Circulating markers of gut barrier function associated with disease severity in primary sclerosing cholangitis

Short title: Gut barrier function and disease severity in PSC

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PSC, primary sclerosing cholangitis; IBD, inflammatory bowel disease; LPS, lipopolysaccharide; LBP, lipopolysaccharide binding protein; sCD14, soluble CD14; IFABP, intestinal fatty acid binding protein; INR, international normalized ratio; ROC, receiver operating characteristics; AUC, area under the curve; UDCA, ursodeoxycholic acid; CC, cholangiocarcinoma; GBC, gallbladder cancer; CP, Child-Pugh; MELD, model for end-stage liver disease; ERCP, endoscopic retrograde cholangiopancreatography; PBC, primary biliary cholangitis.

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Author contribution

JRH, MT and IS planned the study. MV, THK, BM, JRH and AKD contributed to collection of samples and clinical data. IS, SÅ and EL performed the laboratory analyses. JRH, MK and AKD planned and performed the statistical analyses. AKD wrote the first draft and MK and JRH contributed to the writing of the manuscript. All authors read, critically revised and approved the final manuscript.

ABSTRACT

Background & Aims: One important hypothesis in primary sclerosing cholangitis (PSC) pathophysiology suggests that bacterial products from an inflamed leaky gut leads to biliary inflammation. We aimed to investigate whether circulating markers of bacterial translocation were associated with survival in a Norwegian PSC cohort.

Methods: Serum levels of zonulin, intestinal fatty acid binding protein, soluble CD14 (sCD14), lipopolysaccharide (LPS) and LPS-binding protein (LBP) were measured in 166 PSC patients and 100 healthy controls (HC).

Results: LBP and sCD14 were elevated in PSC compared with HC (median 13662 vs 12339 ng/mL, P=0.010 and 1657 vs. 1196 ng/mL, P<0.001, respectively). High sCD14 and LBP (values>optimal cut-off using receiver operating characteristics) were associated with reduced liver transplantation-free survival (P<0.001 and P=0.005, respectively). The concentration of sCD14 was higher in patients with hepatobiliary cancer compared to other PSC patients and HC. Zonulin was lower in PSC than controls, but when excluding PSC patients with increased prothrombin time zonulin concentrations were similar in PSC and HC. Concomitant inflammatory bowel disease did not influence the results, while IBD patients without PSC (n=40) had lower concentration of sCD14. In multivariable Cox regression, high sCD14 and high LBP were associated with transplantation-free survival, independent from Mayo risk score (HR 2.26 (95%CI 1.15-4.43), P=0.018 and HR 2.00 (95% CI 1.17-3.43), P=0.011, respectively).

Conclusions: PSC patients show increased levels of circulating markers of bacterial translocation. High levels are associated with poor prognosis measured by transplantation-free survival, indicating that ongoing gut leakage could have clinical impact in PSC.

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Keywords: primary sclerosing cholangitis; gut leakage markers; sCD14; LBP; LPS; zonulin.

KEY POINTS BOX

- **Key point 1. The circulating levels of soluble CD14 and lipopolysaccharide-binding protein were higher in patients with primary sclerosing cholangitis compared with healthy controls.**
- **Key point 2. High levels of the same markers were associated with shorter liver transplantation-free survival in the PSC patients, independent from Mayo risk score.**
- **Key point 3. The concentration of sCD14 was particularly high in patients with hepatobiliary cancer**
- **Key point 4. Overall, the results suggest that impaired gut barrier function may be relevant for disease severity and progression in primary sclerosing cholangitis.**

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic inflammatory liver disease affecting intra- and extrahepatic bile ducts, eventually progressing to end-stage liver disease.[1] There is currently no medical treatment available. In a recent large international cohort study, median liver transplantation-free survival was 14.5 years,[2] while it was 21.3 years in an Dutch population-based study.[3] A feared complication is hepatobiliary malignancy, occurring in 10% of the patients.[2] Approximately 70% of the PSC patients are diagnosed with concomitant inflammatory bowel disease (IBD), predominantly affecting the colon.[1]

The close relationship with IBD is the basis of several important hypotheses of PSC pathogenesis. Recent studies have identified an altered gut microbiota in PSC patients, both in stool and the mucosa.[4] It has previously been hypothesized that leakage of bacterial products across an impaired mucosal barrier in the gut could contribute to the disease. The liver is continuously exposed to a large variety of substances, including nutrients, gut bacteria and bacterial products originating from the gut via the portal circulation. Disruption of the mucosal gut barrier, during e.g. alcohol consumption,[5] inflammation or infection,[6] can lead to increased hepatic exposure to a range of bacterial byproducts and metabolites which in turn can activate the immune system and trigger inflammation both systemically and in the liver.[7] Changes in the gut microbiota have also been associated with increased gut permeability,[8,9] As a proof-of-principle, induction of small intestinal bacterial overgrowth in a rat model has been shown to increase translocation of bacterial products and lead to pathological changes similar to those seen in human PSC.[10] In human PSC, however, intestinal permeability has only been measured in one small study, and no alterations were observed.[11]

The classical marker of bacterial translocation is lipopolysaccharide (LPS), which are glycolipids derived from the outer membrane of gram negative bacteria functioning as pathogen associated molecular patterns, eliciting strong immune responses.[12] LPS-binding

protein (LBP) is an acute phase protein that binds to and correlates with LPS,[13] and facilitates the association between LPS and soluble CD14 (sCD14). Furthermore, sCD14 mediates the interaction of LPS with cells, thereby signaling the presence of gram-negative bacteria.[14] The concentration of sCD14 thus represents a host response to translocation measure, mainly related to monocyte activation. Zonulin is the only known physiological regulator of intercellular tight junctions and has been shown to correlate with intestinal permeability and could thus be used as a measurement of impaired gut barrier function.[15] Patients with inflammatory diseases like celiac disease and type 1 diabetes also show increased serum levels of zonulin compared to controls.[15] Finally, intestinal fatty acid binding protein (IFABP) is a biomarker for intestinal epithelial cell damage both *in vivo* and *in vitro*.[16]

Only limited data and non-conclusive studies are available about some of these markers in PSC.[17,18] Based on the observations described above, we hypothesized that circulating markers of gut barrier function are elevated in PSC compared with controls and that high levels of the same markers are associated with reduced liver transplantation-free survival.

PATIENTS AND METHODS

Patient population and data collection

Diagnosis of PSC was based on typical findings on magnetic resonance cholangiography or endoscopic retrograde cholangiography (ERCP) according to established criteria.[1] Patients were recruited at admission to Oslo University Hospital Rikshospitalet in the period 2008 to 2015. Serum and plasma were collected according to a standardized protocol and stored at -80 degrees until analysis. The time of the first diagnostic cholangiogram defined the time of diagnosis. Duration of disease was defined as the time from diagnosis to serum sampling. Some patients had serum sampled before final diagnosis (i.e. negative disease duration). Clinical and demographic information was acquired from patient records and research databases regarding clinical and laboratory data, including ascites, encephalopathy, variceal bleeding, type of inflammatory bowel disease, colorectal or hepatobiliary malignancy, and medication at the time of serum extraction. Inflammatory bowel disease was diagnosed based on endoscopy and histological findings, according to accepted criteria.[19] Healthy controls (n=100) were included from the Norwegian Bone Marrow Donor Registry, as used in previous studies.[20] IBD patients with a final diagnosis of ulcerative colitis but without PSC (n=40), were included from the Inflammatory Bowel Disease in South-Eastern Norway (IBSEN) population-based inception cohort. Serum was sampled at the 10-year follow-up.[20]

Written informed consent was obtained from each study participant. The Regional Committee for Medical and Health Research Ethics of South-Eastern Norway approved the study (Reference: 2015/2140/REK). The study was carried out in accordance with the Declaration of Helsinki.

Analysis of markers of bacterial translocation and clinical biochemistry

Child-Pugh score, Model for End-Stage Liver Disease (MELD) score and revised Mayo risk score were calculated according to established algorithms.[21-23] Since only data on presence/absence (and not severity) of ascites/ hepatic encephalopathy were available, only one additional point was given for ascites or hepatic encephalopathy when calculating the Child-Pugh score. Serum concentrations of IFABP, zonulin, LBP and sCD14 were analyzed with enzyme-linked immunosorbent assay (ELISA) kits: IFABP and LBP (Hycult biotech, Uden, The Netherlands, coefficient of variation (CV) 8.2% and 7.6%, respectively), Zonulin (Immune diagnostic, Bensheim, Germany, CV 12.5%) and sCD14 (R&D Systems, Abington, Oxon, UK, CV 8.9%). LPS analysis (in plasma) was established after testing several methods. A pilot study of 40 PSC samples using the commonly applied limulus amebocyte lysate colorimetric assay (Lonza, Walkersville, MD, USA) revealed a strong correlation between LPS and bilirubin concentrations (rho=0.75, $P < 0.001$), suggesting that bilirubin interferes with the yellow read-out of the test. LPS analyses were therefore performed using the Pyrochrome Diazo kit (Cape Cod, East Falmouth, MA, USA, CV 15.0%) according to the manufacturer's instructions, and no correlation with bilirubin concentration was found (rho = 0.045, P=0.58). Standard biochemical analyses were performed as part of clinical routine, including liver function tests and international normalized ratio (INR).

Statistical analyses

Data are presented as median (range) unless stated otherwise. The Mann–Whitney U test was applied for continuous variables. Correlation between levels of gut leakage markers zonulin, IFABP, sCD14, LBP and LPS were performed by the Spearman's rank correlation test (rho). Kaplan-Meier plots and log-rank tests were used for survival analyses. The biomarkers of interest were categorized as high and low levels by the median (2 ntiles-option in SPSS). Receiver operating characteristics (ROC) curve analyses were performed to define area under the curve (AUC), sensitivity and specificity, as well as the optimal cut-offs using Youden's

index. When analyzing subgroups, the samples were re-categorized in each stratum. Clinical features and laboratory data from the time of sampling was compared with mortality or liver transplantation with Cox proportional hazards analysis. Statistical analyses were performed using SPSS (version 24; SPSS, Inc., Chicago, IL) and MedCalc (MedCalc Software, Ostend, Belgium). P-values <0.05 were considered statistically significant.

RESULTS

Population characteristics

In total 166 PSC patients (80% male, 73% with IBD) and 100 healthy controls (59% male) were included, with a median age of 41.0 (16.3–72.4) years in the PSC patient group and 39.9 (28.1–56.1) in the control group (Table 1). During a median follow-up time of 3.9 (0.0–8.3) years 74 (45%) of the PSC patients reached an end point; 19 patients died and 55 patients underwent liver transplantation.

Increased sCD14 and LBP concentrations in PSC patients compared with controls

The concentration of sCD14 was higher in the PSC patients compared to controls, median 1657 (885-4095) and 1196 (835–1776) ng/ml, *P*<0.001, respectively (Figure 1A). LBP was also higher in PSC patients than controls (13662 (4856–34311) vs. 12339 (4674–43400) ng/ml, (*P*=0.010), Figure 1B), while zonulin was lower in the patients (32 (6-58) vs. 34 (14–55) ng/ml, (*P*=0.008), Figure 1C). IFABP and LPS were similar in PSC patients and controls (*P*=0.20, Figure 1D and *P*=0.84, Figure 1E, respectively). There was a modest but significant correlation between sCD14 and LBP in PSC patients (rho=0.22, *P*=0.005, Figure 1F), but not in healthy controls (rho=-0.07, *P*=0.46). Similarly, sCD14 and LPS were correlated in PSC patients (rho=0.23, *P*=0.003), but not in healthy controls (rho=0.03, *P*=0.78). There were no significant differences in levels of gut leakage markers between PSC patients with and without IBD, but IBD without PSC had lower levels of sCD14 than PSC patients (Figure 2A and B). PSC patients using ursodeoxycholic acid (UDCA) showed higher levels of sCD14

compared to other PSC patients (1848 (973-3264) vs 1540 (885-4095), *P*=0.005). Patients on prednisolon or 5-aminosalicylic acid treatment showed similar levels of sCD14 as other patients (data not shown).

High sCD14 and LBP predict reduced liver transplantation-free outcome

PSC patients who reached an end point (death or liver transplantation) during follow up showed higher concentrations of sCD14 compared to transplantation-free survivors (2047 (895–4095) vs. 1496 (885–2713) ng/ml, *P*<0.001). Similarly, LBP was also increased in patients with end point during follow up compared to transplantation-free survivors (14302 (4856–34311) vs. 13076 (5013–29557) ng/ml, *P*=0.046). There were no significant differences for LPS, zonulin or IFABP (data not shown).

In order to evaluate the predictive value of sCD14 and LBP, the patients were stratified into high and low concentration by the median and analyzed by Kaplan-Meier plots and log rank tests. Patients with high sCD14 or high LBP concentrations showed reduced liver transplantation-free survival compared with the low concentrations groups (*P*<0.001 and *P*=0.043, respectively). There were no significant effects on survival for LPS, zonulin or IFABP (data not shown). ROC tests were subsequently performed to define the optimal cut-offs in the complete PSC population. The AUC of sCD14 to differentiate patients with and without end-point was 0.76 with an optimal cut-off of >1638 ng/ml, associated with a sensitivity of 74% and specificity of 70% (Figure 3A). LBP yielded an AUC of 0.59, with the optimal cut-off >13942 ng/ml (sensitivity of 55% and specificity of 64%, Figure 3B). Using the optimal cut-offs, sCD14>1638 ng/ml was associated with reduced liver transplantation-free survival, mean 3.5 years (CI 2.7-4.2) vs. 6.8 (6.2-7.4), *P*<0.001, while LBP>13942 ng/ml was associated with a mean survival of 4.3 years (3.4-5.1) vs. 5.8 (5.1-6.5), *P*=0.005 (Figure 3C and 3D).

Concentration of sCD14 was higher in the groups with hepatobiliary cancer

Of the 166 PSC patients, 25 had a diagnosis of hepatobiliary malignancy at baseline, or were diagnosed during follow-up (22 of them had cholangiocarcinoma (CC) and 3 had gallbladder cancer (GBC)). The concentration of sCD14 was higher in the cancer groups than in PSC patients without cancer and healthy controls (Figure 4A), while there were no significant differences for the other markers. When stratifying according to the occurrence of cancer or not, there was still a strong association between high sCD14 and reduced liver transplantation-free survival in the group without cancer (Figure 4B).

Zonulin concentration is confounded by liver synthesis function

Zonulin (encoded by the Haptoglobin gene) is primarily synthesized in the liver. Zonulin concentration was significantly lower (P=0.001) in PSC patients with reduced synthesis function, as defined by increased prothrombin time (INR>1.2), which was available for 134 PSC patients. When only including patients with normal liver function (n=115), patients with PSC no longer had reduced zonulin levels compared with healthy controls (Figure 5A). There were no significant differences in the other markers between patients with high and low INR. High levels of sCD14 were associated with reduced liver transplantation-free survival regardless of high or low INR (Figure 5B and C). When using other measures of advanced chronic liver disease, Child-Pugh score and MELD score, sCD14 was significantly higher in patients with more advanced disease (Child-Pugh B/C vs. A or MELD >=10 vs <10), 2331 (895–4095) vs 1535 (885–2956) and 2420 (895–4095) vs 1574 (885–3264), respectively (both P<0.0001). However, high sCD14 (>1638 ng/ml) was not significantly associated with liver transplantation-free survival in the Child-Pugh B/C and MELD>10 groups (Data for Child-Pugh score shown in Figure 5D and 5E), suggesting that the strongest predictive power is in less advanced disease.

Relationship between cholangitis, cholestasis and gut leakage markers

In PSC, the presence of acute cholangitis may nonspecifically confound the investigated markers. C-reactive protein (CRP) correlated with sCD14 (rho=0.47, *P*<0.001), LPS (rho=0.22, *P*=0.007) and leukocytes (rho=0.20, *P*=0.015). Considering an arbitrary cut-off of CRP>10 mg/L, *n*=40 (26%) of the patients had elevated and *n*=112 (74%) normal CRP (*n*=8 missing values). Elevated CRP was associated with reduced liver transplantation-free survival (log rank *P*<0.001), however, elevated sCD14 was still associated with decreased survival when patients with high CRP were excluded (Figure 6A).

A moderate to strong correlation was observed between cholestasis markers and sCD14, demonstrated for alkaline phosphatase (rho 0.58, *P* <0.0001) and bilirubin (rho 0.63, *P*<0.0001), while only a weak correlation was seen between LBP and bilirubin (rho 0.17, *P*<0.038), Figure 6B-D.

No relationship with genetic variation in the CD14 gene

Other possible confounders are genetic determinants of sCD14 expression. In a previous study in PSC patients, genetic variation in the rs2569190 single-nucleotide polymorphism in the *CD14* gene correlated with sCD14 concentration.[18] However, when using genotype data of rs2569190 available from 72 of the included PSC patients from a previous study,[24] there was no relationship between rs2569190 genotype and circulating sCD14 concentration in the present cohort, irrespective of genetic model (genotype or dominant/recessive, data not shown).

Independent effects of sCD14 and LBP in multivariable Cox regression

Cox regression was performed to assess the relationship between sCD14, LBP and established risk factors in PSC. We found both elevated sCD14 and LBP to be associated with reduced liver transplantation-free survival in PSC, independent of Mayo risk score (which entail age, albumin, bilirubin and AST), the presence of reduced synthesis function (elevated INR) or a

cancer phenotype (occurrence of hepatobiliary cancer), while elevated CRP was not significantly associated with survival in multivariable models (Table 2).

DISCUSSION

In this cross-sectional study of circulating markers related to bacterial translocation, intestinal permeability and monocyte activation, sCD14 and LBP were elevated in PSC patients compared with controls. High levels of sCD14 and LBP were associated with reduced liver transplantation-free survival, suggesting that bacterial translocation may be relevant for disease progression in PSC. Of note, elevated sCD14 was associated with reduced survival even when excluding individuals that were later diagnosed with hepatobiliary cancer, which was associated with the highest sCD14 levels.

Serum concentrations of both sCD14 and LBP were clearly elevated in PSC patients. To our knowledge, there are no previous reports comparing circulating sCD14 and LBP in PSC or other cholestatic diseases with healthy controls. In non-alcoholic fatty liver disease, elevated levels of sCD14 were observed in a study of 113 patients and 21 matched healthy controls, positively correlating with both liver inflammation grade and hepatic sCD14 expression.[25] Elevated sCD14 has also been reported in systemic inflammatory diseases like arthritis [26] and atopic dermatitis.[27] The correlations between sCD14 and LBP, and sCD14 and LPS, suggest that translocation of gut microbial products may in part influence sCD14 concentrations, despite no increase of LPS observed in the systemic circulation. Importantly, sCD14 has been found to be elevated in bile in PSC compared with control subjects undergoing ERCP,[20] indicating that a biliary source of sCD14 is possible. LPS has been reported to accumulate in the biliary epithelium of PSC patients, which also shows hyper-responsiveness to LPS stimulation.[28-30] One could therefore speculate that there is an increase in LPS in portal blood, which is no longer detectable after first passage of the liver.

High sCD14 and LBP were associated with liver transplantation-free survival independent of Mayo risk score. This is in line with a study of 136 Japanese patients with primary biliary cholangitis (PBC) followed for 8.8 years, where elevated sCD14 at baseline was significantly associated with both liver decompensation and liver-related death or liver transplantation.[31] In contrast to our findings, a recent German study in PSC showed increasing sCD14 associated with reduced risk of liver transplantation or death.[18] The sCD14 concentrations in that study correlated with a common genetic variant of CD14; CD14 -260C>T (rs2569190). A similar correlation was not reproduced in the present study, suggesting that there were major differences between the cohorts. The positive correlations between sCD14, LBP and LPS suggest that sCD14 concentrations in our patient cohort, at least in part, are driven by pro-inflammatory stimuli and not host genes. These results are also coherent with the association between LBP and reduced liver transplantation-free survival. The German study excluded patients with advanced disease or acquiring cancer, contributing to the differences between the cohorts.[18] Still, the contradictory observations regarding sCD14 and liver transplantation-free survival are difficult to fully explain and warrant further studies.

A critical question when evaluating markers of bacterial translocation in PSC is the disease stage, since cirrhosis *per se* influences the gut barrier,[32] as well as confounding factors like acute cholangitis and cholestasis. Structural and functional alterations of the gut mucosa that lead to increased intestinal permeability have been described in liver cirrhosis [33] and increased intestinal permeability has been linked to the progression of liver disease and the complications of cirrhosis.[34] In the present study, neither sCD14 nor LBP were affected by the presence of reduced liver synthesis function, as measured by INR. However, reduced liver synthesis function may explain the reduced concentration of zonulin, which is only synthesized in the liver. In contrast, sCD14 was highly elevated and lost predictive power in patients with advanced chronic liver disease as evaluated by Child-Pugh score and MELD, suggesting that the effects of cirrhosis and portal hypertension outweigh any early gut barrier

dysfunction. We are uncertain why the use of UDCA was associated with higher sCD14 concentration. UDCA is of limited use in Norway and one possibility is that it is given mainly to symptomatic patients, who could have higher disease activity.

LBP and sCD14 both correlated with CRP, which is in line with elevated levels observed during acute cholangitis.[35] Elevated sCD14 and LBP concentrations have also been observed in patients with distal obstructive jaundice.[36] The correlations observed in particular between sCD14 and alkaline phosphatase as well as bilirubin could therefore suggest that cholestasis per se increases sCD14, although an alternative cause could be the mechanisms driving cholestasis. The elevated sCD14 levels in PSC, irrespective of concomitant IBD, compared with IBD patients without PSC may also point to the liver as an important driving force. Given the similar concentrations of LPS, IFABP and zonulin (in the group with normal synthesis) in the patients compared with controls, one should therefore be careful before concluding that increased bacterial translocation is the only or major explanatory factor for the present observations. This is in line with the lack of changes in small-bowel intestinal permeability observed in the only permeability study so far published in PSC,[11] although one could argue its relevance given that PSC patients most often experience colonic IBD. However, sCD14 and LBP were similar in PSC patients with and without a diagnosis of IBD, suggesting either that IBD is less relevant, or that an undiagnosed subclinical inflammatory intestinal process is ongoing also in PSC patients without IBD. Of note, the by far highest concentrations of sCD14 were observed in patients with hepatobiliary cancer. It could be speculated that this is partly related to activated tumor associated macrophages, and serves as rationale for further studies related to sCD14 or other macrophage-related markers in the pathogenesis or as clinical biomarkers in cholangiocarcinoma.

Some limitations should be discussed. The cohort is fairly large, but the results are not independently validated. Missing data for some confounders reduced the number of included

patients in the multivariable model. All patients have been included at a tertiary referral center, suggesting that the cohort may represent PSC phenotypes at the severe part of the disease spectrum. All assays were tested and found reproducible in our lab, but may have limitations; e.g. IFABP may be more useful in acute damage and LPS has a short half-life and variability in detection rates.[32] The extensive work to identify an assay not influenced by bilirubin is however a strength of the present study.

In conclusion, sCD14 and LBP were elevated in PSC patients compared with controls. These markers were moderately correlated, and high concentrations were associated with reduced liver transplantation-free survival independent from Mayo risk score, the occurrence of hepatobiliary cancer and reduced synthesis function. This opens the possibility that bacterial translocation may be relevant for disease progression in PSC.

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TABLES

Table 1. Patient characteristics.

	All PSC patients	Liver Tx-free survivors	Liver Tx/death	P
	n = 166	*n* = 92	*n* = 74	
Males, n (%)	132 (80)	74 (80)	58 (78)	0.847
Age, years, median (range)	41.0 (16.3-72.4)	36.3 (16.3-67.3)	48.0 (21.2-72.4)	<0.001
Age at diagnosis, years, median (range)	34.7 (13.2-71.5)	31.1 (13.8-65.8)	40.4 (13.2-71.5)	<0.001
PSC duration, years, median (range)*	1.6 (-0.74-32.1)	0.7 (-0.7-32.1)	2.7 (-0.6-26.5)	0.205
IBD ever, n (%)	121 (73)	72 (78.3)	49 (66.2)	0.051
Follow-up, years, median (range)	3.9 (0.01-8.3)	6.4 (1.8-8.3)	0.6 (0.01-7.3)	<0.001
Liver transplant, n (%)	55 (33)	0	55 (74)	
Death as endpoint, n (%)	19 (11)	0	19 (26)	
Mayo risk score, median (range)	0.06 (-3.0-4.13)	-0.25 (-2.99-3.20)	0.98 (-1.70-4.13)	<0.001
Ursodeoxycholic acid, n (%)	52 (31)	23 (25)	29 (39)	0.061
Prednisolon, n (%)	39 (23)	16 (17)	23 (31)	0.042
5-Aminosalicylic acid, n (%)	54 (33)	27 (29)	27 (36)	0.313
Laboratory data				
ALP, U/L, median (range)	225 (51-1459)	198 (51-869)	283 (78-1459)	<0.001
ALT, U/L, median (range)	90 (14-1008)	79 (14-885)	98 (24-1008)	0.420
AST, U/L, median (range)	75 (16-1683)	59 (16-1219)	99 (20-1683)	<0.001
Albumin g/L, median (range)	41 (23-51)	42 (31-51)	37 (23-48)	<0.001
Total bilirubin g/100 mL , median (range)	23 (3-591)	14 (3-167)	42 (5-591)	<0.001
Creatinine µmol/L, median (range)	65 (18-137)	68 (28-119)	62 (18-137)	0.069
Platelets 10^9, median (range)	283 (22-903)	293 (70-903)	244 (22-819)	0.030
Other information				
Gall bladder cancer, n (%)	3 (2)	0	3 (4)	0.090
Cholangiocarcinoma, n (%)	22 (13)	1 (1)	21 (28)	<0.001

PSC, primary sclerosing cholangitis, Tx, transplantation; IBD, inflammatory bowel disease; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

*A few patients have "negative" disease duration since diagnosis of PSC is set to the first definitive diagnostic cholangiogram.

Table 2. Cox regression analysis of PSC patients.

	Univariate analysis				Multivariate analysis[#]			
	P	**HR**	**95 % CI**	**N**	**P**	**HR**	**95 % CI**	**N**
Gender	0.708	1.11	0.64-1.94	166				
IBD	0.034	1.69	1.04-2.74	164	0.075	0.61	0.35-1.05	131
Mayo risk score	<0.001	1.85	1.56-2.20	154	0.008	1.43	1.10-1.86	131
IFABP (high[*])	0.926	1.02	0.65-1.61	166				
Zonulin (high[*])	0.973	0.99	0.63-1.57	166				
sCD14 (high[*])	<0.001	4.63	2.71-7.91	165	0.018	2.26	1.15-4.43	131
LBP (high[*])	0.006	1.92	1.21-3.04	166	0.011	2.00	1.17-3.43	131
LPS (high[*])	0.212	1.34	0.84-2.13	166				
INR > 1.2	<0.001	4.09	2.28-7.30	134	0.007	2.54	1.29-5.01	131
CRP > 10	<0.001	3.24	2.01-5.23	154	0.38	0.74	0.38-1.45	131
Hepatobiliary cancer (yes)	<0.001	5.79	3.46-9.67	165	0.001	2.86	1.50-5.47	131

[#]All the significant variables ($P < 0.05$) from a univariate cox regression analysis were included in a multivariate model. *For IFABP, zonulin and LPS, median was used as cut-off, while the cut-offs for sCD14 were >1638 ng/ml and LBP > 13942 ng/ml, which were the optimal cut-offs from area under the curve analysis. HR, hazard ratio; PSC, primary sclerosing cholangitis; IBD, inflammatory bowel disease; IFABP, intestinal fatty acid binding protein; sCD14, soluble CD14; LBP, lipopolysaccharide binding protein; LPS, lipopolysaccharides; INR, international normalized ratio; CRP, C-reactive protein.

FIGURE LEGENDS

Fig. 1. Levels of gut leakage markers in primary sclerosing cholangitis (PSC) patients vs. healthy controls. PSC patients (*n* = 166) show a significant increase in (**A**) soluble CD14 (sCD14), (**B**) lipopolysaccharide-binding protein (LBP) and (**C**) decrease in zonulin compared to healthy controls (*n* = 100), while levels of (**D**) intestinal fatty acid binding protein (IFABP) and **(E)** lipopolysaccharide (LPS) levels were similar. (**F**) Scatterplot of sCD14 and LBP levels in PSC patients. Data in A-E shown as median (min–max). Data missing for sCD14 in 1 patient with PSC. NS, not significant.

Fig. 2. Levels of gut leakage markers and inflammatory bowel disease (IBD). (**A**) Increased soluble CD14 (sCD14) in both primary sclerosing cholangitis (PSC) patients with IBD (PSC+IBD, *n* = 121) and without IBD (PSC no IBD, *n* = 43) compared to both healthy controls (*n* = 100) and IBD patients without PSC (*n* = 40). IBD patients (all diagnosed with ulcerative colitis) also showed increased levels of sCD14 compared to healthy controls. (**B**) Only PSC patients with IBD have increased lipopolysaccharide-binding protein (LBP) levels compared to healthy controls, while PSC patients show similar LBP levels as IBD patients without PSC, irrespective of IBD status. Data shown as median (min–max). Data missing for sCD14 in *n* = 1, and IBD status for *n* = 2, all in the PSC group. NS, not significant.

Fig. 3. Identification of optimal cut-offs for soluble CD14 (sCD14) and lipopolysaccharide-binding protein (LBP) and corresponding Kaplan Meier survival analyses. Receiver operating characteristic (ROC) curve tests defined the optimal cut-off in the complete PSC population to differentiate patients with and without end point to be (**A**) 1638 ng/ml for sCD14, associated with a sensitivity of 74% and specificity of 70%, and (**B**) 13942 ng/ml for LBP, associated with a sensitivity of 55% and specificity of 64%. These cut-offs were then used to analyze liver transplantation free-survival in PSC patients stratified by **(C)** high and low

sCD14 (divided by cut-off from the ROC curve test above) and (**D**) by high and low LBP (divided by cut-off from the ROC curve test above). Data missing for sCD14 in *n* = 1.

Fig. 4. Increased soluble CD14 (sCD14) levels in primary sclerosing cholangitis (PSC) patients with hepatobiliary cancer during follow-up. (**A**) sCD14 levels in healthy controls, PSC without cancer, PSC patients with cholangiocarcinoma (CC) and PSC patients with gallbladder cancer (GBC). (**B**) Kaplan Meier survival analysis of PSC patients without hepatobiliary cancer during follow-up (*n* = 140 (84.3%)), stratified by high and low sCD14 (divided by median). Data in A shown as median (min–max). Data regarding hepatobiliary cancer during follow-up missing for *n* = 1, and sCD14 levels missing for *n* = 1 in the PSC+CC group.

Fig. 5. High levels of soluble CD14 (sCD14) is associated with poor prognosis regardless of liver synthesis function while zonulin concentration is confounded. (**A**) Zonulin levels in healthy controls vs primary sclerosing cholangitis (PSC) patients stratified by high (≥1.2) and low (<1.2) international normalized ratio (INR). High sCD14 levels is associated with reduced liver transplantation-free survival in both PSC with (**B**) normal liver synthesis function (*n* = 115, defined by normal prothrombin time (INR ≤1.2) or (**C**) reduced liver synthesis function (*n* = 19, defined by increased prothrombin time (INR >1.2)). When stratifying patients according to Child-Pugh class, (**D**) high sCD14 levels is associated with reduced liver transplantation-free survival in patients in class A, while this is not the case in (**E**) patients in Child-Pugh Class B&C (classes were combined to achieve adequate sample size). Data for INR, sCD14 and Child-Pugh score missing for *n* = 32, *n* = 1 and *n* = 36, respectively. NS, not significant.

Fig. 6. High levels of soluble CD14 (sCD14) is associated with poor prognosis regardless of C-reactive protein (CRP) levels and correlates with liver biochemistry. (**A**) After excluding primary sclerosing cholangitis (PSC) patients with high CRP (*n* = 40, defined as CRP >10),

high sCD14 was still associated with decreased liver transplantation-free survival (n = 112). Correlations between (**B**) cholestasis markers alkaline phosphatase (ALP) and sCD14 and (**C**) bilirubin and sCD14, and (**D**) lipopolysaccharide-binding protein (LBP) and bilirubin. Data on sCD14 and CRP missing for n = 1 and n = 14 PSC patients, respectively.