Anti-TNF-α antibodies improve intestinal barrier function in Crohn's disease

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Abstract

Background and aims: Intestinal barrier function in Crohn's disease patients and their first degree healthy relatives is impaired. The increased intestinal permeability may result in an enhanced mucosal immune response and thereby aggravate intestinal inflammation. Humanised anti-TNF-α antibodies have been shown to be effective in the treatment of active Crohn's disease and in the treatment of enterocutaneous fistula.

The aim of the present study was to investigate the influence of anti-TNF-α antibody (infliximab) treatment on the intestinal barrier function of patients with active Crohn's disease.

Methods: The differential intestinal uptake of lactulose and mannitol was measured to quantify intestinal permeability in patients with long standing active Crohn's disease (n=17) directly before and seven days after treatment with infliximab (5 mg/kg bodyweight). In parallel, intestinal permeability was studied in a healthy control group (n=20). Serum samples were analysed with pulsed amperometric detection after separation on an anion exchange column.

Results: Intestinal permeability was significantly increased in all patients with Crohn's disease (L/M ratio 0.24±0.17) prior to infliximab treatment compared to the control group (L/M ratio 0.01±0.02; p-value <1×10^-7). Treatment of patients with infliximab resulted in a marked decrease of intestinal permeability as measured by L/M ratio from 0.24±0.17 before to 0.02±0.02 (p-value <1×10^-7) seven days after infliximab application.

Conclusions: Treatment with anti-TNF-α antibodies improved impaired intestinal barrier function in patients with Crohn's disease. This effect may correlate to the well documented anti-inflammatory effect of TNF-α blockade in this intestinal disease.

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Intestinal barrier function in Crohn’s disease

1. Introduction

Increased intestinal permeability, as a marker for impaired intestinal epithelial barrier function, is characteristic for patients with active Crohn’s disease (CD).1-5 Several reports indicate that this might not only be a secondary effect of mucosal inflammation but more likely a genetically determined disease-facilitating condition, since an increased intestinal permeability has also been found in healthy first degree relatives of Crohn’s disease patients.6-8 Furthermore, there seems to be a crucial role for NOD2/CARD15 since 3020insC mutations, representing a high risk for Crohn’s disease, have been associated with an impairment of intestinal barrier function in CD patients and their healthy first-degree relatives too.7 Interestingly, abnormal permeability of the small intestine in Crohn’s disease seems also to be associated with CARD15 mutations.9 However the genetic defect alone is not sufficient to induce the disease. Other factors are discussed to explain the barrier dysfunction in CD which include the abnormal composition of the luminal bacterial flora, the altered secretion of antimicrobial peptides and unknown external factors.10-12 The disturbance of the intestinal barrier in turn may trigger an enhanced mucosal immune response, thus aggravating chronic inflammatory bowel disease.

Humanised anti-TNF-α antibodies (infliximab, Remicade®) have been shown to be effective in the treatment of active Crohn’s disease as well as in the treatment of enterocutaneous fistula. In addition, long term treatment with these antibodies has been demonstrated to manage symptoms in patients with active Crohn’s disease not responding to conventional treatment.13-15 In one case, elevated gut permeability manifests even years before the histological diagnosis of CD has been established.16 Furthermore, there is evidence that the permeability of the small intestine reflects disease activity and might indicate relapse under therapy.5,17-20 Therefore determining intestinal permeability in patients with Crohn’s disease and demonstrating the effect of the chosen treatment on this parameter might be pivotal to control the course of the disease.1

Several methods have been developed to determine intestinal permeability.21 Over the recent years, substantial effort has been undertaken to establish non-invasive methods for the in vivo determination of intestinal permeability. Several non-toxic and non-metabolized marker substances such as Polyethylen-glykol (PEG 400),22 inulin,23 51Cr-EDTA24 and several non-toxic and non-metabolized marker substances such as Polyethylen-glykol (PEG 400), inulin, 51Cr-EDTA and the differential uptake of rhamnose/lactulose or lactulose/mannitol25 -27 — that are ingested orally have been used for this purpose. In a clinical setting, the lactulose/mannitol uptake test has been the most widely used test in the last years. In healthy humans, mannitol is taken up by a transcellular mechanism which is independent of paracellular permeability28,29 and the larger disaccharide lactulose is not resorbed. Normally only traces of lactulose appear in the serum or in urine after oral application. Therefore an increased serum concentration of lactulose indicates disturbed paracellular permeability and represents an impaired intestinal epithelial barrier function.

In this study, we investigated the effect of anti-TNF-α antibodies on the intestinal permeability of Crohn’s disease patients directly before and seven days after an infliximab infusion by measurement of lactulose/mannitol uptake.

2. Material and methods

2.1. Patients

All patients had a long history of active Crohn’s disease not responding adequately to conventional therapy. These patients had already received at least one course of treatment with anti-TNF-α antibodies (infliximab) before they were included in the study.

Inclusion criteria were an interval to the last infusion of infliximab of at least six weeks and clinical signs of any deterioration of their clinical condition after the therapy. By choosing these criteria, we aimed to avoid a severe relapse under infliximab therapy resulting in a rather low mean CDAI of the investigated patients of 171. Patients using NSAIDs and or drinking larger amounts of alcohol were excluded since these compounds are known to influence permeability. Patient’s characteristics are listed in Table 1. The healthy control group consisted of 20 age and sex matched individuals.

2.2. Ethics approval

All patients and all individuals of the healthy control group (n=20, mean age: 37 years) gave their informed consent to the study. The study was approved by the local ethics committee (AZ: D314/01).

2.3. Measurement of intestinal permeability

Intestinal permeability was determined using a modified technique as previously described.7,20,30,31 Lactulose (20 g) and mannitol (5 g) were given orally diluted in 300 ml water 2 h before or 7 days after infliximab infusion (5 mg/kg bodyweight). Blood was drawn at four time points (0, 30, 60, 90 min). Serum concentrations of lactulose and mannitol were determined using melibiose as internal standard as described below.

2.3.1. Measurement of lactulose and mannitol serum concentrations

Mannitol and lactulose (4-O-β-D-galactopyranosyl-β-fructofuranose) for oral administration were obtained from Fluka (Seelze, Germany) and Calbiochem (San Diego, Ca, USA) while melibiose for internal standards was supplied from Sigma (Deisenhofen, Germany). The HPLC equipment was supplied by Merck (Darmstadt, Germany) and comprised an eluent degas module, a micro injection valve fitted with a 25 μl loop and a gradient pump. The pulsed amperometric detector (PAD) with a gold electrode and a silver/silverchloride reference electrode, a Carbopac PA100® anion exchange column and a guard column filled with the same material was supplied by Dionex (Idstein, Germany). Data were processed using a Merck integrator D7500. Deionized water with high resistance (18 MΩ) for dilution of samples and eluent was supplied from Merck.

2.3.2. Eluent preparation

HPLC water was degassed by sparkling with helium for 15 min. 50% sodium hydroxide solution was added and gently stirred for 5 min to give the required concentration of 90 mmol/l NaOH.
2.3.3. Sample preparation (serum)
Serum was separated from whole-blood samples by 10 min centrifugation with 3000 U/min and afterwards stored at −20 °C for further analysis. An aliquot of 0.5 ml serum was deproteinized by precipitation in 5% 5-sulfosalicylic acid and 200 μl of the internal standard (melibiose 0.1 mg/l) was added. After a 15 min incubation period (4 °C) the samples were centrifuged at 9000 U/min for 5 min and the supernatants were removed and diluted (1:100) with 90 mmol/l NaOH. 25 μl of these supernatants was then injected onto the anion exchange column.

2.3.4. Analyses of carbohydrates with HPLC and pulsed amperometric detection (PAD)
The samples were eluted with the 90 mmol NaOH eluent at a flow rate of 1 ml/min at 25 °C, separated at the Carbo-pac100® anion exchange column and detected with a pulsed amperometric detector (PAD) using a gold working electrode and a silver/silverchlorid reference electrode. A repeating sequence of three different potentials was used. Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of a gold electrode (E1: 0.05 V, T1: 0.4 s.). The second potential oxidizes the gold electrode to clean the surface between measurements (E2: 0.75 V, T2 0.2 s.). The third potential reduces the gold oxide on the electrode surface back to gold, thus permitting a new detection during the next cycle (E3: −0.15 V, T3: 0.4 s.). Sensitivity on the PAD was set to 100 nC. Calculation of sugar concentration was performed by integrator analysis of the area of the melibiose reference peak and the area of the lactulose and mannitol peaks.

2.3.5. Data analysis and statistics
Differences between groups (CD pre and post infliximab and healthy controls) were determined by quantification of the fold change, which was based on the ratio of the medians of the compared groups. Significance was determined by using the Mann–Whitney U-test, p-values were corrected for multiple testing using the Benjamini–Hochberg correction.32

3. Results

3.1. Intestinal permeability is increased in patients with Crohn’s disease
The patients included in this study had all a long history of active CD which failed to improve under immunosuppressive therapy strategies (refer to Table 1). Intestinal permeability was assessed by differential uptake of lactulose and mannitol in CD patients before and seven days after initiation of infliximab treatment. In parallel sugar uptake in healthy controls was measured using HPLC analysis. Due to the design of the study the initial CDAI was relatively low (mean CDAI: 171; Table 1) and seven days after initiation of infliximab therapy was unusual for reevaluation of CDAI. However even at this early time point all patients clinically responded to infliximab therapy as indicated by lowering of the CDAI (mean CDAI: 153; Table 1).

Mannitol levels, an indicator for transcellular permeability, did not differ significantly between patients with moderately active Crohn’s disease (mean CDAI: 171; Table 1) and healthy controls (603 mg/ml in CD before infliximab and 594 in healthy controls; Fig. 1A). As compared to healthy controls, patients with CD have a significant increased intestinal lactulose uptake (140 mg/ml CD vs. 8 mg/ml control group, p-value < 1×10−7; Fig. 1B) indicating impaired epithelial paracellular barrier function. By comparing the lactulose to mannitol levels (lactulose/mannitol 0.24 in CD and lactulose/mannitol 0.24 in CD)
vs. 0.01 in controls, p-value $<1 \times 10^{-7}$) and statistical testing by a Mann–Whitney U test, we were able to show that the disturbance of the intestinal permeability is highly significant.

### 3.2. Infliximab treatment strongly reduces the increased intestinal permeability in CD

As shown in Fig. 1, intestinal permeability is highly increased in patients with CD compared to a healthy control group. Seven days after an infusion of infliximab (5 mg/kg body-weight), the lactulose serum levels dropped from 140 mg/ml to 10 mg/ml (p-value $<1 \times 10^{-7}$; Fig. 1B) and the L/M-ratio, as a marker for the intestinal permeability, decreased significantly from 0.24 to 0.02 (p-value $<1 \times 10^{-7}$; Fig. 1C). The mannitol serum levels showed no significant changes due to the infliximab therapy (Fig. 1A). As seen in Fig. 1D, there is a high overlap for mannitol, lactulose and lactulose/mannitol ratios between the healthy control group and the infliximab treated CD group in contrast to the CD before infliximab treatment.

### 4. Discussion

The present study demonstrates that anti-TNF-α antibodies are able to restore the impaired intestinal permeability of patients with active Crohn’s disease. Infliximab treatment leads to an improvement of the lactulose/mannitol ratio as an indicator of paracellular epithelial barrier function, indicating a direct effect of the proinflammatory cytokine TNF-α on intestinal barrier function. The patients included in this study had received at least one infusion of infliximab and had symptoms of a relapse of CD with only a moderate CDAI before they were included in the study. These results indicate that lactulose/mannitol ratios are highly sensitive for disturbance of the intestinal barrier. In line with this data, a larger longitudinal study with a higher number of patients and a correlation of histological

![Graph A](image1) ![Graph B](image2) ![Graph C](image3) ![Graph D](image4)

**Figure 1** Intestinal permeability was determined using lactulose and mannitol. Lactulose (20 g) and mannitol (5 g) were given orally diluted in 300 ml water 2 h before or 7 days after infliximab infusion (5 mg/kg bodyweight). Blood was drawn at the time points: 0, 30, 60, 90 min. A) Boxplot of serum concentrations of mannitol using melibiose as internal standard. B) Boxplot of serum concentrations of lactulose using melibiose as internal standard. * indicates p-values $<1 \times 10^{-7}$. C) Boxplot of Lactulose/Mannitol ratios. * indicates p-values $<1 \times 10^{-7}$. D) Lactulose and mannitol quantities in response to infliximab treatment, illustrating how diseased individuals are more similar to healthy individuals after infliximab treatment. Quantified levels in healthy control individuals (green), CD before infliximab treatment (grey) and after infliximab treatment (blue) are displayed. The areas represent the median (darkest shading) the 75th percentile and the 95th percentile (lightest shading) of all measurements, while lactulose levels are plotted on the x-axis and mannitol levels on the y-axis.
changes, clinical course and permeability will be initiated to evaluate if lactulose/mannitol ratios are usable as indicator for mucosal healing\textsuperscript{33} and also for therapy management.

Epithelial paracellular barrier function is mainly regulated by the number and structure of the “tight-junctions” (zonula occludens).\textsuperscript{34} Occludin and the claudin proteins seem to be the most important integral proteins of tight-junctions.\textsuperscript{35} A downregulation of occludin, claudin 3, 5 and 8 and a redistribution of these proteins through TNF-α has been shown to be followed by an impaired epithelial barrier function.\textsuperscript{4,36,37} We were able to show that TNF-α downregulates intestinal expression of the tight-junction protein occludin in a murine GvHD model (Noth et al., in revision). This effect might be mediated in part via myosin light chain kinase triggered caveolin-1-dependent occludin endocytosis and a direct effect of the anti-TNF-α therapy on the expression level of certain tight-junction proteins. Other important aspects to the impaired intestinal barrier in patients with CD like the influence of the abnormal composition of the luminal bacterial flora, the altered secretion of antimicrobial peptides like α-defensin and sIgA are subject of ongoing studies. Thus, it is still unclear if the impaired intestinal barrier function in patients with CD is a primary, genetically determined event that induces, together with other factors, the overwhelming inflammatory response or if it is a primary immune reaction which leads to a barrier defect through the secretion of proinflammatory cytokines like TNF-α. Interestingly, TNF-α is reported to increase transcellular permeability of antigens and other proteins by an undefined mechanism.\textsuperscript{5}

Even if the pivotal role of TNF-α in mediating increased intestinal permeability in CD is evident\textsuperscript{38,39} there are only limited reports and practically feasible tests to measure this central aspect of an anti-TNF-α treatment. One report used measurement of urinary excretion of 51Cr-EDTA after oral intake four weeks after infliximab therapy and showed a strong reduction in the intestinal permeability.\textsuperscript{3} Since the use of a radioactive substance is problematic for the proposed purpose, the lactulose/mannitol uptake is the most frequently used strategy for estimating intestinal permeability in clinical settings. Other factors, which support the use of the lactulose/mannitol test are the existing long clinical experience with these two non-toxic, not metabolized carbohydrates and that possible interfering factors like renal insufficiency or short bowel syndrome are corrected by the measurement of two independent substances. Up to now, there are only limited data on this test in CD\textsuperscript{1,40,41} and results after anti-TNF-α are completely missing. Our study clearly showed that lactulose/mannitol ratios could serve as a good clinical marker for an effect of anti-TNF-α treatment on mucosal permeability. However, we are not able to discriminate between a postulated direct effect of infliximab on the barrier function\textsuperscript{4,42} or an indirect effect on the permeability by dampening the inflammatory response.\textsuperscript{2,43,44} It will be interesting to investigate the correlation of lactulose/mannitol ratios with the findings in the small intestine by ileocolonoscopy and or capsule endoscopy\textsuperscript{55,46} under anti-TNF-α therapy. If lactulose/mannitol ratios are in line with macroscopic and microscopic finding, a non-invasive follow up for CD patients could be achieved by this method, as recently postulated.\textsuperscript{47}

In conclusion, we showed for the first time that an anti-TNF-α therapy in patients with Crohn’s disease significantly improves intestinal barrier function which can easily be monitored by measurement of Lactulose and Mannitol.

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