Original Article

Development of Fibrosis in Acute and Longstanding Ulcerative Colitis

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Abstract

Background: Intestinal fibrosis is a process driven by chronic inflammation leading to increased presence of myofibroblasts and collagen deposition. Although strictures are rarely seen in ulcerative colitis [UC], longstanding disease is believed to cause fibrosis resulting in altered bowel function.

Methods: The presence of fibrosis was studied in colectomy specimens from patients with recent-onset UC refractory to medical treatment [n = 13] and longstanding UC [n = 16], and colon cancer patients without UC [n = 7] as controls. Severity of inflammation was scored according to the Geboes score on haematoxylin and eosin stainings. Immunohistochemistry was performed to detect α-smooth muscle actin, fibronectin and collagen I and III.

Results: Colectomy specimens from patients with acute UC showed significantly more inflammation than those with longstanding disease [19 vs 9 points, p = 0.01]. Both acute and longstanding UC showed a thicker muscularis mucosa than controls [0.10 vs 0.10 vs 0.05 mm, respectively, p = 0.019]. An increase in collagen I and III deposition in the mucosa was observed in UC compared with controls (40% [30–75] vs 25% [10–25], p = 0.033), but this did not differ significantly among acute and longstanding UC patients.

Conclusions: Collagen deposition is enhanced in UC compared with controls. However, UC collagen deposition does not increase significantly over time and does not seem to aggravate the entire fibrotic process.

Keywords: Ulcerative colitis; fibrosis; pathology

1. Introduction

Traditionally, ulcerative colitis [UC] has been defined as a mucosal disease, in contrast with Crohn’s disease [CD] where the intestine is transmurally affected. In CD approximately 70% of the patients develop disease complications with strictures and/or fistulas over time. Contrary to the relatively high incidence of strictures in CD, the frequency of this complication is much lower in UC, varying between 1.5% and 11.2%. Moreover, an important proportion of these reported strictures have been related to the presence of cancer.

Fibrosis is a chronic and progressive process characterised by an excessive deposition of collagen and other extracellular matrix
Fibrosis in Ulcerative Colitis

The fibrotic process is characterised by a cascade of events including epithelial cell damage and repair, angiogenesis and lymphangiogenesis, and activation of immune cells and mesenchymal cells. Mesenchymal cells can differentiate into fibroblasts, myofibroblasts, and smooth muscle cells. These cell types have an interrelated phenotype and can differentiate and dedifferentiate into each other. One of the determining factors is the presence of alpha-smooth muscle actin [α-SMA] and desmin. Fibroblasts synthesise several extracellular-matrix proteins, among which are collagen and fibronectin. Increased deposition of ECM is a result of increased proliferation and activation of fibroblasts. Fibroblasts proliferate in response to inflammatory mediators such as pro-inflammatory cytokines and several growth factors, or in response to contact with T-cells, eosinophils and mast cells. Fibroblasts initiated it may progress independently of further inflammation. Collagen is one of the dominant ECM components synthesised during the process of fibrosis. The most important collagen subtypes are type I and type III. Collagen I gives tensile strength and mechanical stability to the connective tissue, whereas collagen type III provides tissue elasticity and flexibility.

End-stage UC may lead to a ‘lead-pipe colon’, with a shortened, stiff, and narrowed colon with loss of haustra. At microscopic level, the muscularis mucosa can become thicker whereas the muscularis externa thins and relaxes. Moreover, the submucosa thickens due to the deposition of fat. The contraction of hypertrophied muscularis mucosa may pull the mucosa away from the submucosa, which has been proposed to contribute to benign strictures.

In UC, little is known about the occurrence of fibrosis. We therefore investigated whether there is a different fibrotic load in acute, early-onset disease vs longstanding disease, and whether the degree of fibrosis correlates with the severity and extent of inflammation.

2. Material and Methods

2.1. Patient selection

From 2007 to 2012, in total 146 UC patients underwent a colectomy at the Academic Medical Center in Amsterdam, The Netherlands. From this cohort, we selected two subgroups meeting predefined criteria at both ends of the spectrum. In the first group, UC patients had a relatively recent onset and therapy-refractory disease with colectomy within 2 years after diagnosis. In the second group, UC patients had longstanding disease [> 10 years] and these patients underwent colectomy for dysplasia. All the other patients were excluded from this study. A third control group consisted of patients who had a colectomy for colon cancer without previous inflammatory bowel disease (IBD).

The resection specimens were retrieved from the pathology tissue bank. In both the acute and the longstanding UC cases, the most inflamed area was identified by an expert IBD pathologist who was blinded for disease duration and treatment [SM]. In the longstanding UC group and control group, the selected sections were taken at a distance of at least 10 cm from neoplastic lesions in order to exclude tumour involvement. In all UC sections, the most inflamed area was identified by the pathologist. This area was identified to be in the descending colon in 20 patients, in the transverse colon in 9 patients and in the ascending colon in 9 patients.

The medical ethical committee granted a waiver for this study, based on Dutch legislation.

2.2. Histology and immunohistochemistry

Immediately following surgery, colectomy specimens were routinely processed for histology. This includes a protocolised approach where routinely every 10 cm a section is being processed perpendicular to the bowel wall and embedded in paraffin. 4-μm thick sections were prepared and stained routinely with haematoxylin and eosin [H&E]. Back to back, consecutive slides were used for Picosirisus Red staining and immunohistochemistry. For the purpose of the current study, Picosirisus Red stainings were performed as described previously. Briefly, after deparaffinisation and dehydration, slides were incubated for 1 h at room temperature with Picosirisus Red solution [Direct Red 80; Sigma Aldrich, dissolved in saturated aqueous solution of picric acid [Sigma Aldrich]]. Slides were washed in acidified water, dehydrated, cleared in xylene, and mounted with DPX mountant [Sigma Aldrich, St Louis, MO, USA].

For immunohistochemistry, sections were deparaffinised, dehydrated, and immersed in 0.3% H2O2 in methanol. After antigen retrieval and blocking, slides were incubated with the primary antibody dissolved in phosphate-buffered saline with 0.1% Triton X-100 and 1% bovine serum. To detect myofibroblasts and smooth muscle cells, an anti-α-SMA antibody [LS B3933; Lifespan, Seattle, WA, USA; dilution 1:750] was used; to detect the ECM component fibronectin, an anti-fibronectin antibody [ab 23750; Abcam, Cambridge, UK; dilution 1:200] was used; and to detect specific collagen deposition, an anti-collagen I and anti-collagen III antibody [respectively 1310-01 and 1330-01, both from Southern Biotech, Birmingham, AL, USA; dilution both 1:200] were used. After incubation with the appropriate secondary antibody [from Immunologic, Duiven, The Netherlands], slides were counterstained with haematoxylin, dehydrated, cleared in xylene, and mounted.

Immunostained slides were all investigated blind to diagnosis under identical light microscope conditions, including magnification [x100], gain, camera position, and background illumination.

2.3. Histological evaluation

Inflammation was scored by a blinded expert IBD pathologist [SM] using the previously described Geboes histological inflammatory activity score. In this score, structural damage, chronic inflammatory infiltrate, presence of eosinophils and/or neutrophils in the lamina propria or epithelium, crypt destruction, and erosions or ulcerations are assessed. The score ranges from 0 [no inflammation] to 22 [maximum inflammation] points. The score was determined on the selected slides in the most involved region.

Staining intensity was determined on the most involved area of the selected slide using Image J analysis software, available free from the National Institutes of Health at [rsweb.nih.gov/ij]. Results are given as the number of positive stained cells divided by the total surface area as indicated by haematoxylin staining. Positive staining was defined as moderate to strong unequivocal staining. Minimal expression was considered as background staining and not taken into account.

2.4. Statistical analysis

Data were analysed using SPSS software version 20.0 [IBM Inc]. Descriptive data were given as mean and standard deviation [SD] or, for non-parametric data, as median and interquartile range [IQR]. Kruskal-Wallis test with post-hoc analysis or the Mann-Whitney U test were performed to compare data, and correlations were calculated using Spearman’s rank correlation. A p-value of < 0.05 was considered statistically significant.

3. Results

3.1. Patient characteristics

We identified 13 UC patients who underwent a colectomy for acute refractory disease and 16 for longstanding UC between 2007 and
Table 1. Disease characteristics of acute and longstanding ulcerative colitis.

<table>
<thead>
<tr>
<th>Disease location</th>
<th>Acute UC</th>
<th>Late UC</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Disease location</td>
<td>E1</td>
<td>1/14 [7%]</td>
<td>0/16 [0%]</td>
</tr>
<tr>
<td>Disease location</td>
<td>E2</td>
<td>2/14 [14%]</td>
<td>4/16 [25%]</td>
</tr>
<tr>
<td>Disease location</td>
<td>E3</td>
<td>11/14 [79%]</td>
<td>12/16 [75%]</td>
</tr>
<tr>
<td>Previous medication</td>
<td>Prednisone</td>
<td>12/12 [100%]</td>
<td>13/13 [100%]</td>
</tr>
<tr>
<td>Previous medication</td>
<td>5-ASA</td>
<td>10/12 [83%]</td>
<td>15/16 [94%]</td>
</tr>
<tr>
<td>Previous medication</td>
<td>Thiopurines</td>
<td>11/13 [85%]</td>
<td>12/15 [80%]</td>
</tr>
<tr>
<td>Previous medication</td>
<td>Methotrexate</td>
<td>1/12 [8%]</td>
<td>1/14 [7%]</td>
</tr>
<tr>
<td>Previous medication</td>
<td>Cyclosporin</td>
<td>2/13 [15%]</td>
<td>1/14 [7%]</td>
</tr>
<tr>
<td>Previous medication</td>
<td>Infliximab</td>
<td>10/13 [77%]</td>
<td>4/15 [27%]</td>
</tr>
<tr>
<td>Previous medication</td>
<td>Adalimumab</td>
<td>1/13 [8%]</td>
<td>1/15 [7%]</td>
</tr>
<tr>
<td>Previous medication</td>
<td>Trial medication</td>
<td>0/13 [0%]</td>
<td>2/15 [13%]</td>
</tr>
</tbody>
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UC, ulcerative colitis; E1, ulcerative proctitis; E2, left-sided UC; E3, extensive UC; 5-ASA, 5-aminosalicylic acid; ns, not significant.

Figure 1. Increased severity of inflammation in the acute colectomy specimens. Representative examples of the severity of inflammation in acute ulcerative colitis [a], longstanding ulcerative colitis [b], and cancer controls [c], with the corresponding Geboes score for all patients [d]. H&E, haematoxylin & eosin; original magnification in 1, 2, and 3: x100. *p = < 0.05, **p = < 0.01. Median and interquartile range are shown in [d].

Table 2. Histology scores and fibrosis in acute UC

<table>
<thead>
<tr>
<th>Trial medication</th>
<th>Acute UC</th>
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2012. Mean disease duration following diagnosis was 1.08 [± 0.55] years and 20.04 [± 7.54] years, respectively. Before diagnosis, the acute UC patients had symptoms for a shorter period of time only [mean 0.2 years ± 0.16]. The mean age at surgery was 32.7 ± 12.4 years in the acute UC group and 45.0 ± 7.7 years in the longstanding UC. Disease extension did not differ significantly between the two UC groups [Table 1]. Acute UC patients more frequently received Infliximab than those with longstanding UC, but other treatments did not differ [Table 1]. Patients in the late UC group had suffered significantly more flares and were treated with more steroid courses than the acute UC patients [mean duration of the flare 21.25 ± 10.6 months vs 6.77 ± 2.6 months, p = 0.001]. From all the patients who underwent a colectomy for colorectal carcinoma, we selected seven patients without IBD where the tumour was radically removed to serve as a control group (mean age at surgery of 64.1 ± 7.3 years).

3.2. Histology scores and fibrosis in acute UC

In the acute UC colectomy specimens, we found significantly higher inflammatory [Geboes] scores than in the longstanding UC specimens [median duration of the flare 21.25 ± 10.6 months vs 6.77 ± 2.6 months, p = 0.001]. From all the patients who underwent a colectomy for colorectal carcinoma, we selected seven patients without IBD where the tumour was radically removed to serve as a control group (mean age at surgery of 64.1 ± 7.3 years).

3.2.1. α-SMA expression in the submucosa is lower in UC than in controls

Since α-SMA stains myofibroblasts, the muscularis mucosa can be nicely visualised with this staining. In acute UC as well as in longstanding UC, the muscularis mucosa was significantly thicker compared with controls [0.10 vs 0.10 vs 0.05 mm, p = 0.02; Figure 2]; however, between the acute and longstanding UC groups the thickness did not differ significantly. There was no correlation between thickness of the muscularis mucosa and the resection site of the original specimen, either when all patients were grouped together [p = 0.2] or when subdivided for either acute vs longstanding UC, acute UC vs control patients, or longstanding UC vs control patients [p = 0.6, p = 0.1, and p = 0.3, respectively]. Therefore, in none of these comparisons was the thickness of the muscularis mucosa related to the location of the specimen.

Strikingly, the expression of α-SMA in the submucosa was lower in both acute and longstanding UC vs controls (29% [16–38] vs 33% [19–47] vs 54% [42–57], respectively, p = 0.009, Figure 3). In the mucosa, muscularis mucosa or muscularis externa, the α-SMA expression did not differ significantly between the two UC groups and controls.

The expression of fibronectin was higher in the mucosa of longstanding UC patients than in the control patients (54% [44–65] vs 36% [26–48], p = 0.022, Figure 4); however, this did not differ significantly between the acute UC patients compared with the controls or between the acute and late UC. In the other layers of the bowel wall there was no significant difference in fibronectin expression between the different groups.

3.3. More collagen I and III deposition in UC than in controls

Collagen I expression was higher in the mucosa of longstanding UC than in controls (40% [30–75] versus 23% [10–25], p=0.033). Between acute UC and controls this difference showed the same trend, although just did not reach significance [p = 0.06, Figure 5]. In the muscularis mucosa, the collagen I deposition was significantly higher in both acute and late UC when compared with controls (respectively, 30% [11–43] vs 10% [5–15], p < 0.01). In both acute UC patients compared with the controls and between the acute and longstanding UC groups this difference showed the same trend, although just did not reach significance [p = 0.06, Figure 5]. In the muscularis mucosa, the collagen I deposition was significantly higher in both acute and late UC when compared with controls (respectively, 30% [11–43] vs 10% [5–15], p < 0.01).
vs 5% [1–16], p = 0.03; and 28% [13–45] vs 5% [1–16], p = 0.02). However, between the two UC patient groups this did not differ.

Last, in the muscularis externa the deposition of collagen I was higher in both acute and late UC when compared with controls (respectively, 64% [36–83] vs 22% [19–28], p = 0.002; and 76% [28–85] vs 22% [19–28], p = 0.014). Also collagen III expression was higher in the muscularis externa of acute UC patients compared with controls, as well as of late UC compared with controls (respectively, 29% [24–38] vs 10% [8–16], p = 0.001; and 32% [29–39] vs 10% [8–16], p = 0.000). Also when looking transmurally, there was no difference in collagen deposition between the acute and late UC patients.

Sirius Red is a very sensitive stain for collagen but does not allow differentiation between different subclasses of collagen. The intensity of Sirius Red staining in the mucosa was higher in longstanding UC compared with acute UC patients (respectively, 30% [28–37] vs 27% [9–30], p = 0.034). Between the acute UC and control patients this difference showed the same trend, although it did not reach significance (p = 0.07).

In the muscularis externa, the intensity of Sirius Red staining was higher in UC patients compared with controls (34% [29–37] in acute UC vs 26% [17–33] in control patients, p = 0.002; between longstanding UC and control patients this difference showed the same trend, p = 0.097). However, the ratio of collagen III: I staining in the separate layers of the bowel wall and transmurally did not differ significantly.

3.4. Negative correlation between inflammation and collagen I deposition in acute UC

Finally, to investigate if the severity and extent of inflammation correlates to fibrotic deposition, we correlated the baseline patient characteristics and Geboes score to the deposition of α-SMA, fibronectin, Sirius red, collagen I, and collagen III.

None of the baseline characteristics [age, steroid use, anti-TNF medication, disease location] were associated with a higher fibrosis load in either cohort.

In acute UC there was an inverse correlation between the severity of inflammation and collagen I deposition in the mucosa, muscularis mucosa, and muscularis externa [R = -0.63, p = 0.02; R = -0.53, p = 0.05; and R = -0.62, p = 0.02, respectively] and a negative correlation between inflammation and Sirius Red staining intensity [R = -0.77, p = 0.002]. In longstanding UC, however, no such correlation was observed.

Moreover, there was a negative correlation between inflammation and α-SMA positivity in the submucosa in the total UC group [R = -0.51, p = 0.003], and this negative correlation remained in the longstanding UC when the disease duration was separated [R = -0.48, p = 0.05].

4. Discussion

The findings in the current study showed no significant difference in collagen deposition, presence of the ECM component fibronectin, or presence of myofibroblasts between acute and longstanding UC.

Inflammation of the intestine leads to infiltration and local activation of ECM-degrading enzymes such as matrix metalloproteinases and elastase, hence facilitating the infiltration of immune cells. In addition, collagenases also contribute to the degradation of ECM components such as fibronectin and collagen. Subsequently, subepithelial myofibroblasts are attracted to the site of injury, aiding renewed ECM synthesis. Subepithelial myofibroblasts stimulate their own migration by the release of several cytokines and growth factors such as insulin-like growth factor 1 [IGF-1], basic fibroblast growth factor [bFGF], platelet-derived growth factor [PDGF], and transforming growth factor-β [TGF-β]. In addition to these cytokines and growth factors, it is also the fibronectin secretion itself which plays an important role in the migration of intestinal myofibroblasts.

Myofibroblasts are located mainly at the bases of intestinal crypts, immediately underlying the epithelium and the basal...
They express pattern recognition receptors and become activated once they are in contact with bacteria as a result of a disruption of the epithelial barrier, and therefore participate in the intestinal immune response.

They play a role in exerting epithelial cell functions such as migration, proliferation, differentiation, and survival via the secretion of paracrine factors [such as TGF-β, Wnt and Bmp], the release of several metalloproteinases, and the production of ECM molecules.

One previous study showed that the myofibroblasts located in the crypts of inflamed CD tissue lose expression of α-SMA [and become positive for desmin]; however, this was not observed in uninflamed CD or inflamed, non-CD controls. The appearance of desmin positivity probably reflects re-differentiation towards a smooth muscle cell phenotype. Normally, smooth muscle cells are located in the lamina propria and muscularis mucosa. This is in line with our finding that there was less α-SMA positivity in UC colon than controls.

Figure 4. Increased fibronectin expression in acute ulcerative colitis. Representative examples of fibronectin expression in the mucosa in acute ulcerative colitis [a], longstanding ulcerative colitis [b], and cancer controls [c], and quantification for all patients [d]. Number of positive-stained cells divided by the total surface area as indicated by haematoxylin staining. Original magnification in 1, 2, and 3: x100. *p = < 0.05. Median and interquartile range are shown in [d].

Figure 5. Increased expression of collagen I in the mucosa [a], muscularis mucosa [d], and muscularis externa [g]; of collagen III in the muscularis externa [h]; and increased Sirius Red staining in the mucosa [c] and muscularis externa [i] in acute ulcerative colitis compared with colon cancer controls, and in late ulcerative colitis compared with colon cancer controls. No differences were seen in collagen III expression in the mucosa [b] or muscularis mucosa [e], or in Sirius Red staining in the muscularis mucosa [f]. Original magnification in all the stainings: x100, and the expression was determined on the most involved region of the selected slide. Number of positive-stained cells divided by the total surface area as indicated by haematoxylin staining. *p = < 0.05, **p = < 0.01. Median and interquartile range are shown in a-i.
Although in the longstanding UC group there was less inflammation than in the acute UC group, the patients with longstanding UC have had recurrent spells of active inflammation in the past but not at the time of surgery. It could very well be that in the longstanding UC patients the myofibroblasts have disappeared during past episodes of active inflammation, and that this phenotype has not reversed upon remission. Taking into consideration that myofibroblasts are also immune-protective, this alteration of myofibroblasts towards more smooth muscle cells might have implications on the mucosal immune homeostasis.

The collagen III: I ratio allows differentiation between early and late stages of fibrosis. Collagen I gives tensile strength to the connective tissue, whereas collagen III provides tissue elasticity. When collagen deposition is rapid, the ratio of type III collagen to type I collagen is increased. This may be defined as the early stage of fibrosis, characterised by an increase in the accumulation of collagen type III in relation to collagen type I. However, during later stages of fibrosis when active collagen deposition diminishes, the collagen III: I ratio decreases. In our study, however, the ratio of collagen III: I did not differ between acute and longstanding UC in any separate layer of the bowel wall, nor in the cross-sectional colon. However, in absolute numbers there tended to be more collagen I in the mucosa of both acute and late UC than in the control patients, whereas the collagen III had similar expression in all three groups. This is in line with previous results reported by Lawrance et al., who also found that in inflamed UC patients the collagen III staining was less intense than in collagen I. Together with the finding that there is more fibronectin in late UC patients than in controls, this could imply that there is indeed more ECM deposition in UC patients than in controls, but this does not seem to aggravate over time.

The question remains why strictures and stenosis in CD occur frequently, but are rare in UC. It is often assumed that, since UC is a mucosal disease and the inflammatory cytokines only reach into the superficial layers of the colon, ECM synthesis remains superficial. Our data show that UC patients not only had more collagen deposition in the mucosa and muscularis mucosa, but also in the muscularis externa, suggesting that inflammation in UC may affect the fibrotic response throughout the whole bowel wall. This is in line with recently published data showing ECM deposition extending throughout the entire bowel wall in advanced disease. Sponheim et al. showed an increased interleukin [IL]-33 expression in ulcerations of UC patients compared with biopsy specimens from control patients. In this study, IL-33 was mainly expressed in fibroblasts. This also suggested an increase of fibroblast activity in UC.

In a recently published series of UC patients with strictures, these patients had longer disease duration than UC patients without strictures [15.6 vs 8.6 years]. The incidence of benign stenosis in this cohort was 1.5% over 23 years. Moreover, a significant increase in bFGF-positive inflammatory cells was observed in stenotic segments. Most of the stenotic cases were double-positive for both bFGF and myeloperoxidase, suggesting that the cause of colonic stenosis is fibrosis rather than inflammation. Given the incidence of strictures in UC of 1.5% up to 11.2%, we expected at least two patients with a stricture in our cohort. However, we did not find any strictures in the UC patients, even not in the longer disease-duration group. It could be that since these patients were operated on for dysplasia, they did not have currently active disease. Nonetheless, the patients in the late UC group had suffered significantly more flares with steroid dependency than the acute UC patients. Another explanation could be that some of these UC patients with strictures actually had CD. Nevertheless, since fibrosis can proceed independently of inflammation once this process has been initiated, it could be conceivable that in this group also strictures might occur.

This descriptive study has the limitation of its retrospective nature. Although the sections were processed via a protocolised approach where the sections were cut perpendicular to the bowel wall, there was still some variation in the orientation and original localisation of the section. Although limited by relative small numbers, this expected variation in the orientation was similar in all groups and therefore this should not have influenced the results. Moreover, this study is limited by a relative small though well-defined sample size. In order to investigate whether longer disease duration and therefore longer periods of inflammation lead to fibrosis, we have selected two study populations at the extreme end of the time spectrum, namely acute UC and established, longstanding disease. Therefore the total number of patients is relative reduced; however, we expected it to be large enough to detect significant differences. The patients in the control group had no inflammation since they were operated on for colon carcinoma without IBD. An even better control group would be patients operated on for infectious colitis such as Clostridium difficile. However, it is extremely rare that those patients undergo resection.

End-stage UC may lead to a ‘lead-pipe colon’, with a shortened, stiff, and narrowed colon with loss of haustrations. Likewise, it has been known for decades that UC patients suffer from motility problems. Most likely the alterations of the enteric neuromuscular compartment, such as a reduced density of myenteric neurons, glial cells, and interstitial cells of Cajal, are the driving force for the dysmotility in UC, rather than fibrosis. In conclusion, we observed more collagen deposition in the colon wall of UC patients than in controls. Collagen deposition, however, did not appear to increase over time.

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Conflict of Interest

The authors of this manuscript have no significant relationship with, or financial interest in, any commercial companies pertaining to this article.

Author Contributions

All authors have made substantial contributions to the following: concept and design of the study [JdB, GvdB, GD], acquisition of data [JdB, SM, MW], providing patients [WB, GvdB, GD], analysis and interpretation of data [JdB, MW, GvdB], drafting the article [JdB], and final approval of the version to be submitted [all authors]. All authors had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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