# Primary sclerosing cholangitis and the gut microbiota

–

a study on mice, man and microbes

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"*Teach war no more*"

- Joshua Lederberg
1958 Nobel laureate[1]

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# 1 Acknowledgements

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Martin Kummen
Oslo, 2016.

# 2 Abbreviations

| | |
|---|---|
| AIH | autoimmune hepatitis |
| ALP | alkaline phosphatase |
| ALT | alanine aminotransferase |
| AMA | anti-mitochondrial antibodies |
| ANA | anti-nuclear antibodies |
| AST | aspartate aminotransferase |
| AUC | area under the curve |
| BEC | biliary epithelial cell |
| BMI | body mass index |
| CBDD | common bile duct dilatation |
| CCA | cholangiocarcinoma |
| CCL | CC chemokine ligand |
| CCR | CC chemokine receptor |
| CD | Crohn's disease |
| Chao1 | Chao1 bacterial richness estimate |
| CONV-R | conventionally raised |
| DC | dendritic cells |
| DCA | deoxycholic acid |
| DNA | deoxyribonucleic acid |
| eGFR | estimated glomerular filtration rate |
| ERC | endoscopic retrograde cholangiography |
| FDR | false-discovery rate |
| FFQ | food frequency questionnaire |
| FMO | flavin-containing monooxygenase |
| FMT | faecal microbiota transplantation |
| GALT | gut-associated lymphoid tissue |
| GF | germ free |
| HLA | human leukocyte antigen |
| IAC | immunoglobulin G4 associated cholangitis |
| IBD | inflammatory bowel disease |
| IBS | irritable bowel syndrome |
| ICAM-1 | intercellular adhesion molecule 1 |
| Ig | immunoglobulin |
| IL | interleukin |
| INR | international normalised ratio |
| LCA | lithocholic acid |
| LPS | lipopolysaccharide |
| MAdCAM-1 | mucosal vascular addressin cell-adhesion molecule 1 |
| MAIT | mucosal-associated invariant T |
| MDU | minimal disease unit |
| MELD | model for end-stage liver disease |
| MRC | magnetic resonance cholangiography |
| MyD88 | myeloid differentiation primary response gene 88 |
| NASH | non-alcoholic steatohepatitis |
| NF-κB | nuclear factor kappa-light-chainenhancer of activated B cells |
| NOD | nonobese diabetic |
| OTU | operational taxonomic unit |
| p-ANCA | perinuclear anti-neutrophil cytoplasmic antibodies |
| PAMP | pathogen-associated molecular pattern |
| PBC | primary biliary cirrhosis |
| PCR | polymerase chain reaction |
| PDC-E2 | pyruvate dehydrogenase complex E2 |
| PPI | proton pump inhibitor |
| PRR | pattern recognising receptor |
| PSC | primary sclerosing cholangitis |
| RNA | ribonucleic acid |
| ROC | receiver operating characteristic |
| rRNA | ribosomal ribonucleic acid |
| SCFA | short-chain fatty acid |
| SMA | smooth muscle antibody |
| SNP | single nucleotide polymorphism |
| T1D | type 1 diabetes |
| TBB-5 | beta-tubulin isotype 5 |
| TG2 | transglutaminase 2 |
| TLR | toll-like receptor |
| TMA | trimethylamine |
| TMAO | trimethylamine-*N*-oxide |
| TNFα | tumor necrosis factor α |
| UC | ulcerative colitis |
| UDCA | ursodeoxycholic acid |
| VAP-1 | vascular adhesion protein 1 |
| WGS | whole genome sequencing |
| γ-GT | gamma-glutamyl transferase |

# 3 List of publications

This thesis is based on the following papers, which are referred to by Roman numerals:

***Paper I***

Kummen M, Holm K, Anmarkrud JA, Nygård S, Vesterhus M, Høivik ML, Trøseid M, Marschall HU, Schrumpf E, Moum B, Røsjø H, Aukrust P, Karlsen TH, Hov JR. The gut microbial profile in patients with primary sclerosing cholangitis is distinct from ulcerative colitis patients without biliary disease and healthy controls. Gut (published online ahead of print: 17 February 2016). doi:10.1136/gutjnl-2015-310500

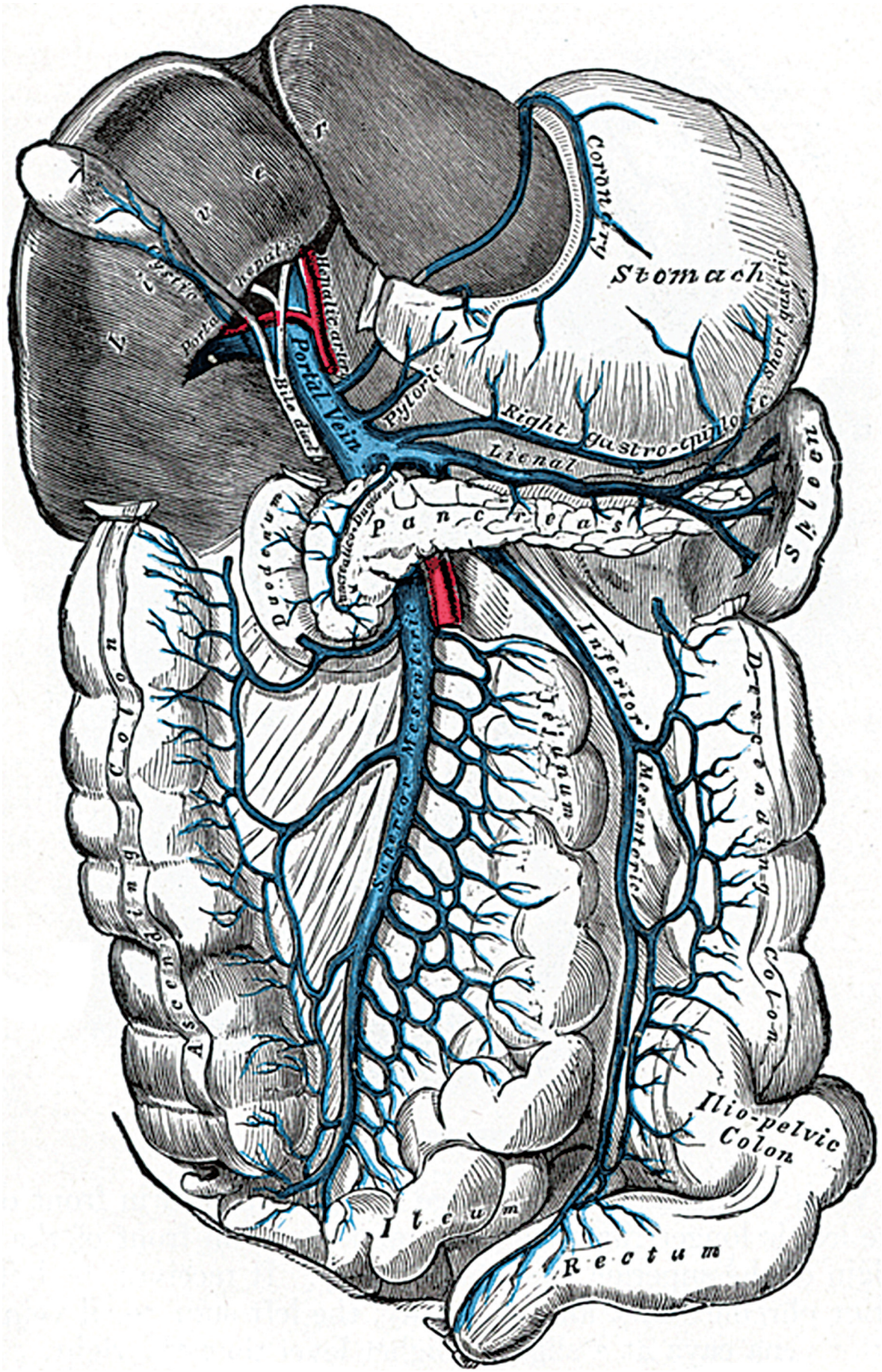
***Paper II***

Kummen M, Vesterhus M, Trøseid M, Moum B, Svardal A, Boberg KM, Aukrust P, Karlsen TH, Berge RK, Hov JR. Microbiota-dependent marker trimethylamine-*N*-oxide (TMAO) is associated with the severity of primary sclerosing cholangitis. (Submitted manuscript).

***Paper III***

Schrumpf E*, Kummen M*, Greiner T, Holm K, Arulampalam V, Baines J, Bäckhed F, Karlsen TH, Blumberg RS, Hov JR, Melum E. The gut microbiota contributes to disease in a mouse model with spontaneous bile duct inflammation. (Submitted manuscript).

*Shared first authorship.


Coronary
Stomach
Hepatic artery
Portal Vein
Bile duct
Pyloric
Right gastro-epiploic
Lienal
Pancreas
Duodenum
Pancreatico-Duodenal
Superior Mesenteric
Inferior Mesenteric
Jejunum
Ascending Colon
Transverse Colon
Ilio-pelvic Colon
Ileum
Rectum

# 4 Introduction

What causes the human liver disease primary sclerosing cholangitis (PSC) has puzzled scientists for a long time, and is still elusive to us today.[2,3] Apart from liver transplantation the treatment options are scarce, and we have few, if any, drugs that can even slow the progression of the disease.[2–4] This has led some to refer to PSC as the "last black box" of hepatology.[4] Nonetheless, in recent decades researchers have been able to shed some light on this dismal situation: Several genetic risk factors have been discovered, as have some environmental factors.[5–12] Some promising clinical trials are also on-going, which could potentially give rise to new drugs that could improve patient care in the future.[2] Although we might not be able to cure PSC, we could hope to give comfort to our patients by discovering means to better ameliorate symptoms, discover new remedies that could slow disease progression, find better prognostic tools and improved biomarkers, and develop better tools for early detection and accurate diagnosis of cholangiocarcinoma (CCA) in PSC. It is in this clinical and academic setting that this thesis has emerged.

The present thesis will discuss the role of the gut microbiota, here used as a collective term for the vast bacterial community that resides in the intestines of all humans and mice, and its role in PSC.

In this introduction I will start by giving a brief overview of PSC, and how it is managed clinically, before introducing the gut microbiota, and a short lead-in to the mechanistic world of animal models used in PSC research. I will then present an overview of some of the known risk factors for PSC together with a summary of the few aspects of PSC pathogenesis that are known. Lastly I will present a short synopsis of the prevailing hypotheses of PSC aetiology and pathogenesis, and elaborate on the link between the gut and the liver, in light of what we know from research in humans and animals.

**Figure 1 (left).** The portal vein (blue) drains blood from the intestines into the liver (top left). The liver is thus the first organ encountered by most molecules originating from the intestines, both 'good' *e.g.* the majority of dietary compounds and 'bad', *e.g.* toxic metabolites. The blood then circulates to the heart and further into the rest of the body, also causing the liver to possibly act as a firewall that mediate the mutualism between the host and its commensal gut bacteria (microbiota).[13] Plate 591 from Henry Gray's "Anatomy of the Human Body" (1918), illustrated by Henry Vandyke Carter, copyright expired.

## 4.1 An overview of primary sclerosing cholangitis (PSC)

PSC is a chronic liver disease of progressive nature, without any effective medical treatment as of today.[14,15] It leads to bile duct destruction and cholestasis, and most patients will gradually advance to end-stage liver disease with need of liver transplantation.[16–18] This, together with better treatment options for other liver diseases, and a low burden of *e.g.* viral hepatitis C and alcoholic liver cirrhosis, has made PSC one of the leading indications for liver transplantation in Norway in recent years.[19–21] PSC was probably first described by Carl Ernst Emil Hoffman, in Basel, Switzerland, in 1867.[3,22] In the middle of the twentieth century several case series emerged that established the link to inflammatory bowel disease (IBD).[23–25] Endoscopic retrograde cholangiography (ERC) became available during the 1970s, making it possible for clinicians to visualise the bile ducts and the biliary tree.[26] This facilitated diagnosis considerably, and several important publications in the beginning of the 1980s contributed to establishing diagnostic criteria, which in part are still used today.[27–29]

### 4.1.1 Epidemiology

PSC is not a very common disease, with a mean annual incidence in Norway of approximately 1.3 per 100,000 inhabitants, and an approximate prevalence of 10 per 100,000.[30] Worldwide there is a striking geographical variance, with reported numbers being a 10- to 100-fold lower in southern Europe and Asia.[4,30,31] A large population-based study of adult inhabitants in Sweden found a prevalence of 16.2 per 100,000, which is the highest prevalence reported.[32] PSC is often referred to as "the disease of the North", but the explanation for this variation is still elusive.[4]

Patients with PSC are also at increased risk of autoimmune diseases (*e.g.* type I diabetes, rheumatoid arthritis and IBD).[33,34] The association with IBD is salient: up to 80% have concomitant IBD, most often diagnosed as ulcerative colitis (UC).[35,36] The observation that the frequency of IBD in PSC increases along the same geographical gradient as the prevalence of PSC itself is also intriguing, with the lowest frequency in Asia (20-37%), ~50% in Southern Europe, and 62-83% in Northern Europe and the US.[3,37] This north-south risk gradient is observed in several autoimmune diseases.[38,39] There is an increased risk of PSC in first-degree relatives of patients with PSC,[40] and there is an approximate 2:1 male:female ratio.[31,41,42] The patients are relatively young at diagnosis, typically in their third or fourth decade of life.[31,32,41,42]

### 4.1.2 Symptoms, signs and diagnosis

The clinical presentation of PSC is variable. Up to one third of patients have no symptoms at the time of diagnosis, and the onset of pathology is thought to precede that of clinical onset by several years.[14,16,17,43,44] Patients without symptoms are identified either serendipitously through the discovery of elevated liver enzymes, or during more selective screening of patients with *e.g.* IBD.[44]

**Symptoms** often presented at diagnosis are; abdominal discomfort in the upper right quadrant, weight loss and pruritus.[16,17,29,43] Fatigue is frequently described as a common symptom in the literature.[2,4,8,43] However, Björnsson *et al.* reported that fatigue was not more common in PSC compared with IBD patients, and they were unable to find any association between fatigue and the severity of PSC. The fatigue was also less pronounced compared with sex- and age-matched healthy controls from the general population.[45]

**Clinical signs** of liver disease in general can be present at diagnosis, *e.g.* enlarged liver (hepatomegaly), enlarged spleen (splenomegaly) and jaundice, but none are PSC-specific.[16,44] Fever and chills are less common, but could arise in the setting of cholangitis.[14] Signs of portal hypertension caused by more advanced liver cirrhosis are more rare and include ascites and haemorrhage from gastro-oesophageal varices.[15] Steatorrhoea and malabsorption of fat-soluble vitamins can occur with prolonged cholestasis.[15]

**Biochemical tests** are often the next step in the assessment of patients with symptoms and signs consistent with liver disease. Alkaline phosphatase (ALP), a biochemical marker of cholestasis, is elevated in most PSC patients and is often elevated at least 3-fold.[14,16] Serum aminotransferase levels are also often elevated, while serum bilirubin is normal in the majority of patients at diagnosis.[14,15] Importantly, if there is a clinical suspicion of PSC, one should not exclude the diagnosis of PSC on the basis of normal biochemical tests alone.

**Autoantibody-screening** is often part of the diagnostic work-up. There are no PSC-specific antibody, but screening can be useful in differential diagnosis.[14,15,17] Perinuclear anti-neutrophil cytoplasmic antibodies (p-ANCA) is the most common antibody found in PSC, but it is also frequently found in UC patients without PSC and in patients with autoimmune hepatitis (AIH).[46] As anti-mitochondrial antibodies (AMA) is detected in 90-95% of patients with primary biliary cirrhosis (PBC), but found in very few patients with PSC, it is most useful when searching for a diagnosis in a cholestatic patient.[46]

**Imaging studies** often start with an abdominal ultrasound. It is not diagnostic and often normal, but can reveal gallstones, cholecystitis, gallbladder enlargement and mass lesions.[14,15] The hallmarks of PSC are: *diffuse and multifocal short strictures or mural irregularities, alternating with normal or saccular dilatations of both the intra- and extrahepatic bile ducts*, illustrated in **Figure 2** below.[2,14,47]

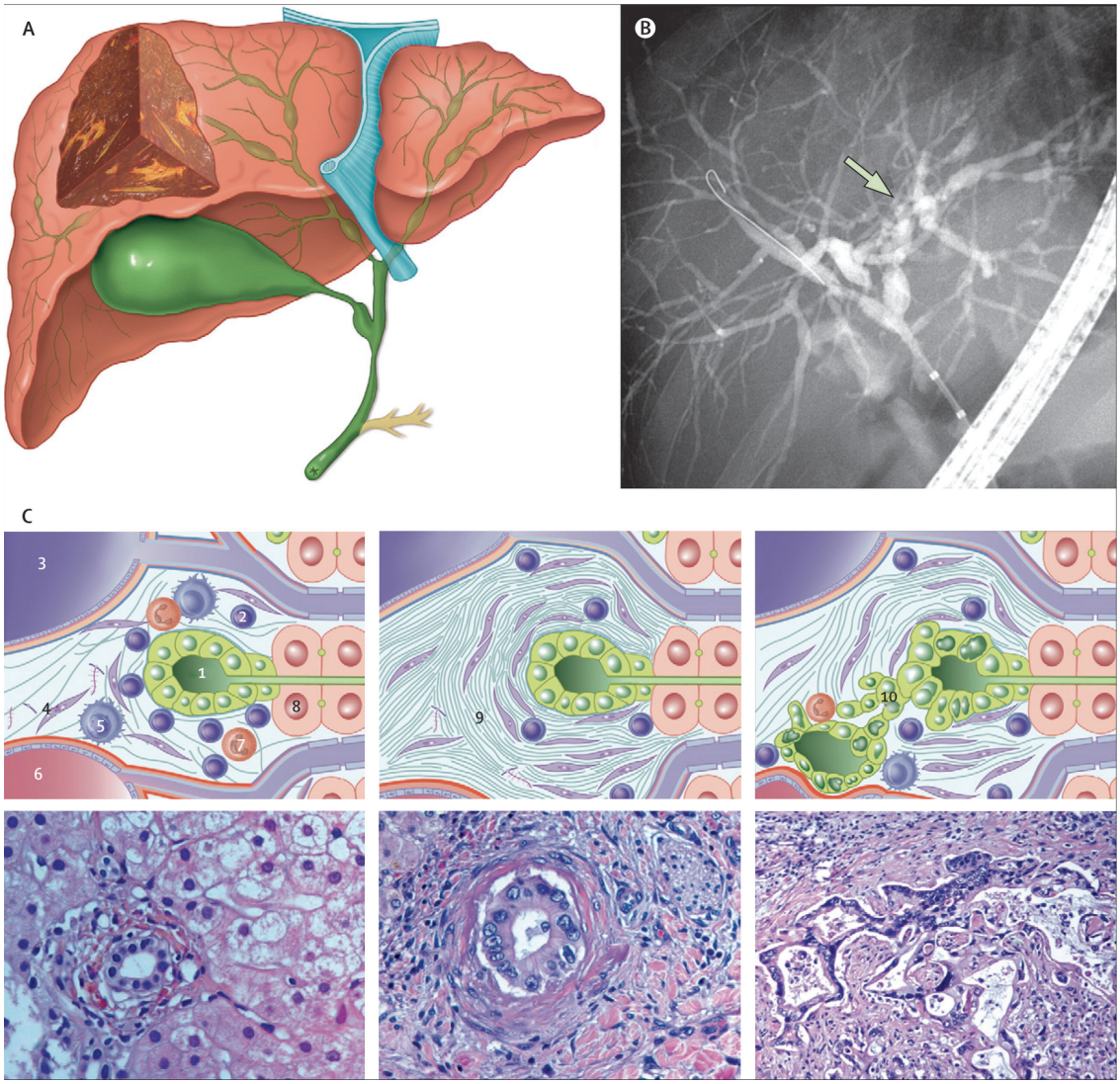



**Figure 2. Pathological themes in PSC. (A)** Diffuse and multifocal short strictures or mural irregularities, alternating with normal or saccular dilatations of both the intra- and extrahepatic bile ducts. The strictures lead to regional cholestasis, patchy affection of peribiliary fibrosis, and ultimately cholestatic liver cirrhosis. **(B)** ERC shows typical features of PSC and a dominant stricture with associated dilatation (green arrow). **(C)** In early PSC, bile ducts show minimum epithelial changes with a few surrounding lymphocytes. The typical lesion (middle) is an obliterative, non-suppurative cholangitis with substantial periductular fibrosis. Dysplastic affection (right) shows severe dysplasia and CCA. CCA, cholangiocarcinoma; ERC, endoscopic retrograde cholangiography; PSC, primary sclerosing cholangitis; 1, bile duct; 2, lymphocyte; 3, portal vein; 4, fibroblast; 5, macrophage; 6, artery; 7, neutrophil; 8, hepatocyte; 9, collagen; 10, cholangiocarcinoma. Reprinted from The Lancet, Hirschfield *et al*, "Primary sclerosing cholangitis".[2] ©(2013), with permission from Elsevier. Reproduced with permission of Kari C Toverud.

An adequate visualisation of the biliary tree by cholangiography is therefore essential (**Figure 2B**). ERC used to be the gold standard for visualisation of the biliary tree, but has now for the most part been replaced by magnetic resonance cholangiography (MRC) as the first choice due to its non-invasive nature and reduced complication risk (**Figure 3**).[14,15,48] ERC is still an essential clinical tool owing to its interventional opportunities. The two methods are equal when it comes to diagnostic accuracy.[48,49]

**Diagnosis of exclusion**. The diagnostic workup in PSC involves excluding secondary causes of sclerosing cholangitis (see **Table 1**, next page).[14,50] One particularly important disease entity is Immunoglobulin (Ig) G4 Associated Cholangitis (IAC), typically characterised by elevated IgG4 levels in the context of autoimmune pancreatitis.[14,15,51,52] There is no consensus on what serum IgG4 cut-off level to use for the diagnosis of IAC, and whether PSC and IAC are actually separate disease entities.[51,53] Nonetheless, it is important to identify patients with IAC, as they may benefit from treatment with corticosteroids.[14,15]

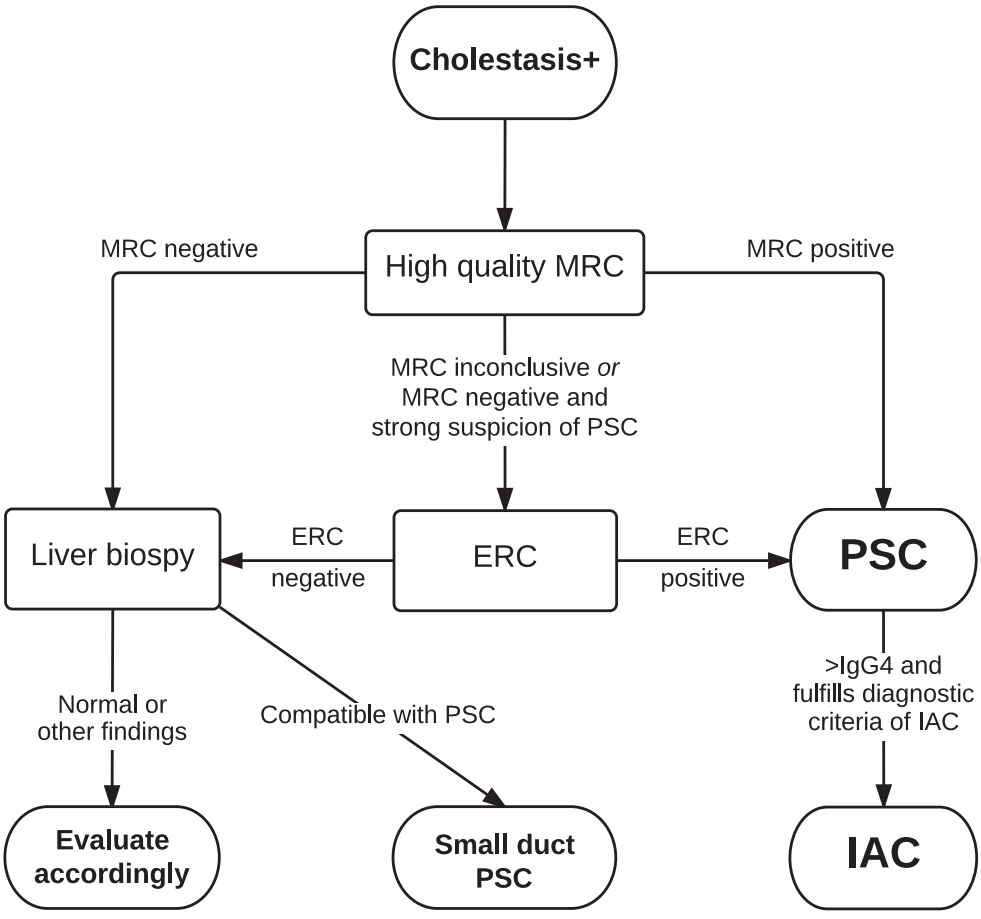



**Figure 3. Flow chart of the diagnostic process in PSC and related cholestatic disorders.** Diagnostic criteria of IAC, see[15]. ERC, endoscopic retrograde cholangiography; IAC, IgG4-associated cholangitis; MRC, magnetic resonance cholangiography; PSC, primary sclerosing cholangitis; >IgG4, elevated IgG4. Adapted from reference[54], © (2013), with permission from Elsevier.

With a cholangiogram indicative of PSC, and exclusion of other secondary causes, a liver biopsy is not required for the diagnosis of PSC, with a few exceptions (**Figure 3**).

**Small duct PSC** is a diagnosis that should be considered in a patient with chronic cholestatic liver disease of unknown aetiology and a normal cholangiogram, but with features suggestive of PSC on histological examination of a liver biopsy.[2,14,55] About 20% of patients with small duct disease will progress to large duct PSC.[2,55] Patients with small duct PSC seem to have a better long term prognosis, and the risk of CCA is minor unless patients progress to large duct PSC.[2,55]

**Overlap syndrome**, a setting where patients show signs of both PSC and AIH at the same time or sequentially, also requires a liver biopsy.[56] 7.4-14% of PSC patients have overlapping features with AIH, and immunosuppressive treatment should be considered for these patients.[56] The International Autoimmune Hepatitis Group has recommended that patients are categorised as AIH or PSC/small duct PSC according to the predominant features of disease, and avoid using scoring systems to establish patient subgroups.[56]

## Table 1. Secondary causes of sclerosing cholangitis

**Mechanical obstruction**
- Choledocholithiasis (+/- infection)
- Polyps
- Pancreatic disease

**Infection**
- Bacterial cholangitis
- Recurrent pyogenic cholangitis

**Immunodeficiency (ID) related**
- Congenital ID
- Acquired ID / AIDS cholangiopathy

**Congenital**
- Caroli's disease
- Cystic fibrosis

**Pancreatic disorder**
- Autoimmune pancreatitis/IAC
- Chronic pancreatitis

**Toxic**
- Intra-arterial chemotherapy

**Ischemic**
- Vascular trauma
- Hepatic allograft arterial occlusion
- Paroxysmal nocturnal hemoglobinuria
- Posttraumatic sclerosing cholangitis

**Others**
- Hepatic inflammatory pseudotumor
- Neoplastic (*e.g.* cholangiocarcinoma)
- Eosinophilic cholangitis
- Metastatic disease
- Portal hypertensive biliopathy
- Sclerosing cholangitis in critically ill patients
- Surgical biliary trauma

ID, immunodeficiency; AIDS, acquired immune deficiency syndrome; IAC, Immunoglobulin G4 associated cholangitis. Table references:[4,14,50].

### 4.1.3 Treatment, follow up and prognosis

**Medical treatment** showing significant effect on 'hard' endpoints, *i.e.* survival or time to transplantation, is not available in PSC.[4] Several immunosuppressive and anti-inflammatory drugs have been investigated, but it has been difficult to show consistent effects.[2] The lack of good disease activity markers makes the evaluation of treatment effects difficult, and an additional challenge in the long term is the low rate of clinically relevant endpoints.[57]

Ursodeoxycholic acid (UDCA), a secondary bile acid produced by gut bacteria, is a main treatment in cholestatic disease.[14,15] It is also used in PSC, but especially high-dose treatment has been a matter of debate, and European and American guidelines diverge in their recommendations at this point.[14,15]

**Procedural treatments** like ERC with endoscopic interventions should be performed on dominant strictures in the bile ducts, as this may improve pruritus, liver biochemistry, jaundice and right upper quadrant pain while reducing the risk of recurrent cholangitis. It can also be combined with sphincterotomy, balloon dilatation or stent placement.[14,15]

**Follow up of** all newly diagnosed patients with PSC should include screening for IBD by colonoscopy with systematic biopsies, if IBD is not already diagnosed.[14,15] There is a 10-fold increased risk of colorectal cancer in PSC patients with UC compared with patients with UC without PSC.[41] As a consequence, it is recommended that colonoscopy be repeated annually for all PSC patients with IBD.[14,15]

The association between PSC and CCA is even more pronounced, with a 160-fold risk increase compared with the general population.[14,58] Patients have a cumulative 10-year CCA incidence of almost 10%, and about half of PSC patients are diagnosed within the first year after diagnosis.[14,15,41,58] With a 5-year survival rate of less than 10%, and the relatively young median age at diagnosis, CCA is one of the most feared complications in PSC.[14,15,41,58] CCA is also notoriously difficult to diagnose accurately in PSC, and there is no reliable diagnostic test capable of detecting CCA at an early stage.[15,59] Screening is therefore not recommended, but ERC with brush cytology should be performed when clinically indicated.[14,15,59]

As for other chronic and progressive liver diseases, PSC in its advanced stage is associated with portal hypertension and oesophageal varices. Endoscopy screening should be performed when patients develop cirrhosis, with appropriate prophylaxis and treatment.[14,15,60] If no varices are detected, endoscopy screening should be repeated every 2-3 years.[60]

**Liver transplantation** is the only treatment option if patients develop end stage liver disease. Other indications for transplantation include impaired quality of life, complications to portal hypertension, concomitant hepatocellular carcinoma and potentially also bile duct strictures and cytological low grade or high grade dysplasia or CCA.[14,15,59,61]

**Prognosis** for PSC patients has proven very difficult to determine, as the clinical course shows large variability.[19] Several models for evaluating prognosis in patients with PSC have been published, and one of the most widely used is the Mayo risk score.[62–64] Age, 3 biochemical parameters (bilirubin, aspartate aminotransferase [AST] and albumin) and whether the patient has ever experienced variceal bleeding is used to calculate the Mayo risk score. However, none of these models, including the Mayo risk score, are recommended as routine in clinical practise,[14,15] as they have limited value in the evaluation of prognosis in the individual patient and are therefore mostly used in clinical research.[19,57]

The overall median survival after diagnosis of PSC is 10–12 years.[4,16] 13-35% of patients with PSC eventually undergo liver transplantation, with a median time from diagnosis of ~6.5 years, although this time seems to have increased in the recent decades.[18,20]

Patients with PSC who undergo liver transplantation have an excellent short term prognosis with one year patient survival exceeding 90%.[19] The long-term prognosis is also good,[20] and survival has increased due to improvement of surgical techniques and generally better care for patients undergoing liver transplantation.[19,21] There is, however, a substantial risk of PSC relapse in the new liver, with estimates varying between a 6-38% recurrence rate.[19,65]

## 4.2 A brief introduction to the gut microbiota

The microbiota is a term used to collectively describe all the microorganisms residing in, or on, a specific area or compartment.[66] Essentially, the term microbiota therefore comprises all bacteria, viruses, archaea and some eukaryotes. *In this thesis the term gut microbiota will generally be used to describe all the bacteria that reside in the gastrointestinal tract of the host in question, excluding e.g. viruses, archaea and eukaryotes, unless otherwise specified.*

The gut microbiota constitutes a vast number of bacteria; in humans it outnumbers the amount of eukaryotic cells in the human body by a factor of 10,[67] and their collective genome (referred to as the microbiome) is almost 500 times larger than the human genome, enabling it to initiate a wide range of metabolic and biochemical activities.[66,68] The gut microbiota is essential for several aspects of human biology including absorption, synthesis and extraction of several metabolites and nutrients, *e.g.* short-chain fatty acids (SCFAs), amino acids and bile acids.[66,69] It facilitates the metabolism of otherwise indigestible polysaccharides and it produces several essential vitamins, especially in the B and K groups.[69] Without the microbiota the development of the intestinal epithelium, the enteric nervous system and the immune system would also be impaired.[66,70] Our ability to protect ourselves against opportunistic pathogens would also diminish, as the microbiota contributes to enhancing barrier fortification, induces IgA production and mediate differentiation, migration and effector functions of cells in the immune system.[66,69,71] The intestinal mucosal immune system is characterised by tolerance to microorganisms rather than responsiveness, and interestingly, so is the liver.[13,72] The relationship between humans and their microbiota is highly mutualistic, and the common concept of 'self' and 'non-self' where most microbes are regarded as pathogens, has been abandoned long ago. This has also led some to state that humans and their bacteria together rather should be regarded as a 'superorganism'.[66] As the 1958 Nobel laureate Joshua Lederberg once wrote; we should "teach war no more".[1]

### 4.2.1 The most common techniques used to study the gut microbiota

Scientists have studied the microbiota since the nineteenth century using cultivation *in vitro*, for a long time the cornerstone of microbial research.[73] These methods are still important for scientists and clinicians, but it turns out that up to 80% of gut bacteria have not been cultured to date.[74,75] New methods using deoxyribonucleic acid (DNA) to identify bacteria in combination with new and cheaper sequencing techniques (often referred to as next-

generation or high-throughput sequencing), among other things, have in combination greatly expanded our ability to survey the microbiota on a large scale, and has led to a surge in published studies on the microbiota in the last decade, illustrated in **Figure 4**.[73]

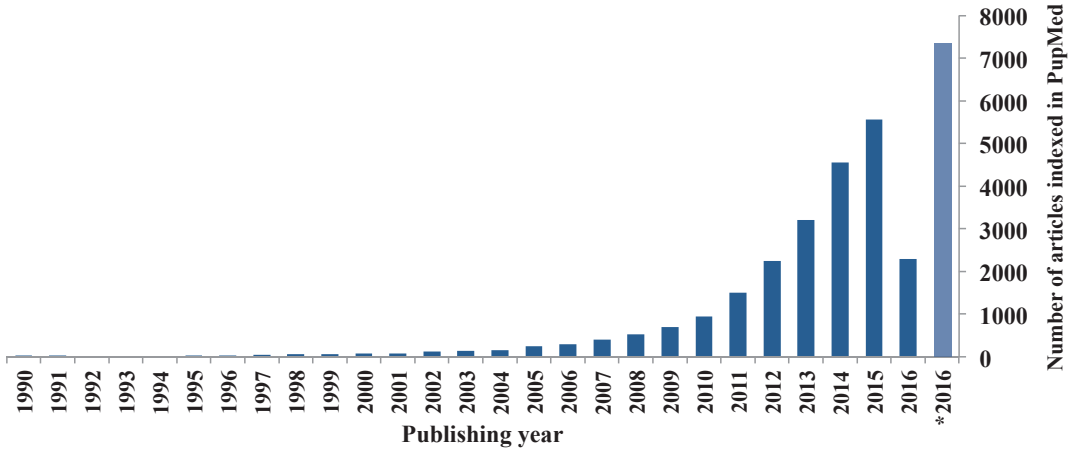



**Figure 4. The 'big-bang' in microbiota research.** Number of articles indexed in PubMed per year from 1990 – 2016 (until 23th of April) matching the search term "microbiota or microbiome". Between 1956 and 1989 the maximum number of indexed articles per year never exceeds 25 (1989). *The estimated publication count for 2016: average daily publication-rate from 1th January – 23th of April multiplied by 366. Data accessed on the 23th of April 2016 from http://www.ncbi.nlm.nih.gov/pubmed/.

There are two main ways of studying the gut microbiota using the bacterial DNA in combination with these new sequencing techniques that have dominated the field, presented simplified below:

**16S rRNA sequencing:** Ribosomes are ancient molecular machines that are responsible for production of proteins in all living cells.[75,76] The ribosomes of all bacteria have a small subunit that contains one ribonucleic acid (RNA) molecule: the 16S ribosomal RNA (16S rRNA).[75] The gene coding for 16S rRNA contains several variable regions that are so *variable between bacteria* that they can be used to identify the bacterial group from which the gene originated, almost like a fingerprint. At the same time these hypervariable regions are flanked by other regions that are highly *conserved between bacteria*.[77,78] In microbiota studies one takes advantage of this by designing genetic primers targeting the conserved regions of this gene, so called 'universal primers'. Then polymerase chain reaction (PCR) is performed on extracted DNA. The PCR amplifies the hypervariable region that the 'universal primers' flank.[75,77] The amplified hypervariable region is then sequenced, and one can use databases trying to identify from which bacterial group the sequence originated.[77] This *provides a compositional overview* of the microbiota.[77,78] This is the method that has been used in the works presented in this thesis (Paper I and III).

**Whole genome sequencing (WGS)**, also referred to as shotgun sequencing, is used to sequence all DNA fragments present in a sample, instead of one small part of the DNA, as in 16S rRNA sequencing.[75,79] Since universal 16S rRNA primers are not used in WGS, one must filter DNA sequences originating from other species (*e.g.* humans) after sequencing.[79] The method is laborious and requires considerable computational recourses. Also, WGS is far more expensive than 16S-based methods. However, it has a few advantages: since all DNA in the sample are sequenced, it is possible to predict *the functional contents* of the bacteria in the samples, and 16S primer bias is avoided.[75,79–81]

### 4.2.2 The basic terms used to describe the microbiota

**Phylogenetics** is the study of the evolution and relationship of individuals or groups of organisms, and **taxonomy** is the classification and naming of organisms. All living organisms can be hierarchically classified into eight major taxonomic levels where *domain* is the most general and *species* the most specific, illustrated in **Figure 5**.[77]

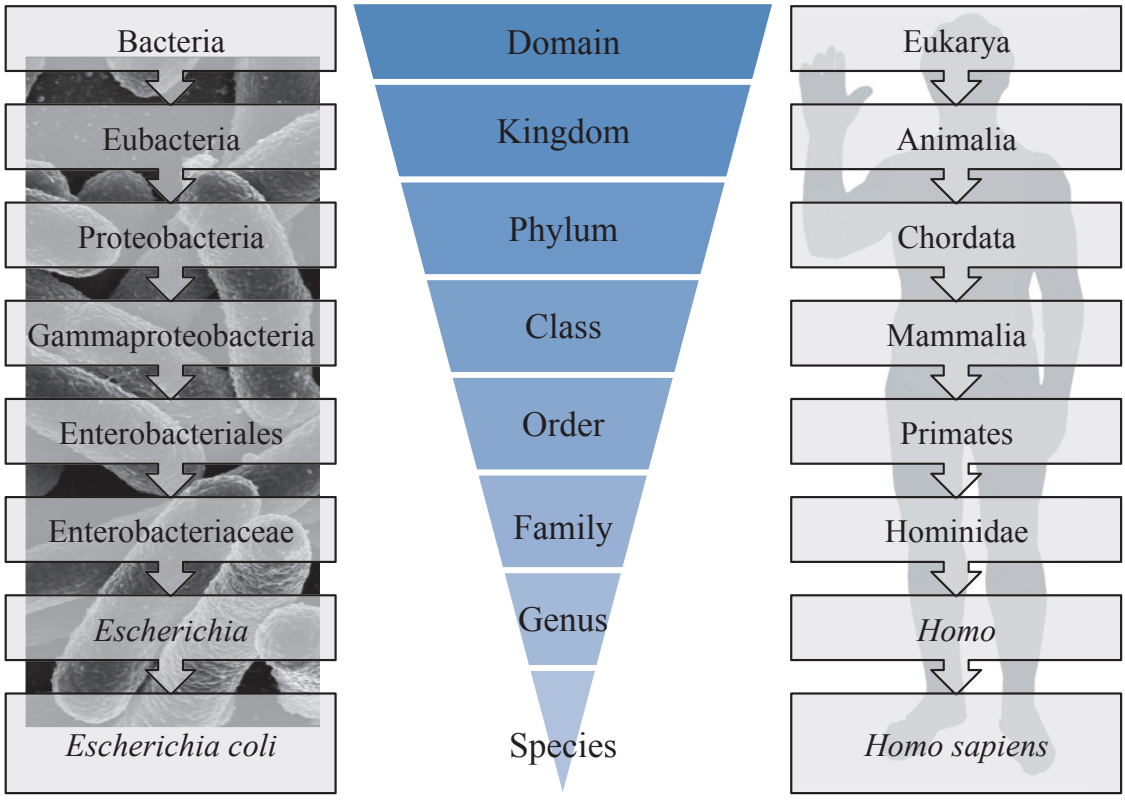



**Figure 5. Taxonomic levels.** Illustrating the hierarchical organisation of taxonomic levels used for classification of different organisms. The bacteria domain is given on the left hand side, and for comparison the taxonomic classification of humans (*Homo sapiens*) is given on the right. Pictures used in the figure are licenced under the Creative Commons Zero licence. The figure is inspired by Tyler *et al.*[77]

**Alpha diversity** (α-diversity) describes the *intra-individual bacterial diversity*.[82] The total α-diversity takes richness and evenness of bacteria into account (**Figure 6, left panel**). There are several different methods for calculating richness, evenness and combinations of the two.[83]

**Beta diversity** (β-diversity) describes the *inter-individual diversity*. Crudely β-diversity tells us something about how different/equal the total bacterial community in different samples are (**Figure 6, right panel**).[82] There are different methods for calculating β-diversity, but UniFrac phylogenetic distance and Bray-Curtis dissimilarity are among the most frequently used.[84]

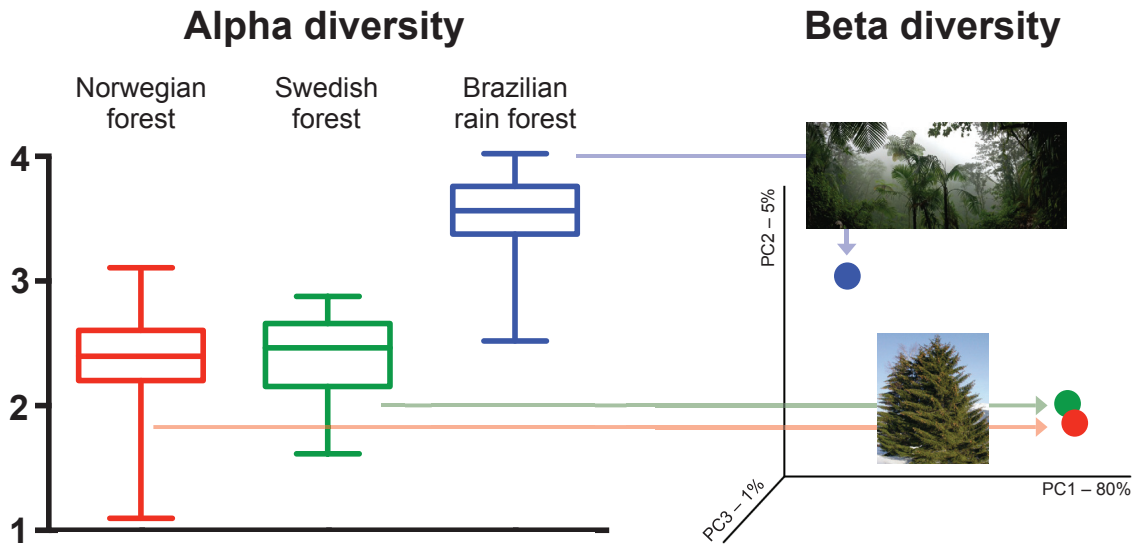



**Figure 6.** Illustrating a (very) simplified explanation of alpha and beta diversity. In this example we will use plant species in forests, comparable to bacterial species in the intestines.

**Alpha (α) diversity:** In most Norwegian and Swedish forests the Norway spruce (*Picea abies*) is the dominating species. One species dominating the ecosystem in this way, resulting in an uneven distribution of species, results in lower *evenness*. This might be compared to the gut microbiota in *Clostridium difficile* colitis. The Brazilian rain forest contains a plethora of different plants, and consequently high plant *richness*, probably higher than its Nordic counterparts. Lets imagine that all the plants in the Brazilian rain forest are also quite equally represented, resulting in great *evenness* as well. Thus; since the Brazilian rain forest shows increased *richness* and *evenness* compared with Norwegian and Swedish forests, it also has greater total α-diversity.

**Beta (β) diversity:** In a principle coordinate plot the total bacterial community of each sample is represented by one circle, and the distance between samples can be interpreted as dissimilarity. Because Norwegian and Swedish forests (in red/green to the right in the plot above) are so equal they cluster together. Since hardly any plant species in the rain forest are found in the Nordic countries it clusters away from these sites. Number on the axis denotes the percentage of the variation in the bacterial community that can be explained by each axis. For simplicity only one sample per forest is used in the plot. Pictures used in the figure are licenced under the Creative Commons Zero licence.

### 4.2.3 The gut microbiota in health and disease

Changes in the gut microbiota have lately been implicated in the pathogenesis of several metabolic, autoimmune, and inflammatory conditions, including gastrointestinal disorders like IBD, but also systemic disorders like diabetes, rheumatoid arthritis, atherosclerosis and obesity.[77,85–90] Changes in both α- and β-diversity, and differences in composition and function of the microbiota between disease groups and healthy controls are often detected.[91] In IBD, both UC and Crohn's disease (CD) have gut microbial profiles which are different from each other, and from healthy controls.[87,88,92] To what degree the changes that are observed are a direct cause of disease, a driver of disease, or just an aggravating bystander is not yet known. There are, however, instances where changes in the microbiota can at least precede the clinical onset of disease.[86,92,93]

### 4.2.4 The gut metabolome in health and disease

The metabolic capacity of the microbiota equals that of the liver, and all together this has given rise to the notion that the gut microbiota cold be regarded as an additional organ, or the 'forgotten organ' as some have put it, which could produce as much as 10% of the detectable compounds in blood.[66,69,71,94]

It is likely that microbial metabolites influence human disease development as well, via *e.g.* the gut-liver axis.[69] In an effort to discover mechanisms that link changes in the composition and function of the gut microbiota to disease development and progression, it has been important to explore metabolites produced by the microbiota (called the intestinal metabolome) and how they may affect human health and disease, in addition to the bacteria themselves.[94]

One example of such a metabolite is trimethylamine-*N*-oxide (TMAO).[95–98] TMAO is a metabolite produced in the liver by flavin-containing monooxygenase (FMO) enzymes from it precursor trimethylamine (TMA), a completely microbiota-dependent volatile gas. Humans cannot produce TMA, but gut bacteria generate TMA from phosphatidylcholine, l-carnitine or gamma-butyrobetaine that humans obtain from dietary sources.[95–98] TMAO has so far been associated with cardiovascular and kidney disease.[95–97,99] TMAO has further been shown to influence cholesterol metabolism, bile composition and lowering of key bile acid synthesis- and transport-proteins, all with a potential role in regulation of inflammation and hepatic metabolic pathways, as well as fibrosis.[89,95,96,100]

## 4.3 Animals in the study of PSC and the microbiota

There has been much progress in the modelling of complex diseases like PSC through methodological advances in recent decades. Despite this, there is no adequate, well characterised and reproducible animal model for PSC so far.[4,101,102] This makes dissecting and understanding the underlying mechanisms in PSC challenging, and it also makes the testing of new treatment modalities more demanding. Although we lack the 'ideal animal model', several models can elucidate certain aspects of the pathology observed in PSC patients:

**Cholestasis** and biliary obstruction are central pathological aspects of PSC disease. Cholestasis can be induced in animal models by obstructing the bile ducts with complete or partial bile duct ligation.[101,103] These models also develop fibrosis, but have several technical challenges.[102] Of note, cholestasis and biliary obstruction are also observed in other diseases *e.g.* PBC, and thus not specific to PSC.

**Cholangitis and biliary inflammation** can be induced by chemical substances, *e.g.* by feeding mice lithocholic acid (LCA), a bile acid that is produced by the gut microbiota.[102] This model also shows signs of segmental bile duct obstruction.[104] Nonobese diabetic (NOD) mice develop diabetes. NOD.c3c4 mice are developed on a NOD background, but do not develop diabetes.[105] Instead they spontaneously develop biliary inflammation in the intra- and extra-hepatic bile ducts.[102] This mouse model has been used in Paper III in this thesis.

**Fibrosis** is another hallmark of PSC, and is seen in several models, including the bile duct ligation models described above.[101,102] All of the most commonly used knockout mouse models for PSC exhibit signs of fibrosis, including the multidrug resistance 2 knockout (*Mdr2*$^{-/-}$) mouse model.[102,106] When the *mdr2*-gene is knocked out of the mice genome it results in an inability to transport phospholipids into the bile. This makes the bile toxic and result in periductal inflammation, fibrosis and cholangitis. Humans have a genetic ortholog to *mdr2* in its genome called *ABCB4* (*MDR3*), which is involved in cholestasis and a wide spectrum of liver diseases, but not PSC.[106,107]

All the models presented above lack the concomitant bowel inflammation seen in most PSC patients. One solution to this limitation is to experimentally induce colitis with dextran sulphate sodium.[2,101,102]

**The use of gnotobiotic mice** is a powerful tool when it comes to investigating the role of microbiota in different models. It has been used for decades,[108] and involves the rearing of animals in a totally germ free (GF) environment, or where the bacterial environment is known. The ability to investigate how disease models evolve without the presence of bacteria,[109,110] when introducing one or a combination of several specific bacteria,[111–113] or even introduce a human flora from patients or healthy individuals,[114] has greatly advanced our knowledge of the gut microbiota in both health and disease, as well as its role in treatment of *e.g.* heart disease and cancer. [115–117]

## 4.4 PSC aetiology and pathogenesis – Part I: What we know

Several secondary causes of cholangitis, like ischemia, infections, toxins and inheritable genetic disorders give rise to both radiological and histological pictures that resemble PSC (see also **Table 1**).[2] This suggests common pathways for injury to the bile ducts. It could also be speculated that what we observe as PSC today, is a clinical picture that actually arises from several different diseases with potentially different aetiologies, but with shared pathogenesis; a 'final common pathway' of biliary injury.[37] The acknowledgment in recent decades of disease entities like IAC also supports this possibility.

### Clinical features

In addition to the clinical features we have mentioned earlier, PSC is characterised by progressive and chronic injury in the small, medium and large bile ducts.[2,37] This obstructs the flow of bile and leads to secondary inflammation with infiltration of lymphocytes, plasma cells, and neutrophils, usually more intense around the bile ducts (see **Figure 2**).[2,37] Cholangiocytes, the epithelial cells lining the bile ducts, react to this injury with upregulation of inflammatory cytokines and adhesion molecules, which contribute to the fibrotic and inflammatory response.[118] This process results in the destruction and loss of bile ducts in parallel with apoptosis, fibrosis and inflammation – a classical 'tragic triad' of several progressive fibrotic diseases, but interestingly these processes are not necessarily closely associated in severity.[2,37] In parallel there is a proliferation of new bile ducts, but these are disorganised resulting in disruption of the otherwise microscopically stringent liver architecture and cirrhosis, ultimately leading to liver failure.[119]

**Genetics**

Family members of patients with PSC have an increased risk of developing the disease themselves.[120] In line with this, case-control studies have identified several specific alterations in the genome (so called single nucleotide polymorphisms [SNPs]) associated with PSC.[121–123] This has established a role for genetics in PSC pathogenesis. Several of the implicated genes are related to the immune system, and accordingly a majority of the genetic risk-loci are also associated with other autoimmune diseases.[5] The interpretation of possible biological implications of risk genes is not straight forward, but these immune-related loci could play an important role in the immune dysregulation often suggested in autoimmune diseases, including PSC.[5,124,125] A total of 20 genetic variations associated with an increased risk of PSC have been reported, and PSC is generally considered a complex genetic disease.[5,123] In this context it is also important to realise that genetics probably also influence the gut microbiota.[126] It is therefore intriguing that one of the genes shown to increase PSC susceptibility, *FUT2*, has been associated with changes in the microbiota in the bile of patients with PSC.[122] Although the list of genetic risk loci might expand in the future, these genes collectively account for less than 10% of the estimated susceptibility to PSC, implying a considerable role for environmental factors in disease development, *e.g.* the gut microbiota.[5,6,123]

**Environment**

Knowing that our genes collectively account for a minor part of the estimated susceptibility to PSC, it might come as a surprise that there are only two established environmental risk factors for PSC: coffee and cigarettes.[7–10,12] Both of them are protective against PSC, but the mechanisms are unknown. Increased bile flow as an effect of coffee has been proposed, but data generally point to a more liver specific health benefit, and not one specific to PSC.[7,127] It has also been suggested that this could be explained by the impact of coffee on the gut microbiota.[128] In this regard it should be noted that in microbiota studies, coffee intake has been associated with increased α-diversity, which again is often associated with a 'healthy' gut microbiota.[129,130] The positive effect of smoking is more elusive. The protective effect observed in PSC is in stark contrast to the health effects of smoking in general.[131,132] One exception is Parkinson's disease where both cigarettes and coffee also have been shown to be protective.[133] Interestingly, a proposed hypothesis for this positive effect, states that cigarettes and coffee changes the composition of the gut microbiota in a way that mitigates intestinal inflammation.[133]

## 4.5 PSC aetiology and pathogenesis – Part II: The hypotheses

Several hypotheses have been proposed throughout the years. One of them is probably not right in the sense that all the others are wrong, and they are by no means mutually exclusive. They are rather partly overlapping, and are all good descriptions of what could potentially be parts of the disease process in PSC. I will start by giving some short background information, before elaborating more on the individual main hypotheses, focusing on the gut microbiota.

The blood from the intestinal circulation drains into the liver via the portal circulation (**Figure 1**), making the liver a kind of 'firewall' that protects the body from possible harmful pathogens and digested substances.[13] Bile acids are secreted from the liver, via the bile ducts into the intestines. In the intestines, primary bile acids are metabolised by members of the microbiota into secondary bile acids. Bile acids are then reabsorbed in the ileum. This enterohepatic circulation is effective, with 95% of bile acids being reabsorbed and returned to the system circulation.[134] Bile acids facilitate the absorption of *e.g.* fat and fat-soluble vitamins. In addition, they are highly active signalling molecules and inflammatory agents that are able to activate signalling pathways regulating several physiological functions, ranging from lipid homeostasis to fibrosis.[134]

The epithelial cells that line the bile ducts are continuous with the epithelial cells of the intestines. The connection between PSC and colitis is consequently thought to be central to disease pathogenesis.[2] The clinical association between PSC and IBD is obvious, with as much as 80% of patients affected.[36] Conversely, 2.3%-4.6% of patients with UC are diagnosed with PSC, although there are data indicating that the true prevalence could be higher.[28,30,135] The prevalence of PSC in CD patients is lower, with estimates varying between 1.2% and 3.6%.[30] In consequence, PSC is considered the most important hepatobiliary disorder associated with IBD. There are, however, several characteristics that differ between IBD without liver disease, and the IBD we observe in patients with PSC. IBD in PSC is characterised by mild inflammation, predominantly on the right side (colon ascendens), with rectal sparing and backwash ileitis, and a mild to moderate clinical course.[2,35,36,136,137] Also genetically, the overlap between PSC and IBD is far from complete. Less than half of the IBD associated genetic loci are also associated with PSC.[5] Overall, these observations have led to the hypothesis that PSC-IBD might actually constitute a distinct disease entity altogether.[2,136,138,139]

### 4.5.1 The autoimmunity hypothesis

PSC could be considered to be an immune-mediated, rather than a classical autoimmune disease, for several reasons, even though the distinction is not clear-cut.[4,46]

First, there is no proof of a disease-specific autoantibody like anti-transglutaminase 2 (anti-TG2) in coeliac disease and the antimitochondrial antibody directed against dihydrolipoyl transacetylase (pyruvate dehydrogenase complex [PDC] E2) in PBC.[125,140] Second, there is a predominance of male patients in PSC, in contrast to the female predominance seen in most autoimmune diseases. Lastly, immunosuppressive treatment has not been successful in treating PSC patients.[2] However, a large number of auto-antibodies have been identified in PSC, *e.g.* p-ANCA, antibodies against biliary epithelial cells (BECs) and antinuclear antibodies (ANA).[46] None of these are neither very sensitive nor specific, and could reflect a more nonspecific dysregulation of the immune system in patients with PSC, as also suggested by genetic studies.[5,46]

The striking north-south risk gradient for PSC and other autoimmune disorders including IBD, has led to speculations as to whether vitamin D could explain parts of the association between sun light exposure and risk of autoimmune diseases.[2,38] Although clearly not being solely responsible for this association, vitamin D has immunomodulatory and anti-inflammatory effects in autoimmune liver disease including PSC, and constitutes a key regulator in liver fibrosis [39,141,142] Moreover, the vitamin D receptor appears to be important in detoxification of bile acids produced by the gut microbiota, thereby protecting the gut from bile acid toxicity, and secondary bile acids constitute key ligands for the vitamin D receptor and other nuclear receptors essential in bile acid metabolism.[143–145]

In support of the autoimmunity hypothesis, patients with PSC also have an increased risk of having concomitant autoimmune disease, and so have their first-degree relatives.[46,120] By far the strongest genetic association in PSC is found within the human leukocyte antigen (*HLA)* gene, which supports an autoimmune component in the pathogenesis.[37] The association is so complex that the *HLA* gene could be considered an immunologic ‘mini-genome’ that we so far are not able to fully comprehend.[5] Furthermore, the portal tracts in PSC are infiltrated by T-cells, with predominant use of a specific T-cell receptor (*TCR*) gene in the liver tissue of PSC patients.[146] This could indicate the presence of a specific antigen in PSC. An antigen could be exogenous, *e.g.* of bacterial or dietary origin, or endogenous. It is also possible that

the antigen is not solely responsible for the immunological reaction, and that *e.g.* an auto-antigen needs a cofactor in a hapten-carrier-like manner, as suggested for gluten and anti-TG2 in coeliac disease.[125] However, no such antigen has been found in PSC and immunosuppressive treatment has so far been unable to slow the progression of disease. Thus one could speculate that there might be agents with a more direct toxic and damaging effect on the bile ducts in PSC, and that immunological mechanisms might play a secondary, though no less important, role in the disease process.

Taken together, this alludes to an important role for the immune system in PSC pathogenesis, where genetic, dietary factors (*e.g.* vitamin D) and the gut microbiota and its metabolites, could also play an important part.

### 4.5.2 The 'toxic bile' hypothesis

The 'toxic bile' hypothesis suggests that an altered ('toxic') bile composition along with defective protection systems in the biliary epithelium could contribute to bile duct injury and cholangitis.[37]

The cholangitis phenotype due to toxic bile observed in mice and humans with defects in the phospholipid transporter ABCB4/MDR3, supports this hypothesis.[106,107] Bile acids are toxic to hepatocytes, and pharmacological manipulation of the bile acid pool with the use of UDCA has been shown to reduce cholestasis in various cholestatic disorders, and is considered standard treatment in PBC.[14,15,37]

Changing bile acid composition by inhibiting the reabsorption of bile acids in the terminal ileum has also been shown to decrease bile toxicity, and reduce progression of sclerosing cholangitis in the *Mdr2*$^{-/-}$ mouse model, in addition to decreased fibrosis and upregulation of anti-inflammatory genes.[147] In cystic fibrosis, the loss of the cystic fibrosis transmembrane conductance regulator in the biliary epithelium leads to modulation of alkalinisation and dilution of the bile, resulting in reduced bile flow, bile duct plugging and cholangiocyte injury that in some cases leads to secondary sclerosing cholangitis (**Table 1**).[148]

Bile acids can also activate endothelial cells with an increase in adhesion molecule expression and recruitment of inflammatory immune cells.[149] As the microbiota is a focal point of human bile acid metabolism with its production of secondary bile acids, this further implicates the microbiota in this hypothesis of PSC pathogenesis.[150]

### 4.5.3 The 'aberrant homing' hypothesis

A range of cells from the immune system resides in the gut, *e.g.* T-cells, IgA-producing B-cells, macrophages and dendritic cells (DCs). DCs in gut-associated lymphoid tissue (GALT) have elongated cellular protrusions that can detect bacteria in the gut lumen directly or indirectly by means of gut epithelial cells known as microfold cells (M cells).[151]

Lymphocyte recruitment to the gut is facilitated by adhesion molecules like mucosal vascular addressin cell-adhesion molecule 1 (MAdCAM-1) and CC-chemokine ligand 25 (CCL25).[151] Data indicate that these molecules are only expressed in the gut under normal circumstances.[151] The DCs and M-cells can also induce lymphocyte expression of CC-chemokine receptor 9 (CCR9) and $\alpha_4\beta_7$–integrin, with their ligands MAdCAM-1 and CCL25, respectively.[72,151,152] This makes it possible for the immune system to promote gut homing of specific 'gut primed' subsets of lymphocytes.

However, in patients with PSC, MAdCAM-1 and CCL25 are also expressed in hepatic tissue, permitting an 'aberrant homing of gut primed lymphocytes' to the liver.[151–153] In line with this, there is enhanced recruitment of lymphocytes to the liver in PSC, facilitated by increased expression of adhesion- and transendothelial migration-molecules like vascular adhesion protein-1 (VAP-1).[152] How this expression of MAdCAM-1 and CCL25 happens, is not well understood. PSC patients show increased expression of VAP-1 during intestinal inflammation, and colitis induces CCL25 expression that correlates with colitis severity and mucosal expression of tumor necrosis factor α (TNFα).[151,154] Overall this has led to the hypothesis that this 'aberrant homing' could be an important driver for liver inflammation in a setting with IBD.

It has also been proposed that products of VAP-1 deamination of dietary amines can lead to expression of MAdCAM-1 by activating nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB).[155] Interestingly, such amines can also be produced by the gut microbiota during metabolic processing of food, and several commensal members of the gut microbiota are known to secrete amines.[89,96,152] This further implicates diet, gut microbiota and microbial metabolites in this hypothesis of PSC pathogenesis.

### 4.5.4 The 'leaky gut' hypothesis

Together with commensal bacteria and a mucus layer, the intestinal epithelial barrier constitutes a 'first line of defence' in the gut.[152] However, the intestinal epithelium is not just a wall towards the outer world, it actively collaborates with the immune system through the expression of pattern recognising receptors (PRRs) and toll-like receptors (TLRs) that react to pathogen-associated molecular patterns (PAMPs), signs of cellular injury and damage.[156] Fluids and dietary substances like nutrients, vitamins, *etc.* will pass through the mucosa, and enter the portal circulation or the lymphatic system before they enter the liver (anatomy illustrated in **Figure 1**). However, even in normal situations, antigens from the gut microbiota will also enter the portal circulation and be presented to the immune system of the liver.[151] In this way the liver constitutes a second line of defence, as it is constantly exposed to microbial antigens.[13] This can possibly explain why the liver also has a certain propensity to microbial tolerance rather than responsiveness, making the liver able to mediate a 'proportionate response' rather than causing a full scale activation of a systemic immune response.[13,72,151,152]

In a setting with chronic intestinal inflammation, like in IBD, the integrity of the epithelial barrier function is compromised, resulting in increased intestinal permeability.[156] This results in a situation with increased hepatic exposure to molecules and metabolites originating from the gut and the gut microbiota.[157] In murine models such exposure has been shown to cause biliary changes resembling PSC, with biliary inflammation, infiltration of mononuclear cells and cholangiopathy.[158] BECs are also able to react to bacterial molecules through TLRs and myeloid differentiation primary response gene 88 (MyD88) and can actively participate in recruitment of lymphocytes to the liver.[152,159] They are thus able to participate directly in hepatic inflammatory and fibrotic processes. BECs can also induce inflammation through a range of signalling molecules.[152] It is therefore possible that in a setting with increased hepatic exposure to both commensal bacteria and bacterially derived molecules through the portal circulation, these protective features of the liver are overwhelmed, with a corresponding 'un-proportionate response' to gut microbiota-associated antigens that could become drivers of hepatic inflammation, or that the immune system in PSC patients are more prone to such non-proportionate responses due to an underlying dysregulation of the immune system.[152] Another possibility is that patients with PSC are more vulnerable to exposure from such gut-derived molecules,[160] or that they elicit aberrant immune responses in some patiens.[161]

Another interesting clinical observation is that while up to 37% of patients suffer recurrent PSC in the new liver after liver transplantation, removal of the colon at or before transplantation, substantially reduces the risk of recurrence,[65,162] indicating that a gut-related process could be essential to recurrent PSC.

That changes in the gut microbiota by itself can induce liver and biliary disease, has been shown in murine models: Lichtman *et al.* induced bacterial overgrowth in the small bowel of rats, and subsequently observed development of hepatic and biliary inflammation, together with bile duct proliferation and destruction, as well as fibrosis around the portal tracts.[163,164] When examined with cholangiography, the rats showed extra-hepatic bile duct dilatations and ectasia of a 'beaded' pattern, archetypical of PSC in humans, and these changes were ameliorated with metronidazole treatment.[47,163,164]

Early treatment trials in PSC used antibiotics, and it was believed that PSC was caused by portal bacteraemia due to bowel inflammation.[165] Some studies have continued this tradition, both with metronidazole, vancomycin and combinations with UDCA. They show improvement of biochemical liver tests, but effect on hard endpoints like time to liver transplantation or death, is lacking.[166–168] Nevertheless, this illustrates that the gut microbiota could be important in PSC pathogenesis, and also that manipulating the gut microbiota could affect disease activity in PSC.

In the last few years, a few human studies exploring the role of the gut microbiota in PSC have been published. They all investigating the microbiota in mucosal biopsies.[169–172] The sample-sizes were quite small, and the number of PSC patients available for comparison in one single study did not exceed 20. This probably contributed to the modest result-overlap between the repoted results.

In conclusion; several of the governing hypotheses, as well as an increasing amount of scientific evidence, point to a possible role of the gut microbiota in the pathogenesis of PSC. This could be elucidated through surveys of microbial composition, function and metabolites in clinical studies, as well as mechanistic studies of more specific components of PSC disease.

# 5 Aims

The overall aim of this thesis is to explore the role of the gut microbiota in PSC. This could potentially elucidate disease aetiology, pathogenesis and possible drivers of disease progression. An increased understanding of these disease elements could also reveal new disease biomarkers, potential intervention and treatment targets and better disease definitions, ultimately leading to better care for our patients.

Data from a range of mechanistic and clinical studies allude to the gut microbiota as a potential key player in PSC disease development, reflected by its potential involvement in several of the dominating hypotheses regarding PSC pathogenesis. We therefore presume that the microbiota is changed in PSC and could affect disease development, and sought to explore this by three separate approaches:

1. Characterise the human gut microbiota in PSC using stool samples (**Paper I**), and secondly:
    a. Study whether, and in what way, the microbiota in PSC is distinct from healthy controls and patients with UC (Paper I).
    b. Determine the effect of IBD-status on the microbiota in PSC (Paper I).

2. Assess the role of trimethylamine-*N*-oxide (TMAO), a microbiota-dependent metabolite, in PSC and explore associations between TMAO and disease progression (**Paper II**).

3. Characterise the luminal and mucosal-adherent microbiota in a murine model with spontaneous biliary inflammation (the NOD.c3c4 strain), and explore how the microbiota affects the biliary phenotype of these mice by comparing conventionally raised and axenic mice (**Paper III**).

# 6 Methodological considerations

Microbiota research in terms of large-scale surveys using high-throughput sequencing, must still be considered an immature field. There are few widely accepted community standards, making some methodological choices particularly challenging.[78] General priorities have therefore been the increase of sample size and the use of standard protocols where possible to facilitate comparison with other studies in the future.

## 6.1 Study design

In Paper I and II we collected samples in a cross-sectional manner. This permits comparison of the different groups, but we are unable to discern whether the changes we detect occur before or after disease onset. With long follow-up time one can, however, relate measurements at sampling to prognosis, as in Paper II.

In Paper I the cross-sectional sampling secured a narrow collection time-span, as seasonal variation is a known potential confounder in microbiota research.[173] Diet is probably the most important driver for this association, but seasonal variation in access to different foods is probably less important in Norway.[173] 85.9% of the samples in Paper I were collected between ultimo May and primo September.

Microbiota studies often involve hundreds of statistical comparisons between study groups. This results in an increased probability of making a type I error (concluding that groups are different when they are not). To address this, in addition to the statistical tools described later, we choose to use a two-panel study design in Paper I; we randomised samples in the primary analyses (PSC versus healthy controls) into either an exploration panel, or a validation panel, and only results that were significant in both panels were reported.

Studies comparing microbial communities of mice, like the work presented in Paper III, are prone to systematic errors like cage effects and community drift independent of genetic background.[78,174] To address this problem, all mice were rederived into a new minimal disease unit (MDU) at the animal facility by cesarean section after the first round of microbiota analyses, and then all microbiota-experiments were repeated after three generations to see if results were reproducible.

## 6.2 Participants

Ideal study groups in a cross-sectional study of humans are equal in all aspects apart from the grouping variable. In an effort to approach this goal case-control-matching with regard to age, sex, medication use *etc.* is often used. There are, however, several challenges related to the establishment of such study protocols. There are also practical considerations that in the end must be weighed against a feasible number of participants. In Paper I and II sample size was prioritised, but some key aspects should be scrutinised during the analyses, *e.g.* age and body mass index (BMI).

**Patients with PSC** were recruited from the Norwegian PSC Research Center (NoPSC) biobank at Oslo University Hospital Rikshospitalet, a tertiary care centre. The diagnosis of PSC was made according to clinical guidelines and typical findings on cholangiography or liver biopsy.[15] All PSC patients included in the analyses had undergone screening for IBD, and diagnosis was based on colonoscopy, histology, and accepted criteria.[175] Time of diagnosis was determined by the first pathological cholangiography, and the duration of PSC was defined as the time from diagnosis to the date of stool or serum sampling in Paper I and II, respectively. Time to event in Paper II was defined as the time from first available serum sample to death or liver transplantation.

In Paper I all non-transplanted patients registred in the NoPSC patient registry at the study start were invited to participate, and all that responded positively were sent sampling equipment. It is therefore possible that our samples could be biased by *e.g.* disease severity. For Paper II all PSC patients included in the NoPSC biobank in the period from 1992-2012 were included in the study, if serum samples were available. If more than one serum sample was available, the oldest sample was included.

**Patient controls with UC** were diagnosed using the same criteria as for the diagnosis of IBD in PSC.[175] In Paper I we recruited patients in an outpatient setting from Oslo University Hospital Ullevål (Oslo, Norway), a secondary care centre, and we only included patients without a medical history of liver disease or unexplained elevation of liver tests. Only patients in clinical remission, as evaluated by the hospital staff, were included. For Paper II serum was collected from a population-based, thoroughly characterised Norwegian IBD-cohort.[176] Since we did not perform screening of the UC patients included in Paper I and II, we cannot rule out the possibility that some suffered from undiagnosed PSC or other liver

diseases. Screening with MRC (or ERC, see **Figure 3**) is the only option to accurately diagnose bile duct disease in these patients. The prevalence of PSC in UC is reported to be 2.3%-4.6%.[28,30,135] In a 20-year follow up of Norwegian IBD patients, large duct PSC was observed in 7.4%, out of which 2.9% were known cases.[177] To not screen UC controls with MRC could therefore be considered a reasonable trade-off, given the apparently low over-all prevalence of PSC in UC.

**Healthy controls** were recruited from the national Norwegian Bone Marrow Donor Registry (Oslo, Norway), and for Paper I these were randomly selected. The use of bone marrow donors as controls have several advantages, as they are screened for several diseases, and are generally considered healthy. They are also relatively young as a group, reducing the chance of unidentified comorbidities, but since they were not screened at study inclusion, there is a possibility that some might have undiagnosed PSC. This problem should be minor, considering the low prevalence of PSC in the general population.[178]

## 6.3 Animal models, housing and logistics

### 6.3.1 NOD.c3c4 and control NOD mice

For the first part of Paper III we used conventionally raised (CONV-R) NOD.c3c4 and NOD mice from The Jackson Laboratory (Bar Harbor, ME). Mice were housed in a MDU at the animal facility at Oslo University Hospital Rikshospitalet, Oslo, Norway. CONV-R NOD.c3c4 and NOD control mice were then rederived into to a new MDU at the same animal facility, and all experiments were repeated. Lastly, CONV-R NOD.c3c4 from this last MDU were rederived into a GF facility.

There is no ideal, standard mouse model for PSC, as elaborated on earlier, all with several limitations.[102] There were three main arguments for using the NOD.c3c4 model in Paper III: (1) It develops a largely immune-driven disease, with a central role of T-cells, and could thus have some advantages when investigating host-microbiota interactions, as opposed to more toxic and acute models of biliary disease.[105] (2) It shows an autoimmune phenotype on a polygenic background, and thus it reflects aspects of PSC disease suggested by genetic studies. This could be considered an advantage over other induced or toxic models of PSC. (3) It is the only known mouse model that spontaneously develops dilatation and inflammation of the common bile duct that is not secondary to infectious agents.[102,179]

### 6.3.2 Germ free (GF) mice

CONV-R NOD.c3c4 were rederived into a GF environment at the Core Facility for Germfree Research at Karolinska Institutet (Stockholm, Sweden) by caesarean sections. To detect potential contamination of the mice, regular monitoring of GF status was performed. GF NOD mice were courteously provided by collaborators at the GF facility at the University of Gothenburg (Sweden) kept under similar conditions as the GF NOD.c3c4 mice in Stockholm. While GF NOD mice from Gothenburg were sampled on site, the GF NOD.c3c4 mice were shipped to Oslo for sampling in sterile containers, as there was no gnotobiotic facility in Norway at the time we conducted the study. It is probably unlikely that this trip alone could give rise to histological or microscopic changes in the organs sampled, or changes to body- or liver-weight. However, although unlikely, we cannot completely exclude an effect of *e.g.* stress-induced hormones on biochemical measurements.

## 6.4 Ethical considerations

Study protocols for projects included in Paper I and II were both in accordance with the 1975 Declaration of Helsinki. Ethical approval for projects in both papers were obtained from the Regional Committee for Medical and Health Research Ethics in South-Eastern Norway, with reference numbers 2012/286b (Paper I and Paper II), 2011/2572 (Paper II) and 2015/2140 (Paper II).

For Paper III all animal experiments were approved by the Norwegian National Animal Research Authority (project licence no FOTS 6809/14) and/or the Ethics Committee on Animal Care and Use in Gothenburg and Stockholm, Sweden. All animal experiments were in accordance with the European Directive 2010/63/EU and The Guide for the Care and Use of Laboratory Animals, 8th edition (NRC 2011, National Academic Press). A clarification in this respect: in line with these directives, individuals working on research projects involving animal testing must complete a compulsory course in laboratory animal science. During the work on this thesis the author only assisted the shared first-author in Paper III (E. Schrumpf) who handled all mice and has completed the compulsory course, and the author only used intestinal tissue already removed from the mice deemed for downstream microbiota analyses on an individual basis. The author was thus not required to complete the course. All mice had *ad libitum* access to water and standard rodent diet.

## 6.5 Collection of participant characteristics and patient data

For Paper I and II patient demographics and characteristics were gathered from patient records, and patient data were also extracted from the NoPSC biobank database. In Paper I a questionnaire was used as supplement to gather anthropometric and dietary data, and other characteristics for all study groups. This could introduce a self-reporting bias, especially important in the healthy control group, but were considered a reasonable trade-off to secure a large sample size. In Paper I all participants with history of bowel resection, a current stoma or that were prescribed antibiotics the last four weeks were excluded, as this has considerable effect on the gut microbiota.[180–182] BMI was calculated as it is associated with changes in the microbiota, and could also give a clue of the long-term total dietary energy intake of the participants.[85,183,184] The cause of death was extracted for relevant PSC patients from the Norwegian Cause of Death Registry (Oslo, Norway) in Paper II.

### 6.5.1 Diet

Participants in Paper I who stated that they followed a specific diet (**Box 1**) were excluded. How to address dietary bias in large-scale clinical studies is unfortunately not straightforward. Only excluding patients with specific diets is a pragmatic approach, and a more thorough dietary survey using *e.g.* food frequency questionnaires (FFQs) or a 24-hour recall questionnaire would unquestionably have been of value. The best alternative would probably have been a FFQ, which is regarded as the most robust method.[185] Dietary surveys are quite expensive to perform and more time consuming for the participants, increasing the risk of participant loss due to increased respondent burden.[185] We do not have tools to objectively measure diet, and the current methods are hampered by self-reporting biases, not even avoided by repeated measurements involving 1-to-1 interviews.[185] In addition, there is still no standardised method for using diet to adjust microbiota data analyses. Our strategy was deemed a reasonable trade-off at the time, to secure a large sample size and increase statistical power, while at the same time considering dietary data at an adequate level.

**Box 1.**
**Excluded diets in Paper I**

| |
|---|
| Vegetarian |
| Vegan (including *e.g.* lacto-vegan *etc.*) |
| Gluten free (*e.g.* coeliac disease) |
| Lactose-/milk-free |
| Low carbohydrate |

## 6.6 Sample collection and storage

### 6.6.1 Blood

In Paper I biochemical analyses were only collected for patients with PSC. We collaborated with 15 regional and local hospitals to retrieve clinical biochemistry from the closest routine control time point from time of stool donation at home (median ~2 months difference). Thanks to national laboratory standards efforts like Norwegian Clinical Chemistry External quality assessment Program (NNK, http://www.nkk-ekv.com/) in cooperation with Norwegian Quality Improvement of Primary Health Care Laboratories (NOKLUS, http://www.noklus.no/) sampling and subsequent measurements are comparable across the different hospital databases in Norway. Although minor differences could still exist between hospitals, they should be negligible. Matched blood and stool samples would have fewer limitations, but was not feasible at the time as participants performed sampling at home.

Serum samples in Paper II were prepared after non-fasting blood was collected, in a standardised fashion following internal biobank protocols, and stored at -70°C. Other biochemical analyses, and measurement of prothrombin time were retrieved from the databases at hospital laboratories if available within 7 days of biobank sampling. In Paper III blood from mice was drawn from the heart directly after they were sacrificed, left in room temperature, centrifuged and finally stored at -80°C awaiting further analyses.

### 6.6.2 Tissue sampling in mice

The weight of the mouse, liver, spleen and caecum were noted when the mice were sacrificed, together with any common bile duct dilatation (CBDD). Liver tissue was fixed in 4% formalin and subsequently embedded in paraffin using standard procedures.

### 6.6.3 Sampling for microbiota analyses

In Paper I, all participants were given a standardised collection devise and a simple procedure facilitating sample preparation at home.[186] It was explicitly emphasised that voiding should be performed prior to sampling to avoid contamination. Sampling was then done using Stool Collection Tubes with Stool DNA Stabilizer (Stratec Molecular GmbH, Berlin, Germany), and the samples were shaken to facilitate homogenisation. The tubes were then sent by mail to the NoPSC biobank to ensure equal processing and adequate

follow-up of missing information on sampling and/or participant, and frozen at a minimum of -20°C (according to the instructions from the manufacturer) awaiting DNA extraction.

An alternative to stool in this setting would be mucosal samples.[77] This is feasible in PSC due to regular screening of patients by colonoscopy, as well as for IBD controls, but is still an invasive procedure with potential complications. Samples from healthy controls are considerably more challenging as most are referred to colonoscopy for evaluation of certain symptoms, and one could therefore argue that they no longer should be considered 'healthy'.[170] An exemption to this is participants in national screening programs involving colonoscopy, but such programs are so far not implemented in Norway, and the participants would also be older than a typical PSC patient cohort. Bowel preparations have a striking effect on the mucosal microbiota.[187] Bowel preparation is essential before the procedure, and there are indications that this effect is not equal between study groups, and that it could have long standing effects.[187,188] Nevertheless, both stool and mucosa inhabit different and distinct niches of the microbiota,[189,190] so both must be explored if we are to fully understand its role in PSC. Besides being non-invasive, the use of stool samples has several advantages as it facilitates longitudinal follow up of a larger number of participants, at a reasonable cost. Another important advantage, although not performed in Paper I, is probably the possibility to perform WGS for identification of functional contents of the microbiota. This is still not cost-effective and has several limitations when performed at DNA extracts from mucosal samples, mainly due to the low bacterial-to-human DNA ratio in these samples.[190,191]

The 'gold standard' method is still fresh stool, with DNA extraction performed on arrival at study centre, or frozen as soon as possible after sampling, all to avoid post-sampling changes to different bacteria in the samples.[87,183] The method is, however, challenging to use in large scale studies involving participants over large distances.[192]

The Stool Collection Tubes with Stool DNA Stabilizer allowed us to greatly increase sample size, something we considered important given the large variation of the human gut microbiota. Importantly, this method has performed at par with more standard methods in comparative studies.[193,194] Samples stored in room temperature for more than 72 hours were excluded according to the manufacturers recommendations. One stool sample was collected per participant. Our experience from other studies supports that double baseline samples are unnecessary in this kind of large-scale surveys, as samples from the same individual taken

at separate time points tend to harbour highly similar gut microbiota profiles,[86] as illustrated by **Figure 7**. Samples were frozen immediately on arrival. An alternative could have been same-day DNA extraction, but this is highly laborious, and freezing *per se* does not appear to have minor impact on the bacterial community.[195]

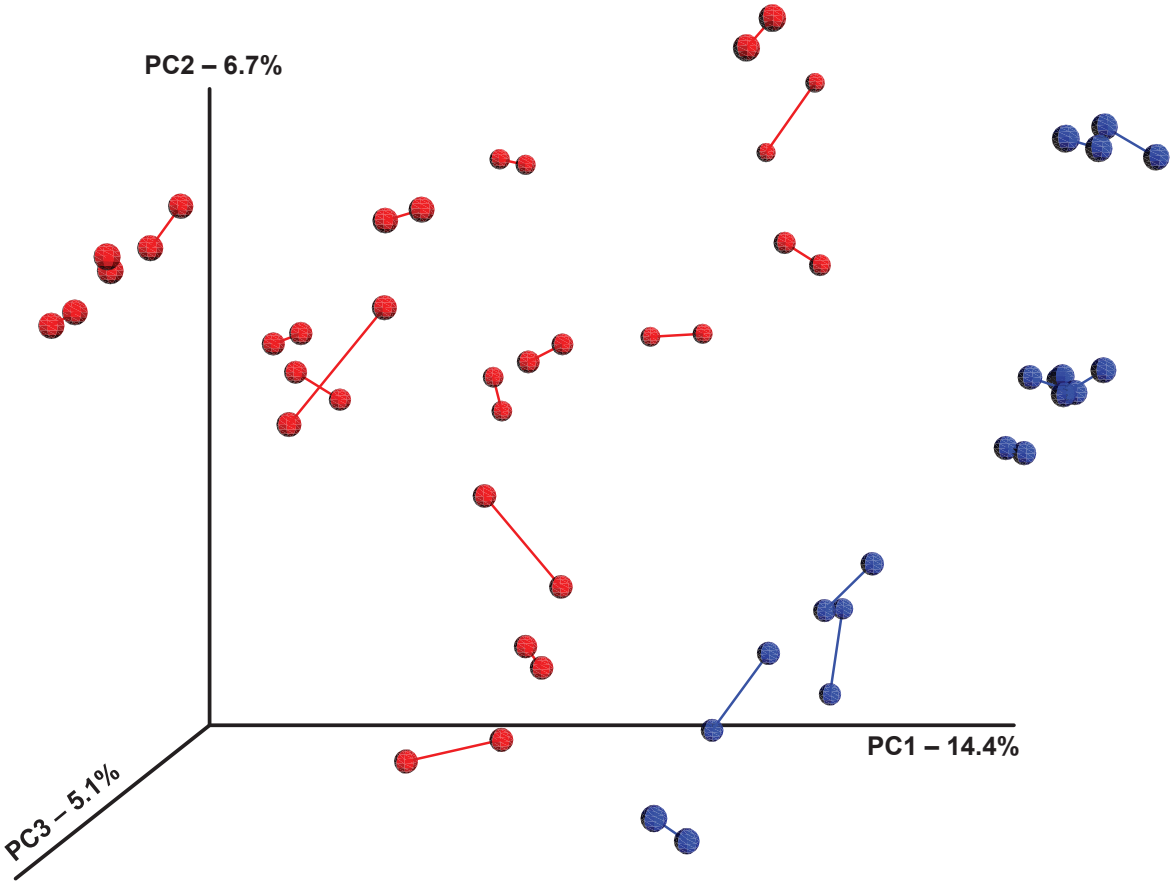



**Figure 7. Double baseline samples.** Illustration of β-diversity based on unweighted UniFrac distance showing highly similar bacterial profiles in samples from the same individual ($r^2$=0.92, p<0.001, 999 permutations). Samples from one individual are connected with a straight line (Cases in red, healthy family controls in blue). Samples were collected and processed using the same methods and protocols as described for Paper I.

In Paper III we sampled caecal contents and ~15 mm of caecal mucosa (whole transverse sections) from mice using disposable sterile equipment. To avoid removal of mucosal adherent bacteria we avoided liquid flushing. Caecal contents and mucosal tissue were then put in separate sterile tubes, snap-frozen in liquid nitrogen and stored at -80°C awaiting DNA extraction. The caecum was chosen as it is easily identified anatomically, thereby securing uniform sampling. Sampling was done at 10 weeks to assure sampling before development of diabetes in NOD control mice, confirmed by fasting blood glucose measurements.

## 6.7 Microbiota I: DNA extraction, library preparations and sequencing

### 6.7.1 DNA extraction

The purpose of the DNA extraction is to expose genomic DNA from the nucleus, without degrading too much DNA in the process. The sample must be cleaned securing that eluted DNA is as representative as possible, with an adequate purity and yield for subsequent processing. It is important to remember that this subsequent processing is not necessarily restricted to the work of the present project with constructions of libraries for 16S rRNA sequencing, but could include WGS and surveys of *e.g.* viruses or fungi in later projects.

It is extremely important to avoid contamination of samples at all steps in the microbiota study pipeline, but the extraction process is perhaps the most critical step in this regard. To address this, equipment and laboratory environment sterilisation procedures were meticulously adhered to, using standardised laboratory protocols. All of the work described in this section was done at one dedicated laboratory bench, by only a handful of people and with extensive use of disposable equipment where possible.

Most protocols for DNA extraction use a combination of chemical, thermic and mechanical methods to disrupt the bacterial cell walls, a process referred to as cell lysis, and cleaning. Final cleaning and elution of the DNA extracts are most often done using a filter column. The use of bead-beating is essential to ensure adequate lysis of especially gram-negative bacteria.[196]

The PSP Spin Stool DNA Kit used in Paper I utilises a combination of thorough homogenisation, thermic and mechanical lysis with bead-beating (using zirconia beads), protein digestion with proteinase K at 80°C to enhance efficiency and specific steps to remove PCR inhibitors. The kit has been evaluated in several studies, and performs very satisfactory compared with other methods, with good DNA yield and recovery of a greater proportion of bacteria in the Firmicutes phylum.[193,194]

For murine samples in Paper III we adopted a well-established protocol from collaborators.[111] In principle one may use similar extraction methods for murine and human samples, either for intestinal contents or mucosa. In this instance it was considered important to use a protocol established in murine samples that included bead-beating for reasons stated above. It was also an advantage that it was applicable to both caecal contents

and mucosa. Chemical lysis was used with a lysis-buffer containing lysozyme (an enzyme that damage bacterial cell walls, Sigma-Aldrich, St. Louis, MO), in addition to thermal disruption and homogenisation with bead-beating using a 0.1 mm zirconia/silica beads mix (BioSpec Products, Bartlesville, OK) and a bead-beater (BioSpec Products). This last step also contributes to thermal lysis, since bead-beating results in heating of the samples that were subsequently put on ice. Final cleaning and DNA elution was done using DNeasy mini DNA extraction kit (Qiagen, Chatsworth, CA).

DNA extracts were checked for presence of high molecular DNA and purity using regular gel-electrophoresis and a NanoDrop instrument (Thermo Fisher Scientific, Waltham, MA). All DNA extracts were then stored at -80°C awaiting library preparations.

### 6.7.2 Considerations concerning sequencing strategy and techniques

Before library preparations, one must choose what type of sequencing to perform, and what sequencing technology to use. In both Paper I and III we applied 16S rRNA sequencing on the Illumina MiSeq platform.

The Illumina MiSeq is considered a next-generation sequencing technique, as opposed to earlier sequencing techniques like classical Sanger sequencing.[75] The MiSeq platform produces shorter reads compared to *e.g.* the older but widely applied 454 technology, but achieves a 10-fold increase in sequencing depth at the same price.[197,198]

This new technology has greatly reduced the total cost of microbiota studies. This has been a contributing factor for the prominent expansion in microbiota research in the recent decade (**Figure 4**). However, this technology has also introduced several new challenges. The most important one being that taxonomic classification sensitivity has been reduced because of the reduced read length. This can be partially addressed by applying paired-end sequencing, as shown in **Figure 8**, where each fragment is sequenced two times; first from one end, and then from the other. If the fragments in the library are shorter than twice the read length the paired reads will overlap. This makes it possible to merge them after sequencing, thus providing one long combined read called a 'contig'.[197,199] We applied paired-end sequencing on the Illumina MiSeq platform in Paper I and III.

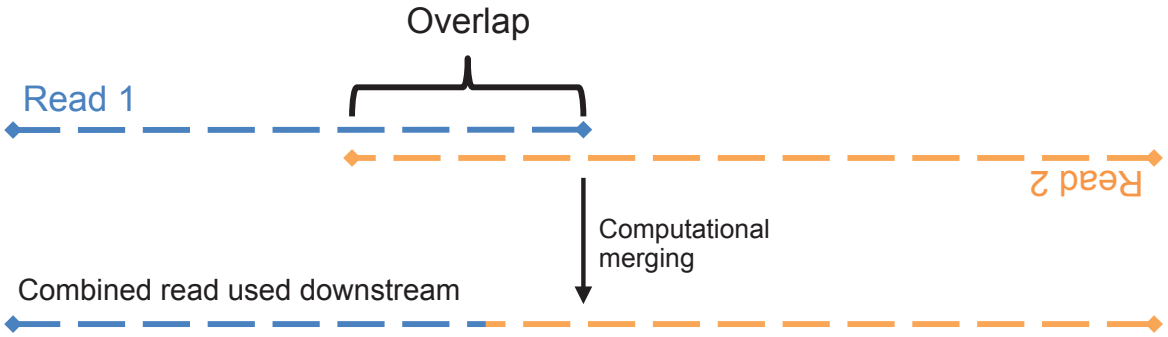



**Figure 8. The concept of a paired-end sequencing strategy.** If the fragments that are sequenced are shorter than twice the read length the paired reads will overlap, thus making it possible to merge Read 1 and Read 2 after sequencing using bioinformatic tools like FLASH as performed in Paper I and III.[199] Two combined reads are called a 'contig'.

**16S rRNA sequencing** has the advantage of being extensively studied, using different universal primer pairs (amplifying a corresponding hypervariable region, or spanning multiple regions, of the 16S rRNA gene). It has also been reasonably easy to adapt compared with the more un-biased alternative of WGS, while allowing sequence comparison and taxonomic assignment at the genus level with the use of well-curated databases, providing a good resolution of the microbial community.[77] This means that species-level resolution is not always possible based on 16S rRNA sequences. Another disadvantage to be aware of is that some organisms have multiple copies of the 16S rRNA gene, which could result in overestimation of the relative abundance of different taxa.[200]

**Choice of 16S rRNA hypervariable region** is the first step of 16S rRNA library preparations. All the hypervariable regions (V1 through V9, and combinations thereof) have been used in different microbiota studies.[75,77,78,201] The PCR amplification of a specific region introduces an unavoidable and general bias, as different regions have different taxonomic coverage (sensitivity) and varying specificity for bacteria during amplification, illustrated in **Figure 9** (next page).[191] One must also consider the length of the segment depending on the read length capability of the sequencing method that will be used.[191] As such there is no ideal primer pair. Both V3-V4 (Paper I) and V4 (Paper III) have been used in several studies, yielding good results, and have good taxonomic coverage for gut microbiota studies in both mice and humans.[77,191,197,202] Importantly, they also have good taxonomic coverage for important taxa like *Bifidobacterium* (genus) and Verrucomicrobia (phylum), in contrast to *e.g.* the V1-V2 region.[77,191,197,202]

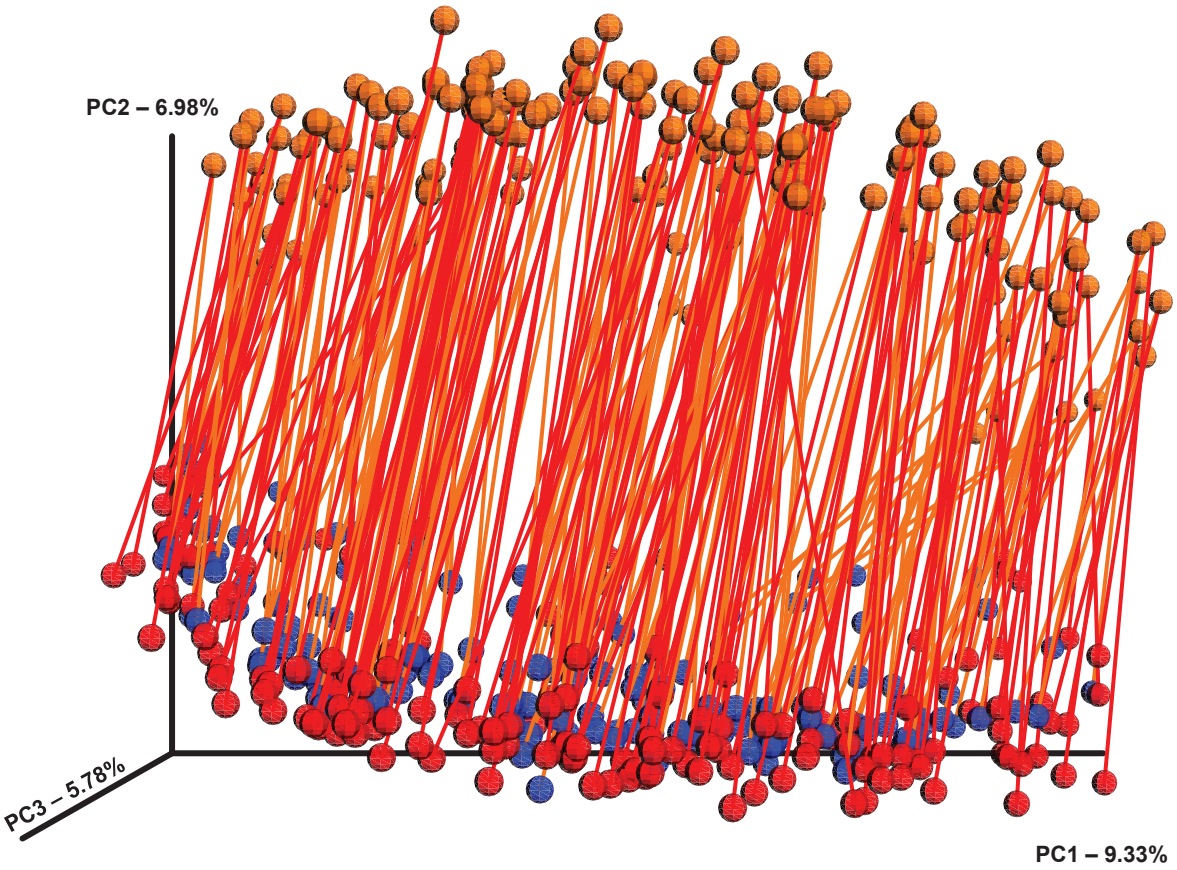



**Figure 9. Illustration of differences introduced by choice of hypervariable region.** The plot is based on unweighted UniFrac distances calculated three times for each sample based on three different sequencing results, respectively, and connected by a straight line.

**(1)** The hypervariable region amplified is the only part of the methods differing between the orange (V3V4) and the blue (V4) dots. The methods are similar to those described for Paper I. This difference clearly represents the most important factor along PC2, and thus has a clear impact on the overall bacterial community.

**(2)** The red and the blue dots are all amplified using the V4 region, and thus cluster closely together. First DNA extracts were processed using our own in-house library preparation protocol (blue dots). For quality control and external validation of the protocol we sent DNA extracts to The Broad Institute (http://www.broadinstitute.org/, Cambridge, MA) for library preparations and sequencing (red dots). The high degree of clustering indicates satisfactory quality of our in-house adaptation of the library preparation protocol based on Kozich *et al.*[197]

### 6.7.3 Library preparations

In Paper I we adopted a library preparation protocol using amplification of the V3-V4 region of the 16S rRNA gene based on a protocol developed by Kozich *et al.* allowing dual-index paired-end sequencing on the Illumina MiSeq platform (San Diego, CA).[197] First, DNA was diluted 1:5 to improve PCR efficiency, and 1 µl was used as template, mixed with 17 µl of Accuprime Pfx SuperMix (Thermo Fisher Scientific), before index primers were added and the PCR initiated. A negative control and a mock community were added to the PCR plate, allowing us to identify parameters that could minimise the sequencing error rate.[197] The forward- and reverse primer design is illustrated and explained briefly in **Figure 10.**

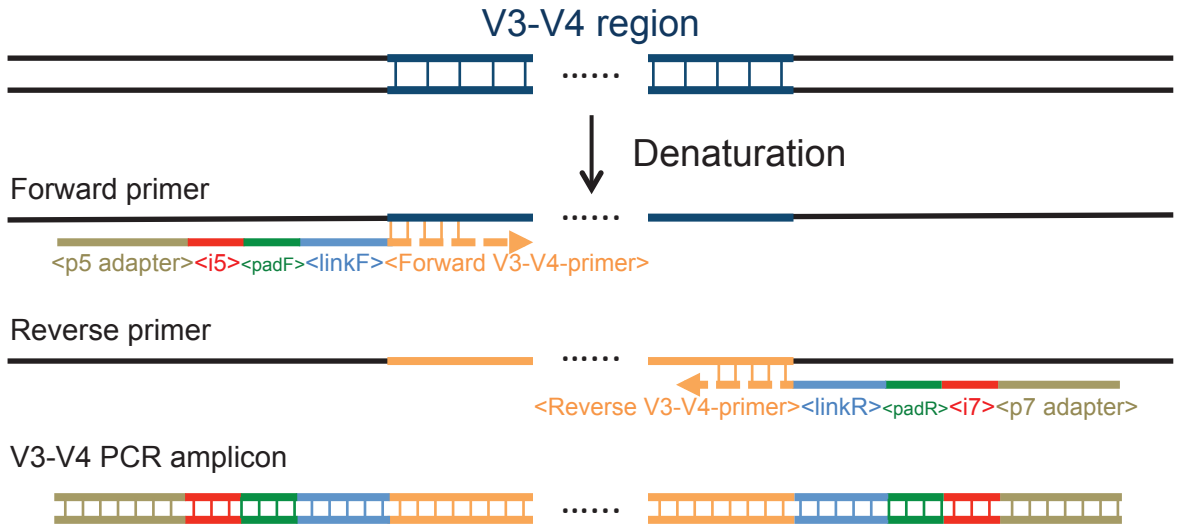



**Figure 10. Illustration of the dual-indexing primer design used during PCR amplification.**[197] Here we use the V3-V4 region of the 16S rRNA gene as an example (as used in Paper I), but the principle can be applied to other regions as well. The primers used each contain 5 elements:

**(1)** Genetic primers: targeting the conserved flanking regions of the hypervariable region of interest, for the forward and reverse primer, respectively.

**(2)** Linkers: selected to share a minimum amount of homology with sequences in a reference database.

**(3)** Pads: used to adjust the estimated melting temperature of the total sequence.

**(4)** Index regions: each combination in the forward and reverse primer is unique for each, allowing us to determine from what sample the read originates.

**(5)** Adapter sequences: these must be appropriate for Illumina, allowing the fragments to adhere to the flow-cell during sequencing. PCR, polymerase chain reaction.

A regular agarose gel electrophoresis was then run to confirm amplification. The PCR products were transferred to a new plate for cleaning and normalisation. Normalisation is an important effort to ensure an equal amount of DNA input from each sample into the amplicon pool, but perfect normalisation will not be achieved. The SequalPrep Normalization Plate Kit (Life Technologies, Carlsbad, CA) provides a plate where each well has the capacity to bind a certain amount of DNA from the amplicon that is added, before removing excess DNA and fluid are washed away using washing buffers. All amplicons on each normalisation plate are then pooled for subsequent quality control.

Each amplicon pool was evaluated with a Bioanalyzer (Agilent Technologies, Santa Clara, CA) using the Agilent High Sensitive DNA Kit (Agilent Technologies) to detect impurities and confirm uniform fragment-sizes. An indication of concentration can also be given. Because each sequencing run has a set sequencing capacity, and we are sequencing several libraries in the same run, it is important that the final pool added to the MiSeq contains libraries with equal DNA concentrations. To determine DNA concentration a KAPA Library Quantification Kit (Kapa Biosystems, London, UK) was used, analysing triplicates of each library. Using internal standards with known concentrations, the average concentration of each library-triplicate is calculated, and equimolar amounts of two libraries were pooled before sequencing.

The use of the SequalPrep Normalization Plate Kit often resulted in low-concentration libraries. The concentration of the library must be >10 nM to avoid the need for modifications of the MiSeq instrument. Thus low-concentration libraries were up-concentrated using Agencourt AMPure magnetic beads (Beckman Coulter, Brea, CA). The beads bind DNA, elution fluid is removed, beads washed, and then DNA is eluted in the volume yielding the desired concentration. The final pooled libraries in Paper I were then transferred to the Norwegian Sequencing Centre (Oslo, Norway) for sequencing.

To ensure quality and externally 'validate' the adaptation of the library preparation protocol we sent 187 DNA extracts to The Broad Institute (http://www.broadinstitute.org/, Cambridge, MA) that performed library preparations and sequencing on the MiSeq using their internal protocols. This sequencing data was then used for comparison against our own, showing satisfactory results **(Figure 9)**.

The DNA extracts in Paper III were submitted to BGI (Shenzhen, China) for library preparations and sequencing. The process is similar to the one used in Paper I, but here the V4 region was amplified,[203] using a standard set of universal primers:

- Forward primer: 515F (5'-GTGCCAGCMGCCGCGGTAA-3')
- Reverse primer: 806R (5'- GGACTACHVGGGTWTCTAAT-3')

### 6.7.4 Sequencing

All samples were quantified again using a Qbit fluorometer (Thermo Fisher Scientific) at the respective sequencing centres, and sequenced using the MiSeq platform. In paper I the version 3 sequencing kit from Illumina was used, while version 2 was used in Paper III. The most important difference is that version 2 provides a maximum read length of 250 base pairs, while the version 3 kit increases this to 300 base pairs. Generally longer read length is considered a positive factor, as long as the error-rate does not increase, but prices also tend to increase with the release of new kits.

The library is added to the sequencer together with PhiX and sequencing primers. Sequencing all the samples in one run in parallel like this is called 'multiplexing'. The adapter sequence lets the DNA-fragment attach to the flow cell. The sequencer reads the DNA sequence of the fragment by detecting light emitted from the different bases that flow by the anchored fragment on the flow cell. Light is emitted when a base attaches to its complementary base on the fragments, and different bases emit light at different wavelengths, allowing the sequencer to determine the base sequence. Each base is also given a quality score used later during bioinformatic quality control.

16S rRNA amplicons have low diversity by nature. This is problematic for the optics of the MiSeq platform, as it detects the light emitted when the bases adhere to the fragment being sequenced, and thus rely on a certain degree of diversity/contrast to detect the wavelength accurately. This is one of the limitations of the MiSeq platform,[191,197] and is resolved by increasing the diversity of the library by adding known genomic DNA, in this case the commonly used PhiX. The downside is simply that since the PhiX DNA is also sequenced and the number of reads per run is fixed, this results in a corresponding decrease in sequencing depth.[202]

## 6.8 Microbiota II: From raw sequences to complete dataset

All reads from all samples are reported together in no specific order. Thus, raw reads were first demultiplexed, and the sequencing centres removed index primers and genomic primers. Reads were then quality trimmed, overlapped and merged using FLASH (concept illustrated in **Figure 8**).[199] The V3-V4 region (Paper I) is approximately 430 base pairs long, providing an overlap of 170 base pairs. The V4 region (Paper III) is entirely covered by both reads yielding increased quality.[197,202] The merged reads were subsequently quality filtered based on truncating reads at three consecutive low quality base calls (phred score < 25) and discarding reads with a truncated length of < 75% of its original length.

The methods used in the rest of this section are identical for Paper I and III, unless specifically noted. We used the Quantitative Insights Into Microbial Ecology (QIIME) platform (version 1.8.0) for quality control and further sequence processing.[204] QIIME is a community developed, open source bioinformatics pipeline. It constitutes a framework that to some degree standardises the post-sequencing workflow. It incorporates bioinformatics software from several different developers, at the same time assuring a certain degree of quality control.

**Operational taxonomic unit (OTU) picking** was performed on reads left after quality control. Sequences were clustered together into OTUs with 97% sequence similarity using a closed-reference approach, with mapping against the GreenGenes database (v13_8).[205] An OTU is thus an artificial construct, and the 97% threshold is merely based on convention, and may be set otherwise. In addition to the closed-reference approach, *de novo* and open-reference approaches can be used as shown in **Figure 11**. In closed-reference OTU-picking, reads that do not get a match in the pre-clustered database are discarded. A lot of reads (~3-6% in our studies), originating from possibly less well-described species, might be lost. However, the method has the advantage of being more specific when assigning taxonomy, is faster to perform and facilitates better comparisons between studies, especially if different primers are used.[83] The GreenGenes database has the advantage of very low chimera levels, but one must be aware that all the available databases may contain errors in sequences and their taxonomic assignment.[77] Chimeras could make up to 45% of sequences in one run and can be found in many 16S databases.[77]

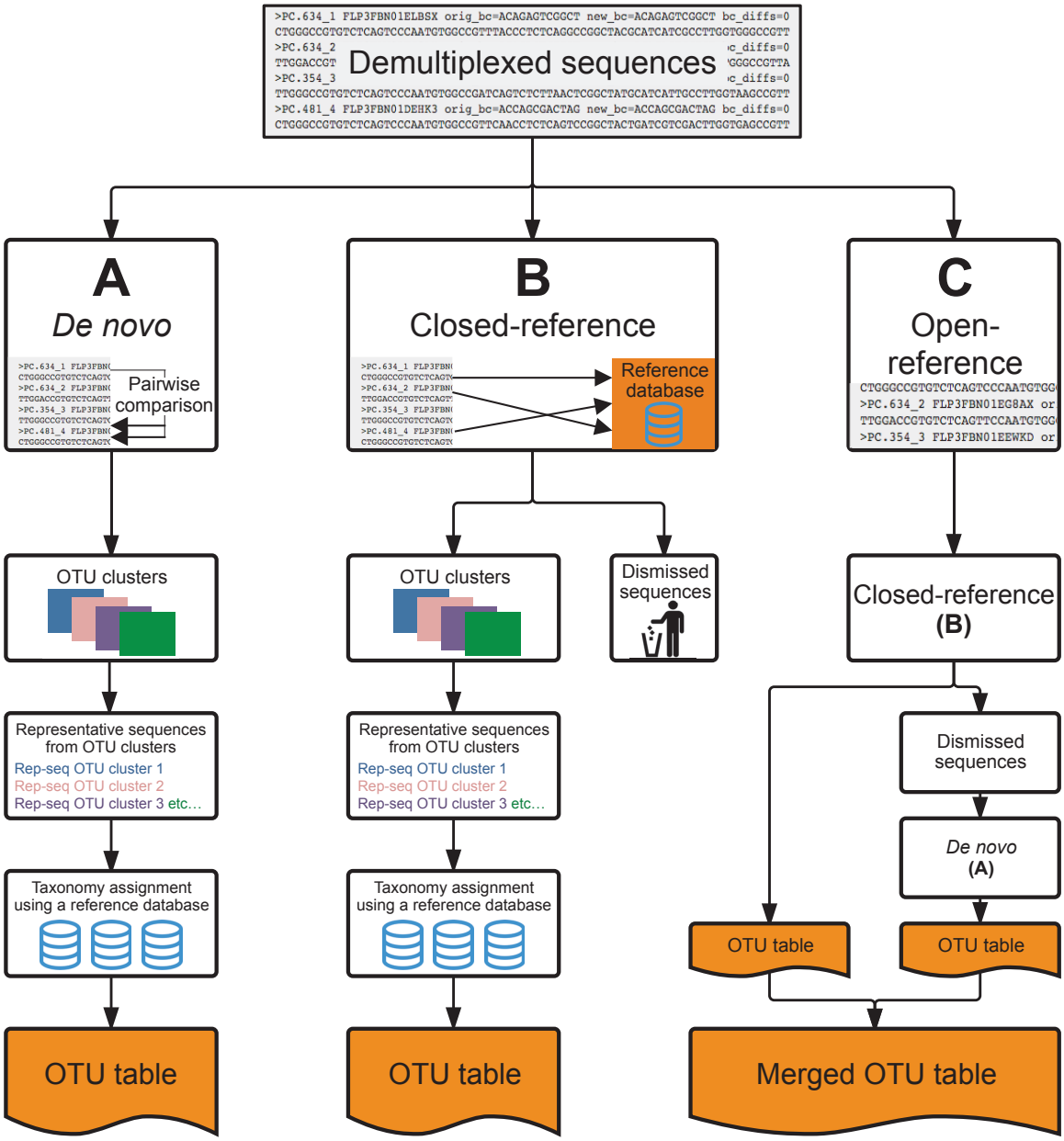



**Figure 11. Workflow for different approaches for making an OTU table in QIIME.**[204]

**(A)** ***De novo*****:** sequences are compared internally and then clustered together depending on a similarity-threshold. A representative sequence from each OTU cluster is subsequently matched to a reference database and taxonomy assigned to the OTU cluster accordingly. It is considerably slower and more computationally intensive than the other methods, but without sequence-loss.

**(B)** **Closed-reference** (used in Paper I and III): sequences are compared directly to representative sequences from a pre-clustered reference database and discarded if no match is found. Taxonomy is directly inherited from the matching reference-OTU. It is computationally fast and facilitates comparison between studies where different methods is used, but is biased toward the reference database and sequences are discarded if no match is found in the reference-database.

**(C)** **Open reference**: here the *de novo* and closed-reference approaches are combined.[83]

OTU, operational taxonomic unit; Rep-seq, representative sequence. Figure is inspired by Navas-Molina *et al.*[83] Pictures used in the figure are licenced under the Creative Commons Zero licence.

**Taxonomy assignment** was done based on the GreenGenes database, which also provides a phylogenetic tree of reference OTUs. The phylogenetic tree is necessary for downstream analyses, like UniFrac. Finally, an OTU table is generated and used in subsequent analyses.

To reduce the number of comparisons and greatly reduce the problem of spurious OTUs, OTUs containing <0.005% of the total number of sequences were discarded at this stage in Paper I, as is also recommended in the literature.[83] However, by doing this we risk missing less prevalent, but potentially important OTUs. In Paper III, OTUs represented in only one single sample in one sample site in each experiment were discarded. In Paper III, OTUs (mapping to the mitochondria family and chloroplast class) misclassified in Greengenes to the Bacteria-kingdom were removed.

After evaluating rarefication curves, samples with <8000 reads were discarded in Paper I, while this was not necessary in Paper III, due to high coverage. This resulted in a mean sequencing depth of 34.490 and 242.046 reads for Paper I and III, respectively.

Because sequencing depth is not equal in all samples, α-diversity (Chao1 bacterial richness estimate [Chao1], Shannon diversity index and phylogenetic diversity) and β-diversity (unweighted UniFrac) were calculated on rarefied OTU tables. This is the main argument for discarding samples with low read-count as described above, as the sample with the lowest read-count decides the rarefication level.

Chao1 has the advantage of being a simple estimate of community richness.[206] Chao1 estimates the total OTU-count one could expect in a sample with infinite sampling. Shannon diversity has the advantage of being frequently reported in the literature, and takes both richness and evenness into account.[82] Phylogenetic diversity is recommended by several authors and exploits phylogenetic information.[83,207]

Generally, β-diversity metrics have the advantage of being robust to noise and low sequence counts, although the latter is less important today.[78] β-diversity metrics can be quantitative (using sequence abundance) like Bray-Curtis and weighted UniFrac, or qualitative (considering only presence-absence) like binary Jaccard or unweighted UniFrac). We used UniFrac in Paper I and III since it is phylogeny based and also has been shown to outperform other metrics in community comparisons.[78,84]

## 6.9 Biochemical analyses

For Paper I and II all routine biochemical analyses, including platelets, creatinine, total bilirubin, albumin, international normalised ratio (INR), Normotest, AST, alanine aminotransferase (ALT), ALP and gamma-glutamyl transferase (γ-GT) were performed in accordance with standard laboratory protocols. For evaluation of p-ANCA status, different methods are in use, and we therefore only obtained this from local databases at Oslo University Hospital to ensure uniform interpretation. For PSC patients, Mayo risk scores were calculated using the revised formula,[62] and renal function was evaluated by estimated glomerular filtration rate (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration equation.[208]

For Paper III, sera from mice were analysed using ADVIA 1800 (Siemens, Munich, Germany) at The Central Laboratory, Norwegian School of Veterinary Science (Oslo, Norway). Non-fasting blood glucose was measured with Accu-Chek Performa (Roche Diagnostics, Basel, Switzerland).

## 6.10 Trimethylamine-*N*-oxide (TMAO) measurements

The measurement of TMAO used in Paper II was done by liquid chromatography–tandem mass spectrometry, a highly accurate platform for targeted metabolic measurements. Calibration curves were made using various concentrations of TMAO together with a fixed amount of the internal standard, allowing quantification of TMAO levels in each sample. The internal stable isotope-labelled standards were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA).

The majority of published TMAO studies have performed TMAO measurements on plasma samples.[89,95,97] We have used serum samples when measuring TMAO in Paper II. As an effort to avoid introduction of a systemic bias to the study, we measured TMAO in 25 participants where both plasma and serum were available. TMAO measurements were highly correlated (Spearman's rho = 0.99, $p<1.8\times10^{-20}$), indicating that this should be a minor issue. Our collaborators at the Department of Clinical Science, University of Bergen (Bergen, Norway) performed all TMAO measurements.

## 6.11 Histology, immunohistochemistry and scoring

For hematoxylin and eosin staining we used standard laboratory protocols on 3 μm sections of formalin-fixed paraffin-embedded murine liver tissue. For Sirius red staining sections were stained with Picro Sirius Red solution 0.1% (Histolab Products AB, Gothenburg, Sweden) for one hour. All sections were scored in a blinded fashion, using the following parameters: portal inflammation, fibrosis, bile infarcts and dilatations of intrahepatic bile ducts. For immunohistochemistry samples blocking was done, followed by incubation with a primary (clone SP7, Abcam, Cambridge, UK) and a secondary antibody (ImmPress Reagent Peroxidase, Vector Laboratories, Burlingame, CA). Staining was then performed with DAB Peroxidase Substrate Kit (Vector Laboratories). We also quantified CD3 positive cells around the bile ducts to characterise lymphocytic infiltrates.[105] This was done in six different 40X fields in each sample. Samples were blinded, cells counted manually, and mean count used in the analyses. An Eclipse E400 Microscope with a DS-Fi1 camera controlled by NIS-elements BR 3.1 software (Nikon, Tokyo, Japan) was used to generate images.

## 6.12 Statistics

### 6.12.1 Basics

For variables meeting requirements of normal distribution statistical significance was calculated with unpaired Student's t-test. For other variables the Mann-Whitney U test was used, this includes comparison of relative abundance of taxa and α-diversity between groups. Relative abundances can be challenging to handle statistically because they are really counting variables bound by 0 and 1. When handled as continuous variables they have a right skewed distribution with zero-inflation, because some taxa are not found in many samples, and handling zero in relative abundance as ‘true absence’ could also be criticised since it could be biased to sequencing depth. Categorical variables were compared using the chi-square test or Fisher’s exact test where appropriate. For correlation analyses, Spearman’s rank correlation test was used. Relative abundance ratios were calculated for illustrational purposes (Paper I, figure 3 and Paper III, Table I and Supplementary Table 2). These ratios were calculated by dividing the mean relative abundance of each bacterial taxon in each category. False-discovery rate (FDR) was calculated according to Benjamini–Hochberg, FDR-corrected p values were denoted $Q_{FDR}$ and were used when performing untargeted screening analyses of different taxa in Paper I.

### 6.12.2 Regression analyses

Linear regression analyses were used in an effort to identify covariates and possible confounders that affected microbiota measurements like α-diversity and relative abundances of different taxa (Paper I). When choosing covariates for the multivariate analyses we considered age, sex, smoking status and BMI as obligate covariates. For subgroup analyses of PSC patients the use of antibiotics, duration of PSC and duration of IBD were also considered obligate. To account for interactions, all other variables with p value <0.10 in univariate analyses were also included as covariates in the multivariate models. Since linear regression presumes a normal distribution, variables with a right-skewed distribution (ALP, AST, ALT, and γ-GT) were transformed by the natural logarithm prior to regression analyses of α-diversity. Relative abundances were arcsine square root transformed, in accordance with the strategy used in the Human Microbiome Project's paper "Structure, function and diversity of the healthy human microbiome".[183] Logistic regression was used as part of ROC-AUC analyses in Paper I, and also here relative abundances were arcsine square root transformed. To explore association between TMAO and other variables and endpoints (death or liver transplantation) in Paper II, we used Cox proportional hazards regression analyses after transforming variables with a right-skewed distribution (bilirubin, ALP, AST, ALT and platelets) by the natural logarithm.

### 6.12.3 Receiver operating characteristic (ROC) area under the curve (AUC) analyses

In Paper I we used ROC-AUC to distinguish phenotypes (method is denoted AUROC in the paper), using only a small selection of taxa at the genus level. To avoid bias to the most abundant taxa, we used each included individual's transformed relative abundances for all bacterial taxa together with multivariate logistic regression coefficient estimates. The performance of the logistic regression models was then evaluated by using them to calculate AUCs, based on the predictive probability of PSC for each individual. A resulting AUC of 1.0 would mean that we could, using this method, predict with a 100% certainty from what phenotype the bacterial profile originates. Correspondingly, an AUC of 0.5 would mean we could just as well flip a coin. Using this kind of analyses on microbiota data was chosen to illustrate how a model, using only the abundance of a few bacterial taxa, performs well at theoretically distinguishing phenotypes. Our collaborators at the Bioinformatics Core Facility at the Institute Medical Informatics, Oslo University Hospital (Oslo, Norway) conducted these analyses, including logistic regressions. ROC-AUC and Youden's index

were used to define the optimal TMAO cut-off in Paper II, and calculations were performed by our collaborator at Department of Medicine, National Centre for Ultrasound in Gastroenterology, Haukeland University Hospital, (Bergen, Norway).[209] Differences between AUCs were compared according to the method of DeLong (Paper I and II).[210]

### 6.12.4 Survival analyses

For visualisation of transplantation-free survival of PSC patients in Paper II we calculated Kaplan-Meier plots. Difference in crude risk was compared by the log-rank test. The cohort has the strength of a long follow-up time, maximum 20.1 years, but there are few patients at risk at this stage, so all participants were censored at 15 years.

### 6.12.5 Comparison of β-diversity

Here we used the PERMANOVA method in QIIME's script *compare_categories.py*. It is a nonparametric method, returning a coefficient called pseudo-F. Since these methods are sensitive to community differences, it is important to interpret significant p values cautiously if coefficients are low, as they might not be biologically relevant. There is a range of tests available for this purpose, and their use is somewhat debated, but PERMANOVA was used due to its relatively widespread use in previous gut microbiota studies and its integration into the QIIME framework.[83,90,95,211,212] It is also important to look for patterns that drive community differences on jackknifed resampling plots with confidence intervals, which was done manually.

### 6.12.6 Power calculations

Power calculations in microbiota studies have not been widely implemented, potentially because this is challenging since large scale microbiota research is a young field, and we still have little knowledge of what effect sizes to expect from the variables we study. The few exceptions are some specific statistical models,[213] and interventional microbiota studies that use common biochemical parameters as readout. The samples size in Paper I and II were decided by the maximum number of samples available, after an effort was made to recruit as many participants as possible, but at the same time introduce strict exclusion criteria as described above.

### 6.12.7 Software

Paper I: Linear regression analyses of α-diversity, Mann-Whitney U, chi-square test, Fisher's exact test, Spearman's rank correlation and comparison of α-diversities were performed in SPSS Statistics for Macintosh (v22, IBM, New York, NY), with one exception; comparison of relative taxa abundances using Mann-Whitney U that was conducted in the statistical programming language R (v3.1.2, https://www.r-project.org). R was also used for all other analyses, including regressions, ROC-AUC and FDR calculations.

Paper II: All calculations were done in SPSS except Youden's index, calculated in MedCalc (MedCalc Software bvba, Ostend, Belgium).

Paper III: All statistical analyses on microbiota data were done using R. All other calculations were performed in GraphPad Prism v5.0b (GraphPad Software, La Jolla, CA).

### 6.12.8 External microbiota analyses tools

In an effort to confirm the results from the primary analyses in Paper I we used the Multivariate Association with Linear Models framework (MaAsLin,[214] v1.0.1, revision 13:4033a2ee4558), and the Linear discriminant analysis effect size tool (LEfSe,[215] v1.0). Both tools were accessed from http://huttenhower.sph.harvard.edu/galaxy/ and standard parameters were used.

**MaAsLin** is a multivariate analysis pipeline based on R. It has the benefit of testing for disease characteristics while controlling for several known or potentially confounding variables. We used age, gender, smoking status, BMI and the number of prescriptions for antibiotics the last 12 months before inclusion as covariates. MaAsLin uses a general linear model at its core in combination with both Bonferroni- and FDR-correction for multiple comparison in different parts of the analysis pipeline. Relative abundances are arcsine square root transformed before analyses to stabilise variation.[214] It also has the advantage of being developed for and used in clinical gut microbiota studies.[92]

**LEfSe** is also validated on human gut microbiota data, and uses linear discriminant analyses to detect the feature (*e.g.* a taxon) that explains most of the difference between the phenotypes of interest. It uses the effect size to estimate the magnitude of how differentiating a feature is for group difference, and then ranks them accordingly, thus

providing a sorted list for further evaluation. It does this by first applying a non-parametric Kruskal-Wallis test, to detect features that are different between groups, and those features left after this screening are then introduced to the linear discriminant analyses to estimate the effect size of each feature.[215] LEfSe puts less emphasis on correcting p values for multiple testing, but it performs well in regard to false positive and negative rates when evaluated using simulation on synthetic data.[215]

# 7 Summary of the results

***Paper I***

**The gut microbial profile in patients with primary sclerosing cholangitis is distinct from ulcerative colitis patients without biliary disease and healthy controls**

Given the strong association between bowel inflammation and PSC, and the fact that the gut microbiota is implicated in several key aspects of central hypotheses for the pathogenesis of PSC, we performed the a large scale gut microbiota profiling study in a cross-sectional cohort of 85 PSC patients and 263 healthy controls, and 36 disease controls with UC.

PSC patients showed a marked decrease in α-diversity measured by several different indices (Shannon diversity index, Chao1 and Phylogenetic diversity) compared with healthy controls ($p<0.0001$), and importantly, this was not associated with use of antibiotics. PSC patients also showed a unique global microbial profile compared with both healthy controls and UC (unweighted UniFrac, $p<0.001$ and $p<0.01$, respectively). α-diversity was identified as an important factor driving these global differences. 12 different bacterial taxa at the genus level showed different levels in PSC and healthy controls. Using linear regression and two published external microbiota analyses tools we confirmed nine of these 12 genera. Eight of these genera were enriched in the microbiota of healthy individuals. However, PSC patients showed enrichment of the *Veillonella* genus, compared with both healthy controls ($p<0.0001$) and UC patients ($p<0.02$). Interestingly, the *Veillonella* genus is associated with other chronic inflammatory and fibrotic conditions. That PSC patients harbour a distinct gut microbiota was further supported by logistic regression and ROC-AUC analyses, where we were able to distinguish PSC from healthy controls and UC patients with an accuracy of 78 and 82%, respectively ($p<0.0001$), using only those genera that separated the phenotypes in the initial analyses. When exploring subphenotypes in PSC, we were unable to unveil any differences in the microbiota according to gender, concomitant autoimmune disease, medication use (including UDCA), or IBD status.

In conclusion, these results clearly indicate the presence of a PSC-associated microbiota. This study provides a rationale for further exploration of *e.g.* the functional gut microbial contents and microbial metabolites in PSC, the potential use of the microbiota as a new treatment target in, and the potential role of the microbiota in PSC disease progression.

*Paper II*

**Microbiota-dependent marker trimethylamine-*N*-oxide (TMAO) is associated with the severity of primary sclerosing cholangitis**

TMA is a metabolite made solely by gut microbes from dietary substances. TMA is converted to TMAO in the liver, and both TMAO and TMA have been implicated in cholangitis by experimental models. In an effort to move from compositional characterisation of the gut microbiota, to detection of possible metabolic pathways by which gut microbiota affect the host in PSC, we aimed to investigate the relationship between TMAO and PSC in a large cohort of well-characterised patients (n=305, followed for up to 20 years).

The data indicated that TMAO is affected by liver function, measured by increased prothrombin time (INR >1.2 or Normotest <70), a finding supported by the literature. PSC with normal liver function (n=197) showed reduced TMAO compared to UC patients (n=90), but similar values to healthy control (n=99). However, TMAO was higher in PSC patients who reached an endpoint (liver transplantation or death) during follow-up, compared with those who did not. Importantly, since TMAO has been associated with cardiovascular disease, only 4.5% of PSC patients with death as primary endpoint were classified with a cardiovascular-related cause of death. Since high TMAO was associated with endpoints, we calculated an optimal TMAO cut-off of 4.1 μM (AUC=0.64, $p<0.001$). PSC patients with high TMAO (>4.1 μM, n=77) exhibited shorter transplantation-free survival than patients with low TMAO (n=120, log-rank test: $p<0.0001$). High TMAO was also associated with reduced transplantation-free survival in multivariate Cox regression (HR 1.87, 95% CI 1.15-3.04, p=0.011), independently of the Mayo risk score (HR 1.74, 95% CI 1.40-2.17, $p<0.001$).

In summary, a metabolic pathway that is diet-, gut microbiota- and liver-dependent (measured by TMAO) is associated with the prognosis of patients with PSC with normal liver function at serum sampling. This is a first step in an effort to investigate how complex environmental factors like gut microbiota and diet affect the host through converging metabolic parameters in PSC. In the future this could help us identify more specific tools facilitating interventions targeting diet or the gut microbiota in the treatment of PSC patients.

*Paper III*

## The gut microbiota contributes to disease in a mouse model with spontaneous bile duct inflammation

With emerging evidence of a connection between gut microbiota and biliary inflammation, we wanted to explore the role of the gut microbiota in biliary inflammation using a spontaneous mouse model that develops biliary inflammation: the NOD.c3c4 mouse.

We first compared mice with and without biliary disease (NOD control mice), and demonstrated that there were substantial differences in the overall gut microbial community of these mice strains. This was accomplished by 16S rRNA profiling of bacterial communities in caecal contents and mucosa. In an effort to validate this finding, and avoid cage- and colony-drift bias, we rederived both NOD.c3c4 and NOD mice into a new animal facility unit and repeated the experiment, with similar results. NOD.c3c4 mice were then rederived into a GF facility and compared with conventionally raised NOD.c3c4 mice. GF NOD.c3c4 mice showed several signs of an ameliorated biliary disease phenotype; less distension of extra-hepatic bile ducts compared with conventionally raised NOD.c3c4 mice ($p<0.01$), reduction of portal infiltrates, and fewer CD3 positive cells around the bile ducts ($p<0.05$).

Overall this implicates the gut microbiota in the development of biliary inflammation and disease in this primarily immune-driven experimental model, and gives the rationale for exploring how more specific manipulation of the gut microbiota might influence these changes.

# 8 Discussion

In the following I will discuss the main findings in this thesis and how they can be interpreted in light of previous research, while emphasising some limitations in the study design and methods used. Lastly, I will present some short conclusions and the implications of the current findings for future PSC research.

## 8.1 Do patients with PSC truly harbour a distinct microbiota?

In Paper I we have tried to answer whether patients with PSC truly harbour a distinct microbiota, using several strategies. PSC patients show a marked reduction in all aspects of α-diversity compared with healthy controls, and this is the most prominent feature driving differences between the two groups.

Antibiotics are used in the treatment of cholangitis, and thus constitute a potentially important confounder in our study.[2,216] It might be reasonable to think that PSC patients use antibiotics more frequently than controls, but this was not the case in our cohort, at least for the 12 months preceding study inclusion. Although we cannot rule out a long-standing effect of antibiotics in the PSC group that could in part explain the observed differences, or reporting bias, PSC patients showed reduced α-diversity irrespective of antibiotics use in the last year prior to inclusion. 87% of the PSC patients did not use any antibiotics at all the last year prior to study inclusion. They still demonstrated a marked reduction in α-diversity compared with healthy controls that had used antibiotics the last year. Importantly, this subgroup of healthy controls demonstrated reduced α-diversity compared with other healthy controls. This clearly indicates that we are able to detect antibiotic-related effects in the data. This is corroborated by the linear regression analyses, where the use of antibiotics during the last year showed a significant association with reduced α-diversity, but with a much smaller beta compared with phenotype (PSC *vs.* healthy). Past antibiotics use did not show any affect within the PSC group alone, on neither α- and β-diversity nor composition. Unfortunately, the few studies that have investigated the long term effect of antibiotics are limited by small sample sizes.[217,218] Although antibiotics inflict long-term compositional changes, α-diversity measures, and especially Shannon diversity, seems quite resilient to these changes over time.[218] In Paper II we do not have information about antibiotics use. As antibiotics are known to reduce TMAO levels,[89] this unfortunately limits our ability to find any supportive data or draw conclusions from this paper. In summary the data still suggest

that antibiotics, to a lesser extent than PSC disease affect α-diversity. This also illustrates how long-term effects of antibiotics on the microbiota, and its host, will be important to investigate in larger prospective longitudinal studies in the future.

The reduced α-diversity in PSC compared with healthy controls was similar to the changes observed in UC controls. Reduced α-diversity is a key feature of IBD reported in several studies, as are changes in bacterial composition and function, both compared with healthy controls and between UC and CD.[87,219] This contrasts our observations in patients with PSC that showed similar α-diversity, β-diversity and composition irrespective of their IBD status. We saw the same when comparing PSC with UC or CD in subgroup analyses. In Paper II we also detect similar TMAO levels in patients with PSC irrespective of IBD-status, although UC controls showed consistently higher TMAO levels compared with PSC. We do not have data on the presence or degree of bowel inflammation at sampling in Paper I. However, the drug-use is rather limited in the cohort, corroborated by the sparse IBD symptoms in PSC patients reported in previous studies.[35,136,137] This indicates that the degree of bowel inflammation in our PSC patients is limited, and further suggests that the observed changes in the microbiota in Paper I are related to PSC and not bowel inflammation. Another possibility is that the inflammation is too sparse to elicit detectable changes in the microbiota, or that IBD may have been overlooked during screening in some cases and therefore potentially influences our results. One last possibility is that what we observe in PSC is a bowel inflammation that is truly different from that of IBD patients without liver disease, which may even be caused by the liver disease itself. This is corroborated by several clinical studies in PSC, and supported by findings from genetic studies, all together alluding to PSC with IBD as a possible unique disease entity.[2,5,35,136,137]

In Paper I we applied a robust two-stage study design, and got similar results using several different methods in addition to confirmation of several key findings using external analysis tools, indicating that the changes we observe are true differences, and not false positive results. We also reproduce key features of the gut microbiota in UC, *e.g.* reduced diversity,[219,220] and depletion of *Akkermansia*,[220,221] in addition to the association between Christensenellaceae and BMI.[126] All together this substantiates that our microbiota data are of high quality. The gold standard would however be replication in a separate cohort from another centre. Other studies on the faecal microbiota in PSC have not yet been published, and data from cholestatic disorders are scarce. There are however published several studies of the mucosal microbiota in PSC as shown in **Table 2**.

**Table 2. Comparison of published studies of the mucosal microbiota in PSC**

| | Rossen *et al.*[170] n (%) | Torres *et al.*[171] n (%) | Kevans *et al.*[#,169] n (%) | Quraishi *et al.*[172] n (%) |
|---|---|---|---|---|
| **PSC patients** | **12 (100)** | **20 (100)** | **19/12 (100)** | **11 (100)** |
| Males | 10 (83.3) | 16 (80.0) | 22 (71.0) | |
| Age (years) | 29.5 (23-56) | 47 (33.5-59.3) | 43/39* | |
| PSC+IBD | 12 (100) | 19 (95.0) | 31 (100) | **11 (100)** |
| PSC+IBD (active) | - (21) | 9 (45.0) | 0 | |
| PSC-UC | 8 (66.7) | 13 (65.0) | 31 (100) | |
| PSC-CD | 4 (33.3) | 6 (30.0) | 0 | |
| PSC-small duct | 1 (8.3) | - | 1 (3.2) | Data published as Letter, thus fewer characteristics were available. |
| Disease duration PSC | 2 (2-12) | 4 (2-12.3) | 1.3/3.0* | |
| Disease duration IBD | 10 (2-38) | 9 (4.8-18.9) | 15/2.2* | |
| UDCA treatment | 9 (75.0) | 10 (50.0) | - | |
| 5-ASA treatment | 8 (66.7) | 8 (40.0) | 16/4* (80/33*) | |
| Mayo risk score | - | 0.03 (-0.63-0.42) | - | |
| Child-Pugh (A/B/C) | 10 / 2 / 0 | - | - | |
| **IBD controls** | **11 (100)** | **15 (100)** | **18/12* (100)** | **10 (100)** |
| Males | 9 (81.8) | 9 (60.0) | | |
| Age (years) | 50.0 (37-67) | 48 (34.5-59.5) | | |
| UC | 11 (100) | 13 (86.7) | 18/12* (100) | |
| CD | 0 | 2 (13.3) | | |
| **Healthy controls (HC)** | **9 (100)** | **9 (100)** | **-** | **9 (100)** |
| Males | 7 (77.8) | | | |
| Age | 65.0 (50-70) | | *Not recruited* | |
| Description | Normal ALP† | Screening colonoscopy | | |
| **Methods** | | | | |
| Biopsies | Snap-frozen | RNAlater | Snap-frozen/RNAlater | |
| Region amplified | Microarray | V3-V4 | V4 | V3-V4 |
| Primers | - | 347F/803R | | |
| Sequencing method | - | MiSeq 2x300bp | MiSeq 2x175bp | MiSeq |
| Clustring/database | - | - | Closed reference/ GreenGenes | |
| **Alpha-diversity** | *vs.* HC | *vs.* HC and IBD | *vs.* UC | |
| Shannon index | Reduced in PSC | No difference | No difference | |
| Richness | Reduced in PSC | - | - | |
| Chao1 | - | - | Reduced in PSC** | |
| Observed species | - | - | No difference | |
| Phylogenetic distance | No difference | - | - | |
| **Beta-diversity** | | | | |
| | No difference (Hierarchical Clustering) | No difference (BC) | - | PSC differed from HC and IBD |
| **Taxa enriched in PSC** | | | *vs.* UC*** | |
| | | Barnesiellaceae<br>*Blautia*<br>*Ruminococcus* | | Lachnospiraceae<br>*Escherichia*<br>*Megasphera* |
| **Taxa reduced in PSC** | | | | |
| | Uncultured *Clostridiales II* | | | *Prevotella*<br>*Roseburia*<br>*Bacteroides* |

Continuous variables are given as median (interquartile range). "-" indicates that data is not available. †Indications for colonoscopy / diagnosis for HC: colorectal cancer screening, rectal bleeding, polyp surveillance / polyposis coli (n=2), diabetes mellitus (n=1), Parkinson's disease (n=1), neurofibromatosis (n=1), prostate cancer (n=1). [#]Cases and controls analysed according to recruitment centre due to geography confounding. *Oslo/Calgary, **only for Oslo cohort, ***none were significant after adjustment for multiple testing with FDR. 5-ASA, 5-Aminosalicylic acid; ALP, alkaline phosphatase; BC, Bray-Curtis; bp, base pair; CD, Crohn´s disease; FDR, false discovery rate; HC, healthy controls; IBD, inflammatory bowel disease; UC, ulcerative colitis; UDCA, ursodeoxycholic acid; *vs*, versus.

The largest study by Kevans *et al.* is an international multicentre-study that recruited UC patients with and without PSC.[169] The authors identified a clear geographical bias in their cohort according to recruitment centre, and analyses therefore had to be done on geographically matched controls, thereby reducing the number of PSC patients available for comparison considerably. Unfortunately, the results from the studies in general are hampered by the modest sample sizes and the lack of uniform control groups, illustrated by the lack of overlap in results. The use of different methods and control groups could to some extent explain these differences. For example, in the study by Rossen *et al.* the healthy control group consists of patients with diabetes, history of colon polyps, Parkinson's disease and prostate cancer.[170] Although the control group in Paper I were not screened for *e.g.* liver disease at inclusion, they were screened for several diseases before inclusion in the registry from which they were recruited by random selection. They are thus regarded as relatively healthy, corroborated by the modest medication use in the cohort. All together this indicates a limited disease- and medication-bias in this study group.

We did not perform age-, gender- and BMI-matching of cases and controls. Compositional features of the microbiota are however quite resilient to perturbations in adulthood.[222,223] Recent data from large European cohorts show that in the age-group constituting the large majority of participants in Paper I, age is positively correlated with α-diversity.[129,224] However, a decline in α-diversity is reported in the elderly, and can probably be explained by dietary changes due to entry into long term care rather than chronological age.[225,226] Data from murine models indicate gender-bias in microbiota studies,[227] contrasting the minor effect shown in human studies so far.[129,183,224] The latter is also corroborated by our results in Paper I. BMI however, is probably one of the most well documented variables associated with changes in the microbiota.[85,114,228] PSC patients and healthy controls differed only in regard to gender in the two primary study-panels in Paper I. We were further unable to detect any differences in BMI between any of the study groups in any panel. However, we did detected signals from BMI in the microbiota data in both linear regression and in the MaAsLin analyses. Overall, although adjusting for covariates in multivariate models cannot replace rigid case-control matching, this indicates that the potential confounding from age, gender and BMI on main findings in this study should be minor.

The problem with apparent lack of overlap between studies (*e.g.* those presented in **Table 2),** especially when it comes to differences in reported taxa, is well known in microbiota research.[88,228,229] Another research field that faced similar challenges in the beginning, and

thus could be used as guidance, is genetic association studies.[5] There are also other similarities; case-control design is the study design of choice, large number of variables are studied, several of the factors identified are detected in both cases and controls and the effect sizes are quite small. Two factors were crucial to obtain valid reproducible results in these studies: a large sample size and the introduction of a two-stage study design with a validation cohort.[230] When designing the study in Paper I we tried to take the experience from genetic studies performed in the past, into account. However, there is a risk of type II errors if we are to strict.[230] Lastly; although the data is not yet published, an abstract by Sabino *et al.* reported on the faecal microbiota of 52 PSC patients with age- and gender-matched controls from Belgium. They replicate several of the key findings in Paper I, *i.e.* reduced α-diversity in PSC, enrichment of the *Veillonella* genus, and similar microbiota in PSC patients irrespective of IBD status, alluding to the robustness of our results.[231]

### 8.1.1 PSC associated changes in the microbiota: cause or consequence?

If we believe that the observed differences are real, the next important question will be whether the changes we observe are primary, or secondary to disease or its treatment? Based on the data in this thesis we can only speculate on the answer.

In addition to the use of antibiotics discussed above, several other drugs could disturb the microbiota, but we were unable to detect such an effect. Moreover, PSC patients without any medication also showed reduced α-diversity, at the same level as other PSC patients. These observations are supported by data from studies demonstrating that host-targeted drugs alter the microbiota to a far lesser extent than antibiotics.[232] High quality trials on the microbiota-related effects of pharmacological agents are unfortunately scarce. 5-Aminosalicylic acid was used by 41% of the PSC patients in Paper I, and has been shown to affect microbial composition in irritable bowel syndrome (IBS), but with modest effect on α-diversity.[233] Data are scarce on changes in the microbiota from UDCA (used by one third of the PSC patients in Paper I). Prednisolone, the third most common drug in the PSC group, did not show any effect on diversity or bacterial composition in dogs, but human studies have so far not emerged.[234] Proton pump inhibitors (PPIs) were the most common drugs used by the healthy controls in Paper I. PPIs' effect on α-diversity is reported to be negligible, while it has more effect on β-diversity.[129,235] Importantly, none of the study groups differed in regard to PPI use, or the other frequent medications used by healthy controls (antihistamines and statins). On a final note, although we cannot exclude that the use of different pharmacological agents could have influenced the results, the effect on α-

diversity is probably minor, supporting a conclusion that PSC disease, to a far greater extent than pharmacological treatment, is associated with reduced α-diversity compared with healthy controls.

So, can changes in the gut microbiota occur before disease onset? The best published data addressing this issue in human diseases so far comes from type 1 diabetes (T1D). Of note, PSC patients are at increased risk of T1D, which is also an autoimmune condition.[86,236] In a study by Kostic *et al.* 33 infants, genetically predisposed to T1D, were followed throughout infancy with detailed clinical, dietary and microbiome mapping.[86] They identified distinct changes in the microbiota (*e.g.* reduced α-diversity) that appeared during the timeframe of seroconversion, but years before clinical disease was evident, as shown in **Figure 12**. This finding replicate an earlier, much smaller study by Giongo *et al.*[93] In line with this, animal models of diabetes show exaggerated immune responses to the commensal microbiota that have been associated with disease progression.[86,112] Interestingly, subsequent microbiota interventions in these studies were able to prevent such responses and pre-empt disease.[86,112]

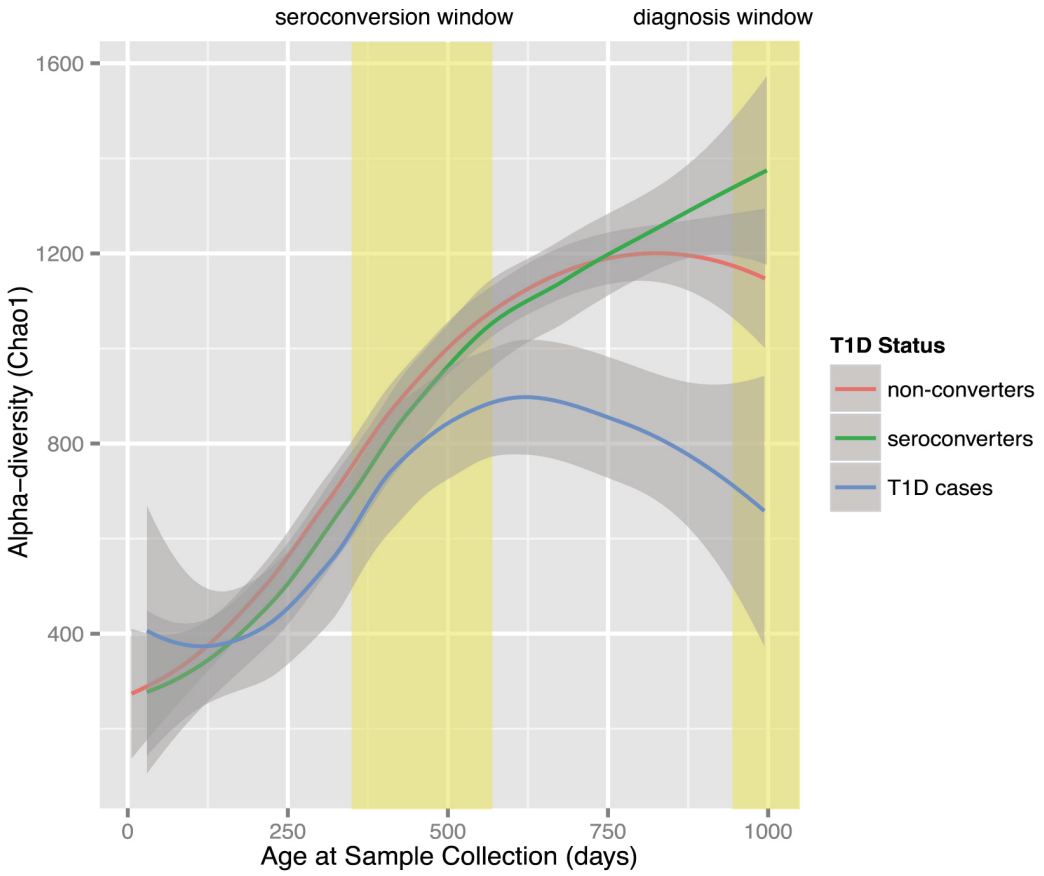



**Figure 12.** In young patients with T1D the normal increase in α-diversity halts, and subsequently drops at seroconversion, years before clinical disease onset. This contrasts the non-converters and seroconverters that do not develop disease. T1D, type 1 diabetes. Adapted from[86], © (2015), with permission from Elsevier.

A study of treatment naïve paediatric CD patients also identified several gut microbiota alterations before clinical onset of disease,[92] although this was not a prospective trial so diagnostic delay could have influenced the results. Together with data from several other autoimmune diseases, this indicates that changes in the microbiota, and maybe reduced α-diversity in particular, could play a pivotal role in development of human immune mediated diseases like PSC, and that this interplay unfolds long before clinical disease develops.[182] In line with this, an increasing amount of data indicates that microbial metabolites could be critical in balancing pro- and anti-inflammatory mechanisms in the host, *e.g.* SCFAs and vitamin B. SCFAs may do this by influencing the generation and differentiation of regulatory T ($T_{reg}$) cells, as shown in murine models with colitis.[237,238] Of note, PSC patients also demonstrate reduced levels of $T_{reg}$ cells compared with relevant controls (healthy and PBC).[238,239] Vitamin B metabolites act as ligands for mucosal-associated invariant T (MAIT) cells,[240] an important agent in liver inflammation.[72]

We were unable to detect any association between α-diversity and PSC duration, age at diagnosis or liver biochemistry. We were further unable to detect an effect from medications and antibiotics use during the last year before inclusion, as discussed earlier. As a result, this could imply that alterations in the microbiota represent a link between the gut and the liver and thus may be involved in development of PSC. Importantly, reduced gut microbial diversity is not a phenomenon related to neither autoimmune diseases nor liver disease in general, as *e.g.* rheumatoid arthritis, non-alcoholic steatohepatitis (NASH) and cirrhosis patients exhibit α-diversity at levels with healthy controls in other studies.[90,241,242]

### 8.1.2 PSC associated changes in the microbiota: do the taxa matter?

The identification of differentiating taxa in Paper I supports that the microbiota in PSC is different from that of healthy and UC controls, further supported by the ROC-AUC analyses showing how the abundance of just a few taxa could differentiate phenotypes with a fair accuracy, as shown in **Figure 13**, comparable to results reported in other autoimmune diseases.[90,92] We further identified an enrichment of the *Veillonella* genus in PSC. *Veillonella* has been associated with increased levels of LCA, a known vitamin D receptor ligand,[145] and several sphingolipids that may be of bacterial origin. Sphingolipids also act as antigens to natural killer T (NKT) cells, and are essential to NKT-cell homeostasis.[86,243] Interestingly, human bile duct epithelial cells are capable of presenting such lipid antigens to NKT-cells.[244]

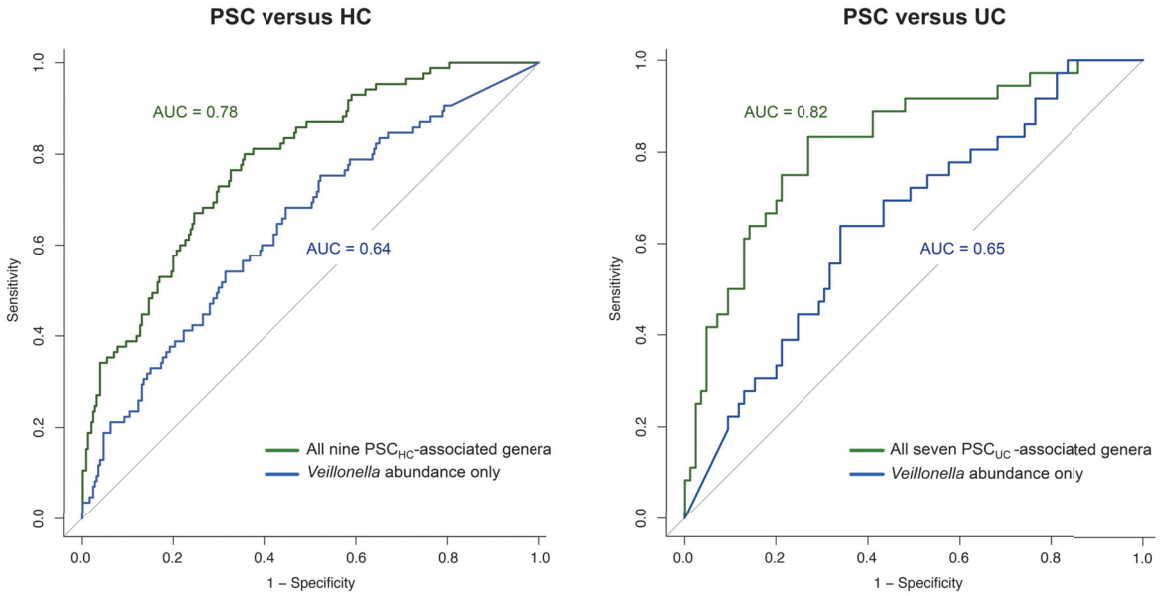



**Figure 13.** Data from Paper I illustrating how we can differentiate phenotypes with a fair accuracy (PSC *vs.* HC: AUC=0.78, and PSC *vs.* UC: AUC=0.82) using only the relative abundance of just a few taxa at the genus level that are altered in the PSC, supporting that PSC patients harbour a distinct gut microbiota compared with relevant controls. AUC, area under the curve; HC, healthy controls; PSC, primary sclerosing cholangitis; UC, ulcerative colitis. Reprinted with permission from BMJ Publishing Group under the author licence.

Of note, *Veillonella parvula* also contains genes involved in vitamin B metabolism, and can thus potentially produce MAIT cell ligands.[240,245] Overall this illustrates multiple possible pathways for *Veillonella*-host interaction potentially important in human bile duct disease. That the *Veillonella* genus could play a role in inflammation and fibrosis is supported by an association with several chronic disorders in humans, as listed in **Box 2**, with the majority being progressive and autoimmune diseases.

| **Box 2**<br>Disorders associated with the *Veillonella* genus |
|---|
| PSC (Paper I)[*] |
| Cystic fibrosis[246,247,*,†] |
| Idiopathic pulmonary fibrosis[248,†] |
| Systemic sclerosis[249,**] |
| Crohn's disease with ileal involvment[250,**] |
| Rheumatoid arthritis[#,90] |
| Liver cirrhosis[241,251,*] |

Material: *Stool. **Mucosa. †Lung. #Saliva.

The association with fibrosis is particularly prominent. Idiopathic pulmonary fibrosis and systemic fibrosis both resemble PSC in that they are progressive, idiopathic fibrotic conditions without a known cure.[248,249] Cystic fibrosis is, on the other hand, a well-recognised cause of secondary sclerosing cholangitis (**Table 1**). Over one-third of CD patients develop intestinal strictures, and interestingly *Veillonella* is associated with relapse in CD patients with ileal disease, a known risk factor for fibrotic stenosis in CD.[92,250,252]

*Veillonella* has further been associated with liver cirrhosis, and *Veillonella* abundance correlate with levels of hydroxyproline (a major constituent of collagen) in stool,[86] but the mechanisms behind these associations are not known. *Veillonella* was enriched in patients progressing to liver transplantation (only n=6) in our data, and correlated with the Mayo risk score. The lack of any association between *Veillonella* and duration of PSC disease or liver tests could suggest that the enrichment we observe in PSC is not only related to cirrhosis *per se*. That *Veillonella* is not specifically linked to cirrhosis is also supported by data demonstrating that the Veillonellaceae family is depleted in cirrhotic patients with NASH.[253] Whether *Veillonella* contributes to the pathogenesis in these diseases, and in that case how, or just thrives in a niche of the microbiota created by changed environment caused by several disease states, warrants further study.

Another genus, *Coprococcus*, was depleted in PSC patients compared with healthy controls in Paper I. This genus has been reported to also be depleted in healthy carriers of the AH8.1 HLA haplotype that is associated with PSC and several other autoimmune diseases.[254] Interestingly, *Coprococcus* has also been associated with reduced levels of TMA, the TMAO precursor, in mice. Moreover, it has been associated with increased levels 'good' fatty acids in humans (*e.g.* omega-3 fatty acids).[86,255] One could therefore speculate that the presence of this particular haplotype affects the microbiota and/or microbiota-related metabolites, and that they individually or together increase the susceptibility to disease like PSC, and that the reverse state might contribute to sustain health. Taken together this emphasises that it will be important to investigate further the interaction between genetics and the microbiota through clinical and mechanistic studies, if these relationships are to be elucidated.[126] This also reminds us of both the complexity of the host-microbiota-environment interactions we are observing, as illustrated in **Figure 14**, and further that one should exercise cautious when interpreting such results, as the majority are just single associations, and not based on mechanistic or longitudinal studies that have been replicated in several studies.[126,256]

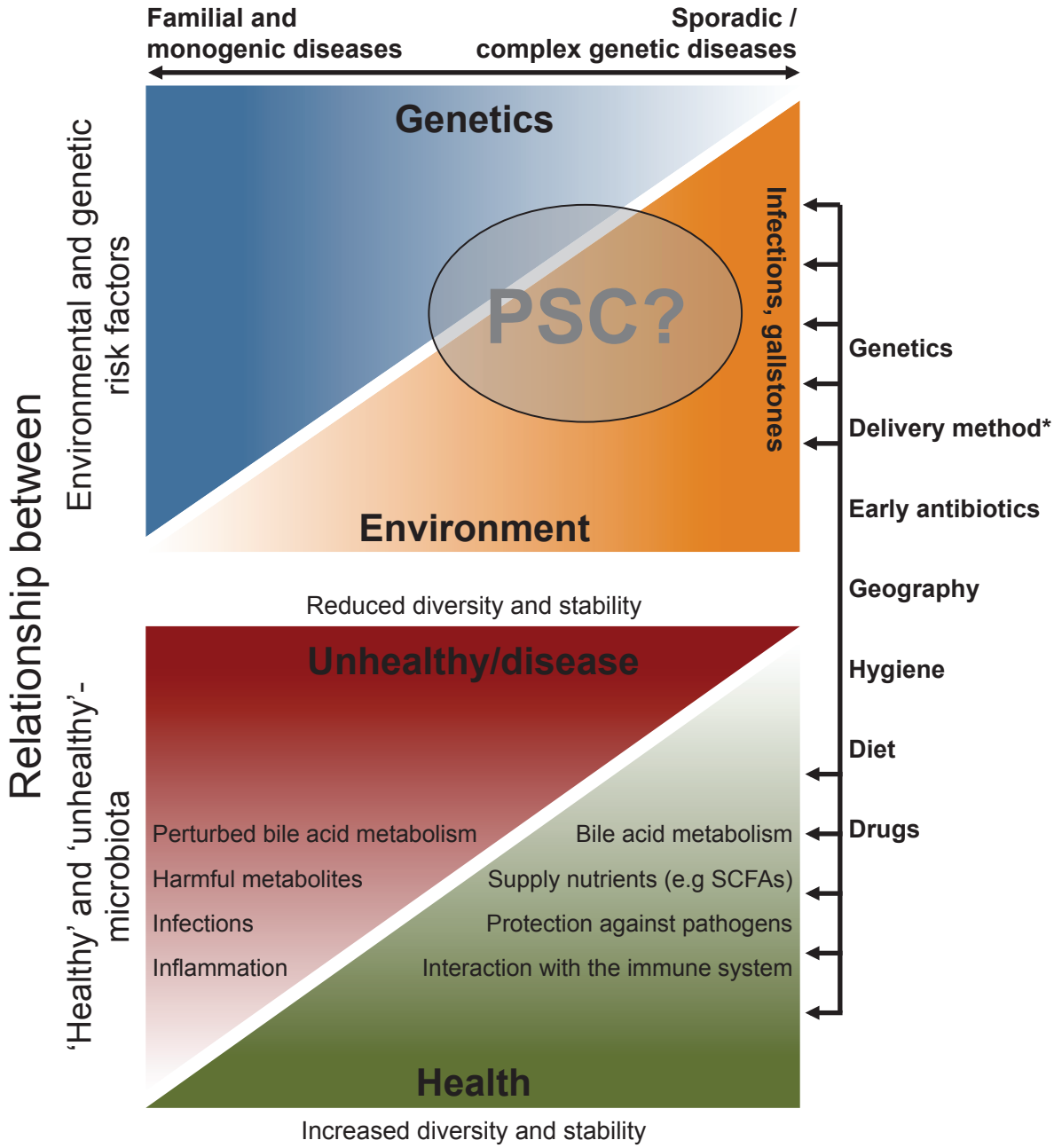



**Figure 14. Relationship and interactions between genetics, the host and the environment in sclerosing cholangitis**. The microbiota holds a key role as it on one hand constitutes an integrate part of the host; essential for development of proper immune functions and production of several essential key metabolites, and on the other hand modifies several other more classical environmental factors like drugs and diet *etc.* The gut microbiota could also be regarded as sole environmental factor. Exactly where PSC belongs in this spectrum is not finally decided, but dozens of risk loci collectively account for a minor fraction of the overall disease risk, leaving a large room for otherwise unknown environmental risk factors in disease development, *e.g.* the gut microbiota.[257] *Delivery method has a dramatic effect on the infant microbiota, but reverse effects are probably more unlikely.[258] PSC, primary sclerosing cholangitis; SCFA, short-chain fatty acid. The top part of the figure is inspired by[259].

Promising as these prospects of further research may seem, there are also arguments for caution in interpreting results based on genus-like taxa in 16S rRNA studies.

First of all; although the genera-level provides us with the best balance between resolution and specificity, it could still be considered a quite crude measurement, at least compared to the species- and serotype/strain-resolution that is often applied in medical microbiology. Each genus could also contain species and strains with possibly distinct functions and metabolic potential.[260]

Second, genus-like taxa categorised as 'unknown', constituting combinations of unknown-OTUs, warrant caution when making comparison between groups, as they do not necessarily contain the same OTU-composition. It is also important to remember that the taxa we report are only artificial constructs based on sequence similarity, dependent on a degree of similarity to reference data to determine taxonomic classification. Third, as we know that different primers have quite different qualities when it comes to 'catching' DNA from certain bacteria, this is important to bear in mind when comparing results from different studies. Collaborations with merging of sequencing data and new OTU-picking using a closed-reference approach (illustrated in **Figure 11**) could overcome this challenge. Another solution to this limitation could be re-sequencing of DNA using WGS. This would also help us overcome the last, and maybe most important point:

Lastly, although the gut microbiota composition shows huge variation between different individuals, the functional content is strikingly similar in comparison, as demonstrated in **Figure 15**. This illustrates that the 'natural selection' in the gut has a propensity for function over taxonomic affiliation.[85,86,183] The latter, in addition to a high diversity of methods in general use, probably also contributes to the lack of overlap in results reported from 16S based microbiota studies in several diseases, including PSC as shown in **Table 2**,[88,228,229] although the small sample sizes could be equally important.

Together these are strong arguments for performing metagenomic profiling (although there are limitations also to this method) of the microbiota in larger international collaborative projects. Thus we would increase resolution and thereby perhaps understand more of the different bacteria and their role in PSC disease and treatment, as demonstrated in *e.g.* type 2 diabetes.[229]

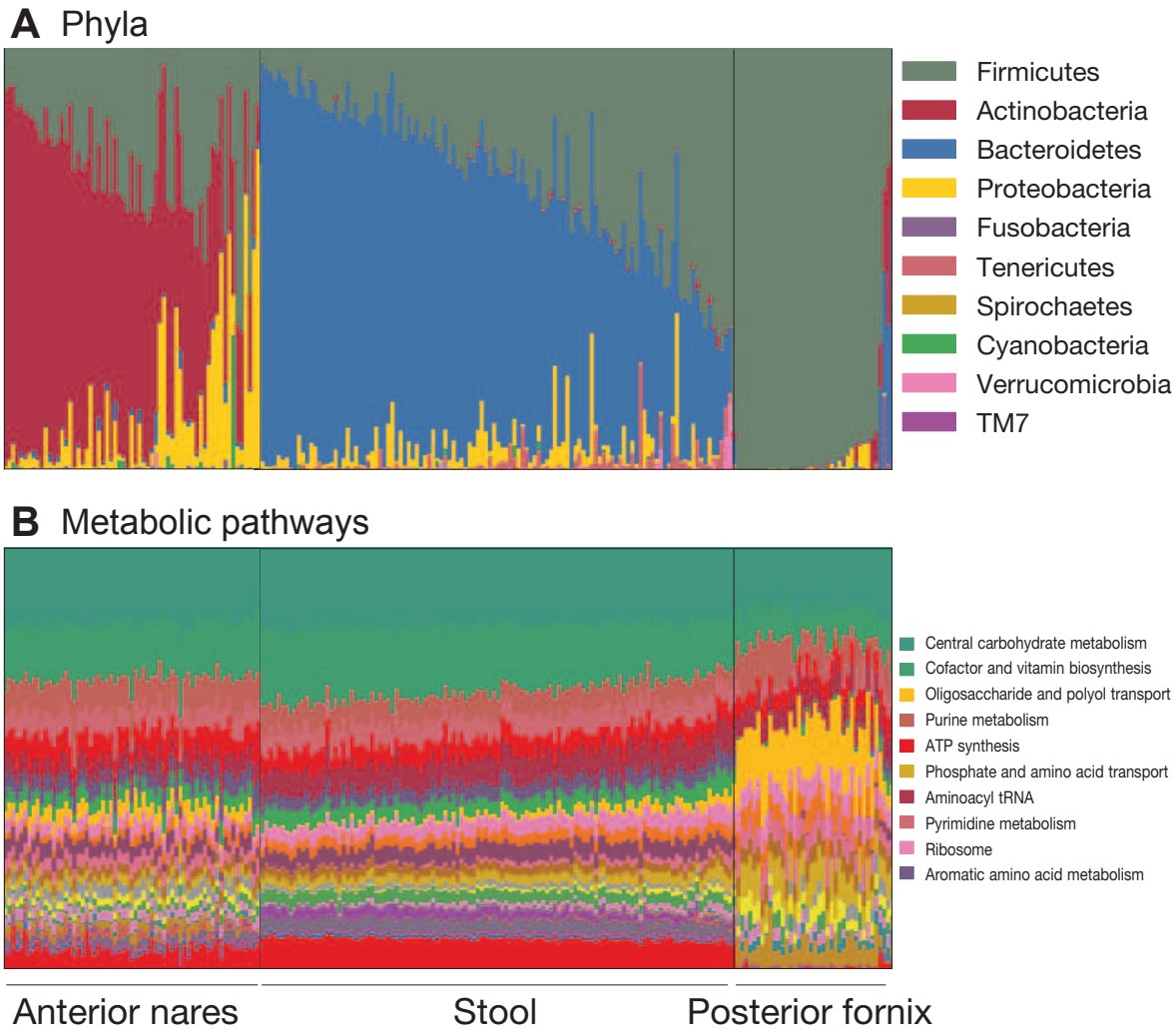



**Figure 15. Composition and function of the human gut microbiota.**

**(A)** The compositional structure of the gut microbiota on the phylum level showing large variability between participants. This contrasts the functional profile detected in the same samples **(B)** from the same participants that show a striking similarity.

The figure is based on microbiota data from The Human Microbiome Project Consortiums paper[183] on the Human Microbiome Project cohort constituted of 242 healthy individuals. Together with data from the European MetaHIT project (124 healthy individuals),[87] these two defining projects have greatly advanced our knowledge of the healthy microbiota in the gut and other body sites.

ATP, adenosine triphosphate; tRNA, transfer ribonucleic acid. The figure is adapted from[183], licenced under CC BY-NC-SA 3.0. Data from some sample-sites (retroauricular crease, buccal mucosa, subgingiva, tongue) have been removed for simplicity, as they show the same pattern.

It is probably unlikely that single bacteria act as solitary major contributors to complex autoimmune diseases like PSC. Another possibility, also supported by the literature, is that groups or networks of bacteria co-occurring in the host could contribute to disease, and to a further extent than single bacteria alone.[261,262] One example of such co-occurrence reported in human disease, *e.g.* dental plaques, atherosclerosis and IBD, is the combined enrichment of *Veillonella* and streptococci.[181,263,264] This co-occurrence might be explained by the production of lactic acid through fermentation by streptococci, which in turn is used as a carbon source by *Veillonella*.[181,263,264] Although not reported in Paper I, this association was also found in our PSC patients (Spearman's rho = 0.42, FDR corrected $p<0.01$). *In vitro* studies of *Veillonella parvula* (probably the most common *Veillonella* species in Paper I) have shown that the combination with streptococci greatly augments IL-8, IL-6, IL-10, and TNFα responses, which is suggested to be specific to this combination of bacteria.[265]

Both *Veillonella* and streptococci are abundant in the oral cavity, and the gut enrichment of such bacteria in liver disease could be explained by altered bile acid production, making the gut susceptible to invasion from such 'foreign' taxa.[251] It has been hypothesised that such displacement of oral taxa to the small intestines contributes to bacterial overgrowth.[251] Such small bowel bacterial overgrowth interestingly leads to biliary inflammation, bile duct proliferation and destruction, as well as fibrosis around the portal tracts in murine models, with extra-hepatic bile duct dilatations and ectasia, as is typically seen in human PSC.[47,163,164,251] Interestingly, these changes were ameliorated by antibiotics. Overall, these data give several possible explanations for the associations between PSC, other fibrotic conditions and *Veillonella.* The data also support that changes in the gut microbiota may play an important role in the pathological process of PSC, and that the role of specific bacteria should be investigated further in mechanistic studies. Hereto it should be mentioned that Björnsson *et al.* were unable to detect small bowel bacterial overgrowth in PSC patients.[266] However, this study is limited by the small sample size (22 PSC patients, 19 healthy controls).

In conclusion, we have identified several PSC-associated taxa that could potentially play a part in aspects of PSC pathogenesis. Thus, there is a need to further investigate the gut microbiota in PSC. This could *e.g.* be done through metagenomic studies using WGS. A complementary approach is to study the gut bacterial metabolome in order to reveal compounds potentially contributing to disease.[94]

## 8.2 Does the microbiota contribute to disease in PSC?

Despite all the possible mechanisms discussed above, the cross-sectional data from Paper I do not provide a definitive answer to the question whether the microbiota contributes to disease in PSC. Correcting for multiple testing we found no associations between the gut microbiota and duration of disease, duration of IBD, biochemical liver tests or severity of PSC (measured by Mayo risk score), except a reduction of unknown genera in the Clostridiaceae family and the duration of PSC disease. An approach to answer this question would be to perform a longitudinal prospective follow-up study with repeated microbiota- and blood sampling, and with detailed dietary mapping. To the author's knowledge no such studies have so far been published.

In an effort to gain some insight into this relationship, we investigated the microbiota-dependent metabolite TMAO in a cohort of PSC patients with up to 20 years of follow-up in Paper II. We identified a clear association between high TMAO levels in PSC patients with normal liver function and more severe prognosis (*i.e.* reduced transplantation-free survival). The association was independent of the Mayo risk score, and was still significant after adjusting for several clinical covariates using multivariate Cox regression. However, TMAO levels did not differ between PSC patients and healthy controls in the cross-sectional assessment when PSC patients with reduced liver function were excluded. This may be true, but it is possible that we are unable to detect a difference because the control group is too small (n=99). The lack of age- and gender-matching to some extent also limits our ability to detect such differences.

The observation that TMAO levels are affected by liver function, and that this could be a result of a deteriorating capacity to transform TMA to TMAO in the liver by hepatic FMOs, is supported by several previous studies.[96,267–269] We used INR and the older Normotest to assess liver function. It could be argued that increased prothrombin time might not be the optimal marker of liver function in cholestatic liver diseases like PSC, although it is widely used for this purpose in Norway and it is also part of the Model for end-stage liver disease (MELD) score. The major objection is probably the possibly impaired absorption of fat-soluble vitamins like vitamin K that could influence INR levels, but his should not be a major issue until very late stages of disase.[270] The fact that diet and the gut microbiota are essential sources of vitamin K and thus possible confounders of INR measurements in Paper II, also highlights the lack of detailed baseline data on these characteristics in this paper.

However, it is not unreasonable to suggest that TMAO will be reduced in advanced liver disease, as it is reported in several other studies, although it makes it less attractive as a biomarker. As a consequence of these considerations we decided to perform all subsequent analyses on the subset of PSC patients with normal liver function. Such sub-group analyses could be problematic, especially in randomised trials, but are a lesser problem in the cross-sectional design of Paper II and the 'PSC-only' analyses, beside the corresponding loss of power.

Patients with PSC could be more vulnerable to exposure from microbiota-derived molecules since cholangiocytes from PSC patients *in vitro* show signs of hypersensitivity to PAMPs such as lipopolysaccharide (LPS) originating from gram-negative bacteria.[160,271] p-ANCA, the most common antibody in PSC, reacts to beta-tubulin isotype 5 (TBB-5) as autoantigen and TBB-5 shares a high degree of structural homology to a bacterial protein called FtsZ, and antibodies cross-react between the two.[46,161] p-ANCA status did not affect microbial composition or α-diversity in Paper I. Of note, FtsZ is common also in healthy controls and patients with PSC seem to harbour antibodies towards both TBB-5 and FtsZ, alluding to an abnormal immune response to gut microbiota-related molecules in susceptible individuals.[161] Bacterial diversity is considered highly important for the immune system to evolve properly and obtain tolerance to antigens originating from dietary sources, the microbiota and oneself.[130,272] Thus, it is possible that the reduced α-diversity in PSC patients reported in Paper I could contribute to abnormal immune responses like the one presented above.

TMAO levels are associated with PSC disease progression in Paper II. Both TMAO and choline leads to progressive fibrosis in extra-intestinal organs in mice.[273] Further, data from animal models show that TMAO and related methylamines (*e.g.* TMA) induce cholestasis, cholangiocyte proliferation and cholangiofibrosis in rats.[274] These are all pathological changes that are hallmarks of human PSC,[2] and it could therefore be suggested that prolonged exposure to such metabolites may contribute to sustain disease progression in PSC patients, and thus explain the poor prognosis associated with high TMAO in Paper II. Another interesting observation in this regard is that methylamine is important for facilitating the aberrant homing of 'gut primed' lymphocytes to the liver seen in PSC.[152,153,155] This is promoted by increased expression of VAP-1, a primary amine oxidase that uses methylamine as substrate,[152] which shows increased expression in PSC patients during intestinal inflammation.[151,154] Moreover, feeding mice methylamine also leads to

VAP-1-dependent expression of MAdCAM-1 in the liver *in vivo,* enhancing recruitment of mucosal lymphocytes to the liver.[155] Manipulating the microbiota with antibiotics, *e.g.* metronidazole (in combination with UDCA) or vancomycin, has also been shown to reduce alkaline phosphatase in PSC patients, but the mechanisms behind this are elusive.[166,167] TMAO has also been shown to influence cholesterol and bile composition and lower bile acid synthesis- and transport-proteins, overall illustrating one of several potential mechanisms for the association between TMAO levels and prognosis in PSC.[89,95,96] Collectively, these data suggest a potentially central role of the microbiota or dietary- or microbiota-dependent metabolites like TMAO and related amines in the pathogenesis of PSC, and could hypothetically explain the association between high TMAO levels and reduced transplantation-free survival in Paper II. In addition, there are data suggesting that increased intake of meat with much phosphatidylcholine (the major dietary source of TMAO), is associated with increased risk of PSC, with the highest risk seen in PSC patients with concomitant IBD.[89,275] Unfortunately, there is a lack of microbiota and dietary data in Paper II. Hence we are unable to identify potential microbial factors that could further explain this association, or find clues to dietary factors that might be harmful contributors to the disease process in PSC.

TMAO has been closely associated with atherosclerosis and clinical endpoints related to cardiovascular disease in several studies.[89,95,99] The poor prognosis of PSC patients with high TMAO was, importantly, not caused by increased cardiovascular burden in these patients. Less than 5% of deaths during follow-up were due to cardiovaskular disease. This is consistent with the observation that PSC patients in general are not at increased risk of ischaemic heart disease.[236] Moreover, TMAO has also been shown to increase dramatically after bariatric surgery, which is intriguing, since it is an intervention shown to reduce risk of cardiovascular disease in several studies.[276] In conclusion this demonstrates that the mechanisms behind the associations between TMAO and cardiovascular disease are not fully understood. It also illustrates the challenges we face in the process of disentangling mechanisms behind clinical co-occurrences, and again remind us that association does not equal causality.[256]

The enzyme mainly responsible for converting TMA to TMAO in murine and human livers, *Fmo3/FMO3*, demonstrates significantly reduced expression levels in males compared to females.[277] In mice the reduction in *Fmo3* levels are primarily due to downregulation by androgens, *i.e.* 'male' sex hormones.[277] Correspondingly, male mice demonstrate increased

plasma levels of TMA, consistent with a reduced capacity to synthesise TMAO from TMA due to reduced *Fmo3* expression.[277] Data further indicate that this increase in TMA is not microbiota-dependent.[277] It is not known whether humans show the same TMA gender-profile with higher levels in males compared to females. However, this would be an interesting lead to explore considering the well-known increased PSC-risk in males. In this regard it is important to note that TMAO levels in our PSC patients did not differ between males and females.

The lack of dietary data and history of antibiotics-use at sampling limits our ability to conclude further on the cause of these associations, but overall the results still indicate that TMAO could be a marker of disease progression. Prospective clinical trials and mechanistic studies are needed to elucidate these latter points further. Interestingly, a recent study by Wang *et al.* proved how targeted inhibition of bacterial TMA production is possible, and importantly, this was possible without bactericidal effects.[278]

Several other microbiota-dependent metabolites besides TMAO could also be important to both local and systemic inflammation.[279] SCFAs are among the most abundant dietary metabolites produced by gut bacteria and appear to have anti-inflammatory properties in multiple immune cells, in addition to a possible important role in regulation of intestinal barrier function.[69,280] Of note, members of the Clostridiales order are the major SCFA producer in the large intestines, and constitute the majority of depleted taxa in PSC patients in Paper I, as is also reported in IBD.[246,281] One could therefore speculate that this depletion could contribute to an increased susceptibility to both intestinal, and potentially also hepatic, inflammation in PSC. Although the studies of the mucosal microbiota in PSC show contradicting results in this regard (**Table 2**), one may argue that the faecal microbiota could elicit a much greater metabolic effect on the host due to its enormous size compared with the mucosal microbiota, especially when it comes to volatile compounds like SCFAs.

Another group of bacteria-derived metabolites that may be of even greater importance in liver diseases like PSC, are secondary bile acids produced by the gut microbiota, *e.g.* LCA and deoxycholic acid (DCA). Bile acids have several immune-regulating functions in the gut and the liver.[151,271] Decreased bile acid deconjugation and modification could also contribute to chronic inflammation, as is shown in IBD.[150] Dietary induced changes to bile acid composition has also been shown to greatly affect colitis susceptibility in mice.[282] It is therefore interesting that several members of the order that contributes most to differences

in taxa between PSC patients and healthy controls in Paper I (Clostridiales) are capable of metabolising bile acids and produce secondary bile acids.[171] A study by Trottier *et al.* showed reduced levels of secondary bile acids in serum of PSC patients compared with controls, in contrast to patients with PBC.[283] Overall, this could reflect a more PSC-specific change in the gut-liver-axis, involving changes in bile acid. Of note, secondary bile acids have anti-inflammatory properties *in vitro*, and data from the *Mdr2*$^{-/-}$ mouse model show that absence of commensal microbial metabolites, *e.g.* secondary bile acids, in GF mice results in exacerbated fibrotic biliary disease.[109,284]

There is no 'perfect' animal model for PSC. Thus we have to utilise different models to explore different aspects of the disease.[101] NOD.c3c4 mice do not develop bowel inflammation, and share characteristics with the human biliary disease PBC.[285] This might have contributed to the modest use of this model in PSC research so far.[101,102] However, NOD.c3c4 mice develop extra-hepatic disease, more typical of PSC, in addition to cystic dilatations of the bile ducts and prominent neutrophil infiltration, which is atypical of PBC.[102] It is also a polygenic autoimmune model. This could be argued to be an advantage compared to toxic and acute models, as it may share more features with PSC, especially in light of what we have learned from genetic studies in PSC.[5,105,123,257] In summary, it is reasonable to conclude that the NOD.c3c4 mice represent an acceptable model for the study of largely immune-driven inflammatory, polygenic cholangiopathies, although it is important to have its limitations in mind when interpreting results.[102,105]

In Paper III, the biliary disease of NOD.c3c4 mice were mitigated when raised in a GF environment, with less extra-hepatic bile duct dilatation, less biliary inflammation and less CD3-positive cell infiltrates around the intra-hepatic bile ducts. In experiments in conventionally raised (CONV-R) animals we also demonstrated large differences in over-all bacterial community composition (*i.e.* β-diversity, unweighted UniFrac) between NOD.c3c4 and control NOD mice in consecutive experiments, and with similar results in both caecal mucosa and contents. In conclusion, these data suggest that the commensal microbiota contribute to disease in this murine model of biliary inflammation.

However, there was no overlap in the differentiating taxa between these experiments. Since the large differences in microbiota between the phenotypes were quickly re-established after rederivation, and the differentiating taxa detected were not the same in each experiment, this could suggest an effect from the biliary phenotype. While this might well be true, it is

probably more reasonable to argue that the experiments are underpowered in regard to detecting differences in relative abundance, to which β-diversity is a more robust measure. In light of this one should probably keep some restraint in interpreting these differences in taxa, although several of them, especially in the Clostridiales order, are associated with both bile acid- and TMA/TMAO-metabolism.[95,171,278,286]

It could also be argued that NOD-mice are potentially problematic control animals, although they genetically are the best alternative, since most NOD mice develop spontaneous T1D.[105,112] Hence it is possible that we detect some differences in the microbiota due to diabetes in NOD mice. The incidence of spontaneous T1D in NOD mice is not 100%, and can be affected by the microbial environment in the animal facility.[112] Still, after birth the microbiota of NOD mice is similar to 'regular' C57BL/6 mice,[287] and the overall bacterial community in NOD mice is quite stable over time, especially compared with the marked differences we observe in β-diversity.[288] Importantly, the NOD mice used in the microbiota analyses were harvested before the development of diabetes, as confirmed by blood glucose measurements. Overall, the risk of confounding from T1D-development in the NOD mice in this study should be minor, although we cannot rule out subtle effects.

The *Mdr2*$^{-/-}$ mouse develops liver fibrosis due to regurgitation of bile into portal tracts and a following induction of portal inflammation and fibrosis, probably due to a direct and harmful effect on the biliary epithelium from bile acids.[102] It could therefore be argued that the *Mdr2*$^{-/-}$ model is a primarily fibrosis-driven model, where a 'toxic' effect on the bile duct epithelium is the dominating driver. This contrasts the primarily immune-driven NOD.c3c4 model used in Paper III, and could thus possibly explain some of the differences in phenotypic development observed in these models when raised in GF environments.

Data from both thioacetamide-treated and carbon tetrachloride-treated mice support the fact that models where fibrosis is a key pathological element show exacerbated disease under GF conditions.[289] These models also display increased liver fibrosis when raised GF, with hepatocyte apoptosis and increased activation of hepatic stellate cells, a known key mediator of hepatic fibrinogenesis, but with no detectable enhancement of inflammatory responses.[289]

We detected less dilatation of the common bile duct in GF NOD.c3c4 mice at nine weeks of age compared with their CONV-R counterparts, a difference that to some extent diminished

at 18 weeks, mainly due to larger dispersion in both groups. The opposite pattern was observed for the inflammatory aspects of the disease, where differences were more pronounced at 18 weeks. Bile duct damage in NOD.c3c4 mice is detected as early as at three weeks of age.[105,290] Data from CONV-R NOD.c3c4 mice indicate that while B-cells play a minor part in this process, they are important in the development of the inflammatory aspects of the disease, which develops at a later stage (>9-12 weeks).[105,290] The prevalence of autoantibodies against *e.g.* ANA and PDC-E2, also increase with age, especially after ~10 weeks, although the prevalence of PDC-E2 is only 53% (in human PBC it is detectable in 90-95%).[105,290] ANA, which is common also in PSC, is detectable in the minority of NOD.c3c4 mice at nine weeks, but the prevalence of ANA increase almost exponentially with age.[46,105,290] In summary, this could partly explain why the results to some degree 'intersect' with increasing age.

The use of GF animals has greatly advanced our knowledge of the microbiota and its effect on its host.[291] There are however some limitations. Since GF mice lack bacterial metabolites and stimuli from birth, they have marked changes in their immune system and enteric nervous system, in addition to reduced mucosal surfaces and organ-size differences when compared with their CONV-R counterparts, as illustrated in Paper III.[70,108] GF animals also have lower fat-mass and weigh less than CONV-R animals, due to altered lipid metabolism.[69] Co-housing experiments, microbiota depletion with non-absorbable antibiotics and metabolomics can be used as complementary approaches.

Although the use of GF mice in Paper III gives strong support for a contribution from the microbiota on the biliary disease in NOD.c3c4 mice, we are limited in our ability to discern whether this is due to *e.g.* absence of bacterial metabolites (as suggested to be important in the *Mdr2*$^{-/-}$ mouse model), changes in bile acid composition or microbiota-dependent changes to the immune system.

## 8.3 Lack of dietary data: the Achilles' heel of microbiota-related studies?

As illustrated by several of the topics discussed above, the most important limitation to Paper II is the lack of dietary data. Although such data would have been of great value also in Paper I, the exclusion of participants who stated that they followed a specific diet, to some extent prevents dietary confounding.

The role of diet and other environmental factors in shaping the gut microbiota remains largely unknown.[129,224] The long-term dietary pattern of healthy individuals is still probably one of the most important drivers of gut microbial communities.[292] Diet is also a potential important driver for differences observed with *e.g.* geography, season and old age. In recent large-scale surveys diet explains ~6% of the variation in the overall gut microbiota composition (Bray-Curtis dissimilarity).[129,224] However, these data also indicate that dietary changes have more impact on β-diversity than α-diversity, the latter being the most prominent affected feature of the gut microbiota in PSC patients in Paper I. Of note, dietary elements associated with a Western-style diet; high total energy intake, snacks, sugar-sweetened soda and high-fat milk, were all associated with reduced α-diversity in the a recent population-based study.[129]

Only excluding patients with specific diets, as we did in Paper I, is a pragmatic approach in an effort to remove participants with a known distinctly deviant dietary pattern compared with the general population, but makes us unable to correct for more subtle effects in the subsequent analyses. While this could be considered a reasonable trade-off to secure a large sample size and increase statistical power, and at the same time including dietary data at an adequate qualitative level, it also prevents us from discovering potentially important drivers of the skewed bacterial profile we observed in PSC, and we cannot exclude that that systematic differences in dietary patterns have affected the results in both Paper I and II. The confounding risk is probably greatest in Paper II, as there is also a lack of a strict fasting blood sampling protocol. There are however experimental data suggesting that major postprandial TMAO-increases are primarily related to dietary challenges with *e.g.* phosphatidylcholine, because the majority of choline in ordinary meals will be reabsorbed in the small intestine before reaching the TMA-generating bacteria in the colon.[293] As fasting status has minimal impact on other common liver function tests, this should be less problematic.[294]

In conclusion, the confounding potential of diet is considerable, and as both diet and microbiota show intricate and complimentary roles in the interplay with each other, and are both important to both host immune-tolerance and inflammation, including such data in further studies should have considerable priority.[238,295] On a final note, as individual effects from dietary substances are probably small, but the additive effect sizes could be substantial, this again underscores the importance of increasing the sample size in future longitudinal studies.[129,224,281,296]

## 8.4 The future: could our findings have clinical relevance?

There are no effective medical therapies available in PSC, and it is therefore natural to reflect on how our findings could be used in exploring new therapeutic options.[2]

**Antibiotics** have a long tradition as a way to manipulate the microbiota in PSC.[165] Vancomycin has shown maybe the most promising results in paediatric patients.[168] As mentioned earlier both vancomycin and metronidazole (in combination with UDCA) have been evaluated in recent trials that have demonstrated reduction of ALP.[166,167] Rifaximin, an oral non-systemic antibiotic, on the other hand, did not have any effect in PSC in a recent pilot-study.[297] The discrepancy in these results might depend on differences in anti-microbial spectrum or what microbiota-dependent metabolic changes they elicit. So far none of these studies have shown a long-term benefit of antibiotics on hard endpoints like liver transplantation or death, but collectively, the data suggest that manipulation of the gut microbes could potentially influence the disease process, but by unknown mechanisms. One possibility is altered bile acid homeostasis in PSC. Others are effects on immune activity, fibrosis or production of bacterial metabolites like TMAO and related metabolites. These are all reasonable candidates that should be explored in future treatment trials.

However, from an ecological point of view, and in a clinical setting with escalating bacterial resistance to known antibiotics and with few new antibiotics in recent decades, long term treatment with antibiotics is not a very appealing alternative.[130,298] In addition, there is an increasing awareness of the underestimated biological cost of antibiotic treatment, especially early in life.[182,272]

An alternative might be to develop highly specific antibiotics against specific pathogens in the future that do not damage essential symbiotic microbial species, and preserve community structure and function. Unless we achieve this, we are nevertheless left with the last, and maybe most important objection: antibiotics reduce α-diversity, the most prominent feature of the 'dysbiosis' observed in PSC. One could argue that the logical goal would be to increase diversity, and thus restore a 'normal/healthy' microbiota (also see **Figure 14**). There are several relevant strategies one could pursue in order to achieve this.

**Probiotics and prebiotics** could be more appealing alternatives, compared to antibiotics. For example, it could be tempting to increase the relative depletion of Clostridiales detected in PSC. As discussed, this might be premature as we would need additional data to pin-point

more specifically what bacteria that are at play, or else the search for the right probiotic could be long. Probiotics have been tested unsuccessfully in PSC previously,[299] but this might be due to exactly this kind of lacking specificity. Studies have lately demonstrated promising strategies for overcoming this challenge; Atarashi *et al.* selected a mixture of Clostridia strains based on their high propensity in enhancing $T_{reg}$ cell abundance and inducing anti-inflammatory molecules.[113] They were further able to demonstrate that this mixture could attenuate disease in animal models of colitis and allergic diarrhoea. Similarly, Buffie *et al.* identified a single human-derived bacterial species (*Clostridium scindens*) that conferred resistance to *Clostridium difficile* infection in mice.[300] Of note, this was due to *C. scindens´* production of metabolites from host-derived bile acids inhibiting *C. difficile*. Data from IBD and dietary enteropathy indicate that a patient's own intestinal IgA response could potentially be used to identify commensal bacteria essential to disease pathology, and that targeted elimination or restoration of such bacteria may reverse or prevent disease development, showing how individual microbiota profiling could be the next step in personalised medicine.[301] On a final note, as we are beginning to understand how diet affects people quite differently, this could hopefully increase specificity in dietary interventions, and could potentially facilitate an increased success-rate in future clinical application of *e.g.* dietary compounds used as prebiotics.[281,296]

**Faecal microbiota transplantation (FMT)** as treatment in PSC has so for not been tested in published trials, but could be a potential alternative in the future. It has been used to restore a healthy gut microbiota in *C. difficile* colitis with excellent results.[302] FMT has been explored in UC and differential response according to pre-treatment microbiota profile has been indicated.[139,303] The results also indicate that some microbiota donors have more 'favourable' properties than others, showing that we need to move towards more standardised regiments in FMT interventions.[139,303] Data also suggest that to achieve remission of colitis, treatment early in disease course might be of utmost importance, and this could be a challenge in PSC.[303]

**Clinical biomarkers.** As demonstrated in Paper I only a few taxa were necessary to differentiate PSC from both healthy controls and UC patients. There are room for improvements in regard to confounding factors, but it is reasonable to suggest that more accurate profiling and probably also metagenomics and/or microbial metabolomics could improve such profiling in the future, as shown in other diseases like *e.g.* type 2 diabetes, CD and rheumatoid arthritis.[90,92,229,304] In type 2 diabetes metagenomics profiling showed better

performance at predicting disease than other established risk factors combined, and performed equally well as most published risk-score systems.[304] This suggests that profiling of microbiota or related metabolites might be valuable in the future also for predicting PSC disease. As changes in the microbiota could be present before clinical disease is evident, as shown in T1D,[86] this could potentially be used to identify PSC patients earlier than today *e.g.* by testing groups with increased risk of PSC, like family members of PSC patients and patients with IBD.

Several challenges exist in the clinical follow-up of PSC patients. The disease course is variable and there is a lack of tools to evaluate the patients according to severity, disease activity and progression. All these factors are important to select the right follow-up and treatment for the individual patient.[253] As shown in Paper II, microbiota-dependent metabolites, *e.g.* TMAO, could be associated with disease progression in PSC. Taken together, these data suggest that an integrated analysis of factors reflecting different aspects of the pathophysiology (*e.g.* fibrosis, inflammation and microbiota) could contribute to personalised medicine by helping us classify patients according to stage and activity by the identification of biomarkers in blood samples, or possibly also stool samples.[305–307]

As there is also a lack of adequate tools for measuring treatment response in PSC, one could imagine that changes to the microbiota or related metabolites could be useful in this regard, as shown in *e.g.* rheumatoid arthritis. Zhang *et al.*[90] performed profiling of gut-, dental- and saliva-microbiomes in a cohort almost the size of the cohort in Paper I, but with less healthy controls. They were able to distinguish patients with rheumatoid arthritis from healthy controls, and importantly, these changes correlated with clinical measures and could be used to stratify individuals on the basis of their response to therapy after only three months. So far this has not been demonstrated in PSC, but alludes to potential benefits from clinical application of microbiota-profiling in the future.

# 9 Conclusions and key challenges for the future

This thesis has explored the gut microbiota in PSC. The faecal gut microbiota of PSC patients has several characteristics separating it from both healthy controls and patients with UC. The most prominent difference between PSC and healthy controls was a markedly reduced bacterial α-diversity, in addition to compositional changes involving several taxa. Using this 'microbial signature' that we detected in PSC patients, it was further possible to differentiate PSC from healthy controls and patients with UC without liver disease with fair accuracy. In contrast, the microbiota in PSC patients with or without IBD were highly similar. That patients with PSC harbour a distinct microbiota could indicate a role of the microbiota in PSC disease. This was supported by a strong association between high levels of a diet- and microbiota-dependent metabolite, TMAO, and shorter transplantation-free survival in PSC patients with normal liver function at sampling. That commensal bacteria can contribute to biliary disease was corroborated by data from a study in mice, were we identified a clear change in the microbiota of mice with biliary disease compared with control mice, and further showed that the mice with biliary disease demonstrated an ameliorated phenotype when raised in a GF environment.

To conclude, our findings suggest that complex dietary and microbial factors are relevant for the underlying pathogenesis in PSC, as well as disease development and prognosis. This also provides a strong rationale for further studies of the microbiota related to pathophysiological mechanisms in disease development. Lastly, this also supports the possibility of microbiota-related clinical utility in PSC, where interventions targeting diet or the gut microbiota could ultimately have the potential to improve patient care.

As emphasised several times in this thesis, a natural next step for descriptive studies of the microbiota in PSC will be to investigate the function of the microbiota using metagenomic methods, in addition to expanding 16S-based studies. It will also be of importance to explore the microbiota of the upper gastrointestinal tract, as both the microbiota and the immune system show large variability throughout the intestines, potentially playing different roles in PSC pathogenesis. When performing these studies, dietary surveys and incorporation of these data in analyses will be paramount. A complementary approach would be surveys of microbiota-dependent metabolites in serum or tissue as part of

metabolic phenotyping, which could be useful not only in PSC, but also in other liver diseases.[306,307]

By moving to functional profiling of the microbiota we could probably also overcome some of the challenges of geographical bias which has been apparent in previous studies on the microbiota in PSC.[169,229] This would facilitate collaboration, which will probably be essential if we are to increase sample sizes in the future, something that should be considered of utmost importance. As we increase statistical power we will also be able to explore important subphenotypes like small duct PSC, and to further explore the relationship between PSC and IBD.

The establishment of collaboration-consortia has proven key to increasing the size of study cohorts in research in general, including PSC,[57,308] and the microbiota community in different fields would probably benefit from the same strategy. This would hopefully also facilitate some standardisation of methods. Fortunately, there are already on-going efforts to address some of these challenges.[309] Such collaboration could hopefully also promote clinical trials exploring interventions targeting the microbiota or related processes in PSC. In addition, prospective and longitudinal trials will be important, to detect changes in the microbiota and how they relates to disease progression in PSC. Sampling of individuals with high risk of developing PSC, with follow-up to potentially detect pre-clinical microbiota changes in PSC would also be highly interesting, but will be challenging to perform. Last, but not least, it will be important to continue and evolve the study of the microbiota and related metabolites in different animal models and mechanistic studies, also building on previous findings.

All these efforts could be important if we are to achieve what should be our most important common goal: better care and treatment options for patients with PSC.

On a final note, although not a theme for this thesis, it will also be important to further explore the role of the other inhabitants of the gut, *e.g.* archaea, viruses, parasites and fungi and the interplay between these 'biomes', if we are to better understand how they interact with us in health and disease.

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(*online only*) Articles published online in their final form that will not be printed. A single page-designator is given by the journal, replacing the page-range.


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