Crohn’s disease (CD) is an chronic auto-immune mediated inflammatory bowel disease (IBD) affecting the entire gastro-intestinal tract.\(^1\) Progression of an inflammatory (non-stricturing/non-penetrating) disease phenotype over time is often marked by the occurrence of fibrosis or fistulae leading to a stricturing or penetrating phenotype.\(^2\) Progression to complications of CD is influenced by luminal microbial factors, behaviour factors such as smoking, the genetic background and the response to anti-inflammatory drugs. Longstanding recurrent transmural inflammatory activity in the gut causes damage to such extent that local repair mechanisms fail to restore the original integrity and function of the gut.\(^3\)–\(^8\) Subsequent exaggerated activation and proliferation of (myo)fibroblasts in the inflammation-affected tissue leads to increased amounts of collagen-rich extra-cellular matrix (ECM), termed fibrosis. Intestinal fibrosis is the result of a disturbed ECM remodelling balance which shifts towards excessive formation of ECM. The excessive ECM formation causes stenosis due to thickening of the luminal wall and is therefore classified as stricturing CD.\(^9\) Intestinal fibrosis is thought to be a protective response in order to limit inflammation and it occurs more frequently in CD (especially in the terminal ileum) than in ulcerative colitis (UC). It also occurs upon radiation injury or in allograft rejection after intestinal transplantation.\(^10,11\) ECM deposition by myofibroblasts and ECM degradation by matrix metalloproteinases (MMPs) that function as collagenases is a continuous process. The expression of MMPs is elevated in IBD.\(^12\) When the ECM deposition/degradation balance shifts to degradation, excessive ECM degradation by MMPs will cause penetrating CD, which is classified as the development of fistulae, abscesses and perforations of the intestinal wall.\(^9\) Both structuring and penetrating CD are major complications of CD that require surgery.\(^9\) Fistula tracts are often surrounded by fibrotic intestinal tissue and therefore structuring and penetrating disease commonly co-exist in the same patient.\(^13,14\)

Strictures and fistulae are considered to be initiated by inflammation, since both entities were never reported in segments of the intestine with no inflammation.\(^15\) Whether chronic formation of fibrosis or fistulae is driven by inflammation, or is also dependent on other factors remains to be elucidated. Long-term epidemiological data shows that the prevalence of complicated stricturing and penetrating CD does decrease over time, suggesting that the increased use of effective immunosuppressives lowered the incidence of these entities to some extent.\(^5,16,17\) 15% of the CD patients at time of diagnosis have stricturing disease, while 7% of the CD patients at time of diagnosis have
penetrating disease. Over a time course of 10 years, notwithstanding the use of immunosuppressants, disease phenotype progresses from non-stricturing/non-penetrating disease to stricturing disease in 27% of the patients with CD, whereas progression to penetrating disease occurs in 29% of patients with CD. Even though endoscopic balloon dilatation and strictureplasty can delay surgery and improve symptoms, patients often require (recurrent) surgery for strictureting or penetrating CD since specific therapies for intestinal fibrosis or fistulae are not yet available. Better understanding of the mechanism and translational models to study intestinal fibrosis and fistulae formation are warranted for developing effective anti-fibrotic (and anti-fistula) strategies.

PATHOPHYSIOLOGY OF FORMATION OF INTESTINAL FIBROSIS

Wound healing is a physiological response which occurs as soon the gut mucosa is injured during inflammation. Excessive wound healing (i.e. fibrosis) in any organ is the result of chronic injury due to different triggers, causing excessive wound healing and thereby a disturbed balance in the formation and degradation of ECM. ECM is increasingly recognized as not only being the result of chronic inflammation, but also as a compartment where cellular processes and molecular interactions take place that contribute to IBD pathogenesis. In intestinal fibrosis, changes in collagen in the ECM are mainly characterised by a relative increase in type III collagen over the amount of type I collagen in the mucosa and submucosa of the intestine. The mechanisms inducing and maintaining the formation of transmural intestinal fibrosis are believed to be comparable with mechanisms inducing fibrogenesis in other organs. However, mechanisms inducing intestinal fibrosis might not be similar to those initiating intestinal inflammation. Mesenchymal cells are activated upon (chronic) exposure to autocrine and paracrine cytokines such as Transforming growth factor (TGF)-β1, Tumor necrosis factor (TNF) and Interleukin (IL)-13, but also in response to damage-associated molecular patterns and to microbe/pathogen-associated molecular patterns. These cytokines are able to differentiate a variety of resident cells as well as circulating cells into myofibroblasts (defined as vimentin and α-smooth muscle actin positive and desmin negative), that migrate to the wound and produce collagens. The differentiation into myofibroblasts and production of collagens has been described for resident intestinal fibroblasts, smooth muscle cells and pericytes. Moreover, epithelial cells can lose the expression of epithelial-specific proteins and develop mesenchymal cell-like properties, such as contractility and the capacity to produce ECM. This physiological process of transdifferentiation (primarily involved in embryogenesis, organ development and tissue remodelling) might also contribute to fibrogenesis and fistula formation.
and is named epithelial-to-mesenchymal transition (EMT).\textsuperscript{29,30} Furthermore, circulating bone marrow derived mesenchymal stem cells (named fibrocytes), which express vimentin and collagen but not yet α-smooth muscle actin, can migrate to areas of fibrosis formation.\textsuperscript{31,32} Within the intestinal muscularis mucosae and muscularis, smooth muscle cells (SMCs) play two distinct roles in the process of intestinal fibrosis.\textsuperscript{25,26} First of all, they differentiate towards a contractile phenotype upon stimulation with TGF-β1, retinoic acid and paracrine stimulation by collagens and proteoglycans. However, upon stimulation with platelet derived growth factor (PDGF)-A/B, insulin like growth factor (IGF) or nitric oxide (among others produced by activated macrophages), SMCs dedifferentiate into a pro-synthetic phenotype which enables them proliferate, migrate and produce ECM.\textsuperscript{25,26} Altogether, SMCs might contribute even more to the actual stiffening and thickening of the intestinal wall compared to the mucosal and submucosal cells that deposit ECM.\textsuperscript{33} Currently, no effective drugs against intestinal fibrosis are available.\textsuperscript{2}

PATHOPHYSIOLOGY OF INTESTINAL FISTULA FORMATION

Fistula formation is characterised by a deficit in the healing of an ulcer. Fistulae are often surrounded by or adjacent to areas of fibrosis, which suggests that these entities share a common pathophysiological mechanism.\textsuperscript{14} Persistent inflammatory activity in these areas (primarily driven by TNF and IL-13), in combination with reduced migration of fibroblasts, increased activity of MMPs and most likely the accumulation of faecal antigens and bacteria in the wound, causes the wound to remain open and become epithelialized.\textsuperscript{34–37} Fistulae are therefore defined as epithelial cell or fibroblast covered tracts between two epithelialized surfaces.\textsuperscript{38} Fistulae in CD typically derive from the intra-abdominal intestine (enteroenteric, enterocutaneous, enterovaginal and enterovesical), but can also derive from the rectum in the perianal area. Perianal fistulae are not specific for CD and can also develop upon infection or as a complication of hidradenitis suppurativa.\textsuperscript{14} The pathophysiology behind CD-associated intra-abdominal fistulae versus perianal fistulae is believed to be the same, whereas the pathophysiology of perianal cryptoglandular fistula of other origin is different.\textsuperscript{38} CD fistulae are surrounded by infiltrated CD45R0 positive T-cells and CD20 positive B cells, non-CD fistulae are mainly surrounded by CD68 positive macrophages, which corresponds to the T-cell mediated (adaptive immunity) origin of CD. In contrast to intestinal fibrosis, CD related fistulae do benefit from immunosuppressive (anti-inflammatory) drugs, such as 6-mercaptopurine (6-MP) and anti-TNF (Infliximab/adalimumab).\textsuperscript{39–41}
Early detection of complications of CD is crucial for adequate treatment. Over recent decades, both clinical and basic/translational studies have tried to find predictors and biomarkers of intestinal fibrosis. Several clinical factors were discovered that have predictive value for disease progression towards a stricturing or penetrating phenotype. For example, the presence of ileal as well as perianal disease is associated with the development of stricturing or penetrating complications.\textsuperscript{17,42,43} Frequent recurring active disease is associated with penetrating disease, indicating again that active inflammation is one of the main drivers in developing penetrating CD.\textsuperscript{17} Furthermore, especially in patients with CD limited to the colon (Crohn’s colitis), associations were found between the prevalence of perianal CD and intestinal fistulization.\textsuperscript{44} Smoking remains the strongest (preventable) risk factor in developing complicated CD. Currently, smoking is associated with a stricturing as well as a penetrating pattern, and patients who smoke have an increased risk of surgical recurrence.\textsuperscript{4,45} When patients quit smoking, the risk of surgical recurrence decreases.\textsuperscript{46} Smoking is also a strong dose dependent risk factor for extra-intestinal manifestations, in both patients with CD and UC.\textsuperscript{47} Furthermore, genetic markers can predict the disease course of IBD. An increase in the number of CD risk alleles is associated with an increased risk for the development of CD with a more severe disease course.\textsuperscript{48} An increase in single nucleotide polymorphisms associated with smoking quantity is associated with an increased risk of surgery in smoking patients with CD, showing that genetic factors and the environment together will influence the disease course of CD.\textsuperscript{49} Recently, a SNP coding for WW domain-containing oxidoreductase was identified as a disease-modifying genetic variant associated with recurrent fibrostenotic CD.\textsuperscript{50}

Next to clinical, environmental and genetic predictors, several serological biomarkers for intestinal fibrosis have been investigated to predict complications of CD. Ryan et al. were able to predict fibrosis or fistulae complicated CD 9-10 years after diagnosis based on anti-Saccharomyces cerevisiae antibody (ASCA) serum levels baseline.\textsuperscript{43} The presence of anti-GP2 IgA and IgG immunoglobulins can predict stenosis complicated CD, but were not entirely specific for stricturing disease.\textsuperscript{51} The presence and accumulation of circulating anti-microbial antibodies, years before Crohn’s disease was diagnosed, was associated with complicated Crohn’s disease at diagnosis or shortly after diagnosis.\textsuperscript{52} None of the above described markers can specifically predict either stricturing or penetrating disease.\textsuperscript{13,53} Serological markers more specific for post-translational modification of collagens might be able to separate stricturing from penetrating CD.\textsuperscript{54}
So far, predictability of drug response to treatment of CD has mainly been studied for biologicals, in particular infliximab (IFX). A sustained response can be predicted based on clinical factors such as disease duration, the age at which IFX is started, prior anti-TNF use and previous surgery.\textsuperscript{55,56} Furthermore, a variety of studies have investigated CRP levels in response to IFX. High baseline CRP levels, as well as a rapid normalization of CRP appears to be predictive for the response to IFX.\textsuperscript{57,58} Other studies however report that baseline CRP levels are higher in patients who are primary non-responders to IFX when compared with patients having a sustained response.\textsuperscript{59} Finally, monitoring and adjusting the IFX dose, based on IFX trough levels in plasma, can be used to guide therapy and improve response.\textsuperscript{60}

None of the above described serological prediction markers are direct markers for tissue response, i.e. are produced at the site of inflammation. Serological biomarkers, which specifically monitor pathology generated post-translational modification such as e.g. protease cleavage or citrullination, are therefore highly desirable.

\textbf{MODELS OF INTESTINAL FIBROSIS}

Over recent years, many comprehensive reviews about intestinal fibrosis pathophysiology, models and treatment have been published.\textsuperscript{13,61} We do therefore not intend to provide a complete overview of the data available, but we will discuss the issues important for this thesis. The mechanism of intestinal fibrosis and the efficacy of anti-fibrotic drugs is studied in a variety of models. The utilisation of (human) intestinal fibroblasts or smooth muscle cells in wound healing assays provide a straightforward way to study wound healing based on both proliferation and migration.\textsuperscript{62–64} Scratch-tests and impedance-based electronic cell monitoring systems can be used to quantify wound healing; they are relatively cheap and well validated. A major disadvantage is however that fibroblasts partially become activated by the (plastic) surfaces on which they are cultured and that therefore the physiological relevance is low. Human intestinal organoids (HIO) provide a multicellular model of mucosal intestinal cells, in which myofibroblasts function and thereby fibrosis can be studied.\textsuperscript{63} HIO respond to TGF-β1 stimulation with an increase in the expression of pro-collagen1α1 and α-smooth muscle actin, which can be blocked by spironolactone.\textsuperscript{65} In this model, the patient’s own cells can be used for personalized drug testing in a multicellular physiological environment of a mucosa simulating organoid. Within the field of regenerative medicine, (human) gastrointestinal tissue is cultured on extracellular matrix bio-scaffolds. Tissue can be decellularized (removal
of cellular components) while the ECM structure remains preserved. Intestinal stem cells are cultured on these ECM scaffolds and are able to differentiate into epithelia, goblet cells, Paneth cells, neuroendocrine cells and enterocytes.\textsuperscript{66,67} This model is currently not validated for the study of intestinal fibrosis. A disadvantage of this model might be that the use of chemicals or enzymes to decellularize the scaffold also modifies and disrupts the ECM.\textsuperscript{67} Intestinal fibrosis is also being studied in several animal models.\textsuperscript{2,61} Fibrosis can be induced by chemicals, such as dextran sulphate sodium and 2,4,6-Trinitrobenzenesulfonic acid, or by (components of) bacteria such as salmonella or peptidoglycan-polysaccharide.\textsuperscript{2,61} Furthermore, intestinal fibrosis can be induced by heterotopic intestinal transplantation and irradiation.\textsuperscript{61,68} SAMP1/YitFc mice develop spontaneous chronic ileal inflammation and fibrosis including perianal disease with a histological pattern that is similar to CD.\textsuperscript{69} Regardless of their relevance to the (patho)physiology of human IBD, the (patho)physiology of disease in animal models will always remain different from the (patho)physiology of CD in human. Furthermore, a disadvantage of animal models are the ethical concerns in regard to the discomfort. Major advantage of an animal model is that mechanisms can be studied in the (patho)physiology of an entire body. Applying the precision-cut tissue slices (PCTS) technique to the (human) intestine might provide a more relevant and translationable model to study the mechanism of intestinal fibrosis and the efficacy of anti-fibrotic drugs.\textsuperscript{70,71}

**BIOMARKERS, MODELS AND MECHANISMS OF INTESTINAL FIBROSIS IN CROHN’S DISEASE**

The aim of this thesis is to provide insight into modulators, biomarkers and models of stricturing and penetrating complications of CD. First of all, we aimed to predict complications of CD and response to therapy using serological biomarkers. Prediction of complications and response to therapy is of increasing necessity in an era of personalized medicine in which an increasing number of medical treatments become available to treat specific disease entities at an early state. Second, we aimed to develop and use new translational models for intestinal fibrosis by applying the PCTS technique to human as well as rat and mouse intestine. Third, we aimed to use animal models to identify the role of pH-sensing receptors as novel factors involved in the process of intestinal fibrosis in order to elucidate new therapeutic targets.

In chapter 2, serological neo-epitopes produced in collagen formation and MMP mediated degradation were studied to differentiate between non-stricturing/non-penetrating, stricturing and penetrating CD. The collagen tissue turnover markers were used in chapter 3 to predict the response to infliximab in patients with CD. In chapter 4, citrullinated fragments of MMP-2, 8 and trypsin-mediated degradation of vimentin, a
protein characteristic for mesenchymal cells which becomes citrullinated by macrophage activity, was studied in order to predict the response to infliximab in patients with CD. In chapter 5, we investigated mRNA expression of proteins involved in post-translational modification of ECM in intestinal fibrosis of patients with CD. This chapter also provides a literature review to find potential anti-fibrotic drugs that target proteins/ enzymes involved in post-translational collagen fibril synthesis and its degradation. In chapter 6, we provide the first data demonstrating that precision-cut intestinal slices made from mouse, rat and human intestine can be used as a translational model to study intestinal fibrosis. In chapter 7, the relevance of the pH sensing receptor OGR1 (ovarian cancer G-protein coupled receptor-1) in human intestinal fibrosis and in a heterotopic mouse model for intestinal fibrosis was studied. Chapter 8 provides a general discussion of the findings described and chapter 9 provides a summary of the results in English as well as in Dutch.
REFERENCES


PART I — Biomarkers of Intestinal Fibrosis