.077	61	52	21	87	54	0.075/0.073
	cut-off=6											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no clinical remission						Mayo endoscopic score>=0					
Sq	46	55	31	70	52		52	60	58	64	57	0.124/0.096
QB	59	51	36	73	54		64	56	52	68	59	0.196/0.093
	Mayo endoscopic score≥1						FC (QB)>250					
Sq	45	55	19	81	53	0.001/0.080	67	58	25	90	60	0.149/0.077
QB	62	49	23	84	52	0.070/0.074	61	49	20	86	51	0.054/0.070
	cut-off=7											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no clinical remission						Mayo endoscopic score>=0					
Sq	61	54	36	76	56		59	57	49	67	58	0.157/0.094
QB	68	45	36	75	52		73	49	52	71	59	0.213/0.088
	Mayo endoscopic score≥1						FC (QB)>250					
Sq	60	53	23	85	54	0.079/0.076	67	52	22	89	54	0.101/0.070
QB	67	41	22	83	46	0.044/0.066	78	44	22	91	50	0.111/0.060
	cut-off=8											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE

	no clinical remission						Mayo endoscopic score>=0					
Sq	61	49	34	74	53		64	54	49	68	58	0.169/0.092
QB	64	37	35	75	48		76	39	48	69	55	0.139/ 0.084
	Mayo endoscopic score>=1						FC (QB)>250					
Sq	60	48	21	84	50	0.048/0.072	67	47	20	88	50	0.073/0.066
QB	71	34	21	83	42	0.029/0.059	83	36	21	91	44	0.091/0.051
	cut-off=9											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no clinical remission						Mayo endoscopic score>=0					
Sq	64	43	33	73	50		66	48	47	67	55	0.127/0.090
QB	82	34	36	81	49		80	34	48	70	54	0.132/0.079
	Mayo endoscopic score>=1						FC (QB)>250					
Sq	65	44	21	85	48	0.048/0.066	72	43	20	88	48	0.074/0.060
QB	81	31	22	87	41	0.058/0.051	83	31	20	90	40	0.061/0.046
	cut-off=10											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no clinical remission						Mayo endoscopic score>=0					
Sq	73	73	36	79	53		70	46	48	69	44	0.154/0.087
QB	85	29	35	81	46		84	30	47	72	53	0.126/0.073
	Mayo endoscopic score>=1						FC (QB)>250					
Sq	75	43	23	88	49	0.092/0.062	72	39	19	88	45	0.005/0.058
QB							83	25	19	88	35	0.035/0.042

Sq, Sanquin; QB, quantum-blue; sen, sensitivity; spe, specificity; PPV, positive predictive value; NPV, negative predictive value; acc, accuracy; K/SE, kappa/standard error.

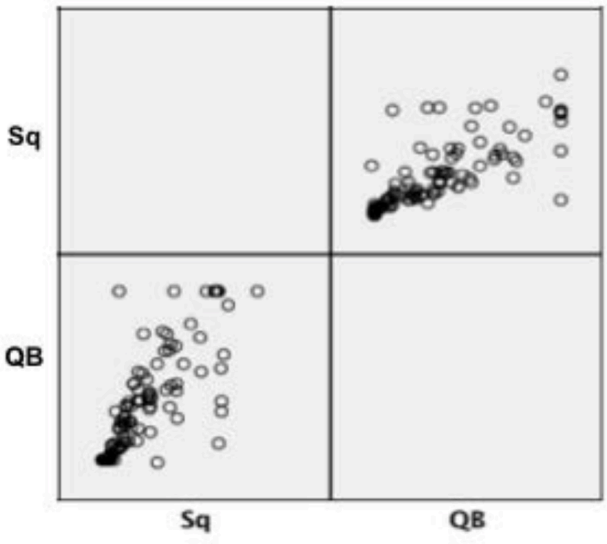
Supplementary Table 2: Performance of each IFX quantification method in the detection of deep remission (including clinical, endoscopic and histological remission, or just clinical and endoscopic remission) and histological remission.

	cut-off=1											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no deep remission (clinical + endoscopic+histology)						no deep remission (clinical + endoscopic)					
Sq	2	91	17	52	50	-	6	95	50	55	55	0.012/0.048
QB	19	75	38	54	50	-	24	79	50	55	54	0.034/0.083
	Geboes score>3.0											
SQ	12	97	67	71	70							
QB	27	81	39	70	63							
	cut-off=2											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no deep remission (clinical + endoscopic+histology)						no deep remission (clinical + endoscopic)					
Sq	10	80	29	51	48	-	18	87	53	56	56	0.054/0.075
QB	23	67	36	52	47		34	76	55	57	56	0.101/0.090
	Geboes score>3.0											
SQ	24	88	47	71	68							
QB	35	75	40	71	62							
	cut-off=3											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no deep remission (clinical + endoscopic+histology)						no deep remission (clinical + endoscopic)					
Sq	20	73	39	52	49	-	27	78	50	57	55	0.051/0.086
QB	29	57	35	50	44	-	46	71	58	60	59	0.169/0.094
	Geboes score>3.0											
Sq	32	80	42	72	65	0.129/0.099						
QB	41	67	37	71	58	0.076/0.098						
	cut-off=4											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no deep remission (clinical + endoscopic+histology)						no deep remission (clinical + endoscopic)					
Sq	22	66	36	50	46	-	35	77	55	59	58	0.118/0.090
QB	33	55	37	51	45	-	48	67	56	60	58	0.154/0.095
	Geboes score>3.0											
Sq	35	76	40	72	63	0.113/0.099						
QB	44	64	37	71	57	0.076/0.097						
	cut-off=5											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no deep remission (clinical + endoscopic+histology)						no deep remission (clinical + endoscopic)					
Sq	34	59	42	42	48	-	43	67	51	59	56	0.097/0.095
QB	46	48	42	53	47	-	56	57	53	60	56	0.128/0.095
	Geboes score>3.0											
Sq	41	65	35	71	57	0.058/0.096						
QB	47	51	31	67	50	-						
	cut-off=6											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no deep remission (clinical + endoscopic+histology)						no deep remission (clinical + endoscopic)					
Sq	44	54	45	53	49	-	49	58	49	58	54	0.073/0.096
QB	46	43	39	50	44	-	60	55	54	62	57	0.150/0.094
	Geboes score>3.0											
Sq	41	54	29	67	50	-						
QB	50	49	32	67	49	-						
	cut-off=7											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no deep remission (clinical + endoscopic+histology)						no deep remission (clinical + endoscopic)					
Sq	46	46	42	50	45	-	59	57	53	63	58	0.157/0.094
QB	54	37	41	50	44	-	68	48	53	64	57	0.160/0.091
	Geboes score>3.0											
Sq	44	47	28	65	46	-						

QB	59	42	32	68	47	0.004/0.084						
	cut-off=8											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no deep remission (clinical + endoscopic+histology)						no deep remission (clinical + endoscopic)					
Sq	50	42	42	50	46	-	63	53	53	64	58	0.163/0.093
QB	65	62	43	53	46	-	72	38	50	61	54	0.096/0.087
	Geboes score>3.0											
Sq	44	42	26	62	43	-						
QB	65	33	31	67	43	-						
	cut-off=9											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no deep remission (clinical + endoscopic+histology)						no deep remission (clinical + endoscopic)					
Sq	58	41	45	53	49	-	65	47	50	62	55	0.116/0.091
QB	67	25	42	48	44		78	35	51	65	56	0.120/0.083
	Geboes score>3.0											
Sq	47	37	25	60	40	-						
QB	74	31	33	71	44	0.031/0.071						
	cut-off=10											
	sen	spe	PPV	NPV	acc	K/SE	sen	spe	PPV	NPV	acc	K/SE
	no deep remission (clinical + endoscopic+histology)						no deep remission (clinical + endoscopic)					
Sq	58	36	43	50	46	-	71	47	52	67	58	0.175/0.089
QB	69	18	40	42	41		84	31	51	69	55	0.144/0.077
	Geboes score>3.0											
Sq	53	35	27	62	41	-						
QB	77	25	33	69	42	0.011/0.065						

Sq, Sanquin; QB, quantum-blue; sen, sensitivity; spe, specificity; PPV, positive predictive value; NPV, negative predictive value; acc, accuracy; K/SE, kappa/standard error.

Supplementary Fig. 1



Supplementary Fig. 2