Use of infliximab and anti-infliximab antibody measurements to evaluate and optimize efficacy and safety of infliximab maintenance therapy in Crohn's disease

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This present PhD thesis is based upon the following publications:

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2. Steenholdt C, Bendtzen K, Brynskov J, Thomsen OO, Ainsworth MA. Cut-off levels and diagnostic accuracy of infliximab trough levels and anti-infliximab antibodies in Crohn's disease. Scand J Gastroenterol 2011;46:310-8

3. Steenholdt C, Al-khalaf M, Brynskov J, Bendtzen K, Thomsen OØ, Ainsworth MA. Clinical implications of variations in anti-infliximab antibody levels in patients with inflammatory bowel disease. Inflamm Bowel Dis. 2012;18(12):2209-17

4. Steenholdt C, Svenson M, Bendtzen K, Thomsen OO, Brynskov J, Ainsworth MA. Severe infusion reactions to infliximab: aetiology, immunogenicity and risk factors in patients with inflammatory bowel disease. Aliment Pharmacol Ther 2011; 34: 51-8

BACKGROUND

INTRODUCTION

Crohn's disease (CD) is a chronic, recurrent inflammatory disorder of the gastrointestinal tract, which appears to originate from a dysregulated immune response to the commensal luminal bacteria flora in genetically susceptible individuals.(1;2) CD has conventionally been treated using anti-inflammatory agents including corticosteroids in case of flaring supplemented with long term thiopurine or methotrexate treatment. Patients with moderate to severe disease activity not treated satisfactorily with these agents may in addition require biologic therapy with tumor necrosis factor (TNF)-α inhibitors such as infliximab (IFX).(3-6) A number of clinical challenges relate to IFX therapy, in particular the optimal management of patients with primary or secondary treatment failure or with severe side effects.(7) This PhD thesis explores the clinical utility of measuring IFX and anti-IFX antibodies (Ab) by newly developed techniques based on radioimmunoassay (RIA), to assist in evaluating and optimizing efficacy and safety of IFX maintenance therapy in CD.

TNF-ALPHA INHIBITORS

Most biological drugs today are proteins or glycoproteins produced in biological systems engineered with the genes of interest. These therapeutic proteins are designed to interfere with a specific biological process involved in disease pathogenesis or to substitute for a lacking or defective protein.(8) The availability of these drugs has introduced a new paradigm in treatment of inflammatory bowel disease (IBD) and other chronic immunoinflammatory diseases such as rheumatoid arthritis and psoriasis, and has resulted in markedly better patient outcomes.(3-5) Monoclonal Ab directed against the pro-inflammatory cytokine, TNF-α, is currently the only registered class of biologic agents for IBD in EU;(9) a second class, which inhibits leukocyte migration to the gut by targeting the α4 integrin adhesion molecule (natalizumab), is registered for CD in USA.(9;10) The two TNF-inhibitors registered for CD therapy in EU are IFX and adalimumab (ADL). IFX is a chimeric immunoglobulin (Ig) G1-κ monoclonal anti-TNF-α Ab consisting of approximately 75% human and 25% murine amino acid sequences.(11) It was the first biological drug to be registered for treatment of CD in the 1990s, and it is often first choice of TNF-inhibitor having been on the market longest and with established efficacy and safety profiles.(4;11-13) IFX is administered intravenously (iv) in doses of 5 mg/kg with induction infusions at weeks 0, 2, and 6, followed by maintenance infusions every 8 weeks.(14) In contrast to IFX, ADL is a so-called fully human IgG1-κ monoclonal anti-TNF-α Ab administered as subcutaneous injections with an induction regimen followed by maintenance injections of 40 mg every other week.(15-18) IFX and ADL target both soluble and membrane-associated forms of TNF-α, thus inhibiting TNF-α from triggering cellular TNF-receptors and

ultimately down regulating inflammation.(19-22) These agents may additionally induce programmed cell death (apoptosis) in certain cells, as well as cytolysis of transmembrane TNF-bearing cells through complement- and/or Ab dependent cytotoxicity.(20-22)

PHARMACOLOGY OF ANTI-TNF BIOPHARMACEUTICALS

Important differences exist between TNF-inhibitors and conventional, chemically synthesized, small molecule drugs.(23) For example, biological TNF-inhibitors are more often than 'small molecules' recognized as a foreign substance by the recipient's immune system.(24) This drug immunogenicity may result in formation of anti-drug Ab.(25;26) Anti-drug Ab generation is usually mediated by T cell recognition of non-self peptides displayed on antigen-presenting cells, followed by B cell activation.(26) In the case of IFX, the main immunogenic component of the drug lies within the murine part of the Fab fragment, but immunogenicity may also originate from other sites on the drug.(27-29) For example, anti-drug Ab may arise from recognition of immunoglobulin allotypes and/or idiotypes (in ADL), from neoepitopes generated by drug aggregation, or from non-human glycosylation.(23;24;29) Accordingly, despite a near-complete molecular 'humanization', ADL may also elicit formation of anti-drug Ab, but the frequency appears somewhat lower than that for IFX.(7;24;30-32) Delivery of biological TNF-inhibitors in principle resembles common vaccination procedures where repeated administrations of non-self proteins are used to elicit an immune response.(23;29;33) In addition, bioavailability and pharmacokinetics of TNF-inhibitors demonstrate a large degree of intra- and inter-individual variation over time.(8;34;35) Awareness of the above characteristics of TNF-inhibitors is rising along with emerging recognition of the advantages of individualized therapy, which in this context means optimizing therapy according to personal needs rather than using standardized regimens deducted from cohorts of patients.(8;23;36) Screening for anti-drug Ab is now a regulatory requirement for the marketing of biological drugs.(37-39)

EFFICACY OF IFX

IFX is routinely used to induce and maintain remission in patients with moderate to severe luminal or fistulizing CD as recommended in international, evidence based guidelines.(3-6;40) Clinical efficacy of IFX is clearly superior to placebo. In a recent metaanalysis which included studies through 2010, the estimated relative risk of failure to achieve remission with IFX in active luminal CD as compared with placebo was 0.68 [95%CI 0.52-090]; estimated relative risk of relapse in patients with quiescent luminal CD treated with IFX as compared with placebo was 0.72 [0.63-0.83]; and estimated relative risk of fistulas remaining unhealed in patients treated with IFX as compared with placebo was 0.62 [0.45-0.81].(41) Similar efficacies have been reported elsewhere.(42-44) IFX may also induce mucosal healing which has been associated with highly favorable outcomes.(45-49) Scheduled maintenance therapy with regular IFX infusions every 8 weeks is superior to an episodic strategy with drug pauses extending more than 12 weeks.(42;50) However, some centers use episodic IFX as a bridging strategy to conventional immunosuppressive agents, largely due to financial restrictions. Whether IFX should be used as monotherapy or in combination with an immunosuppressant, remains controversial.(6;42) Hence, combination therapy has been associated with increased risk of opportunistic infections and lymphomas including the usually fatal, but extremely rare hepatosplenic T cell lymphoma;(51;52) and contin-

ued treatment with immunosuppressives beyond 6 months has not shown benefit over IFX monotherapy.(53) On the other hand, combination therapy is more effective than monotherapy in IFX naive patients, and this may also apply to others.(54-56) IFX is generally used according to a "step-up" principle, in which therapy is initiated in case of insufficient effect of conventional immunosuppressives or steroid dependence.(3-6;41;43) It has been suggested, that use of IFX early in the course of disease as a first line agent following a "top-down" strategy may provide better outcomes and possibly even change the natural course of disease.(4;54;57-59) However, the fact that disease course seems to follow the natural history of CD in patients having discontinued IFX while in complete, long term clinical remission does not support this argument.(60-63)

SAFETY OF IFX

IFX is generally well tolerated but may result in side effects such as opportunistic and non-opportunistic infections, various skin reactions, autoimmunity, and infusion reactions.(64-66) Infusion reactions are classified as acute when they occur during an infusion, and as delayed when they arise after infusions.(67) Acute mild to moderate reactions are self-limiting and resolve spontaneously after temporary cessation of infusion or reduction of infusion rate.(65;67) Acute severe infusion reactions are of particular concern because of the severity and subsequent permanent discontinuation of IFX followed by limited future treatment options.(68) These reactions are reported in up to 5% of IBD patients, and resemble anaphylactic reactions with e.g. hypotension; chest tightness; and respiratory distress with dyspnoea, bronchospasm, or laryngeal oedema; urticaria, or rash.(65;67;69)

CLINICAL RESPONSE TYPES TO IFX

In pivotal placebo controlled maintenance trials with open label IFX, the maximal response was observed at week 10.(7;70;71) Hence, primary non-response is defined as absence of clinical response following IFX induction regimen. It is optimally determined as the absence of a significant drop on a validated clinical activity score such as Crohn's Disease Activity Index (CDAI), in this case defined by a 70 points decrease.(7;72-74) Approximately one third of CD patients are classified as such;(7;70) and it is speculated that primary response failure may arise from e.g. non-TNF-α driven immunoinflammatory mechanisms, individual differences in drug metabolism and elimination, variable drug binding in serum or tissues, presence of innate anti-IFX Ab, or absence of inflammation.(23;75) Recent guidelines fail to give recommendations on strategy for handling this subgroup of patients.(3-6) In addition, a notable fraction of up to about 50% with initial response to IFX induction later lose effect and experience flare of disease during ongoing IFX maintenance therapy.(7;41;76;77) These patients are classified as secondary non-responders. Classification is optimally supported by a documented increase in disease activity by validated scoring indices, e.g. CDAI > 150 points combined with an increase of ≥ 70 from baseline.(7;77) The annual risk of secondary response failure has been estimated to 13% per patient year, but studies are inhomogeneous and incidence is not constant being higher in the first year and subsequently leveling out.(76;77) The extent of primary non-response and secondary loss of response to IFX is biased by use of different definitions of response failure, and of variable timings of outcome reportings.(7;41;76;77) Finally, approximately one third of CD patients starting on IFX obtain a state of long term sustained remission, and are classified as maintained responders.(7;41;70;76) It is currently unresolved if, and notably when,

IFX can later favorably be discontinued in this subgroup.(3;6;78-80)

LOSS OF RESPONSE TO IFX

Treatment of patients with loss of response to IFX maintenance therapy is complicated by a limited number of therapeutic options including few alternative medications.(3-6;19) This, along with the complexity of the disease phenotype and severity of symptoms, often has high impact on patient well-being and costs.(81-84) Response may be recaptured at least on the short term by IFX dose optimization with increased frequency of administrations to every 4 or 6 weeks, or by increased dosing to 10 mg/kg.(6;50;70;75;76;85;86) The different dose optimizing strategies do not seem to differ significantly in the clinical setting,(87;88) but pharmacokinetic models suggest superiority of shortening infusion intervals.(89) Even though some patients are later able to successfully go back to the standard IFX regimen of 5 mg/kg every 8 weeks, others may not respond to dose optimization or later lose response once again.(50) These patients sometimes benefit from switching from IFX to a second (i.e. ADL) or even a third TNF-inhibitor (i.e. Certolizumab Pegol, a humanized pegylated Fab fragment against TNF-α, is registered for CD in USA),(90-94) but efficacy is decreased compared with the primary TNF-inhibitor.(17;75;95-97) Taken together, these empiric observations support current recommendations suggesting to first optimize IFX dosing and then change to ADL in case of loss of response to IFX.(3;42;75;98) However, this strategy is not optimal in all patients, it often takes long time to restore inactivity of disease, and patients meanwhile risk irreversible tissue damage.(42)

Factors associated with a favorable response to IFX include young age, short duration of disease, non-smoking, an inflammatory disease phenotype, disease localization limited to the colon only, and concomitant immunosuppressive treatment.(99-102) Genetic markers in the form of single nucleotide polymorphisms in selected candidate genes belonging to the TNF receptor superfamily members (e.g. TNFRSF1A and 1B) have also been associated with responsiveness to IFX.(103-108) The gene expression profile has been found characteristic for patients with a beneficial response to IFX.(109;110) Among inflammatory markers, a high baseline C-reactive protein (CRP) and early normalization of CRP during IFX induction has been associated with later maintained remission.(111-113) Reports on fecal calprotectin have been conflicting.(114;115) Despite recognition of predictors of clinical response types to IFX, it is not yet possible to avoid loss of response by selecting appropriate candidate patients. Thus, there is an obvious clinical need for development of a more rational therapeutic approach to patients with loss of response to IFX maintenance therapy.

THERAPEUTIC MONITORING OF IFX

In light of the general characteristics of anti-TNF biopharmaceuticals combined with recognition of advantages of prolonged use of IFX in patients with CD, is it speculated that therapeutic monitoring with reliable measurements of drug bioavailability and immunogenicity may help to identify mechanisms for loss of response, and to guide selection of optimal intervention in individual patients. This hypothesis has been supported by relatively consistent findings of associations between detectable IFX in serum and maintenance of remission; and between low or undetectable serum IFX concentrations prior to the next infusion (i.e. trough levels), and insufficient effect or loss of response to IFX.(53;116-121) Data on the clinical importance of anti-IFX Ab is, however,

ambiguous.(122-124) Formation of anti-IFX Ab may occur shortly after IFX initiation as well as after prolonged drug exposure.(70;117;125;126) Anti-IFX Ab have been observed in up to 20% of IBD patients on maintenance treatment, and in up to 60% of patients on episodic treatment.(122-124) The risk of anti-IFX Ab development is reduced by concomitant immunosuppression with thiopurines or methotrexate.(54;85;117;118;123;125-127) Anti-IFX Ab are believed to neutralize IFX by binding to the murine derived part of the Fab fragments and/or to increase clearance of the drug, thus resulting in an inadequate inhibition of TNF-α and potentially in therapeutic failure.(7;27;34) Kinetic studies have supported that anti-IFX Ab may increase clearance of IFX.(128-132) Accordingly, several studies have observed a correlation between anti-IFX Ab, low IFX trough levels, and loss of response.(28;117;119;126;127;130;133;134) Yet, a number of studies have failed to detect these correlations,(13;53;85;118;120;125;135-137) and recent reviews have been unable to confirm a clinically significant impact of anti-IFX Ab on the efficacy of IFX therapy.(122-124) More consistently, anti-IFX Ab have been associated with increased risk of acute infusion reactions.(28;85;117;125;127;133;136) Nevertheless, the nature of acute severe infusion reactions including significance of immunogenicity, usefulness of anti-IFX Ab measurements for risk stratification, and potential impact of immunological cross reactions in case of later treatment with a different TNF-inhibitor remains unresolved.(67;69;122;138)

TECHNIQUES FOR MONITORING IFX AND ANTI-IFX AB

Although associations between clinical efficacy and safety, and serum levels of IFX and/or anti-IFX Ab have been acknowledged, the results are not consistent, and clinical utility of these tests for determining mechanisms of insufficient response, and for deciding on optimal intervention in individual patients has not been established.(23;77;122;139) The cause of the variable and to some extent contradictory findings of currently available data is unknown, but several aspects need consideration. For example, definitions of efficacy and safety parameters have been inconsistent, and timing of sampling with respect to outcome assessment has been highly variable.(77) Differences in patient populations and trial designs may also have led to conflicting results.(122) Of particular note, several techniques have been used to measure IFX and anti-IFX Ab concentrations, and it is unknown if and how these different assays compare.(7;77;122-124) The most commonly used technique is based on solid phase enzyme-linked immunosorbent assay (ELISA) and has a number of limitations.(29;122) Of note, ELISA is unable to detect anti-IFX Ab in the presence of IFX, thereby rendering test results from about half the patients in clinical trials inconclusive.(118;125;136) ELISA is furthermore prone to false positive and false negative test results due to e.g. matrix effects and interference with serum factors, and the inability of bridging ELISA to detect IgG4 anti-IFX Ab.(7;23;29;140) Finally, it is unknown whether ELISA measures bioactive IFX, and if detected anti-IFX Ab are functional and interfere with IFX activity.(23;122;141)

Alternative techniques for detection of IFX and anti-IFX Ab have been developed due to the limitations of ELISA.(7;124) Novel RIAs for this purpose have generated promising results in patients with rheumatoid arthritis, and in one study of patients with CD.(27;119;128;142) These RIAs take place in fluid phase and resemble in vivo conditions better than ELISA's. Furthermore, they are not influenced to the same degree by potential artifacts encountered in solid phase assays.(143;144) The RIA for IFX is functional in the sense that IFX concentration is determined as

the TNF-α binding capacity of the serum.(23;128) RIA for anti-IFX Ab detects all isotypes of immunoglobulin binding to IFX, and measures anti-IFX Ab also in the presence of IFX.(23;128) We hypothesized that monitoring IFX maintenance therapy in CD by measurements of IFX and anti-IFX Ab concentrations by newly developed RIAs would be useful in clinical practice to assist in evaluating and optimizing efficacy and safety.

AIM

The aim of this PhD thesis was to investigate the clinical utility of measuring IFX and anti-IFX Ab by RIA to aid in evaluating and optimizing efficacy and safety of IFX maintenance therapy in patients with CD. In addition, to examine conditions that could potentially influence the significance of IFX and anti-IFX Ab associations with clinical outcomes, including the use of different analytical techniques, different cut-off levels for binary stratification of test results, and differences in timing of measurements. This was investigated in four independent studies with the following objectives:

STUDY I

To compare analytical properties of RIA for measuring serum concentrations of IFX and anti-IFX Ab with those of other commonly used techniques for this purpose including binding assays and cell based bioassays.

STUDY II

To investigate associations of IFX and anti-IFX Ab with clinical response to IFX maintenance therapy. In addition, to establish cut-off levels to optimally distinguish response types.

STUDY III

To investigate variations of anti-IFX Ab over time including clinical implications hereof and importance of timing of measurements when evaluating efficacy of IFX maintenance therapy.

STUDY IV

To investigate etiology and risk factors of acute severe infusion reactions to IFX including the role of immunogenicity.

MATERIAL AND METHODS
STUDY DESIGNS AND POPULATIONS
Study I

Study I was an experimental study comparing basic analytical properties of RIA for detection of IFX and anti-IFX Ab with commonly used ELISA techniques, and further with those of a recently developed cell-based reporter gene assay (RGA), and a new enzyme immunoassay (EIA) for anti-IFX Ab. This study included 13 anti-TNF naive patients with inactive CD and six CD patients with anti-IFX Ab as described in detail below.

Study II-IV

Study II-IV were observational, retrospective, cohort studies of all CD patients treated with IFX at the Department of Gastroenterology on Herlev Hospital until ultimo 2010.145 Study II included patients (n=85) in whom IFX and/or anti-IFX Ab had been determined during IFX maintenance therapy, and with a well-defined clinical response as described below. Study III included patients (n=75) who had developed anti-IFX Ab and in whom anti-IFX Ab had been reassessed at least once at a later time point. Study IV included patients (n=25) who had experienced an acute severe infusion reaction to IFX. Patients were identified by review of files

of all patients treated with IFX during the investigated time period. IFX and anti-IFX Ab analyses were done by RIA.

CLINICAL CLASSIFICATION OF PATIENTS
Study I

Inactive CD was defined as Harvey-Bradshaw score < 5 and without fistula activity or use of oral corticoid steroids within the last 3 months from sampling.(85;146;147)

Study II-IV

Due to the retrospective nature of study II-IV, classification of clinical response to IFX was based on the treating senior gastroenterologist's global evaluation of symptoms and findings (clinical, biochemical, diagnostic procedures etc.) as noted in the patient files, and in accordance with clinical practice and previous studies.(42;148)

Clinical outcome of IFX maintenance therapy (study II) was classified as loss of response, defined as a an initial favorable response to IFX induction and maintenance therapy (i.e. minimum one IFX infusion 8 weeks after the induction series) with clinical remission and no symptoms or findings indicating active disease; and later followed by loss of clinical response with active disease despite dose optimization and finally resulting in discontinuation of IFX. Conversely, maintained remission was defined as a favorable clinical response to IFX induction and followed by a continued clinical response to maintenance therapy with complete clinical remission at time of follow-up.(28;87;119)

Clinical outcome in patients in whom IFX maintenance therapy had been continued despite previous anti-IFX Ab detection (study III) was classified as no clinical response to the ongoing IFX treatment with no improvement of symptoms and findings of active disease; alternatively, as clinical response to the ongoing IFX treatment with complete or partial clinical remission.(149)

Acute severe infusion reactions (study IV) were defined as reactions occurring during IFX infusion which were judged severe by the treating physician, and resulting in immediate and permanent discontinuation of IFX and symptomatic treatment with antihistamines and/or hydrocortisone, and if necessary, epinephrine.(127;133;150)

SAMPLES

Blood samples for IFX and anti-IFX Ab analyses were obtained as trough levels 30 minutes prior to IFX infusion with exception of samples obtained after infusion reactions. Samples were analyzed by Biomonitor A/S (Copenhagen, DK). Test results were calculated as means of duplicate assessments, and samples were retested if the difference was > 20%. All patients routinely received hydrocortisone (100 mg iv), cetrizine (10 mg orally), and acetaminophen (1 g orally), prior to all IFX administrations.(133)

Study I

Blood samples for IFX analyses were obtained from 13 anti-TNF naive patients with inactive CD.(145) IFX (MSD, Ballerup, DK) was added to pooled serum to yield final IFX concentrations of 0, 1, 3, and 9 µg/ml for intra-day testing, and 0 and 3 µg/ml for between-days testing. IFX was also added to each patient's serum to yield a final concentration of 0 and 3 µg/ml for inter-individual testings. IFX measurements were repeated six times on the same day (intra-day assessment), once on six separate days (between-day assessment), or once in each individual (inter-individual assessment), to determine limit of detection (mean background + 3 standard deviations (SD)), reproducibility (coefficient of variation,

(CV%)), inaccuracy (% of expected, bias), correlation, and agreement.

Anti-IFX Ab were determined six times on the same day in CD patients with low, intermediate, and high titer levels respectively (intra-day assessment), or once on six separate days in different CD patients with low, intermediate, and high titer levels respectively (between-day assessment), to determine relative sensitivity, reproducibility, correlation, and agreement.(145;151) Identification of patients with anti-IFX Ab was based on initial screenings using RIA, and followed by randomly selection of six patients to represent different titer levels.

Study II
Results of previously analyzed relevant serum levels of IFX and anti-IFX Ab were obtained from patient files. Timing of sampling in patients with loss of response corresponded trough levels as samples were collected at the time where the patient should have received the next scheduled IFX infusion given the treatment had been continued. Sampling was done at random time points during the maintenance phase in patients with maintained remission. All tests had been done at the discretion of the treating physician.

Study III
Patients who had previously been tested positive for anti-IFX Ab were identified from patient files. These patients were then reassessed for anti-IFX Ab at time of this study, unless a repeat anti-IFX Ab measurement had already been collected routinely by the treating physician. Samples for reassessments were obtained from the department's biobank, in which blood samples were routinely stored from all patients prior to IFX infusions and following an infusion reaction from May 2009 and onward. Before May 2009 samples were obtained sporadically as decided by the treating physician. Patients were asked to give a blood sample at time of study in case of absence of relevant biobank samples.

Study IV
Test results of anti-IFX Ab measurements before initiation of a new IFX treatment series and/or after an acute severe infusion reaction were identified from patient files. Additional relevant samples for anti-IFX Ab tests were obtained from the biobank as described above, and from samples collected at time of this study.

BLINDING
Sample analyses at Biomonitor A/S were done under blinded conditions and without knowledge of efficacy and safety of IFX therapy. Samples in study I were also blinded for concentration of IFX, and for previous level of detected anti-IFX Ab. The treating physician was blinded for results of measurements of IFX and anti-IFX Ab in study II. Classification of clinical response type in study II-IV was done without knowledge of IFX and anti-IFX Ab test results.

ASSAYS FOR IFX AND ANTI-IFX AB
Fluid phase RIA for IFX
Serum was incubated with ^{125}I-TNF-α (Perkin Elmer, Waltham, MA, USA), and after separation of free and IgG-bound tracer using rabbit anti-human Fcγ Ab (Dako, Copenhagen, DK), the pellet activity was measured using a γ-counter (Wallac, Alleroed, DK). Serum concentration of IFX was determined as the TNF-α binding capacity, which was expressed as the equivalent activity of ^{125}I-

TNF-α binding to a reference IFX solution (MSD).(27;119;128;142;152)

Fluid phase RIA for anti-IFX Ab
IFX is a monoclonal Ab which consists solely of κ light chains. The RIA assay used anti-human λ light-chain Ab to distinguish between free ^{125}I-IFX and ^{125}I-IFX in complex with any class of λ-containing human immunoglobulin.(27;119;128;142;152) Thus, serum was incubated with ^{125}I-IFX, and pellet activity was determined after precipitation of immunoglobulin-bound tracer with rabbit anti-human immunoglobulin λ-chain Ab (Dako). Anti-IFX Ab concentrations were generally (study II-IV) expressed as arbitrary laboratory units (U) per ml, where < 10 U/ml was considered negative (i.e. detection limit of the assay). However, all assays for anti-IFX Ab in study I measured anti-IFX Ab concentrations as titers, using variable concentrations of serum supplemented with normal human serum (NHS) to yield a constant 1% final concentration of human serum. A common readout point was in this study used in order to facilitate inter-assay comparisons. This readout point was in each assay (RIA, ELISA, EIA, RGA) defined as the individual assay's mean background activity in medium + 10 SD. Cross reactivity between anti-IFX Ab and ADL was tested in study IV by co-incubation of ^{125}I-IFX and sample as above with and without IFX at 25 μg/ml and ADL at 50 μg/ml, respectively. The limit for positive ADL cross binding was set to 20% of the displaced ^{125}I-IFX obtained by the addition of IFX.(27)

Fluid phase RIA for anti-IFX IgE Ab
Serum in study IV was first depleted of IgE by co-incubation with anti-IgE monoclonal Ab coupled to paramagnetic beads.(153) Then, ^{125}I-IFX was added, and the amount of labeled ligand bound to IgE was determined using a γ-counter (Wallac). Beads were sampled at intervals during the experiment and assessed with anti-Betula verrucosa IgE positive serum and ^{125}I-Betula verrucosa allergen no. 1 (i.e. the major allergen in pollen of birch) to include a positive control.

Solid phase capture ELISA for IFX
Capture ELISA for IFX was carried out as described by Ternant et al.(154) Titer plates were first coated with recombinant human TNF-α (R&D Systems, Minneapolis, MN, USA) followed by addition of serum samples. Rabbit anti-human IgG conjugated with horse-radish peroxidase (HRP) (Dako) was added in order to detect IFX bound to the coated TNF-α. Sample IFX levels were determined by addition of TMB One component HRP microwell substrate (BioFX Laboratories, Owings Mills, MD, USA) and assessment of the color reactions on a spectrophotometer.

Solid phase bridging ELISA for anti-IFX Ab
This double-antigen ELISA assay was constructed as previously described.(117;118;126) Briefly, titer plates were first coated with IFX (MSD) followed by incubation of variable patient serum concentrations supplemented with pooled NHS to yield a constant 10% final concentration of human serum. Then biotinylated IFX was added as detecting Ab, and HRP-conjugated streptavidin (Thermo Scientific, Copenhagen, DK) were added together with TMB substrate before final spectrophotometric analysis.

Solid phase EIA for anti-IFX Ab
This EIA measured binding of IFX to patient IgG pre-absorbed to protein G as previously outlined.(140) In short, titer plates were coated with protein G (Thermo Scientific) which is known to bind selectively to the Fc portion of IgG.(155) Patient serum samples

were added at variable concentrations supplemented with pooled NHS to yield a constant 1% final concentration of human serum, followed by successive addition of biotinylated IFX, HRP-conjugated streptavidin, and TMB substrate. Anti-IFX Ab titers were determined by spectrophotometric analysis. To selectively detect anti-IFX Ab of subclass IgG4, a mouse monoclonal Ab to human IgG4 (AbD Serotec, purchased from Trikem, Skanderborg, DK) was used instead of protein G as coating.

Cell based RGA for IFX and anti-IFX Ab

The RGA for IFX and anti-IFX Ab was based on the iLite™ TNF-α reporter cell line (Biomonitor A/S) originating from human erythroleukemic K562 cells transfected with an NFκB-regulated Firefly Luciferase reporter-gene construct, as detailed in the reference.(156) Briefly, this RGA measures specifically TNF-α- and TNF-β-induced Firefly Luciferase activity. Furthermore, the activity of Firefly Luciferase is normalized relative to another engineered construct encoding Renilla Luciferase activity, which is constitutively expressed by these cells. This makes the TNF-activity measurements less dependent on the number of viable cells and, hence, of putative toxic factors in serum. Firefly Luciferase and Renilla Luciferase activities were determined by luminescence (Promega, Fitchburg, WI, USA). IFX concentrations in serum samples were determined from residual TNF-α activity, and by interpolation on a standard curve of Firefly Luciferase relative to Renilla Luciferase activity obtained by titration of increasing concentrations of IFX against a fixed concentration of human TNF-α (2.0 ng/ml). Anti-IFX Ab titers were determined from residual sample TNF-α activity (as above) using variable concentrations of patient sera supplemented with NHS to yield a constant 20% final concentration of human serum, and mixed with an equal volume of IFX and TNF-α.

BIOCHEMICAL PARAMETERS

Biochemical markers of disease activity were assessed to support clinical classification of patients in study II and III.(157-159) Biochemical tests of blood samples were routinely done at the Department of Clinical Biochemistry at Herlev Hospital (DK). CRP and albumin concentrations were measured by immunoturbidimetry (Konelab Prime 60i, Thermo Scientific). Hemoglobin-, platelet-, and white blood cell (WBC) concentrations were measured by spectrophotometry (Advia 2120, Siemens, DE).

STATISTICS

Fisher's exact test or Chi-squared test as appropriate was used for univariate analysis of discrete variables. Non-parametric tests were used for univariate analysis of continuous variables, except for assessment of biochemical parameters where parametric tests were applied under the assumption of data being normally distributed. Levene's test was used to assess variance homogeneity. Correlations of assays in study I were investigated using linear correlation analysis (Pearson correlation coefficient, R^2) and followed by linear regression analysis in case of significance, or non-linear correlation analysis (Spearman's rank correlation coefficient, r_s) in case of lack of significance. Receiver operating characteristics (ROC) analysis was used to establish cut-off levels for IFX and anti-IFX Ab associated with outcome of IFX maintenance therapy in study II. Multivariable logistic regression analysis in study III and IV included parameters with $p < 0.2$ as identified in univariate analysis. Survival analysis in study III and IV was estimated using Kaplan-Meier statistics. Log-rank test and Cox proportional hazard regression analysis was used to compare survival curves. P-values were two sided and $p < 0.05$ was considered significant. Analyses were done in SPSS version 18 (IBM, NY, USA) and in GraphPad Prism version 5 (GraphPad Software, CA, USA).

ETHICS

All studies were approved by the regional ethics committee (H-D-2009-055) and by the Danish Data Protection Agency (2009-41-3479).

RESULTS

STUDY I

Assays for IFX

Limit of detection was lowest in RIA (0.07 µg/ml), followed by RGA (0.13 µg/ml), and ELISA (0.26 µg/ml). Reproducibilities were generally comparable and all were ≤ 20%. Between-days reproducibility of RIA (8%) was, however, superior to that of ELISA (12%, p<0.05) and RGA (20%, p<0.01). Maximal inaccuracy was 23% in ELISA, 39% in RIA, and 24% in RGA. All assays generally underestimated the concentration of IFX in serum. As shown in Figure 1, there were highly significant linear correlations between ELISA and RIA (R^2=0.98, p=0.001), ELISA and RGA (R^2=0.99, p<0.001), and RIA and RGA (R^2=0.97, p=0.002). However, assays disagreed on absolute IFX sample concentrations by up to 1.55

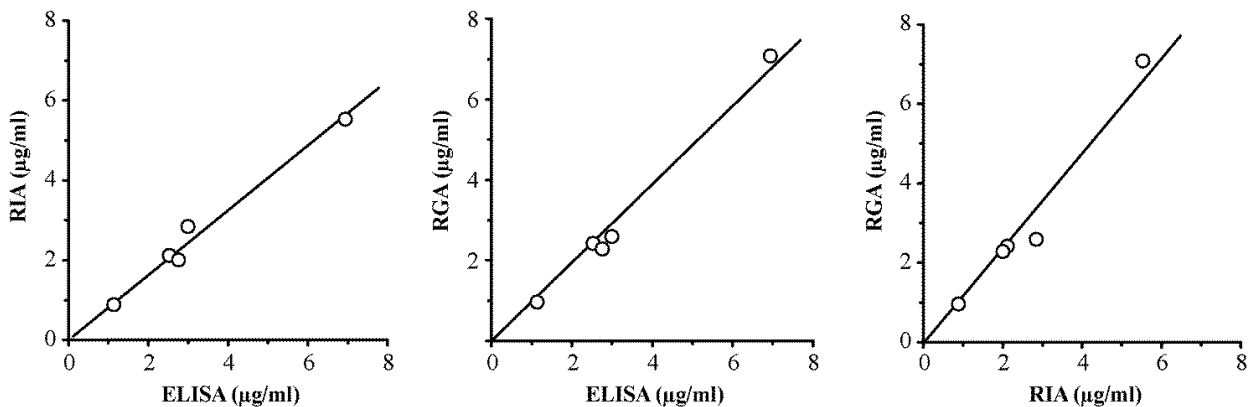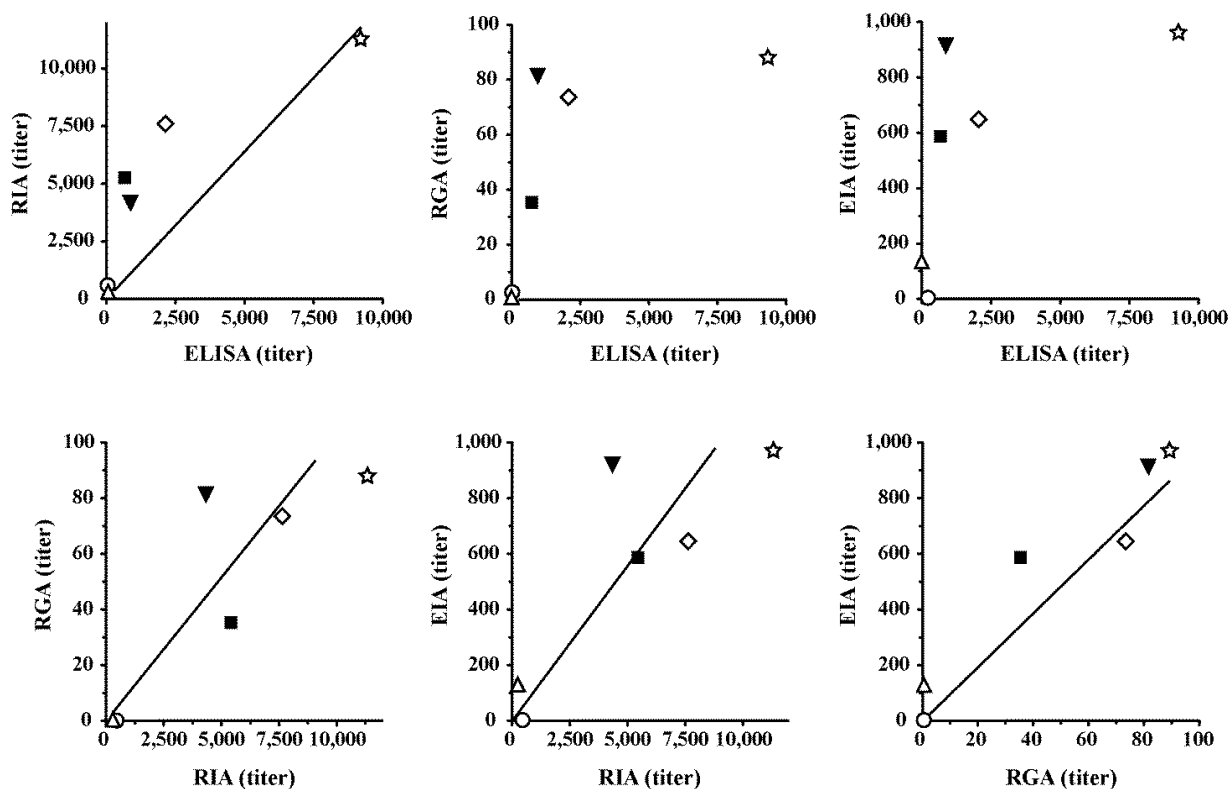**Figure 1**
Mean IFX concentrations of repeated measurements in sera with drug levels of 1, 3, and 9 µg/ml assessed by RIA, ELISA, and RGA. Linear regression lines are shown.

Figure 2
Mean anti-IFX Ab titers of repeated measurements in sera from 6 different patient sera (unique symbols). Linear regression lines are shown.

µg/ml for RIA and RGA, 1.41 µg/ml for ELISA and RIA, and 0.48 µg/ml for ELISA and RGA (p<0.05).

Assays for anti-IFX Ab
The most sensitive assay was RIA (titers median 118-fold higher than in RGA), followed by ELISA (titers median 24-fold higher than in RGA), EIA (titers median 11-fold higher than in RGA), and RGA. RGA gave highly reproducible results (≤ 7%) compared to all others (24-26%, p<0.05). As shown in Figure 2, there was a linear correlation between 4 of the 6 pairs of assays: ELISA and RIA (R^2=0.73, p=0.03), RIA and RGA (R^2=0.75, p=0.03), RIA and EIA (R^2=0.71, p=0.04), and RGA and EIA (R^2=0.93, p<0.01). A non-linear correlation was observed in the remaining 2 of the 6 pairs

of assays: ELISA and RGA (r_s=0.93, p=0.02), and ELISA and EIA (r_s=0.89, p=0.03). This was at least partly due to inability of bridging ELISA to detect anti-IFX IgG4 Ab. Thus, 2 of the 6 sera contained considerable amounts of IgG4 anti-IFX Ab (Figure 2-3, black symbols), and ELISA of these sera revealed only low titers most likely representing non-IgG4 anti-IFX Ab. All assays disagreed on absolute anti-IFX Ab titers with mean difference -500 [-900– -100] in RGA and EIA, and up to 4,500 [600–8,400] in RIA and RGA. A contributing factor to these discrepancies was inability of ELISA to detect IgG4 Ab (Figure 3).

Conclusion
Basic analytical properties of RIA, ELISA, EIA, and RGA for detec-
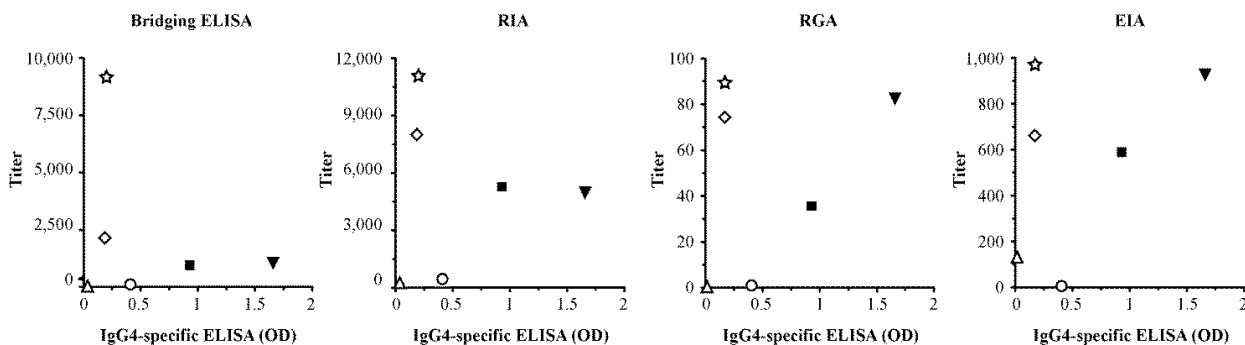
Figure 3
Anti-IFX IgG4 Ab in the 6 sera visualized in Figure 2 as compared with total anti-IFX Ab levels as measured by each type of assay. Same patient-specific symbols as in Figure 2, and with solid symbols for sera with high IgG4 fractions.

tion of IFX and anti-IFX Ab appeared overall comparable. Assays generally agreed on trends, but serum factors and/or matrix effects interfered differently, and ELISA was unable to detect functionally monovalent IgG4 anti-IFX Ab. Of note, exact IFX and anti-IFX Ab concentrations were not comparable between assays, and clinically relevant levels should therefore be determined individually for each type of assay. Accordingly, study II not only explored associations of IFX and anti-IFX Ab as measured by RIA, with clinical efficacy of IFX maintenance therapy; but further established clinically relevant cut-off levels in RIA which associated with response types to IFX maintenance therapy.

STUDY II
Classification of patients
Classification of clinical outcome of IFX maintenance therapy was based on the treating physicians' global evaluation and supported

by biochemical parameters as shown in Figure 4 (data not previously presented). At time of IFX initiation, levels of biochemical parameters were similar among patients with loss of response and maintained remission (p>0.05), and mean CRP and WBC levels were above normal (i.e. CRP <10 mg/l and WBC 3-9 $\times 10^9$/l). At time of follow-up, levels of CRP, WBC, and platelets had decreased significantly, and albumin levels had increased significantly, in individual patients who maintained remission. The corresponding parameters remained largely unchanged in patients with loss of response. Mean CRP and WBC were within the normal ranges at time of follow-up in patients who maintained remission, but not in the others.
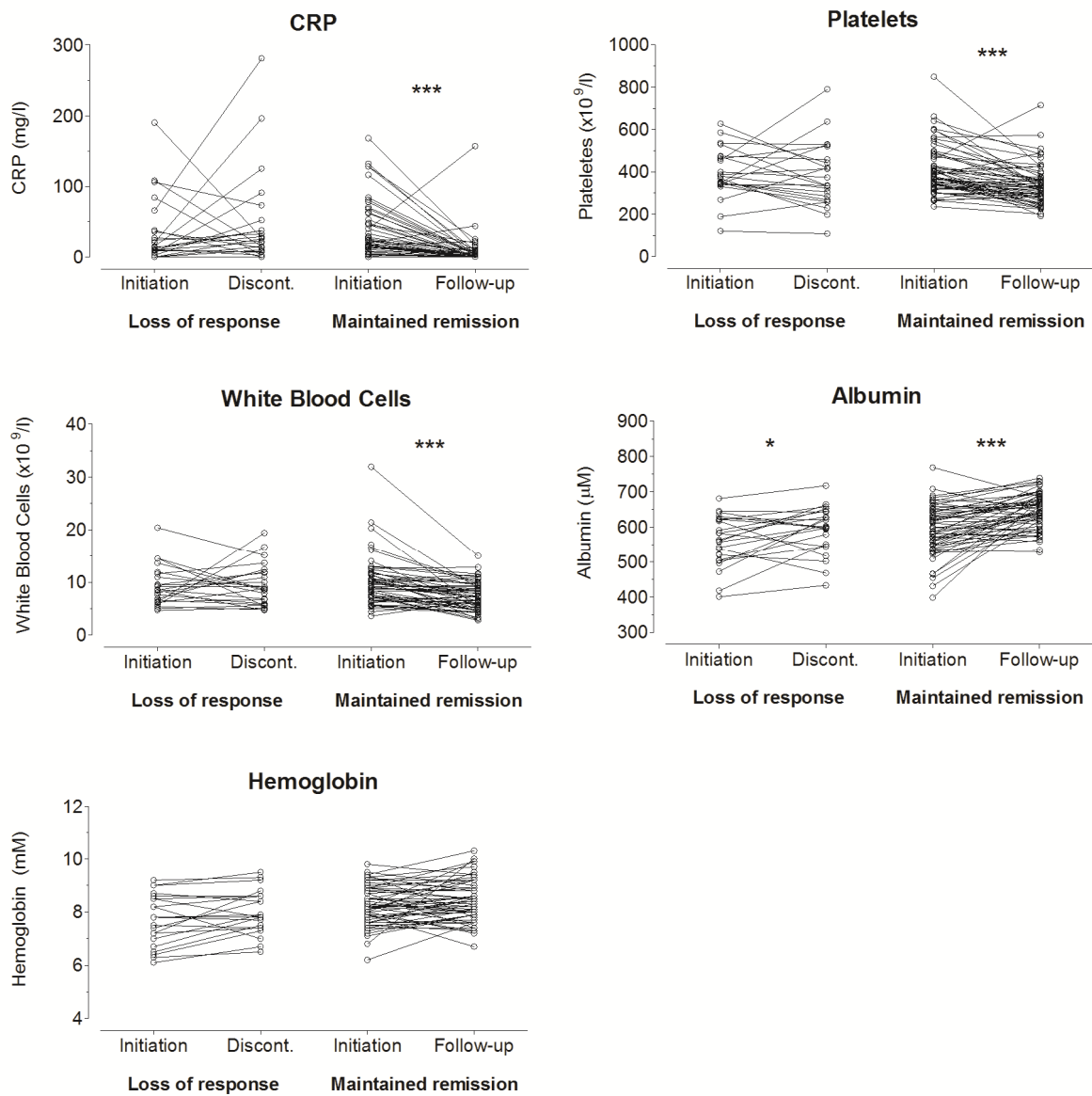
Figure 4
Biochemical markers of disease activity in patients with loss of response (n=24), and maintained remission (n=56) to IFX therapy at time of IFX initiation and at discontinuation/ follow-up, respectively (samples unavailable from 2 patients with loss of response, and 3 patients with maintained remission). * p<0.05; ** p<0.01; *** p<0.001

IFX levels and clinical efficacy

IFX trough levels were significantly higher in patients with maintained remission (median 2.8 µg/ml, IQR 0.8-5.3, n=48) compared to those with loss of response to IFX maintenance therapy (median 0 µg/ml, IQR 0-0, n=21; p<0.0001). An IFX concentration of 0.5 µg/ml provided an optimal cut-off value for discrimination between these groups of patients as defined by a minimal difference between sensitivity and specificity (Table 1).160 Thus, 73% [52-88] of patients with IFX trough levels <0.5 µg/ml had loss of response to IFX maintenance treatment (n=19), while 95% [83-99] with IFX ≥0,5 µg/ml had maintained remission (n=41).(161)

Anti-IFX Ab levels and clinical efficacy

Anti-IFX Ab trough levels were significantly higher in patients with loss of response to IFX maintenance therapy (median 35 U/ml, IQR 12-76, n=26) compared to those with maintained remission (median 0 U/ml, IQR 0-0, n=59; p<0.0001). Detectable anti-IFX Ab (i.e. 10 U/ml) provided an optimal cut-off value for discrimination between patients as defined above (Table 1).(160) Thus, 78% [57-91] of patients with anti-IFX Ab ≥10 U/ml had loss of response (n=21), while 91% [80-97] with undetectable anti-IFX Ab had maintained remission (n=53).(161)

Combined IFX and anti-IFX Ab and clinical efficacy

Combined measurements of IFX and anti-IFX Ab and the use of the above cut-off values for identification of patients with loss of response (i.e. combined test was regarded positive if both tests were positive otherwise considered negative) increased accuracy and specificity (Table 1). Thus, 85% [61-96] with IFX <0.5 µg/ml and detectable anti-IFX Ab had loss of response to IFX maintenance treatment (n=17), while 92% [80-97] with IFX ≥0.5 µg/ml and/or undetectable anti-IFX Ab had maintained response (n=45).

Table 1
Cut-off values for classification of response to IFX

|	Sensitivity	Specificity	AUC^ROC	Accuracy
IFX < 0.5 µg/ml	86% [64-97]	85% [72-94]	0.93 [0.85-1.00]	87%
Anti-IFX Ab ≥ 10 U/ml	81% [61-93]	90% [79-96]	0.89 [0.79-0.98]	87%
Combined: IFX < 0.5 and anti-IFX Ab ≥ 10	81% [57-94]	94% [82-98]	NA	90%

Conclusion

IFX and anti-IFX Ab measured by RIA strongly associated with clinical response types to IFX maintenance therapy. Cut-off values providing optimal discrimination of patients with loss of response or maintained remission were established. Combined assessments of IFX and anti-IFX Ab using the established cut-offs appeared effective for identifying patients with loss of response. Monitoring IFX maintenance therapy by assessments of serum IFX and anti-IFX Ab levels by RIA appeared promising for evaluating and optimizing therapy in CD.

STUDY III
### Anti-IFX Ab variations during ongoing IFX maintenance therapy

The association between anti-IFX Ab and loss of response to IFX maintenance therapy has only recently been acknowledged.(23;75;122;152;162) Accordingly, some patients in our cohort had continued IFX treatment based on an overall clinical judgement by the treating senior physician despite incomplete response and development of anti-IFX Ab. Anti-IFX Ab appeared

functionally active both at the initial detection (not shown) and at reassessment (Figure 5) because the corresponding IFX levels were undetectable or low.
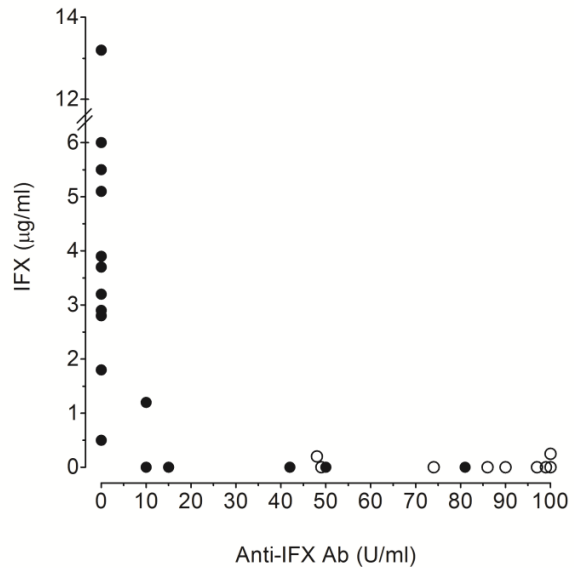
Figure 5
IFX and anti-IFX Ab trough concentrations at time of anti-IFX Ab reassessment during the IFX maintenance phase in patients with previous positive anti-IFX Ab detection. Black dots denotes patients with response (n=17), and white dots denotes patient without response (n=9) to continued IFX therapy.

Anti-IFX Ab had disappeared at reassessment in 11 of 17 patients (65%) with a beneficial clinical response (9 complete and 8 partial) to continued IFX maintenance therapy after a median of 4 [3-5] infusions (Figure 5-6). In contrast, anti-IFX Ab persisted in all 9 patients without clinical response to continued IFX therapy, and concentration had increased from median 27 U/ml, IQR 17-76 at first assessment to 90 U/ml, IQR 62-100 at reassessment, p<0.001. Disappearance was independent of initial anti-IFX Ab titres, IFX dose escalation, and use of concomitant immunosuppression. Biochemical markers of disease activity supported the clinical classification as CRP was significantly higher, albumin significantly lower, and hemoglobin tended to be lower among patients with no response to continued IFX (not shown).
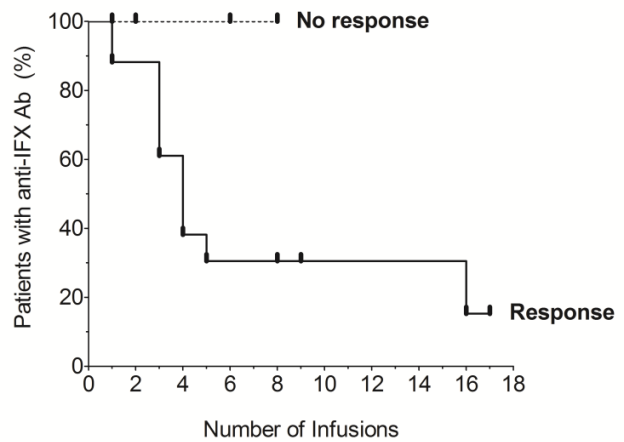
Figure 6
Number of infusions from detection of anti-IFX Ab until disappearance during continued IFX maintenance therapy according to clinical response type. A drop on the Y-axis indicates disappearance of anti-IFX Ab. Vertical bars are censored cases.

Anti-IFX Ab variations after IFX discontinuation

Persistence of anti-IFX Ab at reinitation after a drug pause may have clinical implications for efficacy and safety, but little is known about variations of anti-IFX Ab after discontinuation of therapy. Hence, our cohort of 56 patients with detectable anti-IFX Ab at time of IFX discontinuation were reassessed for anti-IFX Ab at later time points. As shown in Figure 7, the proportion of patients with anti-IFX Ab gradually declined over the years, and with a fraction still having anti-IFX Ab after 3 years. No variables, including Ab concentration at baseline, were associated with anti-IFX Ab disappearance in statistical multivariable analysis.
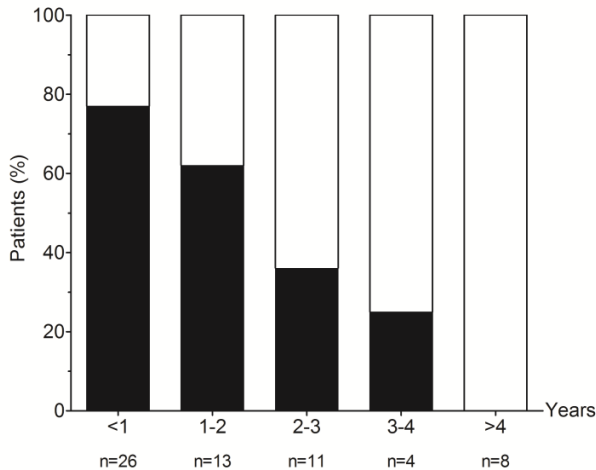
Figure 7
Time until disappearance of anti-IFX Ab in patients who had discontinued IFX shortly after detection of anti-IFX Ab. Black columns denote patients with persisting anti-IFX Ab, and white columns denote patients with undetectable anti-IFX Ab.

Conclusion

Clinical response to ongoing IFX therapy should be taken into account when evaluating the importance of a single positive anti-IFX Ab assessment. Measurements should preferably be repeated at a later infusion in case of an ill-defined clinical response type. The main clinical implication of these findings is that continuation of therapy may be considered in patients with clinical response as anti-IFX Ab later disappear in two thirds of these patients for yet unknown reasons. Anti-IFX Ab can persist for years after discontinuation, and this should be considered at reinitiation.

STUDY IV
Characteristics of acute severe infusion reactions

Twenty-five of the 315 IBD patients (8%) who had received IFX at our center had experienced an acute severe infusion reaction to IFX as judged by the treating physician. IFX was immediately and permanently discontinued in all of these patients, and symptomatic treatment consisted of iv antihistamine (n=22), hydrocortisone (n=6), and/or epinephrine (n=2). Clinical manifestations of reactions as noted in the patient files were acute severe malaise (100%), severe dyspnoea (60%), chest pain (44%), nausea (36%), universal erythema (32%), tachycardia (24%), chills and perspiration (24%), dizziness (16%), and hypotension (12%).

Risk factors

Episodic therapy with reinitiation of IFX after minimum 3 months of drug pause was the only variable associated with reactions in a multivariate model, OR 4.9 [1.9-12.5]. The risk increased if IFX was reinitiated after 4 months (5.5 [2.2-14.1]) or 6 months (7.7

[3.0-19.5]). The proportion of reactions at the second infusion during the second IFX series was significantly higher than at all other infusion time points (p<0.01). In contrast, no infusion time points were associated with increased proportion of reactions among continuously treated patients (p=0.66).

Role of immunogenicity in acute severe infusion reactions

To systematically address the role of immunogenicity in acute severe infusion reactions in individual patients, we measured anti-IFX IgG and IgE Ab after these reactions (n=20) as well as prior to reinitiation of IFX after drug pauses (n=11 out of 18 reactions having occurred during episodic therapy). Samples obtained after reactions were generally collected within one hour (n=14), while a few were obtained up to nine days later (n=6). Anti-IFX IgE Ab were negative in all patients. As false negative results can occur if anti-IFX IgE Ab are bound to IFX at the time of testing, we measured anti-IFX IgE Ab also at multiple time points in 15 patients (median number of assessments 3, IQR 2-4; IQR 7 days before reaction to 35 days after reaction). Again, all tests were negative.

In contrast, a clear association between reactions and high levels of circulating anti-IFX IgG Ab measured shortly after the reaction was observed in individual patients. Thus, 19 of 20 patients (95%) were anti-IFX IgG Ab positive after reactions (Figure 8). Concentrations of anti-IFX Ab were generally higher (median 84 U/ml, IQR 30-100, n=20) than at time of loss of response as observed in study II (median 35 U/ml, IQR 12-76 U/ml, n=26; p<0.05). In the majority (7 of 11 patients) of those patients tested prior to IFX reinitiation, anti-IFX IgG Ab were negative despite development of acute severe infusion reactions in the reinitiation series (Figure 8).
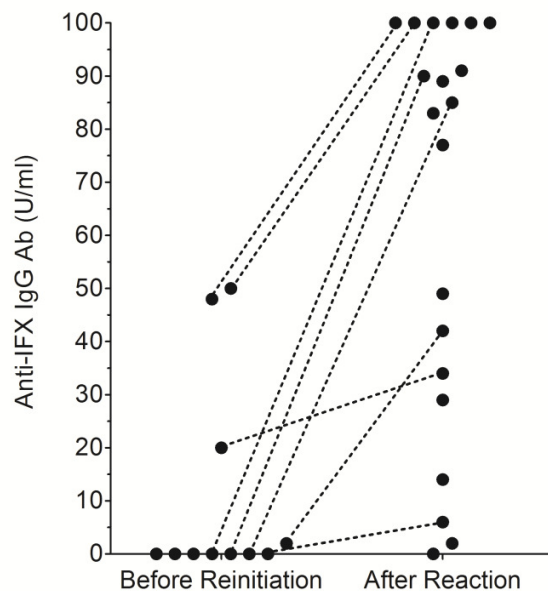
Figure 8
Anti-IFX IgG Ab in individual patients before reinitiation of IFX (n=11) and after acute severe infusion reactions (n=20). Samples from the same patients are connected by dotted lines. Assay sensitivity was increased to detect very low levels of Ab also below 10 U/ml.

All 19 patients with detectable anti-IFX Ab after the acute severe infusion reactions tested negative for anti-IFX Ab cross reactivity with ADL, indicating that switching to ADL following a reac-

tion would be safe. Seventeen patients in fact later received ADL (median duration of treatment 177 days, IQR 56-770), which was tolerated by all, except one. This patient developed a delayed hypersensitivity reaction most likely caused by anti-ADL Ab; however, pre-existing low titers of cross reacting anti-IFX Ab (below the detection limit) cannot be ruled out.(163)

Conclusion
The vast majority of acute severe infusion reactions to IFX appear not to be true IgE-mediated anaphylactic reactions, but rather associated with development of anti-IFX IgG Ab. Risk of reaction is relatively high during episodic therapy, and especially at the second infusion in the second series; but absence of anti-IFX Ab prior to a reinitiation series cannot be taken as evidence that retreatment is safe. Analogous to conditions in patients without reactions,(26;27;128;164) pre-existing anti-IFX Ab after reactions did not cross react immunologically with ADL, and routine investigation hereof appears unnecessary.

DISCUSSION
Anti-TNF biopharmaceuticals have introduced a new paradigm in the management of CD, where a specific immunological disease pathway is modulated to decrease intestinal inflammation.(9) The result has been dramatic by providing markedly better outcomes for patients refractory to, dependent on, or intolerant of conventional medical therapies.(3-6;44) In this era of TNF-inhibitors, treatment goals have optimistically been extended from symptom control to include persistence of clinical remission with mucosal and fistula healing; and avoidance of surgery and hospitalizations.(42;48) However, application of TNF-inhibitors have confronted clinicians with novel challenges such as how to determine optimal treatment strategies in patients with therapeutic failure.(7;42;75) Although current recommendations of dose optimization or switching to an alternative anti-TNF drug may prove effective, a more rational approach which preferably takes the underlying mechanisms into account, is warranted to allow for safer and more effective therapies with better cost-effectiveness.(23;139;165) Hence, TNF-inhibitors are extremely expensive with an estimated total cost of 1.2 billion DKR in 2011 in Denmark of which approximately 350 million DKR was used for IFX and 480 million for ADL.(166) Accumulating evidence indicate, that the biologic nature of TNF-inhibitors with variable drug bioavailability and immunogenicity in individual patients, may play an essential role in therapeutic failures.(7;123;124) Accordingly, and in the case of IFX in particular, measurements of serum levels of drug and anti-drug Ab are increasingly recognized as important parameters to aid in optimizing therapy.(139;165) However, investigations of the clinical utility of measuring IFX and anti-IFX Ab are still limited and with inconsistent reportings of associations with treatment outcomes. It remains yet unresolved exactly how these analyses should be interpreted and implemented in a clinical context.(42;75;122-124;139)

ASSESSMENTS OF IFX BIOAVAILABILITY AND IMMUNOGENICITY
Technical challenges
An important aspect of serum IFX and anti-IFX Ab assessments to monitor therapy in individual patients is the ability of assays to accurately and reliably measure levels of bioactive IFX with TNF-α neutralizing capacity, and functionally active anti-IFX Ab which alter the bioavailability and/or pharmacokinetics of IFX.(37;167-169) There are currently no defined gold standard assays for this purpose, and available data are characterized by the use of a large number of non-standardized techniques.(37;75;122;167;168;170) Detections are generally impeded by the fact that the drug (IFX) is an immunoglobulin in itself, and by the complexity of measuring Ab against Ab.(144) Anti-IFX Ab are predominantly of the IgG isotype,(27;143) and can be neutralizing or non-neutralizing.(25;37;169) Neutralizing anti-IFX Ab are believed to constitute the most serious problem because they directly block the interaction between IFX and TNF-α, and increase drug clearance.(25-28;37;132;144;171) However, non-neutralizing anti-IFX Ab may also have clinical impact and prevent IFX from reaching the inflamed gut due to increased drug clearance by immune complex formation in serum followed by rapid removal from the circulation with or without involvement of the complement system, mainly by splenic removal, by endothelial impact, and by binding to Fcγ receptors on immune effector cells.(23;25;37;169;171) Currently applied binding assays for anti-IFX Ab (e.g. RIA, ELISA, and EIA used in study I) do not distinguish between neutralizing and non-neutralizing anti-IFX Ab;(25;167;169) whereas cell based bioassays (e.g. RGA used in study I) measure only the neutralizing fraction of anti-IFX Ab.(167;169) The precise clinical relevance of these technicalities is incompletely understood, and may also vary between individuals according to the specific nature of the immunological reaction to the drug. However, simultaneous determination of serum levels of IFX and anti-IFX Ab is necessary to adequately evaluate if detected anti-IFX Ab are functionally active, and result in low IFX; and to determine if low IFX is caused by immunogenicity or by non-immunological mechanisms.(143;167;169;172)

ELISA
Clinical investigations in CD have so far primarily been carried out with different types of solid phase capture ELISAs for IFX,(70;117;154) and with bridging ELISAs for anti-IFX Ab.(117;118;126) Cell-based RGA,(156) homogeneous mobility shift assay,(173-175) fluid phase EIA,(121) western blotting,(28) and chromatography(176) have also been used but is less well characterized. ELISAs are relatively simple and easy to use; and a number of in house assays with variable designs have been applied.(7;122;143) However, ELISAs have several notable limitations.(7;75;122;124;139) Thus, sensitivities and specificities are generally low, and it is unknown if detected IFX and anti-IFX Ab are functionally active.(23;27;144;167) False negative IFX and anti-IFX Ab test results may arise from the matrix effects encountered in solid phase assays due to e.g. epitope masking because of protein aggregation. Further, capture ELISA for anti-IFX Ab may not detect anti-idiotypic Ab because idiotopes (constituting the TNF-α binding sites on IFX) are masked by TNF-α on the solid surface.(27;29;143;177) Most importantly, however, false negative findings may arise from the presence of IFX in the serum, as this interferes with detection of anti-IFX Ab, particularly in bridging ELISA.(144;177) Anti-IFX Ab status is therefore reported as inconclusive by investigators using this ELISA if testing is negative and IFX is detectable.(75;126) This is the case in about half the patients in clinical trials, and use of ELISA for anti-IFX Ab detection therefore introduces severe bias and counteracts attempts to draw useful conclusions on the therapeutic relevance of anti-drug Ab.(118;125;136) This is further substantiated by the fact that ELISAs are also prone to report false positive findings for example from neoepitope formation and from non-specific binding of low affinity Ab, including heterophilic Ab, and also from rheumatoid factors and/or activated complement which may cross-bind IgG Fc fractions in bridging ELISA.(23;27;141;143;178)

RIA

This PhD thesis hypothesized that novel techniques based on RIA for measuring IFX and anti-IFX Ab would favorably assist in evaluating and optimizing efficacy and safety of IFX maintenance therapy as the results of these assays have correlated well with clinical outcomes in patients with both rheumatoid arthritis and CD;(27;119;128;142) and because they have important technical advantages over commonly used ELISAs. Hence, reactions take place in fluid phase and mimic the actual conditions in vivo, and assays are less influenced by artifacts encountered in solid phase binding assays as mentioned above. The RIA for IFX measures TNF-α binding capacity which relates to the functional TNF-neutralizing effect of IFX. Importantly, the RIA for anti-IFX Ab detects all isotypes of immunoglobulins, including all IgG subclasses binding to IFX, and with low drug sensitivity.(23;29;128;140;179) Limitations include potential lack of detection of anti-IFX Ab bound to IFX, and inability to recognize κ light-chain anti-IFX Ab. However, anti-IFX IgG Ab express κ- and λ light-chains at a constant ratio, and binding avidities are largely independent of the light-chain isotype.(27;128;140) RIA requires advanced laboratory facilities, but the assays are now commercially available (Biomonitor A/S) and with acceptable response times for clinical usage (test results within 2 weeks).

Evaluation of assays

Study I compared analytical properties of RIA for detection of IFX and anti-IFX Ab with those of two solid phase binding assays (ELISA and EIA), and one cell based bioassay (RGA), in order to identify assays with potential technical benefits. Characteristics of assays were found to be generally comparable, but RIA had a few important advantages, which included a high sensitivity for detection of even small quantities of IFX and anti-IFX Ab, superior between-days reproducibility for IFX, and RIA was the only assay that detected anti-IFX Ab including IgG4 subtypes in all six patients assessed. IgG1 and IgG4 Ab constitute the major isotypes of Ab during prolonged immunizations, and study I is the first documentation that IgG4 Ab is also the predominant IgG subclass in a notable fraction of CD patients with Ab against IFX.(29;180) IgG4 Ab are functionally monovalent and therefore go unnoticed in bridging ELISAs.(27;143;177;181;182) In light of the findings of study I, and in accordance with the theoretical assay considerations mentioned above, it appears that RIAs may have important advantages over ELISAs for monitoring IFX and anti-IFX Ab in the context of evaluating and optimizing IFX efficacy and safety in individual patients with CD. This is substantiated by our recent reporting from a prospective study where IFX and anti-IFX Ab levels were measured by RIA and ELISA in 67 patients with loss of response to IFX (53 luminal, 6 fistulizing, 8 both; median CDAI 284; median Perianal Disease Activity Index 9).(183) Here, results were generally comparable to those of study I with linear correlations between assays, but disagreement on absolute concentrations; and with RIA markedly more sensitive for detection of even low quantities of IFX (detectable IFX in 59 patients with RIA vs 50 with ELISA) and anti-IFX Ab (anti-IFX Ab detectable in 18 patients with RIA vs 6 with ELISA). The optimal assay for IFX and anti-IFX Ab cannot be identified on the basis of study I. Important limitations include lack of assessment of functionality of detected IFX and anti-IFX Ab, unknown anti-IFX Ab concentrations, relatively low number of repeat measurements, and the fact that the ELISAs used may not be extrapolated to all ELISA subtypes.(117;118;126;154) More importantly, no studies have until now compared individual assays' ability to facilitate the ultimate goal of successfully evaluating underlying mechanisms for thera-

peutic failures, and favorably guiding optimal intervention in individual patients.

CLINICAL EFFICACY OF IFX

Validation of assays for IFX bioavailability and immunogenicity

Heterogeneity of currently applied techniques for detection of IFX and anti-IFX Ab, and lack of standardization and reference values necessitates exploration of how different types of assays relate in order to meaningfully compare and extrapolate individual study results.(7;122) Such data have not previously been published. Study I compared IFX concentrations in micrograms per milliliter detected by RIA, ELISA, and RGA, and established using standard curves to allow for direct comparisons. This study further compared detected titers of anti-IFX Ab quantified by RIA, ELISA, RGA, and EIA by titration curves, and with a similar readout point to allow for direct comparisons.(118;119) Assays had markedly different sensitivities, and disagreed on absolute concentrations. The reason for this is unknown but is likely caused by several factors including different interference of serum factors and/or matrix effects, the inability of ELISA to detect IgG4 Ab, the ability of RIA to measure the TNF-α binding capacity of IFX, and of RGA to measure neutralizing activities at the cellular level. These novel findings make it clear, that the use of different assays to monitor IFX therapy may potentially bias findings of clinical importance of serum levels of IFX and anti-IFX Ab; and reported variations of the clinical impact of these parameters may possibly stem from analytical incongruence. Further, previous use of non-validated cut-off values for IFX and anti-IFX Ab may potentially have resulted in false negative reportings of lack of associations with clinical response types.(28;89;117;118;126) Taken together, standardization of assays is needed to allow for more wide routine usage.(7) In addition, adequate basic and clinical validation should be considered a prerequisite when using an assay to monitor individual patients, as severe consequences may result if therapeutic decisions are made on the basis of an assay that does not reflect the situation in vivo.(23;140;165;179) Accordingly, study II observed a strong and independent association of both IFX and anti-IFX Ab as measured by RIA with clinical efficacy of IFX maintenance therapy. Thus, patients who had lost effect of IFX maintenance therapy had significantly lower serum trough levels of IFX and significantly higher levels of anti-IFX Ab at time of therapeutic failure as compared to patients who maintained remission. Even though similar findings have been reported,(28;117;126;128;184) the majority of studies utilizing ELISA have failed to do so or only detected an association with either IFX or anti-IFX Ab,(53;85;118;125;136;137) supporting the view that RIA can be used for clinical evaluations and measures biologically relevant IFX and anti-IFX Ab activities without inconclusive tests.

Utility of IFX and anti-IFX Ab assessments to aid optimizing therapy

Strategies which incorporate measurements of IFX and anti-IFX Ab to enable more effective and rational drug utilizations during prolonged therapies are warranted.(185) Different approaches have been used to relate IFX and anti-IFX Ab levels with clinically relevant outcome measures. Quantifications have generally been analyzed as binary variables (positive versus negative) according to the ability of the given assay to detect the compound, or using non-validated cut-off values; but assay sensitivities and limits of detection varies, and the clinical impact of absolute values have thus far remained largely unexplored.(28;89;117;118;126) In study II, ROC analysis was used to establish cut-off values for IFX (0.5 µg/ml) and anti-IFX Ab (above detection limit) trough concen-

trations which optimally distinguished between patients with manifest loss of response or maintained remission. These cut-off values had relatively high sensitivities and specificities (>80%), and high AUC (~90%); and combined assessments increased the accuracy (from 87% to 90%). It is suggested that the cut-off values may prove useful to aid in discrimination of subgroups of patients on IFX maintenance therapy; and to identify mechanisms for loss of response. Accordingly, an algorithm for evaluating and optimizing treatment in individual patients with documented loss of response to IFX maintenance therapy is proposed (Figure 9).(23;186)

	High anti-IFX Ab	Low anti-IFX Ab
Low IFX	① Bioavailability and/or pharmacokinetic problem due to induced immunogenicity of IFX ⬇ Change to different TNF-inhibitor	② Bioavailability and/or pharmacokinetic problem due to reasons other than immunogenicity of IFX ⬇ Intensify treatment with current TNF-inhibitor. Reassess anti-IFX Ab at later infusions to evaluate window problem
High IFX	④ Unknown problem: Consider non-functional anti-IFX Ab or false positive testings ⬇ Repeat IFX and anti-IFX Ab assessments at later infusions. In case of unchanged results, then handle as the subgroup with a pharmacodynamic problem	③ Pharmacodynamic problem ⬇ Confirm that symptoms are caused by relapse of Crohn's disease and with active intestinal inflammation. If so, then change to a drug with a different therapeutic target than TNF-α

Figure 9
Algorithm for evaluating and optimizing treatment in individual patients with documented loss of response to IFX maintenance therapy.

The key element is to intervene and tailor therapy according to objective pharmaco-immunological evidence obtained from each individual patient considering four principal situations: 1) Immunogenicity with generation of anti-IFX Ab resulting in insufficient TNF-α blockade due to neutralization of IFX and/or increased clearance of IFX. Patients should be changed to a different TNF-inhibitor in order to restore optimal TNF-inhibition. 2) Non-immune mediated inadequate IFX bioavailability to sufficiently inhibit TNF-α activity due to pharmacokinetic changes with e.g. increased drug turn-over (e.g. increased inflammatory load), increased drug degradation, or increased drug elimination/excretion.(187-189) To date, only limited information exists regarding factors other than formation of Ab that influence pharmacokinetics of IFX but factors such as gender, body size, concomitant immunosuppression, and blood levels of TNF, CRP, and albumin may be involved.(185) Patients in this situation should receive increased IFX dosings to restore sufficient TNF-inhibition. 3) Pharmacodynamic issues where loss of response occurs in the presence of high circulating TNF-neutralizing capacity due to TNF-independent disease mechanisms. This is speculated to arise from e.g. activation of alternative immune pathways bypassing TNF-α as one of the central mediators of CD.(98;190) TNF-inhibitors are considered ineffective in this subgroup and are probably best discontinued. Clinical approach includes confirmation of ongoing inflammatory disease activity

and exclusion of abscess formation as well as non-inflammatory complications such as strictures or post-inflammatory irritable bowel which could provoke similar symptoms. Depending on the findings the next step is optimization of conventional immunosuppressive therapy or, if possible, changing to other biologic with a different target than TNF-α in case of inflammatory activity. Patients with stenosis or strictures, insufficient drainage of perianal fistulas, or with abscess formation must be treated for these complications before it is decided whether it is appropriate to continue the biological treatment. 4) Detectable levels of IFX in the presence of anti-IFX Ab may arise from false positive tests or be due to detection of functionally inactive anti-IFX Ab. Blood samples and analysis hereof are suggested to be repeated at later infusions, preferably with an assay for functionally active anti-IFX Ab. In case of unchanged findings patients are considered to represent a subgroup with a pharmacodynamic problem, and should be handled as in group 3. It is suggested that the cut-off values established in study II could aid in distinguishing between the four scenarios for loss of response as described above. The exact numeric value of these cut-off levels should optimally be confirmed in larger and prospective studies as the design of study II had several limitations; i.e. retrospective data, low number of patients, lack of clinical activity index scores and endoscopic evaluations; and due to the underlying assumption that identification of patients with loss of response and maintained remission was equally important. However, previous studies have used a similar approach, biochemical data supported clinical classification of patients, and cut-off values were validated by including data from a second cohort.(119;160) As shown in study I, the established cut-off values in RIA may not necessarily be generalized to other types of assays.

Though intuitively appealing, the algorithm presented above needs to be tested prospectively. We are currently doing this in the Danish study of Optimizing IFX Therapy in CD (DO IT CROHN; www.ClinicalTrials.gov: NCT00851565). Notably, a retrospective study recently added weight to our hypothesis by indicating higher response rates when IBD patients with loss of response and positive anti-IFX Ab were changed to another TNF inhibitor as compared to dose escalation; as well as when patients with undetectable IFX were dose escalated as compared to change of anti-TNF drug.(162) Others have proposed similar or slightly modified versions of this algorithm.(75;77;124;139;162;185;191) It has also been argued, that dose optimization using serial measurements to secure an IFX trough level of 3-7 µg/ml (i.e. use of a therapeutic interval) irrespective of anti-IFX Ab assessments is more relevant, and this is currently being investigated in the TAXIT study.(192) It is also debatable if the concept of cut-off values is in fact applicable to IFX therapy,(193) and whether cut-off levels apply to individuals rather than to cohorts of patients.(42;123;139) Baert et al. found that anti-IFX Ab trough concentration of 8 µg/ml measured by ELISA was optimal for association with duration of therapeutic response to IFX as defined by time to the next infusion(.117) Further, IFX concentrations of ≥12 µg/ml 4 weeks after infusion or >1.4 µg/ml at dosing trough was predictive of duration of response.(117) Vermeire et al. later showed that patients with significant anti-IFX Ab development defined as >8 µg/ml and quantified by this same ELISA, had significantly lower IFX levels already at week 4 after first infusion compared to all others.(126) A recent post-hoc analysis of ACCENT 1 explored the long term clinical efficacy of IFX at one year as a function of week 14 serum IFX trough levels assessed by ELISA. It was reported that sustained response associated with higher IFX levels after the induction regimen (week 14) and with a

cut-off of 3.5 μg/ml to optimally discriminate patients with and without sustained response (sensitivity 54%, specificity 72%).(194) A similar approach has been used in patients with ulcerative colitis, and here a somewhat higher cut-off value of 7.19 μg/ml was found to be optimal (sensitivity 57%, specificity 80%).(195) Based on these findings, the TALORIX study (www.ClinicalTrials.gov: NCT01442025) is currently investigating if measurements of IFX trough levels early in the course of treatment can guide a dosing strategy where sufficient IFX levels as defined above are achieved. In our cohort, anti-IFX Ab were detected early in the course of therapy after a median of 4 infusions, supporting that monitoring drug bioavailability and immunogenicity may be useful even early on and prior to manifest failure. Alternative suggested approaches for relating IFX and anti-IFX Ab assessments with clinical outcomes have been proposed to rely on determination of e.g. AUC, average serum concentrations, or peak levels.(7;124) Along this line, the use of trough levels originates from the limitation of common ELISAs to measure anti-IFX Ab in the presence of IFX, and samples are therefore obtained immediately prior to IFX infusions as IFX serum levels are lowest here.(124) However, it is questionable if this time of sampling is in fact optimal for evaluations of efficacy as trough may be influenced by e.g. changes in dosing regimen and concomitant medications.(185) Peak levels measured shortly after infusions have been indicated clinically relevant, and may enable more robust pharmacokinetic analyses.(121;185) Data on these issues are scarce and need further attention.

Timing of sampling

Not only the technical qualities of assays for detection of anti-IFX Ab need to be acknowledged, when evaluating the clinical importance of immunogenicity of IFX, but also the fact that immunogenicity is a gradual and dynamic process developing and changing over time.(143) Hence, several studies have reported that a low IFX trough levels is precursor for later development of anti-IFX Ab,(120;126;196) suggesting that anti-IFX Ab may be present in the circulation also at time points prior to initial detection, but is undetectable due to e.g. insufficiency to completely clear the drug (window problem).(77) Study III addressed a related aspect regarding the potential importance of timing of sampling by investigating variations of detected anti-IFX Ab during ongoing IFX maintenance therapy. Interestingly, the majority of patients with initial anti-IFX Ab appeared to benefit from continued IFX therapy, and anti-IFX Ab disappeared in two thirds of these responders when reassessed before subsequent infusions. On the other hand, anti-IFX Ab persisted at increased concentrations in all patients with loss of response and subsequent IFX discontinuation. Thus, this subgroup seems to represent patients with loss of response to IFX maintenance therapy because of drug immunogenicity as described in the algorithm above and in Figure 9.(23) The reason for anti-IFX Ab disappearance is unknown, but could be a result of immunological tolerance towards IFX. Alternatively, inability of assays to detect anti-IFX Ab may arise from binding of all anti-IFX Ab in complexes with IFX, or to increased clearance of immune complexes; thus resulting in false negative results. Finally, previous false positive anti-IFX Ab detections cannot be completely ruled out, but this seems unlikely as anti-IFX Ab appeared functionally active and associated with low IFX levels both at initial detection and at reassessment; and several patients had serially negative and/or positive anti-IFX Ab measurements. Novel data from other immunoinflammatory diseases and other biologic drugs support the observation that anti-drug Ab may later disappear or become undetectable.(137;197-200) Casteele et al. ob-

served transient anti-IFX Ab in 14 of 52 IBD patients (27%) and found transiency associated with low titres and IFX dose optimization; and persistence of anti-IFX Ab associated with loss of response and IFX discontinuation.(200) Taken together, it appears that not only clinical response to ongoing IFX therapy needs to be taken into account when evaluating the importance of measurements of IFX and anti-IFX Ab. Hence, timing of sampling is also an important aspect, and a potential bias, when interpreting IFX immunogenicity. This is the case both in the early phase of anti-IFX Ab development, as well as once anti-IFX Ab have become detectable in the circulation. As a consequence, it is suggested to preferably assess IFX bioavailability and immunogenicity on a serial basis with repeated measurements over time to monitor the evolution and secure sound clinical interventions – rather than simply measuring at a single time point. Recent pilot data have supported this understanding.(201)

SAFETY OF IFX

Etiology of acute severe infusion reactions

Immunogenicity of IFX has been suggested to potentially affect safety of therapy,(7;65;67;122;202) and anti-IFX Ab have been associated with increased risk of acute infusion reactions in numerous studies.(85;117;123;125;127;133) Successful desensitization following these reactions also indicate an immunological mechanism.(203;204) However, data on the etiology of acute infusion reactions are limited, and previous studies have examined relatively small cohorts and few events; all subtypes of acute infusion reactions have typically been combined in the analyses; and timing of anti-IFX Ab assessments with respect to reactions has been highly variable.(122-124;202) Acute severe infusion reactions clinically resemble anaphylactic reactions, i.e. a type 1 hypersensitivity reaction mediated by anti-IFX IgE Ab. An IgE mediated reaction to IFX has been reported in a single child with CD and in three patients with rheumatoid arthritis.(134;138) IgE Ab have been associated with acute severe reactions to other biologic agents such as cetuximab, a chimeric mouse-human IgG1 monoclonal Ab against the epidermal growth factor receptor.(205) A study of four CD patients, however, reported normal serum tryptase levels after reactions to IFX indicating that mast cell degranulation and IgE Ab are not involved.(206;207) We observed that all patients except one had high circulating levels of anti-IFX IgG Ab measured shortly after the acute severe infusion reaction. In contrast, anti-IFX IgE Ab were not detected in any of the reactions despite assessments at multiple time points to exclude false negative results. It therefore appears that immunogenicity of IFX in the form of specific anti-IFX IgG Ab is involved in the vast majority of acute severe infusion reactions; and that IgE Ab are not usually involved. In support hereof, newly published data identifies several alternative pathways for development of anaphylactoid reactions including IgG-mediated basophil-dependent mechanisms, and IgG-induced complement activation.(69) Along this line, polymorphisms in the Fcγ receptor IIIb, which mediates the IgG binding to basophils, have been associated with acute infusion reactions in rheumatoid arthritis.(208) Due to the retrospective study design, findings of high levels of anti-IFX IgG Ab after reactions could in principle be a confounder and not necessarily the cause of reactions. Thus, it cannot be completely excluded that at least some acute severe infusion reactions to IFX involve IgE Ab or other immunoinflammatory mechanisms such as cytokine release syndrome.(69;206;209)

Strategies for prevention of acute severe infusion reactions

Consistent with other reports, risk of acute severe infusion reactions was highly increased during episodic IFX therapy and increased with the length of drug pause.(45;150;206;210-213) Observations from study III that anti-IFX Ab persist up to several years after discontinuation of IFX in some patients led us to hypothesize that assessments of immunogenicity prior to reinitiation of IFX could be used to identify patients with increased risk of reactions. However, this did not seem to be the case as most patients were anti-IFX Ab negative prior to reinitation. A relatively short half-life of anti-IFX Ab has been reported elsewhere.(125;149;214) The fact that risk of reaction was substantially higher at the second infusion in the second series suggests, that the first IFX infusion in a retreatment series elicits an immunological response to the drug which manifests as a hypersensitivity reaction at time of re-administration. Following this argument, it should next be explored if anti-IFX Ab measurements following first infusion in a reinitiation series can help identify patients at increased risk of reaction. Other approaches to avoid reactions remain undefined. Concomitant immunosuppression and pre-infusion of corticosteroids have been variably associated with reduction of risk.(68;125;133;150;210;215;216) We were, however, unable to detect protective effects hereof. Of note, increased risk of reactions during episodic therapy is not evident in all patient subgroups as e.g. CD patients having discontinued IFX while in clinical remission respond favorably to reinitation at relapse and without any infusion reactions.(60;62)

MINIMIZING RISK OF IFX IMMUNOGENICITY

Strategies for avoidance of anti-IFX Ab development are attractive when appreciating the potential influence of immunogenicity of IFX on efficacy and safety. Our findings supported numerous previous reports of an increased risk of anti-IFX Ab during episodic treatments and when not applying concomitant immunosuppression.(7;42;122;123;185) It remains unknown if anti-IFX Ab development is actually inhibited by use of concomitant immunosuppression; or if persisting anti-IFX Ab rather become undetectable because they are neutralized and/or their clearance is enhanced due to the elevated drug levels caused by an immunosuppressant's anti-inflammatory effect which may reduce the systemic TNF-load.(140;185) Pre-treatment with corticosteroids prior to IFX infusions to reduce anti-IFX Ab formation is controversial.(133;217;218) Despite all our patients receiving corticosteroids and antihistamines prior to all IFX infusions, we observed relatively high frequencies of anti-IFX Ab development supporting a negligible effect of this intervention.(219) This, however, was not addressed in formal statistical analyses.

STUDY LIMITATIONS

Principal study limitations not mentioned in the previous sections relate primarily to the retrospective design of study II-IV. First, classification of clinical response types was based on an overall clinical evaluation as noted in the patient files, and without strict and well defined objective criteria. Classification should optimally have been supported by validated clinical scoring systems; and by endoscopy and/or diagnostic imaging techniques.(73;74) However, this was not routinely done in our clinic at the time of study, and data were unavailable. To minimize the risk of bias we used independent investigators for clinical classification and data analysis, blinding of the investigator for the test results of interest, and biochemical data to support the classification used. From a practical point of view, the clinician's evaluation of response type and decision regarding continuation or change of therapeu-

tic regimen probably reflects the setting at most institutions, and a similar approach has been used before.(28;42;87;162) Our definition of acute severe infusion reactions accords with previous studies;(125;133;150;210;211) and symptoms reported by our patients were similar to symptoms reported elsewhere.(204;206;219) We cannot rule out, however, that classification of reactions were biased by the retrospective setup to include mild to moderate acute infusion reactions and thus contributing to the apparent high frequency of reactions in our cohort as pointed out by Casteele et al.(220) Alternative explanations for the high frequency of reactions include the relatively high number of episodically treated patients, the small sample sizes of previous studies, use of different definitions of episodic therapy, and study differences of mean number of infusions per patient.(219) A second implication of the retrospective design of study II-IV is that it cannot be completely ruled out, that reported findings regarding IFX and anti-IFX Ab arose from confounding instead of originating from a causal connection. Third, relevant sample material for serum tests was lacking in some cases, and a few samples were obtained at irregular time points. Fourth, a few patients with ulcerative colitis were included in study III and IV (10 of 75 patients, and 4 of 25 patients, respectively). This was done to increase sample size, and under the assumption that the mechanisms of anti-IFX Ab variations, and acute severe infusion reactions would be the same among patients with CD and ulcerative colitis. Of note, removal of patients with ulcerative colitis from the analyses did not affect the reported conclusions. Fifth, in spite of inclusion of patients with ulcerative colitis, studies may still have been underpowered. Finally, retrospective studies should generally be interpreted with caution due to risk of e.g. selection- and reporting bias; and findings should preferably be assessed prospectively.

CONCLUSION

Monitoring IFX maintenance therapy in CD by serum measurements of drug bioavailability and immunogenicity by the novel RIA techniques primarily used here appears useful for clinical evaluations of underlying mechanisms for loss of response and acute severe infusion reactions. Assessments seem promising also to aid in the process of optimizing efficacy and safety of IFX therapy in individual patients, all with minimal costs and inconveniencies. Specific strategies for evaluation and intervention in these subgroups of patients were established based on precise estimations of IFX bioavailability and immunogenicity at relevant time points. These algorithms need prospective clinical validation, as sufficient evidence is at hand to realize the advantages of immunopharmacological guidance for more effective, safe, and cost-effective IFX utilization.

Associations of serum IFX and anti-IFX Ab levels with clinical relevant outcome measures are potentially influenced by the type of assay used, application of non-validated cut-off values for binary stratification of test results as positive or negative, timing of measurements, and transiency of anti-IFX Ab. These conditions should be considered and addressed when interpreting test results; and may have contributed to previous conflicting reports on the clinical usefulness of measuring IFX and anti-IFX Ab.

When monitoring individual patients, is it essential that estimates of IFX bioavailability and immunogenicity reflect the actual situation in vivo, and severe consequences may result if therapeutic decisions are based on an erroneous assay. RIA appears to have some important advantages over commonly used solid phase binding assays for this purpose. However, larger and pro-

spective studies are needed to identify the optimal analytical technique for monitoring IFX therapy in patients with CD

PERSPECTIVES AND FUTURE STUDIES

Accumulating evidence supports that application of a "trial-and-error strategy" with dose optimization and/or change to another TNF-inhibitor in cases of IFX failure will frequently lead to suboptimal therapeutic decisions. A better understanding of the factors that impact pharmacokinetics and pharmacodynamics of TNF-inhibitors is crucial to ensure more efficient dosing regimens and enhanced therapeutic success. The perspectives of monitoring IFX therapy by utilizing regular and objective measurements of drug bioavailability and immunogenicity includes potential benefits of personalized therapy in form of optimized efficacy, safety, and cost-effectiveness, to the benefit of patients, physicians, and society. It remains to be established how exactly these analyses should be incorporated in clinical practice to optimally achieve this. There is urgent need for prospective, randomized trials that investigate strategies for optimal utilization of test results, including validation of the currently proposed algorithm for handling patients with loss of response to IFX maintenance therapy. It further remains to be defined how test results are best used as surrogate markers to characterize clinical relevant outcomes and thus tailor therapies in individuals including the applicability of cut-off values and use of a therapeutic interval versus use of steady state concentration, total drug exposure estimated as AUC, and/or peak levels. Ongoing clinical trials such as DO IT CROHN, TAXIT, and TALORIX will hopefully elucidate some of these issues.

It should be recognized, that timing of blood sampling is important when evaluating test results of IFX bioavailability and immunogenicity in a clinical context. Several related aspects need attention, including how immunogenicity to IFX develops and changes over time; and how to best time sampling and use repeated assessments to accommodate for timely changes in bioavailability and immunogenicity. Aspects of the heterogeneity of currently applied analytical techniques for assessments also need attention; and performances of individual assays to aid in evaluating and optimizing therapy, including identification of optimal assays for therapeutic guidance in individual patients with CD, need to be defined. Clinical utility of monitoring IFX therapy in patient subgroups with primary response failure or long term persistence of remission is largely unexplored and should be addressed to potentially gain higher initial response rates and to help identify patients in whom IFX can favorably be discontinued again. Involvement of immunogenicity in other safety aspects than acute infusion reactions also needs attention. Longitudinal studies using optimized methods for anti-IFX Ab detection are needed to uncover the influence of immunosuppressive therapies on the immunogenicity of IFX. Finally, the extent to which current available findings regarding IFX extrapolate to therapies with other anti-TNF biopharmaceuticals and other immunoinflammatory diseases remains to be explored in detail.

SUMMARY

Background: Infliximab (IFX) is a therapeutic monoclonal antibody (Ab) against TNF-α, which is used to induce and maintain remission in patients with moderate to severe Crohn's disease. Despite its effectiveness, approximately one third of patients experience primary treatment failure, and another one third later lose effect of maintenance therapy. IFX is well tolerated but may result in potentially life-threatening side effects such acute severe infusion reactions. Determining optimal therapy after therapeutic failure is complicated. Recent studies have indicated, that measurements of IFX and anti-IFX Ab concentrations in individual patients may be helpful in this process.

Aim: The aim of this PhD thesis was to investigate the clinical utility of measuring IFX and anti-IFX Ab by novel radioimmunoassay (RIA) techniques. Specifically, the aim was to investigate if these measurements could aid in evaluating and optimizing efficacy and safety of IFX therapy in patients with Crohn's disease.

Methods: An experimental study for comparison of analytical properties of assays for measuring IFX and anti-IFX Ab was applied. In addition, three observational, retrospective, single center cohort studies of all patients with Crohn's disease treated with IFX were carried out.

Results: Serum levels of IFX and anti-IFX Ab measured by RIA strongly associated with clinical response types to IFX maintenance therapy. Cut-off values providing optimal discrimination of patients with loss of response or maintained remission were established. An algorithm for evaluating and optimizing therapy in individual patients with loss of treatment response based on IFX and anti-IFX Ab levels was proposed. Acute severe infusion reactions appeared not to be true IgE-mediated anaphylactic reactions, but rather associated with development of anti-IFX IgG Ab. Risk was increased during episodic therapy, but absence of anti-IFX Ab prior to a reinitiation series did not exclude reactions and assessments hereof could not be used for risk stratification. Several factors may potentially interfere with associations of IFX and anti-IFX Ab with clinical outcome including use of different analytical techniques, different cut-off values for reporting of positive test results, differences in timing of measurements, and transiency of anti-IFX Ab.

Conclusions: Monitoring serum levels of IFX and anti-IFX Ab by novel RIA techniques appears promising for evaluating and optimizing efficacy and safety of IFX therapy in Crohn's disease. Previous conflicting reports on the importance of tests are potentially biased by use of different types of assays, different cut-off values for binary classification of test results, and inconsistent timing of measurements. Prospective validation of proposed treatment algorithms in larger cohorts is warranted.

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