IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF GASTROINTESTINAL DISEASE GENES

Ghazaleh Assadi

Stockholm 2016
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Department of Biosciences and Nutrition

Identification and functional characterization of gastrointestinal disease genes

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Stockholm 2016
To my precious Mother and Father who sacrificed everything to give my brothers and me better opportunities in life. This was possible thanks to you two❤❤❤

“Beginnings are usually scary and endings are usually sad, but it’s everything in between that makes it all worth living”

- Bob Marley
ABSTRACT

The inflammatory bowel diseases (IBD) Crohn’s disease (CD) and ulcerative colitis (UC) are conditions characterized by chronic and relapsing inflammation of the gastrointestinal tract. IBD affects around 2.5 million people of European ancestry and the incidence is increasing worldwide (currently, 1% of the population suffers from IBD in Sweden). IBD patients require life-long medication, hospitalizations, recurring sick-leaves, surgical intervention and may acquire serious complications, such as colorectal cancer. There is as yet no definitive cure, and new treatment modalities are effective, but far from being optimal. A much greater understanding of IBD pathophysiology is therefore needed, in order to delineate improved therapeutic strategies, and to predict disease course and response to treatment.

Although the etiology of IBDs is unknown, current consensus is that they occur in genetically predisposed individuals, primarily due to a dysregulated immune response to gut microbiota. IBD genetic research has highlighted the importance of innate immune interactions with the gut microbiota, the regulation of immune functions, the maintenance of gut epithelial barrier, and autophagy in order to maintain gut homeostasis. However, these discoveries have not yet led to the identification of novel pathogenetic pathways that may be amenable to exploitation for renewed therapeutic intervention. Eventually, this may come from the study of risk genes of unknown function.

The overall aim of this thesis is the functional characterization of novel gastrointestinal disease genes, and in particular the Laccase (multicopper oxidoreductase) domain-containing 1 (LACC1) gene, in order to elucidate the mechanism(s) by which its genetic variation(s) contributes to IBD, and ultimately provide novel opportunities for therapeutic exploitation.

In paper I, we tested a series of LACC1 common variants for association with disease in two Swedish cohorts of IBD and non-systemic juvenile idiopathic arthritis (nsJIA). Significant findings were detected for multiple LACC1 markers in the studied cohorts, thereby expanding previous results for CD to both UC and nsJIA.

In paper II, we identified FAMIN (the LACC1 encoded protein) as a core metabolic regulator of macrophage function. By forming a complex with fatty acid synthase at peroxisomes, FAMIN promotes carbon flux through de novo lipogenesis (DNL) and drives high levels of fatty-acid oxidation (FAO) alongside high levels of glycolysis. As a consequence, FAMIN deficiency causes defects in DNL, FAO, reactive oxygen species production, inflammasome activation, endotoxin-response and bacterial clearance, thereby providing a plausible explanation to the observed disease phenotype in patients with the variants Ile254Val and Cys284Arg.

In paper III, we found higher LACC1 expression in human immune-tissues and cells such as spleen, lymph nodes, monocytes/macrophages, DCs and neutrophils. In addition, FAMIN expression was shown to be regulated by peroxisome proliferator-activated receptor ligands.
In paper IV, we identified a number of potential candidate biomarkers that may be followed up in validation experiments in independent IBD case-control cohorts. Of particular interest, FAMIN serum levels were found to differ between IBD patients and healthy controls, with lowest expression in CD patients. This parallels mouse and human data suggesting reduced FAMIN activity predisposes to disease.

In summary, this thesis characterizes LACC1/FAMIN as a new major player in IBD pathophysiology, identifying novel biological pathways that may be amenable to modulation for therapeutic purposes, while at the same time providing preliminary data of potential exploitation for biomarkers delineation.


LIST OF PUBLICATIONS NOT INCLUDED IN THE THESIS:


* Equal contribution
LIST OF ABBREVIATIONS

AS  Ankylosing spondylitis
ATG16L1  Autophagy-related 16-like 1
CARD  Caspase activation and recruitment domain
CD  Crohn’s disease
cCD  Colonic CD
CeD  Celiac disease
DC  Dendritic cells
DNL  De novo lipogenesis
ECP  Eosinophil cationic protein
EOCD  Early-onset Crohn’s disease
FAMIN  Fatty acid metabolism-immunity nexus
FAO  Fatty-acid oxidation (also known as β-oxidation)
FASN  Fatty acid synthase
FMT  Faecal microbiota transplantation
GI  Gastrointestinal
GWAS  Genome-wide association study
HLA  Human leukocyte antigen
HPA  The human protein atlas
IBD  Inflammatory bowel disease
IC  Indeterminate colitis
iCD  Ileal CD
IFNγ  Interferon γ
IL23R  Interleukin-23 receptor
ILC  Innate lymphoid cell
IRGM  Immunity-related GTPase M
JIA  Juvenile idiopathic arthritis
LACC1  Laccase (multicopper oxidoreductase) domain-containing 1
LCFA  Long-chain saturated fatty acids
LRR  Leucine-rich repeats
MDP  Muramyl dipeptide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro ribonucleic acid</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide-adenine-dinucleotide phosphate</td>
</tr>
<tr>
<td>NET</td>
<td>Neutrophil extracellular trap</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-κB (transcription factor)</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NLR</td>
<td>Nod-like receptor</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide binding oligomerization domain</td>
</tr>
<tr>
<td>nsJIA</td>
<td>Non-systematic JIA</td>
</tr>
<tr>
<td>OCR</td>
<td>Oxygen-consumption rate</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PLA</td>
<td>Proximity ligation assay</td>
</tr>
<tr>
<td>PMP70</td>
<td>70-kDa Peroxisomal membrane protein</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptors</td>
</tr>
<tr>
<td>PRR</td>
<td>Pattern-recognition receptors</td>
</tr>
<tr>
<td>PTPN22</td>
<td>Protein tyrosine phosphatase, non-receptor type 22</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid arthritis</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>S100A</td>
<td>S100 calcium binding protein A</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interference ribonucleic acid</td>
</tr>
<tr>
<td>sJIA</td>
<td>Systematic JIA</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative colitis</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T-helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TNFSF15</td>
<td>Tumor necrosis factor superfamily member 15</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
</tbody>
</table>
1 INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) consists of the two major subtypes Crohn’s disease (CD) and ulcerative colitis (UC), two chronic idiopathic and remittent inflammatory disorders of the gastrointestinal tract (GI tract) \(^1,2\). The most common symptoms of IBD include abdominal pain, diarrhea, fever, weight loss, blood- and/or mucus-containing stool \(^3-5\). CD and UC can occur at any age, but the peak incidence is during late adolescence and early adulthood \(^4,5\).

IBD affects around 2.5 million people of European ancestry and the incidence is increasing worldwide \(^6\) (Figure 1). IBD can be considered as a disease of the West as it was previously uncommon in non-Western areas of the world. However, the incidence and prevalence of IBD is now increasing rapidly due to changes in diet, environment and social norms in industrialized countries \(^6,7\). In fact, many recent studies have reported the increasing incidence of this “Western disease” in Asia, Middle East and even South America \(^8-14\).

![Figure 1. The global prevalence of IBD in 2015. The highest prevalence is found in North America, Australia and parts of Europe. Reprinted by permission from Macmillan Publisher Ltd: Kaplan, G. G. The global burden of IBD: from 2015 to 2025. Nat Rev Gastroenterol Hepatol 12, 720-727, copyright (2015)].

IBD patients require life-long medication, hospitalizations, recurring sick-leaves, and surgical intervention and may acquire serious complications (such as colorectal cancer) \(^15\). There is a dramatic reduction of life quality in IBD patients \(^16\), which consequently results in a substantial economical burden both on the healthcare system and on society as a whole \(^6\). Although the etiology of IBD is still unknown, these complex immunologically mediated diseases are believed to occur in genetically predisposed individuals due to a dysregulated immune response towards environmental triggers, gut microbiota and medication use \(^17,18\). Therefore, it is of great importance to attempt to elucidate the etiology of IBD, with a view to find a more efficient therapeutic management of the disease and eventually a cure.
1.1 THE CLINICAL ENTITIES

IBDs are heterogeneous inflammatory diseases where the inflammation can affect one specific area of the GI tract or several different areas simultaneously. UC is characterized by a continuous inflammation of the intestinal mucosa (Figure 2) and it is limited to the colon/rectum while CD manifests with transmural inflammation involving eventually all the intestinal wall layers and can affect different part of the GI tract in a segmented/patchy distribution. Generally, IBD is divided into three different phenotypes, namely CD, UC and indeterminate colitis (IC). The two main phenotypes, CD and UC, have several overlapping clinical and pathological features, but they can still be distinguished from one another by localization, endoscopic appearance, histology and behavior. In cases where it is difficult to distinguish CD from UC using the diagnostic criteria, the condition is called IC. During the past years, there have been several classification systems suggested for the identification of these phenotypic subgroups.

Figure 2. Endoscopic images of healthy colon (left) and severe ulcerative colitis (right). By courtesy of CH, endoscopist at Gastrocentrum, Karolinska University Hospital, Stockholm, Sweden.

The Montréal classification was introduced as a revised version of the previous ones and for the first time the Montréal Working Party recommended a sub-classification system for UC. The Montréal classification system was the result of a gathering of experts in 2003, to establish an integrated clinical, molecular and serological classification of IBD. The result of this gathering was presented at the 2005 Montréal World Congress of Gastroenterology.

1.1.1 Crohn’s disease

In 1932, articles were published by the three physicians Dr. Burrill Crohn, Dr. Leon Ginzburg and Dr. Gordon Oppenheimer, where they described a condition causing inflammation in the terminal ileum. At the start, this condition was termed regional or terminal ileitis, but later on the entity was referred as Crohn’s disease. CD is a lifelong chronic relapsing immune-mediated disease with unknown etiology. The diagnosis is based on clinical history and physical examination in combination with endoscopic, histological and radiological findings. The Montréal classification of CD has 3 main parts, age at diagnosis, disease location and behavior to differentiate patients into useful clinical categories (Table 1 and Figure 3A). The inflammation in CD is patchy and can involve any part of the...
GI tract from the mouth to the anus, but most commonly involves the distal ileum and colon\textsuperscript{1,29}. A recent large genotype association study showed that predictive models based on genetic risk scores could actually distinguish between iliac and colonic CD (iCD and cCD). Thus it has been suggested that CD should be subdivided, on the base of genetic factors, into iCD and cCD\textsuperscript{30}. The clinical features of CD differ according to disease location and include chronic diarrhea with or without blood and mucus, weight loss, fever and abdominal pain. Disease location is a fundamental feature of CD (Table 1 and Figure 3A), and it is in part determined by genetic susceptibility. It is also the major driver of change in disease behavior over time\textsuperscript{30}. Patients can also display different extraintestinal manifestations such as aphthous mouth ulcers, skin ulcers called pyoderma gangrenosum and inflammation of fat cells under the skin, a condition known as erythema nodosum\textsuperscript{27,31} (Figure 3B). The course of CD consists typically of relapse and remission periods with repeated phases of inflammation that are followed by the development of strictures, abscesses and fistulas\textsuperscript{32}. CD can occur at any age but most frequently the diagnosis is made in patients in their 20s\textsuperscript{32}. CD diagnosis at an earlier stage of life (<40 years) has usually a more aggressive prognosis than a diagnosis later in life (>40 years)\textsuperscript{33,34}. Early-onset CD (EOCD) is often monogenic and associated with a severe phenotype\textsuperscript{35–37}.

**Table 1. Montréal classification for Crohn's disease\textsuperscript{4,23}**

<table>
<thead>
<tr>
<th>Crohn's disease</th>
<th>Age at diagnosis</th>
<th>Location</th>
<th>Behavior</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A1 below 16 years</td>
<td>L1 ileal</td>
<td>B1 non-stricturing, non-penetrating</td>
</tr>
<tr>
<td></td>
<td>A2 between 17 and 40 years</td>
<td>L2 colonic</td>
<td>B2 stricturing</td>
</tr>
<tr>
<td></td>
<td>A3 above 40 years</td>
<td>L3 ileocolonic</td>
<td>B3 penetrating</td>
</tr>
<tr>
<td></td>
<td></td>
<td>L4 isolated upper disease*</td>
<td>p perianal disease modifier</td>
</tr>
</tbody>
</table>

* Can be added to L1-L3

"p" is added to B1-B3 when concomitant perianal disease is present

**Table 2. Montréal classification for extent and severity of ulcerative colitis\textsuperscript{5,23}**

<table>
<thead>
<tr>
<th>Ulcerative colitis</th>
<th>Extent</th>
<th>Anatomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1 Ulcerative proctitis</td>
<td>Involved limited to rectum</td>
<td></td>
</tr>
<tr>
<td>E2 Left sided UC (distal UC)</td>
<td>Involved limited to a proportion of the colorectum distal to the splenic flexure</td>
<td></td>
</tr>
<tr>
<td>E3 Extensive UC (pancolitis)</td>
<td>Involved extends proximal to the splenic flexure</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Severity</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>S0 Clinical remission</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>S1 Mild UC</td>
<td>Passage of four or fewer stools/day (with or without blood), absence of any systemic illness, and normal inflammatory markers</td>
</tr>
<tr>
<td>S2 Moderate UC</td>
<td>Passage of more than four stools/day with minimal signs of systemic toxicity</td>
</tr>
<tr>
<td>S3 Severe UC</td>
<td>Passage of at least six bloody stools/day, pulse rate &gt; 90 beats/min, temperature &gt; 37.8 °C, haemoglobin &lt; 10.5 g/dl, and ESR &gt; 30 mm/h</td>
</tr>
</tbody>
</table>

ESR, erythrocyte sedimentation rate.
Figure 3. Phenotype of Crohn’s disease. (A) Montréal classification by age is A1<16 years, A2 17-40 years, A3>40 years. (B) Major extraintestinal manifestations and associated autoimmune disorders (blue). GI=gastrointestinal. p=perianal disease modifier. p is added to B1-3 when concomitant perianal disease is present. L4 describes upper GI disease and is also used as a modifier that can be added to L1-L3 when concomitant upper GI disease is present.

1.1.2 Ulcerative colitis

Clinical and pathological features of “ulcerative colitis-like” disorders have been described since Hippocrates (460-377 BC), but it was first in 1859 that the British physician Samuel Wilks identified UC as a distinct disease. UC is the more prevalent form of IBD and similar to CD in that it is a lifelong chronic inflammatory disease with unknown etiology. Some of the most common clinical features of UC include blood in the stool, chronic diarrhea, fever and abdominal pain. Montréal classification of UC considers the extent and severity of disease (Table 2). As for CD, the diagnosis of UC is made through a combination of medical history, physical examination as well as macroscopic, microscopic and endoscopic examinations. UC is characterized by inflammation that typically starts in the rectum and spreads proximally in a continuous fashion. However, the inflammation is limited to the colon. In contrast to CD where the inflammation can spread through all the intestinal wall layers, the inflammation in UC is only affecting the mucosal layer. Histologically, a varying degree of infiltration of immune cells, such as lymphocytes, plasma cells and granulocytes, can be seen in the mucosal layers. About 10% of the patients have extraintestinal manifestations, such as arthropathy, episcleritis and erythema nodosum. UC is characterized by periods of relapse and remission. In the same manner as CD, UC may

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* This image was published in Lancet 380 by Baumgart D. and Sandborn W. Crohn’s disease, 1590-1605, copyright Elsevier 2012.
occur at any age but the diagnosis is more common in patients in their 30s \(^{44}\). In addition, there is a second peak of disease onset between the ages of 50-70 years \(^{40}\). Similar to CD, early onset of UC (before the age of 16) has often a more aggressive initial course \(^{5}\).

### 1.2 MANAGEMENT OF DISEASE

There is as yet no definitive cure for IBD and therefore medical treatments are used to ameliorate the life quality of the patient and to achieve a sustained clinical and endoscopic remission \(^{27}\). There are several crucial issues regarding the clinical management of IBD. Apart from the often significant delay until diagnosis, there is also a lack of tools to aid prediction of who will develop severe disease with complications and who will benefit from which therapy. There are a few promising faecal biomarkers, such as calprotectin, lactoferrin, elastase and S100 calcium binding protein A12 (S100A12), that are used as diagnostic tools and have been proven to detect inflammation of the colon \(^{4,5,45}\). In a recent study, regular faecal calprotectin measurements have been shown to aid in predicting IBD relapse \(^{46}\). It is important to note that calprotectin, just as the other biomarkers, detect inflammation in general. The majority of the biomarkers used today originate from neutrophils. Nonetheless, several studies have shown changes in eosinophil numbers, eosinophil protein release and extracellular deposits of eosinophil cationic protein (ECP) as well as elevated faecal ECP and eosinophil protein X (EPX) in UC \(^{47-50}\). Taken together this indicates that eosinophil proteins might be novel biomarkers for UC. However, more studies are needed to determine the dynamics of UC activity and eosinophil response.

The heterogeneity of the disease affects the clinical management of patients and requires a more personalized treatment in order to find a safe therapeutic approach that benefits the individual patient \(^{51}\). Mild to moderate UC inflammation can be successfully treated with the anti-inflammatory 5-aminosalicylic acid (5ASA) compound with a quite safe tolerability profile \(^{5}\). For moderate to severe inflammation steroids, orally or intravenously, remain so far the principal treatment both for UC and CD \(^{4,5}\). In case of steroid-dependency and/or refractory disease and in case of very aggressive inflammation, immunosuppressive agents such as thiopurines and/or biological treatment (such as anti-TNFα) can be used \(^{51,52}\). Unfortunately, none of the therapeutical strategies used today is free from severe side effects. In particular it has been shown that triple therapy with steroid, immunosuppressive and biologicals may give a higher risk of severe infections, while increased risk of malignancy has been observed for long-term therapy \(^{51,52}\). Furthermore, surgery has a central role in the therapeutical strategy for IBD patients. It has to be carefully timed to optimize the condition of the patients before the operation and to decrease risk of complications. Tight collaboration between gastroenterologist and surgeon is highly recommended for optimization of IBD management \(^{40,53}\).

Overall, there is a need to improve the diagnostic criteria, identify predictors of disease course, and establish novel criteria for tailor-made therapy in individual patients. Therefore, there is an urge for biomarker discovery in IBD.
1.3 PATHOGENETIC MECHANISMS IN IBD

During the past 10-15 years several biological pathways have been shown to be involved in the pathogenesis of IBD. The discovery of these pathways was made mainly by the identification of disease-specific genes. The most extensively investigated pathways involve the innate and adaptive immunity, autophagy, the cytokine response and alterations of the gut microbiota composition \(^{54,55}\) (Figure 4).

![Figure 4](image)

**Figure 4.** Key pathways involved in the pathogenesis of disease, deriving from gene discovery in IBD. NOD2, ATG16L1, IRGM and IL23R focused the attention to microbial recognition, autophagy and adaptive immunity. These pathways are mainly associated with CD, whereas UC has been shown to be associated with epithelial barrier genes. Reproduced from New IBD genetics: common pathways with other diseases, Lees, C. W. et al., 60, 1739-1753, Gut copyright 2011 \(^{55}\) with permission from BMJ Publishing Group Ltd.

1.3.1 Immune cells in IBD

The immune system consists of innate and adaptive immunity, functions that protect the host from invading pathogens. The epithelial barrier together with the mucosal layer is the first line defense against the invaders. The activation of the innate immune cells such as antigen presenting cells, phagocytes and granulocytes in turn induces the initiation of the adaptive (memory) immunity \(^{2,3}\). The innate immunity is non-specific and does not elicit long lasting immunity. The epithelial barrier, mucosal layer, neutrophils, monocytes, macrophages, dendritic cells (DCs), natural killer (NK) cells, eosinophils, basophils and the novel family of innate lymphoid cells \(^{56}\) (ILCs; ILC1, ILC2 and ILC3) are the “building blocks” of the innate immune system \(^{57}\). By interacting with each other, the innate immune cells start the inflammatory process through secretion of cytokines, chemokines and antimicrobial peptides. Additionally, this results in phagocytosis of infected cells and pathogens, antigen presentation and activation of the adaptive immunity \(^{3}\). The adaptive immune system consists of cytokine producing T-cells and antibody producing B-cells \(^{57}\). T-cells are divided into different
subtypes depending on the cytokines and transcription factors expressed. The major subtypes include T-helper (T<sub>H</sub>), T-regulatory and T-cytotoxic cells. In contrast to the innate immunity, the adaptive immunity response is specific and long lasting. Although T-cells and the adaptive immune system have a major role in IBD pathogenesis, they will not be discussed further here. Instead, in line with the subject of this thesis, the focus in the upcoming paragraphs will be on the innate immune cells; neutrophils, monocytes/macrophages and the recently IBD implicated ILCs (Figure 5).

**Figure 5. Immune cells and cytokines in the pathogenesis of IBD.** In patients with IBD and in experimental mouse models of colitis, pro-inflammatory and anti-inflammatory cytokines have been shown to be produced by various cells of the mucosal immune system in response to environmental triggers. In particular, dendritic cells (DCs), neutrophils, macrophages, natural killer (NK) cells, intestinal epithelial cells (IECs), innate lymphoid cells (ILCs), mucosal effector T cells (T<sub>H</sub>1, T<sub>H</sub>2 and T<sub>H</sub>17) and regulatory T (T<sub>Reg</sub>) cells produce cytokines in the inflamed mucosa. The key transcription factors and cytokines produced by T helper cell subsets in IBD-affected mucosa are shown. The balance between pro-inflammatory and anti-inflammatory cytokines regulates the development and potential perpetuation of inflammation in patients with IBD. The dashed arrow indicates that ILCs, which produce cytokines that are involved in intestinal inflammation, may respond to IL-18. GATA3, GATA-binding protein 3; IL, interleukin; ROR<sub>γ</sub>t, retinoic acid receptor-related orphan receptor-γt; TGFβ, transforming growth factor-β; TNF tumor necrosis factor. Reprinted by permission from Macmillan Publisher Ltd: Neurath, M. F. Cytokines in inflammatory bowel disease. Nat. Rev. Immunol. 14, 329–342 copyright (2014).

### 1.3.1.1 Neutrophils

Neutrophils are the most abundant polymorphonuclear leukocytes in human blood, generated nonstop in the bone marrow. The daily production may reach up to 2×10<sup>11</sup> cells under the control of granulocyte colony stimulating factor (G-CSF), produced in response to interleukin-17A (IL-17A). IL-17A is synthesized by T-helper 17 (T<sub>H</sub>17) cells that regulate the neutrophil production. Tissue-resident macrophages and DCs regulate the IL-17A release by secreting IL-23. During neutrophil maturation three types of granules are formed and filled with numerous pro-inflammatory proteins. Neutrophils are quickly recruited to infection sites, where they fulfill their antimicrobial duties (Figure 5).
have a critical physiological function to kill pathogens through different mechanisms; phagocytosis, degranulation and by releasing neutrophil extracellular traps (NETs) \(^6^0\). Neutrophils are able to recognize diverse pathogens through cell surface and intracellular receptors, such as Nod-like receptors (NLRs) and toll-like receptors (TLRs), and in this way activate pathways to eliminate the pathogens \(^6^4\). When neutrophils have recognized and engulfed the pathogens in the so-called phagosome, they kill the pathogen by producing reactive oxygen species (ROS) or by secreting antibacterial proteins, such as cathepsins, defensins, lysozyme and lactoferrin \(^6^3\)\(^6^5\). Neutrophils can also after recognition of pathogens simply secrete different antimicrobial proteins and proteases in order to eliminate them \(^6^0\). These antimicrobial proteins can either be secreted into the phagosomes or to the extracellular sites where pathogens are in order to eliminate them. Lastly, upon activation neutrophils can secrete NETs that contains chromatin and granular proteins \(^6^6\). These NETs capture the pathogens in their surroundings and immobilize them, which in turn prevents the spreading of the pathogens and simplifies their phagocytosis. After performing their function, the neutrophils send a “find me” signal to macrophages that can through a process called efferocytosis regulate the phagocytosis of apoptotic neutrophils and in this way efficiently resolve the inflammation. Efferocytosis decreases IL-23 and IL-17 production and diminishes G-CSF production \(^6^7\).

Infiltrating neutrophils play a major role in the pathogenesis of IBD and are found in significant portions in the intestinal wall of IBD patients \(^6^8\). Calprotectin and lactoferrin, which are neutrophil-associated proteins, are found in faecal samples of IBD patients and are therefore commonly used as diagnostic and monitoring biomarkers of IBD \(^6^9\). Recently, Kvedaraite et al. reported that tissue-infiltrating neutrophils are the main source of IL-23 in the colonic tissues of pediatric IBD patients \(^7^0\). With the contribution of neutrophil activity to the pathogenesis of IBD and other inflammatory diseases it would be of considerable value to find targeted therapies capable of modulating neutrophil activity.

### 1.3.1.2 Monocytes/Macrophages

Produced in the bone marrow, monocytes are the mononuclear leukocytes that can mature into macrophages or DCs. Monocytes are abundant in the lymph nodes and spleen and when a pathogen enters the body, they migrate through the bloodstream to the infected site where they differentiate into tissue resident cells \(^7^1\). Monocytes and their progeny have several functions in the immune system, such as antigen presentation (therefore called antigen presenting cells), regulation of tissue homeostasis and repair, phagocytosis, and cytokine production \(^7^1\). Macrophages can also activate nitric oxide synthase, which in turn results in the production of nitric oxide. This gives macrophages cytostatic and cytotoxic activity against many extracellular and intracellular intruders, such as bacteria, fungi, helminthes, viruses and tumor cells \(^7^2\). Macrophages are a very heterogeneous group of cells that can be divided into subgroups depending on the anatomical location and their function \(^7^1\). M1 (inflammatory) macrophages are a class of macrophages that are classically activated in order to protect the host from bacteria, viruses and have antitumor properties. M1 macrophages
have a metabolism that is characterized by increased glycolytic rate and reduced mitochondrial oxidative phosphorylation (fatty-acid oxidation; FAO) compared to un-activated or alternatively activated macrophages, so called M0 and M2 macrophages respectively\textsuperscript{73}. M2 (regenerative) macrophages have anti-inflammatory properties and are involved in tissue homeostasis and repair\textsuperscript{71,74}. M2 macrophages have an oxidative metabolism for survival and to support the cell function\textsuperscript{75,76}.

Inflammatory macrophages strongly regulate the pathogenesis of IBD by producing pro-inflammatory cytokines such as IL-23 and tumor necrosis factor α (TNFα)\textsuperscript{77,78} (Figure 5). It has been reported that CD and UC patients have increased expression of the pro-inflammatory cytokine IL-17, which originates from T-lymphocytes and monocytes/macrophages\textsuperscript{79}. Although, there are extensive indications that macrophages have a pro-inflammatory role in inflammatory diseases, many studies have also shown the immune suppressive roles of these cells\textsuperscript{74}. Activated macrophages produce pro-inflammatory cytokines that have been shown to protect mice from CD by accelerating the clearance of pathogenic commensal bacteria from the mucosal layer of the bowel\textsuperscript{80}. The maintenance of homeostasis of the intestine is thought to be a result achieved by recruited monocytes and resident tissue macrophages, which clear the site of inflammation from apoptotic cells and debris, promotes epithelial repair, antagonizes pro-inflammatory macrophages and produces suppressive cytokines\textsuperscript{80–82}.

Macrophages have also been shown to be highly elevated in adipose tissues in the lymph nodes and intestine of CD patients\textsuperscript{83–86}. These fat depots are called “creeping fat” or “foam cells” and have been found to have a protective role in CD by functioning as an enveloping barrier on the site of inflammation and in this way potentially limiting it. The macrophages have a M2 subtype in the creeping fat and secrete anti-inflammatory cytokines like IL-10, IL-6 and TNFα\textsuperscript{86}.

### 1.3.1.3 Innate lymphoid cells

The ILCs resemble the T\textsubscript{H}1, T\textsubscript{H}2 and T\textsubscript{H}17 cells, with the exception that they are involved in the innate immunity and in tissue formation, repair and remodeling\textsuperscript{87,88}. Three key features define these novel effector cells: the absence of B- and T-cell antigen-specific receptors; the absence of ‘classical’ immune cell markers (besides some NK cell markers); and lastly their lymphoid morphology\textsuperscript{89}. The ILCs accumulate in the mucosal tissues and exert host protective immunity by secreting the same cytokines as their T\textsubscript{H}-cell counterparts (Figure 5). Just like T\textsubscript{H}1, ILC1 contributes to host resistance against intracellular infection\textsuperscript{90}. ILC2 shares the T\textsubscript{H}2 activity against helminth invasion\textsuperscript{91} and ILC3 contributes to host resistance against bacterial and fungal infections by secreting IL-17A and/or IL-22, like T\textsubscript{H}17\textsuperscript{92,93}. In mice models, ILCs have been shown to be the mediators of chronic intestinal inflammation\textsuperscript{94}. Additionally, Geremia et al. found that cells isolated from inflamed colon of patients with CD or UC have increased expression of ILC3 cytokines, cytokine receptors and transcription factors\textsuperscript{95}. Further studies are warranted to elucidate the function of ILCs in IBD.
1.3.2 IBD pathways

There are a number of central pathways that have been discovered to be involved in IBD pathogenesis. These include bacterial recognition intracellular and transmembrane receptors, intracellular catabolic processes, cytokine signaling and host-bacteria interactions (Figure 6).

![Figure 6. Schematic representation of cell-specific signaling pathways mediated by CD susceptibility genes.](image)

The mucus layer and tight junctions associated with intestinal epithelial cells maintain barrier integrity under homeostatic conditions. Disruption of this balance between host-defense immune responses and enteric bacteria is central to the pathogenesis of CD. This figure illustrates signaling pathways involved in inflammation and the potential roles of proteins encoded by IBD disease-associated genes. DC, dendritic cell; MSP, macrophage-stimulating protein; MST1R, macrophage-stimulating 1 receptor (the MSP receptor); NO, nitric oxide; PTGER4, prostaglandin E receptor 4. Reprinted by permission from Macmillan Publisher Ltd: Xavier, R. J. & Rioux, J. D. Genome-wide association studies: a new window into immune-mediated diseases. Nat. Rev. Immunol. 8, 631–643 copyright (2008) 96.

1.3.2.1 Nod-like and toll-like receptors

The pattern-recognition receptors (PRRs) play an important role in IBD since it is crucial to distinguish external pathogens from the commensal gut microbiota 97. The innate immune system uses PRRs to sense the presence of microorganisms and thereafter activate an immune response toward potential infectious threats. When the PRRs detect pathogen-associated molecular patterns (PAMPs) they activate monocytes, macrophages, DCs and neutrophils in order to eliminate the infectious threat 97. PAMPs activate PPRs and lead to a downstream signaling cascade where pro-inflammatory cytokines are produced 98. Inflammasome activation is a consequence of immune responses toward pathogens. Inflammasomes are multiprotein oligomers, which upon activation recruit pro-caspase 1 that in turn induces autoproteolytic cleavage into active caspase-1. Caspase-1 cleaves pro-IL-1β and pro-IL-18, which leads to the generation of the biologically active IL-1β and IL-18. The exact composition of the inflammasome is dependent upon the response-triggering molecule. Most
inflammasomes are formed with NLR family members, a family of the PRRs. There are five families of PRRs but only two of them will be briefly described here.

NLRs (also called leucine-rich repeat (LRR)-containing receptors) are cytosolic receptors that get activated through recognition of different intracellular pathogens. Nucleotide-binding oligomerization domain (NOD) proteins NOD1 and NOD2 are NLRs that are composed of a N-terminal with caspase activation and recruitment domain (CARD), a nucleotide-binding oligomerization domain (NOD), and a C-terminal with multiple ligand-binding LRRs. NOD1 gets activated by binding D-glutamyl-meso-diaminopimelic acid (iE-DAP), a dipeptide primarily found in Gram’ bacteria but also in some Gram’ bacteria. In contrast, upon binding of muramyl dipeptide (MDP; peptidoglycan derived from gram’/’ bacteria) NOD2 undergoes an oligomerization, which in turn activates the adaptor receptor-interacting protein 2 (RIP2). The activation of RIP2 starts a downstream signaling cascade that in the end results in the activation of the nuclear factor-κB (NF-κB) transcription factor. NF-κB belongs to an evolutionary conserved transcription factor family that regulates the induction of gene expression involved in inflammation and immune responses.

TLRs are a class of PRRs with the highest expression on monocytes and neutrophils. TLRs are a family of at least 12 transmembrane PRRs characterized by an extracellular LRR domain, a transmembrane domain and a cytoplasmic Toll/IL-1 receptor (TIR) domain. The extracellular domain recognizes the bacterial ligand through the LRR-containing horseshoe-like structure. Upon ligand binding the TLRs form homo- or heterodimers, recruit adaptor proteins and signal through different pathways downstream in order to activate the NF-κB transcription factor and induce pro-inflammatory cytokine production by monocytes and macrophages.

1.3.2.2 Autophagy

The process of autophagy was described already in the early 1960s, and was initially considered to be an energy recycling pathway activated by nutrient deficiency. However, with the discovery of the association between autophagy and CD there has been a renewed interest in the autophagy pathway and its role in innate immunity and inflammation.

Autophagy is an evolutionary conserved intracellular catabolic process that delivers cellular components to the lysosome for degradation. There has been a rapid expansion of knowledge regarding the autophagy pathway over the last 20 years, driven by basic studies in yeast. These studies have aided in identifying important molecular components and regulators of this pathway.

The process of autophagy involves a survival mechanism induced by external stimuli such as cellular starvation, stress or infection, in order to protect the organism. Thus, the autophagy pathway is induced when the cells need to eliminate damaging content such as bacteria, other pathogens and protein aggregate accumulations. In addition, autophagy occurs at low basal levels in virtually all cells in order to maintain cellular homeostasis.
through protein and organelle turnover. This pathway is then rapidly upregulated when cells are in need of energy and nutrients, for instance during growth factor absence, starvation and high bioenergetic demands\textsuperscript{111}. Furthermore, the autophagy pathway is involved in several different immune processes as it has been shown to be important for regulating self-renewal, maturation and survival of B cells, T cells and haematopoietic stem cells\textsuperscript{112–115}. The autophagy pathway is also essential for the monocyte maturation into macrophages\textsuperscript{116}. Thus, the upregulation of autophagy may facilitate proper regulation of innate immune signaling and enhancement of antigen presentation, in addition to enhancing pathogen degradation\textsuperscript{111,117–119}. Hence, autophagy plays an important role in immune function, tissue remodeling, and disease\textsuperscript{111}.

The core machinery of autophagy consists of over 30 autophagy-related genes (ATGs)\textsuperscript{120}. Recent studies have shown that polymorphisms in ATGs, such as autophagy-related 16-like 1 (ATG16L1), ATG5, immunity-related GTPase family M (IRGM), and NOD2, are associated with an increased risk of IBD\textsuperscript{55,107,108}. However, the role of autophagy in both IBD and innate immunity is complex. In some contexts, autophagy may enhance innate immune responses, whereas in other contexts autophagy may prevent excessive and destructive innate immune responses. Therefore, it has been suggested that the role of autophagy is to balance the innate immune response in such a way that it remains adaptive rather than dysfunctional\textsuperscript{119}. Thus, upregulation of autophagy may be useful in enhancing the antimicrobial innate immunity and at the same time preventing excessive inflammatory responses that may be damaging to the organism. In fact, one study has been performed where one CD patient was treated with sirolimus (rapamycin), which is an immunosuppressant, as a candidate therapy\textsuperscript{121}. Sirolimus was used to treat the patient for 6 months, which resulted in great improvements of the symptoms and endoscopic appearance\textsuperscript{121}. Sirolimus is a drug that inhibits mammalian target of rapamycin (mTOR) and thereby prevents T-cell proliferation\textsuperscript{121}. mTOR is a serine/threonine kinase that is the key for inhibiting autophagy and other signaling pathways that regulate autophagy induction\textsuperscript{122,123}. Taken together, this study implicates defects in the autophagy pathway as a pathogenic mechanism in IBD, and suggests that targeting the members of this pathway may provide novel therapeutic possibilities.

1.3.2.3 The IL-23 pathway

The revolutionary discovery of the involvement of the IL-23 pathway in IBD pathogenesis led to the development of several clinical trials, targeting different genes involved along the pathway. In fact, there are several genes that have been associated with IBD, all positioned along the IL-23 biological pathway.

IL-23 is a cytokine involved in the recruitment and activation of different inflammatory cells essential for the induction of chronic inflammation and granuloma formation, both hallmarks of IBD\textsuperscript{124}. The IL-23 pathway, in combination with the IL-12 pathway (responsible for antimicrobial response to intracellular pathogens), compromise two important immunological
pathways in the regulation of innate and adaptive immunity. The main source of IL-23 in IBD patients is believed to be infiltrating neutrophils in the colon tissue.

IL-12 is a heterodimer, formed by the IL-12p40 and the IL-12p35 subunits, that signals through the IL-12 receptor (IL12R). The IL12R also consists of two subunits, IL12Rβ1 and IL12Rβ2. Activation of the IL-12 pathway leads to phosphorylation of signal transducer and activator of transcription (STAT) family members, which in turn results in differentiation of naïve CD4+ T-cells into interferon (IFN)-γ-producing T_H1 cells. The IL-23 membrane receptor complex is composed of the IL-23 receptor (IL23R) that binds the IL-12p19 subunit and the IL12Rβ1 that binds the IL-12p40 subunit. IL-23 binds to the IL23R, predominantly expressed on memory T-cells, T-cell clones, NK cell lines, and in low levels on myeloid derived cells, such as monocytes, macrophages and DCs. By forming a heterodimeric complex with IL12Rβ1 IL23R regulates the IL-17 producing T_H17 cells. T_H17 cells are important in the host defense against different bacterial and fungal infections, and are involved in the pathogenesis of IBD.

1.3.3 Microbiota

The intestinal bacterial flora, gut microbiota, has been shown to have a significant role in the immune homeostasis. As previously mentioned, IBD is a complex disease and the interplay of genetic, microbial and environmental factors results in a continuous activation of the mucosal immune and non-immune responses. In a healthy individual, the intestinal mucosa is in a state of controlled inflammation regulated by a fine-tuned balance of different T-cell populations. In contrast, in IBD there is an immunological imbalance of the intestinal mucosa, predominantly associated with the cells from the adaptive immune system that react to self-antigens, which leads to chronic inflammatory conditions in the patients. The GI tract is the main site of interface between the host immune system and microorganisms, both symbiotic and pathogenic. Gut symbiotic bacteria are beneficial for the host: they metabolize indigestible compounds, extract vital nutrients from food, defend against pathogen colonization and contribute to intestinal architecture development. In IBD there is an imbalance in the gut microbiota (so-called dysbiosis), specifically there is an increase in the proportion of pro-inflammatory microorganisms and a decrease in anti-inflammatory microorganisms.

The main components of the gut microbiota consist of the two phyla: **Firmicutes** and **Bacteroidetes**, which together make up approximately 90% of the gut microbiota. There are some other less abundant phyla; **Proteobacteria**, **Actinobacteria** (Bifidobacterium), **Fusobacteria**, **Cyanobacteria**, and **Verrucomicrobia**. Reports have shown that IBD patients have altered gut microbiota, where healthy controls had a significantly higher bacterial diversity compared to IBD patients. Frank et al. showed that the abundance of **Firmicutes Lachnospiraceae** and **Bacteroidetes** is depleted in IBD patients; instead several other less abundant phyla are enriched in these patients. Dicksved et al. compared the gut microbiota of monozygotic twins with CD. They showed that the healthy twins had a more diverse gut microbiota composition compared to the diseased twins and that there are...
differences in the composition of Bacteroidetes species in iCD twins compared to cCD and healthy twins. In a review on the role of bacteria in CD, Man et al. compiled results from several studies on microbiota composition in patients, showing that CD patients have a decrease in abundance of Firmicutes and an increased abundance in Bacteroidetes and Proteobacteria. The gut microbiota of UC patients with inactive disease has been shown to be closer to that of healthy individuals, thus there seems to be differences in the influence of faecal microbiota on the pathophysiology of UC compared to CD.

Faecal microbiota transplantation (FMT) has been shown to be a promising treatment option in IBD. The goal of FMT is to restore/normalize the gut microbiota and its interaction with the immune system. There are conflicting results regarding FMT treatment. However, in a recent meta-analysis of FMT treatment in UC patients they reported clinical remission of 30.4% with no difference in administration route or number of infusions. At present there are not enough data on FMT treatment in IBD and more studies are needed in order to establish it as a therapeutic option. However, some of the obtained FMT results are very promising and with more knowledge it might be possible to use the gut microbiota not only for treatment, but also for diagnostics and disease monitoring in IBD patients.

1.4 GENES AND GENETICS IN IBD

The hereditary component of IBD was recognized already in the early 20th century, and we know today that the greatest risk of developing IBD comes from having a relative suffering from the disease. IBD is familial in 5-10% of individuals while the remaining 90-95% have a sporadic form. Several studies have shown that a positive family history is more common in CD patients than in UC patients, and the risk to develop IBD is larger in first-degree relatives, especially in siblings. In addition, twin-studies have revealed that the heritable component is stronger in CD compared to UC, where monozygotic twins show higher phenotypic concordance in CD patients (37%) compared to UC patients (10%). The causative mechanisms of IBD remain elusive, however it has been demonstrated repeatedly that there is a strong genetic component, and with the recent development of molecular genetics there has been a tremendous progress in the field of IBD genetics. In total, 163 IBD loci have been identified through analyses of Caucasian populations, and further meta-analyses including multi-ethnic cohorts, like Asian, Indian and Iranian, led to the identification of additional loci, bringing the current number of risk loci up to 200. These data support the concept that IBD is a genetically complex disease with a large number of genes involved in its pathogenesis.

1.4.1 Genetic history of IBD

The genetic component of IBD has been known for a long time. However, it was not until the advent of genome wide association studies (GWAS) that the identity of the IBD genes started to unravel and with it the understanding of the pathogenic pathways within IBD. In GWAS, allele frequencies of common variants are compared between unrelated cases and controls. GWASs have been used to identify several thousands of loci associated with a large
number of diseases and physiological traits. They reveal associations between specific genomic loci and genetic traits or diseases via a panel of hundreds of thousands to a million markers, so-called single nucleotide polymorphisms (SNPs). These SNPs are designed to tag all known common variants in the human genome. A successful GWAS will result in the identification of one or more genetic variants within a locus, marked by the associated tag SNP, that has biological functions driving the observed association with the disease or trait of interest\textsuperscript{154,155}.

The research field of IBD genetics began in the 1980s with association studies using functional candidate genes and focusing mainly on the HLA genes. Then in the late 1990s a number of linkage studies identified shared chromosomal regions on chromosomes 1, 3, 5, 6, 12, 14, 16, and 19 – subsequently called IBD1-IBD9\textsuperscript{156,157}. Further characterization of these IBD loci led to the identification of several IBD susceptibility genes, such as \textit{NOD2} (also known as \textit{CARD15}) within IBD1\textsuperscript{100}.

It was in 2001 that two independent groups used positional clonal strategy and positional plus functional candidate gene approach to identify the first CD susceptibility gene, \textit{NOD2}\textsuperscript{100,101}. Only four years later in 2005, Yamazaki and colleagues performed the first GWAS for CD and identified several SNPs in the \textit{Tumor Necrosis Factor Superfamily Member 15 (TNFSF15)} gene\textsuperscript{158}. The following year a second GWAS for CD was published, where the authors, in addition to confirming the \textit{NOD2} risk variants, identified risk variants in the receptor for pro-inflammatory cytokine IL-23, namely \textit{IL23R} gene\textsuperscript{159}. Less than 6 months later another very important discovery was made, a non-synonymous SNP in the \textit{ATG16L1} gene was found to be associated with CD\textsuperscript{107}. During the last 10 years, there has been recognition of the fact that larger data sets are needed to find susceptibility alleles that might have only a small or modest contribution to IBD. In order to obtain these large data sets several national and international consortia have been formed, such as for example the International Inflammatory Bowel Disease Genetics Consortia (IIBDGC)\textsuperscript{160}, a world-wide collaboration project with the aim to collect very large datasets from many different countries. The meta-analysis studies resulting from this international collaboration have yielded a vast amount of knowledge on new susceptibility loci, common pathways and genetic differences between UC and CD\textsuperscript{54,153,161–164}.

The identified IBD susceptibility genes have been shown to be part of several different molecular and cellular pathways in addition to being altered during the course of the disease. These pathways involve alterations of gut microbiota composition homeostasis, defects in the receptors of innate immune response toward pathogens, genes involved in autophagy and in the cytokine response. Indeed, GWAS have paved the way for identifying the majority of presently known IBD risk genes and have advanced our awareness of the significance of genetic susceptibility in IBD. Nonetheless, these identified loci explain only a minority of the variance in CD (13.1%) and UC (8.2%)\textsuperscript{153} leaving a large number of discoveries to be made in future studies. Rare variants in monogenic IBD (100% penetrance) have large effect on gene function and are often not detected in GWAS\textsuperscript{165}. The innovation of next generation
sequencing has opened up new possibilities in the field of IBD genetics, with independent rare variants being discovered by deep re-sequencing of GWAS’s loci. Exploiting of this new technology will without doubt aid in discovering additional susceptibility genes, new gene variants and novel pathways important for IBD pathogenesis.

1.4.2 Susceptibility genes in IBD

A number of familial IBD loci have been identified through family studies using nonparametric linkage analysis. Some of these IBD loci have been replicated and confirmed by several GWAS. Through these GWAS it has been confirmed that several immune-mediated diseases share many features. Although many of the identified risk loci are shared between multiple immune-mediated diseases, the pattern of genetic associations with the phenotypes varies. Candidate gene studies have supported the idea of shared susceptibility loci. Historically, the human leukocyte antigen (HLA) region has been implicated in immune-mediated disorders, but more and more genetic loci located outside the HLA region are being described. On the basis of these observations, it is highly likely that subgroups of immune-mediated diseases share etiology and underlying mechanisms.

As mentioned earlier, the two types of IBD, CD and UC, differ in several ways. Perhaps the most striking difference being the fact that CD has a higher family inheritance indicating a difference in the genetic background of the two. However, the clustering of these diseases in certain families and their somewhat overlapping risk loci (70%) also support similarities in their etiology.

1.4.2.1 NOD2

Hugot et al. and Ogura et al. identified the three major CD associated NOD2 mutations, Arg702Trp, Gly908Arg and Leu1007fsinsC (frameshift variant). All these mutations lie either within or near the C-terminal LRR domain, which is important for microbial sensing. These mutations in the LRR domain lead to a decreased capacity to respond to bacteria and therefore impaired clearance of invading bacteria, which in turn may lead to a more severe inflammation since the anti-inflammatory pathway is not activated. Furthermore, as mentioned earlier, it has been shown that NOD2 initiates autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacteria entry. Thus, mutations in NOD2 do not only result in defective bacterial handling and antigen presentation but also in defective autophagy.

Interestingly, the CD susceptibility loci in NOD2 has been found to have a significant protective effect for UC, but the mechanism regarding how this susceptibility allele for CD is a protective allele for UC remains unclear.

NOD2 can, independently of its role in NF-κB activation, also regulate autophagy through intracellular bacterial sensing. MDP activation of NOD2 in epithelial cells induces autophagy and increases the bacterial killing in an NOD2-dependent (and ATG16L1-
dependent) manner, a signaling pathway that is defective in CD patients with the NOD2 variants.

1.4.2.2 TNFSF15

After the NOD2 discoveries, several SNPs in the TNFSF15 gene were identified to be associated with CD. TNFSF15 gene encodes TNF ligand-related molecule 1A (TL1A), which binds to activated CD4+ T-cells and in this way induces the proliferation and differentiation of Th17-cells. These cells in turn produce IFNγ and IL-17, which are important cytokines in the defense against pathogens and in the homeostatic interaction with gut microbiota. Therefore, alterations in the TL1A signaling or expression affects the response to pathogens. Additionally, polymorphisms in the TNFSF15 gene may contribute to altered TL1A production, leading to pathogenesis of other inflammatory diseases.

1.4.2.3 IL23R

Various GWAS have identified several protective alleles for IBD that are associated with IL23R, namely Arg86Gln, Gly149Arg, Arg381Gln and Val362Ile. These protective alleles all have loss of activity, which results in reduced cell surface expression of mature IL23R and consequently reduced IL-23 signaling. In turn reduced IL-23 signaling leads to reduction in pro-inflammatory cytokines. The IL-23 pathway has been of specific interest due to the recent development of IBD antibody based therapies directed against IL23R or IL-12p40, a subunit of both IL-23 and IL-12, with the aim to neutralize the IL-23 pathway.

1.4.2.4 ATG16L1, ATG5 and IRGM

Several GWAS have identified variants in the autophagy gene ATG16L1 to be strongly associated with CD, and variants in the IRGM gene to be associated with both CD and UC. ATG16L1 mediated interaction between ATG5 and ATG12 leads to complex formation. This complex is then delivered to autophagosomes leading to breakdown of the bacteria and the bacterial antigen presentation. Autophagy may control inflammation through several different processes, such as interactions with innate immune signaling pathways, by removing inflammasome agonists and by affecting the cytokine secretion.

1.4.2.5 PTPN22

Protein tyrosine phosphatase non-receptor type 22 (PTPN22) is an enzyme involved in several signaling pathways. PTPN22 gene encodes the lymphoid tyrosine phosphatase (LYP), which is an important negative regulator of T-cell receptor signaling by de-phosphorylation of tyrosine residues from target proteins, and tyrosine phosphorylation has been shown to be important in the regulation of neutrophil function. PTPN22 is a gene that is altered in IBD, where the Arg620Trp variant is protective against CD.
1.4.3 The overlap of IBD with other immune-related diseases

A major feature of the genetic architecture of IBD, both CD and UC, is the percentage (70%) of risk loci that are shared between these two subtypes. With the increasing numbers of GWAS performed for different diseases, it has become evident that there are a large number of susceptibility genes that overlap not only between different complex diseases, but also between complex diseases and monogenic diseases. Particularly interesting is the overlap of IBD risk loci with several genes conveying susceptibility to mycobacterial diseases such as leprosy.

Susceptibility loci are shared between IBD and many other well-known immune-mediated diseases such as ankylosing spondylitis (AS), type 1 diabetes (T1D), celiac disease (CeD), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), psoriasis, multiple sclerosis (MS) and juvenile idiopathic arthritis (JIA) (Figure 7). The overlap between IBD and these immune-related diseases are through a number of different susceptibility genes such as HLA, IL23R and TNFSF15 for AS; HLA for CeD; Laccase (multicopper oxidoreductase) domain-containing 1 (LACC1) and PTPN22 for JIA; IL2RA, IL7R and PTPN22 for MS; IL23R, IL12β and PTPN22 for psoriasis; PTPN22 and TNFSF15 for RA; ATG5, IL-10 and PTPN22 for SLE; IL-10 and PTPN22 for T1D. Interestingly, a PTPN22 variant (Arg620Trp) has been shown to be both a protective gene in CD and a risk gene in SLE. In a similar fashion, variants of the gene TNFSF15 have been shown to be risk factors in CD and protective in leprosy.

These observations add complexity to the genetics of immune-related diseases and emphasize the need for more knowledge on susceptibility gene function in different contexts. Nevertheless, the vast overlap of susceptibility genes point to shared biological mechanisms and pathways between many of the different immune-related diseases, for example when it comes to response to bacterial infections and inflammation.

Leprosy is a chronic granulomatous infectious disease caused by Mycobacterium leprae (M. leprae) that causes nerve damage in the host. Leprosy is of particular interest since there is a very strong genetic overlap with IBD. There are only a few genes identified for leprosy so far and all of these genes are in fact also IBD associated genes. The shared genes between the two diseases include NOD2, TNFSF15, RIP2, LRRK2, IL23R, IRGM and LACC1, which are all confirmed IBD/leprosy genes in a number of cohorts. Both leprosy and CD have granuloma formation as a clinical feature and mycobacterial infection has been implicated in CD as a causative factor. IL12β is confirmed as a known susceptibility gene for mycobacterial infection in CD and leprosy. Additionally, the known CD and leprosy susceptibility genes, NOD2, RIP2 and IRGM, may cause deficiency of mycobacterial recognition and clearance through autophagy. Therefore, despite the fact that CD and leprosy are different clinical entities, they are both chronic inflammatory diseases and there is indicative evidence that these two diseases share common pathogenic mechanisms.
Figure 7. Schematic presentation of susceptibility gene-overlap between IBD and other immune-related diseases. The susceptibility genes given in the figure is a selection, more genes have been described in the literature. Grey lines indicate existence of overlap in susceptibility genes between the connected diseases, however these genes are not the focus here. AS: Ankylosing spondylitis; CeD: celiac disease; JIA: juvenile idiopathic arthritis; MS: multiple sclerosis; IBD: inflammatory bowel disease; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; and T1D: type-1 diabetes mellitus.

JIA is a chronic inflammatory childhood arthropathy, with enduring joint damage and lasting functional limitations and disabilities. There is significant evidence for genetic involvement behind this complex disease. JIA has a lot in common with other immune-related diseases like RA and also IBD. One gene previously implicated in JIA is PTPN22, that is associated with increased risk of different phenotypes of JIA in different populations. Recently, a rare missense mutation in LACC1, a prototypic IBD gene of uncharacterized function, was found in a monogenic form of systemic JIA (sJIA). LACC1 is an example of a gene causing more than one immune-related disease. Remarkably, the same rare missense mutation was also detected in EOCD. The fact that LACC1 common variants are associated with both IBD and leprosy, whereas its rare variant is causative in both sJIA and EOCD implies that LACC1 is a major candidate gene for regulation of key immune functions.

1.5 LACCASE (MULTICOPPER OXIDOREDUCTASE) DOMAIN-CONTAINING 1

For more than a decade our research lab has been working at the genetic and functional characterization of IBD susceptibility genes. Recently, we focused considerable research efforts on the LACC1 gene. This was identified by GWAS as a CD risk gene already in 2008 and it has surfaced as a particularly interesting candidate gene with potential to reveal novel pathways in IBD pathophysiology.

LACC1, previously named Chromosome 13 open reading frame 31 (C13orf31), encodes a protein that lacks homology with any known mammalian protein. LACC1 is located on chromosome 13 (13q14.11) and contains 7 exons. The encoded protein consists of 430 amino acids and has a molecular weight of approximately 48 kDa. The LACC1 protein is unique in that it contains a laccase domain, which is similar to bacterial multi-copper polyphenol
oxidoreductases (POs, also known as laccases). Laccases are enzymes that catalyze the oxidation of a broad range of aromatic and phenolic substrates\textsuperscript{223}. These enzymes have also been shown to be the key components of the insect immune system\textsuperscript{224}. The conserved laccase domain is located in the C-terminal of the human LACC1 protein (Figure 8). Furthermore, computational gene function prediction identified phospholipid binding (24\%) and lipid binding (20\%) properties as the most significant molecular functions.

Figure 8. LACC1 gene structure. LACC1 has a size of 14,650 bases and consists of 7 exons (yellow). The introns are indicated by black lines and the exons encoding the laccase domain are in red. 5' and 3' UTRs are given in purple.

IBD has a strong genetic component and despite the increasing number of susceptibility loci/genes identified, our knowledge of disease pathogenetic mechanisms is still limited. The genes that have been found to be associated with IBD are all part of different pathways, such as microbial sensing (NOD2)\textsuperscript{225}, T-cell immunity (IL23R)\textsuperscript{159} and clearance of pathogens through autophagy (ATG16L1, IRGM)\textsuperscript{107,183}. Polymorphisms in some of these genes have been reported to be associated with changes in gut microbiota composition and this emphasizes the link between microorganisms and inflammation in IBD\textsuperscript{226,227}. The IL23R and NOD2 may be of particular importance, since they appear to explain the largest fractions of genetic variance\textsuperscript{54}. Although as yet, it is not recommended to test for genetic variants in IBD for clinical purposes, the increasing knowledge of IBD susceptibility genes and their function will most certainly contribute to the individualization of IBD therapy in the not too distant future. In addition, the need for better diagnosis and the possibility to predict disease course is most eagerly anticipated. This may be achieved with the identification of well-characterized biomarkers and the characterization of the genetic background in different individuals. Possibly, the characterization of novel IBD susceptibility genes, such as LACC1, with hitherto unknown function, may lead to discoveries of pathogenetic pathways that can be translated into the clinic.
2 AIMS OF THE THESIS

The overall aim of this thesis is to characterize the novel LACC1 gene, and to understand its role in IBD and other inflammatory diseases. This thesis is based on the results generated in the four papers below, which will be discussed and referred to as paper I-IV throughout the thesis.

The specific aim for each paper is:

Paper I. To test the hypothesis that LACC1 common SNPs, in addition to CD, are associated with UC (the other major form of IBD), and non-systemic JIA (nsJIA).

Paper II. To investigate LACC1 biological function, its role in immunity and inflammation.

Paper III. To characterize LACC1 expression and subcellular localization in human primary cells, cell lines and tissues.

Paper IV. To screen the IBD risk proteome in IBD patients and controls, in order to identify serum candidate targets for future IBD biomarker-profiling efforts.
3 RESULTS AND DISCUSSION

This section briefly summarizes the main findings of the thesis, and their interpretation. Detailed information can be found in papers I-IV.

3.1 PAPER I: LACC1 COMMON POLYMORPHISMS ARE ASSOCIATED WITH UC AND JIA

At the initiation of this study, the biological function of LACC1 was unknown. Nevertheless, it had been shown in previous studies that LACC1 common polymorphisms were associated with CD, leprosy and possibly UC [54,162,164,181,188–192,228]. More recent evidence revealed that a rare LACC1 missense mutation (Cys284Arg) can cause monogenic forms of both EOCD and sJIA [37,206]. While the relevance of LACC1 in IBD (CD) was known, the link to sJIA was novel, and prompted us to explore whether LACC1 common SNPs are associated also with the complex nsJIA form. For the purpose of the target candidate gene investigation, we genotyped 11 SNPs (selected based on their functional (coding variants) and tagging properties; Figure 9) and tested their effect on disease risk in 3855 Swedish individuals, including 1124 CD patients, 1297 UC patients, 229 nsJIA patients and 1205 healthy controls.

![Figure 9. Linkage disequilibrium map and characteristics of the studied LACC1 SNPs.](image)

Left: Linkage disequilibrium structure generated with Haploview 4.2 analysis of genotyping data from the control population. The numbers in each box correspond to r²-values between SNPs. Center: SNPs (Marker) and SNP position (Chr pos (Hg19)) are listed with alleles at each locus (SNP, minor allele in lower case) and minor allele frequency (MAF) in the European population was retrieved from Ensembl. Right: position of each SNP within the LACC1 genomic region, and corresponding effect on messenger RNA (mRNA). Reprinted by permission from Macmillan Publisher Ltd: Assadi G. et al. LACC1 polymorphisms in inflammatory bowel disease and juvenile idiopathic arthritis, Genes Immun. 17, 261-264 copyright (2016) [229].
Variants of LACC1 are associated with increased risk of IBD, CD, UC and nsJIA

In paper I, we described significant associations of the LACC1 variants with CD, UC, IBD and nsJIA. Significant association with CD was detected for eight SNPs, which were also significantly associated with increased risk of IBD. Six SNPs showed significant association with increased risk of UC in our Swedish cohort. Most notably, seven out of the eleven selected SNPs were found significantly associated also with an increased risk of nsJIA. The direction of the genetic risk effects was the same for CD, UC and nsJIA, indicating that similar pathogenetic mechanisms likely involve LACC1 polymorphisms in the predisposition to these conditions.

In conclusion, we detected false discovery rate corrected significant associations with individual markers in the studied cohorts, thereby expanding previous results for CD to both UC and nsJIA. This is the first study showing that LACC1 polymorphisms are significantly associated with increased risk of nsJIA, and future studies are warranted to validate our findings in larger cohorts. The findings in paper I add to previous studies that showed the association of LACC1 variants with CD, EOCD, sJIA, leprosy and possibly UC, thereby justifying further investigation into the role of LACC1 in immune-related diseases. These data are in line with evidence that common molecular mechanisms and pathways are involved in the pathophysiology of these diseases, which only share some of their clinical features. The overlap of several susceptibility loci among inflammatory diseases has aided in the understanding of various pathophysiological mechanisms, and therefore the characterization of LACC1 molecular function has the potential to shed new light upon the pathogenesis of IBD. While the characterization of LACC1 function is the scope of the following papers II and III, future detailed molecular analyses are warranted to decipher the precise mechanism(s) by which LACC1 SNPs affect the expression or function of the corresponding protein (FAMIN; the LACC1 encoded protein), thereby ultimately affecting risk of several diseases.
3.2 PAPER II: IDENTIFICATION OF FAMIN AS A MACROPHAGE METABOLIC REGULATOR

From previous studies, $LACC1$ has been confirmed as a susceptibility gene for both IBD and leprosy, two diseases where the immune response to bacteria is key to the pathogenesis. It has been shown that $LACC1$ is highly expressed in macrophages, the immune cells that defend the host from both extracellular and intracellular bacteria. In paper II, we aimed at a functional characterization of $LACC1$ using in vivo and in vitro model systems. At the same time, we investigated the potential mechanisms by which $LACC1$ causative coding variants affect disease risk. Throughout paper II we have chosen to refer to the $LACC1/C13orf31$ encoded protein as FAMIN (fatty acid metabolism-immunity nexus) due to our findings regarding its biological function(s).

**FAMIN co-localizes with peroxisome markers and interacts with fatty acid synthase**

Using confocal microscopy, we could show co-localization of FAMIN with the peroxisome markers 70-kDa peroxisomal membrane protein (PMP70) and catalase in macrophages, and this co-localization was further confirmed with proximity ligation assay (PLA). In order to find FAMIN-interacting proteins, we performed an in vitro (immunoprecipitation) protein-protein interaction screen, where we identified fatty acid synthase (FASN) as a FAMIN binding partner. The interaction between FAMIN and FASN was also detectable in macrophages using in situ PLA (Figure 10). FASN is a cytoplasmic protein that associates with the membranes of different subcellular compartments. FASN is important for de novo lipogenesis (DNL) where it catalyzes the synthesis of long chain saturated fatty acids (LCFAs) from acetyl-CoA, malonyl-CoA and nicotinamide adenine dinucleotide phosphate (NADPH). It has been shown previously that FASN co-localizes with PMP70. Thus, in macrophages FAMIN is localized to the peroxisome where it interacts with FASN.

![Image of FAMIN and FASN interaction](Figure 10. FAMIN interacts with FASN and localizes to peroxisomes. Proximity ligation assay of FAMIN and FASN (yellow) in THP-1 derived macrophages. The nucleus is stained with DAPI (blue). Scale bars, 5 µm. Reprinted by permission from Macmillan Publisher Ltd: Cader, M. Z. et al. C13orf31 (FAMIN) is a central regulator of immunometabolic function. *Nat. Immunol.* 17, 1046-1056 copyright (2016).)
FAMIN is a regulator of metabolic function and bioenergetic state in macrophages

Since FAMIN interacts with FASN on peroxisomes, we hypothesized that FAMIN could affect FASN-dependent cellular functions such as DNL. To investigate the function of FAMIN we generated knockout Lacc1 (the mouse homologue of human LACCI) mice (mFamin−/−) and studied the effects of FAMIN absence on macrophages. In brief, macrophages from mFamin−/− mice differed from wild type (wt) in i) lower availability of fatty-acyl-CoA for FAO (also known as β-oxidation), ii) higher extracellular levels of citrate (indicating defective DNL), iii) less oxidative capacity, iv) lower levels of total cellular ATP and phosphocreatine. Hence, we concluded that in macrophages FAMIN functions as a regulator of i) synthesis of endogenous fatty acids (through DNL) and ii) mitochondrial oxidation. Likewise, FAMIN also controls glycolytic activity and overall ATP regeneration, and in doing so affects cellular energy availability in macrophages. Thus, FAMIN is localized to the peroxisome where it interacts with FASN, influencing macrophage cellular metabolic pathways (Figure 11).

Figure 11. Schematic illustration of the cellular metabolic pathways showing FAMIN and FASN co-localization at the peroxisome and their putative involvement in fatty acid metabolism. ACLY: ATP citrate lyase; CPT1a: carnitine palmitoyltransferase-1a.


FAMIN knockout macrophages have defective clearance of bacteria

With the observed effect of FAMIN absence on macrophage metabolism, we then investigated macrophage immunological function under the same (knockout) conditions, and observed inefficient intracellular bacterial killing in mFamin−/− macrophages. The same decrease in intracellular bacterial killing was seen in human macrophages, where FAMIN had been silenced using siRNA targeting LACCI mRNA. ROS are important for macrophage bacterial killing, and in fact mitochondrial oxidation is crucial for the generation of ROS. In line with previous mFamin−/− data and mitochondrial activity, we observed a significant decrease in ROS content in mFamin−/− macrophages. Furthermore, there was a decrease in inflammasome activation in mFamin−/− macrophages when stimulated with lipopolysaccharide (LPS). This implies that full inflammasome activation in macrophages may be FAMIN-dependent and requires FAO. Inflammasomes are the immune system sensors that induce
inflammation and sepsis \(^{239}\), and in \(mFamin^{\text{c}}\) mice injected with LPS sepsis profiles were significantly more pronounced. Taken together, the \textit{in vivo} experiments confirmed the importance of FAMIN for bactericidal and inflammasome function in macrophages.

\textit{LACC1 coding variants impair FAMIN function}

Two \textit{LACC1} coding variants have been reported in association with disease, namely the common Ile254Val (rs3764147\(^{G/G}\)) variant is increased in CD patients compared to controls, while the rare Cys284Arg variant has been found in familial cases of EOCD and sJIA. To investigate the potential effects of \textit{LACC1} coding variants on FAMIN function we used the CRISPR-Cas9 gene-editing system on wild type C57BL/6N mice. Wild type C57BL/6N mice carry the \(mFamin^{p254Val}\) variant with a valine at position 254 that corresponds to the human CD and leprosy risk variant rs3764147\(^{G/G}\). We generated mice homozygous for i) the p254Ile (CD and leprosy non-risk variant, rs3764147\(^{A/A}\)) with a substitution of isoleucine for valine at position 254 (\(mFamin^{p254Ile}\)) ii) the rare missense mutation at amino acid position 284 changing a conserved cysteine into an arginine (\(mFamin^{p284Arg}\), p284Arg).

In summary, \(mFamin^{p254Ile}\) macrophages displayed the highest glycolysation rate, basal oxygen consumption rate (OCR), maximal uncontrolled-OCR and extracellular ROS (eROS), whereas \(mFamin^{p254Val}\) had intermediate levels of all these. The \(mFamin^{p284Arg}\) macrophages had the lowest metabolic flux and showed diminished levels of eROS, comparable with the \(mFamin^{\text{c}}\) macrophages. Moreover, we studied macrophages and neutrophils from healthy human donors carrying either the risk haplotype p254Val or the non-risk haplotype p254Ile. Isolated p254Val human macrophages had lower extracellular ROS (eROS) production compared to p254Ile, confirming the results from the mouse macrophage studies. Isolated human neutrophils showed a similar pattern as the macrophages, extending FAMIN importance to neutrophil function as well. All together, these results imply that p254Val leads to partial loss and p284Arg to a complete loss of FAMIN function, thus linking diminished or lack of FAMIN biological activity to disease predisposition.

To summarize \textit{paper II}, we identify FAMIN as a core metabolic regulator of macrophage function. FAMIN forms a complex with FASN at peroxisomes and promotes carbon flux through DNL, and drives high levels of FAO alongside with high levels of glycolysis. As a consequence, FAMIN deficiency causes defects in DNL, FAO, ROS production, inflammasome activation, endotoxin-response and bacterial clearance.

The discovered critical role of FAMIN and FAO for macrophage function adds a metabolic pathway to the various pathways already implicated in immune-related diseases. Metabolic pathways have recently emerged as important determinants of immunological function \(^{75}\). It has been long known that macrophage adhesion and phagocytosis can be affected by the ratio of saturated and unsaturated fatty acids \(^{240}\), possibly through the effects of fatty acids on the macrophage cell membrane \(^{241}\). More recently, it has been shown that inhibition of the mitochondrial citrate carrier (CIC/SLC25A1), a protein essential for both FAO, macrophage activation and inflammatory responses \(^{242,243}\), causes citrate to accumulate within
mitochondria thereby leading to both reduced ROS and reduced prostaglandin production in macrophages \(^{229}\). Citrate plays an important role in fatty acid synthesis and high levels of citrate are known to inhibit glycolysis \(^{244}\). Similarly, FAMIN deficiency was shown to increase intracellular levels of citrate and reduce rates of glycolysis in macrophages (paper II). Hence, this provides additional evidence that macrophage immune function is heavily dependent upon metabolic pathways.

We also show that the metabolic mechanisms of FAMIN were affected by genetic variation in \(LACC1\). The studied \(LACC1\) coding variants lead to reduced or loss-of-function functional properties of FAMIN. Homozygosity of the substitution of cysteine for arginine at position 284 (Cys284Arg), which is known to cause EOCD and sJIA \(^{37,206}\), resulted in a loss of FAMIN function and limited the tolerance to endotoxin, while the substitution of isoleucine for valine at position 254 (Ile254Val), associated with risk for CD and leprosy \(^{54,188}\), was hypomorphic. In conclusion, in paper II we uncovered a metabolic pathway that controls macrophage immune function and can play a role in predisposition for inflammatory and infectious disease.
3.3 PAPER III: FAMIN IS A PPAR REGULATED PEROXISOME-ASSOCIATED PROTEIN

In paper III, we wanted to further characterize the expression, subcellular localization and regulation of LACC1 in human primary cells, cell lines and tissues.

LACC1 expression in immune-related tissues and cells

In order to study the endogenous expression of LACC1 and the LACC1-encoded protein FAMIN in THP-1 cells, quantitative real-time PCR (qRT-PCR) and WB analyses were applied. The LACC1-negative HeLa cell line was transfected with different LACC1 expressing plasmids in order to characterize FAMIN antibodies to use for further experiments. Although all antibodies tested showed specific detection of FAMIN, the strongest signal-to-noise ratio was obtained from the E-12 FAMIN antibody and therefore it was chosen for the following experiments.

We then tested LACC1 mRNA expression under PMA differentiation of THP-1 cells. A 50-fold upregulation of LACC1 mRNA expression was observed in response to PMA differentiation in THP-1 cells compared to undifferentiated cells. An additional 2-fold upregulation of LACC1 mRNA expression was seen with LPS stimulation. To investigate LACC1 gene expression in human tissues, qRT-PCR analyses were performed on human tissue cDNA panels from the digestive and immune systems. The analyses showed relatively equal expression of LACC1 in all tissues, but with somewhat higher expression detected in the immune-related tissues, lymph nodes and spleen. A more extensive characterization of FAMIN expression in human white blood cells, by flow cytometry analysis, showed high expression in monocytes, neutrophils and DCs (myeloid and plasmacytoid). In contrast, B- and T-cells did not show any detectable FAMIN expression.

Detailed analysis of FAMIN subcellular localization

THP-1 derived macrophages were used to perform a thorough characterization of FAMIN subcellular co-localization, using a panel of antibodies directed towards different organelle markers. Results obtained confirmed our previous finding (paper II) of co-localization of FAMIN with peroxisome markers PMP70 and catalase. Thus, FAMIN appears to be confined to the peroxisomes. In addition, some punctuate co-localization with endomembrane structures (endoplasmic reticulum (ER), early endosomes, lysosomes, trans-Golgi and mitochondria) was detected. No co-localization could be observed with the structural components (fibrillarin, β-tubulin, NUP98, CENP-A and histone H3) of the cell.

In vitro analysis of FAMIN laccase activity

We established an assay to measure FAMIN laccase activity based on a standard spectrophotometric methodology, where laccase activity was determined by time-series measurements of absorbance changes. For this purpose, we used a recombinant human FAMIN protein (C-terminal MYC/DDK-tagged) and analyzed its activity using four different
phenolic substrates. None of the substrates were oxidized with the recombinant human FAMIN, while the fungal laccase from *Trametes vesicolor* oxidized all substrates.

*Peroxisome proliferator-activated receptor (PPAR) ligands downregulate FAMIN expression*

In order to find potential *LACC1* regulatory pathways, we screened 35,000 gene expression microarray data and identified 33 genes co-expressed with *LACC1* under a number of perturbations. These 33 genes were then used to run a gene-set enrichment analysis (GSEA) to highlight eventual *LACC1*-relevant biological pathways using the Kyoto Encyclopedia of Genes and Genomes (KEGG 2015) reference knowledgebase. The GSEA identified the HSA03320 PPAR signaling pathway as the one most enriched for *LACC1* co-expressed genes. This prompted us to further explore this finding at the experimental level, by testing the effect of PPAR ligands on FAMIN protein expression. We observed that 24hrs treatment with PPARα (WY14643) and PPARγ (rosiglitazone) ligands downregulated FAMIN expression in PMA-differentiated THP-1 cells (Figure 12).

![Figure 12](image)

**Figure 12. Western blot showing PPAR-ligand downregulation of FAMIN expression.** PMA-differentiated THP-1 cells were treated with PPAR ligands WY14643 or rosiglitazone (ROSI). A clear downregulation of FAMIN (48kDa) could be detected after 24hrs with both treatments. β-actin (42kDa) was used as control.

**PPARα ligand treatment has no effect on peroxisome protein expressions**

Because of its localization to peroxisomes, we explored whether depletion of FAMIN expression leads to defects in these organelles. We set up an in vitro model system using siRNA-mediated downregulation of FAMIN expression in THP-1 cells, which resulted in a complete knockdown. However, FAMIN depletion did not have any detectable effect on the expression of peroxisome markers PMP70 and catalase, neither did it affect the number or size of peroxisomes. This was reproduced (no difference between FAMIN\(^+\) and FAMIN\(^-\) cells) when cells were stimulated with the PPARα ligand WY14643, which interestingly induced a slight increase in peroxisome number (independent of FAMIN expression).

To summarize **paper III**, we provide confirmatory and novel results that contribute to the understanding of *LACC1* function and involvement in the pathogenesis of immune-mediated diseases. Interestingly, the high *LACC1* expression pattern detected in spleen, lymph nodes, monocytes, DCs and neutrophils resembles the expression pattern of *protein tyrosine phosphatase non-receptor type 22 (PTPN22)* \(^{231}\), a protein shown to be involved in susceptibility to immune-mediated diseases including JIA \(^{245}\). A SNP-SNP interaction for *PTPN22* (rs2476601) and *LACC1* (rs3764147) has been shown to confer increased risk of UC in IBD patients \(^{246}\), indicating that these two genes may have overlapping mechanisms of disease predisposition, through neutrophils or macrophage-specific functions.
Very little was known earlier on the expression patterns and the biological function of FAMIN. In the first study on FAMIN function, we presented data showing that FAMIN, located at the peroxisomes, controls macrophage bioenergetic capacity by promoting DNL and FAO (paper II). The peroxisome aspect was also highlighted in the present study through an extensive fluorescence microscopy analysis of FAMIN co-localization (paper III). We showed that FAMIN appears to be confined to peroxisomes and, to a lower extent, endomembrane structures like those found at the level of the ER. The punctuate localization of FAMIN to the ER may correspond to nascent FAMIN detected before transport to the peroxisomes which would then be consistent with the previous finding of unfolded FAMIN (LACC1) mutant (Cys284Arg) retained in the ER (paper II). Peroxisomes have a key role in cell lipid metabolism and FAMIN-dependent FAO triggers inflammasome activation and ROS production (paper II). Also some of the defense potential of macrophages and neutrophils is exerted through the peroxisome content (antimicrobial peptides, peptidases and hydrolases). Therefore, maintaining peroxisome homeostasis would be essential for the host defense, something that is implied from the observation of decreased peroxisome numbers in intestinal epithelial cells from CD patients, showing negative effects on FAO.

The siRNA-mediated knockdown of FAMIN expression did not reveal any direct effect on peroxisome numbers. However, GSEA analysis of LACC1 co-expressed genes highlighted the PPAR signaling pathway as the top enriched KEGG process. PPARs are transcription factors involved in the control of carbohydrate and lipid metabolism, but have also been shown to play important anti-inflammatory roles. Impaired expression of PPARγ has been detected in both UC and CD. In a mouse model of IBD, animals deficient in macrophage-specific PPARγ expression fail to recover after treatment with the PPARγ agonist pioglitazone. The synthetic PPARα ligand WY14643 and the PPARγ ligand rosiglitazone induced FAMIN downregulation in THP-1 macrophages (paper III), suggesting the involvement of PPAR pathways in the modulation of LACC1 expression. One may speculate that the observed PPAR-driven downregulation of LACC1 expression reflects a potential feedback-loop mechanism to control (FAMIN-dependent) FAO rate. At the same time, this may represent one genuine mechanism through which PPARs are able to modulate inflammation, since downregulation of LACC1 may also result in reduced NLRP3 inflammasome activation and pro-inflammatory production of ROS. Of interest, it has been shown that M1 macrophages can be shifted to M2 phenotype by activation of the PPAR pathways. Given that LACC1 expression has been detected at higher levels in murine bone marrow-derived M1 compared to other macrophage subtypes (paper II), it may be so that subtype switches involve PPAR-driven changes in LACC1 expression. However, the regulation of FAMIN expression by PPARs needs a more thorough investigation. While the precise molecular mechanisms through which LACC1 exerts its biological effects remain to be elucidated, these data expand the current knowledge by providing a resource of experimental conditions and investigative tools that may be exploited in future LACC1 functional studies.
3.4 PAPER IV: LACC1 LEVELS IN SERA ARE CORRELATED TO DISEASE

In paper IV, we set up a proteomic high-throughput approach to screen for potential candidate biomarkers of diagnostic and/or prognostic value in IBD. Genetic risk effects often appear to be mediated by allelic differences (risk vs. non-risk variants) in the modulation of mRNA expression (expression quantitative trait loci; eQTLs), something that may be reflected in serum protein profiles of patients compared to controls. The protein targets that were screened corresponded to genes mapping within the known 163 IBD risk loci, in addition to neutrophil-associated and inflammatory proteins. In this paper, we took advantage of the Human Protein Atlas (HPA) large repository of antibodies to identify proteins that might be of future use for clinical diagnosis and prognostic value. We focused on i) proteins that would enable the distinction between IBD patients and healthy individuals; and ii) markers that may be useful to differentiate between UC and CD.

**IBD risk proteome screening**

In total 365 antibodies directed against 218 unique target proteins were used for the analysis, where sera from 100 IBD patients (CD N = 49 and UC N = 51) were compared with sera from 50 healthy controls. When comparing the IBD patients with the controls, we observed that antibodies directed against S100 calcium-binding protein A9 (S100A9) and serum amyloid proteins A1-A2 (SAA1 and SAA2) were among the top hits in the analyses. Also among the top 15 candidate proteins, we found a number of T-cell regulatory proteins (CARD11 and BTNL2)\(^{255,256}\). A few of the proteins in the top 15 have been shown previously to be present in sera (CNTF, IL2RA, LNPEP, SAA and S100A9)\(^{257-261}\). Interestingly, the protein with the highest significant difference between patients and controls was LACC1 (also known as FAMIN), when detected with HPA040150 antibody.

**LACC1 levels are lower in CD and UC patients compared to controls**

The detected sera levels of LACC1 protein were higher in healthy controls compared to CD and UC patients. When comparing CD with UC, lower levels of LACC1 protein were observed in sera from patients with CD. The results were similar for the two anti-LACC1 antibodies (HPA040150 and HPA061537), however the anti-LACC1 HPA040150 antibody showed generally reduced and more homogeneous results. In order to better characterize this antibody, we applied WB analysis of LACC1-transfected cell extracts and confirmed its specificity, making it suitable for use in future development of validation tools such as sandwich assay and immune-capture mass-spectrometry (IC-MS). Of particular interest, the observed LACC1 downregulation in IBD patients compared to healthy controls, with lowest expression observed in CD patients, is in concordance with the results obtained for the coding variants (Ile254Val and Cys284Arg) in paper II, in that reduced or impaired LACC1 (FAMIN) expression correlates with disease.

Other proteins, such as gluthatione peroxidase 4 (GPX4), ubiquilllin 4 (UBQLN4) and NOD2, showed similar trends as LACC1, when comparing CD with UC. These proteins are involved
in protection from oxidative stress, regulation of protein degradation through autophagy and intracellular bacterial recognition, respectively \(^{54}\).

To summarize **paper IV**, we found serum levels of the LACC1 protein product to differ between IBD patients and healthy controls and to a lower extent also between CD and UC patients. This warrants further analyses of LACC1 expression in independent cases and controls for eventual consideration of this target as a candidate biomarker to combine with other biological predictors. In this context, and in the light of our recent results on LACC1 (FAMIN) and fat metabolism (**paper II**), it is interesting to note that a recent study reported the potential use of plasma ether lipid levels to differentiate CD from UC \(^{262}\). This implies that fat metabolism may have a role in IBD, and that in the future a related blood test may be developed to distinguish between CD and UC. The identification of specific disease biomarker panels for IBD is indeed an intense area of investigation, and other protein targets have also been recently highlighted \(^{263}\).
4 CONCLUDING REMARKS

The work presented in this thesis mainly relates to the characterization of a novel IBD risk gene, \textit{LACC1}, and explores its biology and functional role(s) in IBD. This section further discusses the significance of the present findings and possible future directions.

IBD is known to be a disease of the Western part of the world, however the incidence and prevalence is now increasing worldwide \textsuperscript{6}. Over the past 10 years, which encompass the GWAS era, enormous progress has been made in IBD genetics, highlighting pathways and plausible pathogenetic mechanisms of disease. However, despite this surge of knowledge, the precise etiology of IBD is still mostly unclear.

This thesis, whose original main aim was the identification and functional characterization of novel gastrointestinal disease genes, contributes with valuable new information to improve our understanding of IBD etiology and pathophysiology: from the initial identification of the CD gene \textit{LACC1} as a genetic risk factor also in UC and nsJIA (paper I), to an in-depth functional characterization of its protein product FAMIN (paper II), including its expression in different cultured and primary cells and human tissues (paper III), and finally the discovery of its differential expression in sera form IBD patients versus controls, which is of diagnostic biomarker potential (paper IV). Using these studies as a stepping-stone, we now have tools and knowledge to further characterize this important gene.

In paper I, we identified a number of variants in \textit{LACC1} associated with IBD, CD, UC and nsJIA. Knowledge about the different variants and the associated phenotype could potentially be used to identify individuals at risk for disease. IBD is a complex and heterogeneous disease where patients could benefit from tailor-made treatments where identification of specific genetic risk profiles could contribute to a personalized medical management, i.e. aid in the selection of specific treatments for individual patients \textsuperscript{264}.

A major finding in this thesis (paper II and III) is the co-localization of FAMIN with FASN at the peroxisomes in macrophages. The complex formed between FAMIN and FASN at the peroxisome was shown to influence a number of cellular pathways that are key to macrophage metabolism, with \textit{LACC1} loss-of-function and/or IBD-risk variants leading to defects in i) DNL, ii) FAO, iii) inflammasome activation iv) mitochondrial and NADPH-oxidase dependent production of ROS and v) bactericidal activity. Thus, paper II provides a molecular foundation to explain the observed disease phenotype in IBD patients carrying the Ile254Val and Cys284Arg \textit{LACC1} variants. Furthermore, it provides novel insight into metabolic pathways that may be targeted by future IBD treatments, for example by regulating FAMIN expression or inducing FAO.

Our focus in paper II was mainly on macrophage function, therefore at this point we can only speculate about the role of \textit{LACC1} in neutrophil function/metabolism. Because of the key role of neutrophils in IBD \textsuperscript{68,265}, it would be of great interest to investigate the bactericidal function of these cells in the absence of FAMIN. Therefore, future endeavors could be aimed at i) characterizing \textit{LACC1} function in neutrophils and other immune cells, preferably from...
IBD patients carrying risk variants, ii) fine-mapping the \textit{LACCI} region in order to identify new rare variants, iii) identification of substances that upregulate the expression of FAMIN and iv) testing the effect of such substances on macrophage and neutrophil metabolism/function to investigate potential novel therapeutics.

The observation that PPAR ligands affect FAMIN expression (paper III) opens up the possibility that PPAR ligands may influence macrophage or neutrophil function through modulation of FAMIN expression. PPARs are activated by a number of natural (fatty acids) and synthetic ligands, and have a role in regulation of lipid and lipoprotein metabolism, glucose and energy homeostasis, thereby influencing many different cellular functions 266. Additionally, they modulate immune and inflammatory response in distinctive tissues and cells 267,268. There are a number of PPAR agonistic and antagonistic drugs available that could be tested for their effect on FAMIN and macrophage metabolism and function, as well as in our mouse models (\textit{mFamin} \textsuperscript{−/−}, \textit{mFamin}\textsuperscript{p254Ile}, \textit{mFamin}\textsuperscript{p254Val} and \textit{mFamin}\textsuperscript{p284Arg}). Ultimately, such investigations could lead to novel therapeutic management of IBD.

In paper IV, we identified differentially expressed serum proteins in IBD versus controls and in CD versus UC. The serum proteins identified were mainly associated with neutrophils, while coming mostly from known IBD risk loci. These results confirm the importance of neutrophil biology in IBD, and represent a list of candidate biomarkers that may be further investigated and validated in independent case-control cohorts. Proteomic profiles may be used as biomarkers, however an “omics” combination of proteome, genotype and transcriptome profiling may be best suitable in distinguishing between different diagnoses. Recently in IBD, exciting novel data have been reported for microRNA (miRNA) 269,270, which show potential to be used as CD versus UC biomarkers 271. miRNA are stable small non-coding RNAs that regulate gene expression post-transcriptionally and that can be found in most biological fluids 269. In the clinical setting, non-invasive tests are of great value and, although in preliminary stages of validation, these recent advances may allow the development of novel diagnostic tools. There is little doubt that future integrative approaches, where several data layers are combined into unified patients’ profiles, will have a considerable impact on the diagnosis and treatment of IBD, implementing “precision medicine” approaches to patients management.
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