Research Article

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Novel serum and bile protein markers predict primary sclerosing cholangitis disease severity and prognosis

Mette Vesterhus[1,2], Anders Holm[1], Johannes Roksund Hov[1,3,4,5,6], Ståle Nygård[7,8,9], Erik Schrumpf[1,3], Espen Melum[1,4,5,6], Liv Wenche Thorbjørnsen[1], Vemund Paulsen[4], Knut Lundin[3,4], Inge Dale[10], Odd Helge Gilja[2,11], Serge J.L.B. Zweers[12], Morten Vatn[13], Frank G. Schaap[14], Peter L.M. Jansen[14], Thor Ueland[5], Helge Røsjø[9,15], Bjørn Moum[3,16], Cyriel Y. Ponsioen[17], Kirsten Muri Boberg[1,3,4], Martti Färkkilä[18], Tom H. Karlsen[1,3,4,5,6,*,†], Fridtjof Lund-Johansen[6,19,†]

[1]*Norwegian PSC Research Center, Department of Transplantation Medicine, Division of Cancer Medicine, Surgery and Transplantation, Oslo University Hospital Rikshospitalet, Oslo, Norway;* [2]*National Centre for Ultrasound in Gastroenterology, Haukeland University Hospital, Bergen, Norway;* [3]*Institute of Clinical Medicine, University of Oslo, Norway;* [4]*Section of Gastroenterology, Department of Transplantation Medicine, Oslo University Hospital, Norway;* [5]*Research Institute of Internal Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway;* [6]*K.G. Jebsen Inflammation Research Centre, University of Oslo, Oslo, Norway;* [7]*Bioinformatics Core Facility, Institute for Medical Informatics, Oslo University Hospital, Oslo, Norway;* [8]*Institute for Experimental Medical Research, Oslo University Hospital and University of Oslo, Oslo, Norway;* [9]*K.G. Jebsen Cardiac Research Centre and Center for Heart Failure Research, University of Oslo, Oslo, Norway;* [10]*Calpro, Oslo, Norway;* [11]*Department of Clinical Medicine, University of Bergen, Bergen, Norway;* [12]*Tytgat Institute for Liver and Intestinal Research, Academic Medical Center, Amsterdam, The Netherlands;* [13]*The Institute of Clinical Epidemiology and Molecular Biology (EpiGen), Campus Ahus, Institute of Clinical Medicine, University of Oslo, Oslo, Norway;* [14]*Department of Surgery, NUTRIM School of Nutrition, Toxicology and Metabolism, Maastricht University, Maastricht, The Netherlands;* [15]*Division of Medicine, Akershus University Hospital, Lørenskog, Norway;* [16]*Division of Medicine, Department of Gastroenterology, Oslo University Hospital, Oslo, Norway;* [17]*Department of Gastroenterology and Hepatology, Academic Medical Center, Amsterdam, The Netherlands;* [18]*Department of Medicine, Division of Gastroenterology, Helsinki University Hospital, Helsinki, Finland;* [19]*Dept. of Immunology, Oslo University Hospital Rikshospitalet, Oslo, Norway*

Background & Aims: Prognostic biomarkers are lacking in primary sclerosing cholangitis, hampering patient care and the development of therapy. We aimed to identify novel protein biomarkers of disease severity and prognosis in primary sclerosing cholangitis (PSC).

Methods: Using a bead-based array targeting 63 proteins, we profiled a derivation panel of Norwegian endoscopic retrograde cholangiography bile samples (55 PSC, 20 disease controls) and a Finnish validation panel (34 PSC, 10 disease controls). Selected identified proteins were measured in serum from two Norwegian PSC cohorts (n = 167 [1992–2006] and n = 138 [2008–2012]), inflammatory bowel disease (n = 96) and healthy controls (n = 100).

Results: In the bile derivation panel, the levels of 14 proteins were different between PSC patients and controls (p <0.05); all were confirmed in the validation panel. Twenty-four proteins in the bile derivation panel were significantly (p <0.05) different between PSC patients with mild compared to severe cholangiographic changes (modified Amsterdam criteria); this was replicated for 18 proteins in the validation panel. Interleukin (IL)-8, matrix metallopeptidase (MMP)9/lipocalin (LCN)2-complex, S100A8/9, S100A12 and tryptophan hydroxylase (TPH)2 in the bile were associated with both a PSC diagnosis and grade of cholangiographic changes. Stratifying PSC patients according to tertiles of serum IL-8, but not MMP9/LCN2 and S100A12, provided excellent discrimination for transplant-free survival both in the serum derivation and validation cohort. Furthermore, IL-8 was associated with transplant-free survival in multivariable analyses in both serum panels independently of age and disease duration, indicating an independent influence on PSC progression. However, the Enhanced Liver Fibrosis (ELF®) test and Mayo risk score proved to be stronger predictors of transplant-free survival.

Conclusions: Based on assaying of biliary proteins, we have identified novel biliary and serum biomarkers as indicators of severity and prognosis in PSC.

Lay summary: Prognostic biomarkers are lacking in primary sclerosing cholangitis, hampering patient care and the development of therapy. We have identified inflammatory proteins including calprotectin and IL-8 as important indicators of disease severity and prognosis in bile and serum from patients with primary sclerosing cholangitis.

Keywords: PSC; Biomarker; Prognosis; Calprotectin; IL-8.

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* Corresponding author. Address: Norwegian PSC Research Center, Division of Cancer, Surgery and Transplantation, Oslo University Hospital Rikshospitalet, Postboks 4950 Nydalen, N-0424 Oslo, Norway. Tel.: +47 23 07 3616; fax: +47 23 07 3928.

E-mail address: t.h.karlsen@medisin.uio.no (T.H. Karlsen).

† These authors jointly supervised the work.

Introduction

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by progressing inflammation and fibrosis of the intra- and extrahepatic bile ducts, leading to cirrhosis in the majority of patients. PSC is frequently associated with inflammatory bowel disease (IBD) and other immunological diseases. To date, liver transplantation is the only curative treatment in PSC, as there is no medical therapy of proven benefit to halt disease progression. The disease course is highly variable between PSC patients, with transplant-free survival ranging from 12–21 years in different cohorts [1,2].

Predictors of outcome in terms of liver transplantation or death have been proposed, mainly based on biochemical variables (e.g. bilirubin or alkaline phosphatase) or clinical signs (e.g. variceal bleeding or ascites) [3], but currently there are no validated prognostic tools to reliably estimate the prognosis in the individual patient [4,5]. Furthermore, the lack of validated biomarkers reflecting relevant disease inflammatory activity is a major hurdle for clinical trials struggling to demonstrate effect of new therapeutic options. A position paper was recently published by the International PSC Study Group aiming to define surrogate endpoints for clinical trials based on a consensus process [6]. Biomarkers reflecting liver fibrosis including the serum-based Enhanced Liver Fibrosis (ELF®; Siemens Medical Solutions Diagnostics Inc., Tarrytown, NY, USA) test have been reported to predict prognosis in PSC [7]. Although promising biomarkers, a critical question is whether the proposed markers reflect disease activity or disease stage.

In this study, we aimed to explore whether inflammatory biomarkers or biomarkers of fibrosis other than ELF® represent biomarkers of disease activity or disease stage in large-duct PSC. As bile is in immediate contact with the primary target of the disease, i.e. the cholangiocytes (of the bile ducts), we hypothesized that the biliary protein pool would be more likely than serum to accurately reflect disease activity. In order to investigate this we performed an exploratory analysis of a large number of putative biomarker proteins in bile, using a custom multiplex antibody array platform and applying a two-step analysis with validation of significant results in an independent panel. Secondly, we investigated the prognostic potential of key proteins identified in bile, in sera from two independent cohorts by a similar two-step derivation-validation analysis.

Materials and methods

Patient population and data collection

PSC was diagnosed based on typical cholangiographic findings according to acknowledged criteria after the exclusion of secondary causes of sclerosing cholangitis, and excluding small duct PSC [8,9]. The first pathological cholangiography defined the time of PSC diagnosis. For the PSC patients, we queried patient records and research databases for information on clinical and laboratory data, including fever, cholangitis, jaundice, ascites, encephalopathy, esophageal varices, variceal bleeding, IBD status, colorectal or hepatobiliary malignancy, and medication at the time of bile or serum extraction. IBD diagnosis was based on colonoscopy and histology findings. Diagnoses of ulcerative colitis (UC) and Crohn's disease were established by accepted criteria. We retrieved updated information on liver transplantation dates and indications (Table S1) by December 31st 2012 from the Nordic Liver Transplant Registry and data on all-cause death by the same date from the Norwegian Death Registry.

We included two panels of bile samples; an exploratory derivation panel (bile panel-1), and an independent validation panel (bile panel-2) (Fig. S1A). We selected bile samples from PSC patients based on clinical and laboratory data in order to constitute groups of varying disease activity, applying the original Amsterdam score [10] to categorize the PSC patients into groups of "mild" or "advanced" disease (Amsterdam scores 0–2 or $\geq$3, respectively) based on revision of endoscopic retrograde cholangiography (ERC) images. Bile panel-1 consisted of bile samples extracted at ERC from 55 large-duct PSC patients (16 mild, 37 advanced) and 20 disease controls (details in Fig. S1) retrieved from the NoPSC Biobank, Oslo, Norway (Table 1). Bile panel-2 included bile samples extracted at ERC from a Finnish cohort of 34 PSC patients (12 mild, 22 advanced) and 10 controls (Table 1).

For the serum analyses, we adopted a two-step study design including two PSC panels recruited during two different, well-defined time periods (Fig. S1B). The panels were not overlapping. Statistically significant results obtained in serum panel-1 were tested in serum panel-2. Serum panel-1 included serum samples from 167 large-duct PSC patients (median follow-up time of 4 years [range: 0–20.1] from time of serum sampling) collected during 1992–2006. Serum panel-2 included 138 independent PSC patients (median follow-up time of 2.2 years [range: 0.0–4.3] from serum sampling) were collected during 2008–2012 from the NoPSC Biobank. Control serum panels of 100 healthy controls and 96 UC cases from a population based Norwegian cohort were retrieved for comparison [11]. The characteristics of the study populations are shown in Table 2.

Standard biochemical analyses were retrieved from clinical routine laboratory databases including C-reactive protein, white blood count, platelets, creatinine, total bilirubin, albumin, international normalized ratio (INR), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma-glutamyltransferase (GGT). Mayo risk scores were calculated using the algorithm for the revised Mayo risk score [12].

Ethical approval

The protocol was in accordance with Declaration of Helsinki and approved by the regional committee for research ethics in South Eastern Norway (reference number 2011/2572). All study participants provided written, informed consent.

Bile sample preparation

The samples were thawed and 100 µl of bile was added to 150 µl PBS containing 0.1% Tween 20 and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, Missouri, USA), then centrifuged at 4 °C for 10 min at 14,000 rpm. CleanAscite™ (100 µl, Biotech Support Group, NJ, USA) and Protein G Sepharose (50 µl; Sigma-Aldrich) were used for removal of lipids and immunoglobulins, respectively. Proteins were labelled with amine-reactive biotin (NHS-PEO_4-Biotin 1 mg/mL, Thermo Fischer Scientific, IL, USA) for 45 min on ice and separated on a Superdex 200 10/300 GL SEC column coupled to an Äkta Purifier (GE Lifesciences). The mobile phase was PBS containing 0.05% Tween 20 at a flow rate of 0.5 ml/min. After a retention volume of 8 ml, 24 fractions of 0.5 ml each were collected. The fractions were aliquoted and stored at −70 °C until analysis. The samples and fractions were kept cold at all times during the preparation procedure to limit proteolysis.

Antibody array analysis

The production of the bead-based antibody array has been described in detail elsewhere [13]. The antibodies used are commercially available and have been evaluated thoroughly in-house with regard to specificity prior to the current study [14]. A comprehensive list of the antibodies used is provided in Table S2. Antibody-coupled beads were mixed, aliquoted and stored at −70 °C. Mixtures of beads with up to 1728 different color codes were added to SEC fractions containing biotinylated bile proteins. After overnight incubation, the beads were washed three times with PBS containing 1% Tween 20 (PBT). Streptavidin-conjugated phycoerythrin was added to label captured biotinylated proteins (SA-PE, Jackson Immunoresearch). The beads were washed in PBT and analysed with an LSRII flow cytometer (BD Biosciences, San Jose, California, USA) as described previously [13]. Flow cytometry files were analysed with a customized software capable of automatically reading the fluorescent bar codes of microsphere subsets and exporting the median SA-PE fluorescence in text format [15]. The data output is reactivity profiles for all antibodies in the array where molecular size is on the x-axis and fluorescence intensity on the y-axis. Specific target capture is visualized as peaks in the reactivity profile. In order to obtain quantitative data we calculated the peak areas using scripts in Microsoft Excel.