Universidade de Lisboa

Faculdade de Medicina de Lisboa

ISAMB - Instituto de Saúde Ambiental

Evolution and Precision of Point-of-Cares in Therapeutic Drug Monitoring in IBD

Cátia Sofia Lourenço Rocha

Supervisor: Doutor Fernando Magro

Co-supervisor: Doutora Maria José Diógenes

Tutor: Doutora Patrícia Machado

Tese especialmente elaborada para obtenção do grau de Doutor no ramo de Ciências e Tecnologias da Saúde, especialidade de Saúde Ambiental

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ABSTRACT

The advance of knowledge about the pathogenesis of inflammatory bowel disease (IBD) has allowed for the discovery of potential treatments aimed at biological targets. Thus, in the early 90's, IBD treatment entered a new era, with the development of biological therapies. However, some patients do not respond to induction treatment (primary loss of response) or lose response during maintenance treatment (secondary loss of response) over time. This loss of response may be related to these treatments being highly immunogenic, thus leading to the development of anti-drug antibodies (ADAs), which can neutralize drug-target binding or increase drug clearance, resulting in sub-optimal drug concentrations and shorter response times. Usually, this loss of response is managed empirically. However, this empirical approach increases the risk of irreversible tissue damage and health care costs, and may delay effective IBD treatment. Therefore, therapeutic drug monitoring (TDM) is an emerging strategy in the treatment of IBD patients. Assessing both drug and ADAs levels can help clinicians adjust therapy on individual basis. However, its proper use depends on knowledge of the pharmacokinetic properties that influence loss of response and on the correct use of methods for detecting drug and ADA levels.

The global aim of this work was to understand the impact of the methodological approach of TDM and the consequent biases in its interpretation in IBD patients treated with anti-TNFα drugs. This work demonstrated that the presence of the drug influences the detection of ADAs and this impact is methodology-dependent. Therefore, the inability of some assays to determine ADAs in the presence of the drug may hamper the clinical interpretation of TDM. Moreover, this work also demonstrated that the methodologies used for TDM of Remicade® can also be used to monitor the levels of Flixabi® (Remicade-biosimilar). In addition, we also demonstrated that Remicade®, Remsima® and Flixabi® show a high cross-immunogenicity, which supports their high similarity, but prevents their exchange, as an efficacious therapeutic option, in patients who do not respond to anti-TNFα therapy.

This work shows that TDM can be advantageous in: i) identifying medication adherence problems in patients who have lost response, allowing clinicians to discriminate between pharmacokinetic and pharmacodynamic reasons for treatment failure; ii) identification of the most appropriate dosing regimen to achieve the optimal response with minimal toxicity; iii) help clinicians identify patients who will and will not benefit from treatment.

However, the use of TDM should always be integrated with the identification of possible methodological biases and the clinical assessment of the patient.

Key-words: Inflammatory bowel diseases; Biological Therapies; Anti-drug antibodies; Biosimilars; Therapeutic drug monitoring.

RESUMO

O avanço do conhecimento acerca da patogénese da doença inflamatória intestinal (DII) permitiu a descoberta de potenciais tratamentos visando alvos biológicos. Por esta razão, no início da década de noventa, o tratamento da DII entrou numa nova era com o desenvolvimento das terapias biológicas. No entanto, uma parte dos pacientes não responde ao tratamento de indução (perda primária de resposta) ou perde a resposta durante o tratamento de manutenção (perda secundária de resposta) ao longo do tempo. Essa perda de resposta pode estar relacionada com estes tratamentos serem altamente imunogénicas, levando assim ao desenvolvimento de anticorpos anti-fármacos (AAF), os quais podem neutralizar a ligação do fármaco ao alvo ou aumentar a depuração do fármaco, resultando em concentrações sub-ótimas e menor duração de resposta. Normalmente, essa perda de resposta é gerida de forma empírica. Contudo, esta abordagem empírica aumenta o risco de danos irreversíveis nos tecidos e os custos de saúde, e pode atrasar o tratamento eficaz da DII. Assim, a monitorização terapêutica de fármacos (MTF) é uma estratégia emergente no tratamento de pacientes com DII. A avaliação dos níveis de fármaco e dos níveis de AAF podem ajudar os médicos a ajustar a terapêutica de forma individualizada. Contudo, o seu uso adequado depende do conhecimento das propriedades farmacocinéticas que influenciam a perda de resposta e do uso correto dos métodos para a deteção dos níveis de fármaco e AAF.

O objetivo geral deste trabalho foi compreender o impacto da abordagem metodológica da MTF e consequentes vieses na sua interpretação em pacientes com DII tratados com anti-TNFα. Este trabalho demonstrou que a presença de fármaco influencia a deteção de AAF e esse impacto varia de acordo com a metodologia utilizada. Portanto, a incapacidade de alguns ensaios em determinar AAF na presença do fármaco pode dificultar a interpretação clínica da MTF. No decorrer deste trabalho também foi demonstrado que as metodologias utilizadas para MTF do Remicade® também podem ser usadas para monitorar os níveis de Flixabi® (biosimilar do Remicade). Além disso, demonstrámos também que o Remicade®, Remsima® e o Flixabi® têm uma alta imunogenicidade cruzada, o que suporta a sua elevada similaridade, mas impede que sejam utilizados sequencialmente, enquanto opção terapêutica eficaz, em pacientes que não respondem à terapia com anti-TNFα.

Este trabalho mostra que a MTF poderá ser vantajosa em vários aspetos: i) identificação de problemas de adesão à medicação entre os casos de pacientes que perderam a resposta, permitindo aos médicos discriminar entre razões de farmacocinética e farmacodinâmica responsáveis pelo insucesso do tratamento; ii) identificação do regime de dosagem mais

apropriado para atingir a resposta ótima com toxicidade mínima; iii) ajudar os médicos a identificar os pacientes que beneficiarão e os que não beneficiarão com o tratamento.

Contudo, a utilização da MTF deverá ser sempre integrada com a identificação de possíveis vieses metodológicos e a avaliação clínica do paciente.

Palavras chave: Doenças inflamatórias intestinais; Terapias Biológicas; Anticorpos Anti-fármaco; Biosimilares; Monitorização Terapêutica de Fármacos.

LIST OF THESIS PUBLICATIONS

1. **C. Rocha**, P. Lago, S. Fernandes, L. Correia, F. Portela, A. I. Vieira, M. Patita, B. Arroja, P. Ministro, C. Alves, C. C. Dias, and F. Magro on behalf of Portuguese IBD Study Group (GEDII). Rapid test detection of anti-infliximab antibodies: performance comparison with three different immunoassays. ***Ther Adv Gastroenterol.*** 2020; Vol. 13: 1–12. DOI: 10.1177/1756284820965790

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3. F. Magro, **C. Rocha**, A. I. Vieira, H. T. Sousa, I. Rosa, S. Lopes, J. Carvalho, C. C. Dias and J. Afonso, on behalf of Portuguese IBD Study Group (GEDII). The performance of Remicade®-optimized quantification assays in the assessment of Flixabi® levels. ***Ther Adv Gastroenterol.*** 2018, Vol. 11: 1–9. DOI: 10.1177/1756284818796956

LIST OF ABBREVIATIONS

ADAs	Anti-drug antibodies
ADCC	Antibody-dependent cell-mediated cytotoxicity
AHLC	Anti-human lambda chain assay
APCs	Antigen presenting cells
ASA	Aminosalicylates
ATIs	Anti-infliximab antibodies
AZA	Azathioprine
bELISA	bridging ELISA
BSA	Bovine Serum Albumin
CD	Crohn Disease
cELISA	competitive ELISA
CHO	Chinese Hamster Ovary
CRP	C- reactive protein
DCs	Dendritic cells
ECCO	European Crohn's and Colitis Organisation
ELISA	Enzyme-Linked Immunosorbent Assay
EMA	European Medicines Agency
Fab	Antibody variable region
Fc	Constant fragment
FcR	Constant fragment receptor
FcRn	Neonatal Fc receptor
FcγR	Fc gamma receptors
FDA	Federal Drug Administration
HMSA	Homogeneous mobility shift assay
HPLC	High pressure liquid chromatography
HRP	Horseradish Peroxidase
IBD	Inflammatory bowel disease
ICC	Intraclass correlation coefficient
IFNs	Interferons

IFNγ	Interferon gamma
IFX	Infliximab
Ig	Immunoglobulin
IgG	Immunoglobulin G
IgG4	Immunoglobulin G4
IgM	Immunoglobulin M
IL	Interleukin
LFA	Lateral flow-based assay
LPS	Lipopolysaccharide
Luc	Luciferase
mAb	Monoclonal antibody
MHC I	Major histocompability complex class I
MHC II	Major histocompability complex class II
6-MP	Mercaptopurine
Nab	Neutralizing anti-drug antibody
NFκβ	Nuclear factor kappa B
NK	Natural killer cell
NODs	Nucleotide-binding oligomerization domain-containing molecules
non-Nab	non-Neutralizing anti-drug antibody
NSAIDs	Non-steroidal anti-inflammatory drugs
PD	Pharmacodynamics
PK	Pharmacokinetics
PNR	Primary non-response
PRRs	Pattern recognition receptors
QB	Quantum Blue rapid test
RA	Rheumatoid arthritis
RES	Reticuloendothelial system
RGA	Reporter Gene Assay
RIA	Radioimmunoassay
SDs	Standard deviations
SFPE	Semi-fluid phase enzyme immunoassay

sTNF	Soluble TNF
TCRs	T cell receptors
TDM	Therapeutic Drug Monitoring
Th cells	T helper cells
TLRs	Toll-like receptors
TMB	3,3’5,5’ tetramethylbenzidine
TMDD	Target-mediated clearance
tmTNF	transmembrane TNF
TNFR	Tumour necrosis factor receptor
TNFα	Tumor necrosis factor alpha
UC	Ulcerative Colitis

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I.

INTRODUCTION

1.1. INFLAMMATORY BOWEL DISEASE

Inflammatory Bowel Disease (IBD) is a chronic inflammatory disorder of the gastrointestinal tract caused by an inappropriate inflammatory response to intestinal microbes in a genetically susceptible individual[1]. It includes two major forms: crohn's disease (CD) and ulcerative colitis (UC). Although the clinical features are similar, they may be separated by disease location and characteristics of inflammation[2,3].

CD is a transmural inflammatory disease of the mucosa that can affect any part of the gastrointestinal tract (from the mouth to the anus). There are different CD phenotypes including inflammatory, structuring, and penetrating, and the symptoms are variable (e.g.: diarrhoea, abdominal pain, weight loss, nausea, and sometimes fever or chills). Patients may have one or more of these symptoms during the course of their disease. UC is a relapsing non-transmural inflammatory disease that is restricted to the colon. Patients typically present symptoms such as bloody diarrhoea, passage of pus, mucus or both, and abdominal cramping during bowel movements[2].

1.2. EPIDEMIOLOGY

IBD has emerged as a public health challenge worldwide, with substantial variation in different countries and regions. Usually, IBD tends to be more common in industrialized countries than in non-industrialized ones. Globally, between 1990 and 2017, the number of individuals with IBD increased from 3.7 million to more than 6.8 million cases[3]. The highest prevalence rates occur in Europe and North America. In Europe, the prevalence of UC and DC is currently 505 per 100,000 inhabitants and 322 per 100,000 inhabitants, respectively. The incidence of UC in Europe ranges from 0.9 to 24.3 per 100,000 inhabitants/year, while for CD it varies between 0.5 to 10.6 cases per 100,000 inhabitants/year[3,4]. In Portugal, the prevalence of IBD has been estimated to be 146 patients per 100,000 inhabitants in 2007. In the same time period, the prevalence of UC and CD was 71 and 73 patients per 100,000 inhabitants, respectively[5].

An increasing incidence of IBD has been identified in newly industrialized countries and it is expected to increase. This increasing global burden of IBD will bring important challenges to health-care systems around the world, as they work to care for this complex and costly disease, accounting for substantial costs to the health care system and society[6]. Thus, it is imperative to invest in the study of this pathology which has a profound impact on the patients' quality of life, as well as for health care systems, providing valuable information for decision making.

1.3. ETIOLOGY AND PATHOGENESIS

The etiology of IBD remains unclear. However, the most consensual pathogenesis model suggests a complex interaction between environmental factors, genetic factors, changes in the gut microbiota, and an abnormal immune response (**Figure 1**)[7].

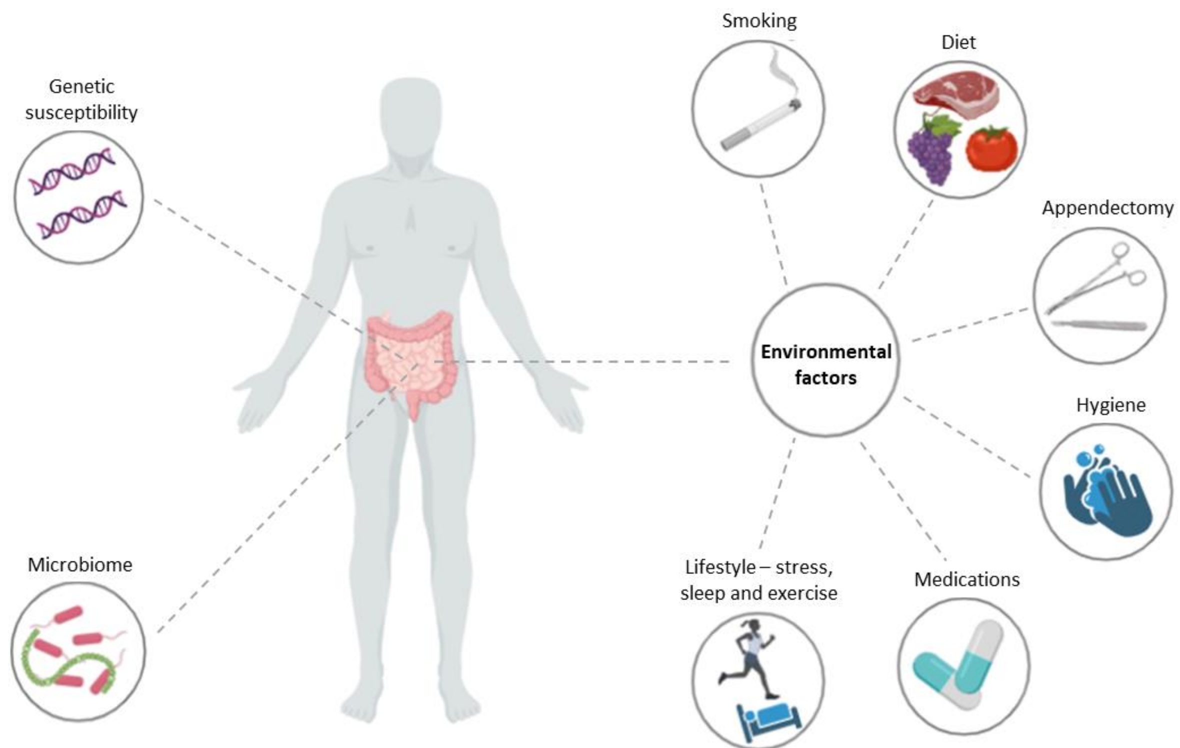

Figure 1 Risk factors involved in IBD pathogenesis: environmental influences, genetic predisposition (leading to immunological abnormalities) and dysbiosis of the gut microbiome.

However, given none of the aforementioned risk factors alone is sufficient for the development of the disease, it has been posited that complex interactions between these factors lead to the development of IBD.

1.3.1. GENETIC RISK FACTORS

The role of genetics in IBD was initially suggested in family and twins' studies, since approximately 5.5-22.5% of IBD patients have another relative with the disease[8]. The risk of developing IBD with one parent having the disease is 2-3 times higher than in the general population. If both parents have IBD, the risk of their children developing the disease before the age of 28 is higher, reaching 33 %. The relative risk (RR) of a sibling of patients with CD are 13-36 times higher than the general population, while that a sibling of a patients with UC, the risk is 7-17 times higher[8,9]. In general, the risk of developing IBD is 2-3% and 0.5-1% in patients with relatives with CD and UC, respectively[8]. Studies with twins represent the strongest epidemiological argument of the genetic contribution to the etiopathogenesis of IBD[10]. For CD, the concordance rates in monozygotic twins are 20–50% whereas that for dizygotic twins is 10%. In UC, the concordance rates are lower: 16% for

monozygotic and 4% for dizygotic twins, suggesting a weaker heritable component for this disease[7,8]. The previous data, supporting the role of genetics in IBD susceptibility, suggest that this role is more significant in CD than in UC.

Over the past 20 years, the genetic knowledge of IBD has grown considerably. At least 163 loci have been identified – 110 were associated with both forms of the disease, indicating common mechanisms, whereas 23 were related to UC and the other 30 to CD[11,12]. Overall, the genes identified can be clustered in three categories: i) innate immune response (e.g.: *NOD2*, *ATG16L1* and *IRGM* genes); ii) regulation of the acquired immune response (e.g.: *IL23R*) and iii) mucosal barrier genes (e.g.: *ECM1*, *CDH1, HFN4A* and *LAMB1*)[13,14].

Innate immunity plays a key role in the host's immune response through participation in microbial recognition at the intracellular level[15]. The nucleotide-binding oligomerization domain containing 2 (*NOD2*) was the first susceptibility gene described for CD. This gene encodes an intracellular protein present in monocytes, macrophages, Paneth cells and intestinal epithelial cells, which acts as a sensor for bacteria recognition[16,17]. This recognition activates the nuclear factor kβ (NF-k_{β}) and triggers the production of antimicrobial substances (e.g.: α-defensins), fundamental to prevent host aggression, namely intestinal bacterial translocation[16–18]. Mutations of the *NOD2* result in the disruption of the NF-k_{β} pathway and in the production of α-defensins[19–21]. Apparently, NF-k_{β} also plays a central role in CD through the regulation of transcription of genes that encode pro-inflammatory cytokines such as tumour necrosis factor α (TNFα)[22]. Recently, *NOD2* has been implicated in the regulation of autophagy (innate process of homeostasis that allows the recycling of organelles and contributes to the intracellular removal of microorganisms), apoptosis, and the production of type I interferons (IFNs)[23,29]. In CD, there is an association between homozygotes and a 20-40 fold increase risk, while in heterozygotes this increased risk is 2-4 fold[8].

Several genes associated with an increased CD susceptibility regulate autophagy (e.g.: *ATG16L1*, *IRGM*)[24]. Moreover, genes involved in acquired immunity (namely in the regulation of pathways involving IL-17 and IL-23) have been associated with an increased risk of IBD[25,26]. For example, the *IL23R*, *IL12B*, *STAT3*, *JAK2* and *TYK2 genes* increase susceptibility to both CD and UC, whilst others are only involved in the risk of CD (e.g.: *IL-27* and *TNFSF15*)[25,26]. Other genes involved in the epithelial barrier function (e.g.: *ECM1*, *CDH1*, *HNF4A* and *LAMB1*) have been specifically associated with increased susceptibility to UC[27,28].

The identification of an increasing number of susceptibility gene loci indicates that genetic components are important factors in the pathogenesis of IBD. The identification of genetic variants aims to define a specific disease phenotype to help follow clinical progression and eventually develop new targeted therapies[1,2,7]. However, it is estimated that, of all the identified loci, only 25% explain

the heritability in IBD[1,2,7], suggesting that other shared environmental and/or epigenetic factors may be involved.

1.3.2. ENVIRONMENTAL RISK FACTORS

The changing epidemiology of IBD over time and geography suggests that environmental factors have a role in inducing or modifying disease expression. Environmental factors such as: i) smoking, ii) diet, iii) appendectomy, iv) hygiene, v) medication and vi) lifestyle (stress, sleep and exercise) have all been associated with IBD development[29].

i) Smoking

Smoking was the first risk factor consistently associated with IBD. Harries *et al* were the first to describe the association between smoking and UC[30]. Various published case-control studies have shown that current smoking is protective against UC and smoking cessation is associated with an increased risk of this pathology[31]. In fact, some authors report that the risk increased significantly 2-5 years after smoking cessation and can remain elevated over 20 years[32]. It has also been reported that current smoking is associated with low hospitalization rates, relapse rates, reduction in colectomy rates, and a decreased need for steroids, suggesting a less severe clinical presentation and a better long term prognosis than in non-smokers[31].

In contrast, smoking increases the risk of developing CD and this is associated with the number of packs smoked per year. CD patients who smoke have a poorer disease course than non-smokers, with higher disease recurrence and hospitalization rates. These patients are also more likely to develop complications, worse response to treatments, and a greater need for immunosuppressive and surgical interventions[31]. As in UC, smoking has a temporary impact, and its cessation results in a decreased risk of CD[32], and decreased need for steroids and immunosuppressive therapy[30–32]. In fact, it has been estimated that after 2 years of smoking cessation, the disease activity and the therapeutic requirement of former smokers are the same as those who have never smoked[33,34]. The risk of CD appears to be twice as high in current smokers, compared to individuals who have never smoked, with a dose-response effect. In addition, ex-smokers have an increased risk of CD of lesser magnitude[32].

Overall, the studies carried out showed unanimity regarding the association of smoking with an increased risk of CD and a reduced risk of UC. However, there are some controversies regarding whether exposure to tobacco smoke in the prenatal period or in childhood is a risk factor for the development of CD and UC[35–37,38].

The mechanisms that mediate these smoking effects remain unclear. Nevertheless, some authors described that the putative mechanisms in UC may involve a response to the carbon monoxide present in cigarette smoke causing the reduction in TNFα and IL-8 expression, increasing the production of IL-10, causing hypoperfusion of the rectum and acutely damaged colonic tissue[31,39]. In CD, the increased carbon monoxide from cigarette smoke may cause impairment in vasodilation capacity in chronically inflamed micro vessels, resulting in ischemia, and perpetuating ulceration and fibrosis[39].

ii) Diet

Epidemiological studies have suggested that dietary factors play an important role in gut inflammation and the risk of developing IBD. Diet modulates the intestinal microbiome, affecting gastrointestinal permeability and acting as food antigens[40,41].

In fact, the diet is influenced by environmental and cultural practices. Some studies report that a high animal protein intake (e.g.: red meat, processed meat, poultry, dairy) may be associated with IBD risk and risk of relapse. Animal proteins can modulate inflammation through the action of specific amino acids or their metabolites in the immune function, such as the haem group contained in meat or a high lipid content, which may be associated with an increased risk of IBD. In addition, it has also been proposed that meat may be a vehicle for bacteria with a relevant role in the development of IBD, and the antibiotics used in its production may also affect the intestinal homeostasis of consumers[42].

The high intake of saturated fat, total lipids, trans-unsaturated fatty acids (trans fats), n-6 polyunsaturated fatty acids (omega-6 PUFA) have been associated with an increased risk of UC and CD[40,43]. Although it has been proposed that this may be due to the pro-inflammatory properties of omega-6 and trans fats, it is not fully established. On the other hand, the higher intake of dietary n-3 polyunsaturated fatty acids (omega-3 PUFA) (e.g., fish oil), as well as a high ratio of omega-3:omega-6, seem to have a protective effect on UC[40,43]. This may occur due to the anti-inflammatory and immunoregulatory role of omega-3 PUFA. However, the same conclusions regarding CD are not shared by all studies, and there is no clear association between those factors and this pathology[43,44].

As already established, the fermentable fiber ingested through food is metabolized by bacteria in the intestinal flora, producing short-chain fatty acids, which have anti-inflammatory properties. Likewise, fiber plays an important role in maintaining this flora and, therefore, the intestinal barrier function[43,44]. Vegetables and fruits, in addition to being a source of fiber, are rich in antioxidants, being able to protect against oxidative stress[45]. Several reports are consistent in stating that the high intake of dietary fiber, particularly fruits and vegetables, is associated with a 73%-80% decreased risk

of CD[43,44]. Regarding UC, there is some evidence of a protective effect[46], but other studies show a less evident or even absent effect[44,47].

iii) Appendectomy

Similar to smoking, appendectomy demonstrates a divergent effect on CD and UC. A consistent association between appendectomy and CD has not been observed. However, some studies show that appendectomy is a risk factor for the development of CD[28,48], while others show no association[49]. Regarding UC, appendectomy seems to be protective[50,51], but the exact mechanism remains unclear. A case-control study showed that patients who had an appendectomy had a significantly lower incidence of UC than controls[52]. This relationship was not observed for appendectomies performed for non-specific abdominal pain (that is, appendix is found to be normal on post-operative pathology). In addition, the protective association to UC was only observed when surgeries were performed before the age of 20 years[52].

iv) Hygiene

Good hygiene was first proposed by Strachan[53] to explain the dramatic increase in autoimmune diseases. The principle underlying this hypothesis is that abnormal immune responses, such as autoimmunity and allergy, are the result of improvements in personal hygiene, which reduced the exposure to microbial stimulation resulting in decreased enteric microbiota diversity and dysbiosis. In fact, different studies support this hypothesis in the IBD context. For example, people with siblings and a large family, drinking unpasteurized milk, living on a farm and exposed to pets (particularly early in childhood) have a lesser risk of developing CD or UC. In developing countries, measures for increasing hygiene have been associated with an increased risk of IBD. The hygiene hypothesis is not applicable to all populations, being more relevant in societies undergoing increasing affluence or migrating from less to richer countries[29,44,54].

v) Medication

Several studies reported that some drugs, such as oral contraceptives, antibiotics, and non-steroidal anti-inflammatory drugs (NSAIDs), can cause or worsen IBD.

Oral contraceptives may influence the risk of IBD through its oestrogen related effects. These modify the intestinal permeability and the levels of oestrogens and endogenous androgens, enhancing the development of autoimmune diseases mediated by Th1 and Th2 cells, and modifying the composition of the intestinal flora. Thus, the use of oral contraceptives containing oestrogens are associated with an increased risk of developing UC and CD, which is independent of dose reduction,

but reversible with discontinuation of therapy. However, other authors describe that the risk related to combined contraceptives is only significant for CD, while others show no association[29,54,55].

Antibiotics can alter the composition of the gut microbiome by decreasing the species diversity and protein expression. Although this disruption is not permanent, it often triggers an aberrant immune response and a cascade of inflammatory reactions in genetically susceptible individuals. In this context, exposure to antibiotics has been suggested to contribute to the development of IBD. Some studies reported a positive association between antibiotics exposure and the development of CD. The association is greater for CD than UC, and is stronger for exposure in the first year of life compared with later use. A dose-response relationship also exists and contributes to a greater increase in disease risk[29,54,55].

NSAIDs have been identified as a risk factor due to mechanisms that involve changes in the innate and acquired immune responses and in the disruption of the intestinal barrier. However, few studies have been conducted to clarify the existence of this association. A prospective study revealed that the regular use of acetylsalicylic acid is associated with a 6 times higher risk of developing CD, while this relationship has not been documented for UC. Other studies have shown evidence that the frequent use of NSAIDs, in high doses and during prolonged periods, increases the risk of UC and CD[29,54,55].

vi) Lifestyle: stress, sleep and exercise

Sleep disorders have become a prevalent health problem. Recent studies have shown that sleep disturbances and disruption of the circadian cycles trigger the activation of pro-inflammatory cytokines, which can lead to pathologies such as IBD. Different studies reported that there is a bidirectional association: increased disease activity might disrupt sleep and, in turn, poor sleep quality might exacerbate inflammation. Both prolonged (more than 9 hours) and reduced (less than 6 hours) duration of sleep were associated with increased risk of UC, but not modify the risk of CD. On the other hand, impaired sleep quality was associated with increased histological activity and risk of clinical relapse[29,54,56]. However, there is still a paucity of information regarding this topic.

Psychological stress and depressive symptoms may also play a role in CD and UC pathogenesis, since they can alter the immune function and the gut microbiome. Studies carried out in this field showed that stressful events and depressive symptoms increased the risk of IBD (especially CD), such as surgery or failure of infliximab therapy, whereas patients with low stress had fewer relapses. However, results are not consistent. There are several pathways through which stress can influence gut inflammation, via the hypothalamus–pituitary–adrenal axis and the autonomic nervous system, resulting in the production of proinflammatory cytokines, activation of macrophages, and modification of the gut microbiome, and alteration of intestinal permeability[29,54,56]. Regarding to

depressive symptoms, observational studies support an increased risk of IBD. In patients with established disease, depression has been associated with elapse, hospitalization, surgery, reduced responsiveness to immunosuppressive therapy and impairment of quality of life[55].

The association between regular physical activity and a reduced risk of developing IBD have been demonstrated. This evidence was greater for CD demonstrating a 44% decrease in risk[57]. This association appears to be independent of the effect of body mass index (BMI), so this will not be a confounding factor[57]. However, data regarding the beneficial effects of exercise on intestinal inflammation or prevention of relapse are limited. Recent studies posit that regular physical activity induces mechanisms of autophagy and immune regulation, responsible for reducing inflammation through the release of chemokines (e.g.: myokines) during skeletal muscle contraction[29,56].

1.3.3. MICROBIOME

The human microbiome consists of more than 10^{14} symbiotic microbial cells that play an important role in human health and disease. The human gut microbiota is a dynamic and diverse community of commensal bacteria, fungi and viruses, and it has even been considered to be an "essential organ", carrying approximately 150 times more genes than those found in the entire human genome. Important advances show that the gut microbiota establishes a symbiotic relationship with the host, contributing to the normal functioning of the digestive system (namely in the digestion, absorption and storage of nutrients), for the prevention of colonization by pathogens, and also to stimulate the secretion of antimicrobial substances[29,54,55,58]. In the healthy human, Firmicutes and Bacteroidetes represent the most prevalent phyla, while the Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia phyla are less abundant. Some changes in the human gut microbiota may occur due to factors such ase infant transitions, age, genetics, ethnic background, environmental exposure, diet and lifestyle. However, the relative importance of each factor is still unclear. Any change inf the gut microbiome composition can alter the microbial equilibrium (termed dysbiosis) and is associated with a variety of gut pathogenesis and intestinal inflammation. In general, dysbiosis is defined as the imbalance of intestinal flora between beneficial and pathogenic microorganisms. It is a process that is characterized by the modification of the composition of the intestinal flora, reduction in bacterial diversity, increase of the pathogens and alteration of the microbial functional capacity[29,54,55,58] .

Some studies suggest that dysbiosis occurs in IBD, and a significant difference between the microbiome of healthy individuals and IBD patients has been confirmed, particularly a decreased microbial diversity and a relative abundance of specific bacterial taxa. The pattern most associated with IBD is a decrease in the abundance of bacteria belonging to the Firmicutes and Bacteroidetes

phyla, while there is an increase of bacteria from the Proteobacteria and Actinobacteria phyla. There is evidences to support that in IBD patients, adherent-invasive *Escherichia coli* (*E. coli*), *Fusobacterium* species, *Ruminococcus gnavus* and the Pasteurellaceae and Veillonellaceae families are increased, and *Clostridium* groups IV e XIVa, *Bacteroides* species, *Suterella* species, *Roseburia* species, *Bifidobacterium* species and *Faecalibacterium prausnitzii* (*F. prausnitzii*) are decreased[29,54,55,58].

Compared to healthy controls, CD and UC patients demonstrated a strong microbial imbalance at different taxonomic levels. At the phylum level, both conditions exhibit a decreased abundance of Firmicutes and an increased abundance of Bacteroidetes and Proteobacteria. For Actinobacteria, a decrease in CD patients and an increase in UC patients has been observed. Some studies explored how the different taxonomic levels belonging to each of the four main phyla (Firmicutes, Proteobacteria, Bacteroidetes, and Actinobacteria) were changed in IBD. For the Firmicutes phylum, the abundance of two classes (Clostridia and Erysipelotrichia) and three families (Ruminococcaceae, Christensenellaceae and Erysipelotrichaceae) were reduced in CD, whereas the levels of two other classes (Negativicutes and Bacilli) and five families (*Veillonellaceae*, *Lactobacillaceae*, *Acidaminococcaceae*, *Streptococcaceae* and *Peptostreptococcaceae*) were increased. In UC patients, the classes Clostridia, Negativicutes and Bacilli, and the families *Ruminococcaceae*, *Lachnospiraceae*, *Veillonellaceae*, *Streptococcaceae* and *Peptostreptococcaceae* were increased and three other families (*Acidaminococcaceae*, *Christensenellaceae* and *Lactobacillaceae*) were reduced.

For the Proteobacteria phylum, an imbalance in *Enterobacteriaceae* and *Burkholderiaceae* families has been observed. *Burkholderiaceae* abundance was increased in both CD and UC, while the abundance of *Enterobacteriaceae* was increased in CD and decreased in UC.

In CD, and for the Bacteroidetes phylum, it was observed that the abundance in the Bacteroidia class and in the *Bacteroidaceae* and *Rikenellaceae families were reduced*, while the *Prevotellaceae* family was increased. In UC patients, the only change was in the abundance of the *Rikenellaceae* and *Tannerellaceae* families, which were decreased, and the *Prevotellaceae* that was increased as in CD.

For the Actinobacteria phylum, the abundance of the Coriobacteriia class and *Coriobacteriaceae* family were increased in both diseases, whereas the Actinobacteria class and *Bifidobacteriaceae* family were reduced[29,54,55,58,59].

In terms of lower taxonomic levels, the relative abundance of *Bacteroides*, *Faecalibacterium*, *Roseburia*, *Blautia, Ruminococcus*, and *Coprococcus genera* were decreased in CD. In fact, the most assessed organism in IBD was *F. prausnitzii*, a beneficial bacterium with anti-inflammatory properties, that has been described as decreased in CD and UC. This taxon, along with *Roseburia hominis* (another "protective" taxon decreased in CD and UC), can produce butyrate, which has anti-inflammatory properties. On the other hand, the *Enterobacteriaceae* family is increased in IBD. Two members of this family, *Enterococcus* and *E. coli*, are increased in CD and UC[29,54,55,58,59].

In summary, it has been shown that various phyla, families and species differ between both diseases, and between the two diseases and healthy controls. However, it is unclear whether the alterations in the microbiota are the cause of the intestinal inflammation or a consequence of it, and precisely how these bacteria contribute to IBD pathogenesis is also hitherto unknown.

1.3.4. ABNORMAL IMMUNE RESPONSE

The immune system is responsible for protecting against a wide variety of infectious agents. IBD is characterized by an excessive and abnormal immune response against commensal flora in genetically susceptible individuals, which involves both innate and adaptive immunity.

The innate immune response is the first line of defence. It is nonspecific and does not confer long-lasting immunity (memory). Nevertheless, it is rapid and essential in the elimination of 99.9% of pathogenic organisms. Innate immunity comprises different components: i) anatomical barriers (mucous layer and epithelial barrier); ii) receptors that detect conserved structural motifs of microorganisms (pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain-containing molecules (NODs)); iii) different cell types including neutrophils, dendritic cells (DCs), monocytes, macrophages, and natural killer (NK) cells, that enable rapid and effective inflammatory responses against microbial invasion[60,61]. When innate mechanisms are not sufficient to fight an infection, the adaptive immune response is mobilized.

Adaptive immunity depends upon the specific recognition of antigens by B or T cell receptors, and, as a result, this type of response is slower than innate immunity [60,61]. The adaptive form develops a highly specific immune response, that requires more time to evolve but provides immunological memory since specificity for the antigen is the result of a complex maturation and development of immune cells[62]. Key players of the adaptive immune response are T cells. Normally the components of the adaptive immune system cooperate with each other and with the molecules and cells of the innate immune system to mount an effective immune response, which is capable of eliminating the invading pathogens[60-62].

Immune dysregulation is characterized by epithelial damage (abnormal mucus production, defective repair); expansion of inflammation driven by intestinal flora and a large number of cells that infiltrate the lamina propria, including T and B cells, macrophages, dendritic cells and neutrophils; and a failure of immune regulation to control the inflammatory response. The activation of these cells leads to the secretion of pro-inflammatory cytokines (TNFα, IFNγ, IL-6, IL-12, IL-21, IL-23, IL-17, etc.) and anti-inflammatory cytokines (IL-10 and TGF-β) in the lamina propria. The imbalance between pro- and anti-inflammatory cytokines that occurs results in disease progression and tissue damage. CD is generally referred to as increased production of IL-12, IL-23, IFN-γ and IL-

17, while UC is generally characterized by increased production of IL-13, IL-5 and IL-9. Thus, the inability to ensure a balance between pro- and anti-inflammatory cytokines can generate chronic inflammation. Cytokines are considered the main contributors in IBD pathogenesis and can be potential therapeutic targets. Therefore, their role in the initiation, mediation and control of intestinal inflammation and tissue damage have been intensively studied[60,61,63–65].

1.4. THE IMPACT ON PUBLIC HEALTH

IBD has a great influence on patient's quality of life, especially in severe or in active phases of the disease[66,67]. It is proven that the quality of care provided, namely courtesy, continuity of care, information provided, autonomy, accommodation, and costs, has an important role in the patient's quality of life. In many cases, this disease interferes with the patient's work and even with their personal relationships, since the symptoms are unpredictable and uncomfortable. In this sense, health professionals must be aware of the psychological burden associated with IBD. It is crucial for the health professional to recognize the importance of the patient associations of IBD and to refer patients to them, as it is beneficial to have contact with people who suffer from the same disease and with whom they can share their experiences[68,69].

Not all cases are diagnosed in the first year after the onset of the first symptoms, which also contributes to decreased quality of life. These cases of late diagnosis overburden the health system. Therefore, it is important that access to specialized physicians is facilitated and that protocols are developed to reduce the time from symptom onset and a definite diagnosis. Since most patients go to the hospital emergency room several times before diagnosis, it is important to raise awareness among IBD healthcare providers regarding the symptoms that may be associated with the disease[70].

1.5. TREATMENT

IBD treatment was initially focused on the management of symptoms. However, in the last years, the main goal of treatment is to change the natural course of the disease, to achieve mucosal healing, induce and maintain disease remission, prevent complications, hospitalization and surgery, and improve the patient's quality of life. The choice of therapy is dependent on the disease type, location, severity, relapse frequency, presence of extra-intestinal manifestations and the patient's general clinical condition[71–73]. The approach is individualized according to the patient's symptomatic response and tolerance to medication[71–74].

1.5.1. CONVENTIONAL THERAPIES

The conventional strategy for treating IBD is a "step up" scheme where there is a staggering of drugs according to the severity, response and location of the disease. The conventional strategy comprises: i) anti-inflammatory (aminosalicylates); ii) corticosteroids, and iii)immunosuppressants (thiopurines (azathioprine and 6-mercaptopurine), methotrexate and cyclosporine[75].

Aminosalicylates (ASA) are the option to induce and maintain remission of mild to moderate UC. ASA are less effective for maintaining remission in CD[76–78]. The most frequently prescribed ASA are mesalazine, sulfasalazine, olsalazine and balsalazide[79]. Although this class is safe and well tolerated by most patients, it still has side effects. For example, headache, nausea, epigastric pain, and diarrhoea are the most common and dose related side effects of sulfasalazine, occurring in 10-45% patients. However, the interruption of treatment could improve the symptoms. The other formulations are better tolerated, and the effects are less frequent and milder. Another disadvantage is its slow onset: the maximum effectiveness can only be seen at 4 weeks[79–82].

Corticosteroids are usually initiated when there is no response to ASA and in patients who are experiencing disease flares. These drugs are highly effective in inducing remission in IBD. However, they have no proven efficacy in maintaining remission and should not be used for this purpose[82,83]. The risk of significant side effects increases with prolonged use. Some examples of side effects are hyperglycaemia in patients with coexisting diabetes, bone loss, venous thromboembolism and poor wound healing[84,85], which makes their continued use unsustainable. In this situation, these drugs should be withdrawn gradually, since there is no additional benefit in maintaining this therapy. Thereafter, the clinicians should consider escalation to immunomodulators[86] or biological therapies[87].

Immunosuppressants such as methotrexate and thiopurines (azathioprine (AZA) and mercaptopurine (6-MP)) are used to maintain long-term remission in patients who have frequent relapses and are or have become corticosteroid intolerant or refractory. Immunosuppressants

have a slow onset of action in CD, so their utility for the rapid induction of remission is limited. However, they have been shown to maintain a remission in CD patients, and are able to induce mucosal healing. Some studies show that treatment with AZA and 6-MP results in an approximately 40% steroid-free remission rate at 1 year[82,83,88]. Patients refractory to these drugs can be treated with biological therapies, such as infliximab, adalimumab, golimumab and certolizumab pegol.

1.5.2. BIOLOGICAL THERAPIES

Biological therapy is the use of molecules (including derivative and recombinant forms) produced by cells of the immune system or by cells that participate in inflammatory reactions. The agents used for biological therapy are designed to specifically target a biological phenomenon, gene, protein, or group of genes or proteins thought to be involved in the disease[89].

The first class of biologic agents investigated and approved for use in IBD were the anti-TNFα agents, which inhibit the cytokine TNFα that is central in the mediation of systemic inflammation. This class of biologics, characterized by a relatively quick onset of action, is highly effective in both the gastrointestinal and extra-intestinal manifestations of the disease, and remains the most commonly used biologic class in the treatment of IBD. So far, four different anti-TNFα agents are approved for use in IBD (**Figure 2**)[73] and can be used for both induction and maintenance of remission[90]. Although all these drugs neutralize TNFα activity, each have different characteristics and routes of administration.

Infliximab (IFX) is a chimeric (combination of human and murine) immunoglobulin G1 (IgG1) monoclonal antibody (mAb) that binds to TNFα[90]. This drug is approved for use in both CD and UC. IFX is administered as an intravenous infusion with a weight-based dosing (standard 5 mg/kg) and its usually posology is every 4–8 weeks. Adalimumab and golimumab are anti-TNFα human IgG1 mAbs. Adalimumab is approved for use in both CD and UC, while golimumab is approved for UC only. In contrast to IFX, adalimumab and golimumab are administered subcutaneously every 2 and 4 weeks at a fixed dose, regardless of body weight (standard maintenance dose 40 mg and 100mg, respectively). Certolizumab pegol is a monovalent Fab fragment of a humanized anti-TNFα antibody that is conjugated to polyethylene glycol (PEG) to increase the serum half-life, thus reducing the requirement for frequent dosing and possibly reducing the immunogenic nature, and lacks the fragment crystallizable region (Fc region). It is approved for use in CD only and is also administered subcutaneously at a fixed dose (standard dose 400mg every 4 weeks) (**Figure 2**)[73].

Although these drugs are approved for IBD therapy, the mechanism of action is still under discussion. However, the TNFα neutralization has been proposed as the primary mechanism (**Figure**

2 and **Figure 3a**)[91,92]. The TNF antagonists (anti-TNFα agents) bind to transmembrane TNFα (tmTNF) and soluble TNFα (sTNF) directly neutralizing the biological effects of TNFα, preventing the amplification of the inflammatory cascade. The affinity to sTNF has been reported to be higher than the affinity to tmTNF for some TNF antagonists.

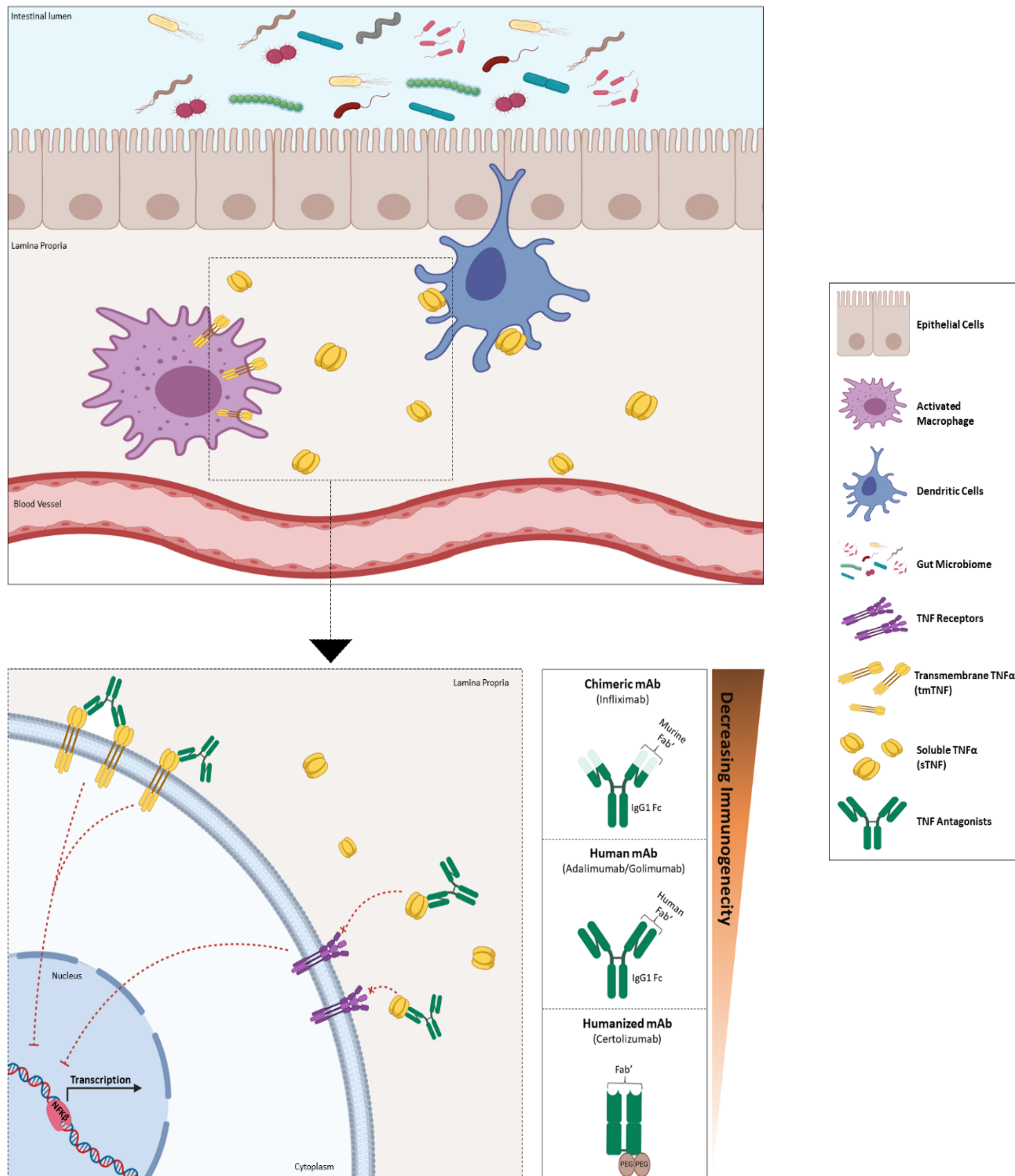

Figure 2 Illustration of the TNF antagonists approved for use in IBD and TNFα neutralization mechanism.

All TNF antagonists have the same target but they are not all equally effective, suggesting there may be different associated mechanisms of actions. Indeed, some studies suggest more complex functions (**Figure 3**): i) outside-to-inside signalling; ii) modulation of the immune system; iii) direct

apoptosis; iv) indirect apoptosis and v) Fc mediated apoptosis (reverse signalling, cell cytotoxicity by activating antibody-dependent cell-mediated cytotoxicity (ADCC), or by activating complement-dependent cytotoxicity (CDC))[91,92]. Briefly, the outside-to-inside signalling consists in the reverse intracellular signalling cascade induced by the binding of TNF antagonists to tmTNF, that acts as a receptor. Its downstream effects include induction of apoptosis (induced G0/G1 cell cycle arrest) and suppression of proinflammatory cytokine expression (**Figure 3b**). TNF antagonists induce the formation of regulatory macrophages in an Fc region-dependent manner. The induced regulatory macrophages inhibit proliferation of activated T cells and produce anti-inflammatory cytokines (**Figure 3c**).

The programmed cell death (apoptosis) of immune cells is a fundamental mechanism of resolution of inflammation. Apoptosis can be either direct or indirect. TNF antagonists induce direct apoptosis of monocytes and T cells in a caspase-dependent way. Indeed, by binding to its receptors (tumour necrosis factor receptor, TNFR), TNFα leads to the formation of death-inducing signalling complex that activates caspase-8 (initiator protease of the extrinsic apoptosis pathway). Caspase-8 activates other downstream caspases resulting in cell death (**Figure 3d**). Indirect apoptosis occurs by binding of TNF antagonists to tmTNF, preventing the TNFR2 activation. Usually, TNFR2 activation leads to Nf-$_{k}$β induction and up-regulation of proinflammatory cytokines production, such as IL-6, and subsequent T cell resistance to apoptosis. Therefore, the induction of apoptosis is dependent on inhibition of TNFR2 signalling (**Figure 3e**). The apoptotic death is a fundamental aspect of the resolution of an inflammatory infiltration and one of the key mechanisms that prevent the development of chronicity of inflammation.

The Fc region of an antibody can mediate different effector functions. In CDC, TNF antagonists bind to a target cell and are subsequently bound by complement. The resulting activation of the complement cascade will result in the lysis of the target cell (**Figure 3f**). ADCC is a mechanism of action of TNF antagonists that have a Fc region. In ADCC, the TNF antagonist binds to the target cell,and in this process the Fc domain is recognized by the Fc receptor of effector immune cells, typically NK cells. These cells then release cytotoxic proteins that subsequently result in lysis of the target cell (**Figure 3f**).

All these theories are based on the observation that TNF antagonists can bind to activated T cells, macrophages and monocytes.

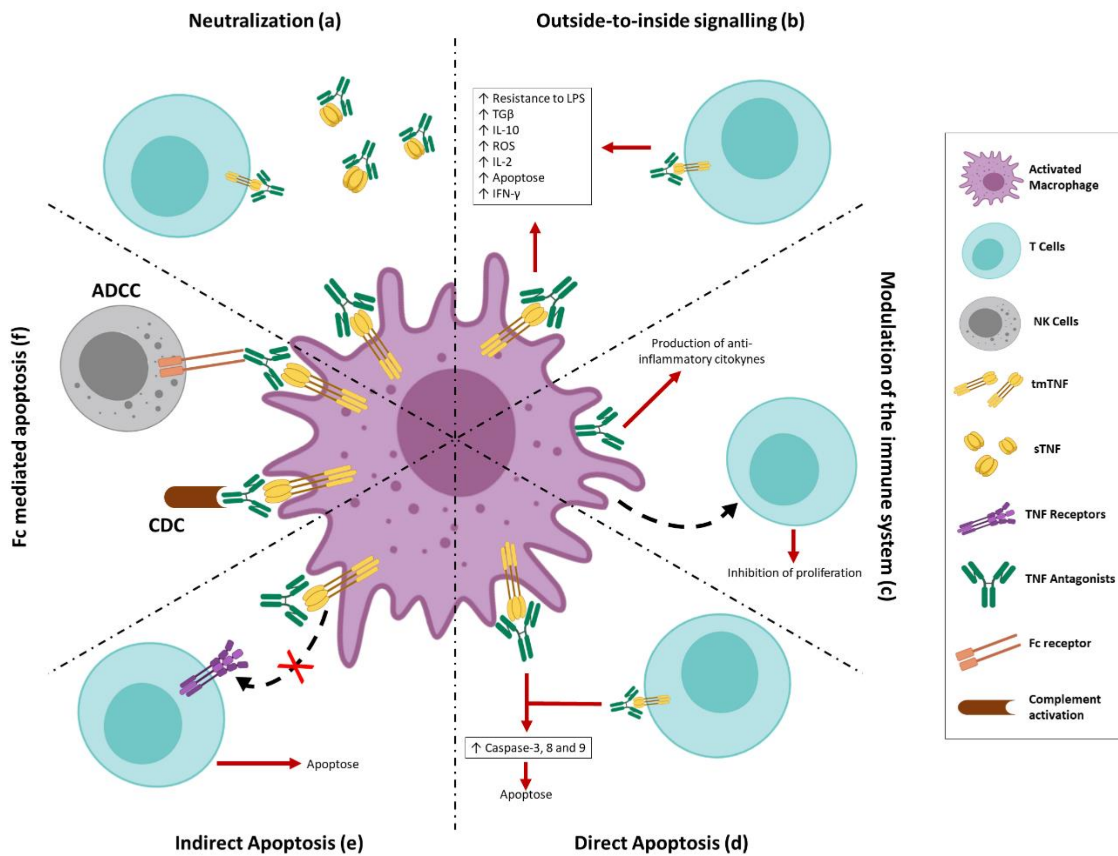

Figure 3 Schematic illustration of possible mechanism of action of TNF antagonists in IBD. Binding to tmTNF and sTNF neutralizes the biological effects of TNFα **(a)**, preventing amplification of inflammation. Some TNF antagonists induce outside-to-inside signaling via tmTNF **(b)** and also cause induction of apoptosis **(d** and **e)**. TNF antagonists reduce proinflammatory T cells subsets and induce regulatory macrophages facilitating inflammation resolution **(c)**. TNF antagonists binding to tmTNF and their Fc regions, mediating antibody dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC), have been suggested **(f)**. NK cells: Natural killer cells; tmTNF: transmembrane TNF; sTNF: soluble TNF; TNFR: TNF receptor; TNF: Tumour necrosis factor; FcR: Fragment crystallizable receptor; LPS: Lipopolysaccharide; TGF-β: Transforming growth factor beta; IL: Interleukin; IFNγ: Interferon gamma.

1.5.3. BIOSIMILARS

Biosimilars (or therapeutic proteins) are biologic products that are highly similar to a previously approved reference biologic drug in terms of safety, purity, and efficacy[93]. Biosimilars are not an exact copy of their originators. Biosimilars are derived from living organisms resulting in minor differences among production batches, due to an extremely complex production process. Therefore, they are expected to have a few structural or functional differences (for example, conformational changes, side-chain additions or subtractions, oligomerization or glycosylation)[94], but these differences are described as having no impact on their clinical properties[95–97]. The number of biosimilars approved by global regulatory agencies, such as the European Medicines Agency (EMA) or the United States Federal Drug Administration (FDA), is increasing. It is expected that biosimilars have a lower cost than innovative medicines and there is a need for Health Authorities to reduce health expenses. These drugs account already for almost 20% of the total pharmaceutical salesmarket[98].

To date, six original biologic therapies are currently available for IBD across the world: four TNF antagonists (IFX, adalimumab, golimumab and certolizumab pegol) and two anti-integrin molecules (natalizumab and vedolizumab)[72]. Recently, some biologic therapies, such as infliximab and adalimumab, have expired their patents, and several pharmaceutical companies developed new similar products (biosimilars). Remsima® and Inflectra® were the first biosimilars to the original Infliximab-Remicade® approved by regulatory agencies (2013 – EMA and 2016 – FDA). Another biosimilar has been produced (Flixabi® (termed SB2)) and approved by regulatory agencies (2016 – EMA and 2017 – FDA)[99].

A key distinction between the reference product and biosimilars is that the latter can potentially be approved for all the indications of the reference product without explicit safety and efficacy testing for each indication. This process, called extrapolation, is the regulatory and scientific process of extending efficacy and safety data derived from one approved therapeutic indication for which the biosimilar has been clinically tested to other indications for which the innovator product is authorized[72]. Importantly, extrapolation can be considered only on a case-by-case assessment of the known mechanisms of action, pharmacokinetics, immunogenicity, and other factors[100,101]. Biosimilars to Infliximab have received marketing authorization though extrapolation of efficacy and safety data (**Table 1**).

Table 1 Summary of some extrapolation studies of biosimilars in IBD.

Study Type **Randomized, double-blind study**	**Main Observations**
PLANETAS[102]	Phase 1 study in ankylosing spondylitis. To compare the pharmacokinetics, safety and efficacy of CT-P13 and Remicade (originator). This study shows that CT-P13 has an equivalent pharmacokinetics profile, and comparable tolerability, safety and efficacy compared to the originator. Comparable immunogenicity was observed in patients who switched from the originator to CT-P13.
PLANETRA[103]	Phase 3 study in rheumatoid arthritis. To demonstrate equivalence in efficacy and safety of CT-P13 compared with the originator when co-administered with MTX. The results showed no clinically meaningful differences in the efficacy, safety or pharmacokinetics profile between CT-P13 and the originator. Comparable immunogenicity was also observed in patients who switched from the originator to CT-P13.

Although the approval of biosimilars for IBD has been based on extrapolated data, controlled trial data have emerged examining the use of biosimilars in IBD (**Table 2**).

Table 2 Selected pertinent studies on the use of biosimilars in IBD

Study Type	Main Observations
Controlled Trials	
NOR-SWITCH[104]	The only published trial for biosimilars in IBD showed non-inferiority of CT-P13 for maintenance of remission in subjects previously stable on the originator. No differences in adverse effects, inflammatory markers, anti-drug antibodies, or clinical remission.
CT-P13 vs Infliximab for Crohn's disease[105]	Phase 3 non-inferiority double blind clinical trial. Biologic-naïve subjects randomized to initiate the originator or CT-P13, and continue vs switch at week 30, assessing efficacy at weeks 6, 30 and 54. No differences in efficacy (clinical response), safety, or immunogenicity were identified.
Observational Studies	
PROSIT-BIO[106]	One of the early observational cohort studies of CT-P13 comprising biologic-naive and originator patients. No significant safety or efficacy differences were observed.
Meyer et al. 2019 French Equivalence Study[107]	The largest observational cohort of biosimilars in IBD to date. To compare the effectiveness and safety of CT-P13 and originator in originator-naive patients. The primary outcome was a composite end point of death, CD-related surgery, all-cause hospitalization, and switch to another biologic. Effectiveness of CT-P13 is equivalent to the originator. No difference was observed for safety outcomes.
Systematic review and meta-analyses of studies	
Efficacy and safety of CT-P13. Komaki et al. 2017[108]	To evaluate the efficacy and safety of CT-P13 in IBD patients. Excellent clinical efficacy and safety profile was observed, supporting its use in IBD treatment.

The rationale underlying the approval process is that the pre-clinical and clinical trial data for which the biosimilar was formally tested supported its mechanism of action, pharmacokinetics, immunogenicity, and toxicity as being sufficiently similar to infliximab as used for IBD. The extrapolation process aims to accelerate the availability and use of the biosimilar products and to reduce costs by avoiding replicative clinical trials for each clinical indication for which the originator is approved[100]. Based on scientific extrapolation, data support that biosimilars appear to have similar safety, efficacy, and immunogenicity as the originator. There is also evidence for the use of biosimilars for both treatment initiation and switching as safe, effective, and potentially less costly alternatives. However, it is uncertain if antibodies against the original drug may cross-react with biosimilars[93,100].

1.6. LOSS OF RESPONSE TO TREATMENT

The primary goals of TNF antagonists for IBD are induction and maintenance of clinically defined remission. More recently, infliximab's ability to induce "mucosal healing" has also been described.

Moreover, TNF antagonists have also demonstrated an improvement in patients' quality of life and a reduction in surgeries and hospitalizations[109–111]. However, approximately 10–30% of patients do not respond to the induction therapy (primary non-response, PNR) and up to 40% of patients lose response during treatment (secondary loss of response, LOR)[112]. Distinct mechanisms may be responsible for these treatment failures. Loss of response may be related to immunogenicity (development of antibodies against TNF antagonists), and pharmacokinetic, pharmacodynamic, or other factors may increase drug clearance[113].

1.6.1. PHARMACOKINETICS AND PHAMACODYNAMICS

Pharmacokinetics (PK) is defined as the movement of drugs through the body that describe a drug's exposure by characterizing absorption, distribution, bioavailability, metabolism, and excretion as a function of time, whereas pharmacodynamics (PD) is described as the body's biological response to drugs[114–117].

Compared to conventional small molecules drugs, TNF antagonists have unique characteristics that make their PK and PD quite complex[114]. TNF antagonist drugs available on the market are from IgG isotype such as IgG1, IgG2, and IgG4, which in general have as PK characteristics slow clearance, long half-life, and limited tissue distribution. This long half-life offers the advantage of less frequent administration in patients compared to small molecules. mAbs have limited oral bioavailability due to their limited penetration across the intestinal epithelium and susceptibility to enzymatic degradation by proteases and peptidases in the intestinal lumen[115]. Therefore, mAbs are administered by an intravenous, subcutaneous and occasionally intramuscular route[114]. The intravenous route allows the administration of a large volume of the drug, resulting in less variability in drug exposure and less immunogenicity, due to the immediate central distribution and slower elimination. Intramuscular administration uses smaller injection volumes and the distribution appears to occur by lymphatic drainage, resulting in individual absorbance variability. In turn, drug distribution via subcutaneous administration occurs mainly in the peripheral blood, with low tissue penetration due to the large size and the hydrophilic nature of the antibodies, and, therefore, the volume of distribution is small[116,117]. This type of pathway is usually more immunogenic[116–118].

Elimination of mAbs through the kidney is considered insignificant, since the typical mAb molecular weight, 150 kDa, is higher than the glomerular filtration threshold (55 kDa)[115]. Thus, the clearance pathways of mAbs (**Figure 4**) seem to occur by: i) a non-specific way through pinocytosis and proteolysis in the liver and in the reticuloendothelial system (RES) ; ii) a target-mediated specific clearance through of degradation of internalised antibody-antigen complex or iii) an elimination of internalised antibodies by receptor-mediated endocytosis process[112,113,116,117].

There are at least two distinct mechanisms that contribute to non-specific clearance of mAbs: pinocytosis (fluid-phase endocytosis) in cells, and proteolysis in the liver and in RES, with subsequent removal from circulation. Given the intracellular uptake via pinocytosis and proteolysis does not differentiate which proteins in the surrounding of a cell are taken up for degradation, a protective mechanism for IgG molecules is necessary to maintain their plasmatic concentrations in order to support their physiologic function to provide long-term immunity[114,118]. One key mediator of the pinocytosis is the neonatal Fc receptor (FcRn, IgG receptor FcRn or Brambell receptor), which protects the internalised antibody from rapid intracellular catabolism[115]. FcRn is functionally expressed in liver cells, monocytes and/or macrophages of the RES, dendritic cells, and endothelial cells. After endocytosis, the IgG antibody (Fc domain) binds to FcRn within the acidic environment of the endosome. The IgG antibody is returned to the cell surface and dissociates from FcRn in a physiologic environment (pH 7.4) and is released into systemic circulation (**Figure 4a**)[114,118,119]. This binding to FcRn results in protection from degradation, thereby prolonging the half-life of mAbs (IgG antibody). In fact, it has been observed that the elimination half-life of IgG1, IgG2, and IgG4 is 18–21 days, which is substantially longer than the half-life of other proteins with similar molecular weights. However, the IgG3 molecules have a substantially lower binding affinity to FcRn, thus exhibiting a half-life of 7 days. Proteins that are not bound to FcRn undergo proteolytic degradation in the lysosome (**Figure 4a**)[114,118]. On the other hand, mAbs clearance through proteolysis in the liver and RES is mediated through binding of the Fc region of the mAbs to Fc gamma receptors (FcγR)-expressing cells, such as Kupffer cells in the liver, and monocytes and/or macrophages of the RES, followed by degradation in lysosomes. The FcγR- mediated elimination pathway is not saturable for mAbs therapeutics. Therefore, FcγR binding alone is not expected to impact mAb PK when nonspecific clearance is dominant[115].

Target-mediated clearance (or target mediated drug disposition, TMDD) refers to the elimination of mAbs through the interaction between them and their target (sTNF or tmTNF), that triggers the internalisation of the antibody into a vesicle and subsequent intracellular catabolism by proteases[114,118]. TMDD decreases with the saturation of the target, which is dependent on mAbs doses (reduces with increased doses). When above the saturation dose level, TMDD becomes insignificant and the clearance of mAbs is mediated through the nonspecific FcRn pathway (pinocytosis) (**Figure 4b**)[114,115,118,120]. Multiple factors affect TMDD, e.g., binding affinity of mAbs, antigen density, turnover rate, and internalisation rate[114,115,118,120].

Receptor-mediated endocytosis results from the interaction of cell surface receptors (Fc-gamma receptors, FcγR) with the Fc domain or one of the Fab binding domains of mAbs. FcγR are expressed on immune cells, including monocytes, macrophages, myeloid progenitor cells and

dendritic cells. Binding of mAbs to FcγR triggers the endocytosis of the complex and subsequent intracellular degradation (**Figure 4c**)[114,115,118,120].

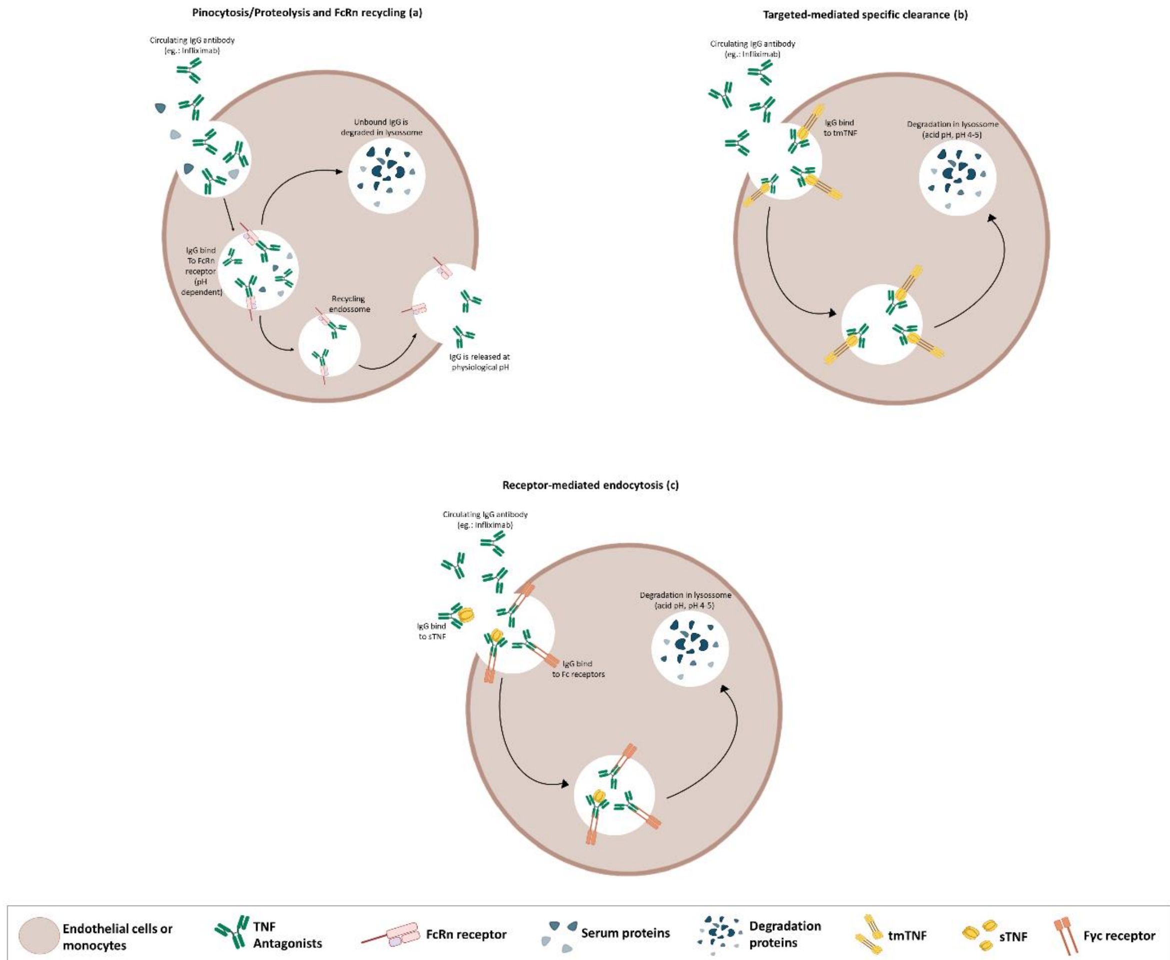

Figure 4 FcRn mediated IgG antibodies drug (TNF Antagonists, e.g.: Infliximab) recycling pathway and removal via pH dependent binding. **(a)** IgG circulating in the blood is taken up by endothelial cells or monocytes through either fluid phase pinocytosis or proteolysis. Once inside the cells, IgG binds to FcRn in the acidified endosomes. IgG that binds to the receptor migrates to the cell surface where the IgG encounters a physiological pH environment and is released back into the blood. IgG that is not bound to FcRn (due to competition with other IgG) will be sorted to lysosomes for degradation. **(b)** Target-mediated clearance (TMDD); IgG antibodies drug bind to their target (sTNF or tmTNF) that triggers the internalisation of the antibody into a vesicle and subsequent intracellular catabolism by proteases. **(c)** IgG antibodies drug contain pH-agnostic Fc domains and can bind to Fc receptors at physiologic pH, triggering receptor-mediated endocytosis. In this case the IgG antibodies do not unbind the Fc receptor upon exocytosis, instead lingering at the cell surface.

Many other factors, such as antibody properties (hydrophobicity, charge, glycosylation patterns), inter-subject variability (disease status, degree of inflammation, body size, genetic polymorphisms, concomitant medication and comorbidities), and immune-mediated response generated by mAbs administration can impact the absorption, distribution, clearance, efficacy and safety of mAbs[115,118,121]. For example, studies have shown that low albumin is associated with increased clearance and, together with high C-reactive protein (CRP) levels, are markers of high

inflammatory load[122,123]. Another example is the degree of inflammation, that increases the clearance by different mechanisms: i) increase in IgG catabolism by the RES; ii) high levels of TNFα will consume the TNF antagonist drug faster; iii) during active intestinal inflammation there is an increase in intestinal permeability and consequent faecal loss of the TNF antagonist drug[117,124]. The individual variability of Fc receptors may also influence PK, determining the degree of exposure of therapeutic antibodies[125,126]. Furthermore, the formation of anti-drug antibodies (ADAs) was reported in an accelerated mAbs clearance and consequently impact response to treatment[117].

1.6.2. IMMUNOGENICITY

Treatment with mAbs drugs can generate an immune response, which may cause the development of endogenous antibodies against foreign proteins (a process termed immunogenicity). The formation of ADAs could potentially affect PK, safety, and efficacy of mAbs drugs due to hypersensitivity reactions and, in some cases, lead to therapy failure[117,127].

Antibody formation is triggered by the interaction of three type of cells: antigen presenting cells (APCs), Th cells and B cells. Briefly, the drug is recognized and engulfed by APCs, through pinocytosis, receptor-mediated endocytosis or phagocytosis. Inside the APCs, in lysosomes, the drug is cleaved into fragments (peptides). Each peptide binds to the major histocompatibility complex class I or class II (Major histocompatibility complex class I (MHC I) or Major histocompatibility complex class II (MHC II)), and those who bind with "strongly enough" affinity will be transported to the cell surface for antigen presentation. Then, the T cells will recognize the MHC-antigen complex, through T cell receptors (TCRs). This interaction, together with co-stimulatory signals provided by the APC through CD80 and CD86 molecules, fully activates the specific Th cell. Subsequently, the Th cells divide and generate a set of pro-inflammatory cytokines that recruit naïve B cells. Immunoglobulin M and Immunoglobulin D receptors present in naïve B cells bind to the epitope of the MHC-antigen-TCR complex, and this interaction induces B cell clonal expansion and differentiation into antibody-secreting plasma cells and memory B cells[117,128].

ADAs that are generated in patients treated with mAbs can be stratified into two main categories: neutralizing ADAs (Nabs) and non-neutralizing ADA (non-Nabs) (**Figure 5**). Nabs bind to and/or modify epitopes that are functionally relevant for ligand-receptor interaction and hence inhibit the drug activity soon after the administration of the drug (**Figure 5a**). Non-Nabs bind to sites on the drug that do not affect target binding and thereby do not impact the drug's pharmacodynamic activity, though its clearance from circulation can still be affected. (**Figure 5b**)[129,130]. TNFα antagonists help to reduce the inflammatory responses by targeting both membrane-bound and soluble TNFα.

Therefore, the neutralization of tmTNFα and sTNFα prevents its ability to bind to its receptor, inhibiting its biological activity.

Commonly, Nabs are generally considered to be more clinically important than non-Nabs, as they directly reduce drugs' efficacy. However, Non-Nabs may indirectly reduce therapeutic efficacy by compromising bioavailability and/or accelerating drug clearance from circulation. Some studies report that both types of ADAs can form immune complexes upon binding the drug, which are cleared by the RES, thus shortening half-life and reducing serum levels[130,131,132]. In fact, the formation of two immune complexes with different size, probably reflecting dimers (a drug molecule linked to a single therapeutic antibody) and tetramers, have been detected. Large immune complexes seem to be cleared from circulation more quickly than small complexes. These results suggest that large immune complexes are eliminated quickly in the liver, while small ones are not and remain in circulation for an extended period of time. Therefore, the type of immune complex also influences both the half-life of the therapeutic protein and treatment efficacy[130]. Currently, many questions related to the development of ADAs remain unaddressed, including the relative frequency of neutralizing and non-neutralizing ADAs, what portion of the ADA exhibits neutralizing capacity, and if the ADAs present in serum are neutralizing or not. Usually, in the clinical context, ADAs levels are assessed with assays that measure total ADA levels, not distinguishing between Nabs and non-Nabs. Since Nabs can trigger clinical effects, specific and sensitive in vitro methods are needed for their detection[130,131,132]. Two types of assays can used: a cell-based assay or a non-cell-based competitive ligand binding assay (**Section 1.7.1.2.2. ADAs DRUGS ASSAYS**)[133–135136–138].

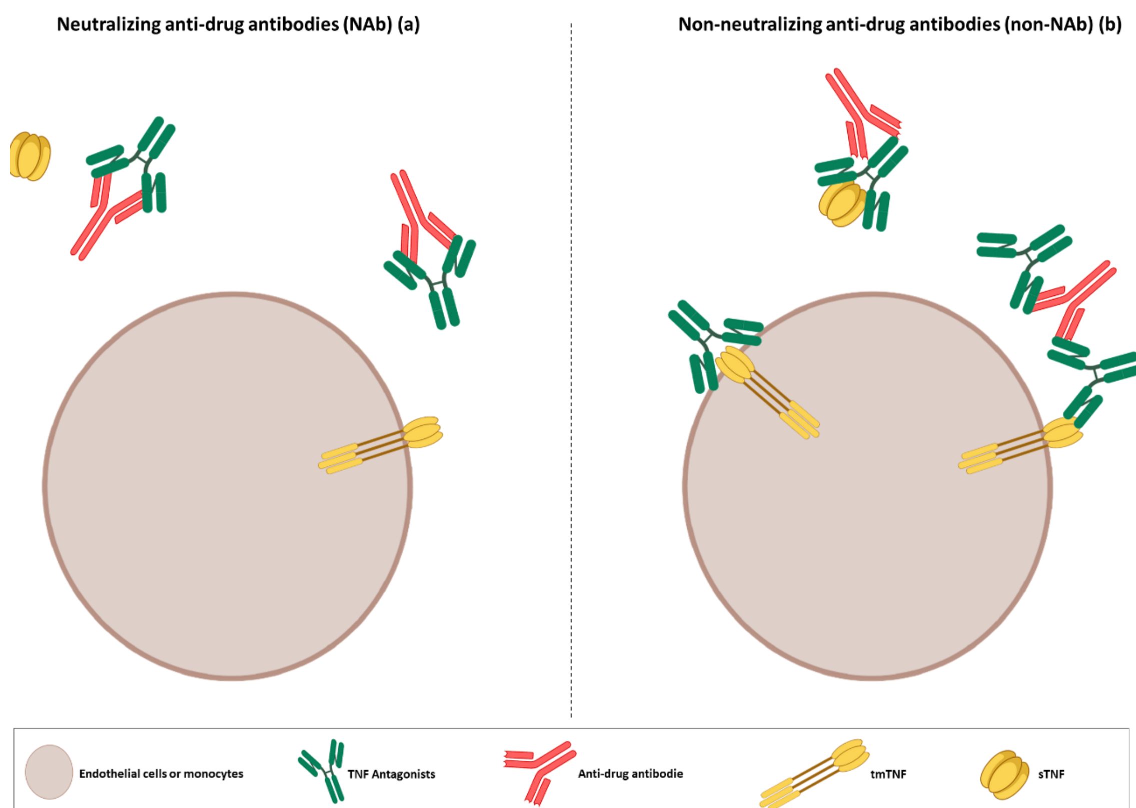

Figure 5 Classification of anti-drug antibodies (ADAs, Y-shaped red symbol) by their mechanism of action. ADAs can be classified as neutralizing (NAbs) or non-neutralizing (non-NAbs) by evaluating the impact on pharmacological drug function. TNF Antagonists (drug) and tmTNF/sTNF (targets) interact at a specific site of the drug (active site). When the drug binds to the target it elicits a cellular response (such as cell proliferation, cell death or cytokine release). **(a)** In the presence of NAbs, this binds to the drug's active site, blocking the interaction of drug and target, thereby inhibiting the cellular response. **(b)** Non-neutralizing ADAs bind at sites of the drug, but do not inhibit the binding of the drug to its target, and hence do not interfere with its pharmacological function.

Several factors may influence immunogenicity: drug's characteristics, patient's genotype and immune system activity, drug dosage, frequency of administration, route of administration and co-treatment with immunomodulator agents[130,139].

The nature of the TNF antagonist drug has been related to the degree of immunogenicity, that is, immunogenicity decreases with the level of humanization of the antibody (fully-human IgG < humanized IgG < chimeric IgG < murine IgG)[117]. The size, structural complexity, and protein sequence variation of the drug may also affect immunogenicity. Therapeutic mAbs are produced in mammalian cell lines (human and non-human), plants, bacteria, yeast and virus. Therefore, during production, minor differences in post-translational modifications, such as glycosylation, may impact 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[140]. This is the main reason why it is preferable to use human cell lines, as the pattern of post-translational modifications is as similar as possible to the human physiological (but may not be the same, as most

cell lines used are immortalized). Additionally, the advance of “omics” technologies has recently given rise to new alternatives, in particular for Chinese Hamster Ovary (CHO) cells. This cells are involved in the production of over 70% of recombinant biopharmaceutical proteins, most of them being monoclonal antibodies (mAbs) [140,141].

Genetic differences, type of disease and the immune status of the patient can also trigger a significant immune reaction. Some studies suggest that patients with a highly active immune system have an increased risk of ADAs formation. For example, patients with Rheumatoid Arthritis (RA) who develop ADAs have higher baseline disease activity than those who do not[130].

The treatment regimen used may also influence ADAs formation. For example, the use of high doses can reduce immunogenicity and induce immunological tolerance through exhaustion of the immune response. Furthermore, subcutaneous administration is more immunogenic than intravenous administration. Other studies also suggest that the risk of ADAs formation increases with increasing treatment duration. However, combination therapy with immunomodulators has been described to inhibit the formation of ADAs. In patients with RA or CD, co-administration of azathioprine, mercaptopurine, hydrocortisone or methotrexate was associated with a reduced frequency of ADAs formation[130]. Thus, the dose optimization and the use of co-medication could help to reduce immunogenicity.

1.7. THERAPEUTIC DRUG MONITORING

When TNF antagonist treatment fails, clinicians need to act immediately to ensure treatment recovery, since long periods of uncontrolled disease may lead to disease progression and increase the risk of irreversible tissue damage[142,143]. The clinical-based approach to deal with lack of response depends on patients' symptoms and a "trial and error" strategy, following the European Crohn’s and Colitis Organisation (ECCO) Guidelines[144,145]. This strategy is a step-by-step process until a response is achieved. First, intensification of the current TNF antagonist regimen (increasing dose or decreasing interval between administrations) is recommended. In case of failure, switching to another TNF antagonist is advised; and finally, switching to another class of drugs. Concomitant treatment with immunosuppressive agents, corticosteroids, and surgery is also suggested. In this approach, a standard treatment is applied to all patients who lose response. However, such a complex disease, where several genes, cytokines and metabolites are involved, may respond differently depending on the individual[82,146,147]. It has also been reported that some patients experience adverse effects, while others do not. In conclusion, an unsuccessful approach leads not only to a negative impact on the

clinical status of patients, but also to a significant financial burden, since TNF antagonists represent the highest health cost for hospitals, being higher than the costs of surgery orhospitalization[148,149].

In this sense, rather than applying the same treatment to all patients, adjusting treatment according to individual characteristics can be a great advantage. Therefore, therapeutic drug monitoring (TDM), by measuring ADAs and drugs levels, can be used to maximize the effectiveness of the drug and achieve a the highest therapeutic benefit with the least adverse effect[73,150]. TDM can also help to identify the underlying cause of therapy failure, which may include PK/PD factors and immunogenicity. Consequently, gastroenterologists began to use TDM to guide dose adjustment when a loss of response or an infusion reaction occurs. TDM can be done in two different ways: i) reactive, when a patient has an clinical recurrence of the underlying disease, or ii) proactive, when a patient is in remission and the goal is to prevent future flares related to subtherapeutic drug concentrations or the development of antidrug antibodies[151]. Reactive TDM is described as a cost-effective strategy compared with empiric dose escalation. This is due to effective triaging of patients to identify those who would benefit from a dose escalation and those who should change therapy. However, it is unknown if a proactive TDM strategy is cost-effective. Many specialists believe that proactive TDM leads to o timely escalation of therapy, higher rates of clinical remission, reduced immunogenicity, reduction of loss of response and, consequently, reduction of costs[152]. A recent systematic review concluded that there was insufficient evidence to support one method of monitoring therapeutic drugs (Reactive vs. Proactive) in relation to the other, particularly concerning clinical remission rates, although recognizing that there was a potential for cost savings when using a reactive TDM approach. As a consequence, the present guidelines state that there is insufficient evidence to introduce the routine use of proactive TDM in the treatment of IBD patients[144,151].

1.7.1. TDM PROCESS

TDM comprises four main steps: 1) blood sampling; 2) measurement techniques; 3) communication and interpretation of the results, and 4) clinical decision-making support (e.g.: dose adaptation if needed).

1.7.1.1. BLOOD SAMPLING

This first step is very important because there are many possible biases: inaccurate sampling and/or erroneous administration time, erroneous dose administration, or even analytical errors. Since TDM is currently based on trough concentrations, blood samples should be collected before the start of the following drug administration. In this sense, there should be an adequate scheduling

of the visits. The manner of sample collection and their storage must be considered to ensure that the laboratory measurements of concentrations is the most correct possible. Laboratory measurements are typically performed on serum, hence the use of serum tubes with a clot activator and gel separator is recommended. In order to avoid haemolysis, the serum should be removed from the clot as soon as possible (within 4 hours). Regarding storage, the following precautions must be taken: if the serum sample is analysed within 1 week, it can be stored at room temperature; however, if the aim is to make further analyses, the serum sample must be stable and can be stored at -20ºC for at least 1 year. In this case, samples should be aliquoted to avoid repeated freezing and thawing cycles[153–155].

1.7.1.2. MEASUREMENT TECHNIQUES

The availability of assays to measure drug and/or ADAs concentrations are necessary for the performance of TDM which has led to the development of different assays to assess these concentrations. Different binding assays based on immunochemical processes, including immunoassays in solid or liquid phase, have been developed. However, it is necessary to be aware that the available assays have different limitations and can produce different results.

1.7.1.2.1. ANTI-TNFα DRUG ASSAYS

Anti-TNFα drug levels may be determined by different methodologies: i) solid-phase enzyme-linked immunosorbent assay (ELISA), homogeneous mobility shift assay (HMSA), lateral flow-based assay (LFA) and fluid-phase radioimmunoassay (RIA). An overview of these assays are illustrated in **Figure 6**, **Figure 7**, **Figure 8** and **Figure 9**, respectively.

ELISA is the most commonly used technique. In ELISA, the drug is captured on a plate and detected using a secondary antibody. The principle of ELISA is illustrated in **Figure 6**. The antigen (TNFα drug) is immobilized onto the wells of a microplate through incubation for a few hours. After several washes to remove unbound excess of the antigen, a blocking buffer (usually, 1% bovine serum albumin (BSA)) is added to block nonspecific binding sites. Afterwards, diluted serum samples are added to the plate and the anti-TNF-α drug present in the serum samples will bind to the antigen immobilized on the plate. After an hour of incubation for complete binding, the plate is washed again to remove unbound material. For quantification, an antibody (anti-Fc domain) linked to a reporter enzyme (horseradish peroxidase, HRP) is added to the plate (1 hour of incubation). Subsequent, the plate is washed again and an appropriate substrate to the enzyme (tetramethylbenzidine) is added, producing a measurable product. In the presence of this substrate a colorimetric product is formed, and the reaction is stopped with an acidic solution. The absorbance of the colorimetric product is

measured at a specific wavelength, using a microplate reader equipment. Usually, ELISA requires expensive equipment, highly trained analysts, a batch of samples and is time consuming (~8 hours)[134,156,157].

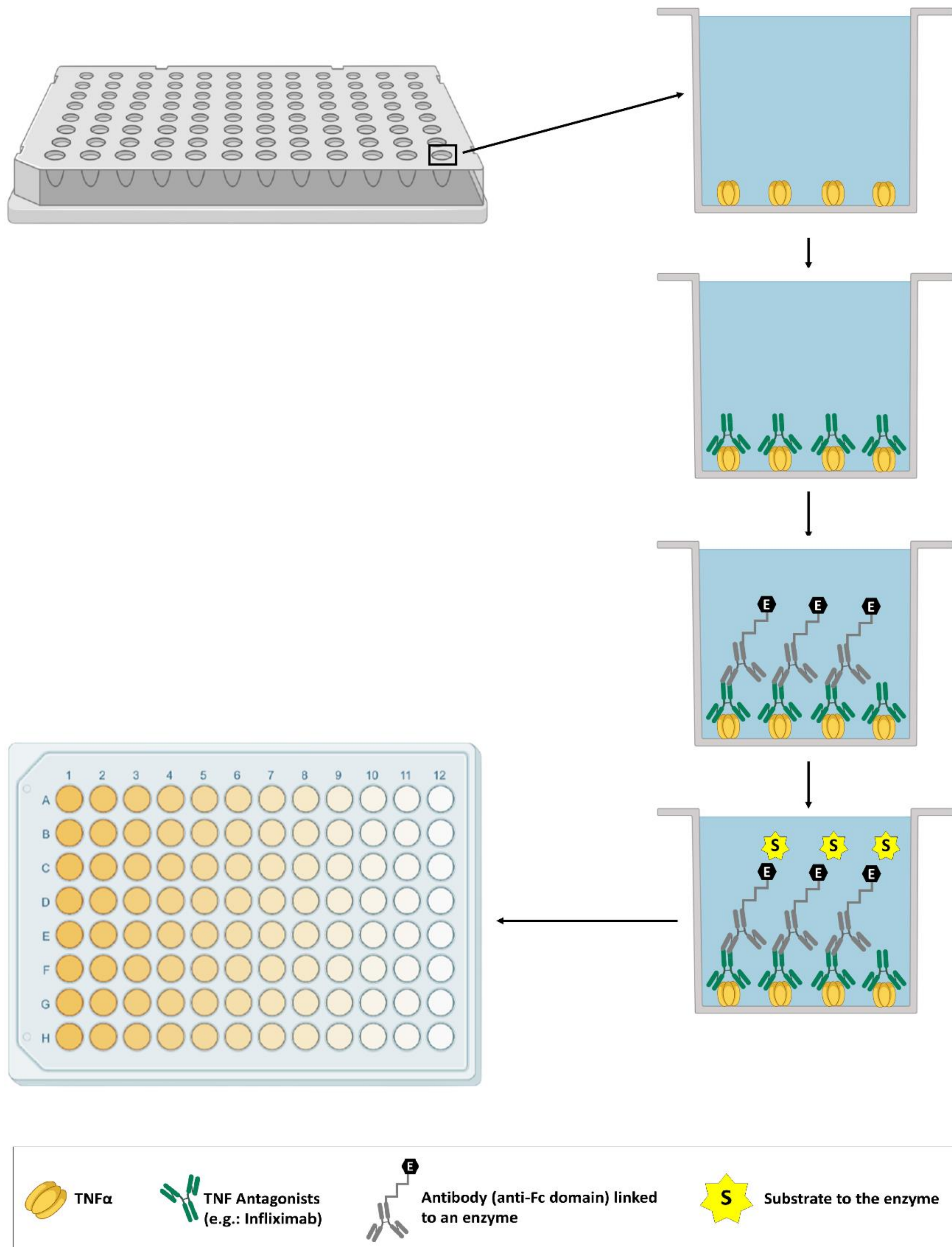

Figure 6 Overview of the ELISA principle for drug measurement. **Step 1)** An antigen protein (TNF-α drug) is immobilized on the surface of a microplate well. **Step 2)** The anti-TNF-α drug present in serum samples binds to the antigen immobilized in the plate. **Step 3)** An antibody linked to an reporter enzyme is added. **Step 4)** An appropriate substrate to the enzyme is

added, producing a measurable product. **Step 5)** Addition of an acidic solution stops the reaction, producing a yellow colour. The yellow colour visible in the plate indicates that the target is present. The higher the colour intensity, the higher the concentration of the target.

HMSA is a high pressure liquid chromatography (HPLC) mobility assay. Briefly, a fluorescent labelled TNFα complex is incubated with the serum sample and subsequently injected into the chromatographic column. The free drug in serum conjugated with the fluorescent labelled TNFα complex (immune complex) is resolved by molecular size separation in the chromatographic column and the peaks are quantified by fluorescence allowing for the identification of the immune complexes (**Figure 7**). The concentrations are determined from a standard sample curve with known concentrations of the immune complexes. The dominance of HPLC as a premier analytical technique is no accident. The most prominent advantage is its applicability to diverse analytes types, from small organic molecules and ions to large biomolecules and polymers. However, considering that if this technique is not optimized, it turns out to be a very time-consuming and also complex process. It is also necessary specific equipment which turns out to be very expensive as well as teams of very experienced technicians[134,135,156].

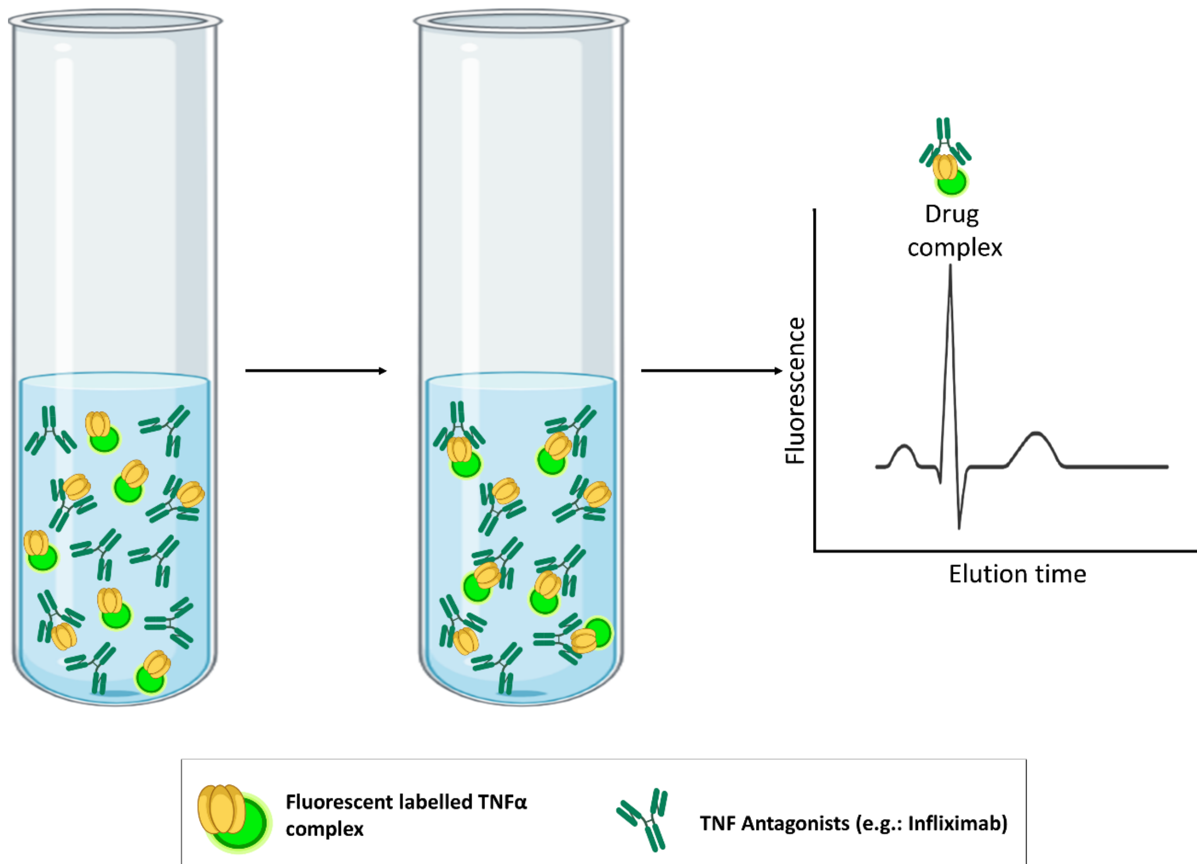

Figure 7 Schematic illustration of the HMSA principles for drug measurement. HMSA depends on the association of fluorescent-labelled drug (TNFα complex) added to serum that binds to the anti-TNFα drug (e.g.: Infliximab) creating immune complexes. HMSA detects these immune complexes by size-exclusion high-performance liquid chromatography (HPLC), and allows for their quantification through fluorescence.

In the last few years, rapid assays have been launched on the market. These assays are based on lateral flow-based assay (LFA) technology for the detection and quantification of drug in a liquid sample, which allows results in just 15 minutes. LFA operates on the same principles as ELISA. In LFA, the components of the sample migrate across a membrane, via capillary force, and are then subjected to an immunochemical reaction (between antibody-antigen)[158,159]. LFA consists of four parts (**Figure 8a**): i) a sample pad (on which the sample is deposited); ii) a conjugate pad (on which antibody labelled to colloidal gold is present); iii) reaction membrane (usually a nitrocellulose membrane, that contains the test and control lines for the antigen-antibody interaction); and iv) an absorbent pad (which retains waste). In this assay, two antibodies are immobilized on the reaction membrane (primary antibody – on the test line, and secondary antibody – on the control line). There is a third antibody that is marked with a colloidal gold and is placed between the sample pad and the reaction membrane. When the serum sample with the drug is added to the cassette, the sample migrates through the membrane, binding first to the antibody labelled to colloidal gold. This immunocomplex (drug–colloidal gold) formed continues through the chromatographic flow moving

to the test and control zones. When the immunocomplex makes contact with the primary antibody, they bind, and a positive reaction is observed by the red line in the test zone. The excess of free colloidal gold binds to the secondary antibody and a red line is formed in the control zone. This control line ensures that the test is a valid test (**Figure 8b**). In a non-drug serum sample, the 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 8c**). The qualitative and quantitative features can be evaluated by the naked eye or with the aid of portable devices. For colorimetric detection, the quantitative test results are based on the reading by focusing light and measuring the reflected light attenuation by the surface of the membrane in the test line and control line[158,159].

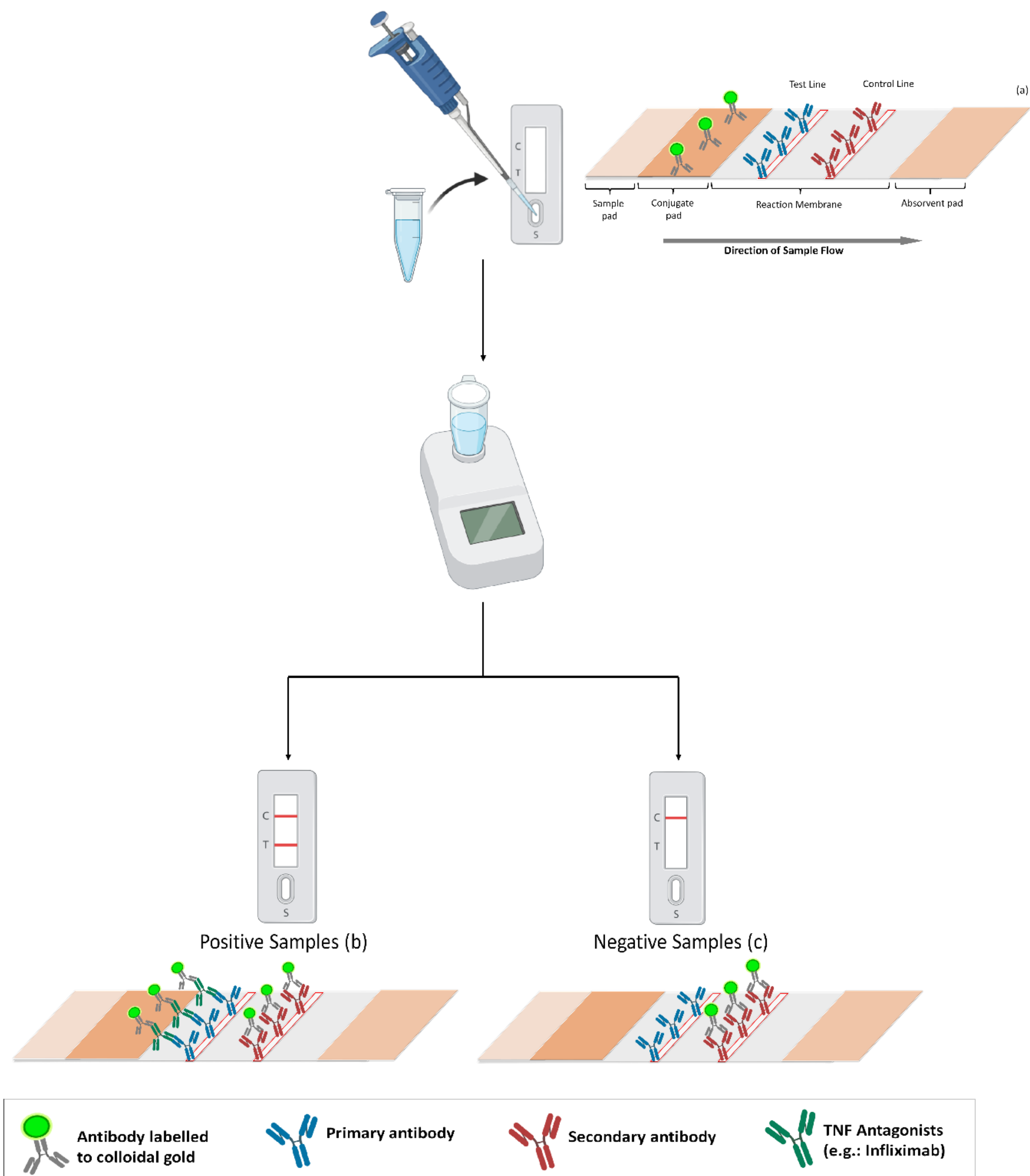

Figure 8 Representative configuration of a lateral flow-based assay (LFA) . **(a)** LFA is usually composed of a sample pad, conjugate pad, reaction membrane and adsorbent pad. The sample is deposited on the sample pad and migrates towards the conjugate. The colloidal gold conjugated antibodies bind to the target analyte and migrate to the test line, where the bound target analyte is captured. Possible results of the test are shown: **(b)** positive sample – is indicated by the red signal in the test line, and **(c)** negative sample – is indicated by the lack of signal in the test line. The control line should be visible independently of the test line result.

Still, more assays are available such as the radioimmunoassays (RIA). These assays are less used due to their complexity, safety concerns (handling and storage of radioactivity, disposal of radioactive waste, and the half–life of the radioactive labels), and also for the high cost of the equipment and

reagents. This resulted in RIA being replaced largely by non–isotopic enzyme-linked immunoassay methods. The basic principle of RIA is competitive binding. It is based on high sensitivity in vitro assay techniques to measure the concentrations of antigens by the use of antibodies. The target antigen is labelled radioactively ("TRACER") and bound to its specific antibodies. A serum sample (containing unlabelled antigens) is added to initiate a competitive reaction between the labelled and unlabelled antigens with the specific antibodies. As the concentration of unlabelled antigen rises, more of the unlabelled antigens binds to the antibody, displacing the labelled antigen. The bound antigens are then separated from the unbound ones, and the radioactivity of the bound antigens is measured (**Figure 9**). These assays use the same principles of ELISA, but in a liquid-phase assay and with a radio-labelled antibody for detection[134,135].

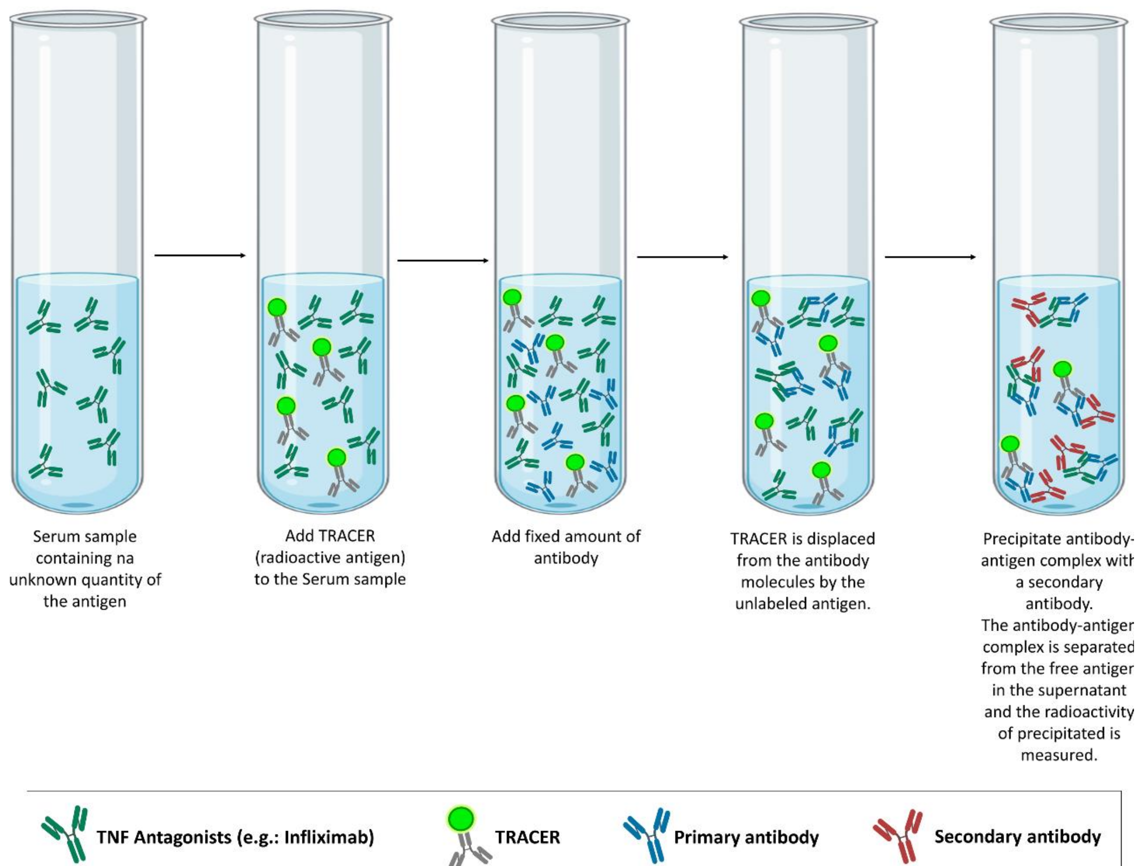

Figure 9 Illustration of RIA principle for drug measurement. RIA involves the separation of a protein (from a mixture) using a specificity of antibody–antigen binding and quantification using radioactivity. A radioactive antigen ("TRACER") competes with a non-radioactive antigen for a fixed number of antibodies or receptor binding sites. This assay includes a precipitation step using a second antibody to separate bound and free fractions, and allows for the quantification of the bound fraction using a gamma (γ) counter.

1.7.1.2.2. ADAs DRUGS ASSAYS

All the previously described assays can also be used for the measurement of ADAs, with some specific changes for that purpose. Bridging ELISA (bELISA) is one of the most common commercial assays for ADA levels assessment. This assay is based on the predominantly bivalent nature of ADAs (double-antigen format), that allows ADAs to form a complex bridge (i.e., cross-link) between the immobilized drug on the plate and the enzyme-labelled drug that is added in the detection step. Briefly, the unlabelled drug is first immobilized onto the wells, and diluted ADAs in a serum sample are allowed to bind, followed by the usual washing step to remove unbound ADAs. Enzyme-labelled drug is then added to complete the bridge, and a colorimetric substrate is added to visually detect the presence of specific ADAs (**Figure 10a**)[129,160,161]. The results for ADAs are reported as microgram per millilitre on the basis of calibrations made with affinity-purified polyclonal rabbit anti-mouse IgG F(ab'). However, this assay cannot determine ADAs below a certain cut-off level because the drug interferes with the assay and the technique is also less reliable for the detection of low-affinity ADAs than high-affinity ADAs dueto the repeated washing steps involved[162,163]. Other drawbacks are also described for this methodology, such as the occurrence of false positives and false negatives. False positives may result by non-specific binding of other immunoglobulins, rheumatoid factors, anti-allotypic antibodies that are able to mimic the bridge, and/or low-affinity antibodies, including heterophilic antibodies in patient serum (**Figure 10b**)[164]. Drug presence may also cause false negatives, because the antibody is already bound to the drug and cannot bind to the immobilized drug in the plate, being lost in the washing steps (**Figure 10c**). Additionally, this assay cannot detect IgG4 ADAs, an IgG isotype that dominates after prolonged immunizations and that is functionally monovalent, and therefore cannot bridge in this type of binding assays (binds to the immobilized drug but is unable to bind to the enzyme-labelled drug) (**Figure 10d**)[135,160,164]. Some researchers report ADAs status as "inconclusive" if the drug is detectable in serum and test negative for ADAs. This has been estimated to be the case in up to half of the patients in the clinical setting[135,160,164,165].

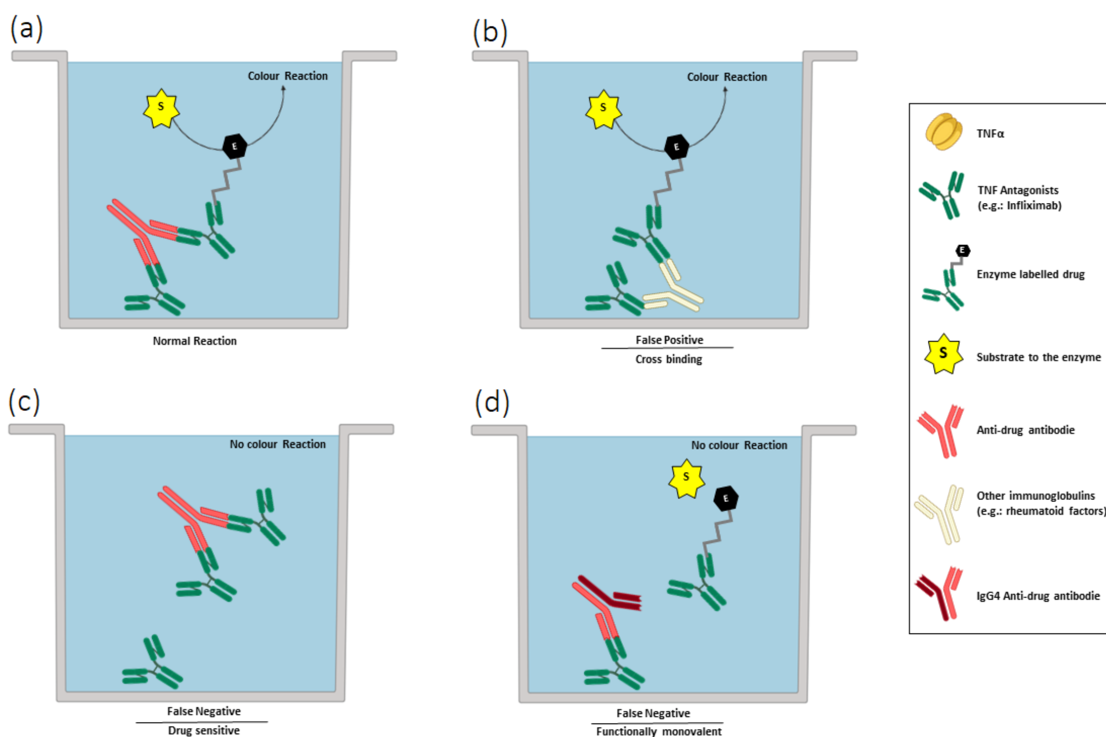

Figure 10 Illustration of bELISA for ADAs detection. **(a)** bELISA depends on the bivalency of IgG ADAs and the ability of these immunoglobulins to bridge to the drug preabsorbed to a plastic well and to the enzyme-labelled drug added in the detection phase. **(b)** False positives can arise from cross-binding of the anti-TNF drug Fc fragments with other immunoglobulins such as rheumatoid factors or anti-allotypic antibodies. **(c)** False negatives can be caused due to the drug sensitivity of the assay implicating that ADAs bound to the anti-TNF drug in patient serum do not bind to the anti-TNF drug preabsorbed to the solid phase **(d)** or false negatives can also occur due to failure to detect functionally monovalent IgG4 anti-drug antibodies.

Given these difficulties with the bridging assay format and drug tolerance issue, alternative assays have been developed: anti-human lambda chain assay (AHLC), semi-fluid phase enzyme immunoassay (SFPE), Radioimmunoassay (RIA), homogeneous mobility shift assay (HMSA), and cell-based reporter gene assay (RGA)[156,157,164].

The anti-human lambda chain assay (AHLC) is based on a sandwich ELISA where TNFα is used as the coating agent, incubated with the anti-TNF drug, and followed by differential dilutions of the tested serum. Aλ light chain antibody labelled to enzyme is used for the detection step (**Figure 11a**). This assay can detect ADAs with a λ constant domain and is believed to be less drug-sensitive, as only one free epitope is required for the capture of bound antibody[157,166,167]. Therefore, the principle of this assay is to detect ADAs comprising a λ light chain, thus avoiding cross reactivity with the drug that comprises the κ light chain. There are also some limitations to this assay. False negative tests may arise when detecting anti-idiotypic ADAs, since the TNFα preadsorbed to the plate binds to idiotypes in the anti-TNFα drug, precluding the binding of anti-idiotypic antibodies (**Figure 11b**). Drug presence may also hamper ADAs detection with the AHLC assay (**Figure 11c**)[135,168] .

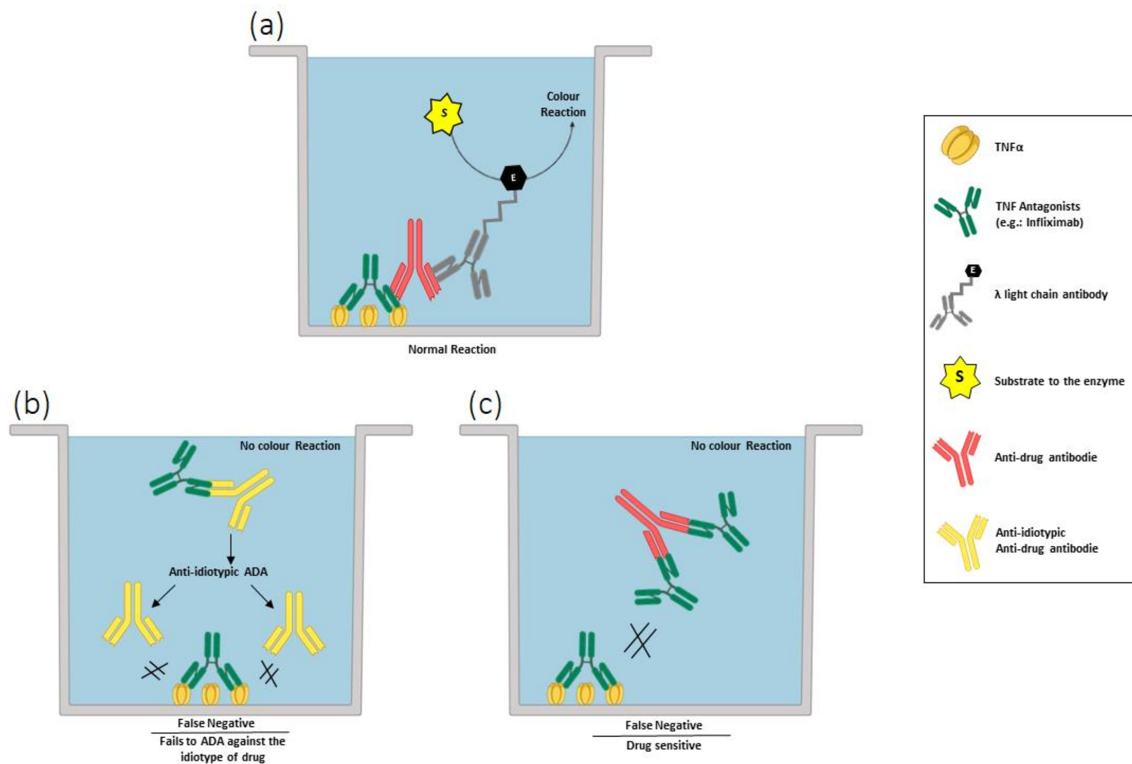

Figure 11 AHLC illustration. **(a)** ADAs comprising a λ light chain, bound to the anti-TNF agent captured on TNF-α-coated plastic wells, are detected by an enzyme-labelled anti-human λ light chain antibody. **(b)** False negatives may arise from failure to detect anti-idiotypic ADA **(c)** and also due to drug sensitivity of the assay. .

The semi-fluid phase enzyme immunoassay (SFPE) is an ELISA assay where the patient serum and biotinylated drug are pre-incubated on acid buffer to dissociate the drug-ADA complex. After the complex dissociation at low pH, acidified samples are incubated with a drug-biotin conjugate, which immobilize ADA to the plate pre-treated with streptavidin, and a horseradish peroxidase (HRP) labelled drug conjugate is used for the detection step (binds to ADA and colour develops) (**Figure *12*a**). However, if ADA binds to double HRP-labelled drug conjugate (**Figure *12*b**) or to double biotin-labelled drug conjugate, false negatives may occur (**Figure *12*c**)[135,164].

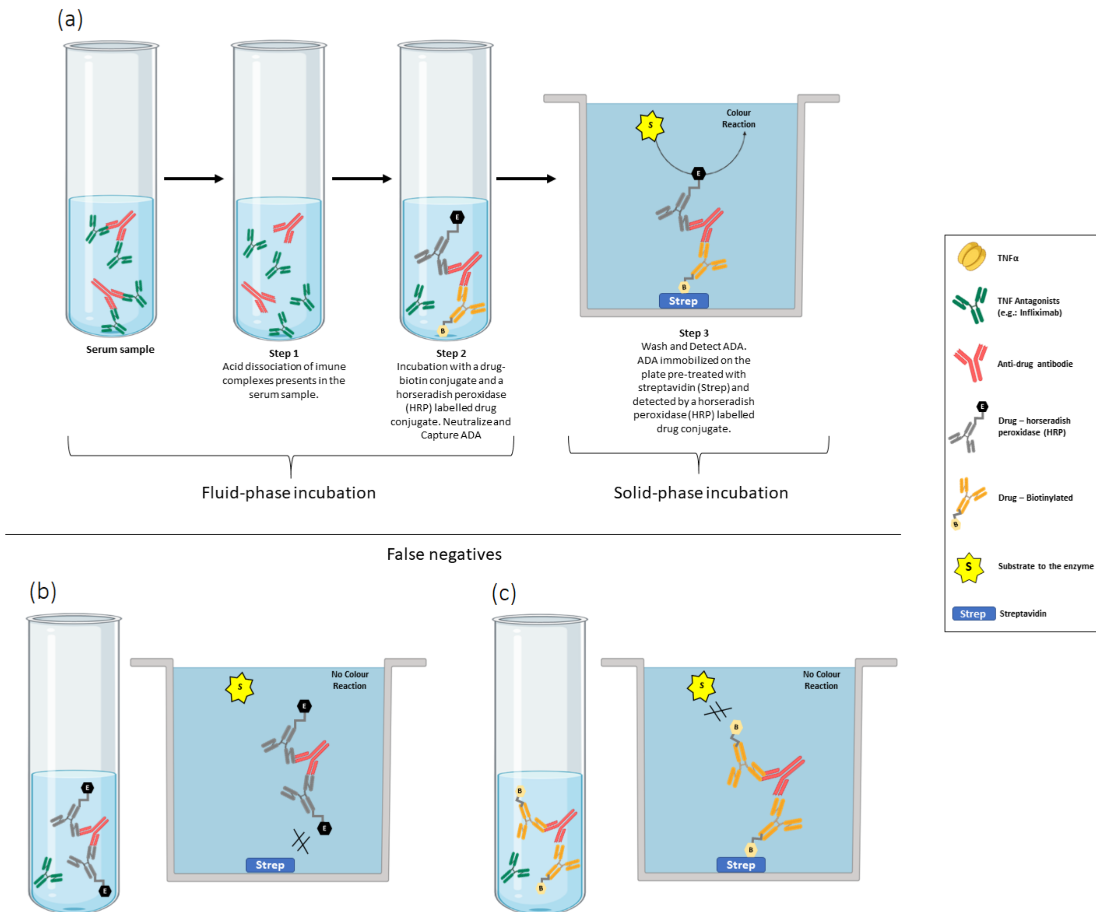

Figure 12 Illustration of the SFPE for ADAs assessment. **(a)** First, the dissociation of the drug-ADA complexes occur on acid buffer, following an incubation step with drug-biotin and drug-HRP for immobilization and detection of ADAs, respectively. **(b)** False negatives occur when ADAs bind to double HRP-labelled drug **(c)** or to double biotin-labelled drug.

In the radioimmunoassay (RIA), the serum sample with ADAs is incubated with a radio-labelled drug which emits radiation and that, after centrifugation, can be measured by a gamma counter. This methodology is highly sensitive, measures ADA also in the presence of limited amounts of drug, and detects all immunoglobulin isotypes of ADA, including IgG4. As previously described, the major limitation is the handling of radioactive material that make this assay less attractive, as well as the necessity for specially trained staff, radioactive waste disposal and advanced laboratory facilities[134,135]. Another fluid-phase assay and most commonly used is the previously described HMSA. For ADAs detection an extra step is added: acidic treatment to dissociate the drug-ADA complex, prior to incubation with the fluorescent-labelled drug. Chromatographic separation in the column allows to detect ADAs that bind to the fluorescent-labelled drug. HMSA exhibits high sensitivity, accuracy and ability to detect all immunoglobulin isotypes of ADA, even in the presence of high serum

drug levels or concomitant MTX therapy. However, it is an expensive method, with the need for costly laboratory equipment and trained personnel[134,135,156].

It should be noticed that all solid-phase assays have several noteworthy limitations, resulting in false-positives and false-negatives results. However, liquid-phase methodologies have been described as being less artificial, better reproducing in-vivo conditions. An important distinction between the ELISA-based tests and the RIA and HSMA assays is that the former cannot detect ADAs in the presence of drug. However, all these assays have in common that none of them provides information about the functionality of ADAs (whether they are neutralizing or not). Therefore, a cell-based reporter gene assay (RGA) was developed.

RGA represents one of the most commonly used cell-based assays and its principles are illustrated on **Figure 13**. Briefly, TNFα is added to the reporter cells (that carry a TNFα inducible NFκβ regulated firefly luciferase reporter-gene construct) and binds to its receptor (TNFR). The reporter gene is activated by the intracellular signalling that is initiated through activation of Nf-κβ, and generates firefly luciferase expression, which is normalized to the expression of the Renilla luciferase gene carried within the same reporter cell. Cells are then lysed and, by the addition of a luciferase-catalysed substrate, light emission is quantified. In order too measure drug activity, the serum sample with drug is added to the cells, pre-incubated with TNFα. If present, the anti-TNFα drug will block the activity of TNFα, and the amount of the anti-TNFα drug present will inversely correlate to the amount of luminescence produced by the cells. On the other hand, in order to measure the concentration of Nabs, the serum sample with ADAs is pre-incubated with known concentrations of the anti-TNFα drug. If the serum has non-Nabs, the anti-TNFα drug will block the activity of TNFα, and it will not induce luminescence in the cells. If the serum contains Nabs, these will block the activity of anti-TNFα drug and TNFα will induce luminescence of the cells. Thus, Nabs levels will correlate with the amount of luminescence produced by the cell (**Figure 13**)[133–135]. Some advantages of this assay are the absence of cross-reactivity, increased sensitivity, and high degree of precision. On the other hand, the assay is time consuming (2 days or more), difficult to standardize, costly, and labour-intensive, with the added disadvantage of the amount of serum and cytokines within the serum that cells can tolerate[138].

Figure 13 Illustration of RGA for Nabs assessment. TNFα is added to the target cells that initiate intracellular signalling through the surface TNF-receptor (**Control**), thus activating the cytoplasmic NFκβ. This activates several inflammatory genes, including an inserted reporter-gene construct (Firefly luciferase, Luc). When TNFα is preincubated with a serum sample containing an anti-TNFα drug and then added to the cells, the drug, if functional, neutralizes the effect of TNFα, and no intracellular signal is initiated (**Patient serum with non-NAbs**). When TNFα is preincubated with a serum sample containing anti-TNFα drug and NAbs and then added to the cells, the drug does not interfere with TNF-mediated signalling, resulting in a luminescence signal (**Patient serum with NAbs**). After cell lysis and addition of substrate, luciferase-catalysed light emission can be quantified.

For all the reasons mentioned above, alternative assays have been developed to avoid these limitations. Until now, some studies have utilized competitive-based assays (competitive ELISA, cELISA) for the detection of Nabs[136–138]. Briefly, the serum samples (ADAs positive or negative) are incubated with the labelled-drug (drug linked to HRP). After an incubation of usually 30 minutes, the serum samples are added to a plate pre-incubated with TNFα for 1 hour. If the serum sample has Nabs, these bind to the HRP-drug complex and prevents binding of the drug to the plate coated with TNFα. A 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate is added to generate a detectable signal (a reaction occurs between the TMB substrate and HRP, producing a measurable colour that correlates with the analyte level). Therefore, in samples positive for Nabs a decrease in the signal will be detected, whilst in samples negative for Nabs, the drug is free and can bind to the TNFα coated on the plate, thus increasing the signal[136–138]. This assay combines a simple protocol, low cost, and shorter analysis time (compared with RGA). However, there are no studies published to date about the comparability of these assays with RGA and with the clinically confirmed detection threshold.

Current techniques used in ADAs measurement require multiple samples and patient appointments; reporting takes several weeks, delaying the decision-making process. Therefore, tools that allow for a rapid monitoring have been increasingly targeted and developed. Rapid tests have been developed with the aim of allowing a better and personalized administration of the drugs based on actual trough levels. In ADAs assessment, the rapid tests used have the same principle that was previously described for drug detection. However, these technologies require further validation. Currently, any methodology seems adequate, but the data published so far is insufficient to ascertain a cut-off level for ADAs. Studies have shown that ADAs assays had a significantly different sensitivity. Therefore, it will be necessary to define a standard assay or establish different cut-off levels for different assays. It is also important to recognize that the assays' heterogenicity may decrease TDM accuracy, and consequently may lead to erroneous clinical decisions.

1.7.1.3. COMMUNICATION AND INTERPRETATION OF THE RESULTS

A correct communication and interpretation of the TDM results must be taken into account. Drug and/or ADAs measurements, dose regimen (administration and timing), blood collection time, and assays characteristics (e.g.: drug sensitive, tolerant ADA assay). Then, the suitability of the drug's plasma concentration should be evaluated, taking into account the target concentration, the expected clinical response, and the suspected side effects. Finally, the dosage of the treatment can be adapted, if necessary.

Another aspect that should not be overlooked and that will have to be taken into account is that the same type of assay used for the patient's follow-up should be maintained, because the use of different assays can lead to different results for the same patient. Thus, it is necessary to bear in mind which assays are being used, the sensitivity of these assays, and the way the results are reported (units of measure). This is important, since laboratories do not provide dosage recommendations and also because concentration-based dosing is not always supported by the biopharmaceuticals leaflet. Thus, clinical decision-making based on measured concentrations is performed by clinicians following evidence from the literature or from their own clinical experience[154,155].

1.7.1.4. CLINICAL DECISION-MAKING SUPPORT

Measuring the drug and ADAs concentrations can guide clinicians in the process of dosing adjustment. Overall, if the plasmatic concentration of the drug is below the therapeutic range, an increase in the dose, interval shortening, or a combination of both strategies, can be considered. These interventions have been integrated in TDM algorithms[154,169]. TDM algorithms are usually decision matrices that suggest an intervention based on drug and ADAs concentrations (

Table 3)[170]. The clinician must decide whether to change the treatment strategy or not, taking into account the information given by TDM.

Table 3 TDM algorithms based on drug (Anti-TNF therapy) and ADAs concentrations

		Anti-drug antibodies	
		Low/Absent	**High**
Anti-TNF therapy	**Low**	Intensify the therapy Shorten interval Consider add immunomodulators	Switch to another anti-TNF therapy
	High	Switch to another drug	Repeat drug and ADA testing Test for neutralizing antibodies Switch to another drug

The interventions considered appropriate according to the TDM algorithm are: 1) patients with low drug concentrations and low/absent ADAs levels: insufficient drug bioavailability due to ADAs should be considered, so in this case dose escalation (shortening of dose interval or dose intensification) is recommended; 2) patients with subtherapeutic drug levels and high ADAs levels should switch to another anti-TNF (switch in class) due to an insufficient drug bioavailability due to nonimmune mediated pharmacokinetics; 3) patients with supratherapeutic drug levels and low/absent or high ADAs levels should switch to another drug (non-anti-TNF biologic=switch out of class) because the TNFα inhibitors are not effective[150,154,171,172]. TDM is a tool to help clinicians in optimal dosage determination but should always be considered critically in the context of the specific clinical situation.

1.7.2. LIMITATIONS OF TDM

There are several barriers before TDM can be implemented in the clinical routine and emerge as the new standard of care. These include cost issues, time interval between serum sampling and test results, proper interpretation and application of the results, and the assay used.

To overcome some of these barriers, rapid assays have been developed. However, and despite the extensive effort and several publications in the field of rapid tests, these are not as widely used in the practice of TDM as their predecessors, the commonly used immunoassays. There may be different reasons that hamper bridging this gap between the clinical and scientific communities. The

first challenge is the lack of standardization and validation of these new technologies. This is not only a technical challenge, but also an economic and practical one, as this technical step requires a strong collaboration between academic, industrial and medical partners. In this regard, the ideal time for developing a TDM protocol could be during the drug development stages, where it can be coupled with the development of PK databases. This collaboration can follow the successful steps of prior analytical methods such as immunoassays. TDM has shortcomings such as inconsistencies in drug and antibodies assays used including specificity, sensitivity, accuracy, precision, reproducibility, and limit of detection/quantification. Other limitations of TDM include variations in laboratory reporting methods of assay results; inconsistencies in the validation of the therapeutic windows; limited accessibility to equipment; limited trained/skilled personnel; and high costs[173].

In addition, more prospective data concerning induction and maintenance treatment for all available anti-TNFs (originators and biosimilars) are also needed. This is of particular importance, since previous randomized controlled studies, such as TAXIT[169] and TAILORIX[174], focused exclusively on maintenance therapy with infliximab and probably failed to achieve the primary outcome due to methodological issues with the study design, therapeutic window and/or the duration of follow-up.

II.

RESEARCH OBJECTIVES

The importance of measuring drug and ADAs levels to adjust therapy is unquestionable. The aim of this thesis was to understand the impact of the methodological approach of TDM in patients with IBD under anti-TNFα drugs treatment. To this end, three major aims were defined and are separated by chapters as follows:

Chapter 1 – Influence of methodology on the assessment of drug levels

- To understand the impact of different assays on results and consequent biases on TDM interpretation (studies I and II);
- To evaluate the performance of assays previously developed and optimized for IFX in the assessment of IFX-biosimilars levels (study I).

Chapter 2 – Influence of methodology on the assessment of ADAs levels

- To understand the impact of different assays on ADAs measurement and clarify if these assays influence the results, interpretation, and management of the disease by clinicians (study III).

III.

CHAPTER 1 - Influence of methodology on the assessment of drug levels

Study I: “The performance of Remicade® - optimized quantification assays in the assessment of Flixabi® levels”
Therap Adv Gastroenterol. 2018 Sep; 11:1-9

Study II: “Accuracy of the new rapid test for monitoring adalimumab levels”
Therap Adv Gastroenterol. 2019 Jan; 12:1–11

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Original Research

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

Ther Adv Gastroenterol

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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®.

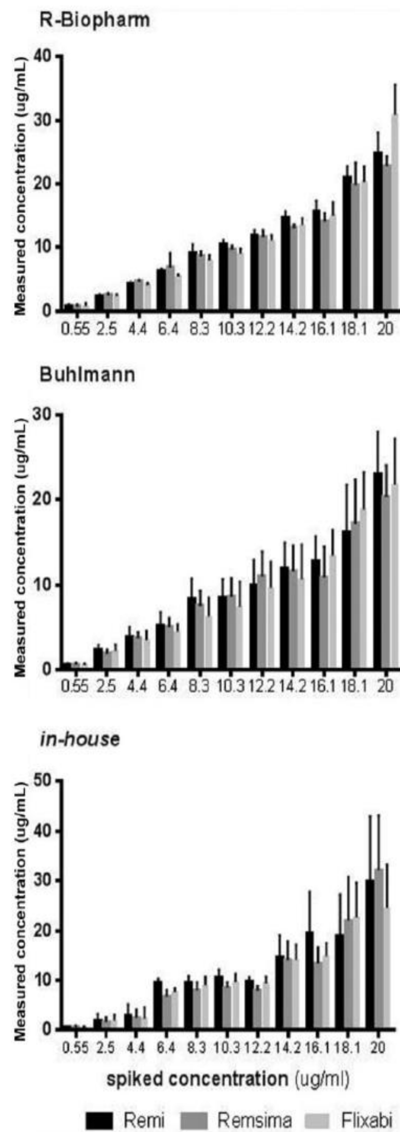

Figure 1. Remicade, Remsima and Flixabi-spiked samples measured by R-Biopharm, Buhlmann and the in-house assays.
Buhlmann, Quantum Blue® infliximab: quantitative lateral flow assay; R-Biopharm, RIDASCREEN® IFX monitoring.

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.

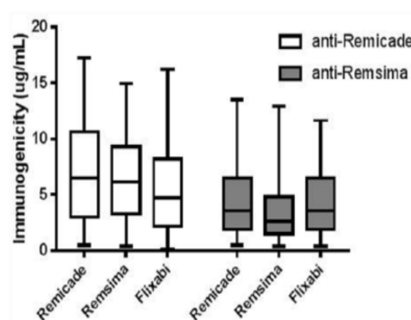

Figure 2. Reactivity of Remicade, Remsima and Flixabi to anti-Remicade and anti-Remsima sera.

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.

Drafting of the manuscript has been done by J. Afonso. 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 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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Accuracy of the new rapid test for monitoring adalimumab levels

Cátia Rocha, Joana Afonso, Paula Lago, Bruno Arroja, Ana I. Vieira, Claudia C. Dias and Fernando Magro, on behalf of Portuguese IBD Study Group (GEDII)

Abstract
Background: The loss of response to adalimumab (ADL) has been related to low serum concentrations at trough. Currently, most methods commercially available for the quantification of ADL are enzyme-linked immunosorbent assay (ELISA) based, with a turnaround time of approximately 8 h, delaying the target dosage adjustment to the subsequent infusion. In this study, we aimed to evaluate the performance of the newly available rapid-test ADL quantification assay by comparing it with three established ELISA methods, using spiked samples and a set of clinical samples.
Methods: Spiked samples from control donors and 120 serum samples from inflammatory bowel disease (IBD) patients undergoing ADL therapy were quantified using lateral flow Quantum Blue® Adalimumab and, the ELISA formats from Immundiagnostik, R-Biopharm and an in-house assay.
Results: The rapid-test assay had intraclass correlation coefficients of 0.590, 0.864 and 0.761 when comparing with the Immundiagnostik, R-Biopharm and in-house assays, respectively. For the five therapeutic windows, the accuracy was high: ADL rapid test compared with the Immundiagnostik (58–88%); R-Biopharm, 68–89%; and in house, 60–88%; and kappa statistics revealed 0.492–0.602, 0.531–0.659 and 0.545–0.682, respectively.
Conclusions: The Quantum Blue® Adalimumab assay can replace the commonly used ELISA-based ADL quantification kits and it is a reliable alternative to these methods. This rapid-test assay enables the quantitative determination of ADL serum trough level in only 15 min. The developed assay allows measurement of ADL over a wide range. Hence, it represents a valuable tool for the clinician to assess the ADL trough level.

Keywords: adalimumab, Quantum Blue® Adalimumab, therapeutic drug monitoring

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Introduction

Biological therapies using antitumour necrosis factor alpha (anti-TNFα) monoclonal antibodies (mAbs) are extensively prescribed for inflammatory bowel disease (IBD) patients. Adalimumab (ADL) was the first fully humanized anti-TNFα mAb approved for the treatment of IBD. This mAb is able to bind with a high affinity and specificity to TNFα, thereby blocking its interaction with the p55 and p75 cell-surface TNF receptors.[1–3] ADL mechanisms of action include the downregulation of pro-inflammatory cytokines, the induction of T-cell apoptosis and the reduction of leucocyte and lymphocyte migration to the sites of inflammation.[4,5]

Despite ADL proven efficacy in the treatment of IBD, about 10–30% of all patients fail to respond favourably to the induction phase of the ADL therapy (primary nonresponders) and up to 50% of initial responders lose their clinical response over time (secondary nonresponders).[6,7] This loss

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of response has been related to low serum trough ADL concentrations; in fact, different studies have reported a clear correlation between serum drug concentrations and clinical response, with IBD patients in clinical remission demonstrating higher median levels of ADL than those with active disease.[8] Still, an optimal ADL cut-off value, from a therapeutic perspective, remains to be established; whereas some investigators argue that levels above 4.9 µg/ml[9–11] can predict clinical remission, others suggest that levels above 5.85 µg/ml[12,13] and above 7.5 µg/ml[14,15] are able to predict clinical response. In the absence of a specific cut-off, an ADL therapeutic window of 5–12 µg/ml is a generally accepted desirable goal.[16–18]

Several factors can interfere with the pharmacokinetics and pharmacodynamics of therapeutic mAbs: albumin, body weight, sex, smoking, disease severity and immunogenicity (with formation of antidrug antibodies) are known to alter mAb availability and overall metabolism.[9,19,20] Therapeutic drug monitoring (TDM) comprises assessment of drug and antidrug antibody levels during mAb therapy. This knowledge allows the optimization of treatment by adjusting drug serum levels in such a way that a minimal efficacious dosage is maintained while adverse effects are minimized. Moreover, TDM can also be used to determine the causes of loss of response to treatment (i.e. low serum levels of the drug or high levels of antidrug antibodies, among other scenarios), allowing informed decision making concerning the patients' therapy. In addition to its therapeutic benefit, TDM can also avoid unnecessary therapeutic interventions, consequently enhancing cost effectiveness of the therapeutic process.[21–24]

TDM relies on an accurate quantification of the drug and antidrug antibodies from the patients' serum, and several methods have been developed to achieve it. The most common one is the enzyme-linked immunosorbent assay (ELISA), in which the biopharmaceutical is captured on a plate and detected using a secondary antibody; alternative methods include those based on fluid-phase radioimmunoassay, reporter-gene assay, enzyme immunoassay, homogenous mobility shift assay and liquid chromatography–tandem mass spectrometry.[25–28] However, these techniques require highly trained staff. Moreover, the time from sampling to result reading is often too long to allow an immediate therapeutic adjustment, which is therefore delayed till the patients' next consultation. Considering this, the development of rapid-test assays is highly useful: these systems allow an on-site and almost immediate reading of the analytical results. The Quantum Blue® Adalimumab (BÜHLMANN Laboratories AG, Schönenbuch, Switzerland) test is a new assay for ADL quantification: this rapid test allows quantitative determination of the drug levels from patients' serum in 15 min, allowing an immediate drug dosage adjustment (should it prove necessary).[29]

In this study, we aim to evaluate the performance of the Quantum Blue® Adalimumab quantification assay. To do so, its performance was compared with that of three established ELISA methods in the assessment of exogenously spiked and clinical samples.

Material and methods

Samples and patients

Clinical samples were obtained from IBD patients immediately before an ADL infusion (trough levels): blood samples were collected, centrifuged, and serum samples were kept at −80°C until being processed. Spiked samples of known ADL concentrations (1.5, 4.8, 8.0, 11.3, 14.5, 17.8, 21.0, 24.3, 27.5, 30.8 and 34.0 µg/ml) were generated by diluting the appropriate amount of exogenous ADL (Humira®, AbbVie Inc., North Chicago, IL, USA) into a pool of serum from control donors.

This study was approved by the Ethics Committees of all involved institutions, and all patients signed a written informed consent prior to their participation.

ADL quantification assays

ADL quantifications using the Quantum Blue® Adalimumab assay, hereafter referred to as Quantum Blue® Adalimumab, were carried out adhering to the manufacturers' instructions. Briefly, serum samples were diluted 1:20 and 80 µl aliquot was loaded into the port of the test cartridge. After incubation for 15 min, the cartridge was read and the results were shown on the Quantum Blue® Adalimumab reader display. The test information and calibration curve for each specific cartridge lot was provided with a chip

card to each test kit. According to the manufacturer, this kit has the following analytical characteristics: the limit of detection is 0.8 μg/ml, and the lower and upper limits of quantification are 1.3 μg/ml and 35 μg/ml, respectively.

The following assays were used as standards in the ADL quantification: the IDKmonitor® ADL drug level ELISA kit (Immundiagnostik AG, Bensheim, Germany), hereafter referred to as Immundiagnostik; the RIDASCREEN®ADM Monitoring (R-Biopharm AG, Darmstadt, Germany), hereafter referred to as R-Biopharm; and an in-house procedure. The quantifications using Immundiagnostik and R-Biopharm were performed strictly following manufacturers' instructions, whereas the in-house procedure was carried out as previously described by Ben-Horin et al.[30,31] Briefly, serum samples were diluted (1:100) 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 (Fc) 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. Lastly, the samples' absorbance was read at 450/540 nm, and the ADL concentration was quantified by interpolating the absorbance values in a standard curve built with known concentrations of exogenous ADL. According to the manufacturer, the Immundiagnostik kit has the following analytical characteristics: the lower and upper limits of quantification are the limit of blank × sample dilution factor and the highest concentration of the standard curve × sample dilution factor, respectively. For the R-Biopharm, the lower and upper limits of quantification are 0.5 and 12 μg/ml. 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. Whenever the results obtained were above these limits of quantification, samples were additionally diluted to obtain linear results within the measuring range of the assays.

Each spiked concentration was repeated between 9 and 13 times and analysed in duplicate. All measurements were carried out by the same researcher.

Statistical analyses

Continuous variables were described using the median, percentile and minimum/maximum value. The reliability between methods was assessed by calculating the intraclass correlation coefficients (ICCs) and the corresponding 95% confidence intervals (CIs). Values less than 0.5 are indicative of poor reliability, values between 0.5 and 0.75 indicate moderate reliability, values between 0.75 and 0.9 indicate good reliability and values greater than 0.90 indicate excellent reliability.[32]

Moreover, Bland and Altman plots were used to compare the different techniques. For analysis purposes, the results of all patients' samples measured by the Quantum Blue® Adalimumab for which the concentrations were below or above the limits of quantification (1.3 μg/ml and 35 μg/ml, respectively) were considered to be those same limits. The accuracy and kappa statistics were computed based on five different therapeutic windows of ADL (⩽3 μg/ml, 3–6.85 μg/ml, >6.85 μg/ml;[33] ⩽4.90 μg/ml, >4.90 μg/ml;[10,11] ⩽5.85 μg/ml, >5.85 μg/ml;[12,13] ⩽7.50 μg/ml, >7.50 μg/ml;[14,15] and ⩽5 μg/ml, 5–12 μg/ml, >12 μg/ml[16–18]). Statistical significance was considered whenever p values were below 0.05. All data were arranged, processed, and analysed with SPSS® version 24 (Statistical Package for Social Sciences, IBM Corp., Armonk, NY).

Results

Study population

This cohort enrolled 120 samples of 89 IBD patients under ADL therapy whose clinical characteristics are depicted in Table 1. The female proportion of the population was 42%, and 22% of all patients were current smokers. A total of 95.5% of the patients were Crohn's disease patients. All patients were on maintenance phase. A total of 36 patients were medicated with azathioprine (40.4%) and 76 patients (85.4%) were or had been under steroids. Moreover, 35 patients were dependent on steroids and three were steroid resistant.

Quantitative analysis

In order to compare the quantification assays' intravariability and recovery rates, 11 exogenously spiked samples ranging from 1.5 to 34 μg/ml were

Table 1. Cohort characterization.

	n	%
Sex		
Male	47	52.8
Female	42	47.2
Smoking status[a]		
Never smoked	44	49.4
Former smoker	18	20.2
Smoker	22	24.7
Disease		
Crohn's disease	85	95.5
Ulcerative colitis disease	4	4.5
Treatment phase		
Induction	0	0.0
Maintenance	89	100.0
Concomitant drugs		
Azathioprine	36	40.4
Steroids	76	85.4
Corticodependent	35	39.3
Corticoresistant	3	3.4

[a]Data not available for all subjects. Missing values for smoking status, *n* = 5.

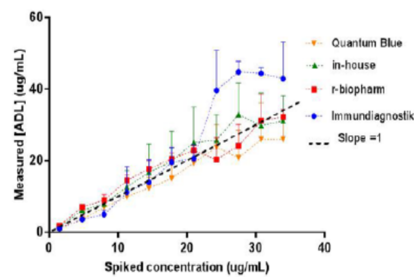

Figure 1. ADL quantification of exogenously spiked samples (µg/ml).
ADL, adalimumab.

quantified using the different methods (Figure 1). The results suggest that the assays yield closer results for lower concentrations, with the standard deviations ranging from 0.3 to 10.6 for the Immundiagnostik, 0.1–7 for the R-Biopharm, 0.74–9.31 for the in-house method, and 0.38–9.66 for the Quantum Blue® Adalimumab. Intrasample variability was calculated for each assay showing a mean of intra-assay coefficient of variation (CV) of 24%, 13%, 29% and 24% for Immundiagnostik, R-Biopharm, in house and Quantum Blue® Adalimumab, respectively. The average recovery rates were 111% (range 65–163%), 113% (range 84–149%), 110% (range 92–132%) and 85% (range 76–98%) for the Immundiagnostik, R-Biopharm, in house and Quantum Blue® Adalimumab methods, respectively.

Table 2 shows the ICCs between the spiked and the measured ADL concentrations for each method, as well as the average differences. The results show that all assays have a high ICC (above 0.927), which means they have values close to the theoretical concentrations. In accordance with recovery rates, the Immundiagnostik and in-house assays have a negative average difference between the spiked and measured concentrations, and the 95% CI did not include 0, which means that these assays tend to overestimate drug concentration, whereas the opposite is seen for the Quantum Blue® Adalimumab, which has a positive average difference and excludes 0, meaning this assay tends to underestimate drug concentration. All assays have the tendency to measure accurate values in the range from 0 to 20 µg/ml and reach a plateau at higher concentrations.

Moreover, in order to test the Quantum Blue® Adalimumab assay in a real-life context, both this and the reference assays were used to quantify the ADL amount in 120 serum samples of IBD patients being medicated with this mAb. All patients were in the maintenance phase of ADL therapy. The ICCs and the average differences between the different assays are shown in Table 3. The highest ICCs were found between the R-Biopharm the Quantum Blue® Adalimumab (0.864) and the Quantum Blue® Adalimumab and in house (0.761), showing a good agreement between these pair of assays. The highest average difference was found between the Immundiagnostik and the R-Biopharm (10.20), in house (13.93), and Quantum Blue® Adalimumab assay (13.34), respectively, and the 95% CI did not include 0, showing that the concentrations measured by Immundiagnostik were consistently higher than

Table 2. ICC between the theoretical and measured concentrations of exogenously spiked samples.

	ICC		Difference	
	ICC	CI 95%	Average	CI 95%
Quantum Blue® Adalimumab	0.982	0.933–0.995	2.71	0.95; 4.48
In-house assay	0.989	0.958–0.997	−1.31	−2.84; 0.22
R-Biopharm	0.984	0.940–0.996	−0.60	−2.31; 1.13
Immundiagnostik	0.927	0.727–0.980	−4.67	−9.74; 0.39

ICC, intraclass correlation coefficient; CI, confidence interval.

Table 3. ICC and differences found upon comparing the different ADL quantification assays in clinical samples.

	ICC		Difference	
	ICC	CI 95%	Average	CI 95%
Quantum Blue® Adalimumab				
In-house assay	0.761	0.658–0.834	0.59	−0.48; 1.66
R- Biopharm				
Quantum Blue® Adalimumab	0.864	0.805–0.905	3.13	2.20; 4.06
In-house assay	0.693	0.559–0.786	3.72	2.38; 5.07
Immundiagnostik				
Quantum Blue® Adalimumab	0.590	0.411–0.714	13.34	10.86; 15.81
R-Biopharm	0.530	0.326–0.673	10.20	7.50; 12.90
In-house assay	0.610	0.440–0.728	13.93	11.47; 13.94

ADL, adalimumab; ICC, intraclass correlation coefficient; CI, confidence interval.

those measured by the other assays. Bland–Altman plots were computed to establish whether those differences were related to certain concentration ranges (Supplementary Figure S1). And indeed, these plots suggest that the differences between the methods increase for higher concentrations, although rarely exceed the ±1.96 SD interval. This is especially evident for the Immundiagnostik ELISA test that shows very good correlations with other tests at concentrations below 20 μg/ml, but very poor correlations above this concentration.

Overall, the Quantum Blue® Adalimumab correlates robustly with the R-Biopharm assay over a large concentration range, while this correlation only holds true for concentrations below 15 μg/ml when compared with the in-house and Immundiagnostik ELISA tests.

Qualitative analysis

To analyse these results from a clinical perspective, ADL levels were stratified according to five possible therapeutic windows: below 3 μg/ml, between 3 and 6.85 μg/ml, above 6.85 μg/ml;[33] below or above 4.90 μg/ml;[9–11] below or above 5.85 μg/ml;[12,13] below or above 7.50 μg/ml;[14,15] below 5 μg/ml, between 5 and 12 μg/ml, and above 12 μg/ml.[16–18] The agreement analyses for these categories are shown in Table 4.

Table 4. Kappa/accuracy between the different methods using different cut-offs.

	⩽3 μg/ml; (3–6.85 μg/ml); >6.85 μg/ml	⩽4.90 μg/ml; >4.90 μg/ml	⩽5.85 μg/ml; >5.85 μg/ml	⩽7.50 μg/ml; >7.50 μg/ml	⩽5 μg/ml; (5–12 μg/ml); >12 μg/ml
	Kappa (accuracy)	Kappa (accuracy)	Kappa (accuracy)	Kappa (accuracy)	Kappa (accuracy)
Quantum Blue® Adalimumab					
In-house assay	0.594 (73%)	0.682 (88%)	0.627 (83%)	0.545 (78%)	0.495 (60%)
Immundiagnostik					
Quantum Blue® Adalimumab	0.602 (79%)	0.570 (88%)	0.507 (85%)	0.492 (78%)	0.477 (58%)
R-Biopharm	0.774 (91%)	0.866 (98%)	0.699 (93%)	0.648 (88%)	0.691 (80%)
In-house assay	0.439 (65%)	0.415 (79%)	0.336 (72%)	0.363 (69%)	0.297 (43%)
R-Biopharm					
Quantum Blue® Adalimumab	0.616 (80%)	0.531 (88%)	0.659 (89%)	0.581 (82%)	0.569 (68%)
In-house assay	0.401 (63%)	0.340 (77%)	0.363 (73%)	0.401 (71%)	0.318 (44%)

Comparisons showed that the agreement was higher for the pair Immundiagnostik and the R-Biopharm in the five therapeutic windows, with a substantial to an almost perfect strength of agreement according to the kappa interpretation of Landis and Koch.[34] In the case of the Quantum Blue® Adalimumab assay, the kappa statistic revealed that the levels of agreement with the standard assays were moderate to substantial in the five therapeutic windows.

Discussion

The use of TDM for ADL in the treatment of IBD is becoming increasingly common. In cases of nonresponse (primary or secondary), TDM can provide information about the cause of treatment failure. Therefore, TDM is a valuable tool that allows the adjustment of a drug's therapeutic dosage according to the plasma concentrations found in each patient, hence, improving patient care and healthcare resource optimization. The recent development of a rapid ADL quantification assay (Quantum Blue® Adalimumab) holds the promise of revolutionizing the TDM-based therapeutic algorithms, by allowing an immediate adjustment of the ADL dosage. However, and to ensure accuracy, one has to evaluate whether this assay has a performance comparable with those already in use. This study arises in that context, with the aim of validating the Quantum Blue® Adalimumab by comparing its performance with that of three already-established ELISA assays, using both exogenously spiked and patients' extracted samples.

The results show that all methods have an acceptable performance as assessed by their average recovery percentage and appear to be similar in quantification of ADL. However, and looking at the individual results, the Immundiagnostik assay has a wide variation of recovery, measuring consistently high values for concentrations above 24.3 ug/ml, whereas the Quantum Blue® Adalimumab seems to underestimate ADL concentrations. Regarding the quantitative analysis of the results obtained from the clinical samples, the comparison of the different assays has shown that the R-Biopharm was the closest to the Quantum Blue® Adalimumab, with an ICC of 0.864, but the former kit measures consistently higher values than the latter. This result is supported by the concentrations obtained from the spiked samples, in which in all but the 10 μg/ml sample, higher values were obtained when using

R-Biopharm *versus* Quantum Blue® Adalimumab. The differences found upon comparing the different methods, as well as those between measured and nominal values, are most likely attributable to the ELISA method itself and may include differences at the detection step. Antibodies that bound to the TNFα–drug complex immobilized in the plate differ substantially between the ELISA systems. While the in-house assay uses a HRP-conjugated anti-Fc antibody for the detection step, R-Biopharm uses a HRP-conjugated MA-ADM40D8 antibody generated specifically for ADL.[35] Moreover, surface aggregation and denaturation of reagents, which are likely to mask epitopes or show new epitopes, may also interfere with the ELISA system performance. Also, the dilution step may affect results, since serum matrix effects may also interfere with drug level assessments (e.g. presence of complement components, cross-reactive immunoglobulins and rheumatoid factors).[36]

Bland–Altman plots of measurements of patients' serum locate the differences between the Quantum Blue® Adalimumab and the other assays in different concentration ranges (Supplementary Figure S1). Overall, the lateral flow Quantum Blue® Adalimumab rapid test, and the different ELISAs measure similar levels of ADL at low concentrations but diverge at concentrations above 20 μg/ml. This difference is most accentuated in comparison with the Immundiagnostik assay, which systematically measures higher values than other assays at high concentrations. The Bland–Altman plots of the in-house assay show a bimodal distribution compared with other assays, which may explain the poor kappa/accuracy values with all other assays over the various therapeutic cut-off concentration levels (Table 4).

The analysis of results from a qualitative perspective is likely more important in this context, as the placement of a patient within a certain range of ADL concentrations will be reflected in the clinical decisions made if TDM is applied. If the drug concentration is below the therapeutic range in a patient, dose increase, interval shortening, or a combination may be considered. Besides treatment intensification, TDM also includes the possibility of providing insight for reducing the exposure when needed, by re-establishing the standard dosing interval or dose de-escalation.[37,38]

In this regard, and in the absence of a well-defined therapeutic window for ADL, the samples were stratified according to five therapeutic windows (⩽3 μg/ml, 3–6.85 μg/ml, >6.85 μg/ml;[33] ⩽4.90 μg/ml, >4.90 μg/ml;[9–11] ⩽5.85 μg/ml, >5.85 μg/ml;[12,13] ⩽7.50 μg/ml, >7.50 μg/ml;[14,15] and ⩽5 μg/ml, 5–12 μg/ml, >12 μg/ml[16–18]). Considering the range that is used, concentrations below this range are considered infratherapeutic (the patient should escalate the dosage), while concentrations above this range are considered supratherapeutic (the patient can safely de-escalate the dosage). According to our results, the results are substantial to almost perfect for the Immundiagnostik–R-Biopharm comparison, and moderate to substantial for the Quantum Blue® Adalimumab–Immundiagnostik and R-Biopharm–in-house comparisons. However, when in house is involved, results tend to be weaker. These results can be explained by the amplitudes of each method being very different: in the Immundiagnostik, the amplitude varies between 0.2 and 47 μg/ml; R-Biopharm varies between 0 and 35 μg/ml; and Quantum Blue® Adalimumab varies between 1 and 35 μg/ml, while the in-house assay varies between 0.10 and 20 μg/ml. Besides, it has been observed that in general the kappa lowers as the cut-off increases, likely as a result of greater dispersion of measurements at higher concentrations.

According to our results, the clinical decision based solely on ADL quantification varies according to the therapeutic window chosen. Thus, considering the different cut-offs: ⩽3, 3–6.8, >6.85 μg/ml; ⩽4.90, >4.90 μg/ml; ⩽5.85, >5.85 μg/ml; ⩽7.50, >7.50 μg/ml; and ⩽5, 5–12, >12 μg/ml, the clinical decision would be of a similar nature in 79%, 88%, 85%, 78% and 58% of the patients, respectively, using the Quantum Blue® Adalimumab instead of the Immundiagnostik assay; in 80%, 88%, 89%, 82% and 68% of the patients, respectively, using the Quantum Blue® Adalimumab instead of the R-Biopharm; or in 73%, 88%, 83%, 78% and 60% of the patients, respectively, using the Quantum Blue® Adalimumab instead of the in-house assay. Our results show that the specific percentages vary if a different therapeutic range is used. In this sense, it is important to emphasize that the decision making should not only be based on ADL quantification, but these decisions should be integrated into patients' clinical context, considering the presence of symptomatology and other disease markers. More studies are needed to integrate patients'

symptomatology with the performance of the Quantum Blue® Adalimumab in the context of TDM, as well as to define specific therapeutic limits of ADL that should be applied with this method.

The Quantum Blue® Adalimumab for the ADL monitoring in IBD patients is quite advantageous, in the sense that the test response time is 15 min compared with approximately 4 h taken by routine ELISA-based kits (Immundiagnostik, R-Biopharm and in-house assays), allowing the physician to optimize treatment immediately as opposed to delaying possible therapeutic interventions to the next administration. Although the role of TDM assessment during the ADL therapy is not clearly defined, more studies from this perspective are being developed and have been demonstrating the benefits of optimizing the ADL therapy in the treatment of IBD and other inflammatory diseases. In fact, these studies have shown that adjusting ADL doses and administration intervals are a clinically powerful and cost-effective strategy.[37,39–41] For these reasons, there has been increasing effort by the scientific and medical community in research for the development of novel rapid-test assays and monitoring of IBD patients.

Another important advantage of this commercial rapid test is its user friendliness; in fact, these assays can be operated by any nurse, physician, or researcher, unlike other ELISA kits that require highly trained personnel and specific laboratory facilities. Besides, as a rapid-test method, samples can be analysed upon their collection (i.e. no transportation is needed), and it is designed to be used individually (as opposed to other ELISA kits designed to work in a batch-fashion). Unfortunately, there is no commercially available ADL antibodies Quantum Blue® Adalimumab at the moment. Emerging data increasingly emphasize that the presence of ADL antibodies may lead to ineffective subtherapeutic levels of ADL and contribute to loss of response by increasing drug clearance or blocking the effect of the drug.[13,41–43] So, monitoring these levels during therapy should be integrated into the management of patients receiving ADL.

For this study, spiked samples and a large number of serum samples obtained from a multicentric and heterogeneous cohort were used and are therefore a representation of reality. However, our study has a few limitations that should be taken into account: the ADL quantification was always made by the same researcher, which hampers the assessment of the interassay variability; serum matrices often contain components such as rheumatoid factors and other proteins, which could lead to bias during sample analyses, mainly in ligand-binding assays, as well as the presence of ADL antibodies that may differentially interfere with the ADL quantification in each kit (that was unaccounted for). In this study, as the focus was on assessing whether the Quantum Blue® Adalimumab treatment could replace the ELISA-based kits, the analytical performance characteristics of the Quantum Blue® Adalimumab were not considered. However, this performance has been evaluated by the manufacturer and is clearly indicated in the instruction manual accompanying the kit: Quantum Blue® Adalimumab limits of quantification are 1.3–35.5 μg/ml, which may hold a disadvantage when compared with the ELISA-based kits.

In conclusion, the Quantum Blue® Adalimumab is a reliable alternative to the commonly used ELISA-based ADL quantification kit. In fact, the rapid test allows a fast and accurate assessment of ADL levels, which in turn contributes towards proactive and cost-effective therapeutic management of IBD patients. This rapid assay for ADL quantification represents a valuable tool for the fast implementation of tailored therapeutic solutions.

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FM: study concept and design; analysis and interpretation of data; drafting of the manuscript;

study supervision; critical revision of the manuscript for important intellectual content. CR: drafting of the manuscript; quantification assays; analysis and interpretation of data. JA: drafting of the manuscript, quantification 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 & Dohme, Abbvie, Vifor, Falk, Laboratorios Vitoria, Ferring, Hospira and Biogen.

Supplemental material

Supplemental material for this article is available online.

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IV.

CHAPTER 2 - Influence of methodology on the assessment of anti-drug antibodies levels

Study III: "Rapid test detection of anti-infliximab antibodies: performance comparison with three different immunoassays"

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Rapid test detection of anti-infliximab antibodies: performance comparison with three different immunoassays

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Abstract

Background and Aims: Therapeutic drug monitoring (TDM) of infliximab (IFX) and anti-infliximab antibodies (ATIs) is essential for treatment optimisation in inflammatory bowel disease (IBD) patients. The aim of this study was to estimate and compare the agreement and accuracy between a new rapid test and three established enzyme-linked immunosorbent assays (ELISAs) to quantify ATIs levels, and to evaluate the impact of exogenous IFX on the performance of these assays.

Methods: We analysed 200 serum samples from 57 IBD outpatients in IFX induction or maintenance therapy at six IBD centres in Portugal. ATI levels were quantified using the rapid test Quantum Blue® (QB) Anti-Infliximab (Bühlmann) and three established ELISAs: In-House, Theradiag (Lisa Tracker Anti-Infliximab), and Immundiagnostik (IDKmonitor Infliximab). ATIs were quantified in patients' serum samples and spiked samples with exogenous IFX, based on analytical and clinical cutoffs. Qualitative agreement and accuracy were estimated by Cohen's kappa (k) with 95% confidence intervals.

Results: ATIs quantification with clinical cutoffs showed a slight agreement between QB rapid test and In-House [k = 0.163 (0.051–0.276)] and Immundiagnostik [k = 0.085 (0.000–0.177)]. Regarding IFX/ATIs status, the QB rapid test showed a substantial agreement with Theradiag [k = 0.808 (0.729–0.888)] and a fair agreement with In-House [k = 0.343 (0.254–0.431)] and Immundiagnostik [k = 0.217 (0.138–0.297)]. The QB rapid test could not detect ATI-positive levels in samples with exogenous IFX at 5–300 μg/ml. Interference on ATIs detection was observed at exogenous IFX ⩾30 μg/ml for In-house and Immundiagnostik assays.

Conclusion: QB rapid test is only suitable to detect ATI-positive levels in the absence of IFX.

Keywords: antibodies, drug monitoring, enzyme-linked immunosorbent assay, immunoassay, inflammatory bowel diseases, infliximab, point-of-care systems

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Introduction

Infliximab (IFX) is a therapeutic monoclonal antibody against tumour necrosis factor alpha (TNF-α). IFX is effective in lowering disease activity and inducing clinical remission in patients with inflammatory bowel disease (IBD).[1] However, up to 30% of patients fail to respond to induction treatment (primary loss of response), and 50% of patients may lose response during maintenance treatment (secondary loss of response), many during the first year.[2,3] This loss of response to IFX therapy may occur due to several reasons, including the development of anti-drug antibodies (ADAs).[4] ADAs may neutralise drug-target binding and increase drug clearance, resulting in sub-optimal concentrations of active drug and shorter duration of response.[5–7] Previous studies have shown that up to 44% of IBD patients treated

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with IFX develop anti-IFX antibodies (ATIs), depending on dosing schedules, concomitant use of steroids or immunomodulators, and measurement methods.[8–12] When loss of response occurs, physicians usually change the therapeutic strategy by increasing the dosage or frequency of the current drug therapy, switch to another TNF-α antagonist, or switch to a different class of drug with another mode of action.[13] However, this empirical approach increases the risk of irreversible tissue damage and healthcare costs, and could delay effective IBD treatment.[1,14] Therefore, the assessment of drug and ADA levels, also known as therapeutic drug monitoring (TDM), is essential to define future therapeutic strategies. The specific assessment of IFX and ATI levels allows physicians to understand the reasons for unresponsiveness, identify patients that will most benefit from the dose adjustment of current IFX therapy or from switching to another drug,[7,14,15] and reduce delays in effective treatment.[1,14] TDM is thus essential to define therapeutic strategies in IBD patients, improving clinical outcomes and minimising IBD-related complications.

TDM has led to the development of methods for quantification of IFX and ATI levels with different applications and limitations. Of particular concern is that some methods quantify both IFX and ATIs, whereas others are specific for only one of these quantifications, which may have a significant impact on TDM's results and interpretation.[3,16,17] Several commercial kits measure IFX levels in the patient's serum, most of them relying on enzyme-linked immunosorbent assays (ELISAs).[16,18,19] However, these assays have a turnaround time of approximately 8 h, which might impair immediate adjustment of IFX therapy. In contrast, recent developments in rapid point-of-care tests allow the semi-quantitative assessment of ATI levels from the patient's serum within minutes.[20,21] In fact, the recent development of a rapid test to ATIs quantification (Quantum Blue® Anti-Infliximab, Bühlmann) promise a fast detection of ATIs (15–20 min turnaround time) on a single sample.[20] These assays facilitate TDM and immediate adjustment of the IFX dosage. Nevertheless, the use of point-of-care tests for the quantification of ATIs in clinical practice is still limited by a lack of data and there is a need to evaluate their inter-assay heterogeneity and accuracy.[22] Also, the impact of detectable IFX levels in patients' serum on the TDM is understudied, particularly for the quantification of ATI levels by already established ELISAs.[3,16,17,23] This indicates a need to evaluate and compare the performance of the various assays currently used in clinical practice to quantify ATI levels, to improve clinical decision-making based on TDM.

With this study we aimed to estimate and compare the accuracy and agreement between a new rapid test and three different established ELISAs for quantifying ATI levels in the serum of IBD patients. We also aimed to evaluate the impact of exogenous IFX on the performance of the four assays. We selected the recently commercially available rapid test Quantum Blue® Anti-Infliximab (Bühlmann) and the established In-House, Lisa Tracker Anti-Infliximab (Theradiag), and IDKmonitor Infliximab (Immundiagnostik) assays for quantification of ATI levels using analytical or clinical cutoff levels.

Methods

Patients and sample collection

This was a multicentre, non-interventional, retrospective study. From July 2016 to August 2019, 200 clinical samples were collected at six IBD centres in Portugal from 57 IBD patients attending routine outpatient consultations. The study population comprised patients who were adults (⩾18 years), male or female, diagnosed with moderate-to-severe active Crohn's disease or ulcerative colitis, primary responders to IFX induction doses were assessed clinically and endoscopically, and received at least three IFX maintenance doses.

The clinical samples were obtained from patients undergoing the induction or maintenance treatment phase, and immediately before the infusion of a new IFX dose. Collected baseline sociodemographic and clinical data included birth date, date of diagnosis, sex, smoking status, diagnosis of Crohn's disease or ulcerative colitis, and concomitant IBD-related medication. Blood samples were collected, centrifuged, and serum samples were kept at −80°C until being processed.

Potentially eligible samples were identified based on the previous quantification of ATI levels in our laboratory using our reference method (In-House assay). Samples were consecutively chosen to cover clinically relevant cutoff points for ATI negativity (<1.7 μg/ml) and positivity (⩾1.7 μg/ml)

defined in the literature.[17,24] ATI samples were included according to the following cutoff levels: negative, <1.7 μg/ml; low, 1.7–2.9 μg/ml; intermediate, 3.0–9.9 μg/ml; and high, ⩾10 μg/ml. Trough IFX concentrations were previously measured for all samples as part of the clinical routine using Quantum Blue® Infliximab (Bühlmann, Schönenbuch, Switzerland). More detailed information about the assays and protocols can be found in Supporting Information The study was conducted in accordance with the Declaration of Helsinki and was approved by the Ethics Committees of each centre. All patients signed a written informed consent before their participation.

Quantification of ATI levels

All samples were analysed with the rapid point-of-care test Quantum Blue® Anti-Infliximab assay (Bühlmann, Schönenbuch, Switzerland), hereafter referred to as QB rapid test, according to the manufacturers' instructions (Supporting Information). The ATI levels were calculated as IgG equivalents to the monoclonal reference antibody used for standardisation (μg_{eq}/ml) and hereafter expressed as μg/ml. The following three ELISAs were used as comparators: In-House assay, Lisa Tracker anti-Infliximab (Theradiag, Croissy Beaubourg, France), and IDKmonitor Infliximab total ADA ELISA (Immundiagnostik, Bensheim, Germany). The quantifications using Theradiag and Immundiagnostik were performed following manufacturers' instructions,[25–27] whereas the In-House assays were carried out as previously described by Ben-Horin *et al.*[17,28] (Supporting Information). The lower and upper detection limits for ATI levels described by the manufacturers were as follows: QB rapid test, 0.6–12 μg/ml; In-House assay, lower limit of 1.2 μg/ml; Theradiag, 0.01–0.2 μg/ml; and Immundiagnostik, higher average optical densities >10 antibody units (AU)/ml were classified as positive. All kits and samples were used and processed by the same technician.

Because the four assays tested have different technical characteristics, detection limits and expression of results, the test positivity cutoffs for a qualitative evaluation of the ATI levels are difficult to establish. Therefore, we used analytical and clinical cutoffs to test ATI-positive (ATI+) levels. The analytical cutoffs were based on the lower detection limits described by the manufacturers for each assay, while the clinical cutoffs used clinically relevant ATI+ levels defined in the literature.[17,24] Using the analytical cutoffs, ATI+ levels were defined as ATI levels ⩾0.6 μg/ml for QB rapid test, ⩾1.2 μg/ml for In-House, ⩾0.01 μg/ml for Theradiag, and ⩾10 AU/ml for Immundiagnostik. Using the clinical cutoffs, ATI+ levels were defined as ATI levels ⩾1.7 μg/ml for QB rapid test, In-House and Theradiag, and ⩾10 AU/ml for Immundiagnostik.

Exogenous IFX in ATI-positive serum samples

To assess the impact of IFX in the quantification of ATI levels, exogenous IFX (Schering Plough, New Jersey, USA) was added to ATI+ serum samples with undetectable IFX concentrations (IFX–) <0.4 μg/ml. ATI+ serum samples with low, intermediate, and high ATI levels were selected – six different samples were selected for each ATIs group. Serum samples with ATI+ levels and IFX- concentrations were preincubated with several exogenous IFX concentrations (5, 10, 15, 30, 100 and 300 μg/ml) for 30 min at room temperature, as previously described by our group.[23] The therapeutic range of IFX concentrations was considered to be between 0 and 100 μg/ml.[29] ATI levels in samples with different IFX/ATI levels status were then quantified by the four assays as described above.

Statistical analyses

Categorical variables were described as absolute (*n*) and relative frequencies (%), and continuous variables were shown as the median and interquartile range (IQR). The quantitative agreement between assays could not be assessed because data was reported using different and arbitrary units (AU/ml). Therefore, the qualitative agreement of ATI levels or IFX/ATI levels status between pairs of assays was determined using Cohen's kappa (k) coefficients and accuracy with 95% confidence intervals (CIs). The Cohen's k coefficients were categorised according to the criteria of Landis and Koch: ⩽0.000 no agreement, 0.000–0.200 slight, 0.210–0.400 fair, 0.410–0.600 moderate, 0.610–0.800 substantial and 0.810–1.000 almost perfect agreement.[30] Accuracy percentages of 0–4% were considered no accuracy, 4–15% minimal, 15–35% weak, 35–63% moderate, 64–81% strong and 82–100% almost perfect accuracy.[31] Accuracy is the agreement between value found and an excepted reference value and the agreement refers to the

closeness of two measured values, not to whether those values are correct or not (estimated by the kappa coefficient).

IFX/ATI levels status were stratified in four combinations of detectable (IFX+) or undetectable exogenous IFX and ATI-negative (ATI-) or ATI+ levels as follows: IFX+/ATI-, IFX+/ATI+, IFX-/ATI+, and IFX-/ATI-. To assess the impact of exogenous IFX concentrations on the quantification of ATI levels, graphical analyses plotted the mean of six measurements from six different samples (one measurement per sample), of ATI levels *versus* increasing exogenous IFX concentrations in spiked serum samples, by quantification assay, for each group of patients' serum samples with low, intermediate, or high ATI+ levels. Statistical analysis was performed using SPSS version 24.0 (IBM Corp, Armonk, NY) and the graphical representation was performed using GraphPad Prism version 8.3.0 (GraphPad Software, Inc., San Diego, CA).

Results

Study population

This study analysed 200 serum samples collected from 57 IBD patients under IFX therapy. Table 1 shows the baseline demographic and clinical characteristics of the patients. Briefly, patients had a median age at diagnosis of 29 (19–36) years, 56.1% were female, 57.9% never smoked, 14.0% were current smokers, and 28.1% were former smokers. A total of 70.2% of patients had Crohn's disease and 29.8% had ulcerative colitis; 22 patients (38.6%) were under concomitant immunosuppression (azathioprine or methotrexate).

Agreement for ATI+ levels

Qualitative agreement and accuracy of the QB rapid test and three established ELISAs was determined by quantifying the ATI levels in patients' serum samples and stratifying the results into analytical and clinical cutoffs.

When stratified by analytical cutoffs for ATI+ levels (QB rapid test ⩾0.6 μg/ml, In-House 1.2 μg/ml, Theradiag 0.01 μg/ml, and Immundiagnostik 10 AU/ml), ATI+ levels were detected in 48 (24.0%) samples with the QB rapid test, 161 samples (80.5%) with In-House, 65 (32.5%) samples with Theradiag and 158 (79.0%) samples with Immundiagnostik. As shown in Table 2, a moderate agreement was found between the QB rapid test and Theradiag ($k = 0.489$), while a slight agreement was observed between the QB rapid test and In-House ($k = 0.160$) and QB rapid test and Immundiagnostik ($k = 0.139$). Comparisons between the remaining assay pairs revealed fair agreements (Table 2).

Based on clinical cutoffs for ATI+ levels (QB rapid test, In-House, Theradiag ⩾1.7 μg/ml and Immundiagnostik > 10 AU/ml), QB rapid test detected 30 (15.0%) samples, Theradiag did not detect ATI+ samples, and In-House and Immundiagnostik detected the highest number of samples, 140 (70.0%) and 160 (80.0%), respectively. A total of 32% of the values negative with our threshold (<1.7 μg/ml) turn out positive with lower limit of quantification cutoff are under 0.010 μg/ml. In fact, these values might reflect only the intra-variability of the assay rather than represent the presence of antibodies. Although the In-House and Immundiagnostik assays detected an approximate number of ATI+ samples, not all samples matched. ATI+ levels were confirmed by both assays in 123 samples (61.5%). All 30 ATI+ samples identified by the QB rapid test were also positive in both the In-House and Immundiagnostik assays. As can be seen from Table 2, using the clinical cutoffs, a slight agreement was found between the QB rapid test and In-House ($k = 0.163$) or QB rapid test and Immundiagnostik ($k = 0.085$). The comparison of the In-House *versus* Immundiagnostik pair showed a fair agreement ($k = 0.289$). The k coefficient could not be calculated for the comparisons with Theradiag as this assay did not detect ATI+ samples.

Agreement for trough IFX and ATI levels status

The accuracy and agreement of IFX/ATI levels status between pairs of assays were also evaluated. The patients' serum samples were divided into four IFX/ATI levels status, using both analytical and clinical cutoffs, resulting in IFX+ concentrations in 90 (45.0%) or 80 (40.0%) of the 200 samples, respectively. The number of IFX/ATIs levels status for each assay in addition to the comparisons between tests can be assessed in Supplementary Table S1.

As shown in Table 3, considering the analytical cutoffs, the QB rapid test did not detect IFX+/

ATI+ samples and Theradiag detected only one, corresponding to one of the IFX+/ATI+ samples detected by In-House. All assays were able to detect samples with the remaining IFX/ATI levels status. Overall, a strong accuracy was found between the In-House and the Immundiagnostik assays (80%) with a substantial agreement ($k=0.661$). A strong accuracy was also found between the Theradiag and the QB rapid test (75%) or In-House assays (70%) with a substantial agreement ($k=0.625$) or a moderate agreement ($k=0.531$), respectively (Table 3).

Regarding the clinical cutoffs for the quantification of ATI levels, only the In-House and Immundiagnostik assays detected IFX+/ATI+ samples (Table 3). Comparing with the analytical cutoffs, the In-House and Immundiagnostik assays identified a similar number of samples in each IFX/ATI status. Conversely, the QB rapid test and Theradiag identified a higher number of IFX-/ATI- samples and a lower number of IFX+/ATI– samples. All assays were able to identify the remaining IFX/ATI status samples except for the Theradiag, which did not detect IFX–/ATI+ samples. An almost perfect accuracy was found between the pair QB rapid test and Theradiag (89%) with an almost perfect agreement ($k=0.808$). The pair In-House and Immundiagnostik showed a strong accuracy (72%) and a moderate agreement ($k=0.531$).

Table 1. Characteristics of patients with IBD treated with infliximab.

	Patients ($n=57$)
Age at diagnosis, median (IQR), years	29 (19–36)
Gender, *n* (%)	
Female	32 (56.1)
Male	25 (43.9)
Crohn's disease, *n* (%)	40 (70.2)
Ulcerative colitis, *n* (%)	17 (29.8)
Smoking status, *n* (%)	
Never smoker	33 (57.9)
Former smoker	16 (28.1)
Current smoker	8 (14.0)
Concomitant IBD-related medication, *n* (%)	
None	21 (36.8)
Azathioprine	19 (33.3)
Steroids	9 (15.8)
Methotrexate	3 (5.3)
Oral 5-aminosalicylates	5 (8.8)
Time under biological therapy, median (min–max), months	6 (1–20)
IFX mg/kg, median (min–max)	6 (5–10)
Number of IFX received, median (min–max)	3 (0–12)
Dose intervals, median (min–max)	7 (5–8)
Dose optimization, *n* (%)	
No	47 (82.5)
Yes	10 (17.5)
Albumin g/l, median (min–max)	41.9 (29.3–66.4)

IBD, inflammatory bowel disease; IFX, infliximab, IQR, interquartile range; *n*, number of patients.

Effect of exogenous IFX on ATI quantification

The impact of IFX on the quantification of ATI levels was evaluated by measuring spiked ATI+ serum samples (5, 10, 15, 30, 100 and 300 µg/ml IFX) with the four assays, based on the clinical cutoffs for ATI+ levels.

Figure 1 displays the results in the samples with low ATI levels (1.7–2.9 µg/ml). No impact of exogenous IFX was evident in the Immundiagnostik assay. An IFX concentration of 30 µg/ml influenced the In-House assay by an additive concentration-effect; however, this influence was not evident at higher concentrations. In contrast, both the QB rapid test and Theradiag assays could not detect ATI+ levels (>1.7 µg/ml) in samples with all IFX concentrations. Moreover, the QB rapid test indicated invalid values in the samples containing exogenous IFX concentrations >30 µg/ml.

Figure 2 presents the results in the samples with intermediate ATI levels (3.0–9.9 µg/ml). The impact of exogenous IFX was more evident in the Immundiagnostik and In-House assays with a decrease in ATI levels. In the presence of 30 µg/ml IFX, these assays were influenced by an additive concentration-effect, however, only the

Table 2. Qualitative agreement between ATIs+ levels: comparison between assay pairs stratified by analytical and clinical cutoffs.

Assay comparison	Accuracy (95% CI)	Cohen's kappa (95% CI)
Analytical cutoffs[a]		
QB rapid test *versus* In-House	51 (44–57)	0.160 (0.102–0.217)
QB rapid test *versus* Theradiag	75 (69–81)	0.489 (0.384–0.595)
QB rapid test *versus* Immundiagnostik	43 (36–50)	0.139 (0.086–0.192)
In-House *versus* Theradiag	70 (63–75)	0.403 (0.301–0.505)
In-House *versus* Immundiagnostik	80 (73–85)	0.388 (0.235–0.541)
Theradiag *versus* Immundiagnostik	67 (60–73)	0.375 (0.276–0.474)
Clinical cutoffs[b]		
QB rapid test *versus* In-House	49 (41–56)	0.163 (0.051–0.276)
QB rapid test *versus* Theradiag	85 (79–90)	–
QB rapid test *versus* Immundiagnostik	35 (29–42)	0.085 (0.000–0.177)
In-House *versus* Theradiag	34 (27–41)	–
In-House *versus* Immundiagnostik	72 (65–78)	0.289 (0.133–0.445)
Theradiag *versus* Immundiagnostik	20 (15–26)	–

[a]ATIs+ levels: ⩾0.6 µg/ml for QB rapid test, ⩾1.2 µg/ml for In-House, ⩾0.01 µg/ml for Theradiag, and ⩾10 AU/ml for Immundiagnostik.
[b]ATIs+ levels: ⩾1.7 µg/ml for QB rapid test, In-House and Theradiag, and ⩾10 AU/ml for Immundiagnostik.
ATIs+, anti-infliximab antibodies-positive; CI, confidence interval; QB, Quantum Blue.

Immundiagnostik assay could detect ATI+ levels at the higher concentrations of exogenous IFX. The QB rapid test and Theradiag assays could not detect ATI+ levels with IFX concentrations from 5 to 30 µg/ml or with all concentrations, respectively. The QB rapid test indicated invalid values in samples containing IFX concentrations >100 µg/ml.

Figure 3 shows the results in the samples with high ATI levels (⩾10 µg/ml). The In-House and Immundiagnostik assays were able to detect ATI+ levels at all exogenous IFX concentrations. As described above, both assays showed an additive effect at 30 µg/ml IFX and ATI levels decreased at 100 µg/ml IFX. Similarly, the QB rapid test and Theradiag assays could not detect ATI+ levels in samples with IFX concentrations from 5 to 300 µg/ml or with all concentrations, respectively.

Discussion

IFX is an effective therapy to the treatment of IBD.[32–34] However many patients may lose response to treatment due to ATI.[35] The ATIs measurement is crucial to adjust the therapy or switch to another drug. The most commonly used assays to evaluate ATI levels are ELISAs,[36] which are very time-consuming. Therefore, the development of a rapid anti-IFX test allows a rapid quantification of ATIs, increasing the effectiveness of TDM and the immediate adjustment of the drug.[20,21] In these present study, we evaluated and compared the qualitative agreement and accuracy of one rapid point-of-care test and three established ELISAs. Moreover, the impact of IFX on the quantification of ATI levels by the four assays was evaluated. IFX and ATI levels were measured in 200 serum samples from 57 IBD patients undergoing induction or maintenance therapy with IFX.

By using analytical and clinical-based cutoffs for defining ATI+ levels, we showed that the In-House and Immundiagnostik assays detected similar numbers of ATI+ samples with both cutoffs. On the other hand, the QB rapid test and Theradiag assays detected a higher number of ATI+ samples using the analytical cutoffs compared with the clinical ones. These results suggest a high prevalence of false negatives for the QB rapid test and Theradiag assays using clinical cutoffs. This finding is consistent with previous data obtained with the Theradiag assay.[17,27,37] Several factors could explain these observations, such as drug interference underestimating ATI levels.[16,17,38] The presence of IFX in the patient's serum interferes with the binding of the marked IFX to the captured ATI, leading to false-negative results.[17,27,37] The assays' inability to detect ATI in the presence of IFX may render inconclusive test results.[17,27,37]

We next focused on the quantification of ATI levels in patients' serum samples with different status for trough IFX and ATI levels (detectable or undetectable). We showed that the In-House and

Table 3. Qualitative agreement regarding the IFX/ATIs levels status: comparison between assays pairs stratified by analytical and clinical cutoffs.

Assay comparison	n (%)				Accuracy (95% CI)	Cohen's kappa (95% CI)
	IFX+/ATIs+	IFX+/ATIs–	IFX–/ATIs+	IFX–/ATIs–		
Analytical cutoffs[a]						
QB rapid test *versus* In-House	0 (0.0%)	42 (21.0%)	46 (23.0%)	3 (1.5%)	45 (39–53)	0.299 (0.211–0.388)
QB rapid test *versus* Theradiag	0 (0.0%)	89 (44.5%)	46 (23.0%)	15 (7.5%)	75 (68–81)	0.625 (0.535–0.715)
QB rapid test *versus* Immundiagnostik	0 (0.0%)	50 (25.0%)	46 (23.0%)	0 (0.0%)	43 (36–50)	0.275 (0.185–0.360)
In-House *versus* Theradiag	1 (0.5%)	42 (21.0%)	94 (47.0%)	2 (1.0%)	70 (63–76)	0.531 (0.433–0.629)
In-House *versus* Immundiagnostik	30 (15.0%)	22 (11.0%)	107 (53.5%)	0 (0.0%)	80 (73–85)	0.661 (0.568–0.753)
Theradiag *versus* Immundiagnostik	0 (0.0%)	49 (24.5%)	95 (47.5%)	0 (0.0%)	68 (61–74)	0.507 (0.407–0.606)
Clinical cutoffs[b]						
QB rapid test *versus* In-House	0 (0.0%)	52 (26.0%)	30 (15.0%)	15 (7.5%)	49 (41–57)	0.343 (0.254–0.431)
QB rapid test *versus* Theradiag	0 (0.0%)	80 (40.0%)	0 (0.0%)	90 (45.5%)	89 (84–93)	0.808 (0.729–0.888)
QB rapid test *versus* Immundiagnostik	0 (0.0%)	41 (20.5%)	30 (15.0%)	1 (0.5%)	35 (29–42)	0.217 (0.138–0.297)
In-House *versus* Theradiag	0 (0.0%)	52 (26.0%)	0 (0.0%)	15 (7.5%)	34 (27–41)	0.219 (0.142–0.296)
In-House *versus* Immundiagnostik	13 (6.5%)	24 (12.0%)	105 (52.5%)	1 (0.5%)	72 (65–78)	0.531 (0.428–0.634)
Theradiag *versus* Immundiagnostik	0 (0.0%)	41 (20.5%)	0 (0.0%)	1 (0.5%)	20 (15–26)	0.129 (0.069–0.189)

[a]ATIs+ levels: ≥0.6 μg/ml for QB rapid test, ≥1.2 μg/ml for In-House, ≥0.01 μg/ml for Theradiag, and ≥10 AU/ml for Immundiagnostik. IFX+ levels: ≥0.4 μg/ml.
[b]ATIs+ levels: ≥1.7 μg/ml for QB rapid test, In-House and Theradiag, and ≥10 AU/ml for Immundiagnostik. IFX+ levels: ≥0.4 μg/ml.
ATIs, anti-infliximab antibodies; ATIs+, ATIs-positive levels; ATIs -, ATIs-negative levels; CI, confidence interval; IFX, infliximab; IFX+, IFX -positive levels; IFX -, undetectable IFX levels; QB, Quantum Blue; *n*, number of matching samples between assays for each IFX/ATIs status in a total of 200 samples.

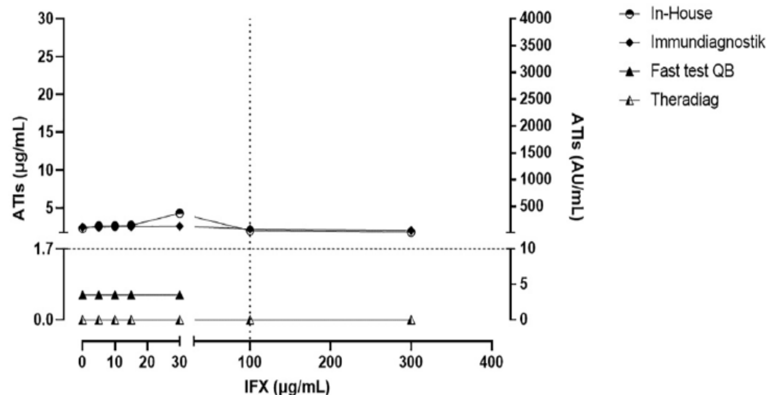

Figure 1. Low anti-infliximab antibodies levels (1.7–2.9 μg/ml) quantified by QB rapid test, In-House, Theradiag and Immundiagnostik assays in the presence of exogenous infliximab. The horizontal dotted line is the lower limit for positive levels of ATIs using the clinical cutoffs (1.7 μg/ml for QB rapid test, In-House and Theradiag, and 10 AU/ml for Immundiagnostik). The vertical dotted line is the upper limit of the therapeutic range of infliximab concentrations (0–100 μg/ml).
ATIs, anti-infliximab antibodies; IFX, infliximab; QB, Quantum Blue.

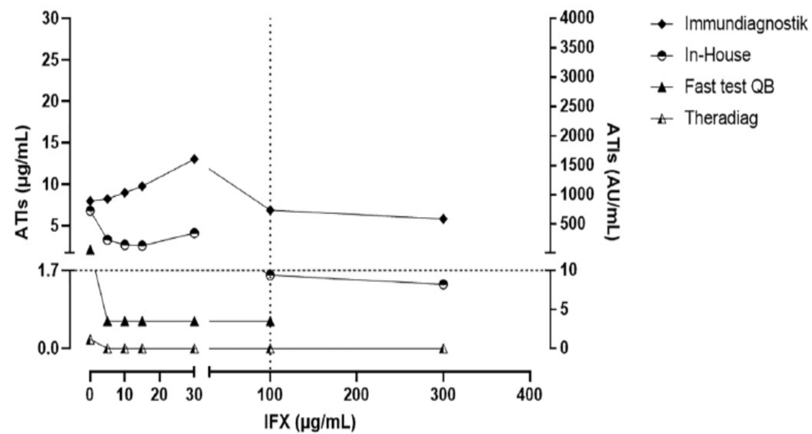

Figure 2. Intermediate anti-infliximab antibodies levels (3.0–9.9 µg/ml) quantified by QB rapid test, In-House, Theradiag, and Immundiagnostik assays in the presence of exogenous infliximab. The horizontal dotted line is the lower limit for positive levels of ATIs using the clinical cutoffs (1.7 µg/ml for QB rapid test, In-House and Theradiag, and 10 AU/ml for Immundiagnostik). The vertical dotted line is the upper limit of the therapeutic range of infliximab concentrations (0–100 µg/ml). QB rapid test indicated invalid values in some samples in the 100 µg/ml IFX concentrations.
ATIs anti-infliximab antibodies; IFX, infliximab; QB, Quantum Blue.

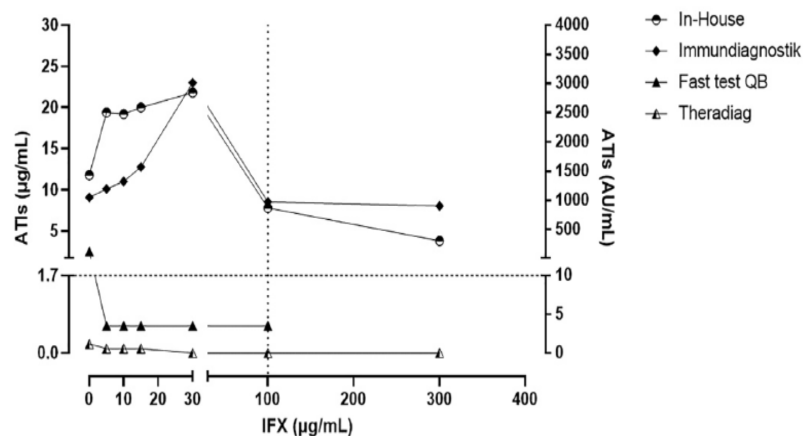

Figure 3. High anti-infliximab antibodies levels (⩾10 µg/ml) quantified by QB rapid test, In-House, Theradiag, and Immundiagnostik assays in the presence of exogenous infliximab. The horizontal dotted line is the lower limit for positive levels of ATIs using the clinical cutoffs (1.7 µg/ml for QB rapid test, In-House and Theradiag, and 10 AU/ml for Immundiagnostik). The vertical dotted line is the upper limit of the therapeutic range of infliximab concentrations (0–100 µg/ml).
ATIs, anti-infliximab antibodies; IFX, infliximab; QB, Quantum Blue.

Immundiagnostik assays were more accurate and could detect ATI+ samples in the presence of IFX. However, in the presence of IFX, the QB rapid test did not accurately detect ATI+ levels using both analytical and clinical cutoffs. A kappa analysis to the IFX-/ATIs samples was also performed and the QB rapid test improves its capacity to detect ATIs in the absence of the drug

(Supplementary Table S2). Furthermore, there was a disagreement between the QB rapid test and the In-House or Immundiagnostik assays in the quantification of ATI+ samples. These findings have clinical relevance and reinforces that the QB rapid test is affected by drug interference. Then, our results show that the QB rapid test and Theradiag measure only free ATIs s detecting a lower amount of ATI+ samples when compared with In-House Immundiagnostik assays. The ability to detect ATI in the presence of the IFX is important, as it was shown that IBD patients with both good IFX trough levels (⩾3 µg/ml) and ATI+ levels have significantly higher levels of C-reactive protein and less mucosal healing during treatment,[7,39] which indicates a reduced control of inflammation mediated by these antibodies even when drug levels are adequate. Our results show that the disagreement increase when the samples had a double-positive or double-negative status, probably related to the specific limitations of each assay. This disagreement can also occur due to the cutoff point chosen to discriminate the ATIS positive from the ATIs negative. This led us to define two different approaches – clinical and analytical approach. The clinical approach seems to highlight the assays' differences. Disagreement increased when samples had double-negative status, probably related to the fact that the QB rapid test detect a greater number of ATI- than the remaining assays. This disagreement could be explained due to the specific limitations and characteristics of each assay.

QB rapid test and Theradiag are drug-sensitive ATI assays, while In-House and Immundiagnostik are drug-tolerant ATI assays. Drug-sensitive ATI assays measures only free antibodies not bound to infliximab, detecting a lower amount of ATI+ samples when compared with drug-tolerant ATI assays. A recent study shows evidence that there is a different clinical interpretation of results when using drug-sensitive *versus* drug-tolerant assays.[40] The choice of the cutoff to discriminate positive *versus* negative also enhances disagreement.

To better understand the impact of IFX on the quantification of ATI, we performed additional experiments using IFX- serum samples incubated with different concentrations of exogenous IFX. We were able to evaluate which IFX concentrations decreased each assays' ability to quantify ATI+ levels. Notably, the addition of exogenous IFX concentrations corresponding to concentrations detected in clinical practice resulted in undetectable ATI levels by the QB rapid test. Using clinical cutoffs, this test could not detect ATIs in serum with intermediate (3.0–9.9 µg/ml) and high (⩾10 µg/ml) ATI+ levels in the presence of 5–300 µg/ml exogenous IFX concentrations. In contrast, the Immundiagnostik and In-house assays were slightly affected by the lowest concentrations of exogenous IFX. Also, these assays were able to detect ATI up to 300 µg/ml of IFX in serum with low, intermediate, and high ATI+ levels. We have previously described the same drug concentration dependency in these assays.[23]

These results show that the ATIs detected are affected by the drug. In this sense, our results show that the QB rapid test and Theradiag are drug-sensitive assays and the In-House and Immundiagnostik are drug-tolerant assays. Clinicians who use these data should have a general understanding of the assay methods to be able to interpret and implement the results. Therefore, these assays should not be interchangeably, and their results should not be directly compared.

The main limitation of this study was the measurement of ATIs levels performed on a single plate and only once, not allowing conclusions about inter and intra assays variability. Furthermore, all the ATI assays used in this study are non-functional assays (not detecting the neutralizing antibodies). In this study, patients were not followed up and it was not possible take conclusions about the relationship between the drug response and the ATI status. Moreover, with emerging reports on transient antibodies, it would be prudent to first ascertain the antibody persistence before making clinical decisions based on a single measurement of ATI levels.[41] Further prospective studies with larger patient cohorts are needed to confirm and validate the findings of this study. Although the findings should be interpreted with caution, a key strength of this study is the large number of serum samples obtained from a multicentric and real-world heterogeneous cohort of IBD patients. Finally, the wide range of ATI+ levels allowed to evaluate the assays' performance both at low and high levels. However, it is important to distinguish clinically between patients with ATIs < 3.7 µg/ml and > 10 µg/ml, since patients with low ATIs levels are more susceptible to dose optimisation while patients with

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Supplemental material

Supplemental material for this article is available online.

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V.

RESULTS AND DISCUSSION

INFLUENCE OF METHODOLOGY ON THE ASSESSMENT OF DRUG LEVELS AND ANTI-DRUG ANTIBODIES LEVELS

With the increased awareness of TDM potential, different assays have been developed for the quantification of drugs and ADAs. Several studies use different assays which represent a challenge to compare results and define thresholds. This is due to the assay's heterogeneity, which may constitute an important bias for the clinical application of TDM. Therefore, in **Chapter 1** and **Chapter 2** we aimed to understand the impact of the different assays on drug and ADAs measurements and their consequent implication in the interpretation of results.

CHAPTER 1 – INFLUENCE OF METHODOLOGY ON THE ASSESSMENT OF DRUG LEVELS

1.1. RAPID TEST FOR DRUG ASSESSMENT

In the last years, rapid tests for drug assessment that would potentially allow clinicians to determine drug concentrations quickly, have been developed. These rapid tests have a rapid turnaround time (approximately 15 min) providing faster results than the established-ELISA assays (approximately 8 hours to have a result). In addition, to perform the ELISA assays, a larger volume of samples (approximately 40 samples), highly trained professionals and specific laboratories, are required. The use of a rapid test instead of an ELISA assay contributes to a faster and more personalized treatment of the patient, avoiding months of delay in successful treatment.

Study II was the first study to validate the Quantum Blue® Adalimumab assay comparing its performance with three already-established ELISA assays. Spiked serum samples from control donors and 120 serum samples from IBD patients undergoing ADL therapy were quantified by the Quantum Blue® Adalimumab assay and the three already-established ELISA assays (R-Biopharm, Immundiagnostik, and In-House). All assays had an acceptable performance as assessed by their recovery percentage (111%, 113%, 110% and 85% for the Immundiagnostik, R-Biopharm, In-House, and Quantum Blue® Adalimumab, respectively) and appeared to be similar in the quantification of ADL (all assays had a high intraclass correlation coefficient (ICC), above 0.927). Regarding qualitative analysis, the paired comparisons revealed the highest ICC for the pair Quantum Blue® Adalimumab – R-Biopharm (ICC 0.864) over a large concentration range. However, the Bland-Altman analyses carried out allowed us to establish that some differences between the assays were related to certain concentration ranges. This analysis suggested that the differences between the assays increase for higher drug concentrations, although rarely exceed the ±1.96 SD interval. This is especially evident for the Immundiagnostik, which showed good correlations with other tests at concentrations below 20µg/ml, but above these concentrations systematically measures higher values than other assays. In general, the Bland-Altman analysis locates the differences between Quantum Blue® Adalimumab

and the other assays in different concentration ranges. For a better evaluation of the behaviour of these assays from a clinical point of view when applying TDM, quantitative analysis were performed. Therefore, ADL levels were stratified according to five therapeutic windows: **i)** ≤3µg/ml; between 3 and 6.85µg/ml, above 6.85µg/ml; **ii)** below or above 4.90µg/ml; **iii)** below or above 5.85µg/ml; **iv)** below or above 7.50µg/ml; **v)** below 5µg/ml, between 5 and 12µg/ml and above 12µg/ml. These different ADL cut-off values were used since an optimal therapeutic window has not yet been established. The researchers suggest different therapeutic windows associated with a clinical response. Some researchers suggest that levels above 4.9µg/ml[175,176] can predict clinical remission, others suggest that levels above 5.85µg/ml[177,178] and above 7.5µg/ml[179,180] are able to predict clinical response. In the absence of a specific cut-off, an ADL therapeutic window of 5–12µg/ml is a generally accepted desirable goal[181–183]. Results showed a greater agreement for the pair Immundiagnostik-R-Biopharm in all five therapeutic windows, with a substantial strength of agreement. In the case of Quantum Blue® Adalimumab, the levels of agreement with the established assays were moderate to substantial in the five therapeutic windows. However, when the In-House method was involved, the results tended to be weaker. These results can be explained by the ranges of each assay being quite different: Immundiagnostik has a range that varies between 0.2 and 47µg/ml; R-Biopharm between 0 and 35µg/ml; Quantum Blue® Adalimumab between 1 and 35µg/ml, and In-House varies between 0.10 and 20µg/ml. In addition, it was observed that the agreement decreases as the therapeutic window increases, probably due to the greater dispersion of the measures at higher concentrations. In conclusion, our results showed that specific percentages vary if a different therapeutic range is used, and that the Quantum Blue® Adalimumab is a reliable alternative to the commonly used ELISA-based ADL quantification kits. Overall, it was shown that the different assays have a good general agreement, but there is a notable variability in drug concentration between trials, so clinicians should be aware of this variability when measuring drug levels. Therefore, it is important to emphasize that the TDM approach should not be solely based on ADL quantification, but should be integrated into the patient's clinical context, considering the presence of symptoms and other disease markers to help in the decision process. More studies are needed to integrate the patient's symptomatology with the performance of Quantum Blue® Adalimumab in the context of TDM, as well as to define specific therapeutic limits of ADL that should be applied with this method. It should also be noted that the same assay should be used to measure drug levels for the same patient whenever a measurement is needed.

1.2. ASSAYS FOR BIOSIMILARS AND CROSS-IMMUNOGENICITY

Biological therapies have revolutionized the treatment of IBD, but despite their fundamental role in the treatment of IBD and other autoimmune diseases, these therapies are substantially

expensive. In fact, biological therapies are currently the main cost drivers in IBD units[184], accounting for an impressive 85.7% of total costs[185]. Therefore, biosimilars have become an attractive alternative – molecules that are highly similar to a reference biologic drug in terms of structural, functional, biological, safety, purity, efficacy and clinical terms[186]. With an expedited regulatory process, biosimilars to infliximab may reduce costs by up to 40%[185]. Despite some controversy regarding the extrapolation of clinical indications[187], two Infliximab (Remicade®) biosimilars have been approved in Europe and in the USA. CT-P13 (Remsima™) was the first biosimilar to infliximab that obtained regulatory approval with no significant differences in terms of efficacy or safety. The second biosimilar approved by EMA (in May 2016) and the FDA (in April 2017) was SB2 (Flixabi®)[188]. Biosimilars offer the potential to reduce costs and improve patient outcomes by increasing accessibility and facilitating treatment with anti-TNFs early in the course of the disease. In addition, they can be combined with other strategies like TDM to optimize the dose and provide better outcomes costs[189]. However, to safely use TDM in patients treated with Flixabi and Remsima, it must be determined whether the assays developed and optimized to quantify Remicade are equally accurate in quantifying its biosimilars. Therefore, **Study I** aimed to: i) validate the utilization of Remicade-optimized TDM assays for the quantification of Flixabi and Remsima; and ii) determine the presence of Remicade, Remsima and Flixabi cross-immunogenicity. A commercial ELISA assay (RIDASCREEN®IFX monitoring, hereafter referred to as R-Biopharm), an In-House, and a rapid test (Quantum Blue® infliximab, hereafter referred to as Buhlmann) were analysed.

Healthy donors sera spiked with known Remicade, Remsima and Flixabi concentrations (generated by diluting the appropriate amount of each drug) were quantified using the three assays. Cross-immunogenicity between Remicade and its biosimilars was also assessed. IBD patients under Remicade and Remsima therapy were recruited, but no clinical samples of patients under SB2 therapy were available since this drug was not commercialized in Portugal at the time of the study. Briefly, to assess ADAs, Remicade, Remsima or Flixabi were added to a plate precoated with TNFα. Afterwards, diluted serum samples (anti-Remicade or anti-Remsima) were added to the plate and incubated for 60 min at room temperature. At the end of the incubation time, ADAs levels were determined using an In-house method previously described[157,190,191].

This study showed that R-Biopharm, In-House and Buhlmann assays measure similar amounts of each drug at any given concentration, with the standard deviations (SDs) being larger for the Buhlmann method. R-Biopharm and Buhlmann assays were slightly more accurate to quantify Remsima than when measuring its originator Remicade. In the case of Flixabi, the In-House was more accurate. The values obtained in the drug measurement with the different assays were rather similar, and the differences found tended to be greater when the drugs' concentrations were above the critical values considered in the therapeutic window, which should not have an effect in clinical

practice[192–194]. Overall, Buhlmann slightly underestimated the three drugs when compared to R-Biopharm, while the In-House slightly overestimated Remicade and Flixabi when compared to Buhlmann. The small differences observed may be due to small changes in the structure of the biosimilars, which can be attributed to differences in the biological synthesis of compounds (e.g.: different cells lines or growth media), storage and transport[186,195,196]. Overall, the results agree with the ones described in the literature for Remsima – methods optimized for Remicade perform equally well when measuring biosimilar levels[197–200].

ADAs have been associated with increased drug clearance and prevent these agents from inhibiting their target cytokine, decrease treatment effectiveness, induce loss of response, and increase the risk of infusion reactions[127,201]. Immunogenicity is a significant concern for biologic drugs as it can affect both safety and efficacy. Hence, ensuring the equivalence of immunogenicity between a biological and its biosimilar is fundamental[202,203]. Cross-immunogenicity is the ability of ADAs to react against compounds other than the one that originated their appearance, and is of the utmost importance from a clinical point of view. In fact, when an anti-TNFα therapy fails due to the presence of ADAs, the absence of cross-immunogenicity should be considered as a criterion for choosing a second anti-TNFα agent. In this context of immunogenicity, it has been shown that Remsima invokes immunogenicity in the same proportion of patients as Remicade[196]. In contrast, Flixabi showed higher rates of ADAs than Remicade in equivalent clinical studies[204]. However, there is a paucity of information regarding the bioequivalence of Remsima and Flixabi in relation to cross-immunogenicity with Remicade and potential immunogenic adverse events. Therefore, in this study, cross-immunogenicity between Remicade, Remsima and FLixabi was evaluated.

This study showed that Remicade, Remsima and Flixabi react in a similar way to anti-Remicade and anti-Remsima sera. Our results showed that the ICCs between the reactions of different drugs to anti-Remicade and anti-Remsima serum was close to 1.0, showing an extremely high cross-immunogenicity. These results are in line with the ones previously published about the cross-immunogenicity of Remsima and its originator[187,190,200]. In this study, we demonstrated the existence of cross-immunogenicity between Remicade, Remsima and Flixabi. This reinforces the similarity between these drugs and the idea that these drugs probably share a common epitope, as well as some clinical implications in considering switching from the original drug to the biosimilar: a patient medicated with Remicade or Remsima, whose therapy fails due to the presence of ADAs, experiencing adverse events or therapeutic failure, would not benefit from switching to Remicade, Remsima or Flixabi.

In conclusion, this study was, at the time, the first to demonstrate that quantification assays optimized (R-Biopharm, Buhlmann and In-house) for Remicade can be used to measure its biosimilars

(Remsima and Flixabi) in an accurate way. Moreover, we have demonstrated the existence of cross-immunogenicity between Remicade, Remsima and Flixabi.

CHAPTER 2 - INFLUENCE OF METHODOLOGY ON THE ASSESSMENT OF ANTI-DRUG ANTIBODIES LEVELS

2.1. RAPID TEST FOR ANTI-DRUG ANTIBODIES ASSESSMENT

Therapeutic failure has been attributed in part to inadequate serum drug concentrations and the formation of ADAs. Different studies have reported that ADAs presence is correlated with a lower drug concentration, and an increase in infusion reactions[205]. Therefore, TDM should also be based on ADAs assessment. However, ADAs assessment is usually more complex than drug levels evaluation due to the presence of the drug itself. Notwithstanding, several assays have been developed, validated, and made commercially available for use in hospitals and reference laboratories. Some of them are able to measure both drugs and ADAs, whereas others are specific for one of these measurements. For the fast and easy detection of anti-infliximab antibodies (ATIs) response, a lateral flow test was developed and preliminary evaluated.

In **Study III** three different ELISA assays (In-House anti-human lambda chain assay, semi-fluid phase enzyme immunoassay (SFPE) from Immundiagnostik, and a bridging ELISA from Theradiag) and a new rapid test to quantify ATIs levels (semi-quantitative sandwich immunoassay from Quantum Blue® Anti-Infliximab (Bühlmann), referred as QB rapid test), were compared. Furthermore, the impact of Infliximab (IFX) on ATIs quantification by each assay was also assessed. IFX and ATIs levels were measured in 200 serum samples from 57 IBD patients undergoing IFX therapy. The four assays feature different technical characteristics, detection limits and reporting of results, which makes it difficult to establish the positivity cut-offs for a qualitative evaluation of ATIs levels. Therefore, two cut-offs (analytical and clinical) were used to test ATIs-positive (ATIs+) levels. The analytical cut-offs were based on the lower detection limits described by the manufacturers for each assay, while the clinical cut-offs were based on the clinically relevant ATIs+ levels defined in the literature.

The overall comparison showed that the agreement varies according to the used cut-off: analytical cut-offs for ATIs+ levels showed a moderate agreement between the QB rapid test and Theradiag (k=0.489), whilst a low agreement was observed between the QB rapid test and In-House (k=0.160), and the QB rapid test and Immundiagnostik (k=0.139). Based on clinical cut-offs for ATIs+ levels, a low agreement was found between the QB rapid test and In-House (k=0.163) and between the QB rapid test and Immundiagnostik (k=0.085). In this analysis the k coefficient could not be calculated for the comparisons with Theradiag because this did not detect ATIs+ samples. Both

analytical and clinical-based cut-offs to define ATIs+ levels showed that the In-House and Immundiagnostik assays detected a similar numbers of ATIs+ samples. On the other hand, the QB rapid test and Theradiag detected a higher number of ATIs+ samples using the analytical cut-offs compared with the clinical cut-offs. These results suggest a high prevalence of false negatives for the QB rapid test and Theradiag using the clinical cut-offs. False negatives may arise due to the presence of the drug, thus underestimating ATIs levels. QB rapid test and Theradiag use labelled IFX as the detection antibody, and therefore the presence of the drug in the patient's serum may compete with the detection of ATIs, leading to false-negative results.

In order to understand the impact of the drug on ATIs quantification, additional experiments were carried out using IFX negative (IFX-) serum samples incubated with different concentrations of exogenous IFX. For this aim, different exogenous IFX concentrations (5, 10, 15, 30, 100 and 300µg/ml) were added to ATIs+ serum samples with undetectable IFX concentrations (≤0.4µg/ml). With these experiments, we were able to assess which IFX concentrations decrease the ability of each assay to quantify ATIs levels. This study was the first to describe the inability of the QB rapid test to detect ATIs+ in samples with IFX levels higher than 5µg/ml, and established which IFX concentrations decrease the assay's ability to detect ATIs. Similar results were observed for Theradiag, confirming what had already been described by Kopylov et al[168], that the bridging ELISA format was unable to detect ADAs in the presence of the drug. In contrast, the Immundiagnostik and In-House were slightly affected by the lowest concentrations of exogenous IFX. These assays were also able to detect ATIs+ in samples with IFX concentrations up to 100µg/ml, which correspond to the upper limit of the therapeutic concentrations of patients under IFX[172,206]. This drug concentration dependence was also previously described by our group. Still, Immundiagnostik was the assay less influenced by the presence of drug in the serum. This may be explained by the initial acid buffer treatment that this assay uses to dissociate IFX-ADAs immune complexes. This study also showed that for high concentrations of ATIs (≥10 µg/mL) the presence of IFX was less significant. This work demonstrates that: i) assays are not only limited by the drug levels in serum but also by ADAs levels; ii) the QB rapid test and Theradiag are drug-sensitive assays and the In-House and Immundiagnostik are drug-tolerant assays, and iii) the assays analysed in this study should not be used interchangeably, and their results should not be compared directly.

CLINICAL IMPACT OF METHODOLOGIES BIASES

Different studies show that serum IFX trough levels are correlated with clinical response, clinical remission and mucosal healing in IBD patients[207–209]. Low serum drug concentrations and the

presence of ADAs are associated with poor clinical outcomes (e.g.: infusion reactions, loss of clinical response)[206,210,211]. As has been described throughout this work, different analytical assays are currently used to measure drug levels and ADAs. However, the variability in results among methodologies makes it difficult to compare data from different studies. Currently, there are no gold standard assays available and the assay heterogeneity may constitute an important bias for the clinical application of TDM, hampering the comparison of results from different studies[212]. Therefore, comparing assays is critical to understand and interpret data of different clinical studies, before they are used for clinical decision-making based on a predefined therapeutic algorithm[213].

Studies have shown that depending on the binary status drug levels/ADA levels of serum from patients treated with anti-TNFα drugs, different clinical decisions are made. In study III, we assessed the impact on clinical decisions when different assays are used to define the binary status of patient serum. A high to complete disagreement was obtained when the QB rapid test and Theradiag were compared with the Immundiagnostik or In-House for double positive status (IFX+/ADAs+) and double negative status (IFX-/ADAs-). In these situations, erroneous therapeutic decisions may occur. The intensification of treatment and shorting interval in a double-negative scenario (IFX-/ADAs-), as well as the change of drug class or the concomitant use of immunomodulators in a double-positive scenario (IFX+/ADAs+), should take into account that these status scenarios are assay-dependent. Regarding the rapid test analysed in this study, a reasonable approach to tackle this issue could be using the QB rapid test to quantify ATIs levels only after performing another assay to quantify IFX levels in patients' serum samples, since the QB rapid test can be used for the quantification of ATIs levels in serum samples with undetectable IFX levels (≤0.4μg/ml) but should not be used in samples with IFX concentrations ⩾0.4μg/ml. However, in the IFX+/ATIs– or IFX–/ATIs+ status, the agreement between assays is significantly higher. In these status the choice of the assay will probably have little influence on therapeutic decisions to change drug class (IFX+/ADAs-) or to change the anti-TNFα antibody (IFX-/ADAs+). These results are in line with what our group had observed previously in a similar study[191]. Therefore, the inability of some assays to determine ADAs in the presence of the drug may difficult the clinical interpretation of TDM. Several studies have described the importance of ADAs on the disease course and their influence on drug levels[214]. For example, Casteele et al reported that patients with IFX>3 μg/mL and positive ADAs have significantly higher levels of CRP and less mucosal healing, indicating a reduced control of inflammation mediated by the presence of ADAs[215]. In another study, Casteele et al also described that patients treated with anti-TNFα, who initially produce ADAs, later develop tolerance to the drug. This tolerance is mediated by an activation of the regulatory immune response[216]. However, ADAs can be considered transient due to false negatives at the re-evaluation time, which have little or no loss of clinical efficacy or safety problems[214,216]. The transience of ADAs has been reported by several studies, reinforcing the idea

that in some cases ADAs are present in the circulation only temporarily. It should be noted that transient ADAs have been described to appear at lower levels than persistent ADAs. In addition, transient ADAs can develop after years of anti-TNFα therapy, while persistent ADAs are generally detectable in the first year. This can impact the role of TDM and lead to misinterpretations and, therefore, integrating TDM into the clinical context and biomarkers is essential[217]. Kopylov et al also showed that patients with double positive status (IFX+/ADAs+) may indicate an increasing immune response to IFX, which would result in future low trough drug levels and loss of response[168]. Double positive status can also occur due to the presence of transient antibodies. Approximately 30% of patients have ADAs that disappear on subsequent re-evaluation during maintenance with IFX[216,217]. Transient antibodies have thus been described as having little clinical significance, since they do not correlate with loss of response.

VI.

CONCLUSIONS AND FUTURE PERSPECTIVES

The implementation of TDM in IBD clinical practice has evolved over the years. Initially, the focus was only on measuring and reporting drug levels and now both drug and ADAs levels were considered as important for the clinical response. Therefore, 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 the assay's limitations and the potential methodological biases in results' interpretation.

TDM is thus considered an emerging strategy to optimize the biological treatment of IBD. Therefore, TDM strategy should be recommended in different scenarios: i) loss of response, guiding physicians to discriminate among PK and PD reasons for therapy failure; and ii) predict loss of response, combining TDM with biomarkers' assessment and individual clinical evaluation (clinical and endoscopic data). This strategy will not only allow the prediction of a patient's future response, but it will also allow physicians to use the information to optimize drug dosage, both to induce and maintain a clinical response in the future. Overall, the main and novel findings of this thesis were:

- ✓ Not all Infliximab originator-optimized quantification assays can be used to measure IFX-biosimilars in an accurate way;
- ✓ Cross-immunogenicity occurs between Infliximab originator (REMICADE) and both IFX biosimilars (REMSIMA and FLIXABI);
- ✓ New methodologies (rapid tests) can safely substitute old methodologies (ELISAs) in drug measurement;
- ✓ New methodologies (rapid tests) have a significant impact in ADAs measurement;
- ✓ Impact of IFX on ATIs levels is different in different assays;
- ✓ Serum double positive (IFX+/ATIs+) and double negative (IFX-/ATIs-) samples lead to higher disagreement between assays.

TDM for IBD offers the opportunity to improve the effectiveness of biological products, allowing dosage and/or therapeutic changes. When implementing TDM, drug levels and ADAs can be measured, offering insight into resistance and response. Overall, TDM assays can be standardized and, although a good correlation is observed for most assays, a difference in the absolute concentration of the drug can be observed. Also, and due to the lack of universal standards, ADA levels cannot be quantitatively compared between assays. For most TDM-based treatment algorithms, measuring the drug's serum concentration is the first step. However, subsequent ADAs measurement can be useful to explain undetectable or low concentrations of the drug. The dynamics between ADAs and drug concentrations may be more informative than a single ADAs measurement, due to the known limitations of sensitivity in the presence of the drug and the non-comparable cut-offs/thresholds between assays.

TDM has shown to be advantageous to: i) identify problems with medication adherence among cases of non-adherent patients; ii) identify the most appropriate dosage regimen to achieve optimal response with minimal toxicity; iii) help clinicians to identify the patients who will benefit and who will not benefit from treatment; and iv) help clinicians to make decisions related to increase or decrease dosage, changing infusion intervals, or change the class of therapeutic mAb, thus maximizing clinical benefit and minimizing potential side effects.

To measure these parameters, ELISA assays, which have been widely used due to their simplicity, stand out. However, these assays have a response time of approximately 8 hours, which can impair immediate therapy adjustment. In contrast, recent developments in rapid tests could deliver results in a much shorter time (15–20min turnaround time). These rapid tests have other advantages such as: i) convenience – testing and consultation including possible treatment changes takes place in the same visit; ii) clinical benefits – swifter diagnosis or exclusion of diagnosis, more appropriate treatment and improved treatment outcomes; iii) economic benefits – fast and assertive therapeutic decisions could be implemented, leading to a better control of disease activity and possibly clinical remission, which is currently the main therapeutic goal.

VII.

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