Results of the 4th Scientific Workshop of the ECCO (Group II): Markers of intestinal fibrosis in inflammatory bowel disease

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Abbreviations: ASCA, anti-Saccharomyces cerevisiae antibody; ATG16L1, autophagy-related protein 16-1; BAL, bronchoalveolar lavage; bFGF, basic fibroblast growth factor; BNP, brain natriuretic peptide; CD, Crohn’s disease; CF, cystic fibrosis; CCL, chemokine (C–C motif) ligand; CKD, chronic kidney disease; COMP, cartilage oligomeric protein; CT, computed tomography; CTE, computed tomographic enterography; CTLA4, cytotoxic T-lymphocyte antigen 4; CTGF, connective tissue growth factor; DLG5, disks large homolog 5; DSS, dextran sulfate sodium; ECCO, European Crohn’s and Colitis Organization; ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; HBV, hepatitis B virus; HSP47, heat shock protein 47; IBD, inflammatory bowel disease; ICAM, intercellular adhesion molecule 1; ICTP, intercellular adhesion molecule 1; ICP, carboxy terminal cross-linked telopeptide of type I collagen; IL, interleukin; INR, international normalized ratio; IP, interferon gamma-induced protein; IPF, idiopathic pulmonary fibrosis; KL-6, Krebs von den Lungen-6; MALDI–TOF-MS, matrix-assisted laser desorption-ionization time-of-flight mass spectrometry; MC, mesenchymal cell; miRNA, microRNA; MMP, matrix metalloproteinase; MR, magnetic resonance; MRI, magnetic resonance imaging; mRSS, modified Rodnan skin score; MT, magnetization transfer; MUC, mucin; NOD, nucleotide-binding oligomerization domain containing; OMUS, obliteration muscularization of the submucosa; PAI-1, plasminogen activator inhibitor-1; PDGF, platelet-derived growth factor; PNP, amino terminal procollagen; PG-PS, peptidoglycan-polysaccharide; SMA, smooth muscle actin; SP, surfactant protein; SSc, serum of systemic sclerosis; TERT, telomerase reverse transcriptase; TGF, transforming growth factor; TIMP, tissue inhibitor of matrix metalloproteinase; TNBS, trinitro-benzene sulfonic acid; TNF, tumor necrosis factor; UC, ulcerative colitis; UEI, ultrasound elasticity imaging; US, ultrasonography; VEGF, vascular endothelial growth factor.

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1. Introduction

The natural history of inflammatory bowel disease (IBD) is highly heterogeneous and frequently complicated by intestinal fibrosis and stricture formation. This appears to be the case for both entities of IBD, ulcerative colitis (UC) and Crohn’s disease (CD). More than 30% of CD and about 5% of UC patients develop a distinct fibrostenosing phenotype with progressive narrowing and potential for intestinal obstruction. Intestinal stricture formation is a frequent indication for surgery in CD and strictures frequently recur leading to repeated surgeries. It appears that both entities of IBD and in particular CD exhibit a progressive nature with changes in disease behavior throughout the disease course. In CD, which is a transmural disease, chronic mucosal inflammation induces remodeling of the entire intestinal wall. This process is a cascade of events that includes epithelial cell and intestinal damage and repair, angiogenesis and lymphangiogenesis and activation of immune...
cells and mesenchymal cells (MCs). MCs include fibroblasts, myofibroblasts and smooth muscle cells and are the major source of extracellular matrix (ECM) components. It is difficult to predict which patients will develop a fibrostenosing phenotype (though a majority will do so eventually) and how rapidly they will progress. No specific therapy to prevent or treat intestinal fibrosis is known. To enable progress in this area, it is essential to identify markers of intestinal fibrosis, in order to (1) stratify patients into different levels of risk before the development of fibrosis, and (2) detect early stages of fibrosis before clinical symptoms have occurred. An optimal fibrosis marker should detect early stages of fibrosis, identify trajectory of fibrosis development, be predictive of future fibrosis, be predictive of and responsive to the effect of anti-fibrotic therapies and be predictive of non-responsiveness to anti-inflammatory therapies. Accomplishing these goals will open the door for targeted anti-fibrotic therapy, and the ability to test candidate anti-fibrotic therapies in clinical trials.

This review first summarizes briefly the current status of markers for intestinal fibrosis, evaluating three distinct areas: clinical phenotypes, serologic markers, and genetic markers. Next, available markers from other fibrotic diseases will be discussed. The main component of the manuscript focuses on novel approaches to identify and develop markers of fibrosis, what gold standard to use for trial endpoints, and to which clinical situations these can be applied to.

### 2. Currently available markers of intestinal fibrosis

No specific and accurate predictors or diagnostic tools for intestinal fibrosis exist and to date no marker of fibrosis is in routine clinical use. Several targets have been tested for this purpose (Table 1). Genetic signatures are attractive as they are stable, present long before the disease onset and are not affected by alterations in the disease course. Several genes have been evaluated for their association with fibrostenosing CD. Alternations in the nucleotide-binding oligomerization domain containing 2 (NOD2) gene, the first discovered and best explored genetic variant, are weakly associated not only with CD fibrosis, but also with ileal disease location and fistulizing disease and hence lack specificity. Other genetic variants have been described as being linked to fibrostenosis, such as those in the matrix metalloproteinase (MMP)-3 gene or in the rs1363670 locus near the interleukin (IL)12B gene. Interestingly, an increasing number of risk alleles, including NOD2, IBDS, disks large homolog (DLG)5, autophagy-related protein 16-1 (ATG16L1), and IL23 receptor (IL23R), confer an increasing amount of risk alleles for NOD2, IBDS, DLG, ATG16L1, and IL23R gene variants are predictive of non-responsiveness to anti-inflammatory therapies. Accomplishing these goals will open the door for targeted anti-fibrotic therapy, and the ability to test candidate anti-fibrotic therapies in clinical trials.

<table>
<thead>
<tr>
<th>Genetic</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD2</td>
<td>7</td>
</tr>
<tr>
<td>MMP-3</td>
<td>8</td>
</tr>
<tr>
<td>rs1363670</td>
<td>9</td>
</tr>
<tr>
<td>Increasing amount of risk alleles for NOD2, IBDS, DLG, ATG16L1, and IL23R</td>
<td>10</td>
</tr>
</tbody>
</table>

### Clinical

- Need for corticosteroids during first flare: 11
- Early disease onset: 11
- Perianal fistulizing disease: 11
- Small bowel disease location: 3

### Serologic

- Anti-microbial antibodies: 14,15
- ECM molecules (Fibronectin, collagen propeptides, laminin): 16–19
- Growth factors (YKL-40, bFGF): 17,20

**Table 1** Currently available markers of intestinal fibrosis.

<table>
<thead>
<tr>
<th>Reference</th>
<th>NOD2</th>
<th>MMP-3</th>
<th>rs1363670</th>
<th>Increasing amount of risk alleles for NOD2, IBDS, DLG, ATG16L1, and IL23R</th>
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<tbody>
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<td>7</td>
<td>8</td>
<td>9</td>
<td>10</td>
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The most widely used criteria to predict fibrostenosing CD are clinical factors. These include the need for corticosteroids, early disease onset, perianal fistulizing disease or small bowel disease location. These factors however are encompassing many different disease phenotypes. On the other hand, the Montreal classification merely identifies fibrosis after it has become clinically apparent and can only be used as a descriptor rather than a predictor. Thus, alternative, noninvasive predictive tools are required. One predictive tool that might be of use as a biomarker is a panel of serologic markers.

Circulating antibodies against microbial products are found in some patients with IBD, such as anti-Saccharomyces cerevisiae (ASCA) among others. These are believed to arise from aberrant immune responses towards the luminal microbiota. These antibodies are qualitatively and quantitatively associated with, and predictive of a more complicated disease phenotype, including fibrostenosis.

However, they are not specific for this phenotype, but rather predict complicated CD, including fistulizing disease and the need for surgery. Extracellular matrix molecules and growth factors, such as laminin, collagens, collagen propeptides or telopeptides, basement membrane components or fibronectin, YKL-40 (also known as human cartilage glycoprotein 39, a chitinase-like protein), basic fibroblast growth factor (bFGF) and others have been investigated as biomarkers of fibrosis, with inconclusive or negative results.

### 3. Markers of fibrosis from other fibrotic diseases

As no existing marker of fibrosis showed specific promise in IBD in our systematic literature review, we reviewed markers in fibrotic diseases of extra-intestinal organs, evaluating whether they could be relevant for further study in IBD, focusing on markers found in fibrosis of the liver, lung, kidney, and skin (Table 2).
3.1. Liver

Liver biopsy remains the gold standard to evaluate fibrosis, but is an expensive, invasive procedure with potential side effects and is limited by sampling bias. Non-invasive methods for indirect determination of liver fibrosis are already established and in clinical use, making this arena the most advanced in the field of markers of fibrosis. These markers have been mainly used in hepatitis B or C, and scores have been proposed, sometimes in combination with other markers of liver disease, like transaminases, albumin, bilirubin, and international normalized ratio (INR). Examples include the amino terminal procollagen III (PIIINP), a cleavage product of a collagen precursor which is significantly correlated with the histological stage of liver fibrosis; hyaluronic acid, a high molecular weight glycosaminoglycan, and markers from other fibrotic diseases.

### Table 2: Examples for markers of fibrosis from other fibrotic diseases.

<table>
<thead>
<tr>
<th>Liver</th>
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<tbody>
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<td>Enolase-1 (α-enolase) and thrombospondin-1 (TSP-1)</td>
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<td>Lung</td>
<td>Reference</td>
</tr>
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<td>Krebs von den Lungen-6</td>
<td>Serum and BAL 104,105</td>
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<tr>
<td>Surfactant protein-A and -D</td>
<td>Serum 105,106</td>
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<td>Serum and BAL 35</td>
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<td>MMP1 &amp; 7</td>
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<td>Number of myofibroblasts</td>
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<tr>
<td>CTGF</td>
<td>Urine 69</td>
</tr>
<tr>
<td>PAI-1</td>
<td>Urine 72</td>
</tr>
<tr>
<td>Collagen IV</td>
<td>Urine 70</td>
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which is an essential component of ECM in virtually every tissue in the body and substantially increased in hepatic fibrosis; and tissue inhibitor of metalloproteinase 1 (TIMP-1) which inhibits interstitial collagenases and metalloproteinases that are capable of degrading ECM. Liver and serum TIMP-1 increase in parallel with the progression of the liver disease. Therefore TIMP-1 has been considered useful in hepatic fibrosis. Another endogenous inhibitor of proteases, serum cystatin C, also correlates with the stage of liver fibrosis in chronic liver disorders.

In addition to these molecular approaches, liver fibrosis has been studied with markers of tissue structure using ultrasound and magnetic resonance (MR). Transient elastography, an ultrasound based technique, has the ability to measure tissue stiffness in an area of 3 cm². It is an easy to perform, widely operator independent, easy to learn approach. This technique has shown high accuracy in determining fibrosis and cirrhosis of the liver. MR elastography uses the same principles as transient elastography, but can assess the whole liver. However, MR elastography is more costly, takes longer, and has limitations in certain patients, including those with iron overload. Imaging and serologic markers have been combined as well producing increased accuracy.

A detailed overview of the state of the field of liver fibrosis markers has been published by Duarte-Rojo and colleagues.

### 3.2. Lung

Given the poor outcomes and lack of effective therapies for patients with lung fibrosis, an intense effort to identify markers of fibrosis has been undertaken to facilitate the development of novel treatment approaches. These markers could act as surrogates for clinically meaningful outcomes. Imaging via high resolution CT scan is sufficient for diagnosis of pulmonary fibrosis without employing specific imaging sequences for fibrosis. Most markers of fibrosis are experimental and from the serum or blood, but the accessibility of the lung offers the option to evaluate direct disease processes by obtaining a bronchoscopy with bronchoalveolar lavage (BAL).

Several markers of fibrosis with direct relevance and mechanistic plausibility for fibrosis in general have also been investigated in pulmonary fibrosis: MMPs 1 and 7 were increased in idiopathic pulmonary fibrosis. Chemokine (C–C motif) ligand 18 (CCL18) is chemotactic for fibroblasts, stimulating their collagen production. In idiopathic pulmonary fibrosis the CCL18 serum level was elevated. YKL-40, which appears to have mitogenic effects on fibroblasts, was increased in the serum and bronchial lavage fluid of patients with pulmonary fibrosis and was associated with survival time in IPF. Osteopontin is a glycoprotein involved in tissue repair through profibrogenic activity of fibroblasts and is elevated in BAL and plasma of patients with IPF compared to controls. Periostin, an ECM protein, was significantly increased in the serum of patients with idiopathic pulmonary fibrosis compared to healthy controls, and was inversely correlated with patients’ pulmonary function. Connective tissue growth factor (CTGF) exerts profibrotic effects on fibroblasts and is elevated in IPF plasma. Heat shock protein 47 (HSP47), a collagen-specific molecular chaperone that mainly functions in biosynthesis and the secretion of collagen was significantly higher in the serum of patients with acute exacerbations of pulmonary fibrosis compared to those with stable disease. Multiple genetic variants are linked to susceptibility for IPF, such as polymorphisms within telomerase reverse transcriptase (TERT), IL-1, IL12p40, MMP1, NOD2 and others.

An additional novel conceptual approach has been pursued in pulmonary fibrosis, by identifying circulating fibroblast precursors, the so-called fibrocytes, as markers of disease. Fibrocytes are believed to be a minor but significant component of the pathogenesis of pulmonary fibrosis. Circulating fibrocytes are elevated compared to controls in IPF and were increased during exacerbations. In addition the relative percentages of T-cell subsets (CD4/CD28 and Tregs) may have utility as a prognostic markers of fibrosis.

Detailed reviews of the state of the field of pulmonary fibrosis markers have been published by Huang et al. and Vij and Noth.

### 3.3. Kidney

The same principles applied in the liver and lung also apply to the skin in scleroderma. Multiple proteins related to transforming growth factor β (TGF-β) activity have been examined in the serum or plasma of patients with scleroderma. Cartilage oligomeric protein (COMP), thrombospondin, MMP-9, CTGF, and osteopontin are increased in serum or plasma of systemic scleroderma (SSc) patients and some of these marks change with disease severity. Matrix components and matrix turnover products have been evaluated as well, such as the carboxy terminal telopeptide of type I collagen, amino terminal procollagen I (PINP), PIIINP or TIMP1. Inflammatory mediators, such as cytokines, chemokines and markers associated with adaptive immune cell activation, including IL-6, IL-8, IL-10, IL-13, CCL2, CCL3, CCL4, soluble CD30 (sCD30) or cytotoxic T-lymphocyte antigen 4 (CTLA4) are elevated in SSc, indicating a link between inflammation and fibrosis in SSc.

SSc offers unusual accessibility of tissue for marker measurement directly in skin biopsies. Examples include the determination of the number of myofibroblasts or different TGF-β related gene expression markers. Clinical scores, such as the modified Rodnan skin score (mRSS), ultrasound, or skin MRI to measure dermal thickness have been developed to evaluate the severity of scleroderma, and if responsive to anti-fibrotic therapies, could be used in clinical trials.

Detailed reviews of the state of the field of scleroderma fibrosis have been recently published by Lafyatis and Moinzadeh et al.

### 3.4. Kidney

While the inciting agents in renal fibrosis are often different from the liver, lung, and skin, the fibrotic process appears to be shared. While lab markers of renal function can inform about progression of renal disease they do not necessarily reflect fibrotic burden and they can be influenced by a wide variety of factors.
Unique to the kidney is the accessibility of urine as a direct, kidney-specific read-out for renal fibrosis, allowing the identification of local markers of fibrosis. Urine levels of TGF-β1, CTGF and collagen IV increase with progression of chronic kidney disease (CKD). \(^{68-71}\) Urine plasminogen activator inhibitor-1 (PAI-1) has also been shown to correlate with renal fibrosis in patients with diabetic nephropathy. \(^{72}\)

Ultrasound imaging has also entered the field of kidney fibrosis. Using the doppler ultrasound technique to calculate the ‘resistive index’ and ‘atrophic index’ has shown promise in predicting time to dialysis and survival in chronic kidney disease. \(^{73}\)

One of the challenges of universal fibrosis markers is that they may be nonspecific, so that a patient with Crohn’s disease may appear to have elevated serum markers of intestinal fibrosis when they have fibrosis in a different organ (skin, liver, lung, kidney, etc.). There may be benefit in obtaining markers of intestinal fibrosis from the gut (biopsies) or its output (stool) to increase the specificity of markers for intestinal fibrosis.

In summary, we can learn from fibrotic diseases of the liver, lung, kidney and skin. Multiple potential biologic and imaging markers are already in clinical use. While organ-specific markers, such as creatinine for renal disease or alveolar epithelial cell specific proteins in the lung, likely will not be helpful markers of intestinal fibrosis, multiple markers with a direct link with fibrogenesis have been identified. As fibrotic mechanisms are shared across organs, these provide possible candidates for use in the intestine as well.

4. Clinical Situations Where Markers of Fibrosis Should be Used

After defining an optimal marker of fibrosis, and discussing existing and potential markers of intestinal fibrosis, we evaluated the specific clinical situations in which future markers could be used in intestinal fibrogenesis (Table 3).

5. Approaches for Identifying Markers of Fibrosis in Inflammatory Bowel Disease Patients

All existing markers carry limitations and no current strategy for the development of novel markers of fibrosis is established. As none of the current markers will likely fulfill all needs of a perfect marker of fibrosis a quest for additional targets should continue. Markers of fibrosis can be considered the most critical missing link in the development of novel therapeutics of intestinal fibrosis. We next evaluate strategies to develop novel markers in IBD-associated fibrosis and provide examples from investigations in other organs.

5.1. Which Body Compartments/Fluids Should be Investigated in the Quest for Novel Fibrosis Markers?

Most of the currently examined markers for intestinal fibrosis focused on genes, clinical factors or serology. However, reviewing the literature from IBD as well as other fibrotic diseases outside of the gastrointestinal tract reveals other potential sampling sources that could be useful for markers of fibrosis research, and screening tools that may be helpful in identifying new markers. Potential body products that may be useful are stool, saliva, breath, or urine samples. Those may reflect the mediators and cells that are active in fibrosis, without the need for invasive procedures such as endoscopy and biopsies, or surgery.

5.2. Which Techniques Could be Used to Identify Novel Fibrosis Markers?

Using single marker candidates remains a viable option. However, this might not reflect the complexity of the underlying disease process and hence likely lacks accuracy. Available screening tools, using whole classes of molecules, instead of just single markers or a small panel of biomarkers, are virtually endless, but emphasis has been put on the ‘omics’ arena, specifically: proteomics, genomics, metabolomics and transcriptomics. Examples of screening tools and body compartments explored can be found below.

5.3. MicroRNA (miRNA) Analysis

miRNAs are important post-transcriptional regulators and are aberrantly expressed in several fibrotic diseases, such as systemic sclerosis. miRNAs with pro- or antifibrotic properties were found to be dysregulated in skin fibrosis and were associated with disease activity and severity. \(^{74}\) Chen et al. reported that miR-200b is overexpressed in the serum of CD patients with fibrosis, and conversely administration of miR-200b could partially protect from fibrogenesis in vitro. \(^{75}\)

5.4. Proteome Analysis

The proteome is the entire set of expressed proteins in a given type of cell, tissue, or organism, at a given time, under defined conditions. One study of urine from methotrexate-induced hepatic fibrosis patients revealed multiple proteins associated with hepatic fibrosis, including N-cadherin, inter-alpha-trypsin inhibitor heavy chain H4, haptoglobin and serotransferrin. Proteomic methods were also used to screen serum samples from patients with hepatitis B virus infection. Two-dimensional electrophoresis (2-DE) and matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) identified 27 differentially expressed proteins in serum from hepatic fibrosis compared to hepatitis B virus (HBV) carriers. \(^{76}\) Examining the concentration of 92 proteins in IPF five candidates (MMP7, intercellular adhesion molecule 1 (ICAM1), IL8, vascular cell adhesion molecule 1 (VCAM1), S100A12) showed that they were associated with disease progression and mortality. \(^{77}\) A study of the saliva of asthma and cystic fibrosis patients found that biomarkers including human VEGF, interferon gamma-induced protein 10 (IP-10), IL-8, epidermal growth factor (EGF), MMP-9, and IL-1β could be identified in subpicomolar range. It was found that four of the six proteins were significantly elevated in asthma and cystic fibrosis (CF) patients compared with healthy controls. \(^{78}\)
5.5. Transcriptomic Screening of Mucosal Samples

The transcriptome is the set of all RNA molecules, including mRNA, rRNA, tRNA, and other non-coding RNA produced in one or a population of cells. This technique was performed in a study of chronic kidney disease progression. Among the targets identified, periostin, an extracellular matrix protein, presented a significantly higher mRNA expression in more advanced renal fibrosis.79

5.6. How to Find New Markers of Fibrosis?

Regardless of etiology, various biological factors are involved and interact in the process of chronic remodeling and fibrosis in the intestine. Thus these may be used as markers in Crohn’s disease in a single candidate approach. In line with a systems biology approach, however, we suggest that it could be necessary to quantitatively analyze the interactions of all components of a biological system in order to define fibrotic processes in Crohn’s disease. The future could well lie in the creation of a biological "profile" of the pathology in intestinal fibrosis, integrating all the above techniques in distinct body compartments.

6. Potential Trial Endpoints for Intestinal Fibrosis Marker Evaluations

The leap in technologies now available allows the quantification of a wide variety of mediators in body compartments with high accuracy. However the lack of a clear definition of fibrosis useable for trial endpoints has limited progress in the field. Currently all available endpoints rely on clinically apparent strictures that are confirmed by cross-sectional imaging.

6.1. Endoscopy

Full thickness histopathological sections to assess fibrosis generally require surgical specimens. While this is an endpoint of the fibrotic process, it is not a helpful fibrosis marker. Endoscopy, typically performed in order to assess mucosal healing, could in the future enable follow-up of fibrosis. This could be achieved by determination of luminal diameter — visually or with devices allowing quantification. Novel optical techniques including confocal endomicroscopy might also be adapted to measure components of intestinal fibrosis. Thus, subepithelial myofibroblasts, ECM components, and other cell types may be visualized with or without the use of specific stains. As more insights are gained, endoscopy could become a useful tool in the assessment and the follow-up of fibrosis.

6.2. Histopathology

Endoscopic biopsies are accessible during endoscopies and could serve as potential endpoints for marker trials. The predominant connective tissue protein in the intestine is collagen. Type I is the most abundant in the body and its
function is to give tensile strength. Type III is associated with tissues that require motile structure, type IV is the major component of epithelial basement membranes and type V is pericellular and can be produced by smooth muscle cells. The presence and composition of collagens have been investigated in intestinal fibrosis. Intestinal strictures in CD are characterized by an increase in type V collagen. Collagen types IV and V are elevated in the muscularis propria and around ganglia, while collagen type III is extensively present in ulcerations. Moreover, in CD, a significant increase in submucosal type III collagen fiber content has been shown in stenosed intestine, with a particular increase in the outer aspect of the submucosa. Collagen composition may also allow clues as of the temporal relation of wound healing. 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, characterized by an increase in the accumulation of collagen type III in relation to collagen type I. In contrast, during the late stage of fibrosis, when active collagen deposition diminishes, the ratio of type III collagen to type I collagen decreases.

Accumulation of myofibroblasts and alterations of the enteric nerves are associated with fibromuscular obliteration of the submucosa, and with thickening of the muscularis propria. Obiterative muscularization of the submucosa (OMUS) has been observed in about one-third of small intestinal resection specimens of Crohn's disease, usually in stricturing disease. OMUS is especially associated with small bowel strictures, which are themselves closely associated with submucosal fibrosis.

To use biopsy histology in order to measure fibrosis is actually neither simple nor feasible, given the limited depth of sampling and the possible sampling error. It is however possible to look at different intestinal histology components as surrogates for deeper tissue layer fibrosis, such as using staining for myofibroblasts (\(\alpha\text{SMA}\)), tenascin, and for collagen subtypes. This could also include non-mesenchymal genes, such as epithelial keratins, as suggested in animal models of colitis.

### 6.3. Functional Cell Assays

Intestinal mesenchymal cells, the main producers of ECM in intestinal fibrosis, can be readily isolated and cultured from biopsies of primary human tissue. They can then undergo in vitro evaluations, such as measurement of matrix production or proliferation that could potentially act as markers of the success or failure of anti-fibrotic therapies. This approach could be limited if rapid changes in the cell phenotype occur in culture, and may require immediate analysis of gene expression (i.e. via single cell real-time PCR).

### 6.4. Radiology

As mentioned, endoscopic mucosal biopsies are superficial and are currently not informative about deeper layers of the bowel. Edema is related to activity and wall fibrosis may be associated either with active or with inactive disease. New imaging techniques are being developed and tested in animal models to detect intestinal fibrosis and assess their potential translation for human use. The high MRI T2 signal of the pathologic bowel wall is directly associated with the presence of edema in the submucosal layer (active disease). In general, T2-weighted imaging directly expresses the amount of fluids within the pathologic wall, more sensitively and specifically than other imaging modalities, including ultrasonography and CT. On the other hand, low T2 signal was correlated with the presence of fibrosis in the intestinal wall. The amount of collagen and fibroblasts is associated with a reduced T2 signal in the bowel wall, predominantly in the submucosal and muscularis propria layers. Therefore, T2-signal intensity of the intestinal wall was directly associated with: (i) the degree of edema in the submucosal layer, (ii) dilatation of sub-mucosal lymphatic vessels, (iii) and signs of mucosal inflammation and ulcers. A high T2 signal of the pathologic intestinal wall was most associated with the degree of submucosal thickening and edema at histology. It was reported that 97.9% accuracy for the diagnostic of fibrotic stenosis in CD was achieved by MRI T2-signal intensity. The fibrostenosing phenotype is related to variable degrees of wall fibrosis and it is characterized by a low T2-wall signal associated with variable degrees of wall thickening and bowel dilatation. Following gadolinium-chelate injection, variable degrees of layered wall enhancement may be detected.

Magnetization transfer (MT) is another new imaging modality. When applied to rats with peptidoglycan-polysaccharide (PG-PS)-induced fibrosis, the mean MT ratio in rats with late phase fibrosis was higher than that in animals with early inflammation and the MT ratio showed a correlation with the amount of tissue fibrosis. Nevertheless, MRI data in murine ileitis or colitis are very limited. T2 relaxometry was also able to discern between the effects of different cycles of dextran sulfate sodium (DSS) and correlated with the observed histological changes, allowing in vivo monitoring of disease status. Tissue edema is associated with higher water content per pixel. Since water has a higher T2 than normal tissue, pixels with substantial tissue edema will be associated with an increased T2. Thus, the shift of the colon to lower T2 values with more cycles of DSS can in part be explained by a decrease of active inflammation over time. The shift in T2 is likely due to a combination of a slower water content due to a reduction in inflammation and progressive fibrosis. The sensitivity and specificity of MT in the detection of human intestinal fibrosis have not yet been determined.

The use of ultrasonography (US) in the assessment of intestinal fibrosis has been increasing. Contrast-enhanced US, for evaluation of mural inflammation in CD, with histopathology as the reference standard, showed significantly negative association between the color Doppler grade and the pathologic fibrostenotic score. Contrast enhanced ultrasound could be useful in distinguishing fibrotic from inflammatory strictures. Ultrasound elasticity imaging (UEI) is a noninvasive method that allows characterization of intestinal tissue on accurate estimates of tissue motion (speckle tracking) between two frames before and after deformation of the tissue. In UEI the tissue is pushing with an ultrasound transducer, and the tissue deformation with real-time ultrasound images produce the excitation. Preliminary results in an animal model demonstrate that UEI can...
detect intestinal stiffness changes as a result of fibrosis development, with reasonably high sensitivity and reproducibility.\textsuperscript{100} When applied to resected bowel segments from TNBS animals to look for evidence of inflammation and fibrosis,\textsuperscript{101} it was able to differentiate acutely inflamed vs. chronic fibrotic changes, and between unaffected and fibrotic intestine in a pilot study of Crohn's disease patients.\textsuperscript{102} In this small sample of 7 patients with Crohn's disease, UDI had 100% sensitivity and specificity in differentiating between fibrotic and normal intestine.\textsuperscript{102} It was feasible in humans, and the transcutaneous UEI accurately measures the tissue properties of stenotic segments of the bowel in patients with CD when compared with the gold standard of tissue elastometry.\textsuperscript{102}

Computed tomography enterography (CTE) findings of mesenteric hypervascularity, mucosal hyperenhancement, and mesenteric fat stranding predict tissue inflammation. However, small bowel strictures without CTE findings of inflammation do not necessarily predict the presence of tissue fibrosis, and inflammation and fibrosis often occur together in the small intestine in a mixed phenotype in Crohn's disease.\textsuperscript{93} Sensitivity and specificity of CTE for intestinal fibrosis in Crohn's disease have not been reported using a gold standard of surgical pathology. Therefore, caution should be used when using CTE criteria to predict the presence of scar tissue.\textsuperscript{93}

6.5. Defining a Gold Standard for Use in Intestinal Fibrosis Research

At this point in time there is no gold standard to detect fibrosis as an endpoint in clinical investigations; however, using more than one method looking for different pathways is advisable. We believe that MRI for transmural evaluation, endoscopy for luminal narrowing, and histopathology for mucosal and submucosal characterization all offer value, and should be evaluated in combination in developing and testing future markers for intestinal fibrosis studies.

Prospective studies for the evaluation of endpoints for trials are needed to clarify the role of histopathology in assessing transmural fibrosis and to create and validate a histologic score for intestinal fibrosis. We need to further define whether there is truly intestinal fibrosis without inflammation as the currently available studies might present a selection bias. No standard exists to define the amount and type of fibrosis required to classify the specimen as predominantly fibrotic. Because of selection bias in surgery, we do not know if there is an association of all clinically strictureting disease and a histologically predominant fibrosis phenotype. Further studies are needed to distinguish between inflammatory and fibrotic strictures.

7. Summary and Outlook

This summary reflects the discussions and ideas raised during the European Crohn's and Colitis Organization (ECCO) Scientific Workshop 4 on intestinal fibrosis. Surprisingly, while being one of the most troubling issues in the care of IBD, specifically CD patients, this is one of the least investigated and least therapeutically developed areas in IBD. In order to advance research in the field of IBD fibrosis, we reviewed IBD markers of fibrosis, and markers of fibrosis in extraintestinal fibrotic diseases. Additionally, fibrosis endpoints, as may be seen by histology, endoscopy and imaging studies were discussed. Finally, we investigated the potential for discovery of markers of fibrosis using rapidly developing omics technologies. We believe that further research in this field should pursue the development and validation of markers, imaging studies, and histology in order to have a common denominator to assess fibrosis in future studies and predict clinical outcomes. We also illustrate key research questions through several clinical scenarios.

Conflict of interest statement

None of the authors has a conflict of interest to disclose.

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References


81. Graham MF, Diegelmann RF, Elson CO, Lindblad WJ, Gotschalk N, Gay S, et al. Collagen content and types in the intestinal


