



Trough s-infliximab and antibodies towards infliximab in a cohort of 79 IBD patients with maintenance infliximab treatment



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Abstract

Background and Aims: The anti-TNF antibody infliximab is effective in inducing remission in Crohn's disease as well as in ulcerative colitis and many patients are treated for several years with sustained clinical remission. However, the role of monitoring s-infliximab and antibodies towards infliximab during maintenance treatment remains unclear. Our aim was to correlate serum drug levels and antibodies to clinical activity, CRP, albumin and concomitant immunosuppression in a cohort on maintenance infliximab treatment.

Methods: We included 79 patients with Crohn's disease or ulcerative colitis who had responded to infliximab and received maintenance treatment (4–69 infusions) in this retrospective study. Infliximab levels and antibodies towards the drug were analyzed with in-house-developed ELISA assays.

Results: The mean s-infliximab was significantly higher in patients in remission (4.1 µg/mL) as compared with disease flare (mean 1.8 µg/mL); $p < 0.001$. The s-infliximab showed a significant negative correlation with Harvey–Bradshaw index ($r = -0.21$; $p < 0.05$). Serum-infliximab progressively decreased with the number of accumulated infusions ($p < 0.05$). In patients with undetectable trough levels, 55% of the patients with concomitant immunosuppressive were positive for antibodies against infliximab, as compared with 94% of patients on monotherapy. Patients with undetectable serum-infliximab were in clinical remission at 25% of the visits.

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Conclusions: The trough level 4.1 µg/mL may serve as cut-off for clinical remission. Drug trough levels decreased during treatment and almost all patients with undetectable s-infliximab and monotherapy had developed antibodies against the drug.

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1. Introduction

Infliximab (IFX, Remicade) is a chimeric monoclonal IgG₁ antibody against Tumor Necrosis Factor (TNF), an important cytokine in the intestinal inflammation seen in inflammatory bowel disease (IBD). The drug is effective in inducing remission in Crohn's disease (CD) as well as in ulcerative colitis (UC), and many patients are treated for several years with maintained clinical remission.^{1–3} The immunogenicity of biologics in IBD was first identified in a study by Baert et al., suggesting that patients may develop antibodies to IFX (ATI), particularly when IFX was administered on episodic basis.⁴ However, even with scheduled treatment, 6–17% of the patients develop antibodies to IFX.^{5,6} The correlation between infusion reactions and ATI is evident and three studies have demonstrated significantly lower trough s-IFX levels (s-IFX just before the next infusion) in patients with ATI, probably due to increased clearance of the drug.^{7–10} ATI formation as well as low s-IFX has been associated with loss of response (LOR).^{4,11–13} However, a clear correlation between clinical efficacy of IFX in terms of mucosal healing and ATI has been difficult to establish and overall, the success of the treatment seems to be more closely related to s-IFX than ATI levels. Trough levels of IFX have even been suggested to have a strong predictive value for response during induction.^{11,13,14}

The role of monitoring s-IFX and ATI in the clinic has been debated. The level of evidence in the area is quite low due to a limited number of studies with small cohorts, retrospective design and different methodological approaches.^{10,12,15} When introducing a patient to biologics, analysis of drug trough levels at week 14 has been suggested in order to establish control of the induction phase and obtain decision basis for dose escalation in case of poor response.¹⁴ In the situation of loss of response (LOR), which appears in up to 70% of patients on IFX, analysis of trough s-IFX and ATI might help the treating physician in the decision of escalating IFX dose or switch to another drug.^{7,12,16} However, the value of drug monitoring has been questioned in this situation since studies have reported that ATI do not always lead to abrogated response to increased dosage of IFX.^{10,15} Observations that the ATI status may be transient in up to 28% of the ATI-positive patients may explain the difficulty in interpreting these data.^{4,14} The issue of discontinuing IFX in IBD patients with sustained clinical and endoscopic remission has been addressed in the STORI trial.¹⁷ Low trough s-IFX has been proposed to be one of the predictive markers for sustained remission after removal of IFX in these patients. The analysis might therefore be useful when identifying patients eligible for discontinuation of biologics.¹⁷

In previous studies, the patients have often been recently introduced to IFX or have lost response to the treatment. In our report, we have included 79 patients with Crohn's disease or ulcerative colitis who have responded to IFX and receive maintenance treatment. Our aim has been to correlate serum IFX levels and ATI analyzed by our in-house-developed assays to clinical activity indices, CRP, albumin and concomitant immunosuppression in this maintenance cohort of IBD-patients.

2. Materials and methods

2.1. Patients and samples

Patients were recruited from a population of maintenance IFX-treated patients with IBD (63 with Crohn's disease, 15 with ulcerative colitis, and one with indeterminate colitis). All patients but nine had received induction therapy and all were considered to benefit from maintenance treatment. The mean dose was 5.3 ± 1.4 mg/kg with a mean interval of 57.2 ± 20.8 days. This is a retrospective study with prospectively collected samples. Blood tests were obtained immediately before the next infusion of IFX (trough level). See Table 1 for patient characteristics. The sera were separated and handled according to standard operative procedures and stored at –20 °C until analysis. CRP (mg/L) and albumin (g/L) were registered at every infusion, with the exception of two and 31 out of 204 visits respectively. Calprotectin (µg/g) was collected in 56 out of the 79 patients and the calprotectin values were considered to reflect the inflammatory activity of the patient during the

Table 1 Background characteristics of the patient cohort.

Total number of patients	79
Gender (male)	50 (63%)
Age at inclusion (median, years)	36 (range 18–74)
Weight (median, kg)	78 (range 40.4–114.5)
Diagnoses	
Crohn's disease	63 (80%)
Ulcerative colitis	15 (19%)
Indeterminate colitis	1 (1%)
Concomitant immunosuppression	
Thiopurines	26 (33%)
Methotrexate	2 (3%)
Mycophenolate mofetil	1 (1%)
Disease activity parameters	
Harvey–Bradshaw index (median)	1 (range 0–13)
Mayo score (median)	0 (range 0–10)
CRP (mg/L)	
Median (range)	1 (1–97)
Mean (SD)	6.5 (11.2)
Calprotectin (µg/g)	
Median (range)	155.5 (20–3749)
Mean (SD)	587 (852)
Albumin (g/L)	
Median (range)	38 (27–44)
Mean (SD)	37.2 (3.7)

observation period. Harvey Bradshaw index (HBI, for Crohn's disease) and Mayo score (for ulcerative colitis) were calculated for each visit. The patient was considered in remission when HBI < 5 or Mayo score < 3 and CRP < 3 mg/L.

2.2. Infliximab ELISA

Concentration of infliximab in serum samples was determined with an in-house developed ELISA. The method was thoroughly validated based on guidelines from Clinical and Laboratory Standards Institute (CLSI). Similar methods have been published before by several groups.^{4,18–20}

The micro-titer plates used were Nunc Maxisorp F96 (Thermo-Fisher Scientific, Roskilde, Denmark). The plates were coated with 100 ng/mL, 50 μ L/well, of recombinant human TNF-alpha (R&D Systems, Minneapolis, USA) in 0.05 M sodium carbonate buffer pH 9.6. The plates were put on a shaker at room temperature (RT) for 2 h and incubated overnight in the cold (+4 °C). The plates were washed 3 times in phosphate buffered saline (PBS) plus 0.05% pH Tween 20 and blocked with PBS + 1% BSA (Sigma, St. Louis, MO, USA) and 0.05% Tween 20 (blocking buffer) for 1 h at RT. Following an additional wash, six standard dilutions (0.40–100 ng/mL) of infliximab (Schering Plough, New Jersey, USA) in blocking buffer were added to the plate simultaneously with serum aliquots spiked with defined concentrations of infliximab (internal controls) and serum samples, diluted 1/500 in blocking buffer. All additions were made in duplicates. The plates were incubated on a shaker at RT for 1 h and washed four times followed by addition of alkaline phosphatase conjugated goat anti-human IgG Fc-specific (Sigma, St. Louis, MO, USA) diluted 1/10,000 in blocking buffer. After incubation for 1 h on a shaker at RT, the plates were washed four times. Substrate (p-nitrophenyl-phosphate, 5 mg/mL in

1 M diethanolamine with 0.5 mM Mg, pH 9.8) was added. Color development was monitored at 405 nm and the final reading was taken when the highest standard reached an OD of approximately 2.0. The standard curve was constructed using a 4-parameter curve fit and the concentration of samples and controls was calculated from the standard curve. Lower and upper limits of quantification were 0.2 μ g/mL and 55 μ g/mL, respectively (compensated for serum dilution 1/500). The total uncertainty of measurement did not exceed 14% CV as determined by measurements of spiked negative control sera (ANOVA).

2.3. Inhibition ELISA for ATI detection

Antibodies to infliximab (ATI) were analyzed with an in-house developed ELISA, based on the inhibition of binding of labeled infliximab to TNF-alpha coated to the ELISA plate. A similar ELISA has been described previously.¹⁸

Alkaline phosphatase was coupled to infliximab by using a commercial kit; Lightning-Link from Innova (Innova Biosciences Ltd., Cambridge, UK). The plates (see above) were coated, 50 μ L/well, with 100 ng/mL of recombinant human TNF-alpha (R&D Systems, Minneapolis, USA) in 0.05 M sodium carbonate buffer pH 9.6. The plates were put on a shaker at room temperature (RT) for 2 h and further incubated overnight in the cold (+4 °C). The plates were washed three times in phosphate buffered saline (PBS) plus 0.05% pH Tween 20 and incubated with blocking buffer for 1 h at RT. 20 μ L of standard (goat anti-human IgG, see above) at a concentration of 23 μ g/mL and serum samples were incubated with 180 μ L of alkaline phosphatase conjugated infliximab for 1 h at RT. Following an additional wash of the coated and blocked plate, 50 μ L in duplicate was transferred from the incubated sera and standard to the TNF-coated plate which thereafter was incubated, with

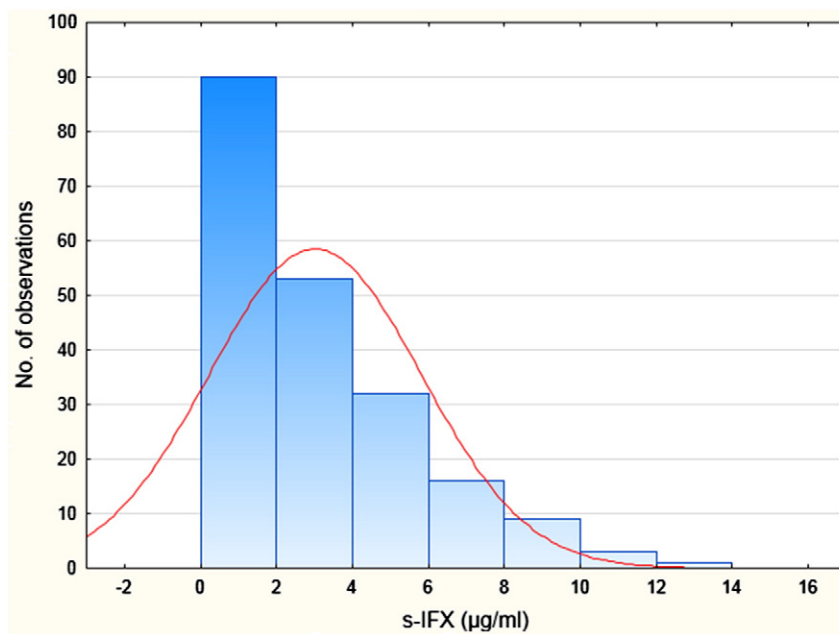


Figure 1 The distribution of s-IFX levels in 204 serum samples from 79 maintenance-treated IBD patients (1–5 samples per patient, median 2), obtained immediately before the next scheduled infusion. Mean s-IFX trough level was 3.0 μ g/mL (<0.2–13.0).

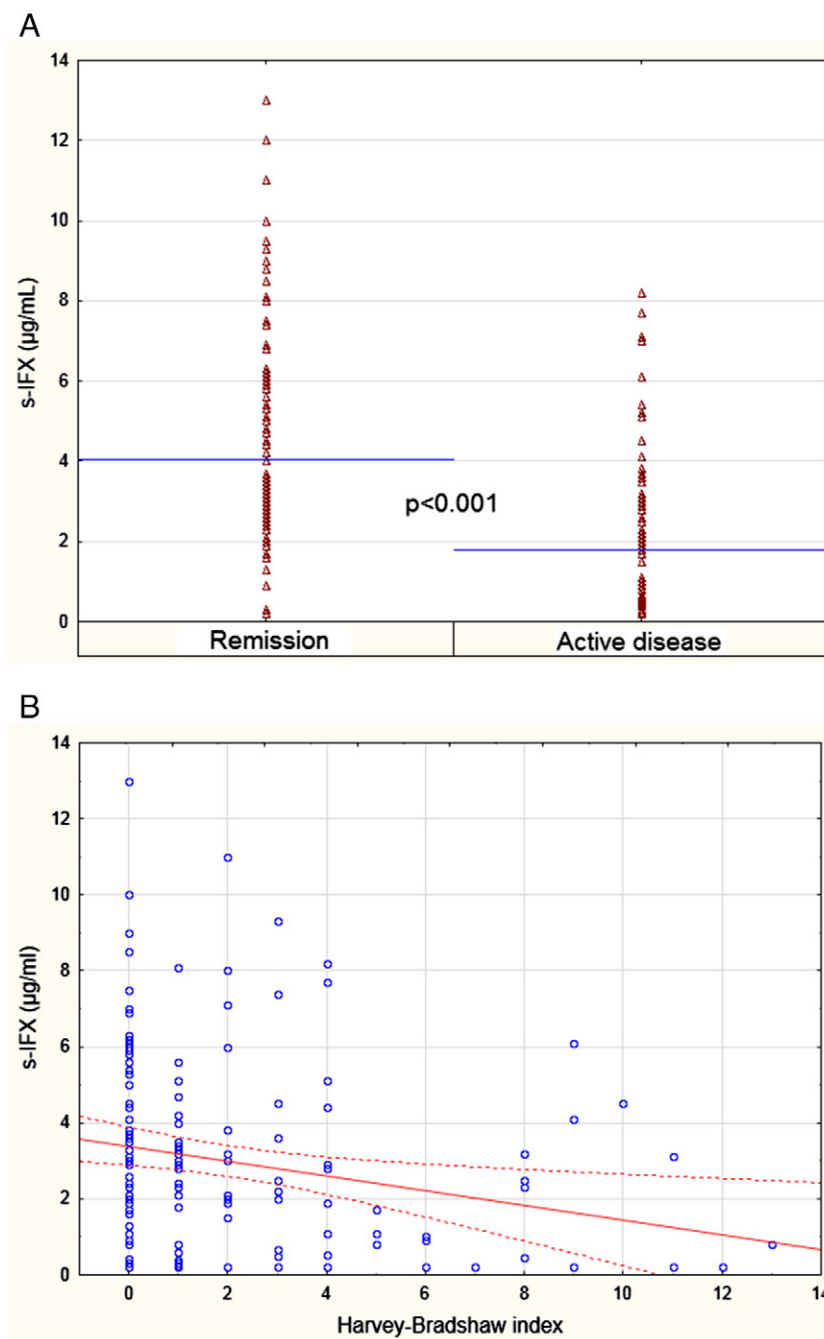


Figure 2 The mean s-IFX level is higher in patients with clinical remission. In the samples obtained during clinical remission mean s-IFX was 4.1 µg/mL, as compared with 1.8 µg/mL in samples obtained during active disease (A, $p > 0.05$, two-tailed t-test). B: In patients with Crohn's disease ($n = 63$), s-IFX levels showed a negative correlation with the Harvey–Bradshaw index (B, $p < 0.05$, linear regression analysis).

shaking, for an hour at RT. After another four cycle wash, substrate (see above) was added and color development at 405 nm was monitored. The final reading was taken when OD of the blank reached 2.5. The results were transformed to % inhibition by normalization of the samples' OD to that of the standard (100% inhibition), using the formula $(OD\ blank - OD\ sample) / (OD\ blank - OD\ standard) \times 100$. Lower limit of detection was set to the mean value plus 2 standard deviations obtained from the measurements of 120 normal control sera.

ATI could only be detected in the absence of the drug due to interference of infliximab in the ELISA assay.

2.4. Statistical analysis

Correlations between clinical parameters, routine chemistry and s-IFX were assessed with simple linear correlation analysis. Intergroup comparisons with respect to diagnosis,

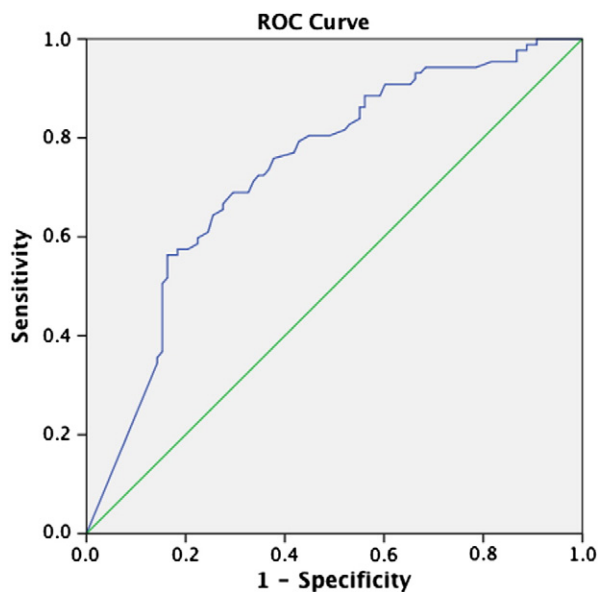


Figure 3 Receiver operator curve (ROC) of s-IFX trough levels predicting clinical remission. Area under the curve = 0.737 (s.e. = 0.037; $p < 0.001$).

disease score and use of immunosuppressive drugs were performed with the two-tailed t-test. ATI positive and negative patients were compared using Mann–Whitney U and Fischer's exact tests. Analyses were performed with the Statistica® software, version 11. Significance was set at $p < 0.05$.

2.5. Ethics statement

The study was approved by the local Ethics Committee. Blood samples were obtained with informed and written consent from all individuals in accordance with the Helsinki II Declaration.

3. Results

A total number of 204 serum samples were collected from the 79 patients (1 to 5 samples per patient, median 2). The patients have received a mean number of 25 infusions (4–69). All samples were taken immediately before the next scheduled infliximab infusion and analyzed for s-IFX (trough level) with an in-house developed ELISA. In addition, routine chemistry tests were obtained and clinical parameters for calculating HBI or Mayo score were recorded.

Mean serum-sIFX trough levels were $3.0 \mu\text{g/mL}$ (range <0.2 – 13.0), displaying a left-shifted Gaussian distribution (Fig. 1). The s-IFX level correlated negatively with the number of days since the previous infusion ($r = -0.18$; $p < 0.05$). There was no significant difference in trough levels between patients with or without concomitant immunosuppressive medication. In association with 185 of the samples, the clinical disease activity score was assessed (Harvey–Bradshaw for CD and Mayo score for UC). The patients were found to be in remission at 98 of the

visits with no significant difference in remission rates between CD and UC patients. The mean s-IFX was significantly higher in the samples obtained during remission ($4.1 \mu\text{g/mL}$) as compared with sera taken in association with a disease flare (mean $1.8 \mu\text{g/mL}$); $p < 0.001$ (Fig. 2A). As expected, these groups also differed significantly in CRP levels (mean 1.2 versus 12.7 mg/L ; $p < 0.001$), f-calprotectin (255 versus $984 \mu\text{g/g}$; $p < 0.001$) and albumin (mean 38.9 versus 36.0 g/L ; $p < 0.001$). The s-IFX showed a weak but significant ($r = -0.21$; $p < 0.05$) negative correlation with the Harvey–Bradshaw index in the patients with Crohn's disease (Fig. 2B). The receiver operator curve (ROC) analysis of s-IFX showed that a trough level above $4.1 \mu\text{g/mL}$ predicted the remission with 87% sensitivity and 44% specificity (Fig. 3).

s-IFX correlated with the inflammatory burden of the patients, as assessed by CRP ($r = -0.32$; $p < 0.05$; Fig. 4A) and albumin ($r = 0.44$; $p < 0.05$; Fig. 4B). We also observed that s-IFX progressively decreased with the number of accumulated infusions of the drug ($r = -0.25$; $p < 0.05$; Fig. 5). Approximately half of the samples were obtained before the 20th infusion. The mean s-IFX in these samples was $3.5 \mu\text{g/mL}$, as compared with $2.4 \mu\text{g/mL}$ in those obtained after the 20th infusion ($p < 0.01$). However, there was no significant difference in disease activity or inflammatory burden between these two groups. Moreover, no significant difference was seen in the use of concomitant immunosuppressives or induction regimens.

One potential reason for low trough levels is the development of antibodies against infliximab. In order to measure such antibodies (ATI), we developed an ELISA based on the inhibition of binding of labeled infliximab to plate-bound TNF-alpha. As is the case with most available immunoassays for detection of anti-drug antibodies, the presence of residual drug in the circulation interferes with the analysis, and ATI detection was therefore confined to patients with unmeasurable s-IFX. In 51 samples in the present study, corresponding to 28 individual patients, 21 with CD; 6 with UC and 1 with indeterminate colitis, s-IFX was below the detection limit of the assay ($<0.2 \mu\text{g/mL}$). Forty-nine of these sera were subsequently analyzed for the presence of ATI and 39 (80%) were found to be positive. Translated to individual patients; 22 out of 28 patients (79%) tested positive for ATI on at least one occasion. In ten patients ATI was detected in more than one sample and in four patients, the ATI-detection was transient with positive as well as negative tests. In all six patients with no ATI the analysis was only performed at one occasion. There were no significant differences in clinical disease activity scores, CRP or albumin between ATI positive and negative samples. Interestingly, at 25% of the visits, the patient was judged to be in clinical remission ($\text{H-B} < 5$ or $\text{Mayo} < 3$, and $\text{CRP} < 3 \text{ mg/L}$), despite undetectable s-IFX. This was corroborated in the analysis of individual patients with non-detectable trough levels, of whom five patients were in remission at all visits (18%), five patients were in remission at least at one but not all visits and 15 patients were exclusively classified as active (54%). Activity indices were missing in three patients.

Concomitant use of other immunosuppressive medication has been reported to reduce ATI formation.^{4,5} Among the 28 patients with undetectable trough levels, 11 patients were on immunosuppressive drugs (excluding corticosteroids); ten used azathioprine and one had methotrexate. Five of these patients (45%) tested negative for ATI, as compared with only one ATI-negative patient in the group of 17 patients

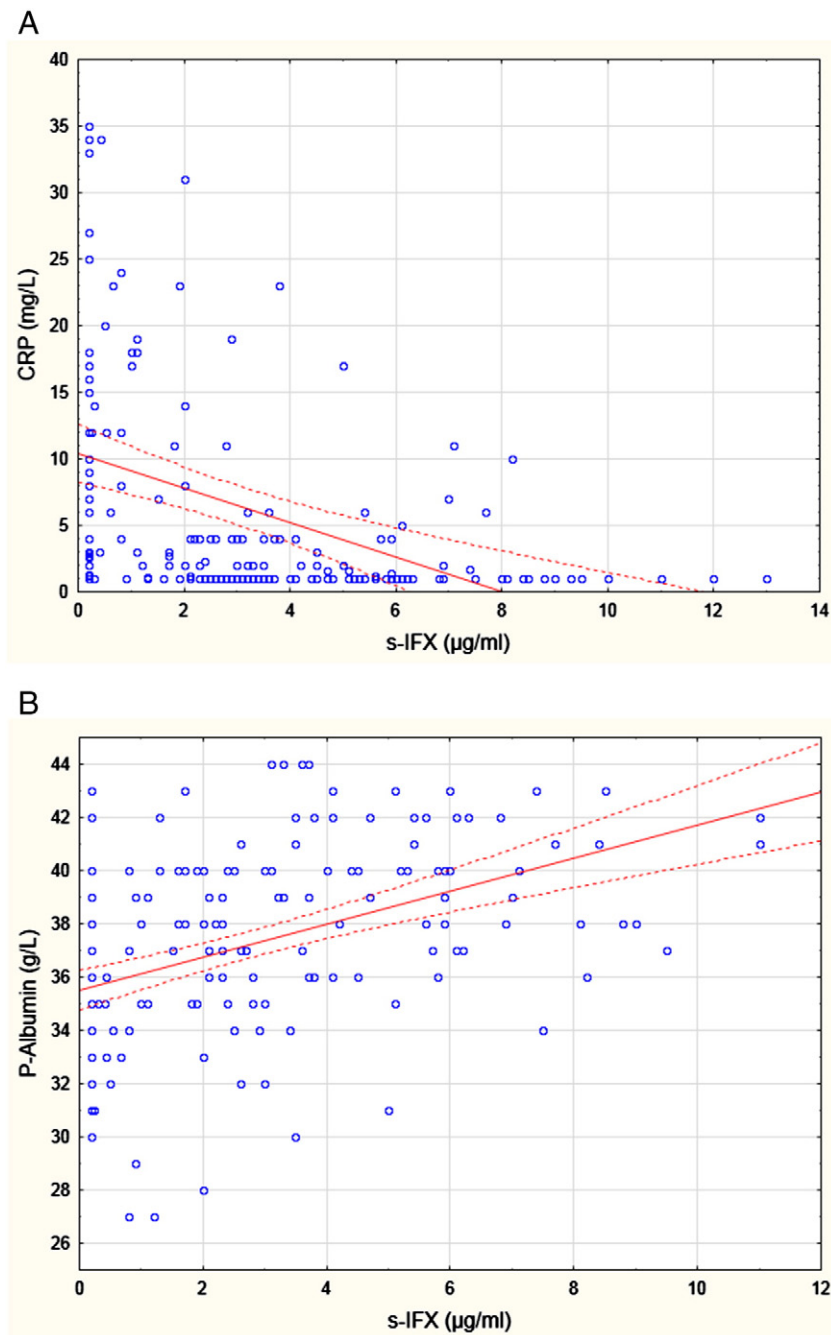


Figure 4 Serum-IFX is correlated with the inflammatory burden of the patients. Serum-IFX showed a negative correlation with CRP levels (A, $p < 0.05$, linear regression analysis) and positive correlation with serum albumin (B, $p < 0.05$, linear regression analysis).

without immunosuppressives and undetectable trough levels ($p < 0.05$, Fisher's exact test; Fig. 6).

4. Discussion

In the present study we have developed in-house assays for IFX trough levels and antibodies towards IFX. We have correlated these analyses with clinical parameters in a cohort of 79 patients with Crohn's disease or ulcerative colitis who have

responded to IFX and continued with maintenance IFX treatment.

The use of drug monitoring has been debated and technical problems have clouded the application of trough levels and especially ATI in the clinical context. A clear correlation has been found between s-IFX and clinical remission, CRP, and endoscopic improvement.¹¹ Moreover, in UC, s-IFX below 1.4 µg/mL is clearly associated with colectomy.¹³ On the other hand, it has been difficult to establish any strong correlation between ATI and the aforementioned clinical

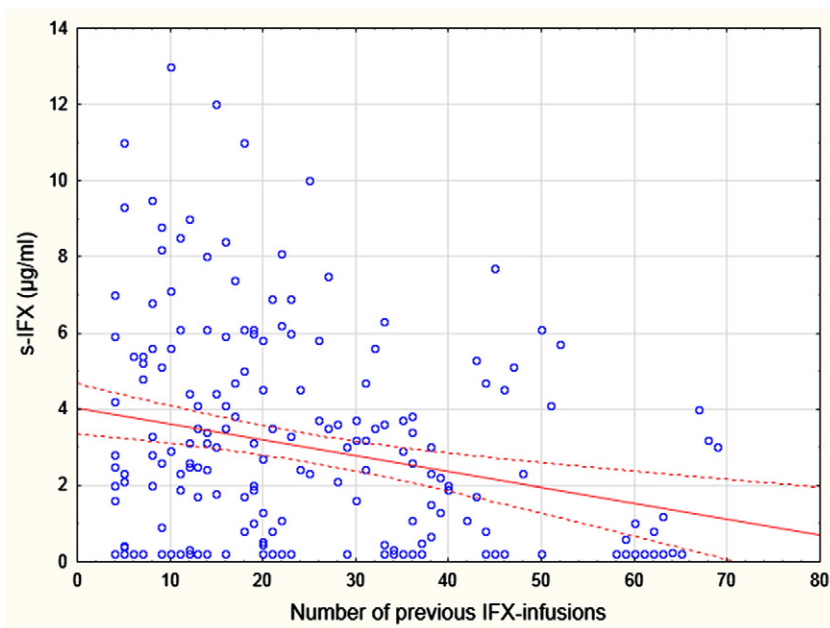


Figure 5 The s-IFX levels correlated negatively with the number of accumulated infusions of infliximab ($p < 0.05$, linear regression analysis).

parameters, maybe due to technical difficulties measuring ATI when IFX is low but present. In a recent meta-analysis covering 10 studies with 668 patients, loss of response is the only clinical parameter correlated to antibodies towards IFX.¹²

The correlation of s-IFX and clinical activity is evident in this maintenance cohort. Not only CRP and albumin, but also Harvey Bradshaw index corresponds to the level of circulating infliximab. The observation that low s-IFX levels are seen in patients with high CRP and low albumin suggests a higher rate of elimination of the drug by the intense inflammation. This may imply a need for adjusting the IFX dosage to the inflammatory activity. In this context, we have calculated the mean level of s-IFX corresponding to clinical remission to 4.1 µg/mL. This trough level might be a target for treatment and individual adjustment of IFX dosage to reach this minimum concentration may improve response. The ulcerative colitis patients were included in the calculation of medium trough levels in the entire study population. However, the UC cohort was too small for any comparison between UC and CD.

One of the interesting observations in this study is the negative correlation between the accumulated number of infusions and mean s-IFX levels. This may indicate a time-dependent increase in the clearance of the drug, perhaps due to development of antibodies or other mechanisms of increasing rate of elimination. Even though there was a significant decrease in trough levels after 20 infusions as seen in Fig. 5, no differences were seen regarding CRP, albumin or clinical activity. Therefore, it is difficult to draw any major conclusions regarding the clinical implication but the observation may call for further studies regarding infliximab levels in patients on long-term maintenance IFX.

In the maintenance cohort, we could not observe any difference in s-IFX in patients with concomitant immunosuppression compared to patients on monotherapy. However, ATI was significantly lower in patients with the addition of immunosuppression as seen in Fig. 6. This observation supports the important role of immunosuppression for

inhibition of antibody development and in the light of a recent publication by Ben-Horin et al., the addition of azathioprine may be an important tool for eliminating ATI in patients with loss of response.^{4,5,21}

In conjunction with earlier reports, we could not see any significant differences in clinical parameters between ATI+ and ATI- patients.^{11–13} The fact that ATI cannot be analyzed in the presence of IFX might be an important reason for not observing such a correlation. The lack of correlation may also be due to the low number of ATI- patients in the subgroup analyzed for ATI. Four out of the five patients with undetectable trough IFX were ATI+ during maintenance IFX treatment. Excluding patients with concomitant immunosuppression, 94% of the patients with low s-IFX had circulating ATI. This

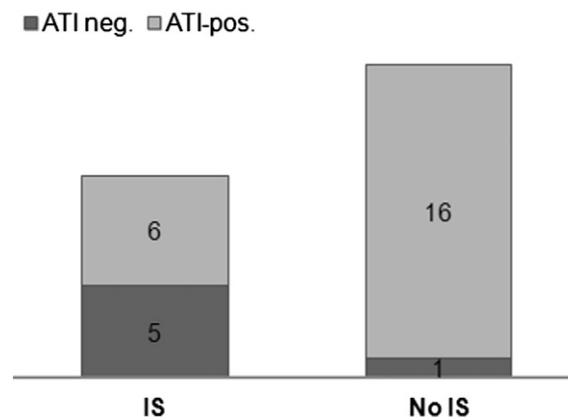


Figure 6 ATI is less common in patients with concomitant immunosuppressive treatment. Twenty-eight of the patients had one or more samples with s-IFX < 0.2 µg/mL, which motivated analysis for antibodies against infliximab (ATI): 5 out of the 11 patients with immunosuppression (IS) tested negative for ATI, as compared with only one of the 17 patients without IS ($p < 0.05$, Fisher's exact test).

observation might imply that patients with undetectable s-IFX could be regarded as ATI+. However, although it would seem sufficient to measure serum levels alone, one must consider other causes than the formation of antibodies as the reason for undetectable drug levels, such as high inflammatory activity, low albumin or high body weight. Therefore, it is advisable, when possible, to measure both drug levels and antibodies.

In 28 out of the 79 patients (35%) with maintenance IFX, the trough levels were undetectable on at least one occasion. Surprisingly, in 25% of these visits, the patients were still in clinical remission. The STORI trial identified low IFX trough level together with other clinical parameters of remission as an indicator for safely stopping IFX treatment.¹⁷ Thus, our results may suggest that many patients with low s-IFX could be eligible for trying to stop the drug, but prospective studies are required to answer this question.

In this study, we show that drug monitoring is useful in IBD-patients with maintenance IFX-treatment. We have established the trough level 4.1 µg/mL as mean concentration for clinical remission. We have demonstrated a progressive decrease in trough levels during maintenance treatment. Moreover, almost all of the patients on monotherapy and undetectable s-IFX were ATI+, while only 55% of patients on concomitant immunosuppressives were positive for antibodies. The observation that almost one out of five patients on maintenance IFX treatment are in clinical remission despite undetectable trough levels warrants further studies regarding the possibility to stop treatment in these patients.

Conflict of interest

Loa Davidsdottir has received lecture honoraria from MSD. Ragnar Befrits and Michael Eberhardson have received lecture honoraria from MSD and AbbVie and participated in national advisory boards.

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We thank study nurse Annika Olsson and Eva Berglund for their skillful help obtaining blood tests and clinical parameters. PM developed the assays, carried out data analyses, performed statistical analysis and drafted the manuscript. LL carried out data analyses, performed the statistical analysis and revised the manuscript, US developed the assays, participated in the design of the study, carried out data analyses and drafted the manuscript, LD carried out data analyses and drafted the manuscript, and JN developed the assays, participated in the design of the study and revised the manuscript. RB participated in the design of the study, carried out data analysis and revised the manuscript; ACW developed the assays, participated in the design of the study, carried out the sample analyses and drafted the manuscript. ME participated in the design of the study, carried out data analyses and drafted the manuscript. All authors read and approved the final manuscript.

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