

# Pre-existing IgG antibodies cross-reacting with the Fab region of infliximab predict efficacy and safety of infliximab therapy in inflammatory bowel disease

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## SUMMARY

### Background

Infliximab (IFX) is a chimeric murine/human anti-TNF antibody (Ab) used for the treatment of Crohn's disease (CD) and ulcerative colitis (UC). Loss of response is common and associated with development of anti-IFX Abs during ongoing therapy. However, human anti-murine immunoglobulin Abs are common and may cross-react with the murine part of IFX.

### Aim

To investigate if Abs binding to IFX's Fab region (IFX-Fab) are present in IBD patients before exposure to IFX, and whether they predict efficacy and safety of IFX therapy.

### Methods

Observational, retrospective cohort study of patients with CD ( $n = 29$ ) and UC ( $n = 22$ ).

### Results

Pre-treatment levels of IFX-Fab reactive IgG Abs were significantly lower in CD patients in remission after 1 year of maintenance IFX (median 91 mU/L,  $n = 8$ ) than in the rest of the patients (639 mU/L,  $n = 21$ ;  $P < 0.01$ ), and lower than in patients with secondary loss of response in particular (692 mU/L,  $n = 7$ ;  $P < 0.01$ ). A cut-off concentration of  $<439$  mU IFX-Fab reactive IgG Ab per litre comprised all patients who later obtained long-term sustained remission on IFX (sensitivity 100%, specificity 67%). Similar trends were observed in UC. The pre-treatment levels of IFX-Fab reactive IgG Abs were markedly higher in patients developing infusion reactions to IFX (1037 mU/L,  $n = 7$ ) than in the remaining patients (349 mU/L,  $n = 44$ ;  $P = 0.036$ ).

### Conclusions

IFX-Fab reactive IgG antibodies present in serum from IBD patients before infliximab therapy associate with lack of long-term efficacy and safety. Assessments of such antibodies may help clinicians to choose between treatment with infliximab and more humanised agents.

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## INTRODUCTION

Infliximab (IFX) is a chimeric murine–human IgG1 monoclonal antibody (Ab) directed against tumour necrosis factor (TNF)-alpha. IFX has proven highly effective in inducing and maintaining remission in moderate to severe luminal or fistulising Crohn's disease and ulcerative colitis,<sup>1–4</sup> as well as in the treatment of other chronic immunoinflammatory disorders.<sup>5</sup> Regrettably, about one third of patients with inflammatory bowel disease (IBD) lack effect of IFX induction and are classified as primary nonresponders. Another third of patients become unresponsive to IFX over time and discontinue the treatment.<sup>6–8</sup> The remaining patients achieve long-term remission under IFX therapy.<sup>9</sup> The underlying mechanisms and predispositions for these response types remain largely unknown.

It is of clinical interest to identify markers of efficacy and safety of IFX before the initiation of therapy or at an early phase of the treatment regimen.<sup>10</sup> Nonresponding patients may suffer irreversible disease progression during ineffective IFX treatments resulting in potentially severe outcomes; and the sheer cost of the failed treatment puts tremendous pressure on public health budgets. Young age, short duration of disease, nonsmoking, an inflammatory disease phenotype, disease site limited to the colon only and concomitant immunosuppressive treatment all indicate a favourable response to IFX.<sup>11–14</sup> Among inflammatory markers, an elevated baseline C-reactive protein (CRP) level with early normalisation during IFX induction treatment has been associated with later sustained remission.<sup>15</sup> Of particular relevance in this context, however, is the development of specific anti-IFX Abs during ongoing IFX therapy as a predictor of clinical efficacy and safety of IFX. Thus, anti-IFX Abs may increase clearance of IFX, resulting in low IFX trough levels and subsequent secondary treatment failure.<sup>16–19</sup> Furthermore, anti-IFX Abs may elicit acute severe infusion reactions to IFX.<sup>20</sup> Development of specific anti-IFX Abs has been observed in 5–18% of IBD patients on maintenance IFX treatment and in up to 61% of patients receiving episodic treatment.<sup>6, 21–23</sup> Concomitant immunosuppressive therapy seems to reduce the risk of anti-IFX Ab development.<sup>24</sup>

It is well recognised that sera from some healthy individuals contain Abs against murine immunoglobulins, leading occasionally to false-positive results in two-site (sandwich) immunoassays using murine Abs as capture- and detecting Abs.<sup>25, 26</sup> The reported prevalence of such Abs varies between 1% and 80% depending of the assay

used.<sup>25, 26</sup> In view of these reports, we examined whether serum from patients with IBD naïve to treatment with biological drugs, and from healthy controls, contains pre-existing Abs cross-reacting with the Fab portion of IFX (IFX-Fab), which is known to be the major immunogenic part of IFX<sup>27</sup>, and in which the murine sequences of the drug are found. We hypothesised that such pre-existing Abs might have a clinical impact on efficacy and safety of IFX therapy in patients with IBD.

## MATERIALS AND METHODS

### Study design and patients

This was an observational, retrospective, single centre, exploratory study of all IFX-treated IBD patients at a Danish university hospital (Herlev Hospital) from January 1999 to April 2012. The study population comprised patients without previous biological treatments and for whom blood samples were available before the initiation of IFX therapy. From May 2009 onwards, blood samples were obtained before IFX infusions as part of a local drug monitoring programme. Before May 2009, samples had been obtained sporadically, as decided by the treating physician.

The primary objective of the study was to assess any association between levels of Abs reacting with IFX's Fab portion, as determined before IFX exposure, and long-term clinical efficacy, defined by clinical remission at 1 year of IFX maintenance therapy with regular infusions every 4–12 weeks. Secondary objectives were to determine any association of pre-existing IFX-Fab reactive Abs with well-characterised clinical efficacy and safety response types to IFX. The patients were identified by review of files of all IBD patients who had received IFX during the designated time period and who had had a minimum of 1 year of follow-up, unless IFX had been discontinued within the first year due to complete remission, incomplete effect or adverse events. The study was approved by the regional ethics committee (HD-2009-055), and all patients consented.

### Classification of response to IFX

Outcome of IFX treatment was determined according to the treating physician's global evaluation as noted in the patient file. Symptoms and clinical findings were evaluated by a senior gastroenterologist, and a decision to discontinue IFX was made on an ad-hoc basis. Clinical outcome was classified in accordance with previously studies<sup>28, 29</sup>:

i. *nonresponse* was defined as no clinical response to IFX induction infusions at weeks 0, 2 and 6, followed by discontinuation of IFX.

ii. *Loss of response* was defined as an initial favourable response to IFX induction and maintenance therapy, with clinical remission and no symptoms or findings indicating active disease, followed later by loss of clinical response with active disease, despite dose optimisation, and finally resulting in discontinuation of IFX within the first year of therapy.

iii. *Maintained remission* was defined as a favourable clinical response to IFX induction, followed by a sustained clinical response during maintenance therapy with complete clinical remission at 1 year.

iv. *Partial response* was defined as a beneficial clinical response at 1 year of IFX therapy, but without complete clinical remission.

v. *Infusion reaction* to IFX was defined as either acute reactions occurring during IFX infusion, or delayed reactions occurring after discontinuation of IFX, typically after 7–14 days.

## Serum and plasma analyses

**Samples.** Blood samples were obtained 30 min before IFX infusions. Serum was collected after centrifugation and stored at  $-80^{\circ}\text{C}$  until analysis. Plasma collected in EDTA containing tubes (BD Biosciences, Plymouth, UK) from 50 donors (23 males, 27 females, age 22–62 years) attending the Blood Bank at Odense University Hospital, Odense, Denmark, was used as controls. The donors fulfilled the requirements for Danish blood donors, i.e. did not have a known history of autoimmune disease or cancer, they were not on medication and did not have ongoing infection. The samples were stored at  $-20^{\circ}\text{C}$  until analysis.

**Pepsin digestion of IFX.** Intact IFX was digested by pepsin into  $\text{F(ab')}_2$  portions and fragmented Fc portions. The digestion and the separation of  $\text{F(ab')}_2$  and Fc fragments was performed using commercially available kit #44885 (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Following dialysis, only  $\text{F(ab')}_2$  portions with an apparent MW of 110 kD were present in the solution (Figure S1A). These were used as antigens in the ELISA described below.

**ELISA for IFX-Fab reactive Abs.** Serum samples were analysed under blinded conditions for their content of Abs directed against the Fab portion of IFX. Polystyrene

microwell plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight with 100  $\mu\text{L}$   $\text{F(ab')}_2$  (2  $\mu\text{g/mL}$ ) of IFX, washed ( $\times 3$ ) and blocked with washing buffer (PBS, 10 mM EDTA, 0.05% Tween-20, pH 7.4) for 30 min at room temperature. A pool of 50 plasma samples was used as calibrator (1000 mU/L). The calibrator and proband samples were diluted in washing buffer in a two-fold dilution series starting at 1:10 and 1:20, respectively, and ending at 1:5120. After incubation overnight at  $4^{\circ}\text{C}$ , the wells were washed ( $\times 3$ ), and the detecting Ab, biotinylated monoclonal antihuman Fc-gamma chain Ab, raised in-house (or polyclonal rabbit antihuman Fc-epsilon, -alpha and -mu chain, respectively), was added to each well at a final concentration of 1.5  $\mu\text{g/mL}$ . This was followed by incubation at room temperature for 1 h, washing ( $\times 3$ ) and incubation for 30 min at room temperature with 100  $\mu\text{L}$  of horseradish peroxidase (HRP)-conjugated streptavidin (diluted to 1:3000). After washing ( $\times 3$ ), the wells were incubated with substrate, o-phenylene-diamine (0.4 mg/mL; Kem-En-Tec, Taastrup, Denmark) for 15 min, and the reaction was stopped by the addition of 1 M  $\text{H}_2\text{SO}_4$ . Optical density was measured at 490–650 nm using a  $V_{\text{max}}$  Kinetic Microplate Reader (Molecular Devices, Wokingham, UK), and the data were processed using the SoftMax Pro software (Molecular Devices). No signal was observed when the detecting Ab was added to wells that had not been incubated with serum samples (Figure S1B), confirming that no antigenic parts of the Fc portion of IFX were present during coating.

The assay for IgG Abs reacting with the Fab portion of IFX fulfilled requirements for parallelism between the individual curves (Figure S1C). This was not the case for Abs of other isotypes. The coefficient of variation (CV) was below 0.06 (intra-assay) and 0.08 (inter-assay). No unspecific binding was observed when human serum albumin was used as coating agent (Figure S1C). On the basis of this negative control (mean + 3 s.d.), the detection limit for the assay was determined to be 34.3 mU/L. Quantification of IFX-Fab reactive IgG was based on a 4-parameter analysis using Softmax Pro software (Molecular Devices).

**Bead array for circulating immunoglobulin classes and subclasses.** Serum levels of immunoglobulin isotypes and IgG subclasses were measured using the Bio-Plex Pro Assay for Immunoglobulin Isotyping (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

**Biochemical parameters.** Biochemical tests of blood samples were regularly performed during ongoing IFX therapy at the discretion of the treating physician. For the purposes of this study, data on biochemical markers of disease activity in terms of CRP and haemoglobin were retrospectively obtained from patient files corresponding to the time of IFX initiation and after 1 year of IFX maintenance therapy. If IFX was discontinued in the first year of therapy, biochemical markers at the time of IFX discontinuation were used.

### Statistical analyses

Descriptive statistics were calculated as percentages for discrete variables and median with interquartile range (IQR) for continuous variables, unless otherwise stated. Fisher's exact test or the Chi-square test was used for univariate analysis of discrete variables as appropriate. The Kruskal–Wallis test or Mann–Whitney test was used for unpaired univariate analysis of continuous variables, and the Wilcoxon signed-rank test was used to assess paired data. Receiver-operating characteristics (ROC) analysis was used to determine cut-off levels for IFX-Fab reactive IgG optimally associated with clinical remission after 1 year of IFX therapy. Time until discontinuation of IFX was estimated using survival statistics including Kaplan–Meier curves, and the log-rank test was used to compare survival curves. Analyses were done in SPSS v.18 (IBM, Somers, NY, USA) or GraphPad Prism v.5 (GraphPad Software, San Diego, CA, USA). All *P* values were two-sided and *P* < 0.05 was considered significant.

## RESULTS

### Study population

The study population comprised 51 anti-TNF- naïve IBD patients: 29 patients with Crohn's disease (Table 1) and 22 patients with ulcerative colitis (Table 2).

### Prevalence of pre-treatment IFX-Fab reactive IgG

Serum levels of IgG Abs reacting with the Fab portion of IFX were assessed by ELISA using a pool of plasma from 50 healthy blood donors as calibrator (Figure S1). The value of this pool was designated to contain 1 U (1000 mU) per litre. The median levels of IFX-Fab reactive IgG were 349 (IQR 150–1070) mU/L in patients with Crohn's disease and 582 (IQR 223–940) mU/L in patients with ulcerative colitis (Figure 1). In comparison, a median level of 645 (IQR 340–1460) mU/L was found in the 50 healthy controls, when tested individually. As regards sera from the controls, the signals obtained using

**Table 1 | Characteristics of patients with Crohn's disease**

|                                                           | Remission<br><i>n</i> = 8 | No remission<br><i>n</i> = 21 | <i>P</i>     |
|-----------------------------------------------------------|---------------------------|-------------------------------|--------------|
| Male (%)                                                  | 5 (63)                    | 8 (38)                        | 0.406        |
| Age at diagnosis – median years (IQR)                     | 24 (19–33)                | 22 (19–39)                    | 0.938        |
| Disease phenotype:                                        |                           |                               | 0.729        |
| Luminal disease only – <i>n</i> (%)                       | 5 (63)                    | 13 (62)                       |              |
| Fistulising disease only – <i>n</i> (%)                   | 1 (13)                    | 1 (5)                         |              |
| Both luminal and fistulising disease – <i>n</i> (%)       | 2 (25)                    | 7 (33)                        |              |
| Age at 1st IFX infusion – median years (IQR)              | 39 (30–56)                | 37 (25–54)                    | 0.518        |
| Disease duration at 1st IFX infusion – median years (IQR) | 12 (7–19)                 | 7 (3–14)                      | 0.147        |
| IFX induction regimen – <i>n</i> (%)                      | 8 (100)                   | 20 (95)                       | 1.000        |
| Total number of IFX infusions – no. median (IQR)          | 8 (6–10)                  | 4 (3–7)                       | <b>0.024</b> |
| IFX dose escalation* – <i>n</i> (%)                       | 3 (38)                    | 4 (19)                        | 0.357        |
| Concomitant immunosuppression† – <i>n</i> (%)             | 5 (63)                    | 9 (43)                        | 0.427        |
| Type of concomitant immunosuppression:                    |                           |                               | 0.211        |
| Azathioprine – <i>n</i> (%)                               | 5 (100)                   | 5 (56)                        |              |
| Mercaptopurine – <i>n</i> (%)                             | 0 (0)                     | 3 (33)                        |              |
| Methotrexate – <i>n</i> (%)                               | 0 (0)                     | 1 (11)                        |              |
| Corticosteroids oral – <i>n</i> (%)                       | 0 (0)                     | 2 (10)                        | 1.000        |
| Infusion reaction to IFX                                  | 0 (0)                     | 4 (19)                        | 0.552        |

Bold letters: Significant difference between subgroups (*P* < 0.05); IFX, Infliximab; IQR, Interquartile range.

\* All patients received IFX dosing 5 mg/kg. Dose escalation was done by decreasing the interval between infusions.

† Azathioprine, mercaptopurine or methotrexate in standard doses during ongoing IFX therapy.

plates coated with F(ab')<sub>2</sub> regions of IFX correlated strongly with signals obtained using plates coated with F(ab')<sub>2</sub> regions of mouse IgG (data not shown), suggesting that the IFX-Fab reactive IgG belongs to the group of commonly occurring anti-mouse IgG Abs described by others.<sup>25, 26</sup>

IgE binding to the Fab portion of IFX was not detectable in any of the patient samples, and measurements of

**Table 2 | Characteristics of patients with ulcerative colitis**

|                                                           | Remission<br><i>n</i> = 10 | No remission<br><i>n</i> = 12 | <i>P</i>         |
|-----------------------------------------------------------|----------------------------|-------------------------------|------------------|
| Male (%)                                                  | 6 (60)                     | 5 (42)                        | 0.670            |
| Age at diagnosis – median years (IQR)                     | 27 (20–36)                 | 28 (19–38)                    | 0.821            |
| Age at 1st IFX infusion – median years (IQR)              | 36 (28–48)                 | 39 (22–47)                    | 0.974            |
| Disease duration at 1st IFX infusion – median years (IQR) | 6 (1–16)                   | 4 (1–13)                      | 0.722            |
| IFX induction regimen – <i>n</i> (%)                      | 10 (100)                   | 9 (75)                        | 0.221            |
| Total number of IFX infusions – no. median (IQR)          | 8 (7–9)                    | 4 (2–5)                       | <b>&lt;0.001</b> |
| IFX dose escalation* – <i>n</i> (%)                       | 3 (30)                     | 0 (0)                         | 0.078            |
| Concomitant immunosuppression† – <i>n</i> (%)             | 7 (70)                     | 2 (17)                        | <b>0.027</b>     |
| Type of concomitant immunosuppression:                    | NA                         |                               |                  |
| Azathioprine – <i>n</i> (%)                               | 7 (100)                    | 2 (100)                       |                  |
| Mercaptopurin – <i>n</i> (%)                              | 0 (0)                      | 0 (0)                         |                  |
| Methotrexate – <i>n</i> (%)                               | 0 (0)                      | 0 (0)                         |                  |
| Concomitant 5-ASA – <i>n</i> (%)                          | 4 (40)                     | 3 (25)                        | 0.652            |
| Corticosteroids oral – <i>n</i> (%)                       | 3 (30)                     | 2 (17)                        | 0.624            |
| Infusion reaction to IFX                                  | 0 (0)                      | 3 (25)                        | 0.221            |

Bold letters: Significant difference between subgroups ( $P < 0.05$ ); IQR, Interquartile range; NA, Not applicable.

\* All patients received IFX dosing 5 mg/kg. Dose escalation was done by decreasing the interval between infusions.

† Azathioprine, mercaptopurine or methotrexate in standard doses during ongoing IFX therapy.

IgA and IgM did not fulfil the requirements for parallelism or low background levels.

### Classification of clinical outcome

Patients with Crohn's disease and ulcerative colitis were classified as being in complete clinical remission at 1 year of IFX maintenance therapy ( $n = 8$  and  $n = 10$  respectively) or not ( $n = 21$  and  $n = 12$ ). Classification of patients was based on the treating physician's global evaluation and supported by biochemical parameters (Figure 2). CRP levels decreased significantly and

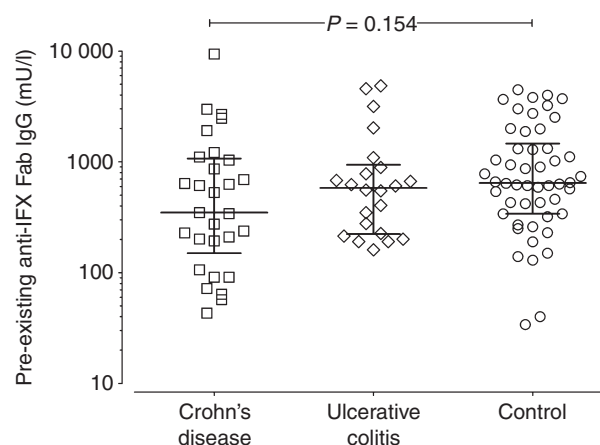


**Figure 1 | Levels of pre-existing IgG Abs cross-reacting with the Fab region of Infliximab (IFX) in anti-TNF-naïve IBD patients and healthy controls. The symbols indicate circulating levels of IgG Abs binding to F(ab')<sub>2</sub> fragments of IFX, as assessed by the ELISA described in materials and methods and in Supporting figure 1. Horizontal bars and error bars indicate median and IQR.**

haemoglobin levels increased significantly during IFX treatment in patients in remission, but not in patients who did not achieve remission. Characteristics of patients with and without remission at 1 year were generally comparable, see Table 1 and 2. A noteworthy exception was, however, that within the group of patients with ulcerative colitis, concomitant immunosuppression was administered to 7 of 10 patients (70%) in remission, compared with only 2 of 12 patients (17%) who did not achieve remission ( $P = 0.027$ ). The number of IFX infusions was higher among patients who were in remission after 1 year than in patients who were not, as IFX was discontinued in the latter group because of insufficient effect.

### Pre-existing IFX-Fab reactive IgG as a predictor of long-term efficacy of IFX

Serum levels of IgG Abs to the Fab region of IFX measured before the initiation of therapy were significantly lower among patients with Crohn's disease in clinical remission at 1 year (median 91 mU/L, IQR 61–228) than in patients who did not achieve remission (median 639 mU/L, IQR 252–1562;  $P = 0.0014$ ) (Figure 3). No difference was found between these groups with respect to the levels of total IgG, IgG subclasses, IgA or IgM in patients achieving remission (data not shown).

A similar trend was observed in patients with ulcerative colitis. Thus, ulcerative colitis patients in remission

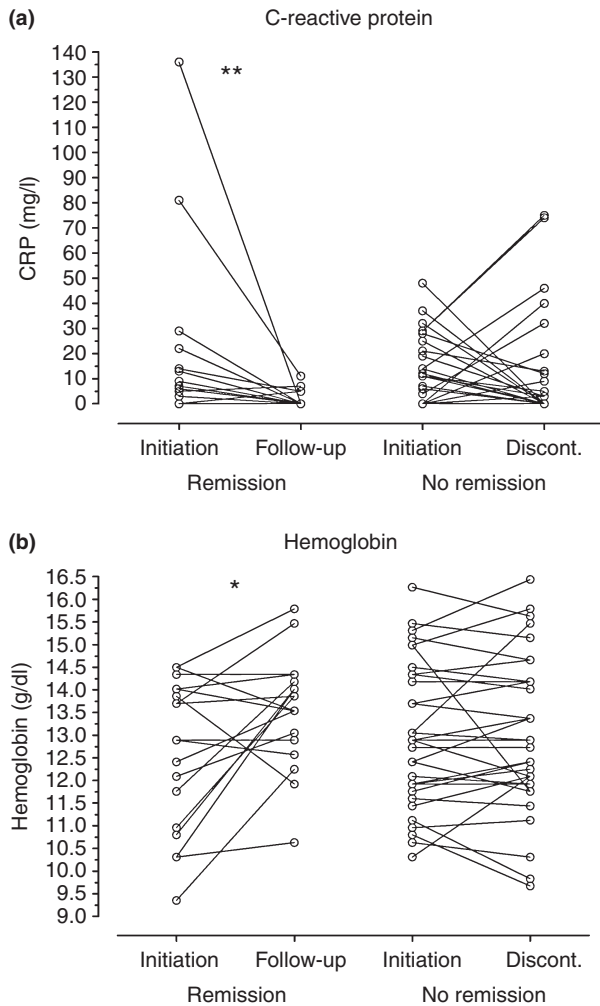


**Figure 2** | Changes in biochemical markers of disease activity during treatment of IBD patients with Infliximab (IFX). Shown are C-reactive protein (a) and haemoglobin levels (b) measured at the time of IFX initiation and at follow-up ( $n = 18$  in remission and  $n = 33$  not in remission). \* $P < 0.05$ ; \*\* $P < 0.01$ .

at 1 year had a relatively low IFX-Fab reactive IgG level of 377 (199–1301) mU/L (Figure 3). The corresponding level in patients who did not achieve remission was 646 (346–1041) mU/L ( $P = 0.291$ ).

#### Levels of pre-existing IFX-Fab reactive IgG associate with long-term remission

Receiver-operating characteristics analysis was applied to determine cut-off levels of pre-treatment IFX-Fab reactive IgG that provided optimal distinction between patients obtaining remission on IFX within 1 year of therapy and those who did not (Figure S2). The area under the ROC curve for patients with Crohn's disease

was 0.89 (95% confidence interval: 0.78–1.00), thus characterising an effective test ( $P = 0.001$ ). In contrast, the area under the ROC curve for ulcerative colitis was 0.64 (0.39–0.88) and not significantly different from 0.5, which characterises an ineffective test ( $P = 0.277$ ). Thus, pre-treatment levels of IgG Abs reacting with the Fab portion IFX-Fab appeared useful for prediction of long-term clinical efficacy in patients with Crohn's disease.

The cut-off levels of IFX-Fab reactive IgG that best differentiated between Crohn's disease patients with or without remission at 1 year are given in Table 3. A cut-off value of <439 mU/L appeared clinically relevant, and identified all patients in long-term remission on IFX (100% sensitivity) and with relatively high specificity (67%). Thirteen of 29 patients with Crohn's disease had levels of IFX-Fab reactive IgG below this cut-off value. Eight of these patients (62% of patients with values <439 mU/L) achieved remission within a year, and five (38%) did not. All 16 patients with levels above this cut-off failed to achieve remission within a year. As shown in Figure 4, this cut-off value separated two subgroups with clearly different duration of active disease ( $P = 0.003$ ); half of the patients with levels <439 mU/L had obtained remission after median 8 months of IFX maintenance therapy.

Applying alternative cut-off values of 233 mU/L for optimal differentiation between patients in remission or not in remission (i.e. sensitivity = specificity), or  $\geq 61$  mU/L for identification of all patients not in remission (i.e. 100% sensitivity), resulted in significant differences between survival curves of patients with IFX-Fab reactive IgG levels above or below these cut-offs ( $P = 0.016$  and  $P = 0.030$  respectively). However, as shown in Table 3, clinical usefulness of these cut-off values was limited due to the relatively low sensitivities and/or specificities.

#### Association between pre-treatment IFX-Fab reactive IgG and clinical efficacy response types

There was an overall difference in pre-treatment levels of IFX-Fab reactive IgG between different efficacy subgroups of patients with Crohn's disease ( $P = 0.017$ ). As shown in Figure S3A, significantly lower Ab levels were found in patients who obtained complete clinical remission (91 mU/L, IQR 61–228) than in patients with loss of response to IFX maintenance therapy (692 mU/L, IQR 210–1910;  $P < 0.01$ ). A less pronounced, albeit borderline significant, difference was observed between primary nonresponders (308 mU/L, IQR 203–987) and patients with complete remission ( $P = 0.05$ ). In contrast,

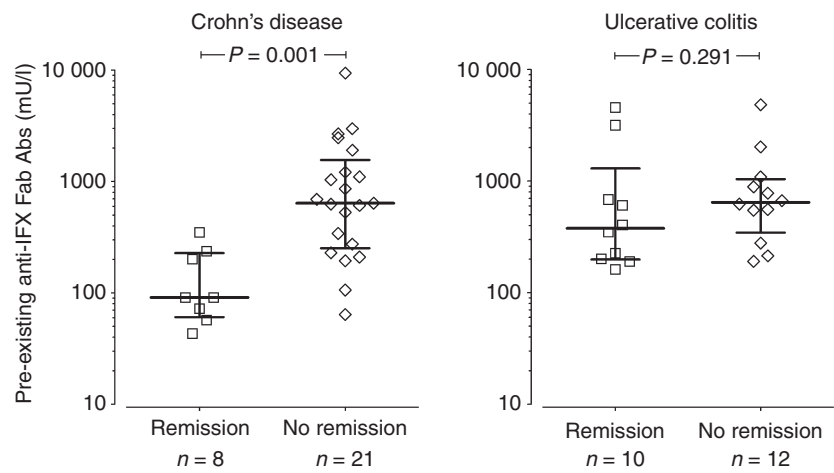


**Figure 3 |** Association between pre-treatment levels of IFX-Fab reactive IgG Abs and clinical response. Patients with Crohn's disease (left panel) or ulcerative colitis (right panel) were grouped according to clinical status (remission or no remission) 1 year after the initiation of IFX therapy. Shown are the levels of IgG Abs cross-reacting with the Fab portion of IFX in sera collected prior to the treatment. Horizontal bars and error bars represent median and IQR.

| Table 3   IFX-Fab Ab cut-off levels for differentiation of Crohn's disease patients with and without remission at 1 year |                       |                        |                        |
|--------------------------------------------------------------------------------------------------------------------------|-----------------------|------------------------|------------------------|
| Description                                                                                                              | Cut-off level* (mU/L) | Sensitivity % (95% CI) | Specificity % (95% CI) |
| Identification of patients in remission                                                                                  | <439                  | 100 (63–100)           | 67 (43–85)             |
| Identification of patients not in remission                                                                              | ≥ 61                  | 100 (84–100)           | 25% (3–65)             |
| Optimal differentiation between patients in remission or not (sensitivity = specificity)†                                | 233                   | 75 (35–97)             | 76 (53–92)             |

\*Pre-treatment level of IgG antibodies cross-reacting with the Fab region of IFX.

†Values below cut-off indicate that patients are in remission (sensitivity).

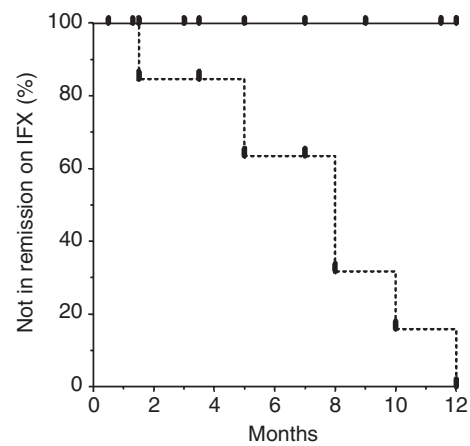


**Figure 4 |** Pre-treatment levels of IgG Abs reacting with IFX-Fab predict clinical response to IFX in Crohn's disease. The length of time without clinical remission on IFX was stratified according to pre-treatment serum levels of IgG Abs cross-reacting with the Fab portion of IFX, using a cut-off value for optimal identification of patients in remission at 1 year of IFX therapy (<439 mU/L,  $P = 0.003$ ). The dotted lines denote patients with Ab levels below the cut-off value and the solid line denote patients with higher values. Vertical ticks marks censored cases.

there was no overall difference between efficacy subgroups of patients with ulcerative colitis.

Association between pre-treatment IFX-Fab reactive IgG and safety

IFX is generally well tolerated, but occasionally results in side effects such as infusion reactions. Seven patients ( $n = 4$  with Crohn's disease and  $n = 3$  with ulcerative

colitis) experienced an infusion reaction to IFX. All reactions resulted in immediate and permanent discontinuation of IFX therapy. Three reactions were classified as acute and severe by the treating physician, and the clinical manifestations noted in the patient files were acute

severe malaise, severe dyspnoea, chest pain, tachycardia, hypotension, nausea and/or universal erythema. The remaining four reactions were classified as delayed, and the clinical manifestations were myalgia and/or arthralgia.

Pre-treatment levels of IgG Abs reacting with the Fab region of IFX were remarkably high in all patients with reactions (1037 mU/L, range 529–4840); significantly higher than in patients without reactions (349 mU/L, range 43–9409;  $P = 0.036$ ). Moreover, patients with reactions had substantially higher levels of IFX-Fab reactive IgG than the above-mentioned cut-off levels that distinguished patients with and without long-term remission on IFX.

There was no difference in total IgE levels prior to IFX therapy in patients with (median 0.3 µg/mL, IQR 0.2–1.3) or without reactions (0.3, 0.3–0.6).

### Changes in IFX-Fab reacting IgG during treatment with IFX

For all patients in this study, sera were available at one or more time points after starting IFX therapy. We compared the posttreatment levels of IgG Abs binding to IFX-Fab, which may contain specific Abs to unique epitopes of IFX, with the corresponding pre-treatment levels. Assuming that IFX was equally immunogenic in Crohn's disease patients and ulcerative colitis patients, we divided the 51 patients in this study into two groups: Patients in remission at the end of the 1-year follow-up period, and those who were not. The posttreatment samples available for patients in remission were taken after a median of nine infusions (IQR: 5–11), corresponding to 359 (IQR: 229–462) days (data not shown). In this period, the levels of IgG Abs binding to IFX-Fab increased by 47% from 214 (91–455) mU/L to 315 (IQR: 182–968) mU/L ( $P = 0.207$ ). In comparison, post-IFX samples from patients not in remission were obtained after three (IQR: 2–4) infusions, corresponding to 91 (IQR: 41–132) days. In this short period, the median levels of IFX-Fab reactive IgG Abs increased significantly, by 63%, from 639 (IQR: 276–1159) mU/L to 1041 (IQR: 302–2018) mU/L ( $P = 0.042$ ).

## DISCUSSION

Only about one third of patients with IBD achieve long-term clinical remission on treatment with IFX.<sup>6, 9, 30</sup> Methods for prediction of patient responses to IFX would be a useful tool for clinicians facing the choice between IFX therapy and treatment with other, more humanised therapeutic Abs. We show here that IgG Abs

reacting with the Fab portion of IFX exist in healthy individuals and, at comparable levels, in patients with IBD prior to exposure to IFX. Furthermore, pre-treatment levels of such Abs were associated with both efficacy and safety responses to IFX in patients with Crohn's disease. Similar tendencies were observed in patients with ulcerative colitis.

Relatively low pre-treatment serum levels of IFX-Fab reactive IgG Abs were found in Crohn's disease patients with sustained response to IFX at 1 year. The Ab levels were substantially higher in patients without remission at 1 year, especially in patients who responded to IFX initially, but gradually lost response (secondary nonresponders). The differences in Ab levels between patients with and without maintained response could not be attributed to differences in total IgG (or IgG subclasses), nor to concomitant immunosuppressive treatment. ROC analysis of efficacy data from patients with Crohn's disease suggested that a cut-off value of 439 mU Ab per litre was clinically relevant and distinguished IFX responders from nonresponders (100% sensitivity and 67% specificity). A high sensitivity (at the expense of specificity) would seem desirable in a clinical setting, as few patients who would potentially benefit from IFX treatment would, in theory, be withheld this therapeutic option. Kaplan–Meier analysis revealed a highly significant difference in disease-free condition between patients with Ab levels below and above 439 mU/L. Taken together, it seems possible to define a cut-off level that distinguishes between long-term responders and nonresponders to IFX in Crohn's disease. Prospective clinical studies are, however, needed to define the utility of measuring pre-existing IFX-Fab reactive Abs in a clinical setting.

In support of an association between IgG Abs to IFX's Fab portion and clinical response to IFX, we also observed a marked difference in the median Ab levels between ulcerative colitis patients with and without long-term remission (377 mU/L and 646 mU/L respectively). This difference was, however, not statistically significant, and was most likely due to a statistical type 2 error (lack of power because of a small number of participants). However, IFX is less efficient in ulcerative colitis (presumably reflecting the fact that ulcerative colitis is driven by TNF to a lesser extent than Crohn's disease), which hampers demonstration of differences between subgroups with different levels of IFX-Fab reactive Abs in this patient group.<sup>3, 31</sup> The substantially higher proportion of ulcerative colitis patients in remission receiving concomitant immunosuppressive therapy may also have biased our findings.

The Abs reported in this study are likely to recognise common, well-conserved framework sequences from the murine part of IFX. In support of this notion, the level in normal serum of IgG binding to the F(ab')<sub>2</sub> regions of IFX correlates strongly with the level of IgG Abs binding to F(ab')<sub>2</sub> regions of murine IgG (Palarasah *et al.*, submitted). However, as the Fab part of IFX contains the G1m17 allotype carried by the minority of the Caucasian population,<sup>32</sup> the pre-existing IFX-Fab reactive Abs may also contain antiallotype Abs induced by maternal–foetal IgG transfer. It is unlikely that the pre-existing Abs characterised in this study recognise the TNF-binding part (the paratope) of IFX, which is unique to IFX and to which the patients are naïve. Irrespective of this, however, formation of IFX/anti-IFX complexes (that might activate complement) may facilitate clearance through Fc- and complement receptor-mediated phagocytosis, leading to low serum IFX levels. Subsequently, epitope spreading may occur, and the pre-existing IFX-Fab reactive Abs may thus facilitate the formation of Abs against the paratope, which may lead to secondary nonresponse.

The group of primary nonresponders presumably contains a subgroup of patients without TNF-driven disease, as indicated by observations that primary IFX response failure often occurs in the presence of high circulating drug levels.<sup>33</sup> It was therefore surprising to find a borderline significant association between pre-existing IFX-Fab reactive Abs and primary nonresponse. This may reflect that the group of primary nonresponders is heterogeneous; some patients may fail to achieve a response because of a relatively TNF-independent pathogenesis, whereas others may fail because of relatively low bioavailability of the drug combined with the presence of pre-existing Abs reacting with IFX. Clearly, pharmacokinetic and pharmacodynamic studies are needed to test this hypothesis. In any event, the levels of IFX-Fab reactive Abs observed in secondary nonresponders in this study (median 692 mU/L) were considerably higher than those in primary nonresponders (median 308 mU/L), as expected.

In the group of IBD patients achieving remission after 1 year, the level of IFX-Fab binding IgG Abs increased moderately by 47% within about 1 year. However, the posttreatment levels in this group reached only half the value of the pre-treatment levels of patients not in remission after 1 year. The level of IFX-Fab binding Abs in patients not in remission after 1 year, however, increased by 63% within a median of only 3 months, i.e. at a considerably higher rate. Together with the high baseline levels, this rapid increase in Ab levels may explain inadequate responses or infusion reactions to IFX merely on the basis of increased Ab:IFX ratios.

As stated above, however, the most important factor determining poor responses may be a change in Ab specificity towards the formation of IFX-neutralising Abs, which may occur to a higher extent in patients with strong pre-existing B- and T-cell responses against IFX.

Seven patients (14%) experienced infusion reactions during this study: three were acute and severe, and four were delayed. Notably, these patients had very high pre-treatment levels of IgG Abs reacting with IFX-Fab (median >1000 mU/L) – levels that were considerably higher than the cut-off levels suggested above. Thus, limiting the use of IFX to patients with Ab levels below the cut-off may exclude patients who would experience infusion reactions. Of note, examining a mixed cohort of patients with rheumatoid arthritis, vasculitis or spondyloarthritis, Vultaggio *et al.* observed non-isotype specific Abs to IFX in 8 of 11 patients that developed before the second and third infusions. They found that three patients with severe reactions had IgE Abs to IFX and positive skin testing.<sup>34</sup>

The pre-treatment serum levels of IgG Abs reacting with the Fab portion of IFX tended to be lower in patients with Crohn's disease than the serum levels in ulcerative colitis and the plasma levels in healthy controls. The former difference is in keeping with earlier findings of lower levels of total IgG in patients with Crohn's disease as compared with patients with ulcerative colitis.<sup>35</sup> We did not detect IgE Abs to IFX-Fab in any of the pre-treatment sera, and measurement of IgA and IgM Abs did not fulfil our technical requirements. It cannot be ruled out that Abs of isotypes other than IgG affect clinical response to IFX and other chimeric therapeutic Abs, as has previously been suggested elsewhere.<sup>36</sup>

The study limitations include a relatively small cohort, and lack of standardised evaluations using clinical scoring systems for classification of patients. The treating physician's classification of patients as being in remission or not was, however, supported by significant changes in common biochemical markers of disease activity. About one third of patients in our cohort were classified as in remission after 1 year of IFX maintenance therapy, one third developed secondary loss of response and one third had primary nonresponse. This distribution of clinical response types is in line with previous reports from large-scale clinical trials in Crohn's disease.<sup>6–8, 19</sup> Due to its retrospective nature, the study was not designed to assess changes in IFX-Aab binding Abs over time, as

posttreatment samples were not taken at fixed time points. Moreover, a bioassay for assessment of neutralising anti-IFX Abs was not employed. Finally, IFX trough levels were not available.

To our knowledge, this is the first study to examine the impact of pre-existing IFX-Fab reactive Abs on the clinical outcome of IFX treatment. Several investigators have examined development of specific anti-IFX Abs during the course of IFX therapy, and some have included measurements of pre-treatment levels of Abs without finding positive samples.<sup>21, 27, 34, 37</sup> This is presumably related to the general use of a threshold between positive and negative tests, which defines the majority of healthy controls and IFX-naïve patients as negative. Our assay would operate almost exclusively below such thresholds. Here, we used a pool of 50 healthy blood donors as a calibrator; it is possible for others to test our data using a similar calibrator. However, development of a more uniform standard, for example a given commercially available preparation of purified IgG for intravenous use, would be desirable.

In conclusion, circulating IgG Abs cross-reacting with IFX may be present before IFX treatment in patients naïve to biological agents, and may predict long-term clinical efficacy and safety of IFX in Crohn's disease and ulcerative colitis. If this observation can be confirmed in larger, prospective studies, measurement of these Abs before initiation of IFX treatment, using a generally accessible assay with well-defined cut-off values, might help physicians to select the best type of therapeutic TNF-blocker in individual patients.

## AUTHORSHIP

*Guarantor of the article:* None.

*Author contributions:* Study design and interpretation of data: CS+ BT + CHN; Collection of data: CS +YP + CHN; Analysis of data: CS + CHN; Drafting the manuscript: CS and CHN. All authors approved the final version of the manuscript.

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Novo-Nordisk, Bristol-Meyers Squibb and Biomedica A/S. A Teisner has served as a speaker for MSD. CH Nielsen has served as a speaker for Roche, Bristol-Meyers Squibb, Fresenius Medical Care, Schering-Plough and MSD. K Bendtzen owns stocks in Biomedica A/S. CH Nielsen, Y Palarasah and B Teisner have a pending patent application 'Pre-existing antibodies to therapeutic chimeric antibodies as predictors of clinical responses'. J Brynskov has no interests to declare.

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## SUPPORTING INFORMATION

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

**Figure S1.** ELISA for measurement of IgG Abs reacting with IFX-Fab. (a) Infliximab (IFX) was cleaved by pepsin and F(ab')<sub>2</sub> fragments were isolated using commercially available kit #44885 (Pierce, Rockford, IL, USA). A subsequent SDS-page analysis is shown. Left lane: MW marker (Novex Sharp), lane 1: intact IFX, lane 2: F(ab')<sub>2</sub> fragments of IFX. (b) Full cleavage of IFX was further demonstrated because biotinylated monoclonal antihuman Fcγ-chain Ab (1.5 µg/mL) and subsequently horseradish peroxidase (HRP)-conjugated streptavidin did not bind to plates coated with F(ab')<sub>2</sub> fragments in the absence of proband serum (left column). Human serum albumin (HSA; middle column) served as negative control and intact IFX (right column) as positive control. Mean ± s.d. of four experiments is shown. (c) In the final ELISA, the plates were coated with F(ab')<sub>2</sub> fragments of IFX, calibrator and proband samples were added in two-fold dilution series, and antihuman Fcγ-chain/HRP-streptavidin was used for detection. The calibrator was a pool (*n* = 50) of normal human plasma. A two-fold dilution series of plasma from a representative donor (no. 39) was included to demonstrate parallelism in relation to the calibrator. The dotted line indicates the detection limit defined by plates coated with HSA (negative control).

**Figure S2.** ROC curve of pre-treatment levels of IgG Abs reactive with IFX-Fab in patients with Crohn's disease. The curve was used to determine cut-off levels asso-

ciated with clinical remission at 1 year of maintenance therapy. The dotted line describes an ineffective test.

**Figure S3.** Pre-treatment levels of IFX-Fab reactive IgG Abs and clinical response types to IFX. Medians are shown

as horizontal bars. Two patients with Crohn's disease had a partial effect of IFX maintenance therapy at the time of follow-up and were excluded.

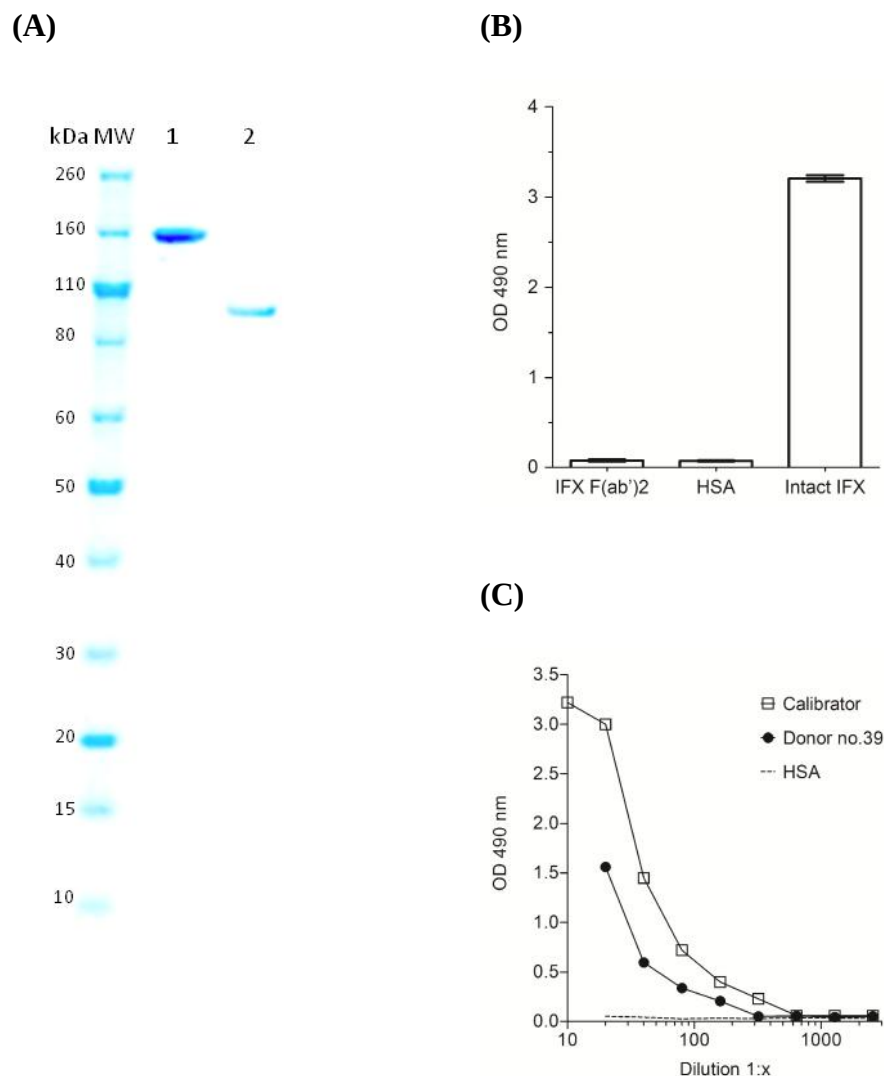
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## SUPPORTING FIGURES

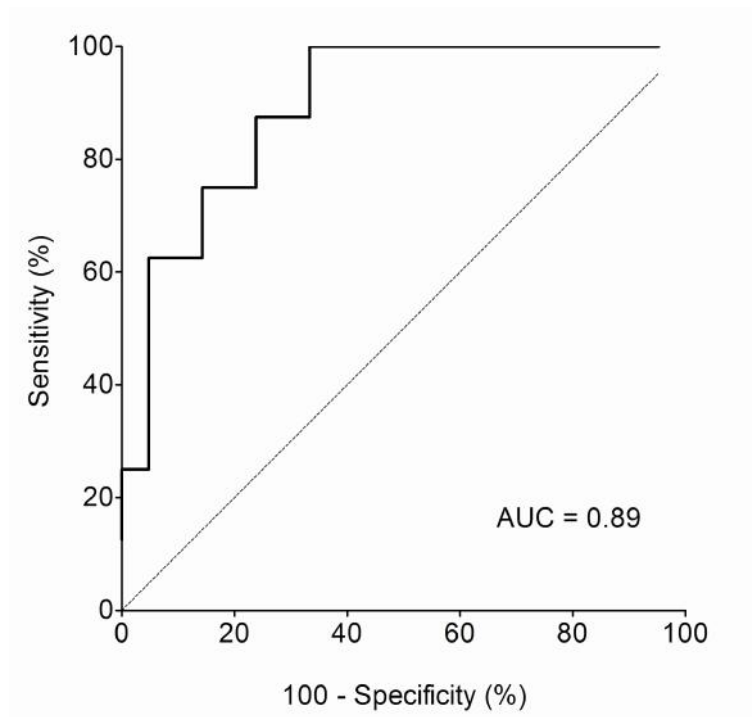
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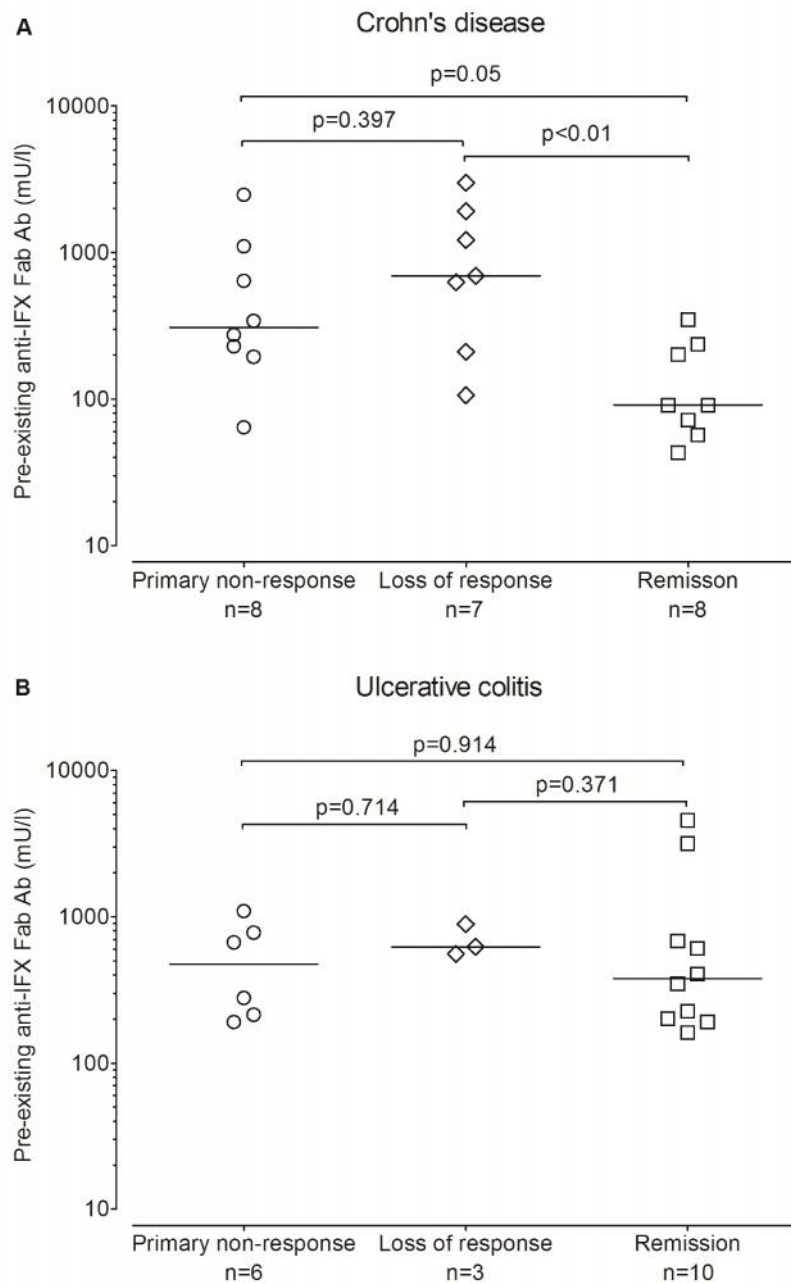
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**Figure S2.**



ROC curve of pre-treatment levels of IgG Abs reactive with IFX-Fab in patients with Crohn's disease. The curve was used to determine cut-off levels associated with clinical remission at 1 year of maintenance therapy. The dotted line describes an ineffective test.

**Figure S3.**



Pre-treatment levels of IFX-Fab reactive IgG Abs and clinical response types to IFX. Medians are shown as horizontal bars. Two patients with Crohn's disease had a partial effect of IFX maintenance therapy at the time of follow-up and were excluded.