



Extracellular Matrix Fragments of the Basement Membrane and the Interstitial Matrix Are Serological Markers of Intestinal Tissue Remodeling and Disease Activity in Dextran Sulfate Sodium Colitis

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Abstract

Background Chronic intestinal inflammation results in tissue damage partly caused by an increase in matrix metalloproteinases (MMP) activity causing degradation of extracellular matrix (ECM) proteins. We studied intestinal tissue remodeling by quantifying ECM protein fragments in serum in dextran sulfate sodium (DSS)-induced colitis, to investigate ECM protein fragments as serological biomarkers of intestinal tissue remodeling and disease activity.

Methods Male Sprague–Dawley rats received 5% DSS in drinking water for 5 days followed by 11 days with regular water. Disease activity index (DAI) was scored daily. Serum was collected on day 0, 6, 7, and 16. ELISAs were used to quantify MMP-derived remodeling fragments of basement membrane type IV collagen (C4M and PRO-C4) and interstitial matrix type III collagen (C3M and rPRO-C3).

Results In DSS rats, serum levels relative to baseline of C4M, PRO-C4, and C3M were elevated ($P < 0.01$; $P < 0.001$; $P < 0.001$) at day 7, which declined at day 16. Levels of rPRO-C3 were lower in DSS rats at day 7 and increased to normal levels at day 16. The ratio between C3M and rPRO-C3 showed an overall degradation ($P < 0.0001$) of collagen type III in DSS rats at day 7, which correlated to the DAI ($r^2 = 0.5588$, $P < 0.0001$).

Conclusion Our data suggest that remodeling of the basement membrane (C4M and PRO-C4) and the interstitial matrix (C3M and rPRO-C3) increased during DSS-induced colitis and declined with reversal of the disease. Thus, serological biochemical biomarkers of the ECM reflect tissue remodeling and could be studied as markers of disease activity in IBD.

Keywords Inflammatory bowel disease · DSS-induced colitis · Serological biomarkers · Extracellular matrix remodeling · Collagens

Abbreviations

MMP Matrix metalloproteinase
ECM Extracellular matrix
DSS Dextran sulfate sodium

DAI Disease activity index
CRP C-reactive protein
TMB Tetramethylbenzidine

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Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are part of the spectrum of inflammatory bowel diseases (IBD), which are chronic inflammatory diseases of the bowel, characterized by fluctuating disease courses with periods of inactive disease and flares of active disease. Disease activity in IBD patients is monitored based on clinical symptoms, endoscopy, and noninvasive biomarkers mainly reflecting the inflammation status. These noninvasive biochemical markers of disease activity are primarily C-reactive protein (CRP), which correlates to some degree with disease activity in CD and UC patients [1]. Furthermore, the fecal biomarkers, calprotectin and lactoferrin, reflecting infiltration of neutrophils in the intestinal wall may be used to assess intestinal inflammation [2].

Chronic intestinal inflammation results in tissue damage partly caused by an increase in matrix metalloproteinases (MMPs) resulting in degradation of the extracellular matrix (ECM) of which fragments are released into the circulation. CD and UC patients have increased expression and activity of MMPs in the intestinal tissue, which relate to severity of inflammation [3–6]. MMP-9 has been reported to be the most abundantly expressed MMP during active IBD, whereas MMP-2 is constitutively expressed in intestinal tissue [3, 7]. Experimental colitis studies in mice show that MMP-9 and MMP-2 are important modulators for the degree of intestinal injury during dextran sulfate sodium (DSS) colitis [8, 9], which underlines the relevance of these MMPs in the DSS model. In addition, serum levels of MMP-9 have been reported to decrease in CD patients who respond to anti-TNF- α treatment [10], which emphasize the pathophysiological role of MMP-9 in the course of active IBD.

The intestinal epithelial cells are situated on a specialized ECM scaffold that is the basement membrane, which is comprised of mainly type IV collagen and laminins. The most abundant collagens in the interstitial matrix, which is situated below the basement membrane, are the fibrillary collagens, type I and type III collagen [11]. During tissue homeostasis, the ECM is constitutively remodeled by the breakdown of ECM proteins mediated by MMPs and deposition of newly synthesized collagen [12–14]. Lower serum levels of the basement membrane protein, type IV collagen, have been reported in IBD patients compared to healthy donors and non-IBD patients [15], and it has been suggested that altered remodeling of the ECM during IBD is not only a consequence of inflammation, but also an active player of disease progression [16]. Fragments of ECM collagens in serum have been proposed as possible biomarkers for disease activity in CD [17]. In addition, serological ECM biomarkers generated by MMPs

are elevated in IBD patients and have shown the ability to differentiate UC and CD patients [18–20] and distinguish disease behavior in CD patients [20].

Since DSS induces the expression of MMPs, we used an acute in vivo DSS-induced colitis model to study the remodeling of the basement membrane type IV collagen and the interstitial matrix type III collagen to assess whether chemically induced colitis causes MMP-generated ECM fragment release into the circulation and whether these fragments reflect disease activity. Therefore, in this study, we investigated degradation and formation of the basement membrane and interstitial matrix (the ECM) in serum as surrogate markers of intestinal tissue remodeling and disease activity.

Methods

Animals

Male Sprague–Dawley rats (ENVIGO), 12 weeks of age, were housed in cages in groups of three. Temperature condition was controlled at 22 °C, and the rats were maintained under a 12:12-h light–dark cycle. The rats had free access to water and food in the entire study period. Four days before study start, the rats were stratified by weight and randomized into two groups, DSS and water controls. All procedures were conducted in accordance with the legislation and under ethical approval of the “*Dyreforsøgstilsynet*” (agreement number: 2017-15-0201-01171).

Study Design

To induce acute colitis, the DSS group ($n = 12$) received 5% dextran sulfate sodium salt (Sigma-Aldrich; $M_r \sim 40,000$, cat: 42867) in their drinking water ad libitum for 5 days. To reverse the disease activity, the DSS rats received regular drinking water the following 11 days. The control group ($n = 9$) received regular drinking water during the entire study. At day 0 ($n = 21$), day 6 ($n = 9$), day 7 ($n = 12$), and day 16 ($n = 12$), blood was drawn from the tail vein and serum was retrieved. Prior to blood sampling, the animals were fasted over night with free access to water (with or without DSS) to minimize the potential effect of collagen fragment deriving from the diet. At day 6, six DSS rats and three control rats were sacrificed, and colon was retrieved for macroscopic and histological analyses. The remaining rats were sacrificed at day 16. Each day, food and water intake were monitored.

Disease Activity Index

Disease activity was assessed each day using the disease activity index (DAI), which combines scores for weight loss,

stool consistency, blood in feces, and rectal bleeding. The DAI used in this study was modified from Shon et al. [21]. Weight loss in DSS rats was scored compared to the mean weight of age-matched controls: score 0 = 0–4.9% weight loss; score 1 = 5–10.9% weight loss; score 2 = 11–15.9% weight loss; and score 3 = 16–20% weight loss. Stool consistency: score 0 = normal and well formed; score 1 = soft and sticky stool visible at base of tail; score 2 = very soft and unformed; and score 4 = diarrhea and watery stool. Blood in feces or rectal bleeding: score 0 = normal color stool; score 2 = reddish color stool; and score 4 = bloody stool or bleeding from rectum. The score from each parameter was included in a daily total DAI score ranging from 0 to 12.

Macroscopic and Histologic Assessment of Colitis

Colon was retrieved from the sacrificed rats. The length was measured (cm), and the most distal 1 cm of the colon was fixated in formalin solution, neutral buffered, 10% (Sigma-Aldrich, cat: HT501128) for 24 h and stored in Dulbecco's phosphate-buffered saline (Sigma-Aldrich, cat: D8537). The tissue was dehydrated through increasing concentrations of ethanol and cleared in toluene before embedded in paraffin. Five- μ m sections of the embedded colon were stained with Masson's trichrome staining to assess structural changes and visualize collagen. Briefly, the tissue sections were deparaffinized and rehydrated, and the sections were stained with Weigert's iron hematoxylin and Biebrich scarlet-acid fuchsin solution, differentiated in phosphomolybdic-phosphotungstic acid solution, and afterward stained in aniline blue and differentiated in 1% acetic acid. Finally, the tissue was dehydrated and cleared in toluene and mounted with PERTEX[®]. The histological specimens were investigated under an OLYMPUS BX60 microscope, and images were obtained with an OLYMPUS DP71 camera.

The histological specimens were scored by a pathologist for the degree of tissue damage in a blinded manner. The tissue damage scoring index used was similar to the one described by Gaudio et al. [22]. Briefly, the parameters that were assessed were (1) destruction of epithelium and glands; (2) dilatation of glandular crypts; (3) depletion and loss of goblet cells; (4) inflammatory cells infiltration; (5) edema; (6) vascular congestion; (7) crypt abscesses; and (8) atrophy. For each parameter, a score from 0 to 3 was reported reflecting the following observations: 0 = absent, 1 = focal, 2 = zonal, and 3 = diffuse. A mean value of three histological specimens covering the 1 cm most distal part of the colon was reported for each rat.

Biomarkers in Serum

Quantifications of degradation and formation fragments of type IV collagen and type III collagen were carried out

by solid-phase competitive enzyme-linked immunosorbent assays (ELISAs). The neo-epitopes measured in this study were C4M, PRO-C4, C3M, and rodent-PRO-C3 (rPRO-C3) [23–26]. Streptavidin-coated 96-well plates (Roche Diagnostics' cat: 11940279) were coated with biotinylated antigen for 30 min at 20 °C while shaking at 300 rpm. Undiluted and 1:2 diluted serum samples, depending on the assay, and standard controls were added to the plates and incubated in the presence of horse-radish peroxidase-conjugated monoclonal antibodies, targeting the specific ECM neo-epitope, for 1 h at 20 °C or 20 h at 4 °C, depending on the assay, while shaking at 300 rpm. Tetramethylbenzidine (TMB) was added to the plates and incubated for 15 min at 20 °C while shaking at 300 rpm and following addition of 100 μ l/well stopping buffer (0.1 M H₂SO₄) to terminate the TMB reaction. Washing of plates with wash buffer was carried out after each incubation. The color change absorbance was read at wavelength 450 nm using a spectrophotometer (SpectraMax M5, Molecular Devices) with 650 nm as reference. A four-parameter mathematical fit model was used for plotting the standard curve.

Statistical Analyses

GraphPad Prism 7.01 was used for all statistical analyses. All serum biomarker levels are presented as mean values of the percentage change in biomarker levels relative to baseline values \pm SE of the mean. The relative to baseline values were tested for normal distribution using Shapiro–Wilk normality test on grouped data. Changes in biomarker levels and differences in colon length were investigated using multiple unpaired t tests. Correction for multiple comparisons was carried out with the Holm–Sidak method and a significance level of $\alpha = 0.05$ was applied. Pearson's and Spearman correlations were used to investigate the relationship between changes in biomarker levels and disease activity.

Results

Rats Receiving DSS Demonstrated Weight Loss and Increased Disease Activity, Which Were Reversed Upon Withdrawal of DSS

Rats receiving DSS in the drinking water lost weight during induction of colitis, with a significant different mean body weight compared to control rats at days 3 and 4 ($P < 0.01$, $P = 0.041$). In the recovery phase, the DSS rats gradually gained weight in parallel with control rats (Fig. 1a). From baseline to the last day of DSS administration (day 5), colitis progressed in the DSS rats, as assessed by the DAI (Fig. 1b). The chemically induced colitis was reversed by administering regular drinking water to the DSS rats, which gradually

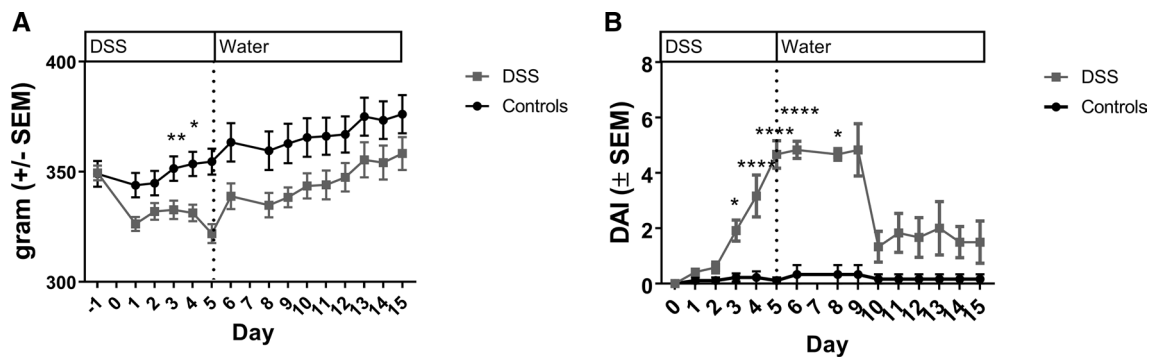


Fig. 1 Body weight changes and disease activity index during DSS-induced colitis and remission of disease. **a** The change in body weight, **b** the change in DAI. The error bars represent the SE of the mean (SEM). *P* values, **P* < 0.05, ***P* < 0.01, *****P* < 0.0001

decreased the DAI score toward the levels of the control rats at the last day of the study (Fig. 1b).

DSS Rats Exhibited Shortening of the Colon and Histological Intestinal Tissue Damage, Which Recovered Upon Withdrawal of DSS

Macroscopically, the colon was significantly shorter in DSS rats compared to controls at day 6 (*P* < 0.01) and day 16 (*P* < 0.05) (Fig. 2a). The colon length of DSS rats terminated after 11 days of regular water (day 16) was significantly longer (*P* < 0.05) than the colon length of DSS rats terminated the day after end of DSS administration (day 6) (Fig. 2a). The histological tissue damage score was significantly increased in DSS rats compared to controls at day 6 (*P* = 0.0357) and day 16 (*P* = 0.0445) (Fig. 2b). In addition, the histological score was significantly higher in DSS rats at day 6 compared to DSS rats at day 16 (*P* = 0.0046) (Fig. 2b). Histologically, it was apparent on day 6 that DSS caused damage of the intestinal tissue. The intestinal crypts

and normal epithelium were lost, and the submucosa had less densely packed collagen (Fig. 3b) compared to controls, which had intact crypt and epithelium along with densely packed collagen in the submucosal space (Fig. 3a). After 11 days of regular water, the DSS rats showed the presence of crypts and more normal mucosa (Fig. 3d) with a more similar tissue structure to the controls at day 16 (Fig. 3c) than DSS rats compared to controls at day 6. However, the submucosa in DSS rats at day 16 was still swollen and showed less densely packed collagen as compared to controls (Fig. 3c, d).

Serological Biomarkers of Basement Membrane Type IV Collagen Demonstrated Increased Remodeling in DSS-Induced Colitis

The biomarker C4M quantifies the amount of MMP degraded type IV collagen alpha 1 chain in serum. There was a significant increase (*P* < 0.01) in the levels of C4M in serum, relative to baseline, from DSS rats compared to

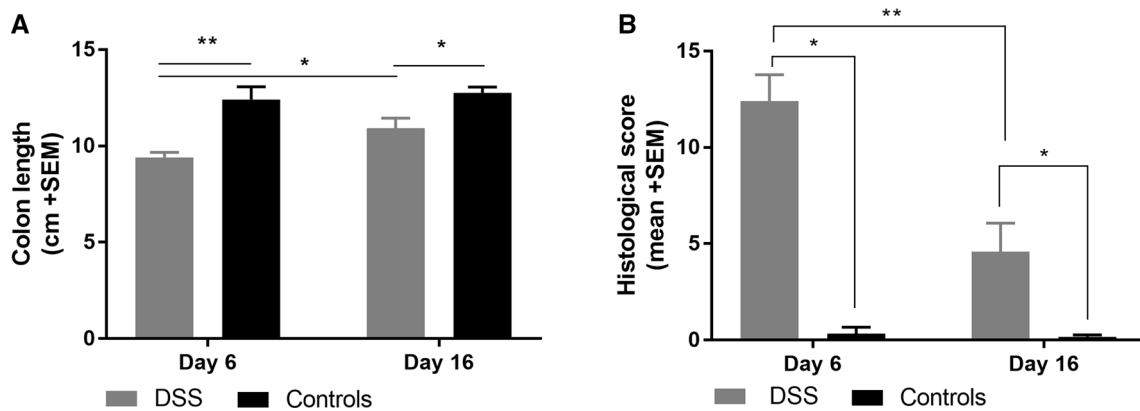
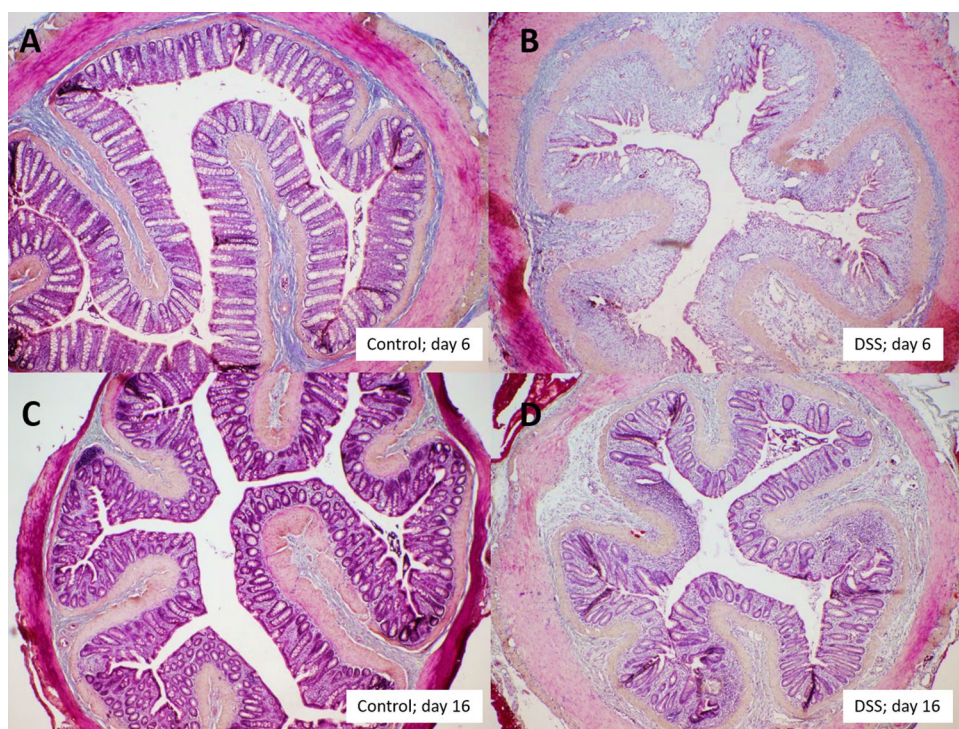


Fig. 2 The difference in colon length and the histological score of tissue damage of DSS rats and controls at day 6 and day 16. **a** The colon lengths are presented in cm, and **b** histological score of tissue damage

is presented as the mean value of three histological specimens from the 1-cm most distal part of the colon. The error bars represent the SE of the mean (SEM). *P* values, **P* < 0.05, ***P* < 0.01

Fig. 3 Histological changes in colonic tissue structure in DSS and control rats at day 6 and day 16. **a** Control rat at day 6, **b** DSS rat at day 6, **c** control rat at day 16, and **d** DSS rat at day 16. The tissue was stained with Masson's trichrome staining, and the color saturation on all pictures was set to 170%



control rats at day 7 (Fig. 4a). In addition, the amount of C4M in serum from DSS rats declined toward the level of control rats at the end of the study after receiving regular water for 11 days. PRO-C4 (type IV collagen formation) serum levels relative to baseline were also increased ($P < 0.001$) in DSS rats at day 7 and decreased toward the levels of control rats at the end of the study (Fig. 4b).

To investigate whether there was increased basement membrane degradation or formation during the DSS-induced colitis, the basement membrane turnover (ratio between degradation (C4M) and formation (PRO-C4) of type IV collagen) was assessed. The turnover of type IV collagen was not dominated by either degradation or formation, nor significantly different between DSS rats and controls during the entire study (Fig. 4c). Furthermore, there was no significant correlation between the turnover of type IV collagen and DAI or the histological tissue damage score (Fig. 4f, i), nor for C4M and DAI or the histological tissue damage score (Fig. 4d, g) and PRO-C4 and DAI or the histological tissue damage score (Fig. 4e, h).

Serological Biomarkers of Interstitial Type III Collagen Demonstrated Increased Degradation and Decreased Formation in DSS-Induced Colitis

Serum levels, relative to baseline, of type III collagen degradation biomarker, C3M, was significantly increased at day 7 ($P < 0.001$) in DSS rats compared to controls

(Fig. 5a). The serum levels of C3M declined toward the levels of control rats at day 16; however, C3M was still significantly increased ($P < 0.01$) in DSS rats compared to controls. The formation of type III collagen was assessed using the formation biomarker rPRO-C3, which showed a tendency of declining in DSS rats after induction of colitis and then increased toward the levels of the controls on the last day of the study, after receiving regular water (Fig. 5b). The turnover of the interstitial matrix type III collagen was assessed by the ratio of the values relative to baseline of C3M and rPRO-C3 (Fig. 5c), which showed a predominant degradation of the interstitial matrix at day 7 ($P < 0.0001$). In addition, the total degradation of type III collagen declined toward the levels of the controls at the last day of the study. The predominant degradation of type III collagen demonstrated a positive correlation ($P < 0.0001$, $r^2 = 0.5588$) with the DAI score (Fig. 5f). In addition, C3M and rPRO-C3 demonstrated a positive ($P < 0.01$, $r^2 = 0.2394$) and negative ($P < 0.05$, $r^2 = 0.1594$) correlation with the DAI score, respectively (Fig. 5d, e). The turnover of type III collagen demonstrated a positive correlation to the histological tissue damage score ($P = 0.002$, $r^2 = 0.44$) (Fig. 5i), and there was a trend of a negative correlation between rPRO-C3 and the histological damage score ($P = 0.094$, $r^2 = 0.16$) (Fig. 5h), but C3M did not show an apparent correlation with the histological tissue damage score (Fig. 5g).

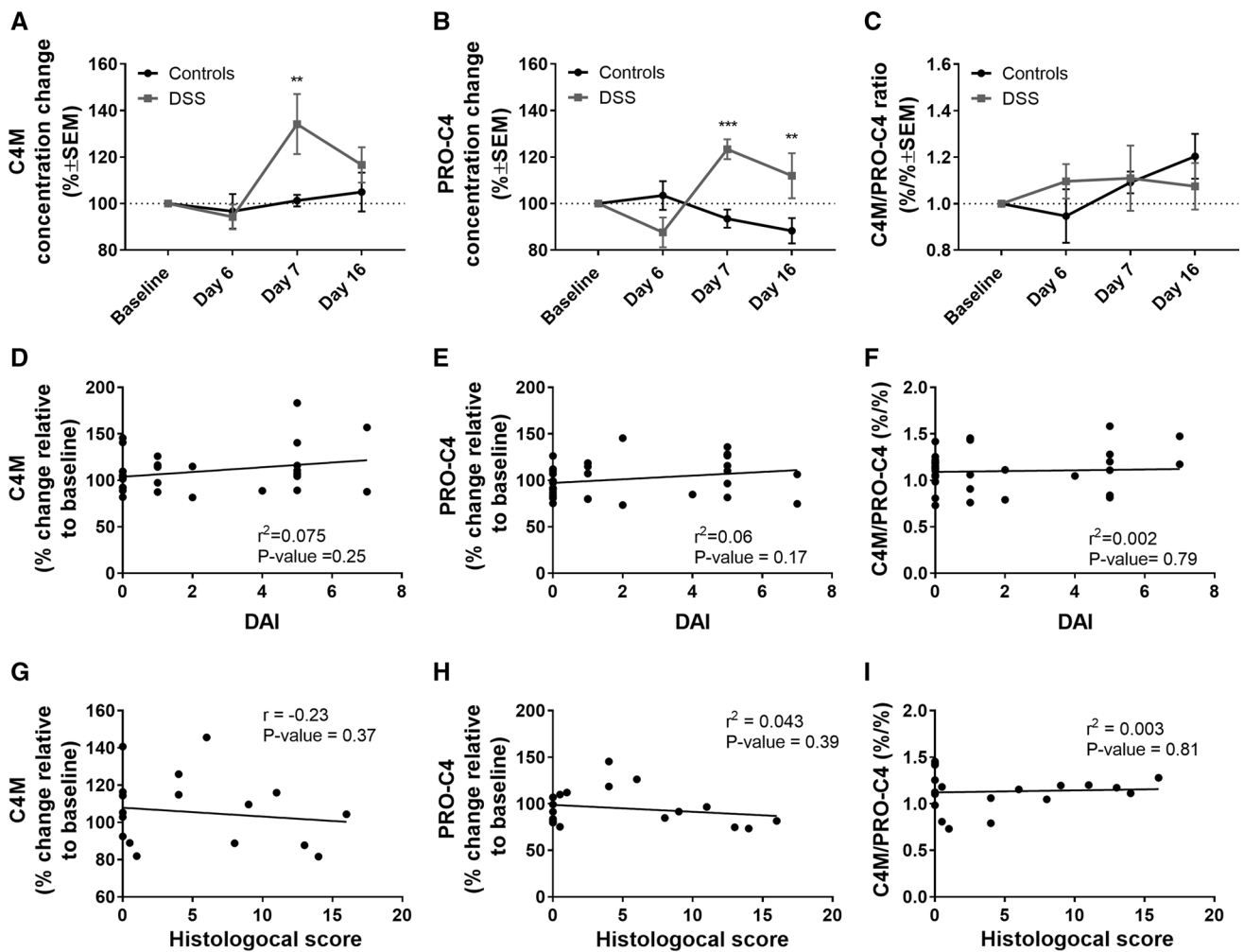


Fig. 4 Serum levels of type IV collagen ECM biomarkers relative to baseline in DSS and control rats and correlations of the biomarkers to DAI and the histological score. The percentage change at days 6, 7, and 16 in C4M (a) and PRO-C4 (b) serum levels relative to baseline values. The turnover of type IV collagen as the ratio of changes in serum levels of C4M and PRO-C4 relative to baseline values at days 6, 7, and 16 (c). Correlations to DAI for C4M (d) and PRO-C4 (e)

alone and correlation between the turnover of type IV collagen and the disease activity index (DAI) (f). Correlations to the histological score for C4M (g) and PRO-C4 (h) alone and correlation between the turnover of type IV collagen and the histological score (i). The error bars represent SE of the mean (SEM). *P* values, **P*<0.05, ***P*<0.01, ****P*<0.001

Discussion

This study presents the first data on serological biomarkers measuring basement membrane and interstitial matrix remodeling in rats with DSS-induced colitis. An exposure to DSS for five days followed by 11 days of recovery provided dynamics with initial colitis followed by a reversal of the inflammation. We observed an increase in basement membrane and interstitial matrix remodeling reflected by degradation and formation of type IV collagen (C4M, PRO-C4) and type III collagen (C3M, rPRO-C3). In addition, the clinical and histological manifestations of disease activity in this model were correlated to the serological biomarkers of the ECM. Our data therefore suggest that serological

biomarkers of ECM remodeling may be used to monitor disease activity during intestinal inflammation.

In response to injury, such as DSS, the intestinal epithelial cells migrate over the denuded epithelial basement membrane, if it is intact, to seal the wound [27]. We found an increase in degradation (C4M) and formation (PRO-C4) fragments of basement membrane type IV collagen in serum from rats receiving DSS compared to control rats. The degradation biomarker, C4M, is generated by MMP-2/-9 cleavage of the type 4 alpha 1 chain [23], which is distributed along the entire crypt villus axis in the intestinal epithelial basement membrane [28]. This suggests an increased intestinal epithelial basement membrane remodeling induced by DSS that may or may not leave the basement membrane

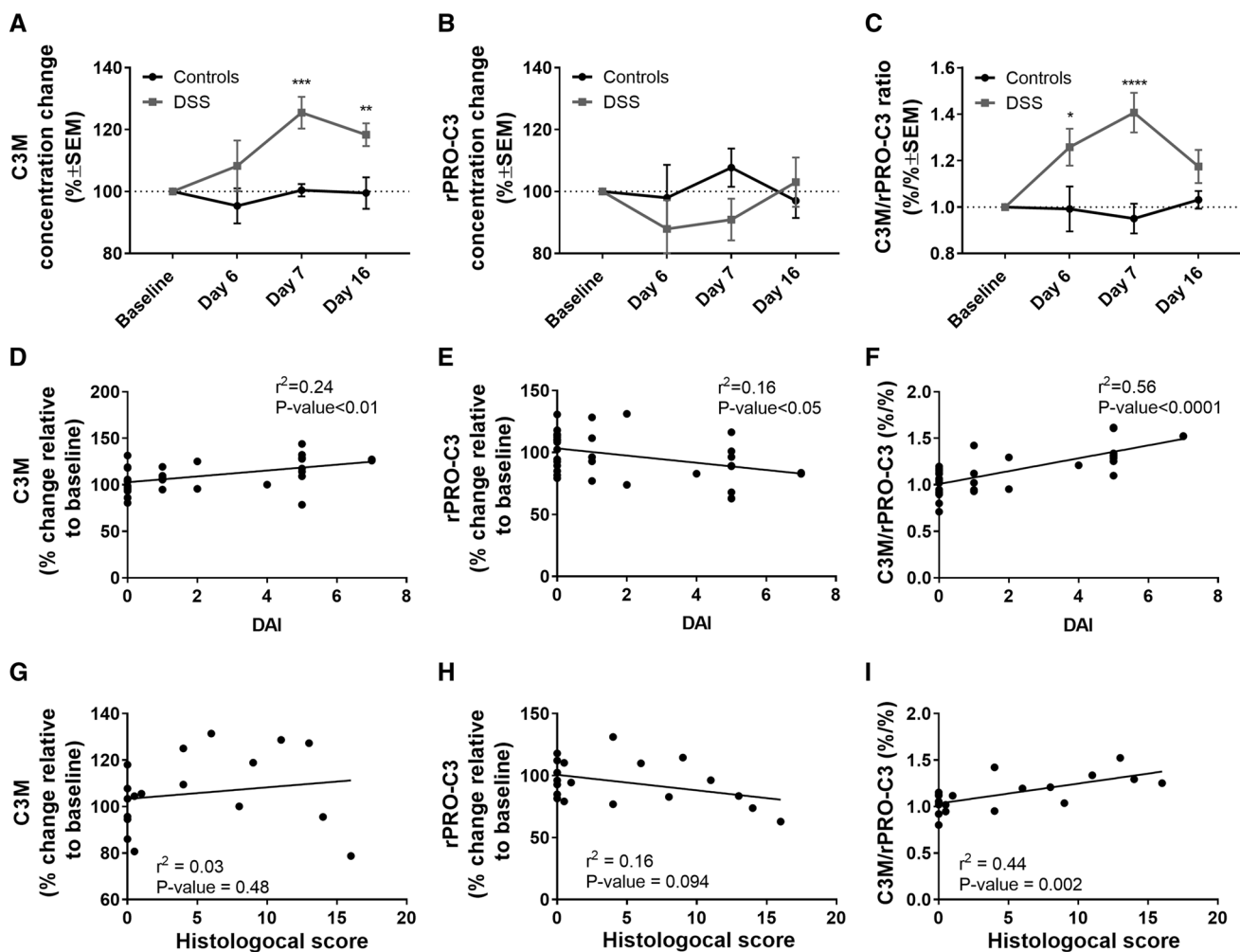


Fig. 5 Serum levels of type III collagen ECM biomarkers relative to baseline in DSS and control rats and correlations of the biomarkers to DAI and the histological score. The percentage change at days 6, 7, and 16 in C3M (a) and rPRO-C3 (b) serum levels relative to baseline values. The turnover of type IV collagen as the ratio of changes in serum levels of C3M and rPRO-C3 relative to baseline values at days 6, 7, and 16 correlations to DAI for C3M (d) and rPRO-C3 (e) alone

and correlation between the turnover of type III collagen and the disease activity index (DAI) (f). Correlations to the histological score for C3M (g) and rPRO-C3 (h) alone and correlation between the turnover of type III collagen and the histological score (i). The error bars represent SE of the mean (SEM). P values, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, **** $P<0.0001$

intact for intestinal epithelial cell migration and adhesion. The amount of type IV collagen fragments in serum reached normal levels at day 16 where DAI was comparable to the control group. The ratio between degradation and formation of type IV collagen did not differ significantly from control rats during induction of colitis and in the period the rats receive regular water from day 5 to 11. In addition, increased DAI and histological tissue damage did not significantly correlate to a basement membrane turnover that was either predominant formation or degradation. Our results therefore indicate that DSS causes an overall increased turnover of the intestinal basement membrane, most likely a strong repair response, which may stay intact during the timeframe of this study. This is in line with a previous report on serum

from IBD patients, in which the type IV collagen degradation (C4M) and formation (PRO-C4) were elevated [18].

The main collagens in the intestinal interstitial matrix below the basement membrane are type I and type III collagen [11]. As MMP-9 is up-regulated in both IBD patients and in DSS-induced colitis animal models [6], we expected to see an increase in the MMP-9 generated neo-epitope of type III collagen, C3M, which also was the case concomitantly with a decrease in type III collagen formation (rPRO-C3). At the end of the study when DAI returned to values comparable to the control group, both biomarkers went toward or were at levels similar to the control rats. These results suggest that DSS has an effect on the deeper interstitial matrix of the intestine and

causes increased remodeling of the tissue. Furthermore, the ratio of type III collagen degradation vs. formation fragments shows a dominating degradation of type III collagen correlating well with DAI and the histological tissue damage, which indicate that the turnover of type III collagen can possibly be used to monitor disease activity. Taken together, the data presented in this study reflect true remodeling of intestinal tissue and are in line with previous reports in human IBD serum samples [18, 29, 20].

DSS has been shown by other research groups to robustly induce the expression of MMP-9 and MMP-2 during colitis in animal studies [8, 30, 31]. This is in complete alignment with the current data, in which we found an increase in type IV collagen degradation fragment (C4M), which is a neo-epitope of MMP-2, MMP-9, and MMP-13 cleavage, in serum from rats receiving DSS. As the expression of MMPs increases due to an increased inflammatory burden during DSS-induced colitis and in active IBD the remodeling of the intestinal tissue is affected. Thus, it is very likely that tissue remodeling correlates well with disease activity and histological tissue damage and as presented in this DSS-induced colitis study, serological biomarkers of basement membrane and interstitial matrix remodeling are in line with that hypothesis.

Administration of 5% DSS during 5 days was used in this study, but it is possible there is a fine balance for the concentration of DSS and the duration of time for administration that will change the inflammatory burden and this could change the basement membrane remodeling profile. Further studies are needed to address this.

The correlation between the histological tissue damage and the biomarkers was not superior to the correlation between the biomarkers and DAI, which was unexpected since the biomarkers are a direct measure of the tissue turnover. However, the biomarker levels showed the highest difference from baseline at day 7 and we only had tissue specimens from days 6 to 16, which may explain why the histological correlation to the biomarkers was not better than to DAI.

Remodeling of the ECM in any organ contributes to the total amount of ECM fragments in the circulation; therefore, when quantifying the DSS-induced intestinal tissue remodeling in serum, we measure the induced intestinal tissue remodeling on top of the steady-state remodeling in other tissues. However, this is accounted for with the healthy controls in this study. In addition, to our knowledge DSS administered alone does not induce any apparent histopathological liver damage [32], which would be the expected secondary organ affected by DSS. This suggests that the serological biomarkers in this model and possible in patients may be accurate biomarkers reflecting the disease pathology and specific tissue remodeling.

Histological assessment of the intestinal tissue revealed that the colonic mucosa had recovered some of its normal structure and emphasis that the type IV collagen measured in serum may reflect excessive repair responses. Rats receiving DSS did still have signs of histological structure damage at the end of the study, after receiving regular water for 11 days. Therefore, future studies will focus on a longer administration period of regular water to assess the biomarker levels in relation to normalization of DAI and true histological healing. The age of the rats as they entered the study (13 weeks) should not be a limitation in the current study as the markers have been evaluated in rats in relation to age [33].

In summary, we report that increased remodeling of the intestinal tissue, quantified with biomarkers of basement membrane remodeling (C4M, PRO-C4), and interstitial matrix remodeling (C3M, rPRO-C3) was increased upon DSS-induced colitis. Thus, increased tissue remodeling, quantified with the serological ECM biomarkers, is associated with disease activity and healing of the intestines. These data suggest that serological biomarkers of basement membrane type IV collagen remodeling and remodeling of the interstitial matrix type III collagen can potentially be used for monitoring tissue damage and disease activity during IBD and in response to treatment.

Author's contribution ML helped in study design, data collection, data analysis, interpretation of data, drafting the manuscript, and critical revision for important intellectual content. TMJ, GIM, AK, MAK, JK, and JHM contributed to study design, interpretation of data, and critical revision for important intellectual content. All authors approved the final draft before the manuscript was submitted.

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Compliance with ethical standards

Conflict of interest ML, TMJ, MAK, and JHM are full-time employees at Nordic Bioscience. TMJ and MAK hold stocks in Nordic Bioscience. Nordic Bioscience is a privately owned small-medium-sized enterprise, partly focused in the development of biomarkers for connective tissue disorders and rheumatic diseases. None of the authors received any kind of financial benefits or other bonuses for the work described in this manuscript.

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