The lectin pathway of the complement system is downregulated in Crohn’s disease patients who respond to anti-TNF-α therapy

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Abstract

Background and aims: The lectin pathway of the complement system is initiated through the recognition of pathogens or altered self-structures by mannan-binding lectin (MBL) or ficolins and subsequent activation of MBL-associated serine proteases (MASPs). Altered ficolin levels may contribute to a dysregulated immune response in Crohn’s disease (CD). A complete analysis of the lectin pathway has not been performed in patients with CD. We hypothesised that the lectin pathway proteins exacerbate inflammation in CD.

Methods: We assessed the lectin pathway proteins in 43 patients with active CD & 350 blood donors by measuring the serum levels of MBL; M-, H-, and L-ficolin; MASP-2; MASP-3; and MAP44. In patients with CD, the blood samples were obtained during induction treatment with infliximab or adalimumab.

Results: Of 43 patients with CD, 32 (74%) were classified as responders. We observed a nearly 50% decrease in median M-ficolin levels between day 0 and weeks 1/7 in the responders (p < 0.001), whereas there was no decrease in the non-responders. MASP-2 levels decreased from baseline to week 1 in both the responders (37%, p < 0.0001) and the non-responders (29%...
1. Introduction

Crohn’s disease (CD) evolves from a dysregulated immune response to the intestinal microbiome in a genetically susceptible individual.1 Aberrant innate immune reactions are critically involved in CD pathogenesis and may contribute to the reduced diversity of the resident intestinal microbiota.2 In CD, defective bacterial sensing and killing have been linked to an impaired production or function of pattern recognition molecules (PRMs), such as the lectins.3 In the cytosolic compartment, genetic variants in the nuclear oligomerisation domain represent the most prominent CD susceptibility locus identified.4 In the extracellular compartment, four soluble PRMs, mannan-binding lectin (MBL) and H-, M- and L-ficolin, recognise pathogen-associated molecular patterns. Reacting with microorganisms, these PRMs subsequently activate MBL-associated serine proteases (MASPs) and consequently the complement system.5 The activated complement system acts against invading pathogens by attracting inflammatory cells to the site of activation, by inducing a membrane attack complex into the membrane of the microorganism, and by mediating opsonisation.

MBL and L- and H-ficolin are predominantly produced in the liver, whereas M-ficolin is produced in monocytes and neutrophils.6 Despite evidence of complement activation via the lectin pathway around sites of CD inflammation and an increased capacity of complement activation in the plasma by MBL, the lectins do not generally act as acute-phase response proteins.7–10

The role of MBL has been studied previously in inflammatory bowel diseases (IBD). Several authors detected MBL gene transcripts in ileal tissue, whereas others only detected negligible amounts.11,12 Low levels of circulating MBL protein have been associated with the CD phenotype, and an increased prevalence of anti-Saccharomyces cerevisiae antibodies (ASCA) and MBL deficiency may accelerate inflammation in experimental colitis.8,11,13–15 However, large studies of MBL protein levels and MBL gene polymorphisms have refuted the role of MBL in CD.16–19

Commercial kits to analyse lectin pathway proteins have recently become available; thus far, however, little attention has been paid to the newly discovered ficolins and their possible role in CD. Fairly constant plasma levels of M-ficolin have been reported in patients with CD and healthy controls. However, studies have been performed in the presence of anti-TNF-α induction therapy. We hypothesised that the lectin pathway of the complement system contributes to the pathogenesis of CD and that changes in lectin pathway protein levels may be associated with clinical outcome.

2. Methods

2.1. Patients

We included 43 patients who had active CD and were scheduled for biologic treatment. CD was diagnosed according to clinical, histopathological, and biochemical criteria. Active disease was defined as a Harvey Bradshaw Index (HBI) ≥ 5 in combination with elevated levels of C-reactive protein (CRP) and faecal calprotectin. Patient characteristics are shown in Table 1. Patients who had been treated with anti-TNF-α agents or corticosteroids or who had changed the dose of immune modifiers (azathioprine or methotrexate) up to 12 weeks before assessment were not included. The decision to treat with either infliximab or adalimumab was made by the prescribing physician independent of study inclusion. Adalimumab was administered subcutaneously at the following dosages: 160 mg on day 0, 80 mg 2 weeks later, and 40 mg every other week thereafter. Infliximab was administered intravenously at 5 mg/kg on day 0, week 2, and week 6. We observed the patients for 7 weeks and obtained venous blood samples at baseline (day 0), week 1, and week 7 after initial treatment. On week 7, we classified patients as responders if they had experienced a reduction in HBI of at least 3 points compared to baseline values. We classified the remaining patients as non-responders. After centrifugation, we stored the serum samples at −80 °C.

2.2. Healthy controls

Blood samples from 348 healthy blood donors (n = 132 women and 216 men) served as controls. Some of the lectin pathway protein data have previously been published together with a more detailed description.7,29 The median age of the healthy donors was 47 years (range, 18–64 years). A thorough medical record of blood donors was obtained.

In the responders only, the level of the inhibitory serine protease MASP-3 increased by 26% from baseline to week 1 (p < 0.001) and remained high at week 7.

Conclusions: Our findings indicated that M-ficolin, MASP-2, and MASP-3 may act in concert to reduce the activity of the lectin pathway, in patients with CD who respond to biological therapy. © 2013 European Crohn’s and Colitis Organisation. Published by Elsevier B.V. All rights reserved.
before every blood donation, and in particular, donors could not give blood when they had on-going infections.

2.3. Ethics

All of the patients provided written informed consent to participate in the study, and the Central Denmark Region Committees on Biomedical Research Ethics (j.no. 1998/4330 with amendment) approved the study protocol. The study conformed to the Declaration of Helsinki.

2.4. Protein measurements

2.4.1. MBL

This MBL assay has previously been described.30,31 The assay estimates the concentration of MBL based on its lectin activity, i.e., binding to a mannan-coated surface. Microtitre plates were coated overnight with 1 μg mannan in 100 μl coating buffer (0.1 M sodium bicarbonate, 0.09% (w/v) sodium azide, pH 9.6). Subsequently, residual binding sites were blocked by adding 1 μg human serum albumin (HSA) per ml Tris-buffered saline (TBS, 10 mM Tris, 145 mM NaCl, pH 7.4) and washed with TBS containing 0.05% Tween 20 (TBS/Tw). Serum samples, three quality controls and a negative buffer control were diluted 100-fold in binding buffer (20 mM Tris, 1 mM NaCl, 10 mM CaCl2, 0.05% (v/v) Triton X-100, 0.1% HSA (w/v), 100 mg heat aggregated human IgG/ml, pH 7.4). To produce a standard curve, pooled citrate plasma from healthy blood donors with a known concentration was diluted 20-fold, after which it was diluted 3.5-fold 7 times, and 100 μl (in duplicates) was added to the microtitre wells and incubated overnight at 4 °C. Dilutions and sample transfer of standard plasma, quality controls and test samples were performed using a PerkinElmer MultiProbe II robotic liquid handling system. After the wash, the wells were incubated with europium-labelled monoclonal (MAb) anti-MBL (Hyb 131-01, Bioporto). Subsequently, the plate was washed, enhancement solution (PerkinElmer) was added to release europium, and a time-resolved immunofluorometric assay (TRIFMA) was performed. The signals were read using a multimode plate reader (Victor X5, PerkinElmer). Inter-assay reproducibility was assessed determining MBL in three different control citrate plasma samples 25 times each (coefficient of variation, 9.9% for 98 ng/ml, 3.2% for 333 ng/ml, 8.4% for 1651 ng/ml). The standard curve covered a dynamic range from 0.1 ng/ml to 182 ng/ml. This means that the lowest measurable MBL level in serum was 10 ng/ml at the 100-fold dilution employed in the assay.

2.4.2. M-ficolin

The quantification of M-ficolin, as previously described,7 uses an MAb specific for M-ficolin (7G1, Hycult Biotech) for both coating and development, which was feasible because the ficolins presented repeated determinants due to their oligomeric structure. The wells of microtitre plates were coated with 1 μg/ml coating buffer of MAb 7G1, and after incubation and washing, the samples diluted 1/60 in 100 μl sample buffer (10 mM Tris, 1 M NaCl, 0.05% Tween 20, 5 mM EDTA, 100 μg heat-aggregated normal human immunoglobulin, 50 μg bovine immunoglobulin and 1 mg HSA per ml) were added to the wells. After incubation and washing, the wells were incubated with biotinylated MAb 7G1 (1 μg/ml TBS/Tw) and after washing with TBS/Tw subsequently developed with 100 ng/ml of europium-labelled streptavidin (1244-360, PerkinElmer) diluted in TBS/Tw, 25 μM EDTA, pH 7.4, and the bound europium-labelled streptavidin measured by time-resolved fluorometry. Dilutions of pooled serum with a known M-ficolin concentration were included on each plate for the construction of a standard curve, as

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Baseline characteristics.</th>
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<tr>
<td></td>
<td>Median [IQR]</td>
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<tr>
<td></td>
<td>Responders (n = 32)</td>
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<tr>
<td></td>
<td>Non-responders (n = 11)</td>
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<tr>
<td></td>
<td>Controls (n = 348)</td>
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<tr>
<td>Age</td>
<td>38 [26–48]</td>
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<tr>
<td>Gender</td>
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<tr>
<td></td>
<td>16 (50%)</td>
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<tr>
<td></td>
<td>Female</td>
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<td></td>
<td>16 (50%)</td>
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<tr>
<td>Smokers</td>
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<tr>
<td>HBI</td>
<td>8 [7–12]</td>
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<tr>
<td>Localisation</td>
<td>Small intestine</td>
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<td>15 (47%)</td>
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<tr>
<td></td>
<td>Colonic</td>
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<td></td>
<td>30 (94%)</td>
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<tr>
<td></td>
<td>Perianal</td>
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<td></td>
<td>10 (31%)</td>
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<tr>
<td>Behaviour</td>
<td>Stricture</td>
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<td></td>
<td>4 (13%)</td>
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<td></td>
<td>Non-perianal fistula</td>
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<td>4 (13%)</td>
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<td>Perianal fistula</td>
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<td>7 (22%)</td>
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<td>CRP (nmol/l)</td>
<td>97 [30–223]</td>
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<td>Faecal calprotectin (mg/kg)</td>
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<td>18 (56%)</td>
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<tr>
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<td>Azathioprine</td>
</tr>
<tr>
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<tr>
<td></td>
<td>Methotrexate</td>
</tr>
<tr>
<td>Budesonide</td>
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<td></td>
<td>0</td>
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<tr>
<td>CRP (reference &lt; 75 nmol/l); faecal calprotectin (reference &lt; 50 mg/kg faeces).</td>
<td></td>
</tr>
</tbody>
</table>

IQR, interquartile range; CRP (reference < 75 nmol/l); faecal calprotectin (reference < 50 mg/kg faeces).
were three internal control sera and a negative buffer control. Inter-assay reproducibility was assessed by 25 times determination of M-ficolin in three different control citrate plasma samples (coefficient of variation, 8.0% for 140 ng/ml, 3.8% for 687 ng/ml, 3.8% for 1527 ng/ml). The standard curve covered a dynamic range from 0.13 ng/ml to 78 ng/ml. This means that the lowest measurable M-ficolin level in serum was 7.8 ng/ml at the 60-fold dilution employed in the assay.

2.4.3. H-ficolin
The assay for H-ficolin was conducted as a TRIFMA, which has been previously described, with minor modifications.25,32 The wells of microtitre plates were coated with 100 ng of MAb anti-H-ficolin antibody (clone 4H5, Hycult Biotech) in 100 μl phosphate-buffered saline (PBS). Serum samples were diluted 100-fold in Tris-buffered of 1 M NaCl, 10 mM CaCl₂, and 0.1% (w/v) HSA, and 100 μl was added to the wells and incubated overnight at 4 °C. After the wash, the wells were incubated with 250 ng/ml of biotinylated MAb anti-H-ficolin antibody (4H5) and developed with 100 ng/ml of europium-labelled streptavidin, followed by time-resolved fluorometry. Normal human standard serum with a known concentration of H-ficolin was used to construct the standard curve. Inter-assay reproducibility of this assay was assessed by 25 times determination of H-ficolin in 3 different control sera (coefficients of variation, 9.6% for 9.8 mg/l, 8.2% for 16.8 mg/l, 11.8% for 24.1 mg/l). The standard curve covered a dynamic range from 0.15 ng/ml to 213 ng/ml. This means that the lowest measurable H-ficolin level in serum was 150 ng/ml at the 1000-fold dilution employed in the assay.

2.5. L-ficolin
L-ficolin was measured using the HK 336 ELISA kit (Hycult Biotech, Uden, The Netherlands), according to the manufacturer's instructions. This standard sandwich enzyme-linked immunoassay (ELISA) is based on a catching MAb anti-L-ficolin and a detecting MAb anti-L-ficolin. The standard curve covered a dynamic range from 15 to 500 ng/ml. This means that the lowest measurable L-ficolin level in serum was 375 ng/ml at the 25-fold dilution of samples we employed in the assay.

2.5.1. MASP-2
To test for the level of MASP-2, we used a sandwich type TRIFMA assay as previously described.31 We coated the microtitre wells with MAb anti-MASP-2 antibody (clone 8B5, Hycult Biotech) in PBS overnight at 4 °C. The wells were blocked and washed, and samples diluted 1/25 in 10 mM Tris–HCl, 1 M NaCl, 10 mM EDTA, 15 mM NaN₃, pH 7.4, 0.05% (v/v) Tween 20 containing 0.01% (w/v) heat aggregated human IgG were added. A pool of plasma with known MASP-2 concentration was used to obtain a standard curve; three different plasma samples were used as internal quality controls and included on each microtitre plate used. After incubation overnight, the wells were washed and incubated with biotinylated MAb anti-MASP-2 antibody (clone 6G12, Hycult Biotech) (1 μg/ml TBS/Tw with 5 mM CaCl₂, 0.01% (w/v) heat aggregated human IgG, 1% (v/v) bovine serum), followed by washing with TBS/Tw and incubation with europium-labelled streptavidin. Bound europium-labelled streptavidin was detected by time-resolved fluorometry after the addition of enhancement solution. Inter-assay reproducibility of this assay was assessed by 25 times determination of MASP-2 in 3 different control sera (coefficients of variation, 5.8% for 85.5 mg/l, 4.9% for 243 mg/l, 2.0% for 586 mg/l). The standard curve covered a dynamic range from 1 ng/ml to 152 ng/ml. This means that the lowest measurable MASP-2 level in serum was 25 ng/ml at the 25-fold dilution employed in the assay.

2.5.2. MASP-3
The test for the level of MASP-3 was performed using a previously described sandwich type TRIFMA.34 In this test, we coated wells with MAb antibody (clone 5FS) (2 μg/ml PBS) to capture MASP-3 in the wells. The samples were diluted 1/100 in 20 mM Tris, 1 M NaCl, 10 mM CaCl₂, 1 mg HSA/ml, 0.05% (v/v) Triton X-100, pH 7.4 and added to the MAb-coated wells; after incubation and washing a biotinylated MAb anti-MASP-3 antibody (clone 38.12.3, Hycult Biotech) (0.25 μg/ml TBS/Tw with 5 mM CaCl₂ and containing 1% (v/v) bovine serum) was added. After the wash, europium-labelled streptavidin was added; then we washed and added enhancement solution and measured the signal obtained by time-resolved fluorometry. We used dilutions of a plasma pool with a known amount of MASP-3 for construction of the standard curve; we used three different plasma samples as internal controls on each plate. Inter-assay reproducibility of this assay was assessed by 25 times determination of MASP-3 in 3 different control sera (coefficients of variation, 8.0% for 740 mg/l, 6.5% for 3148 mg/l, 5.6% for 5801 mg/l). The standard curve covered a dynamic range from 0.6 ng/ml to 533 ng/ml. This means that the lowest measurable MASP-3 level in serum was 60 ng/ml at the 100-fold dilution employed in the assay.

2.5.3. MAP44
The assay for MAP44, previously described by Degn et al.34 was performed with minor modifications. The microtitre wells were coated with MAb mouse anti-human MAP44 antibody (clone 2D5, Hycult Biotech) (5 μg/ml PBS). The wells were subsequently blocked with TBS/Tw. Serum samples, three quality controls and a negative buffer control were diluted 40-fold in binding buffer (Tris-buffered 1 M NaCl, 10 mM CaCl₂, 0.05% Triton X-100, 0.1% (v/v) HSA, 100 μg/ml of each of heat-aggregated human IgG, bovine IgG, rat IgG and mouse IgG, pH 7.4). We used dilutions (1:10) of a plasma pool with a known amount of MAP44 for construction of the standard curve. Samples at 100 μl were added to the wells and incubated overnight at 4 °C. After incubation and washing, the wells were incubated with biotinylated MAb anti-CCP1 antibody (clone 4H2, Hycult Biotech) (0.1 μg/ml TBS/Tw with 5 mM CaCl₂ and containing 1% (v/v) bovine serum). After the wash, 1000-fold diluted europium-labelled streptavidin was added; then, after incubation and washing, enhancement buffer was added. The released europium was measured by time-resolved fluorometry. Inter-assay reproducibility was assessed by 25 times determination of MAP44 in three different control citrate plasma samples (coefficient of variation, 7.1% for 366 ng/ml, 5.4% for 1312 ng/ml, 6.6% for 2336 ng/ml). The
standard curve covered a dynamic range from 3 ng/ml to 170 ng/ml. This means that the lowest measurable MAp44 level in serum was 120 ng/ml at the 40-fold dilution employed in the assay.

2.6. Statistical analysis

The statistical associations between lectin pathway proteins and other variables were analysed by Spearman’s rank correlation with Bonferroni correction for multiple comparison. Wilcoxon’s rank sum test was used to study the differences between patients and healthy controls. In the patients, repeated-measures analysis of variance was performed for over-time-comparison of lectin pathway protein levels. These levels were normally distributed after logarithmic transformation and were included as such in the analyses. The Greenhouse–Geisser epsilon was used to adjust for possible violation of sphericity in the repeated-measures design. Normality was checked by QQ plots and histograms. Two-sided tests were used throughout, and a p value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Clinical characteristics

Clinical and biochemical data are shown in Table 1. Of 43 patients, 32 (74%) were classified as responders according to the criteria defined for the study. The baseline disease severity estimated by HBI and the markers of inflammation were similar in both groups. The non-responders had more active CD inflammation using both clinical and biochemical markers. It is plausible that the patients who were defined as non-responders (p < 0.001) and this decrease was sustained at week 7, whereas there was no decrease in the non-responders (Fig. 1B). This decrease in M-ficolin correlated with the decrease in HBI (r = 0.40, p = 0.01) and was sustained throughout the follow-up period. The MASP-2 levels decreased from baseline to week 1 in both responders (37%, 425 ng/ml to 268 ng/ml, p < 0.0001) and non-responders (29%, 557 ng/ml to 397 ng/ml, p = 0.02); however, this change was not sustained at week 7 (Fig. 2A). In the responders, the MASP-3 levels increased by 26% from baseline to week 1 and remained high at week 7, whereas there was no change in the non-responders (Fig. 2B). Of interest, there were no changes over time in MBL, H-ficolin, L-ficolin, or MAp44 levels (Figs. 1 and 2).

3.2. Baseline lectin pathway protein correlations and levels

In accordance with previous studies, we observed large inter-individual variances in the serum levels of all the investigated soluble PRMs. At baseline, the M-ficolin median level in patients with CD was approximately 60% of the level in healthy controls (1062 ng/ml vs. 1408 ng/ml, p = 0.013) (Fig. 1B), whereas the H-ficolin levels were 36% higher than those in controls (27,389 ng/ml vs. 19,500 ng/ml, p = 0.0001) (Fig. 1C). The median MASP-3 level was 10% higher in patients than in controls (4429 ng/ml vs. 3950 ng/ml, p = 0.04) (Fig. 2B). We observed no differences in MBL, MASP-2, or MAp44 levels between patients and controls (Figs. 1A, 2A, 2B, and C, respectively).

The M-ficolin level correlated positively with both faecal calprotectin (r = 0.55, p = 0.004) and CRP (r = 0.36, p = 0.002). The H-ficolin level was weakly correlated to CRP (r = 0.31, p = 0.045) but not to any other parameters. MBL, MASP-2, MASP-3 and MAp44-levels did not correlate to any of the investigated clinical parameters. We observed no statistically significant differences in any of the median lectin pathway protein levels, depending on smoking status and disease location, and we did not observe any statistically significant differences between patients later classified as anti-TNF-α responders or non-responders (p > 0.10, all).

3.3. Lectin pathway protein levels during 8-week follow-up

We observed a 50% decrease in median M-ficolin levels between day 0 and week 1 in the responders (1150 pg/ml to 571 pg/ml, p < 0.001) and this decrease was sustained at week 7, whereas there was no decrease in the non-responders (Fig. 1B). This decrease in M-ficolin correlated with the decrease in HBI (r = 0.40, p = 0.01) and was sustained throughout the follow-up period. The MASP-2 levels decreased from baseline to week 1 in both responders (37%, 425 ng/ml to 268 ng/ml, p < 0.0001) and non-responders (29%, 557 ng/ml to 397 ng/ml, p = 0.02); however, this change was not sustained at week 7 (Fig. 2A). In the responders, the MASP-3 levels increased by 26% from baseline to week 1 and remained high at week 7, whereas there was no change in the non-responders (Fig. 2B). Of interest, there were no changes over time in MBL, H-ficolin, L-ficolin, or MAp44 levels (Figs. 1 and 2).

4. Discussion

Ficolins and MBL are important components of the innate immune system. These soluble and membrane-bound PRMs bind both pathogens and commensals at mucosal surfaces and may play a pivotal role in the interplay between the host and the microbiome in CD. PRMs have become increasingly interesting because drugs targeting the complement system are under development and may represent a new therapeutic strategy in IBD.35

The present study is the first dynamic study to investigate a range of lectin pathway proteins in active and quiescent CD. We observed that the induction of remission by anti-TNF-α therapy in the patients with active CD was associated with a significant decrease in M-ficolin. This decrease occurred along with a slight decrease in MASP-2, an important activator of the complement system, and a modest early increase in MASP-3, a presumed negative regulator of complement activation.36 Another essential negative regulator of complement activation, MAp44, tended to be increased in the responders compared to both the healthy controls and the non-responders. Collectively, our findings suggest that the net effect of changes in the levels of M-ficolin, MASP-2, and MASP-3 may result in reduced complement activation in patients with CD who respond to biological therapy.

It is difficult to judge the causal relationship of the observed changes, particularly in the light of the low baseline levels of M-ficolin pre-treatment in all patients with CD compared to those in the healthy controls as well as the decrease in MASP-2 in both the responders and the non-responders. We estimated active CD inflammation using both clinical and biochemical markers. It is plausible that the patients who were defined as responders in this study experienced a general reduction in systemic inflammation. Thus, the observed changes in the M-ficolin and MASP levels may reflect the general state of systemic inflammation and may not have resulted from immunological effects at the mucosal level. However, M-ficolin is relatively stable in healthy individuals and is not regarded to be a classical acute-phase protein.7 M-ficolin is released from granulocytes upon bacterial contact. We
speculate that the lower levels of M-ficolin may reflect an altered state of activation of granulocytes in CD.

With the exception of M-ficolin in synovial fluid, most lectin pathway proteins have only been determined in peripheral blood. A limitation of the present study is the confinement of analyses to peripheral blood. Ficolin levels at the site of inflammation at the mucosal level may be more relevant. However, only minute amounts of MBL messenger-RNA have been retrieved from mucosal biopsies. Individual MBL levels are largely determined by genotype, and the intra-individual variability of lectin pathway protein levels is pronounced. The activity of the lectin pathway proteins is determined by the level of functional proteins, and we chose to determine the ficolin, MASP and MBL serum levels. We found that there was no difference in the MBL levels between the healthy controls and the patients, irrespective of the treatment response and specific anti-TNF-α agent. These findings are consistent with those reported in several previous studies.

H-ficolin is secreted in the bile and is present on mucosal surfaces; it may represent an important first-line defence against mucosal pathogens. The low H-ficolin levels may define a subgroup of CD patients with high titres of anti-S. cerevisiae antibodies; however, the H-ficolin levels in a general CD population were not different from those noted in healthy controls. We observed a tendency towards higher levels of H-ficolin in patients with CD, although the difference was not statistically significant.

In conclusion, our findings suggest a downregulation of the lectin pathway of the complement system in patients with CD who respond to anti-TNF-α therapy, mediated by reduced M-ficolin and MASP-2 levels and a parallel increase in MASP-3 activity. Whether these changes are part of the therapeutical effect of anti-TNF-α agents or merely mirror

![Figure 1](image-url)
the state of inflammation is not possible to conclude from our study. Furthermore, we found no evidence that MBL, MAp44, and H- and L-ficolin are implicated in CD.

Conflict of interest statement

None of the authors has any conflict of interest to report that may be related to this submission.

Acknowledgement

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Author contributions: TDS & JA initiated the project. AKD, JFD, JA & CLH enrolled and treated the patients and gathered clinical data. ST made the sample analysis. TDS & CLH made the statistical analysis and TDS & JK wrote the manuscript. All authors read and approved the final manuscript.

References