Biomarkers, Models and Mechanisms of Intestinal Fibrosis

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Chapter 8

General discussion and future perspectives
Intestinal fibrosis is a heterogeneous process involving multiple complex mechanisms. Despite advances in the understanding of the pathophysiology, the incidence of intestinal fibrosis in patients with Crohn’s disease (CD) remains high and therapeutic managing of stricturing CD remains a challenge in clinical practice. Several pharmacologic therapies have become available, but none of them have proven to be able to prevent or even reverse intestinal fibrosis. Surgical resection or strictureplasty are currently the only options to treat stenotic intestinal fibrosis.

Unravelling the mechanisms of intestinal fibrosis and finding anti-fibrotic therapies warrants relevant (animal) models. As discussed in the introduction of this thesis, several (animal) models for intestinal fibrosis are available and the efficacy of therapeutic interventions inhibiting formation of fibrosis was proven in some of them. To demonstrate the efficacy of these anti-fibrotic drugs in patients in a randomized controlled trial, well validated end-points for intestinal fibrosis are essential. Ideally, this should be serological markers that predict the degree of intestinal fibrosis. However, the discovery of markers for intestinal fibrosis is difficult since inflammatory and fibrotic pathways partially overlap and concomitant immunomodulatory therapies can obscure serological molecular changes related to fibrosis. Gaining more insight into the pathophysiology of intestinal fibrosis might identify molecular (serological) biomarkers for intestinal fibrosis. Discovering a serological marker for intestinal fibrosis will have major impact in the management of patients with IBD who have fibrotic complications of their disease. In this thesis, we aimed to explore new biomarkers, models and mechanisms of intestinal fibrosis in Crohn’s disease (CD).

Part I of this thesis aimed at exploring if serological biomarkers for formation and degradation of extra-cellular matrix (ECM) can reflect intestinal fibrosis formation and degradation (chapter 2), and if these markers can predict the response to anti-Tumor Necrosis Factor (TNF) therapy in patients with Crohn’s disease (chapter 3 and 4). We tried to differentiate between inflammatory, stricturing and penetrating CD based on serological biomarkers related to formation and degradation of the main interstitial collagens namely collagen type I, type III, type V and type VI. Enzymatically cleaved N- and C-terminal pro-peptides of collagen quantified by enzyme linked immunosorbent assay (ELISA) were used as serological markers of collagen formation. Matrix metalloproteinases (MMP) cleaved fragments of degraded collagens quantified by ELISA were used as serological markers of collagen degradation. We could differentiate
between penetrating CD versus stricturing and inflammatory CD based on an increase in MMP-9 cleaved collagen type III in penetrating ileal CD, but increased concentrations of MMP-9 cleaved collagen type III were also found in active CD independent of the disease phenotype. Because penetrating disease is among others driven by inflammation (given the fact that anti-inflammatory biologicals are effective in treating fistulising CD), MMP-cleaved products of collagen type III are also upregulated by inflammation and are therefore not effective to differentiate between active or penetrating CD. Further research should aim at discovering whether other collagen degrading enzymes cleaved degradation markers might be specific markers of tissue inflammation in patients with inflammatory bowel diseases (IBD) apart from reflecting whether intestinal fibrosis is formed or degraded. In that case MMP cleaved fragments of collagens could be used to monitor disease activity and to monitor tissue response in order to predict the effect of remission-induction therapy in patients with active CD as described in chapter 3 and 4 of this thesis.

Unfortunately, the in chapter 2 studied ECM formation and degradation markers were not able to differentiate between inflammatory and stricturing CD of the terminal ileum (i.e. to distinct fibrosis from inflammation), which was the aim of this study and needed to prove the efficacy of anti-fibrotic drugs. The generation of MMP degraded fragment of collagens is to such an extent dependent on the degree of inflammation that the degree of intestinal fibrosis present cannot be monitored using these biomarkers. Endoscopic or radiological evaluation to determine the degree of fibrosis is so far also not possible. A validated endoscopic method to classify the degree of fibrosis present is not available and endoscopically taken biopsies do not reveal the presence of transmural fibrosis as only the mucosa and submucosa can be sampled (which also implies that no validated histopathology-based scoring system is available to grade the severity of fibrosis). Furthermore, studies focusing on imaging have shown that the radiologist global impression of whether a stricture was 'active' or 'inactive' was not associated with the presence or degree of fibrosis. Ideally, the level of a (serological) biomarker for intestinal fibrosis would not be influenced by the state of inflammation at that time. However, such a biomarker is not yet available. Up to now, the proposed serologic markers for complicated CD were antibodies against microbial antigens (among others antibodies to the outer-membrane porin C of Escherichia coli) which are detectable years before diagnosis. The presence of antibodies against microbes in serum in the pre-clinical stage of CD might indicate the antecedent loss of intestinal barrier function which might precede CD but does not relate to intestinal fibrosis. Even though these factors can predict the risk of complications and surgery, these cannot be used to monitor progression to fibrostenotic disease and to monitor response to therapy. Also genetic risk factors associated with a fibrostenosing phenotype (i.a. a single nucleotide polymorphism...
(SNP) in the WWOX gene\textsuperscript{16}, cannot be used to monitor progression to fibrostenotic disease as the burden of fibrosis is not reflected in the presence or absence of this SNP. Further research focusing on discovering serum biomarkers reflecting the degree of intestinal fibrosis present or reflecting fibrogenesis to monitor the efficacy of anti-fibrotic drugs, could aim at epigenetic (i.e. miR-200b\textsuperscript{17}) markers, at other serologic immune-assays or at endoscopic molecular imaging. Other potential biomarkers for intestinal fibrosis are proteins derived from post-translational modifications, which take place outside the cell and can be detected in the serum. Post-translational modification of proteins is a non-DNA-coded change to the composition or structure of a protein that generates uniquely modified molecules (proteins) also known as neoepitopes.\textsuperscript{18} Post-translational modification occurs enzyme mediated or spontaneous.\textsuperscript{19} Measuring neo-epitopes formed by disease specific post-translational modifications of a protein (e.g. glycosylation, hydroxylation, citrullination, isomerisation) using immune-assays, provides more pathology-relevant information compared to measuring the (serum) concentration of the protein itself (if at all possible).\textsuperscript{18} Post-translational formation of collagen fibrils is a complex interplay of enzymatic modifications of the collagen fibril. As described in chapter 5 of this thesis, mRNA expression of several genes involved in post-translational modification of collagens are up-regulated in the fibrosis affected area specifically. We observed an upregulation in mRNA of the crosslinking enzymes Lysyl-oxidase (\textit{LOX}, including lysyl oxidase like 1-4). Assuming that an increase in mRNA expression of these genes leads to an increase in protein expression, the enzymatic modifications made by these specific to fibrosis enzymes, may create a fibrosis specific profile. Therefore, serum levels of lysyl oxidase could reflect the degree of intestinal fibrosis present, as was observed for systemic sclerosis, liver fibrosis and atrial fibrosis.\textsuperscript{20–22} Crosslinking of elastin or collagen fibrils by lysyl oxidase is specific for ECM formation and so far has not shown to be regulated by inflammation so far. Further research to find serum biomarkers for intestinal fibrosis might be non-hypothesis driven. An omics (genomics/ transcriptomics/ proteomics/ metabolomics) approach on single human blood cells or intestinal tissue comparing respectively fibrotic disease/areas to inflammatory disease/areas will provide extensive tissue-specific characteristics and drug targets.\textsuperscript{23} Other factors relating to the degree of fibrosis, such as post-transcriptional modification (crosslinking) or for example the presence of receptors for collagens (see chapter 5, e.g. discoidin domain receptor tyrosine kinase 1 or 2, or mannose receptor, C type 2), might be endoscopically detectable. By antibody mediated labelling, these proteins might be detected and quantified with near-infrared fluorescence molecular endoscopy as showed by Nagengast \textit{et al.} and thereby give insight in the degree of fibrosis over inflammation present in an intestinal stricture.\textsuperscript{24}
Radiological and nuclear imaging could furthermore be considered to quantify specifically the degree of intestinal fibrosis. Elastography (a non-invasive method to assess the mechanical properties of tissue, in particular stiffness), is currently routinely used to quantify renal and liver fibrosis.\textsuperscript{25,26} Elastography is also applicable to the intestine.\textsuperscript{27} Baumgart et al. assessed whether ultrasound based real-time elastography can detect gut fibrosis. In a proof of concept study, they found a correlation between pre- intra- and post-operative real-time elastography values of unaffected and fibrosis affected segments.\textsuperscript{27,28} Moreover, a correlation between real-time elastography values and histologic quantification of intestinal fibrosis using Masson trichrome and elastica–van Gieson staining was observed. Furthermore as tested in several clinical studies, the degree of active IBD can be quantified by more advances techniques such as positron emission tomography (PET)/Computed Tomography (CT) or by single-photon emission computed tomography (SPECT)/CT, as comprehensively reviewed by Caobelli et al.\textsuperscript{29} Whereas \textsuperscript{18}F- Fludeoxyglucose (FDG)-PET can be used to quantify the activity and the extent of the disease as it detects metabolism and glycolysis, only \textsuperscript{18}F- FDG-PET alone is not able to differentiate between the fibrotic or inflammatory nature of a stricture.\textsuperscript{29,30} Using SPECT, the degree of fibrosis over inflammation might be quantified by anti-body mediated labelling of extra-cellular ECM proteins of interest to technetium-99m (\textsuperscript{99m}Tc).\textsuperscript{29} Zhang et al. quantified liver fibrosis based on \textsuperscript{99m}Tc-3PRGD2 scintigraphy, which targets integrin α\textsubscript{v}β3 and thereby assessed the activation of hepatic stellate cells.\textsuperscript{31} Activation of intestinal stellate cells is proposed to play a role in intestinal fibrosis.\textsuperscript{32,33} α\textsubscript{v}β3 is present in the intestine and detectable using PET.\textsuperscript{34} Van den Brande et al. were able to predict the clinical efficacy of the anti-tumor necrosis factor therapy infliximab by visualizing and quantifying intestinal apoptosis by \textsuperscript{99m}Tc–annexin V SPECT.\textsuperscript{35} They showed that rapid anti-TNF-induced apoptosis in the gut predicts the response to anti-TNF treatment in patients with active Crohn’s Disease.\textsuperscript{35} \textsuperscript{99m}Tc could be bound to antibodies against e.g. extra-cellular collagen itself, to membrane bound fibroblast activator protein (FAP) or to (pro-)collagen receptors (see above) to confirm and quantify fibrosis (formation). The abovementioned imaging modalities that might be suitable to quantify intestinal fibrosis, could be used to validate serological biomarkers for intestinal fibrosis, to create a well-defined end-point for a clinical trial and to study the effect of anti-fibrotic therapies. An intestinal fibrosis specific biomarker (either serologic/radiologic/endoscopic) which has sufficient accuracy to be used in clinical practice and as end-point for trials evaluating the efficacy of anti-fibrotic drugs in human, remains to be discovered but will have a major impact on managing fibro-stenotic complications of IBD.\textsuperscript{8}

Part I of this thesis also describes that serum levels of markers for formation and degradation of extracellular matrix turnover, measured during the anti-TNF induction phase, can differentiate between clinical
responders and non-responders to anti-TNF treatment. The associations between response to anti-TNF and differences in concentrations of serological markers for collagen formation and degradation, are not reflected in concentrations of CRP. As discussed above, serological markers which quantify the post-translational modification of a protein, can quantify the response of disease affected tissue. The data described in chapter 3 is preliminary and should be considered as a proof of concept. Before markers can be used in the clinic, validation and determination of cut-off values should occur in larger cohorts and for different anti-inflammatory interventions (e.g. vedolizumab, ustekinumab). Within these studies, statistical correction for disease behaviour and e.g. smoking behaviour and by imaging quantified length of the affected area or the degree of fibrosis present, could be performed. It should be confirmed whether concentrations of biomarkers of tissue turnover are e.g. different between patients with active colonic Crohn’s disease compared to those with active ileal disease, and whether the cut-off values to define the response are different between these phenotypes. Furthermore, correlation between colonoscopy and faeces calprotectin, currently the most used non-invasive marker for gastro-intestinal inflammation, and with MRI-enterography or perhaps elastography is needed to validate whether serum concentrations correlate to tissue response and perhaps to (a reduction in) intestinal fibrosis.
As described above, validated biomarkers for intestinal fibrosis are needed to monitor disease progression and to prove the efficacy of anti-fibrotic drugs. On the other hand, relevant and translationable models for intestinal fibrosis are needed to further study disease mechanisms and to study the efficacy of anti-fibrotic drugs in the pre-clinical phase. As described in the general introduction of this thesis, many comprehensive reviews about intestinal fibrosis pathophysiology models have been published recently, all stating that further insight into pathways and mechanisms is needed.\(^8\)\(^,\)\(^36\) Models for intestinal fibrosis vary from cell-culture assays to \textit{in vivo} mouse models and some of those are translatable to the human \textit{in vivo} environment. This thesis describes a new (more) translational model for intestinal fibrosis named precision-cut intestinal slices (PCIS, chapter 6). PCIS are prepared from human as well as from rat and mouse intestine, which gives the opportunity to compare the effect of pro- and anti-fibrotic stimuli in different species. Pharmacological effects of the same substance can be different between species.\(^37\)\(^,\)\(^38\) By slicing and incubating PCIS, a spontaneous fibrosis reaction is induced.\(^39\) This effect is also observed in liver, and kidney.\(^40\)\(^,\)\(^41\) In the study presented in chapter 4 of this thesis, human jejunal PCIS were incubated with and without stimulation with Tgf-\(\beta\)\(_1\). Gene expression of fibrosis markers such as collagen type I and \(\alpha\)-smooth muscle actin generally decreases upon incubation, whereas gene expression of heat shock protein 47 and fibronectin increases. Addition of Tgf-\(\beta\)\(_1\) induced fibrosis markers in mouse and rat PCIS, but not in human PCIS. We concluded that PCIS can be used as a model for the early phase of intestinal fibrogenesis in the intestine. Differences between mouse/rat and human PCIS observed in chapter 4, might be explained by the fact that human PCIS do not contain the full cross section of the intestinal and thereby all cells present in the intestine. Unfortunately, it is technically not possible to prepare human PCIS with a full cross-section of the intestine. This is a disadvantage, since especially Crohn’s disease is typically considered a transmural disease and since Crohn’s disease associated fibrosis also occurs transmural.\(^1\) The differences between human PCIS (which only contain mucosa and a part of the submucosa) versus mouse and rat PCIS (which contain the full thickness of the intestine) in response to incubation and pro-fibrotic stimuli, might be explained by the fact that cells in the muscularis play a more important role in the process fibrosis and stenosis formation in the intestine. Pilot experiments performed with PCIS from ileocecal resection due to Crohn’s disease, and from non-affected ileum and colon obtained from right-sided hemicolectomy due to adenocarcinoma of the colon, showed highly variable gene expression of fibrosis markers upon incubation (data not shown). Especially fresh intestinal tissue from patients with Crohn’s disease was scarce and the disease behaviour phenotypes according to the Montreal classification was heterogenous. It is questionable if the Montreal classification for CD
is discriminant enough to pinpoint different disease mechanisms since severity, length of affected bowel and disease progression in time are not taken into account in this classification. Differences in age, disease duration, treatment strategies and microbiome to which these intestines were exposed (an element which is certainly disregarded in cell culture experiments when antibiotics are added to the culture medium) and in the genetic background of these patients causes variation which can be expected from translational studies. The use of medication before and at time of resection causes high variability in molecular analysis. Ideally, one would use tissue from medication naïve patients with Crohn’s disease who had an initial resection, but this tissue is even more scarce. The variety of different patient related factors, is not the only factor causing variability. Data obtained from the PCIS model might be less reproducible because the human mucosal/submucosal PCIS vary in the degree of mucosa over submucosa due to technical difficulties in dissecting submucosa from muscularis, especially in a fibrotic intestine in which the submucosa is fibrotic. To further improve the PCIS model as a model for intestinal fibrosis, further research should aim at minimizing or stratifying for the above-mentioned patient and reducing technical variables as much as possible. Furthermore, the morphological and biochemical viability of PCIS during incubation should be improved. Incubation time and viability might be improved by optimizing cell culture medium (currently used is Williams Medium E), maybe with the addition of factors needed to culture organoids from intestinal stem-cells. This might reduce necrosis occurring in PCIS (as shown in the histology figures in chapter 4) and making the model more physiological relevant.

Furthermore, the question arises which factors actually cause thickening of the intestinal wall and narrowing of the intestinal lumen during intestinal fibrosis. Whether this is predominantly an increase in extracellular matrix (ECM) content or as well contraction and stiffening of smooth muscle cells in the muscularis and muscularis propria has to be determined. ECM remodelling is increasingly recognized a key event and an active participant in IBD pathophysiology. Intestinal fibrosis (in the muscularis) might become self-perpetuating once mechanisms of ECM deposition and increased ECM stiffness coincide and augment each other. Tissue stiffness can induce fibrosis formation by activation of mesenchymal cells, also in the absence of inflammatory factors. As observed in chapter 5 of this thesis and by others, intestinal stricturing is characterized by increased expression of markers for (vimentin-positive) mesenchymal cells including myofibroblasts, smooth muscle cells (SMC) and fibroblasts; hypertrophy of smooth muscle cells and myofibroblasts; and accumulation of excess ECM proteins. SMC are believed to be able to switch from a contractile phenotype to a less mature synthetic phenotype. This switch is accompanied by a loss of differentiation with decreased expression of contractile markers, increased proliferation as
well as the synthesis and the release of several signaling molecules such as pro-inflammatory cytokines, chemotaxis-associated molecules, and growth factors.\textsuperscript{51,52} This plasticity of intestinal SMCs and to which extent this leads to stricturing of the intestine, could be studied using human PCIS of the submucosa/muscularis/serosa. After optimizing and validation of viability and morphology of the culture of PCIS, therapeutic interventions on (de)differentiation of intestinal SMCs and other anti-fibrotic compounds could be studied. Ideally, human PCIS would be prepared from human terminal ileum and colon as this is where intestinal fibrosis typically occurs.\textsuperscript{1,53} Most likely, intestinal fibrosis occurs in the terminal ileum and colon as this is where Crohn’s disease and ulcerative colitis occur. Furthermore, human PCIS should be prepared from healthy as well as with Crohn’s disease or Ulcerative colitis affected intestine. Comparing human PCIS of disease affected terminal ileum with the non-affected proximal resection margin, with the affected distal colonic resection margin and with non-IBD affected ileum and colon obtained from ileum and colon resection margins due to non-inflammatory condition such as adenocarcinoma under further improved PCIS incubation conditions, might reveal factors specific for intestinal fibrosis that could be used as biomarkers or relevant targets for therapy. Further research should also aim at comparing mucosal/submucosal vs submucosal/muscular/serosal PCIS upon incubation and in response to pro-fibrotic stimuli.

Animal models are needed to obtain further insight into the mechanism of intestinal fibrosis. In the pre-clinical phase, animal models are needed to define and understand the pathophysiology of intestinal fibrosis.\textsuperscript{36} Studying animal models for intestinal fibrosis, provide the opportunity to perform mechanistic studies (i.e. genetically modified animals with a specific knock-out or animals expressing i.e. green-fluorescent protein ubiquitously to observe cell migration into from a transplant recipient into the graft, as was performed in the development of the heterotopic transplantation model for intestinal fibrosis)\textsuperscript{54}, as well as to test potential anti-fibrotic drugs in the pre-clinical phase. Animal models are an indispensable tool to substitute for what cannot be studied in humans for ethical, practical and logistic reasons.\textsuperscript{36} Performing studies in animal models enables the investigation of genetically modified animals lacking a certain receptor, cytokine or for example lacking the ability to initiate V(D)J rearrangement and fail to generate mature T or B lymphocytes.\textsuperscript{36} The model of heterotopic intestinal transplantation induced intestinal fibrosis, which is used in chapter 7 of this thesis, is one of the available models for intestinal fibrosis. Intestinal fibrosis can also be induced in animals (especially in mice) chemically, by bacteria, by radiation and by surgery.\textsuperscript{1} Upon isogenic heterotopic transplantation, an ischemic pro-inflammatory response of chronic auto-immune mediated rejection is induced, in which rapid onset of fibrosis occurs.\textsuperscript{54} Advantages of this model are the rapid onset of fibrosis and the different
Genotypes that can be examined in the same mouse. A disadvantage is that transplanted bowel segments are not perfused and are disconnected from the fecal stream. Human anatomy and the physiology of IBD are therefore only partially mimicked. However, this model does provide a very reproducible way to further unravel (new) pathways of intestinal fibrosis and to screen therapeutic agents. Mechanisms of fibrosis formation are most likely as complex as the mechanisms of intestinal inflammation. As hypoxia and inflammation are two sides of the same coin, inflammation (leading to transcription of Nuclear factor kappa β) leads to activation of hypoxia pathways (leading to transcription of hypoxia-inducible factor (HIF) α and HIF-β) and vice versa. An increase in hypoxia in intestinal mucosa/submucosa upon or after inflammation causing acidification, might perpetuate fibrosis formation by activation of pH-sensing receptors and is potentially a new target for therapy. pH sensing receptor Ovarian-G-protein coupled receptor-1 (OGR-1) is not specific to the intestine as it is expressed as a single 3.0-kb transcript in several tissues, including spleen, testis, small intestine, peripheral blood leukocytes, brain, heart, lung, placenta, and kidney. Acidification of intestinal tissue might not only occur upon inflammation and ischemia, but perhaps as well upon acidification due to changes in the intestinal microbiota in a gut with an impaired barrier function. Also smoking might cause acidification of tissue and might therefore perpetuate fibrosis formation. To which extent all above described factors contribute to actual stenosis and symptoms, through which (so far undiscovered) receptors and mechanisms stenosis is caused and how it can be monitored and reversed, remains an important question and should be further studied. Further research should aim at exploring the role of the other known pH sensing G-protein coupled receptors (GPR4 and T cell death-associated gene 8 (TDAG8)) on intestinal fibrosis, and on the effect of targeting OGR1 using an antagonist on intestinal fibrosis.

In conclusion, a serological marker for intestinal fibrosis is not yet available, but serological markers of extracellular matrix turnover have the potential to reflect intestinal inflammation, intestinal fibrosis and to predict response to anti-fibrotic therapy. Moreover, several relevant and translational models for intestinal fibrosis to test the anti-fibrotic properties of therapies and to study mechanisms are being developed. Models for intestinal fibrosis have revealed and will reveal relevant mechanisms, biomarkers and drug targets. Biomarkers for intestinal fibrosis that predict the effect of anti-inflammatory and anti-fibrotic drugs will have major impact on the management and disease course of CD patients, especially in patients with a fibrostenotic phenotype.
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