Primary sclerosing cholangitis: Surrogate markers of natural history, disease severity, and prognosis

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Thesis for the degree of Philosophiae Doctor (PhD)
University of Bergen, Norway
2023

UNIVERSITY OF BERGEN

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Thesis for the degree of Philosophiae Doctor (PhD)
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Date of defense: 01.12.2023

Year: 2023

Title: Primary sclerosing cholangitis: Surrogate markers of natural history, disease severity, and prognosis

Name: Guri Fossdal

Print: Skipnes Kommunikasjon / University of Bergen

Contents

Scientific environment

The following work was conducted between 2019 and 2022 while I was enrolled in the PhD programme at the Department of Clinical Science, Faculty of Medicine, University of Bergen (UIB), under the supervision of Professor Mette Vesterhus (main supervisor). Professor Eystein Husebye from the Dept. of Clinical Science, UIB, Professor Tom Hemming Karlsen, and PhD Trine Folseraas from the Norwegian PSC Research Centre (NoPSC) at OUS Rikshospitalet, Oslo, acted as co-supervisors.

The research project was carried out in the clinical liver research group at the Department of Medicine at Haraldsplass Deaconess Hospital and at the Department of Clinical Science, University of Bergen, in close collaboration with NoPSC, OUS Rikshospitalet. NoPSC is internationally acknowledged as a leading centre for research on primary sclerosing cholangitis (PSC). We retrieved data and biological samples from the NoPSC biobank and the research registry and biobank for the Network for autoimmune liver disease, established in 2019 and coordinated from Haraldsplass Deaconess Hospital. We also had a close collaboration with professor emeritus Rolf Berge from the Energy and Lipid Metabolism Research group at the Dept. of Clinical Science. Analyses of fibrosis markers and metabolites were performed at UCL & the Royal Free Hospital, London, UK; Nordic Biosciences, Herlev, Denmark; and Bevital AS, Bergen.

The project was funded by a PhD grant from Helse Vest. NoPSC and the Halloran Family Foundation funded the biobanking and laboratory analyses. An open grant from Helse Vest funded the lipidomic analysis in Study III.

Acknowledgements

First and foremost, I wish to thank my main supervisor, Professor Mette Vesterhus. Thank you for accepting me as your PhD candidate for this project. I am truly grateful for all your guidance and help, always quick to respond with constructive feedback. Thank you to the co-supervisors Professor Tom Hemming Karlsen, Trine Folseraas, and Professor Eystein Husebye, for your valuable support along the way. I am so grateful for this opportunity to extend my horizon beyond the daily clinical work and deeply grateful for all your support and collaboration during this time.

I also want to extend my gratitude to NoPSC at Rikshospitalet for sharing your expertise and experience. It has been a true pleasure to get to know my fellow researchers and staff in Oslo. Thank you to Merete Tysdahl for all practical help and for tying social events to the every-day research life. Furthermore, my gratitude goes out to all patients who have participated in our study registries.

I am especially grateful to Lasse Giil for sharing your extensive knowledge and insight into medical statistics. This project has truly benefitted from your engagement. I would also like to thank Professor Emeritus Rolf Kristian Berge at the University of Bergen for your collaboration and introduction to the area of lipidomics.

Furthermore, I wish to thank my fellow PhD candidates at Haraldsplass Deaconess Hospital, Stein-Erik Solvang, Katinka Nordheim Alme, Irit Titlestad, Ragnhild Skogseth, and Christian Alsing. You have all been so welcoming when I started working in Bergen, and it has been a pure joy to take part in such an easy-going and warm environment. Finally, I wish to express a special thank you to research consultant Ida Sangnes for your openness, support, and friendship.

"The Pythagorean doctrine", said Junior. "Do you think it's a disease?", said Twigson. «It sounds so scary». «No, it's a kind of homework», said Junior.

From Junior and Twigson by Anne-Cath. Vestly, Gyldendal Norsk Forlag, 2009.

Abbreviations

AA: Anthranilic acid;

ACLF: Acute-on-chronic liver failure;

AI: Atherogenicity index;

AIH: Autoimmune hepatitis;

AII: Anti-inflammatory index;

ALA: Alpha-linolenic acid;

ALP: Alkaline phosphatase;

ALT: Alanine aminotransferase;

AOM: Amsterdam-Oxford model;

ARFI: Acoustic radiation force impulse;

AUROC: Area under the receiver operating characteristic curve;

ARLD: Alcohol-related liver disease;

AST: Aspartate aminotransferase;

B-MODE: Brightness mode;

BGM: Marker of biglycan degradation;

C3M: Degradation of type III collagen;

C4M: Degradation of type IV collagen;

CCA: Cholangiocarcinoma;

CRP: C-reactive protein;

C_V: Coefficient of variation;

DHA: Docosahexaenoic acid;

EASL: The European Association for the Study of the Liver;

ECM: Extracellular matrix;

eCRF: Electronic case report form;

EFSUMB: European Federation of Societies for Ultrasound in Medicine and Biology;

ELF: Enhanced liver fibrosis;

ELISA: Enzyme-linked immunosorbent assay;

EPA: Eicosapentaenoic acid;

ERCP: Endoscopic retrograde cholangiopancreatography;

FA: Fatty acid;

FIB-4: Fibrosis-4 score;

GGM: Gaussian graph model;

GGT: Gamma-glutamyl transferase;

HA: Hyaluronic acid;

HAA: 3-hydroxyanthranilic acid;

HC: Healthy controls;

HK: 3-hydroxykynurenine;

IBD: Inflammatory bowel disease;

ICC: Intraclass correlation;

IL-2: Interleukin-2;

INR: International normalized ratio;

IQR/M: Interquartile range divided by the median;

KA: Kynurenic acid;

kPa: Kilopascal;

KT-ratio: Kynurenine-tryptophan ratio;

Kyn: Kynurenine;

LPB: Lipopolysaccharide binding protein;

LSM: Liver stiffness measurement;

mNAM: N1-methylnicotinamide;

MRCP: Magnetic resonance cholangiopancreatography;

MUFA: Mono-unsaturated fatty acids;

NAD: Nicotinamide adenine dinucleotide;

NAFLD: Non-alcohol-related fatty liver disease;

NAM: Nicotinamide;

NoPSC: The Norwegian PSC Research centre;

PBC: Primary biliary cirrhosis;

Pic: Picolinic acid;

PIIINP: Propeptide of type III procollagen;

PLP: Pyridoxal 5'-phosphate;

POA: Palmitoleic acid;

PPAR: Peroxisome proliferator-activated receptors

PRO-C3, PRO-C5: Type III and V collagen formation;

pSWE: Point shear wave elastography;

PSC: Primary sclerosing cholangitis;

PUFA: Poly-unsaturated fatty acids;

QA: Quinolinic acid;

ROS: Reactive oxygen species;

SCD: Stearoyl-CoA desaturase;

SCFA: Short-chain fatty acids;

SD: Standard deviation;

SFA: Saturated fatty acids;

SWE: Shear wave elastography;

TCA: Tricarboxylic acid;

TE: Transient elastography;

TFA: Total fatty acids;

TI: Thrombogenic index;

TMAO: Trimethylamine N-oxide;

Trig: Trigonelline;

Trp: Tryptophan;

TIMP-1: Tissue inhibitor of metalloproteinases-1;

UDCA: Ursodeoxycholic acid;

UFGC: Ultrafast gas chromatography;

ULN: Upper limit of normal;

VICM: Citrullinated type III intermediate filament protein vimentin;

VLCFA: Very long-chained fatty acids;

Wt%: Percentage by weight;

Abstract

Background: Primary sclerosing cholangitis (PSC) is an immune-associated liver disease of unknown aetiology characterized by cholestasis, inflammation, and stricturing of the biliary tree, which typically progresses to general liver fibrosis, cirrhosis, and end-stage liver disease. Despite its status as a rare disease, it has been the leading cause of liver transplantation in Norway for decades. The disease course is highly variable and notoriously unpredictable. The lack of established biomarkers to stratify risk and assess disease activity is a major hurdle to developing effective therapy. Hence, new biomarkers are highly warranted to improve patient selection and effect assessment in clinical trials.

Aims: The objective of the present study was to further characterize biomarkers of prognosis in PSC and explore novel potential biomarkers. Thus, we aimed to evaluate the within- and between-patient variability over time in PSC for the two currently most promising predictive markers, the enhanced liver fibrosis test (ELF) and liver stiffness measurement (LSM) (**Paper I**). Moreover, we aimed to identify a panel of multiple biomarkers with improved predictive abilities in people with PSC compared to current clinical risk scores or single biomarkers (**Paper II**). Lastly, we aimed to study markers of mitochondrial function in PSC **(Paper III)**.

Methods: In **Paper I**, we applied a longitudinal mixed model to analyse ELF and LSM by point shear wave elastography in repeated measurements from a prospective patient panel of 113 patients from Bergen and Oslo. In **Paper II**, we used elastic net- and multivariate regression to identify a prognostic multimarker panel for PSC based on cross-sectional, retrospective data from a panel of 138 PSC patients from the NoPSC biobank. In **Paper III**, we performed comprehensive lipidomic analyses and applied spatial regression. Here we explored markers of mitochondrial function cross-sectionally in plasma from 191 patients and 100 healthy controls and in liver tissue from people with PSC and non-cholestatic liver disease controls from the NoPSC biobank.

Main findings: In **Paper I**, we found a significant increase in ELF and LSM over time, which was restricted to the high-ALP group (>1.5 x upper limit of normal) in subgroup analysis. Five years from baseline, about 30 to 40% of patients had reduced LSM and ELF values. A subgroup of 10% of patients showed a concomitant decrease in ELF, LSM, and ALP, underscoring the need to understand and define a clinically significant reduction. Between-patient effects explained 78% of ELF variation and 56% of LSM variation, suggesting that ELF may have superior reliability for risk stratification compared to LSM. In **Paper II**, we illustrated how prognostic biomarkers proposed in PSC seemed to form three groups of tightly intercorrelated variables. We demonstrated the best predictive ability in a panel consisting of biomarkers reflecting different aspects of PSC pathogenesis, i.e., fibrosis (ELF), inflammation (kynurenine-tryptophan ratio; KT-ratio), and a microbiota metabolite (pyridoxal 5'-phosphate; PLP). In **Paper III**, we demonstrated extensive differences in fatty acid profile in plasma from PSC patients compared to healthy controls, including increased mono-unsaturated fatty acids (MUFA), decreased long-chain saturated fatty acids (SFA), total n-3 and n-6 polyunsaturated fatty acids (PUFA). Moreover, our findings clearly indicated mitochondrial dysfunction as a prominent feature in PSC, which was more pronounced with increasing cholestasis and disease stage.

Conclusion: Our findings underscore the need to understand the variation in biomarkers in PSC and establish a definition of clinically significant change. Furthermore, we have demonstrated the potential to improve the predictive abilities in PSC by combining biomarkers reflecting several pathogenic processes, warranting further studies in large and independent patient panels. Finally, we demonstrated lipidomic changes and mitochondrial dysfunction, which need further exploration to identify biomarkers or putative therapeutic targets.

Sammendrag

Bakgrunn: Primær skleroserende kolangitt (PSC) er en immunrelatert leversykdom av ukjent årsak som karakteriseres av kolestase, inflammasjon og strikturer i galletreet og vanligvis utvikler seg til generell leverfibrose, cirrhose og endestadium leversykdom. Til tross for at tilstanden er sjelden, har den i flere tiår vært den vanligste årsaken til levertransplantasjoner i Norge. Sykdomsforløpet er høyst varierende og uforutsigbart. Mangelen på etablerte biomarkører til stratifisering av risiko og sykdomsaktivitet er det største hinderet for å utvikle effektiv behandling. Følgelig er nye biomarkører sterkt etterspurt for å bedre pasientseleksjon og effektmåling i kliniske studier.

Mål: Målsetningen for studien var å karakterisere prognostiske biomarkører ved PSC og identifisere potensielle nye biomarkører. Vi har derfor vurdert variasjon over tid innen og mellom personer med PSC for dagens to mest lovende prediktive markører, «enhanced liver fibrosis test» (ELF) og leverstivhetsmålinger (LSM) (**Artikkel I**). I tillegg ønsket vi å undersøke om et panel med flere biomarkører ga bedret prediktiv verdi ved PSC sammenlignet med nåværende kliniske risikoscorer og enkeltmarkører (**Artikkel II**). Til slutt har vi studert markører for mitokondriefunksjon ved PSC (**Artikkel III**).

Metoder: I **Artikkel I** ble det brukt en longitudinell blandet modell for å analysere ELF og LSM ved skjærebølge-elastografi i repeterte målinger fra et prospektivt pasientpanel med 113 pasienter fra Bergen og Oslo. I **Artikkel II** brukte vi elastisk nettverk og multivariat regresjon for å identifisere et prognostisk multimarkørpanel for PSC, basert på tverrsnittsdata fra et retrospektivt panel med 138 personer med PSC fra NoPSC biobank. I **Artikkel III** utførte vi omfattende analyser av lipidomsetning og anvendte romlig regresjonsanalyse. Her gjorde vi tverrsnittsanalyser av markører på mitokondriefunksjon i plasma fra 191 pasienter og 100 friske kontroller, samt levervev fra personer med PSC og ikke-kolestatiske leversykdommer som kontrollgruppe fra NoPSC biobank.

Resultater: I **Artikkel I** fant vi en signifikant økning av ELF og LSM over tid, men subgruppeanalyse viste at økningen kun forekom i gruppen med høy ALP. Fem år fra baseline hadde ca. 30 til 40% av pasientene reduksjon i LSM og ELF. En undergruppe på 10% av pasientene viste samvariasjon med reduksjon i ELF, LSM og ALP, og understrekker behovet for bedre forståelse og definisjon av hva som utgjør klinisk signifikant reduksjon. Effekter mellom pasienter forklarte 78% av variasjonen i ELF og 56% av variasjonen i LSM, og foreslår at ELF muligens har bedre evne for risikostratifisering sammenlignet med LSM. I **Artikkel II** illustrerte vi hvordan prognostiske biomarkører i PSC dannet tre grupper med tett korrelerte variabler. Vi demonstrerte at et panel bestående av biomarkører fra ulike deler av patogenesen i PSC hadde den den beste prediktive evnen, det vil si fibrose (ELF), inflammasjon (kynurenin-tryptofan ratio; KT-ratio) og en mikrobiell metabolitt (pyridoxal 5'-fosfat; PLP). I **Artikkel III** viste vi at det er uttalte forskjeller i fettsyreprofilen i plasma ved PSC sammenlignet med friske kontroller, inkludert økning av enumettede fettsyrer (MUFA), reduksjon av langkjedete mettede fettsyrer (SFA), total n-3 og n-6 flerumettede fettsyrer (PUFA). Funnene våre indikerte at mitokondriell dysfunksjon er fremtredende ved PSC og mer uttalt med økende kolestase og sykdomsstadium.

Konklusjon: Våre funn understreker behovet for å forstå variasjonen i biomarkører ved PSC og å etablere klare definisjoner av hva som er klinisk signifikante endringer. Videre har vi vist at det er mulig å bedre prediksjonen ved å kombinere biomarkører fra ulike sykdomsprosesser ved PSC. Dette fordrer videre studier i større og uavhengige pasientpaneler. Til slutt har vi vist endringer i lipidomsetning og mitokondriefunksjon, som gir et behov for videre studier for utforsking av biomarkører og mulige behandlingsmål.

List of studies

I. Fossdal G, Mjelle AB, Wiencke K, Bjørk I, Gilja OH, Folseraas T, Karlsen TH, Rosenberg W, Giil LM, Vesterhus M. **Fluctuating biomarkers in primary sclerosing cholangitis: A longitudinal comparison of alkaline phosphatase, liver stiffness, and ELF.** JHEP Rep. 2021 Jul 2;3(5):100328. Https://doi: 10.1016/j.jhepr.2021.100328

II. Fossdal G, Giil LM, Braadland PR, Karsdal MA, Grønbæk H, Husebye E, Karlsen TH, Folseraas T, Hov JR, Vesterhus M. **Multimarker analysis combining markers of fibrosis and inflammation in primary sclerosing cholangitis.** Unpublished manuscript.

III. Fossdal G, Braadland PR, Hov JR, Husebye E, Folseraas T, Ueland PM, Ulvik A, Karlsen TH, Berge RK, Vesterhus M. **Mitochondrial dysfunction and lipid alterations in primary sclerosing cholangitis.** Submitted to Hepatology Communications in June 2023.

1. Introduction

1.1 Preface

Primary sclerosing cholangitis (PSC) is a severe, cholestatic liver disease of unknown aetiology, characterized by inflammatory and fibrotic strictures of the biliary tree, progressing to fibrotic deposits in the liver parenchyma and development of cirrhosis. Moreover, PSC carries a substantial risk of intra- and extrahepatic malignancies. The disease course is heterogeneous but generally progresses toward end-stage liver disease. Curative medical treatment is unavailable, rendering the transplant-free survival time unchanged at 13-20 years[1]. Since most patients are diagnosed in their 30-40'ies, PSC significantly affects life quality, mental health, physical health, and life expectancy[2, 3].

Biomarkers of disease activity and prognosis are sorely needed in PSC since liver biochemistries like ALP and bilirubin do not reflect the disease course well, especially in the early progression of PSC[4, 5]. There is also a need to establish cut-off values for clinical decision-making. Promisingly, new biomarkers have been suggested, including blood-borne molecules and imaging biomarkers such as elastography.

For patients, new biomarkers may provide better individual information about their prognosis. For the clinician, new biomarkers will enable tailored follow-up care and focus on high-risk patients. Moreover, circulatory biomarkers will provide further insight into the various pathogenic pathways in PSC. The slow disease progression seen in many PSC patients calls for replacing solid endpoints with surrogate markers. For the scientist, surrogate markers could facilitate clinical trials and the development of effective therapy[6, 7]. Notably, ELF and transient elastography (TE) are included in the current EASL (The European Association for the Study of the Liver) guidelines for evaluation and follow-up in PSC[8].

1.2 Liver anatomy and physiology

1.2.1 Anatomy of the liver and biliary tree

The liver is organized as hepatocytes radiating from a central vein, forming portal triads containing venules, bile canaliculi, and arterioles. Endothelial cells cover the hepatocytes and the hepatic sinusoids, receiving fluid and solubles from the portal venous blood. The perisinusoidal space between the hepatocytes and sinusoids contains blood plasma and represents the site of hepatic lymph formation. Here the Kupffer cells and hepatic stellate cells represent an essential part of the endogenous immune system of the liver and the formation of connective tissue in liver fibrosis. The cholangiocytes extend throughout the biliary tree, creating a tight barrier through their connections with transmembrane proteins yet allowing the transport of solutes into the bile ducts. Of relevance for PSC, the biliary epithelium also lines the gallbladder wall[9-11].

In addition to the cellular compartment, the liver parenchyma consists of an extracellular matrix (ECM) consisting of the basement layer and interstitial matrix, providing structural support and orientation for the surrounding cells. Under normal conditions, collagen formation balances degradation. Collagen molecules are also involved in signalling processes through their affinity for growth factors[12], and byproducts of collagen are likely to possess paracrine effects[13]. The liver matrix is mainly formed by network-forming collagen like collagen IV and XVIII, and laminin. In contrast, the interstitial part is constituted by larger fibrils of collagen type I, III, V, and VI[13]. Collagen types III and V are first and foremost found around the portal tracts and perisinusoidal spaces[14, 15].

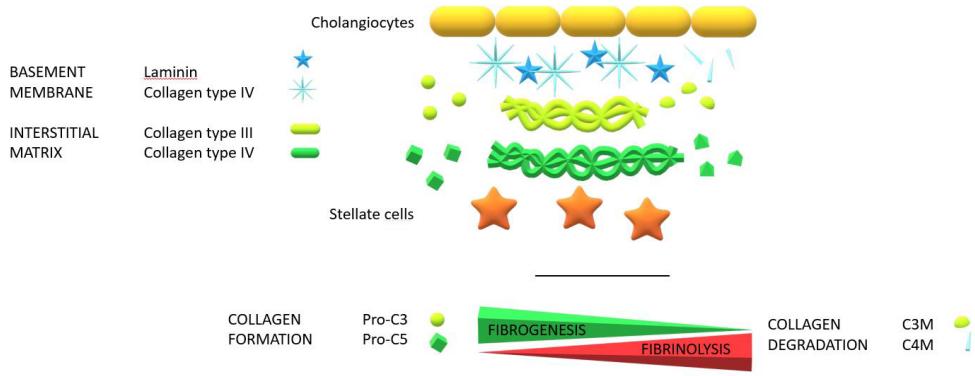

Figure 1. Overview of the extracellular matrix (ECM). The ECM is situated between the cholangiocyte layer and the cellular stroma with hepatic cells. The ECM contains the basement layer and interstitial matrix. The main components of the basement membrane are network-forming collagens like collagen IV and glycoproteins like laminin. The fibril-forming collagens collagen III and V are typical for the interstitial matrix. Pro-C3 and Pro-C5 represent collagen formation related to collagen type III and V, while C3M and C4M arise from the degradation of type III and IV collagen. These byproducts are elevated from augmented fibrogenic and fibrinolytic processes, denoting increased collagen turnover. *Created by G. Fossdal using Paint 3D (© Microsoft 2022).*

1.2.2 Formation of bile

The liver and biliary system secrete around 1000 ml of bile per day, contributing to the degradation of ingested nutrients[10]. The bile consists of 95% water, with the remaining part balanced between organic substances and electrolytes. Degradation of haemoglobin yields bilirubin, accounting for approximately 0.3% of the bile's constituents[16].

Bile acids represent the main disposal route for cholesterol. Through modification, oxidation, and conjugation, cholesterol is prepared for excretion, mainly as CDCA and CA (chenodeoxycholic and chenocholic acid). Bile acids undergo primary conjugation in the liver with the amino acids taurine and glycine, while a minor amount remains unconjugated. The combination of conjugated and unconjugated bile acids achieves different chemical properties. While conjugation is pivotal to avoid

precipitation of bile salts, unconjugated bile acids act as detergents and contribute to the antibacterial function with aid from IgA-secreting B-cells residing in the portal area[17, 18]. In addition, the cholangiocyte $Cl^-/HCO3^-$ pump forms a protective layer of bicarbonate-rich glycocalyx on the apical cholangiocyte membrane, often called the bicarbonate umbrella[19, 20]. Findings also point towards immunological cross-talk between the biliary tree and gut, where bile acids stimulate regulatory T-cells in the large intestine, but also a direct effect of the gut microflora on the bile acid composition[21-23].

Several molecules are involved in bile acid transport, where TGR5 (the Takeda G protein-coupled receptor-5) and FXR (the farnesoid X receptor), function as important regulators of bile acid transport across the cell's outer membrane and nucleus, respectively[17, 24, 25]. The bile acid synthesis is also coupled to the nuclear receptor FXR through negative feedback mechanisms[26, 27]. A family of nuclear hormone receptors, the peroxisome proliferator-activated receptors (PPARs), is central to the orchestration of bile homeostasis. PPARs enhance the enzymatic detoxification of bile acids and protection from toxic bile acids through phosphatidylcholine secretion[28-33]. Furthermore, PPARs are involved in lipid metabolism and anti-inflammatory effects through the downregulation of NF-κB and prevent liver fibrosis from their effect on hepatic stellate cells[34-36].

1.3 Primary sclerosing cholangitis

1.3.1 Epidemiology and demographics

PSC primarily affects younger adults around a median age of 35-40 years, with a tendency for peaking at the ages of 15 and 35[37-41]. There is a significant global variation in the occurrence of PSC, with an increasing gradient towards the Nordic countries[41]. The incidence rate is 0.4-1 in different regions of England, 1.2:100 000 in Sweden, to the highest reported 1.6:100 000 in Finland[40, 42, 43]. The prevalence ranges from 5.6 per 100 000 in the UK to 16.2:100 000 and 31.7:100 000 in Sweden and Finland, respectively[41, 44]. For the Norwegian population, a study from the mid-1990s observed an incidence rate of 1.3 per 100 000 and prevalence rates of 8.5 per 100 000 inhabitants[45]. Several studies point towards an increasing incidence rate[1, 42, 46, 47], but whether this is a de facto increase or due to improved diagnostics is unclear.

Population-based studies have traditionally demonstrated a sex difference with 60-65% male preponderance[37-39, 48, 49]. However, a few population studies, including a Norwegian study screening IBD patients with MRCP, revealed a substantial number of asymptomatic PSC patients and a relatively equal gender distribution[47, 50, 51].

At diagnosis, most patients demonstrate both intra- and extrahepatic biliary affection (about 65%), intrahepatic only (25-30%), or extrahepatic only (about 5%)[37, 42]. Around 10% of the patients are diagnosed with features of autoimmune hepatitis (AIH). The cholangiogram appears normal in about 3-10% of the patients, and the diagnosis is assessed histologically as small duct PSC[42, 48, 52]. PSC occurs concomitantly with IBD in around 60-80%, predominantly ulcerative colitis as seen in 2/3 of the patients. However, ulcerative colitis is more common among male PSC patients[49, 53]. Notably, IBD and PSC can precede each other in either sequence[39, 42, 48].

General mortality is increased by four times compared to the normal population[1]. PSC can be regarded as a precancerous condition, as approximately 20% of PSC patients are diagnosed with cholangiocarcinoma during their lifetime. Population-based studies also show a 150-400 times increased lifetime risk of

cholangiocarcinoma (CCA) compared to the normal population[1, 51 39, 54], and about 1/3 is diagnosed within the first year after diagnosis of PSC[1, 37, 49]. Hepatocellular carcinoma occurs in 2-4% of persons with PSC[54, 55]. The risk of developing colorectal cancer (CRC) is nine times higher than the general population. Colorectal cancer also seems to develop two decades earlier than in patients with ulcerative colitis without PSC[1]. Whether pancreatic malignancies are increased has yet to be clarified[56].

Another frequent finding and concern are gall bladder polyps in approximately 5-11% of the patients. Clinical studies have found that 55-60% of the gall bladder polyps in PSC develop into adenocarcinoma, irrespective of polyp size[57-60]. Up to 3.5% of PSC patients experience gall bladder carcinomas during their lifetime[58]. Consequently, PSC surveillance recommends ultrasonographic gall bladder monitoring and swift referral for cholecystectomy at the presentation of a mass lesion, irrespective of size.

Hepatobiliary malignancies occur more frequently with increasing age at the time of diagnosis. Otherwise, hepatobiliary malignancies do not seem associated with disease stage in PSC, as 1/3 of malignancies are recognized within the first year after primary diagnosis of PSC[1, 49]. Regarding IBD, patients with PSC and ulcerative colitis are at risk of a worsened outcome[53]. On the other hand, epidemiological data have shown less association with liver transplantation, death, or malignancies in small duct PSC and females with PSC[1, 49] .

1.3.2 Pathogenesis and pathophysiology

The disease process in PSC is highly elusive but likely evolving from a combination of genetic susceptibility and environmental factors. The pathogenesis involves an intricate interaction between liver and biliary cells, endogenous immune cells of the liver, and extrahepatic immune cells resident in the gut and vascular system. PSC is believed to run from inflammation to tissue scarring, causing fibrosis of the biliary tree and liver parenchyma, eventually leading to end-stage liver disease or

malignancy (**Figure 1**). From a broader perspective, one could ask whether these pathways play out linearly or in parallel mechanisms.

In PSC, genes related to the adaptive immune response represent the majority of genetic alterations, such as the HLA complex involved in antigen presentation toward T-cells and IL-2 expression[52, 61, 62]. Mutations in regulators of bile acid homeostasis, like the plasma membrane receptor TGR5[63, 64] and the *FUT2* gene, are also of interest, with potential implications for dysbiosis, gut permeability, and adhesion of bacteria to the biliary epithelium[64, 65]. Notably, one epidemiological study found significantly different geographical distribution among the two cholestatic diseases, primary biliary sclerosis (PBC) and PSC, indicating a possible interaction between environmental factors and genetics in susceptible individuals[66].

The composition of bile acids and gut microbiota is altered in PSC compared to healthy controls, regarding reduced biodiversity and increased bacterial species like Veillonella, Enterococcus, and Fusobacteria[67]. Although these bacteria might be elevated in other liver diseases and not disease specific for PSC, they might influence the pathogenesis in PSC and alter the composition of unabsorbed bile acids like the potentially toxic bile acid taurocholic acid[68]. Bile acids are strong detergents and may disrupt protein compounds and cell membranes[69], possibly disturbing the mitochondrial respiratory chain, and resulting in the accumulation of reactive oxygen species (ROS), causing cell damage and apoptosis[70]. Bile acids may also be considered potent signalling molecules through their effect on the FXR and TGR5 receptors, potentially initiating inflammation, as reviewed[67].

Inflammation involves intracellular proteins collectively termed the inflammasome, a term for molecules involved in the inflammatory response. In PSC, inflammation leads to activation of the Kupffer cells[71-73]. Secondly, cross-talk between the Kupffer cells and HSC stimulates the latter to transform into activated myofibroblasts, a main driver of fibrogenesis. The myofibroblasts gain contractile features and secrete excessive collagen and signalling molecules to neighbouring cells. This process

results in thickening of the basement membrane from deposits of type IV collagen and fibril-forming type I and III collagen[13, 74].

The collaboration of T-cells, neutrophils, and activated macrophages[75], stimulates the cholangiocytes into a proinflammatory state and stimulates biliary tree stem cell recruitment into their microenvironment. Subsequently, this inflammatory process is followed by metaplasia and fibrosis, resulting in what is often described as an onion-skin appearance and cholestasis[76, 52]. The bicarbonate-rich secretions from cholangiocytes also turn more mucinous and less protective towards bile acids[76], potentiating further damage to the bile ducts.

The role of inflammatory bowel disease in PSC has yet to be fully understood. However, one likely part of the pathogenesis involves altered expression of adherence molecules normally confined to the gut, alleviating hepatic recruitment of intraepithelial lymphocytes, and activating resident T-cells of the biliary tree[75, 77]. Dysbiosis, referring to a disadvantageous composition of gut microflora, might play a role in T-cell recruitment[78-83]. However, a recent study of adhesion molecules in explanted livers found gut-liver homing of T-cells as a general feature of chronic liver disease[84]. Therefore, changes in the microbial composition may not necessarily be disease-specific for PSC but a modifier of disease development[77].

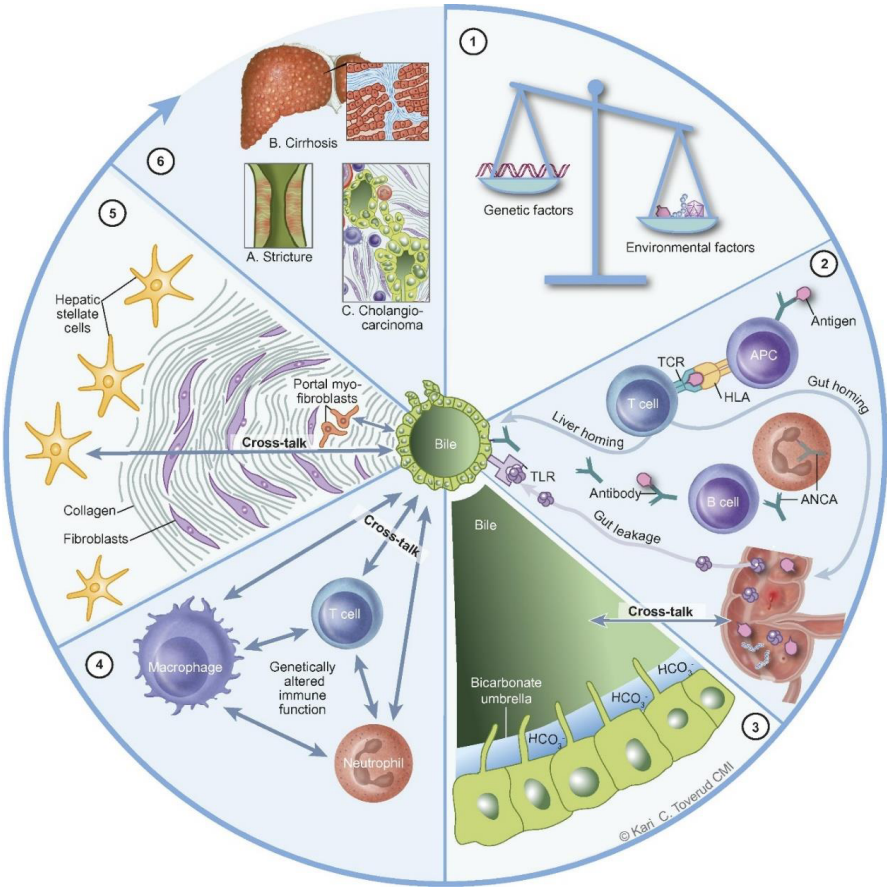

Figure 2. Overview of the pathogenic processes in PSC. Several genetic traits have been connected to susceptibility for PSC, especially variants of the human leukocyte antigen (HLA) on chromosome 6. There is also a possible connection to environmental factors (panel 1). Activated T-cells migrate from the gut to the biliary tree and liver parenchyma, initiating cellular cross-talk between cholangiocytes, hepatic stellate cells, and immune cells, rendering their phenotypes in a proinflammatory and profibrotic state (panels 2 and 4). These changes disturb the bile composition and the protective bicarbonate layer, increasing collagen deposition (panels 3 and 5). Fibrotic strictures appear in the biliary tree and eventually the liver parenchyma, causing end-stage liver disease from cirrhosis or malignancies like cholangiocarcinoma 6). *Figure by Karlsen et al. J Hepatol 2017, reprinted with permission*[52].

1.3.3 Clinical presentation and symptoms

The clinical course in PSC is heterogeneous, and symptoms can fluctuate throughout the disease course without any apparent association with disease progression in PSC. About 50% of the patients at diagnosis, among whom 20% develop symptoms during the first 6-7 years[37, 42]. At the time of diagnosis, the most common findings are upper right abdominal pain in about 25-35%[37, 42] and fatigue in 35%[48]. Less common is jaundice in 20-30%, pruritus in 20-30%, fever and bacterial cholangitis in about 10-15%, while acute or chronic cholecystitis occurs in 25%[42, 48, 58]. PSC patients are also prone to intercurrent events of flares in liver enzymes, pruritus, jaundice, and bacterial cholangitis caused by gallstones or biliary strictures. With advancing disease, PSC patients may experience decompensating events from liver cirrhosis, i.e., episodes of ascites, liver encephalopathy, or variceal bleeding.

1.3.4 Diagnosis and liver biochemistries

Traditionally, elevated ALP and typical findings on biliary tree imaging have formed the basis for diagnosing PSC. Notably, the radiologic manifestations indicating PSC are not pathognomonic, and other aetiologies should be excluded[85]. Magnetic resonance cholangiopancreatography (MRCP) is the modality of choice, but endoscopic retrograde cholangiopancreatography (ERCP) is preferred when endoscopic intervention is indicated. The latter poses a risk for post-ERCP pancreatitis in around 3,5% and biliary perforation upon cannulation in up to 0,5% of the patients[86]. The diagnosis of PSC relies on at least one of three criteria, i.e., elevated ALP or GGT as cholestasis markers, diagnosis of IBD, or a liver biopsy supportive of PSC[85]. However, the International PSC Study Group has recently proposed revised diagnostic criteria with increasing emphasis on imaging and IBD. Consequently, elevated ALP is no longer required for the diagnosis of PSC. Typical findings on the cholangiogram are strictures with or without focal dilatations of the biliary tree[85, 87]. An inconclusive MRCP with sustained clinical suspicion of PSC might favour ERCP for detecting stenoses. By using distal balloon occlusion and

contrast injection, ERCP results in higher intraluminal pressure and enhances visualization of stenoses[8].

The scattered distribution of PSC causes substantial sampling variability on repeated biopsies[88-90]. Thus, current guidelines recommend against liver biopsies for diagnostic purposes unless AIH is suspected from elevated transaminases (>5 x ULN), elevated IgG (>2 x ULN), or verification of small duct PSC[85]. Although image guidance is recommended for liver biopsies, there is a non-negligible risk of adverse events causing perforation injuries resulting in haemorrhage, haemothorax, or death. Studies report major bleeding episodes accompanied by blood transfusion or other clinical intervention among 0.3-4.6% of patients undergoing liver biopsy and death in 0.02-0.04%[91-93].

Clinical studies report ALP elevation in 54-76% of PSC patients at the time of diagnosis[4, 5]. Still, as bilirubin, ALP tends to fluctuate over time and independently from the disease course. Bilirubin and albumin are often normal in blood samples until decompensated liver function in advanced cirrhosis. Despite the alleged relationship to autoimmunity, PSC has no distinctive pattern among autoantibodies. Elevated ANA, SMA, and pANCA are seen at varying frequencies[85, 94], possibly related to binding distinct cellular moieties in the disease process. However, they are not disease-specific for PSC nor autoimmune liver disease, and their prognostic value is unestablished[94]. About 10-25% of PSC patients demonstrate serum IgG4 levels above normal[95-97], but studies have not verified a clear association between IgG4 in blood and periductular deposits with disease progression[96, 97]. The most recent consensus statement recommends a threshold of 4xULN for distinguishing between PSC with IgG4 elevation and IgG4 cholangitis[85].

Features of autoimmune hepatitis, previously called “AIH overlap” in PSC, should be suspected in case of elevated aminotransferases or serum IgG[1, 48, 98]. AIH and PSC should be diagnosed independently per their respective diagnostic criteria. A liver biopsy with findings of at least moderate interface hepatitis is mandatory to confirm AIH in addition to cholangiopathy supportive of PSC[85].

1.4 Biomarkers in PSC

The Biomarkers, EndpointS, and other Tools (BEST) glossary define a *biomarker* as an objectively measurable characteristic related to normal biological or pathogenic processes, anatomic measurements, or response to therapeutic intervention[99]. Although biomarkers are commonly measured in the blood (plasma or serum), they might also include other measurements of molecular, histologic, radiographic, or physiological characteristics. Regulatory authorities such as the U.S. Food & Drug Administration (FDA) adhere to the BEST glossary, defining seven categories of biomarkers according to their purpose: susceptibility, diagnostic, monitoring, prognostic, prediction, pharmacodynamics, and safety.

The lack of established biomarkers in PSC is a major unmet need for clinical follow-up and trial design. This issue obstructs the development of effective therapy and adds to the disease burden among PSC patients. Hence, there is a significant focus on research aiming to identify and establish biomarkers for three purposes: (1) monitoring disease progression for individual patients to ensure personalised information and a possibility for ease of disease burden, (2) tailored clinical trials with stratified patient selection into designated prognostic risk groups, and (3) as surrogate endpoints in clinical trials; i.e., biomarkers associated with solid endpoints such as death or liver transplant or other clinical events. In this way, a measure of change in surrogate markers represents a substitute for the occurrence of a clinical event and a measure of the therapeutic effect of an intervention. However, several factors challenge the establishment of such biomarkers in PSC:

(1) A protracted disease course yielding few solid endpoints in observational studies

(2) The disease course varies largely between patients, hampering the reproducibility of findings across studies

(3) Natural fluctuations in ALP, bilirubin, and symptoms, which are sometimes associated with intercurrent events (i.e., bacterial cholangitis, gallstones) and which are, at least in part, dissociated from the severity of the underlying liver disease.

Various histological and ERCP-based scores may predict clinical outcomes in PSC[100-102]. However, given that PSC patients need long-time monitoring for years, even decades, repeated measurements are necessary and call for noninvasive biomarkers. Various noninvasive biomarkers have been identified in PSC, including imaging modalities and circulatory biomarkers. Regarding noninvasive imaging, various MRI- and MRCP-based scores and spleen length have been proposed to predict clinical outcomes. Presently, liver stiffness measurement assessing liver fibrosis using ultrasound or magnetic resonance-based elastography techniques offer promising results but are not validated standardization between scanner systems or towards surrogate biomarkers[103-108]. LSM from MRI is also prone to varying results in fibrosis depending on the size of ROI determined and general processing of algorithms and data generation[109, 110]. Biomarkers of interest in PSC are listed in **Table 1**.

Before establishing a biomarker in clinical practice or trials, identifying the biomarker represents the first of several steps (**Figure 3**). The biomarker's stability, reliability, and accuracy need assessment to decide its analytical performance. Finally, validation in several independent patient panels is required to determine the association with clinical outcomes, preferably large and prospective panels, to avoid biases inherent to retrospective studies.

Figure 3. Development of new biomarkers. New biomarkers are established after several steps starting from exploration and discovery in biological material via statistical analysis in an explorative and, secondly, a validation panel before implementation in practice. *Created by G. Fossdal using Paint 3D (© Microsoft 2022).*

Table 1. Biomarkers and literature reference. Literature referring to the first published association for a potential biomarker towards clinical outcome in PSC or, if otherwise stated other liver diseases.

	Reference literature
Fibrosis markers	
ELF	Vesterhus 2015[104]
Pro-C3	Nielsen 2018[105]
Pro-C5	
C3M	
C4M	
BGM	Vesterhus 2021[106]
VICM	
Anti-GP2 IgA	Jendrek 2017[107]
Calprotectin	Vesterhus 2017[108]
Autotaxin	Dhillon 2019[109]
Inflammatory markers	
IL-8	Vesterhus 2017, Zweers 2016[108, 110]
CD14	Dhillon 2019[111]
LPB	
CD163	Bossen 2021[112]
CD206	
Metabolic markers	
PLP	Kummen 2021[81]
TMAO	Kummen 2017[80]
Neopterin	Dhillon 2021[113]
KTR	
Clinical scores	
Mayo risk score	Kim 2000[114]
AOM	De Vries 2018[115]
UK-PSC risk score	Goode 2019[118]
Ultrasound elastography	
Transient elastography	Corpechot 2014[119]
pSWE	Mjelle 2020[97]
ARFI	Goertz 2019[120]

Abbreviations: Anti-GP2, anti-glycoprotein-2; AOM, Amsterdam-Oxford model; BGM, marker of biglycan degradation; C3M, C4M, degradation of type III and IV collagen; ELF, enhanced liver fibrosis; HCV, hepatitis C virus; HIV, human immunodeficiency virus; IL-8, interleukin-8; KT-ratio, kynurenine-tryptophan ratio; LPB, lipopolysaccharide binding protein; PLP, pyridoxal 5'-phosphate; Pro-C3, Pro-C5, type III and V collagen formation; VICM, citrullinated type III intermediate filament protein vimentin.

1.4.1 Liver elastography in PSC

Tissue elastography has gained a significant role in noninvasive evaluation of chronic liver diseases, enabling quantification of liver fibrosis with diagnostic accuracy comparable to histology[111-113]. Elastography encompasses a range of methods. Shear wave elastography (SWE) is preferred for liver fibrosis assessment, constituting transient elastography (TE), point shear wave elastography (pSWE), and 2D-SWE.

The SWE methods differ in the way they induce and measure share waves. In TE, a short mechanical impulse is applied to the skin surface, inducing tiny tissue displacements that travel into the liver as a shear wave[114]. Liver stiffness is determined for a liver volume of 1x4 cm at a set depth without visualization of the parenchyma[115]. In pSWE and 2D-SWE, on the other hand, one or several acoustic impulses are deposited inside the liver region of interest (ROI) at a depth selected by the operator during B-mode visualization. Then, a shear wave is initiated within the liver, travelling perpendicularly to the acoustic beam. The generation of acoustic signals inside the liver makes pSWE and 2D-SWE less influenced by ascites or adipose tissue compared to TE[114, 116]. The propagation of shear waves depends on tissue elasticity: increasing velocities reflect increasing liver stiffness[117]. Results may be given as shear wave velocity in m/s; however, commonly, the result is expressed as liver stiffness in kPa, calculated from the measured velocity.

In PSC, studies from 2006 and 2014 have demonstrated an association between TE, histological stage, and clinical outcome[112, 118]. These findings were later validated in independent studies, yielding international guidelines recommending TE for evaluating prognosis in PSC at baseline and during follow-up[8]. A cut-off level at 14.4 kPa for TE was able to exclude all cases of cirrhosis with a 100% negative predictive value[118]. Furthermore, pSWE and 2D-SWE have also demonstrated good feasibility and low interoperator variability in PSC and a good correlation between TE, pSWE, and 2D-SWE platforms[103, 119, 120]. However, the interpretation of results should consider possible confounders. Cholestasis due to biliary obstruction or flares of acute autoimmune hepatitis is particularly relevant in PSC. In addition, alcohol-related hepatitis, congestive cardiac hepatopathy, or recent meal intake are important

differential diagnoses[121-123]. In this regard, pSWE and 2D-SWE are advantageous compared to TE, as the B-mode visualization often indicates such confounders. However, the larger sample volume in TE could possibly favour its role considering the patchy disease distribution in PSC.

1.4.2 Circulatory biomarkers

Serological fibrosis markers

Serum fibrosis markers and liver stiffness measurement are currently the most promising prognostic tools in PSC. Among these, the ELF test is the best validated and recommended for use during diagnosis and follow-up of PSC[8]. ELF is a patented, commercially available fibrosis panel based on an algorithm combining three important constituents of the ECM, all directly involved in fibrogenesis: hyaluronic acid (HA), propeptide of type III procollagen (PIIINP), and tissue inhibitor of metalloproteinases-1 (TIMP-1). The glycosaminoglycan HA is a significant component of the ECM, a potential activator of hepatic stellate cells, whereas PIIINP reflects an unfavourable deposition of fibril-forming collagen III. Secretion of TIMP-1 from hepatic stellate cells inhibits the endogenous collagen-degradation from metalloproteinases[124, 125], contributing to the unbalance towards collagen formation. Biochemically, ELF is therefore suited for detection of earlier stages of fibrosis[124].

ELF was developed from a cohort of approximately 1000 patients with various chronic liver diseases, followed by a 7-year follow-up study of over 450 of these patients to assess its prognostic strength.[126, 127]. ELF correlated well with fibrosis stages and was at least equal to the histological staging of fibrosis for prediction of liver-related outcomes[127, 128], with an approximately linear relationship to the Ishak fibrosis stages[128]. Thresholds for mild, moderate, and advanced fibrosis were established in a mixed patient population with viral and autoimmune liver diseases at 7.7-9.7, 9.8-11.2, >/=11.3, holding a minimum of 80% sensitivity vs. 97% specificity for cirrhosis for the highest threshold[128]. An observed increase within a threshold

might suggest at-risk individuals since an increment of 1 unit is found to double the risk of a liver-related outcome over seven years[127].

ELF is already demonstrated as a strong predictor in PSC, showing an independent association with transplant-free survival in two independent panels; ELF above 11.2 demarcated a high-risk group[129]. The predictive ability of ELF was later validated in a large, international, multicentre study and in other studies [5, 95, 129-131]. Repeated measurements of ELF have demonstrated association to clinical events both from baseline values and by change over time, with 9.8 as the optimal level for prediction of outcome and an increase of 0.19 at three months correlating to the development of fibrosis[95]. Compared to other parameters, ELF has shown less variation within individuals over time than ALP and a strong correlation to liver stiffness measurements[5, 95, 129].

However, despite the strong evidence in favour of ELF, it is conceivable that a biomarker may be tailored for PSC with superior predictive and monitoring properties. Markers of formation and degradation of collagen and other extracellular matrix constituents may reveal early changes in extracellular matrix turnover before the deposition of excessive connective tissue in the liver parenchyma. Consequently, several neo-epitopes from collagen formation (Pro-C3, Pro-C5) and degradation (C3M, C4M) have demonstrated prediction of transplant-free survival in PSC[132, 133]. In the initial stages of fibrosis, basement membrane remodelling is succeeded by changes in the interstitial matrix. While type III collagen represents a large proportion of liver collagen, collagen V accounts for the most significant increase in cirrhosis. Not unexpectedly, both Pro-C5 and C3M are strongly associated with clinical outcomes in PSC[132, 134].

Markers of inflammation

Inflammation is commonly found in liver biopsies in early PSC[90], rendering inflammatory markers of high interest. In a study of an extensive array of antibodies in serum and bile, multivariate analyses demonstrated a list of predominantly inflammatory biomarkers associated with advanced PSC, most importantly calprotectin in bile and IL-8 in bile and serum[135].

IFN-γ-driven pathways, including the kynurenine-tryptophan pathway and neopterin, appear ubiquitously in disease and have been linked to clinical outcomes in liver cirrhosis and PSC[136]. Analyses of bile in PSC have indeed revealed elevated IL-8 and calprotectin involved in neutrophil reaction and activation of monocytes and macrophages[137, 138], in addition to the proliferation of cholangiocytes and fibrogenesis stimulated by IL-8. In line with these findings, livers from PSC patients contain increased numbers of macrophages, especially in the peribiliary areas[139, 140]. Correspondingly, monocyte- and macrophage activation markers like CD14, CD163, and CD206 are associated with clinical outcomes in PSC[141, 142]. Intriguingly, CD163 is tied to the initiation of fibrosis[143].

Markers of metabolism and gut microbiota

Published studies in PSC have drawn attention to the liver metabolization of substrates from gut microbiota[80]. Reduced PLP, the active form of vitamin B6, was reported to be associated with disease progression and transplant-free survival in PSC[144]. Not only does PLP function as a co-factor in the kynurenine-tryptophan pathway, but it also affects cell-mediated immunity, including the intestinal immune response. Another microbial metabolite processed by the liver, TMAO, has demonstrated association towards outcome and is proposed as a potential surrogate marker for advanced PSC[127].

Markers of mitochondrial function

A growing body of evidence has linked oxidative stress and mitochondrial dysfunction to liver diseases of various aetiologies, including primary biliary cholangitis (PBC)[145]. Published studies on bile-duct ligation in mice have linked cholestasis to mitochondrial dysfunction through direct inhibition of the mitochondrial electron transport chain[70, 146-148]. Various parameters related to mitochondrial function show different expressions in PSC, including upregulation of the methylation-controlled J-protein (MCJ) involved in the electron transport chain[70, 149]. These findings suggest a putative role of mitochondrial dysfunction in PSC. We have assessed a wide range of metabolites, including free fatty acids, cholesterol, and triglycerides to main groups of fatty acids, enzymes relating to fatty acid conversion, and molecules aiding the fatty acid transport chain across the mitochondrial membrane. The free fatty acids range from short-chained, via medium and long-chained to very long-chained fatty acids, the latter involving the peroxisomal function. We have also investigated adjacent nicotinamide (NAD) formation pathways from amino acid metabolism. The inverse association of the kynurenine-tryptophan ratio to clinical outcome in PSC further supports this role since the kynurenine-tryptophan-nicotinamide pathway supplies the electron transport chain with NAD^+[136]. However, up to date, no markers of mitochondrial function have been proposed as biomarkers of disease activity or prognosis in PSC.

1.4.3 Ideal properties of a robust biomarker

In general, the ideal biomarker needs to fulfill several considerations:

(1) Reliability. The repeatability is crucial, i.e., fluctuation of a biomarker should reflect a shift in disease activity or stage. A low coefficient of variation (CV) indicates high reliability for biochemical biomarkers, i.e., a low total variation due to well-controlled preanalytical and analytical factors and low biological variation within subjects[150].

(2) Validity. The biomarker should reflect the biological phenomenon studied (e.g., biliary inflammation or liver fibrosis) and correlate with the disease severity or stage, reflected in measurements of high predictive power. Hence, we want high sensitivity to detect true positive cases, high specificity to identify true negative cases, and high values of area under the receiver operator characteristics curve (AUROC).

(3) Availability. A biomarker should be accessible, affordable, and independent of the examinator's experience.

(4) Safety. This point is crucial for repeated measurements in a surveillance setting, such as in the clinical follow-up of patients with PSC.

The ideal predictive biomarker in PSC should be closely related to disease progression independent of flares. A biomarker also needs strong predictability toward clinical outcomes. Prognostication calls for high sensitivity towards early disease progression to enable personal adjustment of patient surveillance. The liver biochemistries currently used to monitor liver disease, i.e., bilirubin and albumin, reflect rather later stages of PSC-related liver disease. This also goes for the Mayo risk score, which largely relies on these parameters, in addition to AST, age, and episodes of variceal bleeding. Although ALP has demonstrated an association with clinical outcome, studies have failed to reproduce a distinct cut-off value across different cohorts[98, 151-153], and it's a tendency to normalize spontaneously over time[5, 153]. Therefore, elevation of ALP is difficult to interpret for clinical decision-making in PSC and an unreliable endpoint in clinical intervention studies. In summary, the ideal biomarker in PSC should be noninvasive, reliable for detecting early disease progression, yet closely related to clinical outcomes.

1.5 Aims of the thesis

The overall aim of this project was to further investigate proposed biomarkers of prognosis in PSC concerning their variability over time and the potential benefit of

combining biomarkers into a prognostic panel, as well as to explore novel potential biomarkers related to mitochondrial function.

1. To investigate the development over time for ELF and LSM compared to ALP.
2. To explore whether a multi-marker panel reflecting several pathways involved in PSC pathogenesis could improve predictive value towards clinical outcome compared to single biomarkers or clinical risk scores.
3. To characterize changes in the lipidomic profile and mitochondrial function in PSC.

2. Methods

2.1 Study design and patient selection

2.1.1 Paper I

A total of 113 patients with large-duct PSC, including eight subjects with features of AIH, were recruited prospectively from 2013-2018 from the two study centres in Bergen and Oslo. The patients were followed with annual study visits to collect liver biochemistries, ELF, and liver stiffness measurements.

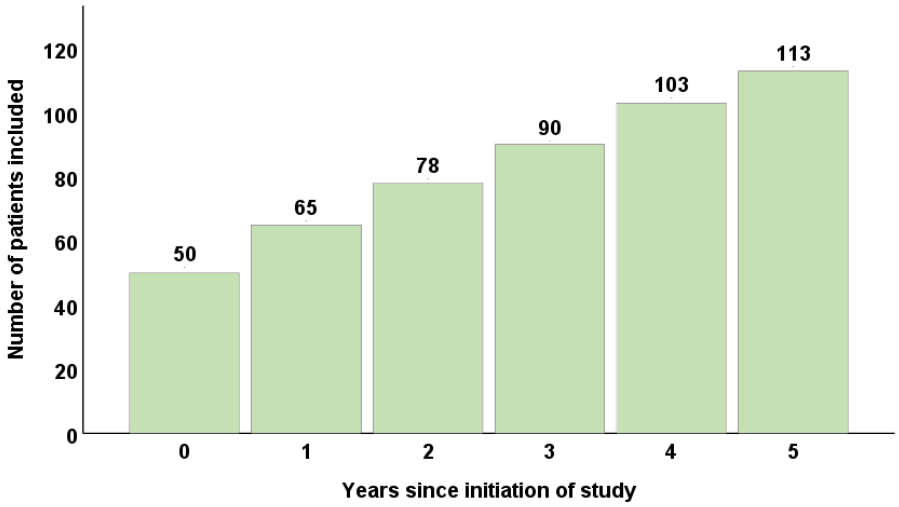

Figure 4. We recruited a total of 113 patients from study start, of which 65 were followed over five years.

2.1.2 Paper II

In this study, we cross-sectionally examined 138 patients included from 2008-2012 and retrieved retrospectively from the NoPSC biobank at Oslo University Hospital Rikshospitalet.

2.1.3 Paper III

In this retrospective cross-sectional study, we compared a population of 191 patients with large-duct PSC to 100 healthy controls, included in 2008-2015. Patients with PSC were retrieved from the NoPSC biobank at Oslo University Hospital, Rikshospitalet.

2.2 Biomarkers

ELF was analysed in studies I and II using the Siemens ELF®Test on an ADVIA Centaur XP analyser (Siemens Medical Solutions Inc., Tarrytown, NY, USA), according to a published algorithm = $2.278 + 0.851 \ln(C_{HA}) + 0.751 \ln(C_{PIIINP}) + 0.394 \ln(C_{TIMP\text{-}1})$. Liver stiffness measurements in paper I were measured in the fasting state using point shear wave elastography (pSWE); for the Bergen cohort using an ElastPQ® Philips iU22 (Philips Healthcare, Andover, MA, USA) scanner (software version 6.3.2.2, convex C5-1 probe) and for the Oslo cohort using Siemens Acuson S3000 (Siemens Medical Solutions USA, Inc., Malvern, PA).

In paper II we collected data regarding serological biomarkers of inflammation, fibrosis, and gut metabolites from the data sets of several previously published studies. Validated competitive ELISA (Nordic Bioscience, Herlev, Denmark) was used for analyses of collagen formation, i.e., PRO-C3 and PRO-C5, for collagen type III and V [133, 154], collagen degradation, i.e., C3M and C4M for collagen III and IV (C3M, C4M), and proteoglycan biglycan (BGM)[155-157]. Anti-GP2 IgA was analysed by immunofluorescence (IIF) (Euroimmun, Germany).

IL-8 and calprotectin were analysed by ELISA kits (R&D Systems Minneapolis, MN, US and Calprolab, Calpro, Lysaker, Norway). Soluble CD163 and CD206 were analysed by an in-house sandwich ELISA technique as described[158, 159]. Concentrations of neopterin, kynurenine, tryptophan, PLP[160, 161], and TMAO were assessed by liquid-chromatography-tandem mass spectrometry (LC/MS/MS) with calibration curves prepared from isotope-labelled internal standards (Cambridge

Isotope Laboratories Inc. ®, Andover, MA, USA). The KT-ratio was calculated from 100*kynurenine: tryptophan (µmol: µmol).

Lipidomic analyses in paper III was carried out by ultrafast gas chromatography (UFGC). Plasma and liver samples were analysed for fatty acids, including saturated fatty acids (SFA), mono-unsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), cholesterol, and triglycerides, as well as enzymes involved in fatty acid turnover. Furthermore, using a platform offered by Bevital A/S (Bergen, Norway), we examined the levels of carnitine metabolites, whereas markers of the kynurenine-tryptophan-NAD pathway were analysed as part of a previous study. Clinical indices were calculated from established algorithms, i.e., the anti-inflammatory index[162], atherogenic and thrombogenic indexes[163]. Liver biopsies were collected from explanted livers from the NoPSC biobank for a total of 49 patients diagnosed with autoimmune hepatitis (AIH; n=11), alcohol-related liver disease (ARLD; n=9), and primary sclerosing cholangitis (PSC; n=25).

Liver biochemistries and clinical data were retrieved from the NoPSC retrospective database (papers II, III) or the prospective database of the National network for autoimmune liver diseases (III). For risk stratification according to advanced PSC, we used Mayo risk score in papers I-III and Amsterdam-Oxford prognostic model for PSC in papers I-II, calculated from their respective published algorithms[164, 165].

2.3 Statistics

Statistical analyses were conducted in SPSS version 26 (SPSS Inc., 2016, Armonk, NY), STATA 16 (StataCorp. 2019, Stata Statistical Software: Release 16.1. College Station, Tx: StataCorp LP) for all analyses. Correlation networks and heatmap were generated in the qgraph package and “pheatmap” packages in R (R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria). Endpoints were defined as all-cause death or liver transplantation for primary endpoints. Secondary endpoints were either death or

liver transplantation due to end-stage liver disease or the development of cholangiocarcinoma. All variables were run through normality tests with the Shapiro-Wilk test and Q-Q-plot. Mann-Whitney U test, Student's t-test, Chi-squared test, Kruskal-Wallis, and ANOVA tests were applied as appropriate. Bivariate correlations were tested by Spearman or Pearson correlation.

Cut-offs for high vs. low levels of selected variables were defined by tertiles or Youden's index from receiver operating characteristics. High-risk patient groups were defined as previous studies in PSC with ELF ≥9.8 as cut-off for severe fibrosis[128, 129], and ALP ≥1.5 x ULN[5, 85, 95, 98]. For liver stiffness with pSWE, we used 1.28 m/s (4.9 kPa) for differentiation between mild and advanced liver fibrosis (F0-2 vs. F3-4)[103, 118]. Odds ratios were analysed with logistic regression. We used a linear mixed model with an unstructured covariance structure for repeated measurements. All variables were established on the same scale from assessing the z-scores by standardizing the predictor and outcome variables to a mean of zero with a standard deviation of one. Intraclass correlation coefficients were estimated in the linear mixed model, and a decomposed mixed model analysis yielded associations between parameters over time. Liver transplantation-free survival was computed from Kaplan-Meier plots and log-rank tests. Regression analyses were performed as univariate and multivariate Cox regression and Elastic net regression analysis.

2.3.1 Statistical considerations

Our studies aimed to estimate change and fluctuation in biomarkers over time as an expression of disease development and use for prognostication. We have also explored biomarkers and combinations of biomarkers as predictors of patient outcomes. We have therefore sought statistical methods capable of finding associations between different types of data, including possible unknown factors that could influence our findings, i.e., confounding effects. Overall, statistical methods handling such questions are termed regression analysis, and the methods applied here will be further presented in this chapter.

Approach to longitudinal analyses

We used the longitudinal mixed model as our statistical model to estimate the magnitude of change over time since it offers analytical strength toward biological variation and upholds statistical power sufficient for the number of participants in our study. Since our model was comparing biochemical parameters with different units, such as elastography measured in velocity, the variables were standardized to the same scale to make the effect sizes comparable. This was done by standardizing to z-scores, i.e., standardizing a variable to a mean of zero with a standard deviation of one: Mean = 0 and SD = 1. The resulting z-score represents the magnitude of change, with negative values representing a decrease and positive values an increase in effect size.

The mixed model is calculated from predictors, representing the means of the observed effects, i.e., the fixed effects, and an estimation of the unobserved parameters, i.e., random effects[166]. The mixed model also accounts for fluctuation within individuals as residual variance[167]. Notably, the residuals are the only variables where a normal distribution is required. Biological variation is approached by assuming a random distribution among the variables at baseline, i.e., a random intercept, and analysing each individual's parameters as a trajectory over time. In this way, each individual functions as their reference, which is a logical approach in biology where severe disease is more likely to continue as such than spontaneous recovery.

The effect of time is assessed by handling time as a continuous variable among the predictor variables. This makes us able to study the effect of time on the data and adjust for any deviation in the time interval between our annual study visits. Variation is assessed from the perspective of

A) between-person or interindividual variation representing variation at a single time point and, therefore, invariant of time
B) within-person or interindividual variation as variation across several time points

Estimating variation over time demands a minimum number of time points in a study. With two time points, every case will give a perfect linear fit. In comparison, three time points or more will enable variation over time. The intraindividual and interindividual variation in our mixed model was estimated from the intraclass correlation (ICC)[168]. As a rule of thumb, high ICC represents a lower degree of variation between the variables, favouring risk stratification. A lower ICC may favour identifying individual changes over time, i.e., prognostication.

We also ran a decomposed longitudinal mixed model to examine whether the variables fluctuated independently or showed concordant fluctuation between variables. This model can point towards common mechanisms behind a simultaneous rise and fall in parameters.

Correlation, collinearity, and the correlation network

The biomarker panels in studies I and II yielded many highly correlated variables. Consequently, we expanded our analyses with a graphical correlation plot to assess and visualize the correlation between pairs and groups of variables. This method is a common approach for correlation analysis in large datasets[169, 170]. We then selected the final predictors from multivariate regression analysis. In general, variables with high degree of correlation will cause collinearity in regression models as a biased effect and reduce the analytical power by increasing standard errors and potentially affecting the p-values[171-173]. A correlation coefficient above 0.5 suggests a hazard of collinearity. The term multicollinearity describes possible collinearity among three or more variables, but not necessarily high correlation coefficients[173].

Correlation represents the linear relationship between two parameters[173], yielding a coefficient between -1 and 1, where values closer to the extremities reflect a strong relationship. In contrast to the term correlation assessing the relationship between two variables, regression addresses their association. In linear regression, this is accomplished by introducing an error (ε) representing the unknown residuals (**Figure**

5) as a measurement for unknown factors causing deviation in a vertical distance for each data point from the perfect straight line.

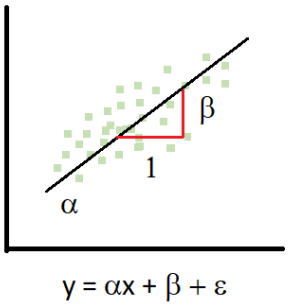

Figure 5. Principle of the linear regression analysis. A straight line is calculated from y = ax + b, (…), representing a relationship between two variables. The fitted black line (y) is calculated from the data points or observations (green dots). The residuals represent the deviation in a vertical distance for each data point from the perfect straight line[173]. *Created by G. Fossdal using Paint (© Microsoft 2022).*

A high degree of correlation can be expected when implementing biomarkers covering the same pathologic processes, i.e., collagen III-formation reflected through Pro-C3 but also a part of ELF. Secondly, high correlation can occur in the case of closely connected pathways, for instance, inflammatory biomarkers and the IFN-γ-driven kynurenine-tryptophan pathway. Graphical correlation plots can suggest possibly related pathways as a hypothesis for further study.

These analyses arrange the output in circles (vertices) and connecting lines (edges)[174, 175]. The connecting lines are termed partial correlation coefficients and represent the linear relationships between two respective variables but also adjust for potential confounding effects among the variables. The confounder effect is estimated by removing one variable at a time and analysing the remaining variables through regression analyses. The thickness of the lines represents the strength of the relationships between two variables. Absent lines represent either very weak or no relationship detected[170, 176].

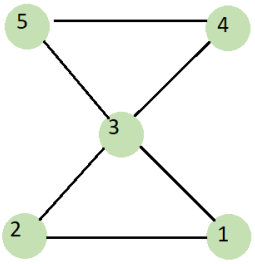

Figure 6. The Gaussian graph model explained: GGM = (V, E), representing vertices (circles) and edges (lines). The vertices are represented by the circles 1, 2, 3, 4, and 5, whereas the edges are represented by each line (1, 2), (1, 3), (2, 3), (3, 4), (3, 5), (4, 5). The lines are calculated as a bivariate correlation adjusted for the contributing effect from the residuals of the remaining variables. *Illustration by Guri Fossdal using Paint (© Microsoft 2022), adapted from Besteman 2017*[177]*.*

We have applied a variant of this network, the Gaussian Graph Model (GGM), which is widely accessible in statistical software packages and visually easy to interpret (**Figure 6**). The term Gauss refers to the assumption of normal distribution among the data[173]. There are few restrictions in the GGM analysis, which is suitable for studying large datasets and new combinations of variables and suiting well for our purposes[170, 175]. Since large data sets can result in large networks, the GGM offers a technique to remove the connecting lines from the smallest partial correlation coefficients in the model. The GGM analysis, therefore, represents an approximation but benefits the visualization of patterns. This is done by the glasso algorithm; a variant of lasso regression for graphical network analyses, by forcing the smallest coefficients to zero[178]. The threshold for omitting an edge in the network can be adjusted in the statistic software. Lack of edges might also be an effect of low sample size and lack of power. Indeed, false negative relationships (lacking line) might occur, giving rise to a type II error by falsely rejecting a null hypothesis[173]. Therefore, the lack of edges does not necessarily exclude the possibility of a relationship between variables[175]. In addition, the network pattern gives us an impression of clustered variables with possible latent covariation and promotes a central arrangement of the strongest variable as reviewed[175], which in **Figure 6** would correspond to node number 3.

Machine learning and regression analysis

In paper II, we estimated the strongest predictors among our biomarkers from multivariate regression analysis. The various multivariate analyses apply different techniques for handling collinearity and multicollinearity. Notably, no model is perfect for any dataset or situation[173]. We considered lasso and ridge regression and the elastic net model. In general, lasso regression selects variables, omitting the ones with the highest correlation, and might be the most robust model in case of fewer predictors in a dataset[173, 179]. However, the lasso regression might be unspecific when variables are rejected from the analysis[180]. Ridge regression handles all variables in the analysis and might perform better than lasso in the case of many predictors and high correlation[179, 180].

Elastic network regression combines lasso and ridge regression techniques to handle collinearity by variable selection and conservation of groups of variables that contribute to a meaningful data structure[181, 182]. The elastic network model is also more robust in handling collinearity than ridge regression but does not fully compensate for these effects and can generate false-positive predictors[183]. The elastic network performs better than lasso in large samples with many predictors[180, 184] and has informally been compared to a “fishing net” that retains "all the big fish”, i.e., the strongest predictors[180]. After running a training analysis, we performed cross-validation to assess which regression analysis achieved the smallest test error, i.e., the best fitting of the model to the dataset. In overfitting, the model will be too eager to follow minor errors or ”noise”, thereby reducing the model's accuracy[173].

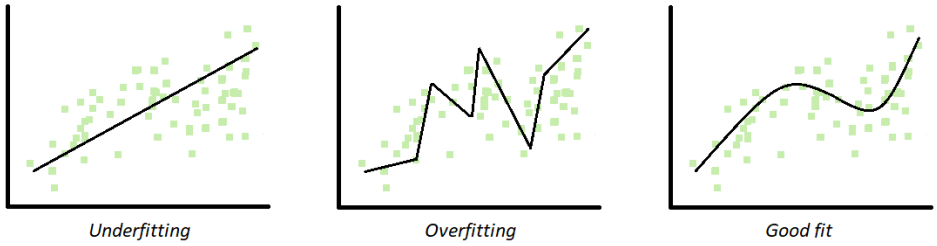

Figure 7. Fitting of a model on training data in machine learning. To the left, few data points are included in the model, resulting in less sensitivity for analysing validation data. The opposite, overfitting, also results in a less accurate model from a surplus of data points, causing "noise" in the model. The right panel demonstrates a balanced fit. *Illustration by Guri Fossdal using Paint (© Microsoft 2022), adapted from James 2021*[173].

Spatial regression analysis

Paper III examined a wide range of parameters, covering single substances, ratios of substances between fatty acids and carnitines, and groups of fatty acids. We pursued to investigate the fatty acid composition in patients with elevated vs. normal bilirubin. We, therefore, sought to find clusters of variables according to phenotype PSC or HC and elevated vs. normal bilirubin in our dataset, i.e., natural groupings of observations from their spatial arrangement among our data. The underlying thought is that observations within proximity could be more associated with each other by shared confounding factors than distant observations. The Ward.D linkage method is a multivariate regression analysis commonly used to address observations' spatial arrangement and their dependent factors. The scattering of observations is estimated from their distance from the mean value, i.e., the sum of squares, yielding a hierarchy of clusters[185]. Since the fatty acids comprised single and groups of fatty acids, we first standardized the variables to the same scale by z-score.

2.4 Ethical considerations

The patients were provided with oral and written information, and written consent was retrieved from all subjects ahead of inclusion. The study visits were conducted as annual follow-up visits as part of clinical follow-up; however, blood sampling included additional tubes for biobanking. This may have caused minimal discomfort. The studies were in accordance with the Declaration of Helsinki. The study of paper I was approved by the Regional committees for medical and health research ethics of Western and South-Eastern Norway (reference 2012/2214/REK VEST and 2008/8670, respectively). Study II was approved by the regional committee for research ethics in South-Eastern Norway (reference 2011/13381). Study III was approved by the regional committees for research ethics in South-Eastern (13381/REK South-Eastern B) and Western Norway (2018/1425/REK Vest).

3. Summary of results

3.1 Paper I

The main objective was to explore the development and variation of ELF, elastography, and ALP in 113 people with PSC over time. Median (IQR) baseline values were ELF 9.3 (1.34), LSM 1.26 m/s (0.52), and ALP 151.5 U/l (197). We noted a percentage of 56, 37, and 50 patients belonging to high-risk groups defined as ALP ≥1.5 xULN, ELF ≥9.8, and LSM ≥1.28 m/s. The median observation time was 4.5 years, and 78 patients (69%) had ≥3 study visits.

We found a significant increase in both ELF and LSM over time, 0.06 SD per year, 95% CI [0.03, 0.20], p=0.005 and 0.07 SD per year, 95% CI [0.02, 0.13], p=0.009, but only in the subgroup of patients with elevated ALP ≥1.5 xULN at baseline. We also found a significant increase for ALP with 0.04 SD per year (95% CI [0.01, 0.07], p=0.011, and bilirubin 0.07 SD per year (95% CI [0.02, 0.12], p=0.007).

The intraclass correlation coefficient (ICC) was 0.86 for ALP, 95% CI [0.82, 0.89], p=0.011, 0.78 for ELF, 95% CI [0.72, 0.83], p=0.005, and 0.56 for LSM, 95% CI [0.47, 0.65], p=0.009. Hence, 78% of the variation for ELF was accounted for by the between-person variation, while between-person and within-person variation contributed fairly equally for LSM.

When testing ELF and LSM against liver biochemistries and clinical risk scores ***(Paper I**, Table 3)*, ELF demonstrated a stronger association than LSM towards liver biochemistries, except for bilirubin: sFE(ALP) 0.47^{ELF}, 0.28^{LSM}, sFE(albumin) -0.39^{ELF}, -0.35^{LSM}, sFE(bilirubin) 0.20^{ELF}, 0.29^{LSM}. ELF was also more strongly associated with the clinical risk scores, i.e., Mayo risk score (sFE 0.48^{ELF} vs. 0.37^{LSM}) and FIB-4 (sFE 0.56^{ELF} vs. 0.42^{LSM}), p<0.001 for all parameters. Furthermore, we found concurrent fluctuations between ELF and ALP, with sFE 0.15, 95% CI [0.11, 0.18], p<0.001 ***(Paper I**, Table 4)*.

A subset of patients with elevated ELF, LSM, and ALP at baseline experienced spontaneous normalization over time. One year into the study, 44.7, 42.7, and 13% had normalized ELF, LSM, and ALP, respectively. The subgroup with baseline ALP ≥1.5 xULN accounted for all cases with a ≥40% spontaneous reduction in ALP. About 10% of the patients experienced a concomitant reduction in ELF, LSM, and ALP at one or more follow-up visits. We registered UDCA treatment at any time among 35% of the patients. Notably, only 25% of the patients on treatment with UDCA experienced a reduction in all three parameters *(**Paper I**, Supplementary Table 3)*.

3.2 Paper II

This study aimed at identifying a panel of multiple biomarkers with increased prognostic capacity toward clinical outcomes. From an exploratory panel of 138 patients, we assessed a range of serological biomarkers already documented as predictors of outcome in PSC, comprising pathways ranging from fibrosis, inflammation, and metabolites from gut microbiota. With various statistical analyses, we wanted to 1) explore their prognostic capacity towards outcome as single markers and 2) identify a final panel of selected biomarkers with increased prognostic capacity.

Correlation analysis examining bivariate and network associations revealed a high correlation between ELF, Pro-C3, Pro-C5, C4M, C3M, CD163, and Mayo risk score *(**Paper II**, Figure 1-2)*. Importantly, KTR and neopterin formed a separate group in the network, implying a strong association between these parameters, albeit independent from the remaining markers.

In addition, we conducted a univariate Cox regression analysis to establish the c-indices for the various biomarkers. Secondly, machine learning in an elastic network analysis selected six biomarkers that all had achieved a c-score at or above 0.65 from the univariate analysis as the set of biomarkers that collectively captured risk best.

Cross-validation in a multivariate regression analysis yielded a final panel consisting of ELF, KTR, and PLP, reflecting different pathways of fibrosis, inflammation, and metabolism. This combination increased the predictive capacity by 4%, as determined by Harrel's C score, compared to a panel of ALP and Mayo risk scores *(**Paper II**, Table 4-5)*.

3.3 Paper III

Multiple studies have shown an association between mitochondrial dysfunction and various liver diseases, including cholestatic liver diseases[186-189]. This prompted us to characterize markers of mitochondrial function in PSC. Our group has previously shown that an elevated kynurenine-tryptophan ratio was associated with clinical outcomes in PSC[136], suggesting that mitochondrial dysfunction may contribute to the pathogenesis. Therefore, we wanted to investigate circulatory and liver-resident metabolites directly reflecting mitochondrial function using mass spectroscopy profiling of fatty acids, carnitine, and acylcarnitines. We also studied metabolites in the kynurenine-tryptophan-nicotinamide (Trp-Kyn-NAD) pathway as a separate pathway of mitochondrial NAD+ generation from amino acid metabolization. The study material consisted of plasma from 190 non-transplant patients with large-duct PSC and 100 healthy controls enrolled in the National Bone Marrow Donor Registry. Liver specimens from 46 patients were divided into 1) PSC (n=24) and 2) non-cholestatic liver disease (n=18), i.e., AIH, ARLD.

Hierarchical clustering showed altered fatty acid profiles in PSC patient plasma compared to healthy controls, especially increased levels of palmitate (C16:0), C18-derived fatty acids, and higher levels of monounsaturated fatty acids (MUFAs) but reduced long-chain saturated fatty acids (SFAs) and polyunsaturated fatty acids (PUFAs). PSC patients with cholestasis had the most pronounced changes. Changes in liver tissue were less pronounced but in line with findings from plasma. Furthermore, PSC patient plasma demonstrated reduced NAD+ synthesis. Overall,

our findings clearly indicated mitochondrial dysfunction in PSC, which was more pronounced in cholestasis.

4. Discussion

4.1 Fibrosis markers in a longitudinal perspective

4.1.1 Methodological considerations

Patient panels

We recruited patients fulfilling the criteria of large-duct PSC or PSC with features of autoimmune hepatitis (AIH) prospectively in our two study centres. Since one of the centres (Oslo University Hospital, Rikshospitalet) is a tertiary centre (the national liver transplant centre), and the other offers non-tertiary patient care, this might introduce selection bias among patients. However, we found no significant baseline differences in liver biochemistries, ELF, or LSM ***(Paper I,*** *Table 1)*.

Biochemical parameters including enhanced liver fibrosis test

9.8, and 11.1 in a mixed panel of liver disease[190]. ELF functions as a combined marker reflecting increased collagen turnover, where we have used a cut-off value of 9.8 for the subanalysis of high-risk patients. This threshold has demonstrated the best ability to discriminate between moderate and severe degrees of fibrosis, with a reported 76% sensitivity and 87% specificity in a mixed population of liver diseases[128], corresponding well to our goal of detecting early disease progression. In our study on ELF in PSC[129], Youden test from our derivation and validation panels yielded an optimal cut-off value of 11.1 and 11.2, with sensitivity/specificity 67.0/82.7 and sensitivity/specificity 72.3/82.4, respectively. This cut-off determined the ability to discriminate between patients with or without the endpoints death or liver transplantation.

In contrast, longitudinal data showed the optimal baseline threshold for ELF in detecting clinical events like disease progression was 9.8, yielding sensitivity and

specificity of 68% and 67%[95]. We therefore adhered to 9.8 as the desired cut-off value for ELF as a marker of early disease progression. Notably, ELF is reported to be significantly higher in males and increases with age, but demographic data were not evaluated further in our analyses[190].

Liver stiffness measurements

We performed liver stiffness measurements using two different pSWE platforms for the respective cohorts, i.e., ElastPQ Philips iu22 in the Bergen cohort and Siemens Acuson S3000 for Oslo. Since we followed the respective cohorts by their designated platform during the study and non-parametric testing (Mann-Whitney) found no statistical difference (p=0.39) in the measurements between the two cohorts, we accepted pooling of the data. Also, there were no significant differences regarding liver biochemistries or clinical severity scores, such as the Mayo risk score.

Our study protocol accounted for several factors of possible confounders for liver stiffness. Firstly, the patients were examined after at least three hours of fasting, as meal ingestion causes increased portal flow and liver stiffness[191, 192]. Cholestasis is another potential bias in liver stiffness measurements[193], but we only found bilirubin >30 µmol/L in six of our 113 patients The patients underwent clinical examination and serological screening prior to inclusion to rule out differential diagnoses like viral hepatitis.

We used pSWE in our studies as this was the only available platform at our center at the start of the study. Although Fibroscan (TE) is the best-validated elastography platform in PSC[112, 118], pSWE has demonstrated an excellent intraclass correlation coefficient (ICC) compared to TE[103] and correlates well with histopathology in PSC[194]. When comparing pSWE with TE using histology as the reference standard, pSWE shows comparable results toward TE in differentiating lower and higher degrees of fibrosis[195-197], supporting the use of pSWE in our patient material.

Elastography measures the speed of propagation of share waves in the tissue. Results are given either as the measured shear wave velocity (SWV) in m/s or as stiffness in kPa, calculated from a mathematical algorithm. Fibroscan (TE) reports the results converted into kPa, whereas we adhered to m/s as the established unit for the platforms used in our studies. Since the conversion to kPa involves simplification with several assumptions regarding tissue properties, velocity expressed in m/s is considered a more direct measurement[198].

The patchy disease distribution in PSC necessitates a sufficient number of measure sites in the liver. A study of liver stiffness in patients with primary biliary cirrhosis (PBC), which has an uneven distribution in the liver, like PSC, revealed significant variation in portal inflammation. They also found a relationship between fibrosis and parenchymal heterogeneity[199].

For transient elastography, the variability measurement interquartile range/median (IQR/M) is recommended below 30% by the European Federation of Societies for Ultrasound in Medicine and Biology (EFSUMB)[198]. A variability criterion for the secondary elastography modalities still needs to be validated, but the EFSUMB guidelines have extended the IQR/M measurement to apply for secondary elastography. Other variability measures based on the mean-based measurements, like the standard deviation (SD) and CV (SD/mean), have been proposed in ARFI and 2D-SWE, respectively[194, 200, 201].

4.1.2 Development of ELF and LSM over time

Our longitudinal mixed model revealed a small but significant increase in both ELF and LSM over five years and a more significant increase in LSM than ELF ***(Paper I,*** *Table 2)*. These findings are in line with previous longitudinal studies of liver stiffness and ELF in PSC[5, 95, 118]. We also detected increased bilirubin and ALP during the study, possibly reflecting disease progression in our cohort during this five-year period. The subgroup defined as high-risk patients with ALP ≥1.5 xULN had elevated ELF and LSM as of baseline. Interestingly, a post hoc analysis could

only identify an increase in ELF and LSM among the patients with baseline ALP ≥1.5 xULN, supporting this cut-off value to identify at-risk patients. Regarding fluctuations between our fibrosis markers ELF and LSM vs. liver biochemistries, i.e., ALP and bilirubin, we found concomitant fluctuations between ELF and ALP (p<0.001**) ***(Paper I,** Table 4)*. Hence, the fluctuations in ELF in our material seem associated with the ongoing changes in the biliary tree.

The changes in LSM in the ALP ≥1.5 xULN subgroup point toward previous studies[95, 118]. The study by Corpechot also did a subanalysis of LSM changes over time according to fibrosis stage and found a significant increase in fibrosis stages F2-F4 but not F0-1. A variant of the longitudinal mixed model allows for the decomposition of associations between parameters over time, testing whether fluctuations in parameters occur independently or as concurrent fluctuations. Fluctuation over time is well-known in PSC for liver biochemistries like bilirubin and ALP[52], but whether this coincides as part of a flare or disease progression is, to our knowledge, not previously described.

4.1.3 Risk stratification and prognostication between individuals

In paper I, we found a high ICC value in ELF compared to LSM, 0.78 vs. 0.56, respectively. The higher ICC favour ELF for stratification between individuals at a single time point. ALP yielded an even higher ICC of 0.86, and the decomposed mixed model demonstrated concurrent fluctuations between ELF and ALP. These findings might imply similar underlying mechanisms for these parameters. Contrarily, ELF demonstrated a superior correlation between other biomarkers and clinical scores compared to ALP ***(Paper I,** Figure 1)*. Despite the fact that ALP achieved a high ICC value, the existing literature is in evident disfavour of this parameter for prognostication and risk stratification due to its inconsistent regarding cut-off values and propensity for spontaneous normalization[5, 151, 202, 203]. Clearly, any value of ICC must be interpreted in a broader context.

We deduce that ELF has the strongest ability to identify at-risk individuals at a single time point. Although a lower ICC may imply a better ability for prognostication, we suspect that the lower ICC for LSM comes from a higher degree of sampling variability and interobserver variation due to variations in the fasting state, probe pressure, and patchy disease distribution. In order to draw firm conclusions about the best prognostic tool, a larger patient material with a sufficient number of solid endpoints would be mandatory for clinical outcome analyses.

4.1.4 Spontaneous normalization of parameters

Since endoscopic and pharmacological intervention can influence LSM and ELF[204, 205], we sought to establish any possible treatment influence on our material. Regarding UDCA, one in three patients (n=39) received treatment on at least one study visit. The low number of patients receiving UDCA did not provide enough power for subgroup analysis per study year. However, we reran the mixed model with UDCA as a categorical variable, defined as UDCA treatment at any time point during study *(**Table 2**, unpublished data)*. We found that patients who received UDCA had higher levels of biomarkers at baseline but also a smaller increment over time than UDCA naïve patients. Although there were insufficient data to perform multivariate analyses adjusted for the biomarker*treatment interaction, we believe that the elevated levels of baseline values were due to confounding factors since UDCA, according to Norwegian clinical practice, is not routinely prescribed in PSC, except for symptomatic treatment of pruritus.

Table 2. Subanalysis vs. to UDCA treatment. Longitudinal mixed model analysis according to treatment status for patients receiving UDCA at any time point during study. UDCA-treated patients presented higher baseline values for all parameters, shown as fixed effect (FE) at baseline. *(Unpublished data)*

		No UDCA			UDCA-treatment		
		FE	*95% CI*	*p*	*FE*	*95% CI*	*p*
ELF	*Intercept*	-0.15	-0.34, 0.04	0.132	0.29	-0.02, 0.59	0.065
	Slope	0.06	0.01, 0.10	0.009	0.01	-0.07, 0.09	0.803
LSM	*Intercept*	-0.13	-0.30, 0.04	0.134	0.17	-0.20, 0.55	0.369
	Slope	0.05	-0.01, 0.10	0.057	0.09	-0.02, 0.20	0.125
ALP	*Intercept*	-0.02	-0.23, 0.18	0.817	0.27	-0.09, 0.63	0.139
	Slope	0.05	0.01, 0.08	0.008*	0.01	-0.04, 0.05	0.883
Bilirubin	*Intercept*	-0.12	-0.33, 0.09	0.271	0.25	-0.08, 0.15	0.530
	Slope	0.07	0.01, 0.13	0.018*	0.04	-0.08, 0.15	0.530

Abbreviations: ALP, alkaline phosphatase; ELF, enhanced liver fibrosis test; ERCP, endoscopic retrograde pancreatography; FE, fixed effect; LSM, liver stiffness measurement.

*=p<0.050

Furthermore, we have evaluated UDCA usage among the patients who experienced either a solitary decline in ALP ≥40% or a concomitant reduction in ELF, LSM, and ALP. Out of 113 patients, we observed a reduction in ALP ≥40% in 15 patients, seven of whom had received UDCA at any time point during study time, and eight did not. We found a reduction in all three parameters in 24 of 113 patients, but only six of these had received UDCA during the study.

Only six patients underwent ERCP with endoscopic interventions (i.e., balloon dilatation or stent placement), totaling ten endoscopic procedures during the study period *(**Table 3**, unpublished data)*. Hence, further subgroup analyses were not applicable, but the data revealed no apparent effect on either ELF or LSM assessed after these procedures. Time from endoscopic intervention to collection of all three parameters, ELF, ALP, and LSM, ranged from 4-35 weeks, with a median of 11.5 weeks. We found a reduction in ALP after three procedures, while only two procedures yielded a decrease in ELF or LSM. Notably, LSM reduction did not coincide with reduced ELF or ALP. In conclusion, although our study was not designed to evaluate treatment effects over time and further statistical analyses were

futile due to inadequate statistical power, we could not decipher a causative effect from UDCA or endoscopic treatment.

Table 3. Biomarkers before and after ERCP. From investigation of our database and patient journals we found six patients who had undergone a total of 10 ERCP procedures with intervention. The table lists the time from intervention to measurement of the parameters. Reduction in any of the parameters appeared independently from another. *(Unpublished data)*

Patient	ELF			ALP			LSM		
	Before	*After*	*Weeks*	*Before*	*After*	*Weeks*	*Before*	*After*	*Weeks*
1	sep.53	okt.14	30	201	274	30	01.okt	0.95*	29
1	okt.14	okt.23	18	274	252*	18	0.95	02.nov	29
1	okt.23	nov.16	14	252	172*	14	02.nov	1.71*	14
1	nov.16	nov.48	6	172	244	6	jan.71	jan.73	8
2	sep.62	9.52*	9	95	93*	9	jan.23	jan.32	9
3	11.okt	10.96*	4	213	324	4	feb.74	03.jul	4
4	okt.78	des.20	12	101	421	12	jan.19	jan.92	12
5	jul.60	jul.87	11	54	60	11	jan.25	feb.17	11
6	08.mar	aug.31	11	86	141	11	01.feb	jan.23	11
6	aug.31	aug.79	35	141	330	35	jan.23	jan.52	35

Abbreviations: ALP, alkaline phosphatase; ELF, enhanced liver fibrosis test; ERCP, endoscopic retrograde pancreatography; LSM, liver stiffness measurement.
*= reduction in the parameter measured after ERCP

4.1.5 ELF and concomitant bowel disease

Most of our patients (75.2%) were diagnosed with IBD at the time, which calls for attention to whether bowel inflammation and fibrosis could affect circulatory biomarkers. A literature search in PubMed did not retrieve clinical studies on the ELF test among IBD patients. Notably, hyaluronic acid, one of the three components of the ELF analysis, has been found as colonic deposits and demonstrated an association with inflammation in IBD as reviewed[206, 207]. Although we did not register IBD activity in our data material, bivariate regression analysis revealed no association between IBD status and ELF level ± 9.8 ($p>0.100$) *(unpublished data)*.

4.2 Establishment of a multimarker panel

We explored a large panel of single biomarkers from a cross-sectional dataset to establish a panel of combined biomarkers with increased predictive power. High correlation and multicollinearity challenged multivariate analyses and were approached by network mapping of correlations and elastic network Cox regression analysis.

4.2.1 Establishment of relationships among biomarkers in a correlation network

From our Gaussian graph model, we extracted three main points *(**Paper II,** Figure 2)*, 1) a strong correlation between several biomarkers, 2) independent groups of biomarkers from their internal relationship, and 3) a central placement of ELF among the predictors examined.

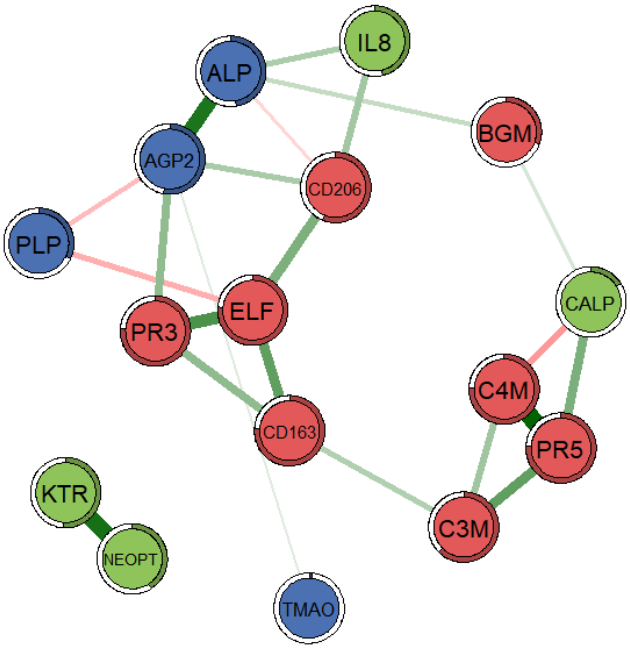

Figure 7. Gaussian graph model. Our analysis found three main groups of biomarkers with higher internal relationships. As anticipated, ELF and Pro-C3 are strongly related due to a partially common pathway through collagen III formation. Importantly, KT-ratio and neopterin represented highly related variables unrelated to the remaining material. (Green lines positive relationship, red lines negative relationship).

Abbreviations: AGP2, anti-glycoprotein 2-IgA; ALP, alkaline phosphatase; BGM, biglycan degradation; C3M, C4M, degradation of type III and IV collagen; CALP, calprotectin; CD163, CD206, cluster of differentiation 163 and 206; ELF, enhanced liver fibrosis; IL-8, interleukin-8; KTR, kynurenine/ tryptophan-ratio; NEOPT, neopterin; PLP, pyridoxal 5'-phosphate; PR3, PR5, PRO-C3 and PRO-C5, type III and V collagen formation; TMAO, trimethylamine-N-oxide.

We found strong relationships between ELF and Pro-C3, as expected since both Pro-C3 and, in part, ELF measure the collagen III level and collagen III formation, respectively. Our results also indicate ELF as a strong predictor by its central placement. However, caution should be drawn to this conclusion, as minor differences in the input of variables in this model can alter the placement of nodes[175]. Further analyses could have helped to shed further light on this conclusion, like comparing AUC values from AUROC analysis, Harrel's C scores from univariate Cox regression analysis, and multivariate regression. Perhaps more interestingly, we found a strong relationship between KT-ratio and neopterin, yet, independent from the remaining network suggesting a pathway separate from the remaining variables in line with the above argument. From this, we hypothesize that parallel pathways contribute to the pathogenesis in PSC and might imply a broader approach for establishing treatment targets. Single fibrosis markers of the basement membrane and interstitial matrix of the ECM seem strongly related, i.e., C4M, Pro-C3, C3M, and Pro-C5.

Interestingly, the inflammatory marker IL-8 was weakly related to the remaining variables. One possible explanation might be that inflammatory markers represent an earlier disease stage in PSC than fibrosis markers, resulting in a weaker association with clinical outcomes for inflammatory markers. Although more advanced statistical methods are needed to evaluate our parameters further, one might speculate that the GGM analysis considers fibrosis markers as stronger predictors than inflammatory markers.

4.2.2 A proposed multimarker panel in PSC

Our final model suggested a prognostic panel including ELF, PLP, KT-ratio, Pro-C3, and C4M with improved predictive capacity. The final panel consisted of a combination of biomarkers from different pathways with less association towards each other, resulting in gained robustness toward multicollinearity. Multicollinearity was especially pronounced between ELF, Pro-C3, and CD163 and might have

obscured the findings of our final model. The strong correlation between ELF and CD163 might result from common pathways within fibrogenesis, where CD163 is known to be involved in the stimulation of hepatic stellate cells from macrophages[143]. Increased CD163 could also represent a response to altered haemodynamic from portal hypertension[208-210].

By machine learning, Pro-C3 and CD163 were left out of our elastic network model, but still, high correlations between ELF and Mayo risk score challenged our model. Therefore, our final model omitted the Mayo risk score, supported by the composition of the Mayo risk score related to later stages of liver disease from variables like variceal bleeding, bilirubin, AST, and albumin. On the other hand, ELF relies on components believed to reflect fibrosis development. Hence, this marker should be more likely to reflect early PSC. Indeed, current guidelines recommend ELF for the evaluation and follow-up of PSC patients[8].

Consequently, our results suggest a multimarker panel in PSC but necessitate further research with validation studies. However, we believe that correlation networks and advanced regression analyses represent promising tools for studying combined biomarkers as clinical predictors.

4.3 Alterations in liver metabolism in PSC

Many liver diseases display signs of mitochondrial dysfunction, such as non-alcohol-related fatty liver disease (NAFLD), acute-on-chronic liver failure (ACLF), liver fibrosis, and PBC[145, 189, 211-213]. Therefore, we wanted to explore indirect markers of mitochondrial function in PSC, including fatty acid profiles, to elucidate whether mitochondrial dysfunction was present in PSC.

4.3.1 Fatty acid and lipid alterations in PSC

In paper III, we studied fatty acid profiles in PSC, especially regarding mitochondrial lipid metabolism. We demonstrated important alterations of the plasma fatty acid profiles in PSC. Using hierarchical cluster analysis of plasma fatty acid profiles, we found two large clusters of patients which separated quite well, one consisting of all healthy controls and PSC patients with bilirubin levels within the normal range and one containing PSC patients with elevated bilirubin *(**Paper III**, Figure 1C)*. Overall, the differences were most prominent in the PSC subgroup with cholestasis.

Our studies in plasma showed significant changes among palmitate C16 and stearate C18 fatty acids, reflected by several single fatty acids and increased enzymatic activities of stearoyl CoA desaturases (SCD-16 and -18, i.e., D9 desaturase C16:0 and C18:0 enzymes) *(**Paper III**, Figure 2C, Supplementary Table 1)*. This finding corresponds to the increased levels of MUFA in people with PSC since these two enzymes stimulate conversion from C16:0 and C18:0 into especially C16:1 n-7 [POA, palmitoleic acid] and C18:1 n-9 oleic acid *(**Paper III**, Figure 2B)* as reviewed[214].

Palmitate C16:0 also serves as a substrate for elongation into fatty acids of longer chain length, such as C18:2n-6 [LA, linolenic acid][215]. We found increased lipogenesis estimated by the C16:0/C18:2n-6 ratio *(**Paper III**, Figure 2C)*. Together with increased MUFA, these findings indicate increased endogenous biosynthesis in PSC. Our findings of reduced long-chain saturated fatty acids (SFAs) *(**Paper III**, Figure 1B, and 2A-B, Supplementary Table 1)* are in line with increased activity of SCD enzymes, as they use SFA for substrates in MUFA synthesis[216].

Fatty acid transport between the peroxisomes and mitochondria is provided by either the carnitine transport shuttle or transmembrane proteins for free fatty acids[217-220]. Indeed, carnitine represents the main mechanism for mitochondrial C16 fatty acid transportation[221]. In addition to carnitine levels, we measured carnitine ratios as they reflect the intramitochondrial amounts of carnitines[221]. We found increased levels of the long-chained palmitoylcarnitine C16, reflected by a reduced C2/C16 ratio *(**Paper***

***III**, Figure 4A),* supporting increased turnover of C16 fatty acids. The alterations in C16 and C18 lipids agree with previous smaller studies in PSC and PBC[222, 223]. Since most liver metabolites were not significantly different between PSC and non-cholestatic liver diseases, our findings could represent more general metabolic changes in liver disease than cholestasis per se.

Fatty acid synthesis is in counterbalance with fatty acid oxidation, i.e., the first step yields malonyl-CoA, thereby inhibiting the beta oxidation[215]. Nonetheless, we found reduced short odd-chain carnitines, indicating increased oxidation of short odd-chain fatty acids. Therefore, intermediates of fatty acid β-oxidation and the end-product acetyl-CoA could have contributed to elucidating the activity from fatty acid β-oxidation in our study.

Another aspect of fatty acids is their potentially damaging effect from the accumulation of metabolites, possibly linking lipid alterations directly to the pathogenesis in PSC. Indeed, studies in vitro have shown palmitate-induced inflammation via the toll-like receptor (TLR4) stimulation, NF-κβ-related pathways, and macrophage activation via CD163[224, 225]. Intriguingly for PSC, studies have linked palmitate to intestinal permeability[226, 227]. Thus, altered metabolism of C16 fatty acids could contribute to a self-propagating state of inflammation, emphasized by the reduction in anti-inflammatory fatty acids among n-3 and n-6 PUFAs. Indeed, supplements with PUFA have ameliorated lipotoxic effects in NAFLD and demonstrated ALP-reduction in PSC. Referring to the increase in palmitoleic acid C16:1 n-7, especially seen in cholestatic PSC patients ***(Paper III**, Figure 3B)*, the inflammatory role of C16 fatty acids appears more nuanced as POA has demonstrated beneficial effects *in vivo* on inflammation via PPARγ[228], suggesting different effects for the various transcription factors of the PPAR family. In this regard, disruption of the palmitate metabolism embraces several known pathways involved in PSC and warrants further studies on the role of C16 fatty acid metabolism in PSC.

Total n-3 and n-6 PUFA were reduced in people with PSC, even if some n-6 PUFA were elevated. The reduction in C18:3n-3 [ALA, α-linolenic acid] and linolenic acid

C18:2n-6 can explain the reduction in total n-3 and n-6 PUFAs, as they represent the most abundant n-3 and n-6 PUFAs, respectively, and are precursors for the longer chained PUFAs[229]. Our findings of altered SFAs, MUFAs, and PUFAs differ from a small study including twenty patients with PSC[230] but are in line with reports of elevated MUFAs in viral and alcoholic liver disease and NAFLD[231]. In general, n-3 PUFAs and n-6 PUFAs are considered to possess anti- and proinflammatory effects[229]. Reduced levels of these two main groups of PUFAs are also found in liver cirrhosis of various etiologies and might play a role in the fibrogenic process[232, 233].

Furthermore, the reduction in ALA in PSC patients might disadvantage the composition of the mitochondrial membranes, as ALA represents a major fatty acid and structural component. ALA also serves as a stabilizer for enzymes of the respiratory chain, a protective substrate from oxidative stress on the endoplasmic reticulum, and prevention of apoptosis in hepatocytes[234].

Analysis of the fatty acid pattern from explanted liver tissue in PSC formed two clusters according to bilirubin levels but less prominent than in plasma ***(Paper III**, Figure 2 vs. Figure 3)*. However, the number of liver samples with PSC was relatively low (n=24) and might have obscured the analysis. This analysis yielded important information but is not directly comparable to our plasma studies since liver tissue from non-cholestatic liver diseases will also be subjected to metabolic alterations compared to plasma samples from healthy controls.

4.3.2 Peroxisomal function in PSC

The peroxisomes serve a myriad of functions (**Figure 8**) and deserve consideration in metabolic studies as providers of

1) Complementary fatty acid oxidation to mitochondrial fatty acid oxidation

2) De novo lipogenesis of very-long chain fatty acids

3) Providers of cholesterol and bile acid conjugation

Ad 1) the peroxisomes serve β-oxidation of very-long chained (>C20) and branched fatty acids into shorter chain lengths/chain-shortened fatty acids for further β-oxidation in the mitochondria, as well as β-oxidation of bile acid intermediates[219]. In contrast to the mitochondrial β-oxidation that runs to completion yielding ATP, H_2O, and CO_2, the peroxisomal β-oxidation results in H_2O_2 (hydrogen peroxide) and heat production but represents an integral and indispensable role in fatty acid turnover. In our material, we found reduced levels of propionylcarnitine C3 and valerylcarnitine C5 *(**Paper III**, Figure 4A)*, handling short the transport of short odd-chain carnitines independent of the carnitine shuttle as for all carnitines of chain-length <C14[215]. Resultingly, degradation of odd-chain fatty acids does not seem hampered in people with PSC.

Ad 2) the peroxisomes are also the site of de novo synthesis of very-long-chain fatty acids from shorter fatty acid intermediates. We did not find a significant reduction in C22:6n-3 [DHA; docosahexaenoic acid] in plasma among people with PSC *(**Paper III**, Supplementary Table 1)*. DHA has previously been shown as an essential peroxisomal substrate and is provided either from the diet or de novo synthesis from alpha-linolenic acid[235]. The peroxisomes handle the final steps to DHA formation before DHA is transferred to the endoplasmic reticulum for synthesis into compounds like phospholipids for incorporation in cellular structures[236]. On the other hand, very-long chained SFAs and n-3 PUFAs were reduced in PSC patient plasma, while very-long chained MUFAs and n-6 PUFAs were increased *(**Paper III**, Figure 1C, 2A-B, Supplementary Table 1)*. Hence, the fatty acid profile regarding DHA and very-long chained fatty acids (VLCFAs) are somewhat ambiguous, and further studies on lipogenesis in PSC needed to clarify whether this is due to altered peroxisomal function or other parts of their synthetic pathways.

Ad 3) cholesterol levels in plasma did not differ between PSC and HC in our study *(**Paper III**, Figure 1, Supplementary Table I)*. Bile acids are elevated in PSC due to biliary obstruction[230, 237], but bile acid synthesis is reduced in advanced PSC[237]. Our findings do not allow us to conclude regarding altered peroxisomal function in PSC.

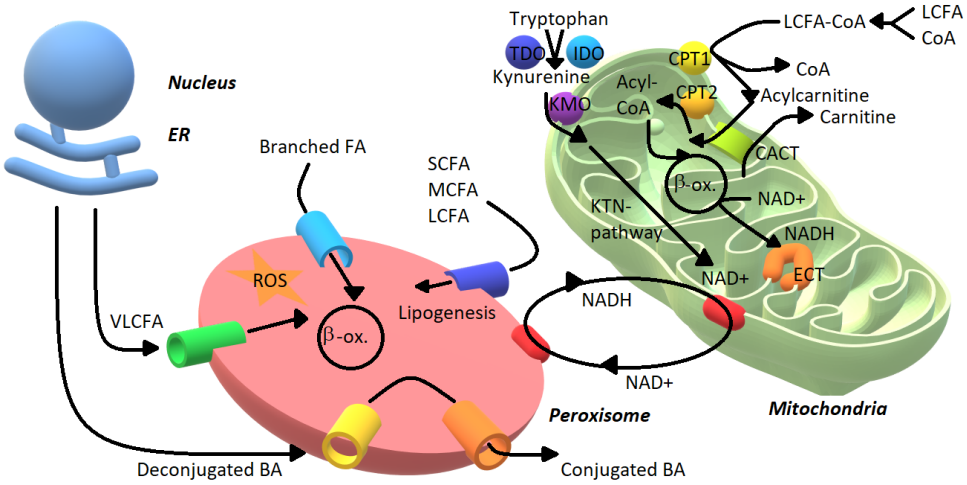

Figure 8. Mitochondrial and peroxisomal main functions illustrated with a simplified overview of the metabolic processes in the liver. The endoplasmic reticulum (ER), with its proximity to the nucleus, is the site of cholesterol synthesis and provides the peroxisomes with substrates like long-chain fatty acids and bile acids, but also antioxidative mechanisms that counterbalance the production of reactive oxygen species from peroxisomal β-oxidation. While the mitochondria provide β-oxidation of short, medium, and long-chained fatty acids, the peroxisomal β-oxidation is necessary for shortening very-long chained and branched fatty acids for further metabolization in the mitochondria. Notably, peroxisomal β-oxygenation relies on shuttling of NAD+ from the mitochondria[219], which is provided from amino acid metabolization through the kynurenine-tryptophan-nicotinamide pathway, β-oxidation of fatty acids or the tricarboxylic acid cycle (the latter not shown). The peroxisomal β-oxidation yields hydrogen peroxide (H_2O_2) and heat but not energy production in the form of adenosine triphosphate (ATP) like mitochondrial β-oxygenation. Transportation of fatty acids ≥14C across the mitochondrial membrane depends on carnitine. The first step requires binding of an acyl group (CoA) for diffusion through the outer membrane, where the fatty acids are converted to acyl-carnitines by the carnitine palmitoyltransferase 1 (CPT1). The carnitine acyltransferase translocase (CACT) provides transport across the mitochondrial inner membrane in exchange for free carnitine[215, 220]. Acyl-carnitines are transformed to free carnitine and acyl-CoA by the carnitine palmitoyltransferase 2 (CPT2) before acyl-CoA enters the β-oxygenation pathway or TCA cycle[220]. Peroxisomes are also the site of bile acid conjugation from cholesterol substrates.

Abbreviations: ECT: electron transport chain; ER: endoplasmic reticulum; BA: bile acid; CACT: carnitine-acylcarnitine translocase; CoA: coenzyme A, CPT1 and 2: carnitine palmitoyltransferase 1 and 2; FA: fatty acid; IDO: indoleamine 2,3-dioxygenase; KMO: kynurenine 3-monooxygenase; KTN-pathway: kynurenine-tryptophan pathway; LCFA: long-chain fatty acid; MCFA: medium-chain fatty acid; NAD: nicotinamide adenine dinucleotide; NADH: nicotinamide adenine dinucleotide in reduced form; ROS: reactive oxygen species; SCFA: short-chain fatty acid; TDO: tryptophan 2,3-dioxygenase; VLCFA: very-long chained fatty acid. *Created by G. Fossdal using Paint 3D (© Microsoft 2022)*

4.3.3 Increased tryptophan clearance and impaired NAM formation in PSC

The kynurenine-tryptophan pathway leads to synthesis of adenine dinucleotide (NAD+) via 3-hydroxykynurenine (HK) and quinolinic acid. NAD is metabolised from NAM, and fuels the electron transport chain in mitochondrial fatty acid oxidation (**Figure 9**)[238]. Our data revealed overall changes in intermediates of this pathway with a significant reduction of NAM *(**Paper III**, Figure* 4B*)*, indicating reduced mitochondrial β-oxidation of fatty acids. Low NAD levels have previously shown effect on triglyceride levels by elevating triglyceride levels from posttranslational modification[239], possibly connecting our finding of diminished NAD to triglyceridemia in PSC with cholestasis *(**Paper III**, Supplementary Table I)*.

In addition to the reduced amount of NAD available for the electron transport chain and peroxisomal β-oxidation[219], downstream metabolites of the kynurenine pathway also possess a direct inflammatory effect from stimulation of immune cells like neutrophils, monocytes, and NK T-cells[240-243]. Inflammatory mediators like TNF-α also represent a direct, negative effect on ATP-synthesis of the respiratory chain[244]. Taken together, altered amino acid metabolism might be a possible inflammatory driver in PSC.

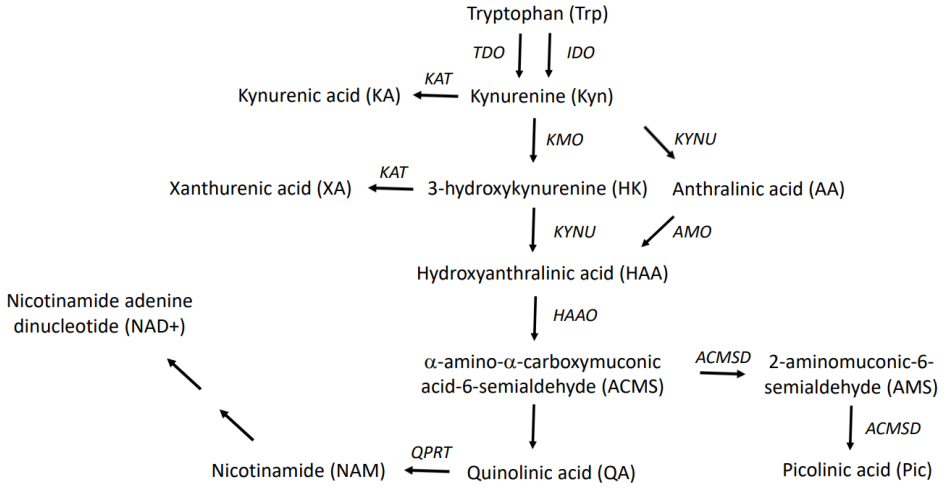

Figure 9. Overview of the kynurenine-tryptophan pathway. Tryptophan conversion through the KT-pathway and the first rate-limiting step via either one of the enzymes tryptophan 2,3-dioxygenase (TDO) or indoleamine 2,3-dioxygenase (IDO), the latter of which we suspect is upregulated in PSC and resulting in an increased kynurenine-tryptophan ratio (KTR). We also found increased quinolinic acid, an intermediate with potential cytotoxic effects[241]. Adapted from Badawi[245].

Abbreviations: ACMSD: □-amino-□-carboxymuconate-□-semialdehyde decarboxylase; AMO: aminocarboxymuconatesemialdehyde decarboxylase; AMS: 2-aminomuconic-6-semialdehyde; HAAO: hydroxyanthranilic acid 3,4-dioxygenase; IDO: indoleamine 2,3-dioxygenase; KA: Kynurenic acid; KAT: kynurenine aminotransferase; KMO: kynurenine 3-monooxygenase; KYNU: kynureninase; QPRT: quinolinate phosphoribosyl transferase, Pic: picolinic acid; TDO: tryptophan 2,3-dioxygenase, Trp: tryptophan; XA: xanthurenic acid.

4.3.4 Metabolic alterations and possible new biomarkers in PSC

Our study demonstrated multiple associations between PSC, cholestasis, and metabolites, including lipids and NAD-related metabolites, pointing towards altered mitochondrial function in PSC. We found increased lipogenesis in MUFA, C16, and C18, with increased transportation and endogenic synthesis of palmitate and stearate fatty esters. Lipogenesis inhibits fatty acid beta-oxidation. Indeed, we found low

NAD for fuelling the electron transport chain. As discussed above, n-3 and n-6 fatty acids were reduced, adding to the variety of metabolic alterations that might be implicated in the inflammatory and fibrotic processes in PSC.

However, the study design did not allow us to conclude regarding the causality of metabolic changes vs. pathological processes, nor whether the changes reflect disease-driving mechanisms or merely an adaption in homeostasis to a disrupted physiological state. Pending future metabolic studies in PSC and giving the vast functions of the mitochondria, one could suggest describing these changes as mitochondrial alterations rather than dysfunction[246].

4.4 Strengths and limitations

overcoming the scarcity of solid endpoints with biomarkers as surrogates for solid endpoints, an issue already addressed in epidemiological research in PSC[247]. The general rarity of PSC, constant fluctuation of symptoms and liver biochemistries, and the highly individual onset time until endpoints challenge the number of study participants necessary for statistically reliable results. In our studies, we have yet to perform a priori power analysis but examined the available data collected from our biobank. Hence, our results are prone to type I errors, i.e., discarding a null hypothesis and stating a significant difference from coincidental effects.

Despite the relatively low number of study participants and scarcity of solid endpoints, the longitudinal methods applied in study I rely on each participant functioning as its control from baseline, increasing resilience towards fewer participants. In paper III, we explored a vast range of variables with descriptive statistics and a spatial regression analysis towards the phenotypes PSC vs. healthy controls and PSC with or without cholestasis. Other confounding factors could have been included, like sex, age, and dietary registration regarding cholesterol and fatty acid biomarkers. Consequently, paper III might have been prone to type II errors by establishing false associations from analysed variables. The long follow-up with a

high proportion of solid endpoints and extensive characterization, including ELF and a broad range of other biomarkers, represent strengths of the patient panels in papers II and III; however, the retrospective study design may have introduced selection bias and missing data. In paper I, the prospective design and repeated sampling represent strengths. In addition, the longitudinal mixed model includes all study participants in the calculations irrespective of missing data within some individuals. This asset contrasts the ANOVA analysis, rendering the longitudinal mixed model more robust. For all three studies, independent prospective validation panels would have strengthened the results.

Possible selection bias could be considered in paper I, as this material consisted of two different patient cohorts, where one of the hospitals functions as a tertiary hospital and transplantation centre. However, subgroup analysis did not find significant differences in the two respective study populations regarding possible confounders like age at inclusion or disease duration, but no significant difference in events of decompensated liver disease or liver biochemistries at baseline. Regarding signs of advanced PSC, we found somewhat inconsistent results, as a smaller proportion of the patients in the tertiary hospital were treated with UDCA but had similar numbers of endoscopic interventions. The gender difference might also contribute to selection bias, as a larger proportion of female patients demonstrate a milder or asymptomatic clinical picture[49], suggesting coupling of patient registries in PSC and IBD to improve identification and recruitment of patients with PSC.

Liver biopsies are not clinically indicated in PSC and were not performed in papers I and II as this was not ethically justified. Histological assessment of fibrosis degree was therefore unavailable for benchmarking our cut-off values. The lipidomic analyses in paper III did offer liver biopsies from patients with PSC but from pre-transplant patients and differed from the respective blood samples. Biopsy material was compared to other patients with non-cholestatic liver disease and not healthy controls, as was the case for plasma analyses. Our biobank material was handled equally in the Oslo and Bergen biobanks and assessed for quality during long-time storage by a specific protocol and not subdued to repeated freeze-thaw cycles. In

paper III, we have emphasized on mitochondrial lipid metabolism. Notably, future studies should proceed with involving peroxisomes and the endoplasmic reticulum as part of the complex system of lipid metabolism. It would also be logical to proceed with biomarkers from other metabolic pathways, like the tricarboxylic acid cycle and amino acid metabolism, function of the electron transport chain (ECT) and handling of reactive oxygen species (ROS), in upcoming studies for to further explore mitochondrial function in PSC.

5. Conclusion

In the present project, we have contributed novel information to the field of prognostic biomarkers in PSC and provided new knowledge regarding mitochondrial function and fatty acid profiles in PSC. We have demonstrated superior stability for the ELF test with less within-patient variability than LSM or ALP. Our findings support ELF as the strongest risk predictor at a single time point and encourage the implementation of ELF in clinical practice. Additional studies are needed to define what magnitude of change represents a clinically significant difference. Also, our demonstration of a subgroup of patients with a concomitant reduction in ELF, LSM, and ALP raises the intriguing question of possible spontaneous resolution of PSC and encourages further prospective studies.

Furthermore, our results are proof-of-concept for combinations of biomarkers from several biological pathways to improve the predictive power of biomarkers in PSC. Therefore, in contrast to the many previous single biomarker studies, future studies should emphasize biomarker panels. Multimarker studies could identify subgroups of patients with increased risk profiles, provided a sufficient patient number per study and independent patient panels. In this way, future studies will offer combinations of biomarkers identifying subsets of PSC patients with different risk profiles toward fibrosis or malignancy for tailored surveillance. Finally, we have demonstrated lipidomic changes relating to fatty acid alterations and mitochondrial dysfunction in PSC, which were more severe in cholestasis. These findings should be further explored, as they could indicate potential novel therapeutic targets and shed light on the mechanism of action for PPARα agonists in PSC.

6. Future perspectives

Our studies have provided new insights regarding prognostic biomarkers in PSC. However, independent and prospective studies should validate our findings, with larger patient panels and adequate follow-up time for sufficient solid endpoints, i.e., mortality, liver decompensation, or hepatobiliary cancer, to allow well-powered endpoint analyses. Due to the rarity of the disease, multicentre studies are necessary to achieve sufficient patient numbers, including contributions from non-tertiary centres, to avoid selection bias.

In order to meet these challenges, we have established a National network for autoimmune liver diseases in Norway and the ScandPSC biobank & patient cohort in collaboration with Karolinska University Hospital. Currently, more than 600 patients are included from 13 active centres across Norway and all seven university hospitals in Sweden, and further expansion is ongoing. Here, patient data and biological samples are prospectively collected at annual visits, enabling analyses of repeated measurements. The data elements of the registry are carefully harmonized according to the recommendations set by the IPSCSG, facilitating international collaboration with other centres currently developing similar initiatives. At present, the number of endpoints is limited due to short follow-ups for most patients. However, in a few years, we expect that ScandPSC will provide a “ripe” material allowing the analyses outlined above. Clearly, there is a need to overcome practical issues and methodological challenges that could otherwise hamper such projects, like harmonization of research protocols and data monitoring to ensure complete datasets.

This body of work has gained further knowledge about fibrosis markers. Adding early fibrosis markers like ELF and elastography for patient follow-up will strengthen surveillance protocols. In this regard, transient elastography holds a central position as the most established method in clinical practice and reliability for differentiating early vs. more advanced fibrosis. In the future, magnetic resonance elastography will likely represent a valuable contribution to assessing fibrosis, as this modality offers possibilities for standardization between platforms and examinators.

This work also inspires us to explore the potential of multimarker analysis further. For instance, one might apply statistical methods like factor analysis to elicit groups of biomarkers associated with specific endpoints. Essential questions will be which combinations of biomarkers are associated with different complications, like cancer development vs. liver fibrosis. Implementing new biomarkers in PSC rests on unravelling their behaviour throughout the disease course. Large patient studies with sufficient clinical endpoints are also necessary to establish relevant cut-off values for clinical studies, underscoring the necessity of nationwide prospective liver registries, biobanking, and international collaboration. The expanding field of artificial intelligence also offers new means of data handling[248].

In conclusion, every patient with PSC should be offered the opportunity to participate in clinical studies. Consolidating collaboration and infrastructure across study centres can overcome the current methodological challenges in the ongoing work, shedding further light on understanding disease development in PSC.

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Papers I-III

Research article

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Fluctuating biomarkers in primary sclerosing cholangitis: A longitudinal comparison of alkaline phosphatase, liver stiffness, and ELF

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JHEP Reports **2021.** https://doi.org/10.1016/j.jhepr.2021.100328

Background & Aims: Primary sclerosing cholangitis (PSC) is a progressive liver disease characterised by fluctuating liver biochemistries and highly variable disease progression. The Enhanced Liver Fibrosis (ELF®) test and liver stiffness measurements (LSMs) reflect fibrosis and predict clinical outcomes in PSC; however, longitudinal assessments are missing. We aimed to characterise the systematic change in ELF and LSM over time in a prospective cohort of patients with PSC, along with their longitudinal relationship to alkaline phosphatase (ALP) and bilirubin.
Methods: We included 113 non-transplant PSC patients (86 males [76.1%]; mean age 43.3 ± 15.7 years) with annual study visits between 2013 and 2019 at 2 Norwegian centres. ELF test, LSM, clinical data, liver biochemistries, and revised Mayo risk score were measured. We used linear mixed-effects models to estimate change over time, intraclass correlations (ICCs), and their relationship with ALP and bilirubin.
Results: At baseline, the median (range) ELF test was 9.3 (7.5–12.9) and median LSM 1.26 m/s (0.66–3.04 m/s). ELF and LSM increased over time (0.09 point/year, 95% CI [0.03, 0.15], $p = 0.005$, *vs.* 0.12 point/year, 95% CI [0.03, 0.21], $p = 0.009$). Between-patient effects explained 78% of ELF variation (ICC 0.78) and 56% of LSM variation (ICC 0.56). ALP also increased and showed the highest ICC (0.86).
Conclusions: ELF and LSM increased over a 5-year period. Longitudinal analyses demonstrated differences regarding within- and between-patient effects, suggesting that the ELF test may have superior reliability for risk stratification compared with LSM in PSC.
Lay summary: Primary sclerosing cholangitis (PSC) is characterised by substantial disease variability between patients and fluctuating liver biochemistries. Hence, new biomarkers are needed to identify individuals with an increased risk of developing end-stage liver disease. We explore the change over time of 2 putative prognostic biomarkers in PSC, the serum Enhanced Liver Fibrosis (ELF®) test and LSMs by ultrasound, demonstrating differences that may reflect differing abilities to discriminate risk.

Introduction

Primary sclerosing cholangitis (PSC) is characterised by multifocal strictures and dilatations of the biliary tree as a result of inflammation and biliary fibrosis, ultimately progressing to end-stage liver disease.[1–3] The natural course of PSC is highly variable, with median transplant-free survival ranging from 13 to 20 years.[2,4,5] A major unmet need is the lack of established biomarkers to (a) gauge changes in disease activity that reflect the pathophysiological processes involved in PSC, (b) identify high-risk patients for risk stratification and prognostication, and (c) evaluate treatment effects before reaching clinical end points. Alkaline phosphatase (ALP) has been applied widely to predict clinical disease progression, to select patients for clinical trials, and as a surrogate outcome marker in treatment studies. Elevated ALP is a consistent marker of poor outcomes at the group level across several studies.[6–9] However, longitudinal fluctuation in ALP limits its use at the individual level. Thus, there is a need to identify more accurate biomarkers with less fluctuation over time.

Keywords: Primary sclerosing cholangitis; Alkaline phosphatase; Elastography; Liver stiffness; Enhanced liver fibrosis test; Biomarker; Risk stratification.
Received 18 December 2020; received in revised form 25 May 2021; accepted 16 June 2021; available online 2 July 2021
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The Enhanced Liver Fibrosis (ELF®) test and liver stiffness measurements (LSMs) are emerging biomarkers for risk prediction and evaluation of treatment effects in clinical trials in PSC.[10,11] They both reflect fibrosis severity but are based on different approaches. The ELF test is a serum-based biomarker panel measuring 3 direct markers of extracellular matrix remodelling and fibrosis.[12,13] In contrast, LSM assesses the physical, viscoelastic properties of the liver using ultrasound-based elastography methods.[14] Both the ELF test and LSM have been shown to predict transplant-free survival in PSC across independent studies.[15–19] However, studies assessing repeated measurements are limited and have not established whether ELF or LSM changes systematically over time in a similar fashion to each other or similar to ALP. Furthermore, it is not known whether ELF or LSM fluctuates together with ALP.

Therefore, we aimed to characterise the longitudinal change in ELF and LSM compared with ALP in a prospective cohort of patients with PSC. We also aimed to evaluate the relative contributions of intra- and interindividual variation for each of these variables using repeated measurements. Finally, we sought to establish the longitudinal associations between ELF, LSM, ALP, and bilirubin.

Patients and methods

Study design

We prospectively included 113 patients with PSC who did not undergo transplantation during 2013–2018 from 2 Norwegian centres: Haukeland University Hospital, Bergen, and Oslo University Hospital, Rikshospitalet, Oslo. The diagnosis of PSC was based on characteristic findings on magnetic resonance cholangiography or endoscopic retrograde cholangiopancreatography according to established diagnostic criteria.[20] The first pathological radiologic finding defined the time of PSC diagnosis. Eight patients with PSC and features of autoimmune hepatitis were included. Patients with small-duct PSC were excluded. Inflammatory bowel disease was diagnosed based on endoscopy and histological findings according to accepted criteria.[21] Clinical and demographic information, including laboratory data, was acquired from patient records and research databases. Liver biochemistry, ELF test, and elastography were sampled annually (±1 month from study visit) from the baseline visit. All patients provided informed written consent. The study was in accordance with the Declaration of Helsinki and approved by the Regional Committees for Medical and Health Research Ethics of Western and South-Eastern Norway (Reference 2012/2214/REK VEST and 2008/8670, respectively).

Laboratory analyses

Biochemical analyses were performed following standard laboratory protocols, including haemoglobin, leucocytes, platelets, international normalised ratio (INR), aspartate aminotransferase (AST), alanine aminotransferase (ALT), ALP, gamma-glutamyl transferase (GGT), total bilirubin, albumin, creatinine, immunoglobulin G4 (IgG4), and C-reactive protein (CRP). The Mayo risk score and the Fibrosis-4 Index for Liver Fibrosis (FIB-4 score) were calculated using published algorithms.[22–24]

ELF test

Frozen serum samples were collected from the 113 patients from 2 biobanks in Bergen and Oslo, following an identical protocol. The ELF test was analysed using the commercially available kit, Siemens ELF®Test, performed on an ADVIA Centaur XP analyser (Siemens Medical Solutions Inc., Tarrytown, NY, USA). The ELF test was calculated according to the published algorithm, including the levels of hyaluronic acid (HA), the propeptide of procollagen type III (PIIINP), and tissue inhibitor of matrix metalloproteinases-1 (TIMP-1), using the following formula: ELF test = $2.278 + 0.851 \ln(C_{HA}) + 0.751 \ln(C_{PIIINP}) + 0.394 \ln(C_{TIMP\text{-}1})$.

Elastography

Point shear wave elastography (pSWE) was performed using an ElastPQ® Philips iU22 (Philips Healthcare, Andover, MA, USA) scanner (software version 6.3.2.2, convex C5-1 probe) and ARFI® Siemens Acuson S3000 (Siemens Medical Solutions USA, Inc., Malvern, PA, USA), in the Bergen and Oslo cohorts, respectively. The examination was performed following international guidelines, including at least 3 h of fasting before examination.[14] Following a B-mode ultrasound scan of the liver and spleen, LSM was measured using a right intercostal approach during relaxed mid-respiration breath-hold with patients in the supine position, with their right hand beneath the head.

A region of interest (ROI) representing a 0.5×1.5 cm sample volume was placed 2–6 cm below the liver capsule in an area where homogenous liver parenchyma could be visualised, avoiding large vessels and bile ducts. LSM was based on the median of 10 acquisitions and considered valid when the success rate was equal to or above 60%. LSM was measured in meters per second (m/s). The published cut-off value of 4.9 kPa (∼1.28 m/s) was used to stratify patients for subgroup analyses.[25] Liver stiffness is expressed as shear wave speed (m/s) or converted into Young's modulus using the equation kPa = $3[(ms^{-1})^2]$.[14] Each patient was followed by a single elastography platform.

Statistics

Values of p <0.05 were considered statistically significant. Continuous variables were evaluated for approximate normality using Q–Q plots and presented as means and SDs or medians and IQRs as appropriate. Because of significant right skewness, logarithmic transformations were applied to liver biochemistries, ELF, and LSM. Transformation resulted in approximate normality as assessed by Q–Q plots, in line with the assumptions of parametric statistical models. The Mann–Whitney U test, Student's t test, and the Chi-square test were applied as appropriate. Correlations at study baseline were tested using the Spearman rank correlation owing to the non-normality of variables and illustrated graphically as a correlation network.

We used a linear mixed model with an unstructured covariance structure for repeated measurement analyses with random intercept and random slope. Intraclass correlation coefficients (ICCs) were estimated from an empty-means linear mixed-effects model. We used a 2-step approach to characterise the associations between LSM, ELF, ALP, and bilirubin in a multilevel context. First, the random intercepts, slopes, and residuals from a multilevel model, either ALP or bilirubin, were estimated and scaled to z-scores. By standardising the variables to a mean of 0 and a standard deviation of 1, the biomarkers are on the same scale with comparable effect sizes. The resulting positive or negative z-score will represent the magnitude of increase or decrease, respectively, in the effect size for all variables. The z-scores were subsequently entered as predictors in a second multilevel model, where they represent between-person differences (random intercepts), between-person linear rate of change (random slopes), and fluctuations (the remaining residuals).[26]

For the relationship between LSM and ELF, we were able to fit a multilevel structural equation model with random intercepts only using both LSM and ELF as separate outcomes. We estimated the correlation between the intercepts and residuals, representing the between-person and within-person correlations. The model was adjusted for time in study. Missing values were assumed to be missing at random. Data were pooled for the 2 different elastography modalities as individual patient trajectories were followed longitudinally using a single platform; there were no significant differences between the 2 cohorts ($p = 0.39$).

Post hoc analyses were performed for defined subgroups. Subgroups for liver fibrosis stages F0–2 and F3–4 were defined using the published cut-off value of 4.9 kPa (~1.28 m/s) for pSWE in PSC.[25] For further subgroup analyses, the cohort was divided according to presumed high-risk profiles at baseline,[8–10,13,15,27,28] that is, ALP ≥1.5× upper limit of normal (ULN); ELF level ≥9.8; and for discrimination between mild and advanced fibrosis corresponding to METAVIR score F0–2 *vs.* F3–4, LSM ≥1.28 m/s, as outlined in Table 1. The analyses were conducted using SPSS version 26 (SPSS Inc., 2016, Armonk, NY, USA) and STATA 16 (StataCorp. 2019, Stata Statistical Software: Release 16.1. College Station, TX: StataCorp LP) for all analyses. The correlation network was generated using the qgraph package in R (R Core Team [2017]. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria).

Results

Patient characteristics are outlined in Table 1. We included 113 PSC patients (86 males; 76.1%). Their mean age at baseline was 43 years (SD 15.7), with a 4-year median duration of PSC and a median follow-up time of 4.5 years. Median time from study visit to LSM was 0 month (SD 1.33 and 2.33 for the Bergen and Oslo cohorts, respectively). Clinical events are listed in Table S1.

Baseline ELF test, liver stiffness, and ALP values

At baseline, the patients had median (IQR) ELF 9.3 (1.34), LSM 1.26 m/s (0.52), and ALP 151.5 U/L (197) (Table S2). There was no significant difference between males and females. There were 37

Table 1. Baseline characteristics of the cohorts of patients with PSC.

Demographics and clinical description	Total	Bergen	Oslo	Reference values	*p* value
Age at study start, x (SD)	43.3 (15.7)	44.6 (16.0)	40.1 (14.6)		0.209
Age at diagnosis, x (SD)	35.3 (14.8)	37.0 (15.1)	31.0 (13.0)		0.045
Males, n (%)	86 (76.1)	58 (71.6)	28 (87.5)		<0.001
PSC duration in years, M (IQR)	4.0 (11)	3.0 (13)	7.0 (9)		0.093
Mayo risk score, x (SD)	-0.5 (0.9)	-0.5 (0.9)	-0.4 (1.0)		0.430
FIB-4 score, M (IQR)	1.1 (1.2)	1.2 (1.5)	0.9 (0.9)		0.808
Decompensated liver disease, n	2	1	1		0.251
Any inflammatory bowel disease, n (%)	85 (75.2)	62 (76.5)	23 (71.9)		0.627
Ulcerative colitis, n (%)	64 (56.6)	45 (55.6)	23 (71.9)		
Crohn's disease, n (%)	12 (10.6)	10 (12.3)	2 (6.3)		
Indeterminate, n (%)	8 (7.1)	6 (7.4)	2 (6.3)		
UDCA treatment at any time, n (%)	39 (34.5)	25 (22.1)	14 (12.4)		<0.001
Patients with endoscopic intervention, n (%)	6 (5.3)	3 (3.7)	3 (9.3)		0.362
Prognostic biomarkers					
Participants above cut-off values					
ALP,* n (%)	52 (46)	36 (44.4)	16 (50)		0.362
ELF,† n (%)	37 (32.7)	22 (33.3)	10 (31.3)		0.428
LSM,‡ n (%)	50 (45)	37 (45.7)	13 (43.4)		0.098
Levels, M (IQR)					
ALP (U/L)	151.5 (197)	149.0 (196)	165.0 (206)	35–105	0.871
ALP by ULN, M (range)	1.4 (0.4, 8.0)	1.4 (0.4, 8.0)	1.5 (0.5, 6.1)		
ELF	9.3 (1.34)	9.3 (1.32)	9.4 (1.45)		0.905
LSM (m/s)	1.26 (0.52)	1.26 (0.48)	1.17 (1.21)		0.373
Other blood tests, M (IQR)					
ALT (U/L)	53.0 (81)	52.0 (66)	74.0 (127)	10–70 (m) 10–45 (f)	0.241
AST (U/L)	48.0 (49)	47.0 (48)	51.5 (75)	15–45 (m) 15–35 (f)	0.633
GGT (U/L)	228.0 (597)	149.0 (565)	238.5 (753)	10–80 (m <40 years)§ 10–45 (f <40 years)§	0.856
Bilirubin (μmol/L)	11.0 (10)	11.0 (9)	12.5 (16)	5–25¶	0.048
Thrombocytes ($\times 10^9$)	245.0 (105)	240.0 (102)	240.0 (111)	145–390#	0.779
Albumin (g/L)	45.0 (5)	46.0 (5)**	44.0 (5)††	see** and††	0.122

Reference values for laboratory parameters are equal for men and women and across study centres unless otherwise specified. P-values were calculated using Student's t-test, Mann-Whitney U test, or Chi-Square test as appropriate.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ELF, enhanced liver fibrosis; f, females; FIB-4, Fibrosis-4 Index for Liver Fibrosis; GGT, gamma-glutamyl transferase; LSM, liver stiffness measurement, M, median; m, males; PSC, primary sclerosing cholangitis; UDCA, ursodeoxycholic acid; ULN, upper limit of normal.
* ≥1.5× ULN.
† ≥9.8.
‡ ≥1.28 m/s.
§ GGT 15–115 U/L for m ≥40 years and 10–75 U/L for f ≥40 years.
¶ Bilirubin ≤21 μmol/L.
** Albumin 39–50 g/L for patients <40 years, 39–48 g/L for patients between 40 and 69 years, and 36–48 g/L for patients ≥70 years in the Bergen cohort.
†† Albumin 36–48 g/L for patients <40 years, 36–45 g/L for patients 40–69 and 34–45 g/L for patients ≥70 years in the Oslo cohort.
# Thrombocytes 145–348×10^9 (m) and 165–387×10^9 (f).

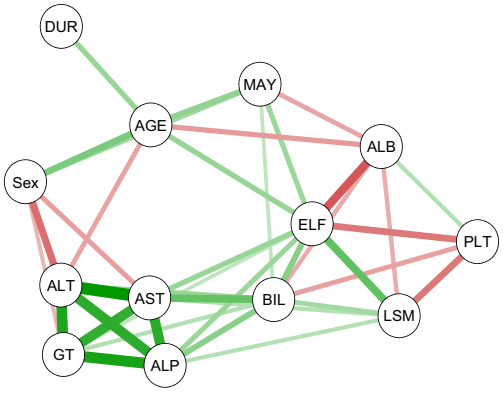

Fig. 1. Correlation network for ELF, LSM, and relevant biochemistries. Correlations at study baseline were tested using the Spearman rank correlation. The strength of correlations is indicated by the widths of the connecting lines. Positive and negative correlations are represented by green and red colour, respectively. The diagram highlights liver enzymes ALT, AST, ALP, and GT as a group with high correlation. ELF and LSM were most strongly correlated with each other and showed correlations with liver enzymes and negative correlations with albumin and platelets. ALB, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BIL, bilirubin; DUR, PSC duration; ELF, enhanced liver fibrosis; GT, gamma-glutamyl transferase; LSM, liver stiffness measurement; MAY, Mayo risk score; PLT, platelets; PSC, primary sclerosing cholangitis.

(33%), 50 (45%), and 52 (46%) high-risk patients defined by ELF test, LSM, and ALP, respectively. Correlation analysis showed a strong correlation of liver parameters, as illustrated by a network diagram (Fig. 1). The liver enzymes ALT, AST, GT, and ALP were strongly correlated; ELF and LSM showed moderate correlation with each other (rho 0.483, p <0.001), and both were correlated with ALP, other liver enzymes, bilirubin, and (negatively) albumin.

Longitudinal change and ICCs

The development over time for the ELF test, LSM, ALP, and bilirubin is illustrated in Fig. 2. Using a linear mixed-effects model, we demonstrated a small but significant increase over 5 years for ELF (0.09 point/year, 95% CI [0.03, 0.15], p = 0.005) and LSM (0.12 point/year, 95% CI [0.03, 0.21], p = 0.009). Scaling of the outcome variables to z-scores demonstrated a slightly larger increase in LSM (0.07 SD per year, 95% CI [0.02, 0.13]) than in ELF (0.06 SD per year, 95% CI [0.03, 0.20]). By comparison, ALP increased by 0.04 SD per year (95% CI [0.01, 0.07], p = 0.011), and bilirubin increased by 0.07 SD per year (95% CI [0.02, 0.12], p = 0.007). The ICC was highest for ALP (0.86) and ELF (0.78), with lower ICCs for bilirubin (0.64) and LSM (0.56). The results are summarised in Table 2.

Longitudinal change over time in high-risk subgroups

Post hoc subgroup analyses of predefined high-risk groups, that is, ELF test ≥9.8, LSM ≥1.28 m/s, and ALP ≥1.5× ULN at baseline, demonstrated a significantly higher baseline ELF level among the high-ALP group compared with the low-ALP group (p = 0.001) and a similar trend for LSM (p = 0.06). Both ELF and LSM increased significantly over time in the high-ALP group (p = 0.014 and 0.022, respectively), whereas they showed no significant increase in the low-ALP group (Fig. 3). However, the interaction between time and the ALP subgroup did not reach significance. There were no significant differences in the change in ELF or LSM over time, according to the baseline risk groups defined by ELF or LSM (data not shown).

Ursodeoxycholic acid (UDCA) treatment was received by 35% of the patients at any time during the study with a median duration of 3.4 years (range 1–6 years) of treatment. Subgroup analysis indicated that ELF and ALP increased significantly over time in UDCA-naïve but not UDCA-treated patients (ELF: p = 0.009 *vs.* 0.803; ALP: p = 0.008 *vs.* p = 0.883), with a similar trend for LSM (p = 0.057 *vs.* 0.125); however, data were insufficient to adjust analyses for the biomarker × treatment interaction. Endoscopic interventions (n = 10 in 6 patients) during the study were not associated with consistent changes in ELF at subsequent visits.

Longitudinal association between ELF and LSM

Using a multi-outcome multilevel structural equation model adjusted for time, we found that the correlation between the random intercepts of ELF and LSM was good (0.79, p <0.001), representing the between-person association between LSM and ELF. In contrast, the correlation coefficient of the residuals was weak (0.24, p = 0.007), representing the within-person association between LSM and ELF.

Longitudinal association between ELF test or LSM and liver biochemistries and Mayo risk score

Over time, liver biochemistries and Mayo risk score were significantly associated with LSM and ELF outcomes (Table 3). ALP showed stronger association with ELF (standardised fixed effect [sFE] 0.47) than with LSM (sFE 0.28). Similarly, ELF showed a stronger association than did LSM with Mayo risk score (sFE 0.48 *vs.* 0.37) and the FIB-4 score (sFE 0.56 *vs.* 0.42). LSM was more associated with bilirubin (sFE 0.29) than was ELF (sFE 0.20), but ELF and LSM showed similar associations with albumin. The effect size sFE can be interpreted similarly in magnitude as correlation coefficients.

Between- and within-person associations between ALP, bilirubin, LSM, and ELF

Variation in the individual means of ALP and bilirubin accounted for most of the association between ALP, bilirubin, and ELF (Table 4). By comparison, variation in the annual rate of change in ALP and bilirubin was not associated with ELF. However, we identified a smaller but significant association between fluctuations in ALP and ELF. For LSM, variation in individual means accounted for most of the association between ALP, bilirubin, and LSM, whereas there was no association with fluctuations in ALP or bilirubin. However, a higher annual rate of change in bilirubin was associated with higher LSM scores.

Spontaneous reductions in ELF, LSM, and ALP

The subpopulation with ALP ≥1.5× ULN accounted for all of the patients with ≥40% ALP reduction at each of the visits in our study. Out of the high-ALP group, a total of 13%, 13%, 10%, and 6% experienced ≥40% ALP reduction at visits 1, 2, 3, and 5 years from baseline, respectively.

In 40% of the total patient cohort, ELF levels decreased from baseline to 5 years, with a mean value of −0.67. A similar proportion of patients (44.7% and 42.2%) showed a reduction in ELF levels within the same range (mean change −0.51 and −0.54) at 1

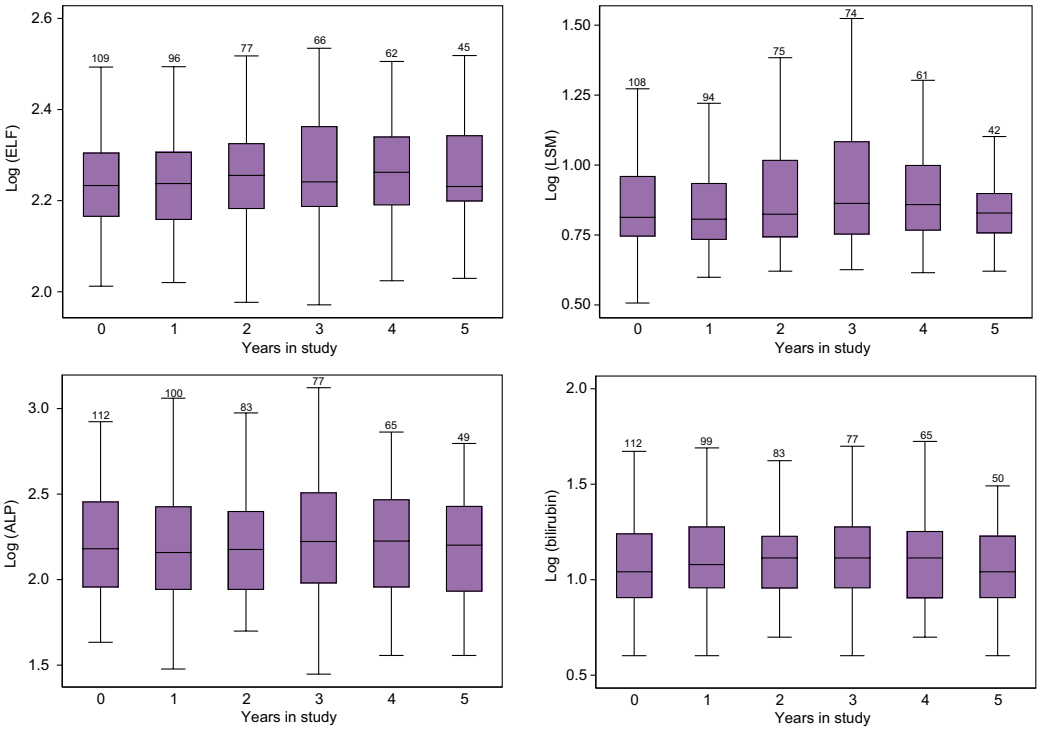

Fig. 2. Development of ELF, LSM, ALP, and bilirubin over time in patients with PSC (n = 113). Boxplot; the lower and upper whiskers represent the first and third quartiles, respectively. Each box is represented by the number of measurements for each parameter per year in study. When applying a longitudinal mixed model analysis considering all available repeated measurements, there was a small but significant increase in ELF and LSM over time (p = 0.005 and 0.009, respectively). ALP, alkaline phosphatase; ELF, enhanced liver fibrosis; LSM, liver stiffness measurement; PSC, primary sclerosing cholangitis.

Table 2. Liver stiffness measures and liver parameters over time.

		Effect size	95% CI	*p* value
ELF	Fixed intercept*	-0.11	[-0.29, -0.06]	0.196
	Fixed slope†	0.06	[0.02, 0.09]	0.005¶
	Crude ICC‡	0.78	[0.72, 0.83]	
	Adjusted ICC§	0.83	[0.77, 0.87]	
LSM	Fixed intercept*	-0.11	[-0.27, 0.06]	0.199
	Fixed slope†	0.07	[0.02, 0.13]	0.009¶
	Crude ICC‡	0.56	[0.47, 0.65]	
	Adjusted ICC§	0.59	[0.48, 0.70]	
ALP	Fixed intercept*	-0.03	[-0.21, 0.16]	0.775
	Fixed slope†	0.04	[0.01, 0.07]	0.011¶
	Crude ICC‡	0.86	[0.82, 0.89]	
	Adjusted ICC§	0.89	[0.85, 0.92]	
Bilirubin	Fixed intercept*	-0.09	[-0.26, -0.09]	0.325
	Fixed slope†	0.07	[0.02, 0.12]	0.007¶
	Crude ICC‡	0.64	[0.55, 0.72]	
	Adjusted ICC§	0.71	[0.62, 0.78]	

ALP, alkaline phosphatase; ELF, enhanced liver fibrosis; ICC, interclass correlation; LSM, liver stiffness measurement.
* The fixed effect at baseline. All variables have been log-transformed and z-scored so that the mean represents the grand mean over 5 years. A negative fixed intercept indicates how much lower the variable is at baseline compared with the grand mean, in standard deviations.
† The fixed slope indicates change in the outcome in standard deviations per year.
‡ The ICC from an empty-means random intercept model.
§ The ICC from a random slope model adjusted for time-in-study.
¶ p value <0.05.

and 2 years from baseline. Reduction in LSM was shown in 34% of the patients at 5 years (mean change −0.29 m/s); similar proportions of patients demonstrated LSM reduction at 1 and 2 years from baseline (42.7% and 36.7%, respectively; mean change of −0.33 to −0.38 m/s). Among the patients with 5-year follow-up time, all remained in the same category concerning low or high levels of ELF or LSM, whereas 16% of the patients moved between categories of low to high ALP as defined by ALP ≥1.5× ULN at baseline). At each follow-up visit (1–5 years from baseline), about 10% of patients featured a concomitant reduction in all of ELF, LSM, and ALP (Table S3), out of which only 25% received UDCA. Six patients received a total of 10 endoscopic treatments during the study period, of which only 2 procedures were followed by significant ALP reductions.

Discussion

To our knowledge, this is the first study to provide an in-depth characterisation of the variation over time in ELF and LSM as well as ALP in a prospective cohort of patients with PSC, allowing differentiation of 'background noise' (random variation) from biological significant variation. ELF and LSM demonstrated a significant but minor increase over 5 years, in line with previous reports in patients with PSC and mild fibrosis.[9,17,27] With the use

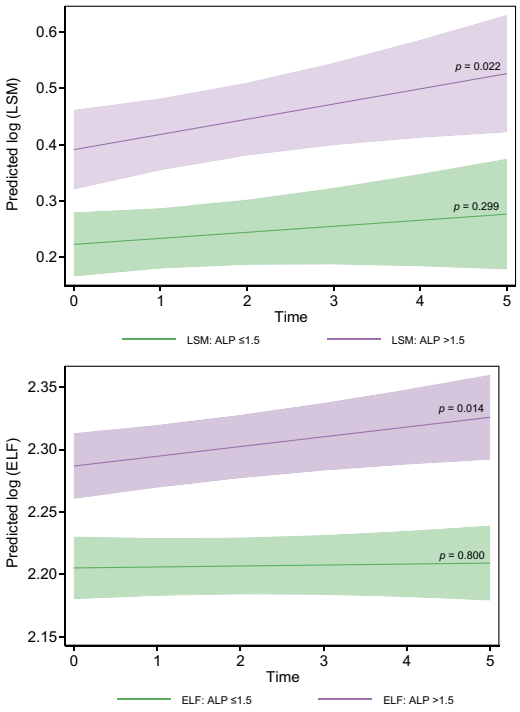

Fig. 3. Linear mixed model analysis of the longitudinal development of ELF and LSM in high and low-risk groups defined by ALP. The high-risk subgroup (ALP ≥1.5× ULN at baseline) showed significantly higher baseline ELF (p = 0.001) compared with the low-risk group, with a similar trend for LSM (p = 0.06). Both ELF and LSM increased significantly over time in the high-ALP group (p = 0.014 and 0.022, respectively), whereas there was no significant increase for ELF or LSM in the low-ALP group. For ELF, there was a trend towards interaction between ALP-defined risk group and time which did not reach significance (p >0.05), whereas for LSM, there was no interaction between risk group and time (p >0.50). ALP, alkaline phosphatase; ELF, enhanced liver fibrosis; LSM, liver stiffness measurement.

Table 3. Associations of ELF and LSM with biochemical markers and clinical scores in a linear mixed-effects model.

Predictor	Outcome	sFE*	95% CI	*p* value
ALP	ELF	0.47	[0.37, 0.56]	<0.001
	LSM	0.28	[0.16, 0.39]	<0.001
Albumin†	ELF	-0.39	[-0.47, -0.32]	<0.001
	LSM	-0.35	[-0.44, -0.25]	<0.001
Bilirubin	ELF	0.20	[0.11, 0.29]	<0.001
	LSM	0.29	[0.18, 0.39]	<0.001
Mayo risk score†	ELF	0.48	[0.40, 0.56]	<0.001
	LSM	0.37	[0.26, 0.47]	<0.001
FIB-4	ELF	0.56	[0.46, 0.65]	<0.001
	LSM	0.42	[0.31, 0.53]	<0.001

Linear mixed-effects models as described under statistics.
ALP, alkaline phosphatase; ELF, enhanced liver fibrosis; FE, fixed effects; FIB-4, Fibrosis-4 Index for Liver Fibrosis; LSM, liver stiffness measurement; sFE, standardised fixed effects.
* sFE calculated as sFE = (FE×SD predictor variable)/SD dependent variable.
† Not log-transformed (all other log-transformed).

of standardised z-scores in a linear mixed model, our results suggest that LSM increased more than ELF and ALP over time. We demonstrated a strong between-person association between LSM and ELF but a weak association for individual fluctuations over time. Overall, in this study, it was indicated that ELF and LSM may stratify similar patients to high-risk groups at baseline, whereas there may be different effects driving change in ELF and liver stiffness over time.

Using ICC analyses yielded by the mixed model, we demonstrated essential differences between ELF and LSM regarding between- and within-person effects influencing variation in these parameters. Whereas ELF showed high ICC, suggesting predominant between-person variation, between- and within-person variations contributed relatively equally for LSM. The relatively stable values within individual patients at repeated measurements for ELF support ELF as a reliable risk stratification marker and may imply that the ELF test is superior over LSM for risk stratification purposes when measured at a single time point. Biologically, this is plausible, as the ELF test reflects 3 direct markers of extracellular matrix remodelling, providing a biological link to disease severity, in contrast to LSM, which represents the sum of several factors affecting liver stiffness.

For a test to be useful for monitoring purposes, the 'noise-to-signal ratio' should be low; that is, any change should reflect a biological difference. Establishment of the magnitude of variation between and within patients is, therefore, a key factor for assessing the qualities of biomarkers. The ICC from the mixed model represents a measure of within- and between-variation in a test at a single time point and longitudinally. In general, a higher ICC value represents a lower degree of variation,[28] reflecting a stronger ability to stratify risk between individuals at a single time point, whereas a lower ICC suggests higher sensitivity to biological variation over time, relevant for monitoring and assessment of treatment effect. However, interobserver variation and other factors may also contribute to lower ICC. Our findings are in line with quality assessments of ELF, which have shown good stability and a low coefficient of variation.[12] The lower within-person variation for ELF compared with that for LSM may partly reflect the inherent differences between patented laboratory assays such as the ELF test compared with ultrasound-based LSM.

As a small note of caution, the ICC of ALP was higher than that of ELF, yet ALP is notoriously fluctuating over time in patients with PSC. This trait is a major challenge, limiting the use of ALP in individual prognostication and monitoring of disease activity. In the decomposed mixed model analysis, we identified concurrent fluctuations in ALP and ELF, which might suggest similar underlying mechanisms behind fluctuations in both parameters. Possibly, ELF may not overcome the problems of individual fluctuation typical for ALP. In favour of ELF towards LSM, we demonstrated stronger associations for ELF with ALP and other liver biochemistries, as well as the Mayo risk score and FIB-4 score.

For LSM, a lower ICC indicated that within-person variation explained a larger proportion of the variability compared with that for the ELF test, reflecting either improved sensitivity to detect biologically relevant changes or increased sampling variability. LSM has previously demonstrated good agreement towards histological stages of fibrosis and clinical outcome in PSC,[17–19,29] and a strong predictive ability for clinical outcomes in independent studies.[17,18] Moreover, the elastography modalities we used (pSWE and ARFI quantification) were reported to

Table 4. Decomposition of longitudinal associations of ELF and LSM with liver biochemistries in PSC.

	Individual means (random intercepts)		Linear change (random slopes)		Fluctuation (residuals)	
	sFE (95% CI)	*p* value	sFE (95% CI)	*p* value	sFE (95% CI)	*p* value
ELF as the outcome						
ALP	0.37 (0.21, 0.52)	<0.001**	0.03 (-0.14, 0.19)	0.768	0.15 (0.11, 0.18)	<0.001**
Bilirubin	0.40 (0.26, 0.54)	<0.001**	0.16 (-0.01, 0.31)	0.052	0.03 (-0.01, 0.08)	0.161
LSM as the outcome						
ALP	0.32 (0.18, 0.46)	<0.001**	0.07 (-0.08, 0.21)	0.384	0.05 (-0.01, 0.11)	0.091
Bilirubin	0.42 (0.30, 0.54)	<0.001**	0.23 (0.10, 0.35)	<0.001**	0.03 (-0.04, 0.10)	0.407

A 2-step multilevel model where first the random intercepts, slopes, and residuals for the predictors ALP and bilirubin were estimated from separate models with time as the predictor. These now represent differences in individual means and individual linear rate of change, and the residuals represent fluctuating deviations from these. These were entered as predictors in a second multilevel model, with ELF or LSM as the outcome and time as the only covariate. **Statistically significant at p <0.001 level.
ALP, alkaline phosphatase; ELF, enhanced liver fibrosis; LSM, liver stiffness measurement; PSC, primary sclerosing cholangitis; sFE, standardised fixed effects.

correlate well with histology[19,30–32] and demonstrated high accuracy in discriminating between lower and higher degrees of fibrosis[31–33] and excellent correlation to TE in patients with PSC.[25] Because of lack of power for end-point analyses, we cannot decipher whether the larger relative contribution of within-patient effects on variability is a result of sampling variability or reflect biological variation over time. Inter and intra-observer variability is an acknowledged possible bias in all ultrasound-based methods.[25,34–36] Furthermore, the patchy disease distribution in PSC and variation in cholestasis may contribute to variations in LSM.[37,38] Based on our results, we cannot rule out that the lower ICC for LSM results from increased measurement variability rather than reflecting a relevant change in fibrosis. The significant linear association between bilirubin levels and LSM over time but no association between their intermediate fluctuations indicates that limited segmental cholestasis in PSC does not severely affect LSM over time. This might suggest that ELF and LSM act as complementary biomarkers, indicative of slightly different aspects of the disease concerning fibrosis and cholestasis.

Interestingly, in a *post hoc* subgroup analysis, we found that patients with an ALP level ≥1.5× ULN at baseline demonstrated elevated baseline levels as well as a significant increase in ELF over time in the high-ALP compared with the low-ALP group. These findings support previous reports proposing this ALP level as an appropriate cut-off level for risk stratification.[6,7,39]

Clinical trials in patients with PSC are suffering from a lack of robust surrogate markers to reliably evaluate the effect of novel therapeutic agents. Reduction in ALP is commonly used as an outcome parameter in pharmacological studies; however, spontaneous reductions in ALP challenge the use of ALP as a surrogate marker in PSC.[7,8,39,40] Although a reduction of ALP by 40% or more is a commonly applied primary outcome, this is questioned by reports of patients showing ALP reductions not supported by reductions in histological fibrosis.[9] In the present study, we found that about 8% of the patients experienced spontaneous ALP reductions of at least 40% at 1, 2, and 3 years of study follow-up. These time points are commonly applied when designing clinical trials, underscoring the challenges of using ALP reduction as a surrogate endpoint. Furthermore, we demonstrated that between one-third and nearly one-half of the patients showed spontaneous reductions in ELF test and LSM, respectively, during the same time frame. Moreover, we identified a subgroup of about 10% of patients at each follow-up visit showing a concomitant reduction in ALP, ELF, and LSM, raising the question of whether the fibrosis level or disease stage may actually regress in PSC. These findings warrant further investigation before considering these biomarkers as surrogate endpoints in clinical trials.

UDCA treatment has been associated with ALP reduction in patients with PSC in clinical studies.[41,42] We did not demonstrate ALP, ELF, or LSM reduction associated with UDCA; however, subgroup analysis showed significant increases in ELF and ALP over time in UDCA-naïve (65%) but not UDCA-treated (35%) patients. Moreover, UDCA users had higher levels of ELF, LSM, ALP, and bilirubin at baseline, suggesting a more advanced disease in this group. Unfortunately, our study was not powered to investigate biomarker × treatment interactions.

Limitations of the study

The major limitation of this study is the limited number of long-term clinical outcomes such as deaths and liver transplantations, precluding end point analyses. Liver biopsies allowing direct assessment of the degree of liver fibrosis were also not available. However, in PSC, liver biopsies are poorly representative owing to the patchy disease distribution, and the procedure carries a risk of adverse outcomes. Current guidelines do not recommend liver biopsies; hence, this was considered unethical.

Conclusion

The ELF test and LSM increased slightly but significantly over 5 years in a prospective panel of patients with PSC. Our longitudinal analyses demonstrated differences regarding within- and between-patient effects, suggesting that the ELF test may be more stable than LSM and is likely to perform better for risk stratification in PSC using single measurements. We advocate that the ELF test may hold practical utility for identification of PSC patients with a high risk of disease progression. ELF and LSM showed a significant increase over time only in patients with ALP≥1.5× ULN, supporting this as a relevant cut-off level for risk stratification. The significance of concomitant reductions in ELF, LSM, and ALP in a patient subgroup warrants further studies.

Abbreviations

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; ELF, enhanced liver fibrosis; FIB-4, Fibrosis-4 Index for Liver Fibrosis; GGT, gamma-glutamyl transferase; HA, hyaluronic acid; ICC, intraclass correlation; IgG4, immunoglobulin G4; INR, international normalised ratio; LSM, liver stiffness

measurement; PIIINP, propeptide of type III procollagen; PSC, primary sclerosing cholangitis; pSWE, point shear wave elastography; ROI, region of interest; TE, transient elastography; TIMP-1, tissue inhibitor of metalloproteinases-1; UDCA, ursodeoxycholic acid; ULN, upper limit of normal.

Financial support
The work is part of the PhD program for GF funded by the Western Norway Regional Health Authority.

Conflicts of interest
W. Rosenberg is one of the inventors and patent holders of the ELF test.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions
Guarantor of the article and supervised the project: MV. Conceived and designed the study: MV, LMG, WR, THK. Collected the biological samples and clinical data: MV, KW, TF. Performed the ultrasound scans and liver stiffness measurements: MV, ABM, IB. Contributed to the liver stiffness measurements: OHG. Contributed to the ELF test laboratory analyses: WR. Designed and performed the statistical analyses: GF, LMG. Contributed to the interpretation of the data.: GF, ABM, TF, THK, LMG, MV. Drafted the manuscript: GF, LMG, MV. Reviewed the manuscript for critical content and approved the final version of the manuscript: All authors.

Data availability statement
Data is available upon request and an appropriate institutional collaboration agreement.

Acknowledgements
The authors thank Liv Wenche Thorbjørnsen for assistance in the collection of serum samples.

Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2021.100328.

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Author names in bold designate shared co-first authorship

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Fluctuating biomarkers in primary sclerosing cholangitis: A longitudinal comparison of alkaline phosphatase, liver stiffness and ELF

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Fig. S1. Patients included.

The total number of patients included since initiation of the study.

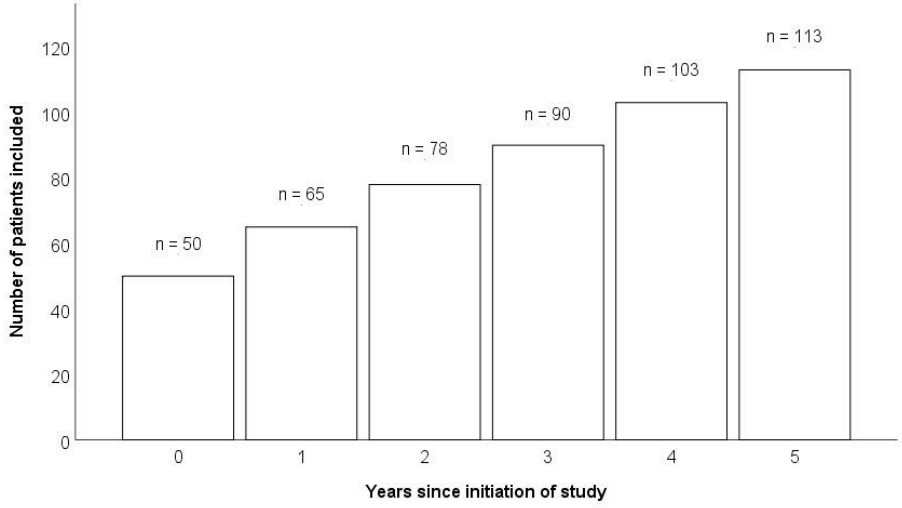

Table S1. Clinical events.
Number of patients in this PSC patient cohort undergoing clinical events in terms of liver transplantation or death since initiation of study.

Indication for liver transplantation (n)		**9**
	Biliary dysplasia	5
	Liver failure	2
	Cholangiocarcinoma	1
	Fatigue	1
Deceased (n)		**6**
	Liver failure	2
	Malignancies	2
	Post-transplant complications	1

Table S2. ELF, LSM, and laboratory values at annual visits. All variables displayed as median (IQR).

	T0	T1	T2			T3	T4	T5
N			80	78	65			50
ELF			9.62 (1.37)	9.47 (1.77)	9.63 (1.58)			9.42 (1.40)
LSM	1.24 (0.49)		1.28 (0.63)	1.39 (0.91)	1.37 (0.58)			1.29 (0.36)
ALP			139 (141)	170 (244)	168 (230)			166 (170)
ALT	53 (81)	49 (83)	62 (106)			63.5 (85)	58.50 (86)	48 (69)
AST	45 (49)	44 (44)	50 (74)			61 (56)	55 (59)	55 (50)
GT	220 (611)	168 (432)	234 (532)			277 (472)	274 (403)	203 (411)
Bilirubin	11 (10)	12 (9)	13 (9)			14 (10)	13 (9)	11 (9)
Albumin	45 (5)	45 (4)	45 (5)			44 (5)	45 (5)	45 (5)
Platelets	237 (104)	229 (100)	227 (96)			231.5 (108)	208 (99)	

Abbreviations: ALP, alkaline phosphatase; ALT, Alanine aminotransferase; AST, Aspartate aminotransferase; ELF, Enhanced liver fibrosis; GT, Gamma-glutamyl transferase; LSM, Liver stiffness measurement;

Table S3 Spontaneous reduction in ALP, ELF, and LSM. Number of patients with spontaneous reduction in either ALP, ELF, LSM or all three parameters. Time represents year from baseline.

Time	1	2	3	4	5
ALP reduction, n (%)	46 (44.7)	39 (43.3)	35 (44.9)	28 (43.1)	28 (56)
ELF reduction, n (%)	46 (44.7)	38 (42.2)	23 (29.5)	24 (36.9)	20 (40)
LSM reduction, n (%)	44 (42.7)	33 (36.7)	24 (30.8)	26 (40)	17 (34)
Reduction in all three risk factors, n (%)	12 (11.7)	12 (13.3)	7 (9.0)	7 (10.8)	5 (10)

Abbreviations: ALP, alkaline phosphatase; ELF, enhanced liver fibrosis; LSM, liver stiffness measurement.

Errata for
Primary sclerosing cholangitis

Surrogate markers of natural history, disease severity, and prognosis

Guri Fossdal

Thesis for the degree philosophiae doctor (PhD)
at the University of Bergen

25/10/2023 Guri Fossdal
(date and sign. of candidate)

26.10.23
(date and sign. of faculty)

Table 1. Biomarkers and literature references. Literature referring to the first published association for a potential biomarker towards clinical outcome in PSC or, if otherwise stated, other liver diseases.

	Reference literature
Fibrosis markers	
ELF	Vesterhus 2015[111]
Pro-C3 Pro-C5 C3M C4M	Nielsen 2018[112]
BGM VICM	Vesterhus 2021[113]
Anti-GP2 IgA	Jendrek 2017[114]
Calprotectin	Vesterhus 2017[115]
Autotaxin	Dhillon 2019[116]
Inflammatory markers	
IL-8	Vesterhus 2017[115], Zweers 2016[117]
CD14 LPB	Dhillon 2019[118]
CD163 CD206	Bossen 2021[119]
Metabolic markers	
PLP	Kummen 2021[81]
TMAO	Kummen 2017[80]
Neopterin KTR	Dhillon 2021[120]
Clinical scores	
Mayo risk score	Kim 2000[121]
AOM	De Vries 2018[122]
UK-PSC risk score	Goode 2019[123]
Ultrasound elastography	
Transient elastography	Corpechot 2014[124]
pSWE	Mjelle 2020[103]
ARFI	Goertz 2019[125]

Abbreviations: Anti-GP2, anti-glycoprotein-2; AOM, Amsterdam-Oxford model; BGM, marker of biglycan degradation; C3M, C4M, degradation of type III and IV collagen; ELF, enhanced liver fibrosis; IL-8, interleukin-8; KT-ratio, kynurenine-tryptophan ratio; LBP, lipopolysaccarhide binding protein; PLP, pyridoxal 5'-phosphate; Pro-C3, Pro-C5, type III and V collagen formation; VICM, citrullinated type III intermediate filament protein vimentin.

Table 3. Biomarkers before and after ERCP. From investigation of our database and patient journals we found six patients who had undergone a total of 10 ERCP procedures with intervention. The table lists the time from intervention to measurement of the parameters. Reduction in any of the parameters appeared independently from another.

Patient	ELF			ALP			LSM		
	Before	*After*	*Weeks*	*Before*	*After*	*Weeks*	*Before*	*After*	*Weeks*
1	9.53	10.14	30	201	274	30	1.10	0.95*	29
1	10.14	10.23	18	274	252*	18	0.95	2.11	29
1	10.23	11.16	14	252	172*	14	2.11	1.71*	14
1	11.16	11.48	6	172	244	6	1.71	1.73	8
2	9.62	9.52*	9	95	93*	9	1.23	1.32	9
3	11.10	10.96*	4	213	324	4	2.74	3.07	4
4	10.78	12.20	12	101	421	12	1.19	1.92	12
5	7.60	7.87	11	54	60	11	1.25	2.17	11
6	8.03	8.31	11	86	141	11	1.02	1.23	11
6	8.31	8.79	35	141	330	35	1.23	1.52	35

Abbreviations: ALP, alkaline phosphatase; ELF, enhanced liver fibrosis test; ERCP, endoscopic retrograde pancreatography; LSM, liver stiffness measurement.
*= reduction in the parameter measured after ERCP

Graphic design: Communication Division, UiB / Print: Skipnes Kommunikasjon AS

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ISBN: 9788230863879 (print)
9788230860281 (PDF)