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Evaluation of Biliary Calprotectin as a Biomarker in Primary Sclerosing Cholangitis

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Abstract: Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease of the bile ducts with limited therapeutic options except liver transplantation. Reliable biomarkers to predict the disease course are unavailable, and currently employed disease activity scores such as the Mayo risk score (MRS) have limitations. The present study aims to evaluate biliary calprotectin as a marker of disease activity and prognosis in PSC.

This is a monocentric retrospective observational study. Calprotectin concentrations were measured by an enzyme-linked immunosorbent assay in bile samples collected by endoscopic retrograde cholangiography from 106 PSC patients and 20 controls. Biliary calprotectin concentrations were compared between the 2 groups. In PSC patients, results were evaluated with regard to the presence of dominant bile duct stenoses, bile microbiology, MRS, survival free of liver transplantation, and necessity for bile duct interventions in the further disease course.

Median (interquartile ranges) biliary calprotectin concentrations were higher in PSC patients than in controls (3646 ng/mL, 249–9748 vs 116 ng/mL, 104–655; $P < 0.001$). In the PSC cohort, higher biliary calprotectin concentrations were associated with the presence of microbes in bile ($P = 0.02$), the occurrence of dominant bile duct stenosis at any time in the disease course ($P = 0.005$), and the necessity for future bile duct interventions ($P = 0.02$). Patients with biliary calprotectin concentrations above a cut-off of 11,610 ng/mL displayed significantly shorter transplantation-free survival than those with biliary calprotectin concentrations $\leq 11,610$ ng/mL ($P < 0.001$). Univariate Cox regression analysis revealed high biliary calprotectin concentration ($> 11,610$ ng/mL) as a risk factor of shorter transplantation-free survival of PSC patients ($P < 0.001$) beside high plasma alkaline phosphatase (ALP) concentration (> 142.5 U/L) ($P = 0.006$), high MRS (≥ 2) ($P < 0.001$), and nonsterility of bile

($P = 0.03$). Multivariate analysis identified only MRS ($P = 0.002$) and ALP concentration ($P = 0.04$) as independent risk factors.

Our data strongly suggest that biliary calprotectin may be a valuable additional marker for disease activity and a predictor of outcome in PSC, so that further studies for evaluation of calprotectin in this disease are warranted.

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Abbreviations: γ GT = γ -glutamyl transferase, ALP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, AUC = area under the ROC curve, CCA = cholangiocarcinoma, CI = confidence interval, CRP = C-reactive protein, ERC = endoscopic retrograde cholangiography, IBD = inflammatory bowel disease, IQR = interquartile range, MELD = Model for End-Stage Liver Disease, MRCP = magnetic resonance cholangiopancreatography, MRS = Mayo risk score, PSC = primary sclerosing cholangitis, ROC = receiver operating characteristic.

INTRODUCTION

Primary sclerosing cholangitis (PSC) is a cholestatic liver disease of unknown etiopathogenesis, which is associated with inflammatory bowel disease (IBD), particularly ulcerative colitis, in $\sim 70\%$ of cases. It is characterized by chronic inflammation and fibrosis of the bile ducts.^{1–3} Although, with an incidence of 0.77/100,000 person-years at risk,⁴ it is a rare disease, research efforts are essential because the diagnosis of PSC implies many potential complications such as cholangitis, cholangiosepsis, cholangiocarcinoma (CCA), colorectal carcinoma, and liver cirrhosis with all its afflictions.^{5–7} As yet, the only cure for PSC remains liver transplantation,⁸ mean transplantation-free survival ranging from 9.6 to 12 years.^{9–12} This is why currently the right selection of patients for this therapy and their position on the waiting list are of utmost importance.

A number of prognostic models including different variables as independent predictors of survival in PSC have been proposed.^{5,11,13–15} However, reliable disease markers for use in clinical routine are still lacking. One parameter that seems to be of prognostic relevance in PSC is serum alkaline phosphatase (ALP).^{16–18} The most common prognostic model for PSC is the Mayo risk score (MRS),¹⁹ which is mainly used as a surrogate endpoint in clinical trials. It has several limitations, for example, like the Model for End-Stage Liver Disease score²⁰ it performs best in end-stage liver disease,¹⁵ whereas there is a special need for tools of risk stratification in the earlier disease course.

The presence of dominant bile duct stenoses has been reported to be a predictor of disease outcome as well.^{5,21,22} To identify dominant bile duct stenoses, it is common routine in many centers to perform magnetic resonance cholangiopancreatography (MRCP) and/or endoscopic retrograde cholangiography (ERC) at regular intervals, as repeated balloon

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AG, DG, and WS conceived and designed the study. AS, PS, DG, YL, and PK-P initiated and managed the bile sample data bank. AG, DG, and CR collected clinical data. YL and PK-P performed calprotectin measurements. AG, JK, and DG analyzed the data. JK performed statistical review of the study. AG and DG wrote the paper. All authors critically reviewed the article.

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dilatations of dominant biliary strictures resulted in improved survival rates in PSC patients.^{23–25} ERC being a risky and costly procedure, it is of great interest to predict disease outcome and thereby better define intervals of necessary intervention.

As PSC is a fibroinflammatory disease,¹ there is a strong rationale for the evaluation of biliary calprotectin as a biomarker in PSC, which may also be useful in the earlier disease course. Calprotectin is a neutrophil granulocyte cytosol protein belonging to the family of S100 proteins and consisting of S100A8 and S100A9.²⁶ High S100A8/A9 levels are characteristic of inflammatory conditions. Calprotectin acts as a chemotactic molecule and is expressed by neutrophils, activated monocytes, and macrophages.^{27,28} Fecal calprotectin is widely used as a marker of disease activity in IBD.^{29,30} Calprotectin concentrations have also been determined in several body fluids, such as ascites, where it may be useful to diagnose spontaneous bacterial peritonitis,³¹ in urine after kidney transplantation,³² in cerebrospinal fluid where it reflects disease activity in multiple sclerosis,³³ and in serum as a biomarker of Crohn disease.³⁴ So far, experience on calprotectin as a marker in PSC is limited and comprises only few patients. In their study including 45 PSC patients, Reinhard et al³⁵ from our group demonstrated that the expression of S100A9 in bile was 2 times higher in PSC patients with high disease activity than in those with low disease activity. Voigtländer et al³⁶ showed in a study with 69 PSC patients that MRS and biliary calprotectin concentrations were correlated. Wang et al³⁷ published that S100A8 in bile may be a biomarker for gallbladder cancer. To our best knowledge, no outcome data have been published with regard to biliary calprotectin concentrations in PSC so far. The objective of this study was to examine the suitability of biliary calprotectin as a marker of disease activity and prognosis in PSC.

METHODS

Eligible patients were retrospectively selected from a data and bile sample bank, which was started in 1987²³ and has been continued to date. All patients included in the present study underwent ERC in the Department of Endoscopy of the University Hospital Heidelberg between September 2006 and August 2011. ERCs were consistently performed by experienced gastroenterologists. All bile specimens from PSC patients with sufficient remnant volumes for the determination of calprotectin concentrations were selected by an investigator (PK-P) who was not involved in further analyses and interpretation of the data. In addition, 20 bile specimens from controls were randomly chosen from the sample bank by the same investigator. There was no overlap of the present cohort with the 1 investigated in the study by Reinhard et al³⁵ from the same group, so that the results on biliary calprotectin presented here are independent of previously published data. The study protocol (ethical committee no. 337/2006) was approved by the local institutional ethics review board (Ethikkommission der Medizinischen Fakultät Heidelberg) on 16 January 2007 and is in accordance with the Declaration of Helsinki.

PSC Patients

The diagnosis of PSC was made according to the American Association for the Study of Liver Diseases practice guidelines based on characteristic biochemical and endoscopic findings.³⁸ Multilocal strictures and dilatations of the intrahepatic or extrahepatic bile ducts were considered to be typical ERC findings. Patients with decompensated liver cirrhosis at the

time of sample acquisition, patients with status post liver transplantation, and patients with the diagnosis of CCA within 6 months of sample acquisition were excluded. According to Stiehl et al,³⁹ a dominant bile duct stricture was defined as a stenosis with a diameter of <1.5 mm of the common bile duct or <1.0 mm of a hepatic duct (within 2 cm of the bifurcation). Major bile duct stenoses were opened by balloon dilatation, whenever possible. Patients underwent physical examination, biochemical blood tests, and abdominal ultrasound at 6- to 12-months intervals. MRCP and/or ERC were performed when any of these noninvasive examinations raised the suspicion of biliary obstruction. At that, ERCs were performed on schedule at individual intervals, depending on the disease course, with shorter intervals in patients displaying dominant bile duct stenoses (usually 6–12 months). MRS was calculated as a marker of disease activity.²⁰ The MRS takes into account patient age, laboratory blood parameters including bilirubin, aspartate aminotransferase (AST), albumin levels, and a history of variceal bleeding to estimate 1- to 4-year probabilities of survival of PSC patients.²⁰ The “low-risk” group comprises patients with MRS ≤0, the “intermediate-risk” group those with MRS between 0 and 2, and the “high-risk” group those with MRS ≥2.

Controls

Controls were patients who underwent ERC but in whom PSC, secondary sclerosing cholangitis, and CCA were excluded. Patients with obvious endoscopic signs of cholangitis were not considered eligible as controls. Therefore, patients with purulent secretion visible during ERC or cholangiographic signs of chronic bile duct inflammation were not included. Thirteen of the controls suffered from choledocholithiasis; 3 received ERC due to the suspicion of bile duct alterations on MRCP, but they displayed completely inconspicuous ERC findings. Three patients had malignant obstructions of the common bile ducts, and in 1, biliary leakage after right-sided hemihepatectomy for living donor liver transplantation was suspected but not confirmed.

Laboratory Blood Parameters

In clinical routine, laboratory blood parameters were in most of the cases determined at the same time as the ERC was performed. They included C-reactive protein (CRP, normal <5 mg/L), leukocyte counts (normal 4–10/nL), ALP (normal 40–130 U/L), bilirubin (normal <1.0 mg/dL), γ -glutamyl transferase (γ GT, normal <60 U/L), AST (normal <50 U/L), alanine aminotransferase (ALT, normal <50 U/L), and plasma albumin (normal 30–50 g/L). Laboratory results were included if they had been determined on the day before or on the day of the ERC of interest. Laboratory results determined after ERC were not included. All laboratory results were retrieved from electronic patient records.

Collection and Storage of Bile Specimens

Intrahepatic bile specimens were obtained by ERC. For endoscopic collection of bile specimens, the papilla of Vater was selectively cannulated. Bile samples were obtained by suction and, whenever possible, before injection of contrast medium and any therapeutic procedure. In patients in whom bile collection was not possible before injection of contrast medium into the bile duct, a volume equivalent to that of the contrast medium was first extracted by suction into a syringe to be discarded before the syringe for the actual bile specimen was attached. This was performed to minimize effects of dilution.

All specimens were immediately snap-frozen in liquid nitrogen and stored at -80°C before further use.

Measurement of Calprotectin Concentrations in Bile

For quantitative determination of biliary calprotectin, the PhilCal calprotectin (myeloid-related protein8/14) enzyme-linked immunosorbent assay kit was used (Immundiagnostik AG, Bensheim, Germany). All bile specimens were diluted 1:100 with distilled water before biliary calprotectin concentrations were determined following the manufacturer's instructions. After initial experiments had been run in triplicate with an intra-assay variability of $<10\%$, further measurements were performed in duplicate. Control samples were analyzed with each run. The detection limit for calprotectin was 3.2 ng/mL. Biliary calprotectin concentrations below the detection limit were defined as 0 ng/mL.

Bacterial Cultures

By selective intubation of the bile ducts during ERC, contaminations from the intestine were considered to be unlikely. Aliquots of all bile specimens were placed in sterile glass tubes containing a medium for aerobic and anaerobic bacterial cultures (BD BBL Port-A-Cul, Becton, Dickinson and Co, Sparks, NV). The material was delivered to the microbiology laboratory of the hospital within ≤ 2 hours. The samples were cultured aerobically and anaerobically at 37°C for 72 hours using blood sugar plates (Colombia II agar base, BBL, Becton, Dickinson and Co) containing horse blood. The anaerobic cultures were set up in anaerobic jars containing 6% carbon dioxide in nitrogen. All cultures were incubated for 72 hours, with the first reading taken after 24 hours.

Collection of Demographic and Clinical Data

Demographic and clinical data were part of the existing data bank or retrospectively collected by review of electronic medical records, if missing. All data were managed in an IBM SPSS data bank (version 22; IBM Corp, Armonk, NY). Demographic and clinical data documented for control patients were sex, age at ERC of sample acquisition, ERC findings, laboratory findings, and diagnosis necessitating ERC. Data extracted from medical records of PSC patients included sex, age at ERC of sample acquisition, age at first diagnosis of PSC, disease duration (defined as the interval between first diagnosis of PSC and ERC of sample acquisition), diagnosis of IBD, laboratory findings, presence of dominant bile duct stenosis at ERC of sample acquisition, presence of dominant bile duct stenosis at any time in the disease course, further balloon dilatation(s) of dominant bile duct stenoses after ERC of sample acquisition, date of death, cause of death, and liver transplantation.

Follow-Up and Outcome Measures

Outcomes of all patients were retrospectively followed up using the electronic clinical documentation system of the hospital until 6 August 2015. Data of patients who discontinued treatment at our hospital were censored at their most recent visits. Outcome measures were transplantation-free survival and necessity of further balloon dilatation(s) of dominant bile duct stenoses after the ERC of sample acquisition. Controls were not followed up.

Statistical Analyses

For all statistical analyses but Cox regression analyses, IBM SPSS (version 22; IBM Corp) was used. Univariate and

multivariate Cox regression analyses were performed with SAS (version 9.3; SAS Institute, Cary, NC).

Data are presented as medians and interquartile ranges (IQRs), and in some cases; minimum and maximum values are indicated in addition. Continuous data were compared using the Mann–Whitney U test. For comparison of categorical variables, χ^2 test was used. For correlation analysis of continuous variables, the Spearman method was employed. Actuarial survival free of liver transplantation, considering death and liver transplantation as events, was estimated by the Kaplan–Meier product limit estimator. Differences between actuarial estimates were tested with the long-rank test. For the determination of cut-off biliary calprotectin and plasma ALP concentrations with regard to transplantation-free survival, the online tool “Cutoff Finder” was used.⁴⁰ The cut-off values were optimized for minimal P values in the log-rank test. Receiver operating characteristic (ROC) curve analyses were used to determine sensitivity and specificity of different cut-off values for biliary calprotectin concentration as predictor of liver transplantation or death. Univariate and multivariate Cox regression analyses were used to identify parameters independently related to reduced transplantation-free survival in PSC patients. Univariate analysis included sex, disease duration, presence of IBD, MRS, presence of dominant bile duct stenosis, biliary calprotectin, plasma ALP, and presence of microbes and specifically *Candida* spp. in bile. In case the univariate analysis yielded a $P < 0.2$ for the type III test, the respective covariate was to be included into the multivariate Cox regression model. To anticipate the potential issue of monotone likelihood, which generally occurs in small samples with substantial censoring of survival times and several highly predictive covariates, Firth bias correction was used to fit all models, and profile-likelihood confidence limits were calculated.⁴¹ Two-sided $P < 0.05$ were considered statistically significant.

RESULTS

Demographic and Clinical Characteristics of the Study Population

In total, 20 controls and 106 PSC patients were enrolled in the study. Among 108 PSC patients who were initially identified for the study, 2 had to be excluded due to ambiguous sample labeling in 1 case and status post liver transplantation in the other. Demographic and clinical characteristics of all patients are presented in Table 1. Both cohorts comprised more males than females (PSC: 75/31, controls: 13/7; $P = 0.61$). Median age of PSC patients at presentation was 40 years (IQR 33–46) and that of controls 50 years (IQR 43–69) ($P < 0.001$). Age and plasma γGT , ALT, and AST concentrations were higher in the control cohort as compared with the PSC cohort ($P < 0.001$, $P < 0.001$, $P < 0.001$ and $P = 0.04$, respectively), whereas plasma CRP concentrations, blood leukocyte counts, plasma ALP, and plasma bilirubin concentrations did not differ between the 2 groups (Table 1). The age at first diagnosis of PSC was 31 years (IQR 23–41), whereas disease duration of PSC at the time of sample acquisition was 6 years (IQR 2–12). Seventy-eight of the 106 PSC patients (73.6%) had IBD. All included PSC patients were under therapy with ursodeoxycholic acid. For PSC patients, median follow-up from the time of sample acquisition was 4.7 years (minimum 0.1, maximum 8.5, IQR 2.5–6.4). Time from first diagnosis of PSC to end of follow-up was 11.4 years (minimum 1.1, maximum 27.6, IQR 8.1–16.8). Ninety among

TABLE 1. Demographic and Clinical Characteristics of the 106 Included Patients With PSC in Comparison With the 20 Controls

	PSC (n = 106)	Controls (n = 20)	Normal Range	P
Sex, M/F	75/31	13/7		0.61
Age at ERC, y	40 (33–46)	50 (43–69)		<0.001
Age at first diagnosis of PSC, y	31 (23–41)	N/A		
Disease duration, y	6 (2–12)			
IBD, y/n	78/28	N/A		
DS at ERC of sample acquisition, y/n	41/65	N/A		
DS ever, y/n	79/27	N/A		
MRS	(n = 61)	N/A		
	–0.37 (–0.83–0.50)			
Plasma CRP concentration, mg/L	(n = 96)	(n = 19)	<5 mg/L	0.74
	3.6 (2.0–12.5)	7.0 (0–15.4)		
Number of blood leukocytes, /nL	(n = 102)	(n = 19)	4–10/nL	0.37
	7.4 (5.3–9.6)	6.5 (4.5–9.8)		
Plasma ALP concentration, U/L	(n = 96)	(n = 18)	40–130 U/L	0.83
	186 (116–299)	191 (110–406)		
Plasma γ GT concentration, U/L	(n = 101)	(n = 18)	<60 U/L	<0.001
	139 (67–228)	484 (270–1186)		
Plasma AST concentration, U/L	(n = 101)	(n = 17)	<50 U/L	0.04
	44 (26–69)	57 (35–183)		
Plasma ALT concentration, U/L	(n = 101)	(n = 18)	<50 U/L	<0.001
	46 (33–80)	139 (53–286)		
Plasma bilirubin concentration, mg/dL	(n = 101)	(n = 19)	<1.0 mg/dL	0.54
	1.1 (0.7–2.1)	1.4 (0.7–3.1)		
Biliary calprotectin concentration, ng/mL	(n = 106)	(n = 20)	N/A	<0.001
	3646 (249–9748)	116 (104–655)		

γ GT = γ -glutamyl transferase, ALP = alkaline phosphatase, ALT = alanine aminotransferase, AST = aspartate aminotransferase, CRP = C-reactive protein, DS = dominant bile duct stenosis, ERC = endoscopic retrograde cholangiography, IBD = inflammatory bowel disease, IQR = interquartile range, MRS = Mayo risk score, N/A = not applicable, PSC = primary sclerosing cholangitis. Results are indicated in medians and IQRs. Groups were compared by the Mann-Whitney U test.

the 106 PSC patients (84.9%) underwent ≥ 1 other ERC at our department after the ERC of sample acquisition.

Biliary Calprotectin Concentrations in PSC Patients and Controls

In controls, biliary calprotectin concentrations ranged from 0 to 11,944 ng/mL, with a median of 116 ng/mL (IQR 104–655), whereas in the 106 PSC patients, they ranged from 0 to 178,856 ng/mL, with a median of 3646 ng/mL (IQR 249–9748). Biliary calprotectin concentrations were significantly higher in PSC patients compared with controls ($P < 0.001$, Figure 1).

Relation Between Biliary Calprotectin Concentrations and Laboratory Blood Parameters

In controls, no significant correlations were found between concentrations of the following laboratory blood parameters and biliary calprotectin concentrations: plasma CRP ($n = 19$, $r_s = 0.43$, $P = 0.07$), leukocyte counts ($n = 19$, $r_s = 0.38$, $P = 0.11$), plasma bilirubin ($n = 19$, $r_s = 0.07$, $P = 0.79$), plasma ALP ($n = 18$, $r_s = 0.07$, $P = 0.78$), plasma γ GT ($n = 18$, $r_s = -0.10$, $P = 0.69$), plasma AST ($n = 17$, $r_s = 0.02$, $P = 0.93$), and plasma ALT ($n = 18$, $r_s = -0.03$, $P = 0.91$). In PSC patients, weak but significant positive correlations were found between plasma ALP and biliary calprotectin concentrations ($n = 96$, $r_s = 0.24$, $P = 0.02$), and between plasma CRP

and biliary calprotectin concentrations ($n = 96$, $r_s = 0.29$, $P = 0.005$). Other laboratory blood parameters at the time of bile sampling, including leukocyte counts, bilirubin, γ GT, AST, and ALT, did not correlate with biliary calprotectin concentrations in PSC patients ($P = 0.06$, $P = 0.61$, $P = 0.18$, $P = 0.10$, and $P = 0.16$, respectively).

Relation Between Biliary Calprotectin Concentrations and Baseline Data in PSC Patients

Biliary calprotectin concentrations in PSC patients did not correlate with disease duration ($r_s = -0.10$, $P = 0.33$), age at first diagnosis ($r_s = -0.11$, $P = 0.27$), or age at ERC with sample acquisition ($r_s = -0.16$, $P = 0.11$). Also, they were similar between male and female PSC patients (3370 ng/mL, IQR 214–8537 vs 3783 ng/mL, IQR 314–11,385; $P = 0.67$). PSC patients suffering from IBD had no different biliary calprotectin concentrations as compared with those without IBD (3848 ng/mL, IQR 223–9789 vs 2411 ng/mL, IQR 269–9424; $P = 0.88$).

Relation Between Biliary Calprotectin Concentrations and Bile Microbiology in PSC Patients

Among a total of 95 PSC patients for whom data on bile microbiology were available, 42 (44.2%) had sterile bile,

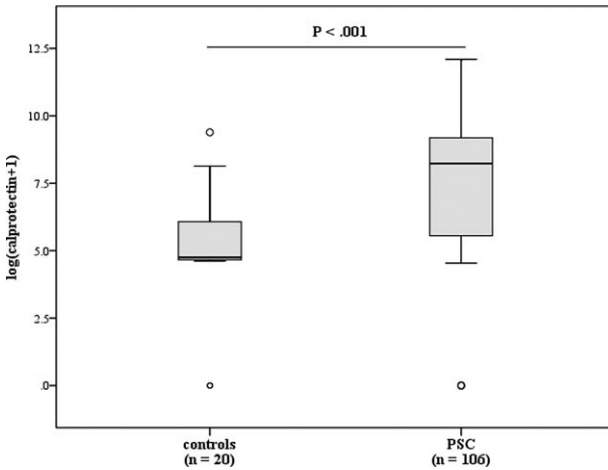


FIGURE 1. Logarithmic application of calprotectin concentrations in bile from controls (n=20) compared with those from PSC patients (n=106). Data are presented as boxplots, indicating medians, ranges, and IQRs. Circles represent outliers. IQR = interquartile range, PSC = primary sclerosing cholangitis.

whereas ≥ 1 bacterial or fungal strain were cultivated from the bile specimens of 53 (55.8%) patients. *Candida* spp. were identified in the bile from 11 PSC patients, whereas no *Candida* was found in the samples from 74 PSC patients, and no information on *Candida* was available in 21 PSC patients. Enterobacteria—independent of other microbes present in the bile—were detected in the specimens in 28 of the 95 (29.5%) PSC patients.

Biliary calprotectin concentrations were higher in the subgroup of PSC patients with nonsterile bile (6165 ng/mL, IQR 446–12,537) as compared with that of PSC patients with sterile bile (1196 ng/mL, IQR 166–7719, $P = 0.02$, Figure 2).

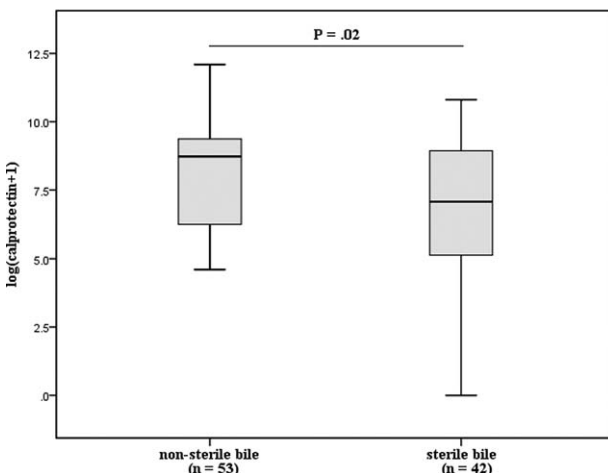


FIGURE 2. Logarithmic application of calprotectin concentrations in sterile (n = 42) versus nonsterile (n = 53) bile specimens from 95 patients with PSC. Bile was defined as nonsterile when ≥ 1 bacterial or fungal strain was cultured. In 11 of the 106 patients, no results on bile microbiology were available. Data are presented as boxplots, indicating medians, ranges, and IQRs. Circles represent outliers. IQR = interquartile range, PSC = primary sclerosing cholangitis.

Calprotectin concentrations in bile specimens that contained ≥ 1 species of enterobacteria, independent of the presence of other microbes in these samples, did not differ significantly from those without the growth of enterobacteria (5468 ng/mL, IQR 544–10,978 vs 3509 ng/mL, IQR 214–9529; $P = 0.57$). Neither was there a difference between calprotectin concentrations in bile with the growth of *Candida* spp. compared with bile specimens without the growth of *Candida* spp. (4678 ng/mL, IQR 378–7027 vs 3646 ng/mL, IQR 207–9684; $P = 0.80$). In comparison, plasma ALP concentrations did not differ between patients with microbes in bile and those with sterile bile ($P = 0.75$). Neither was there an association between the non-sterility of bile and the presence of dominant bile duct stenosis at ERC with sample acquisition ($P = 0.08$) nor between the presence of *Candida* in bile and dominant bile duct stenosis ($P = 0.13$) or the presence of enterobacteria in bile and dominant bile duct stenosis ($P = 0.99$).

Relation Between Biliary Calprotectin Concentrations and the Presence of Dominant Bile Duct Stenoses in PSC Patients

In 41 (38.7%) of the 106 PSC patients, dominant bile duct stenoses requiring endoscopic treatment were detected during the ERC of sample acquisition. Biliary calprotectin concentrations were ~ 3 times higher in PSC patients with dominant bile duct strictures (5276 ng/mL, IQR 646–10,198) as compared with those without (1408 ng/mL, IQR 177–9637, $P = 0.05$, Figure 3A). It was also documented whether a PSC patient had ever presented with a dominant bile duct stenosis, that is, before, during, or after the ERC during which the specimen for calprotectin determination was acquired. The patients who experienced dominant bile duct stenoses at any time (n = 79) had ~ 14 times higher biliary calprotectin concentrations than those who did not (n = 27, 5659 ng/mL, IQR 341–10,372 vs 359 ng/mL, IQR 158–140, $P = 0.005$ (Figure 3B)).

Relation Between Biliary Calprotectin Concentrations and MRS

The MRS at ERC could be calculated in 61 of the 106 (57.5%) PSC patients, and it ranged between -1.85 and 4.09 , with a median of -0.37 (IQR -0.83 – 0.50). In our cohort, there was a weak correlation between disease duration of PSC and MRS ($r_s = 0.26$, $P = 0.04$). According to the MRS, 38 patients (62.3%) belonged to the “low-risk” group, 20 patients (32.8%) belonged to the “intermediate-risk” group, and 3 patients (4.7%) belonged to the “high-risk” group. Biliary calprotectin concentrations were 2660 ng/mL (IQR 202–9687) in the “low-risk” group, 5168 ng/mL (IQR 203–26,544) in the “intermediate-risk” group, and 6821 ng/mL in the “high-risk” group. In spite of this tendency of higher calprotectin concentrations in bile from patients with higher MRSs, comparing biliary calprotectin concentrations between the “low-risk” and the combined “intermediate/high-risk” groups yielded no significant difference ($P = 0.53$). Neither did the Spearman test reveal a significant correlation between MRS and biliary calprotectin concentration ($r_s = 0.14$, $P = 0.29$).

Outcome Analyses

After a median follow-up of 3.7 years from the ERC of sample acquisition (range 0.4–7.3), 5 (4.7%) of the PSC patients died due to PSC without having undergone liver transplantation (3 died from CCA and 2 died from

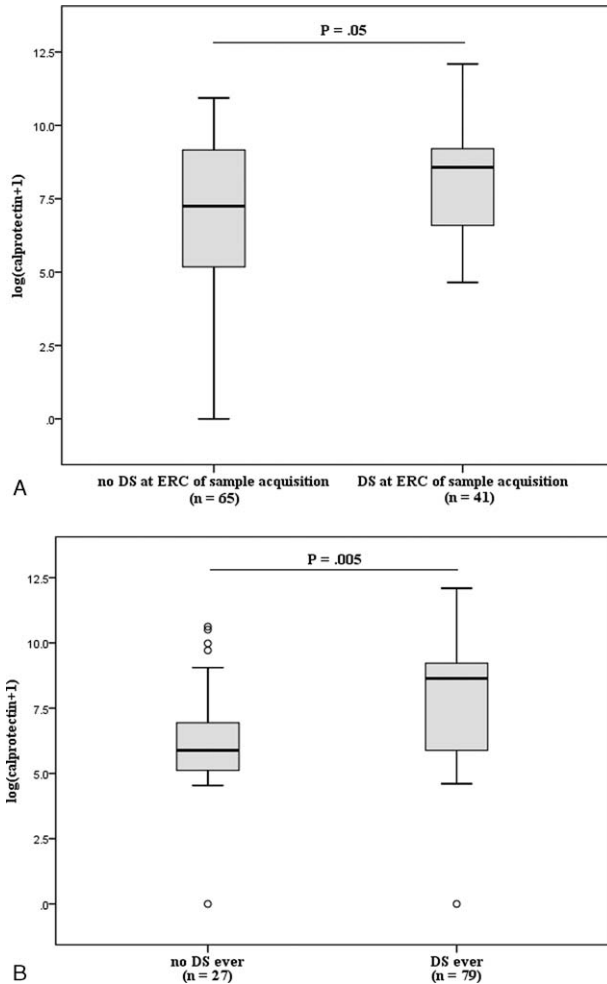


FIGURE 3. Logarithmic application of biliary calprotectin concentrations in PSC patients with dominant bile duct stenosis (DS) versus no dominant stenosis (no DS). (A) The 106 PSC patients were stratified by the absence ($n = 65$) or presence ($n = 41$) of DS at the ERC during which the bile specimens were collected. In (B), patients were stratified by the absence ($n = 27$) or presence ($n = 79$) of DS at any time during their disease course until the end of follow-up. Data are presented as boxplots, indicating medians, ranges, and IQRs. Circles represent outliers. ERC = endoscopic retrograde cholangiography, IQR = interquartile range, PSC = primary sclerosing cholangitis.

complications of liver cirrhosis). Thirty-five (33.0%) of the PSC patients underwent liver transplantation after a median follow-up of 3.6 years (IQR 2.3–5.9) from the time of ERC with sample acquisition. Median transplantation-free survival from first diagnosis in the group of patients who were transplanted or died was 10.6 years (7.7–16.7). Calprotectin concentrations were significantly higher in bile specimens from the 40 patients who reached the combined endpoint of either death or liver transplantation than in bile specimens from the 66 PSC patients who did not (6004 ng/mL, IQR 736–11,631 vs 1222 ng/mL, IQR 176–8456, $P = 0.02$). The cut-off concentration of biliary calprotectin that yielded the lowest possible P value with regard to transplantation-free survival was 11,610 ng/mL (log-rank, $P < 0.001$, Figure 4A). The sensitivity for this cut-off value to

predict liver transplantation or death was only 25.0%, whereas the specificity was 86.4%. The pair yielding the highest combined sensitivity and specificity, which could be identified by ROC curve analysis was 57.5% and 59.1% for a cut-off calprotectin concentration of 4334 ng/mL. However, for this cut-off value, the Kaplan–Meier survival analysis did not yield a significant difference in transplantation-free survival (log-rank, $P = 0.12$). The area under the ROC curve (AUC) was 0.64 (95% confidence interval [CI] 0.53–0.74, $P = 0.02$, Figure 4B). In comparison, AUC for plasma ALP concentrations with regard to transplantation-free survival was 0.69 (95% CI 0.58–0.79, $P = 0.002$).

Investigating other parameters with potential influence on outcome, the Kaplan–Meier analysis in our PSC cohort did not reveal a significant difference in transplantation-free survival between patients with dominant bile duct stenoses and those without, even though transplantation-free survival was shorter in patients with dominant bile duct stenoses (dominant stenosis at ERC of sample acquisition, $P = 0.35$; dominant stenosis ever, $P = 0.44$). In contrast, a comparison of transplantation-free survival between PSC patients with sterile and nonsterile bile yielded a significant difference ($P = 0.03$, Figure 4C), patients without microbes in the bile having longer transplantation-free survival. Also, transplantation-free survival was significantly shorter in the MRS “intermediate/high-risk” subgroup than in the “low-risk” subgroup ($P < 0.001$, Figure 4D). The cut-off plasma ALP concentration that yielded the lowest possible P value with regard to transplantation-free survival was 142.5 U/L. Patients with higher values at ERC of sample acquisition displayed significantly shorter transplantation-free survival than those with lower values ($P = 0.002$).

To identify independent risk factors of reduced actuarial transplantation-free survival, we performed univariate and multivariate Cox regression analyses. The following potential risk factors were included into univariate analysis: sex, age at ERC of sample acquisition, disease duration until ERC of sample acquisition, presence of dominant stenosis at ERC of sample acquisition, presence of dominant stenosis ever, MRS at ERC of sample acquisition, plasma ALP, nonsterility of bile, the presence of *Candida* spp. in bile, presence of IBD, and biliary calprotectin (Table 2). In univariate analysis, plasma ALP concentration >142.5 U/L ($P = 0.006$), intermediate/high-risk MRS ($P < 0.001$), nonsterility of bile ($P = 0.03$) and biliary calprotectin concentration $>11,610$ U/L ($P < 0.001$) were significantly related to transplantation-free survival. These 4 parameters and the presence of *Candida* spp. in bile ($P = 0.12$) were included into multivariate analysis, which revealed only MRS ($P = 0.002$) and plasma ALP concentration >142.5 U/L ($P = 0.04$) as independent risk factors of short transplantation-free survival (Table 2).

With regard to the necessity of further balloon dilatation(s) of dominant stenoses after the ERC of sample acquisition, we analyzed a subgroup of our PSC cohort including only patients who did not reach the combined endpoint of death or liver transplantation and who underwent ≥ 1 other ERC after the ERC of sample acquisition ($n = 55$). Within this subgroup, patients who underwent ≥ 1 other balloon dilatation during follow-up displayed significantly higher biliary calprotectin concentrations (6255 ng/mL, IQR 1295–10,734, $n = 18$) than those who did not (314 ng/mL, IQR 152–7527, $P = 0.02$). In contrast, no significant differences were found between these 2 subgroups with regard to plasma ALP concentrations ($P = 0.52$) and MRS ($P = 0.57$). Among the 55 patients who underwent ≥ 1 other ERC after the ERC of sample acquisition, if only those

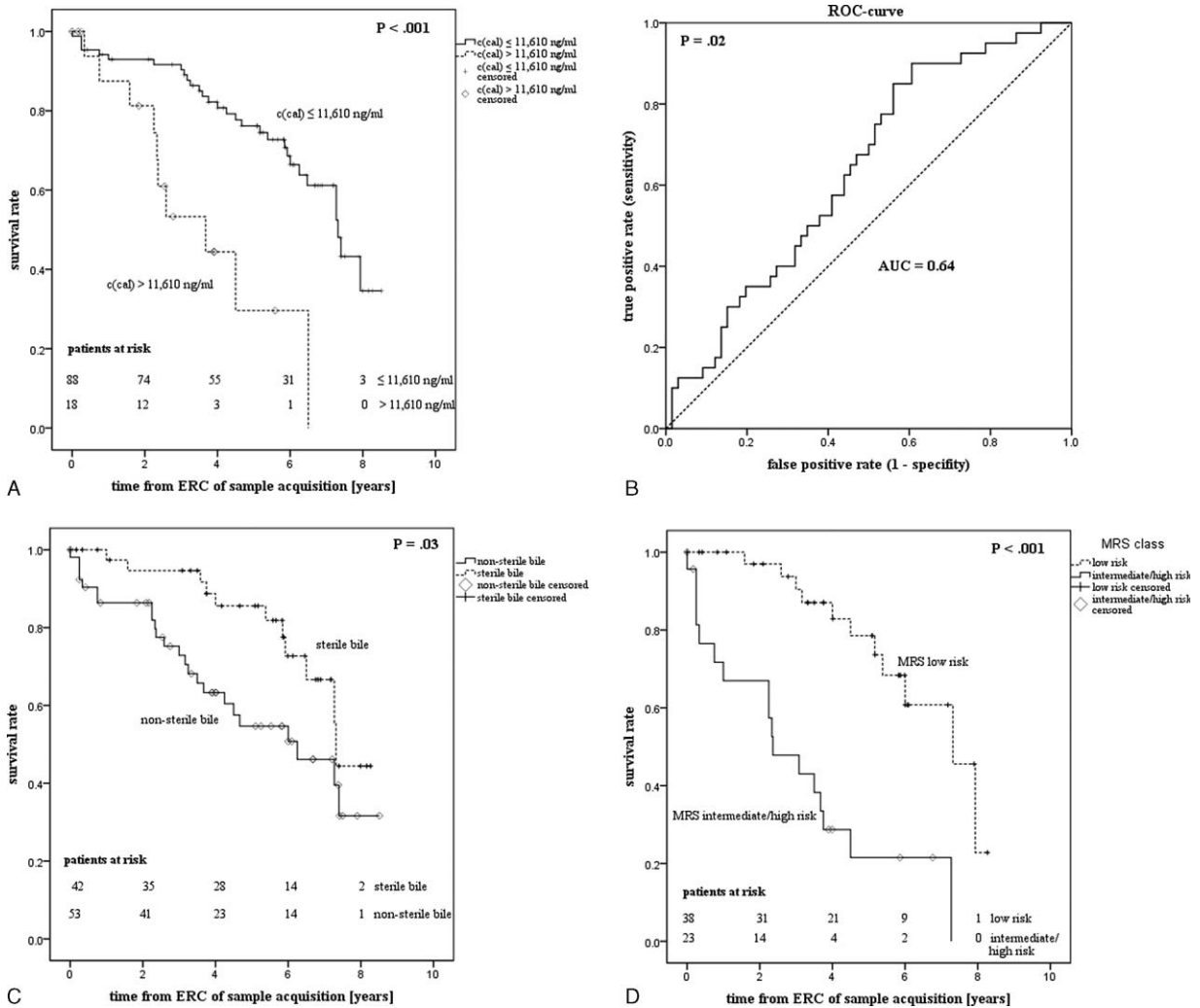


FIGURE 4. (A) Kaplan–Meier estimates of transplantation-free survival between PSC patients with biliary calprotectin concentrations [c(cal)] >11,610 ng/mL or ≤11,610 ng/mL, corresponding to a defined cut-off value with the lowest possible P value in the log-rank test. In all, 40 patients underwent liver transplantation (n = 35) or died from complications of PSC (n = 5). (B) ROC curve for biliary calprotectin to predict the combined endpoint of death or liver transplantation in our PSC cohort. (C) Kaplan–Meier estimates of transplantation-free survival between PSC patients with sterile versus nonsterile bile at the ERC of sample acquisition for calprotectin determination. (D) Kaplan–Meier estimates of transplantation-free survival between PSC patients belonging to the MRS “low risk” versus the combined “intermediate/high-risk” group. MRS was available in 61 patients. ERC = endoscopic retrograde cholangiography, MRS = Mayo risk score, PSC = primary sclerosing cholangitis, ROC = receiver operating characteristic.

who had no dominant stenosis at the ERC of sample acquisition were considered (n = 32), the difference was still statistically significant (P = 0.04).

DISCUSSION

Our study was aimed at identifying the potential value of biliary calprotectin as a marker of disease activity and prognosis in PSC. Its key findings are that biliary calprotectin concentrations are many times higher in PSC patients as compared with controls, and that in PSC patients, high biliary calprotectin concentrations are associated with the presence of microbes in bile, dominant bile duct stenosis, the need for balloon dilatation(s) of dominant stenoses in the further disease course, and shorter transplantation-free survival.

The difference in biliary calprotectin concentrations between the control and the PSC cohort was highly significant, although plasma CRP, ALP, and bilirubin concentrations were similar in the 2 groups, whereas plasma ALT and γGT concentrations were even higher in the control cohort as compared with the PSC cohort. Hence, cholestasis alone does not seem to result in a relevant elevation of biliary calprotectin concentrations, which is also emphasized by the fact that none of the laboratory blood parameters of cholestasis correlated with biliary calprotectin concentrations when analyzed within the control cohort.

Our observation that biliary calprotectin concentrations were higher in PSC patients than in controls confirms results from a study published by Voigtländer et al,³⁶ in which PSC patients without CCA displayed significantly higher biliary

TABLE 2. Risk Factors of Reduced Transplantation-Free Survival in PSC

Risk Factor	Univariate			Multivariate (n = 47)		
	HR	HR (CI 95%)	P	HR	HR (CI 95%)	P
Female sex (n = 106)	0.95	0.48–1.79	0.88			
Age at ERC of sample acquisition (n = 106)	0.99	0.97–1.02	0.66			
Disease duration (n = 106)	1.00	0.95–1.04	0.83			
DS at ERC of sample acquisition (n = 106)	1.35	0.72–2.51	0.35			
DS ever (n = 106)	1.33	0.60–3.41	0.53			
MRS intermediate/high (n = 61)	5.37	2.46–12.48	<0.001	6.24	2.15–20.58	0.002
Plasma ALP concentration >142.5 U/L (n = 96)	4.03	1.67–12.51	0.006	21.50	2.78–2764.86	0.04
Non-sterility of bile (n = 95)	2.16	1.09–4.52	0.03	2.16	0.66–7.79	0.23
Presence of <i>Candida</i> spp. in bile (n = 85)	2.15	0.76–5.09	0.12	2.46	0.63–8.82	0.19
Presence of IBD (n = 106)	1.02	0.52–2.17	0.95			
Biliary calprotectin concentration >11,610 ng/mL (n = 106)	4.19	1.91–8.64	<0.001	1.28	0.35–4.88	0.72

ALP = alkaline phosphatase, CI = confidence interval, DS = dominant stenosis, ERC = endoscopic retrograde cholangiography, HR = hazard ratio, IBD = inflammatory bowel disease, MRS = Mayo risk score, PSC = primary sclerosing cholangitis.

calprotectin concentrations than patients with choledocholithiasis. In their cohort of PSC patients, the median biliary calprotectin concentration was 237 mg/L, whereas it was 3.65 mg/L in our PSC cohort. As—to our best knowledge—there is no calprotectin assay available validated especially for the determination of calprotectin in bile, the assay we used is meant for the measurement of calprotectin in serum, plasma, and urine. Yet, after 1:100 dilution of bile specimens, the measurement of calprotectin concentrations worked with low intra-assay and interassay variability. At present, it remains unclear why median biliary calprotectin concentrations differed nearly by a factor of 100 between the 2 studies. With this discrepancy in mind, calprotectin measurements were repeated several times in our study, and an error caused by dilution during ERC or in the laboratory was excluded. Even if absolute values of biliary calprotectin may not (yet) be comparable between different departments, the statements in the present study will remain unchanged, as all specimens were treated in the same way. We also calculated ratios between biliary calprotectin and total bile acid concentrations in 91 of the PSC patients in whom bile acid concentrations were available (data not shown). The range was comparably high as for calprotectin per bile volume, so that we did not follow this path further. However, before the introduction of biliary calprotectin as a biomarker in PSC, more research would be necessary to validate biliary calprotectin concentrations.

Our study reveals an association between the presence of dominant bile duct stenoses and higher biliary calprotectin concentrations in PSC patients. More importantly, the necessity for future balloon dilatations of dominant bile duct stenoses was also linked to higher biliary calprotectin concentrations but not to higher MRSs or higher plasma ALP concentrations determined at the time when the bile samples were obtained. This observation is of clinical relevance, as it may eventually facilitate the implementation of individual ERC schedules for PSC patients. That would be highly desirable due to the risks and costs of ERC.

Previous studies showed reduced transplantation-free survival in PSC patients with dominant bile duct stenoses as compared with those without.^{5,21,22} In our study, patients with dominant bile duct stenoses at the ERC of sample acquisition

with biliary calprotectin determination had shorter transplantation-free survival than patients without dominant stenoses, but the difference did not reach statistical significance. The fact that we found no association between dominant bile duct stenoses and transplantation-free survival in the current study is possibly due to relatively low numbers of patients with dominant stenoses and the fact that most of the patients underwent endoscopic dilatation early in the course of their disease. Also, previous studies have revealed that bacteriobilia and fungibilia seem to influence the outcome of PSC in a negative way.^{5,12,42} This observation was partly confirmed by our data. However, our data were not significant for *Candida* spp., which may be due to the small number of only 11 PSC patients with the presence of *Candida* in bile in our cohort. As was the case in patients with dominant stenoses, biliary calprotectin concentrations were higher in PSC patients with microbes in bile, whereas there was no association between the presence of microbes in bile and the occurrence of dominant stenoses in our cohort. This is of note as it is hypothesized that bile duct infection in patients with dominant stenoses is partly caused by previous bile duct interventions.⁵ As it was not the subject of our study, we did not evaluate the exact numbers of prior bile duct interventions and changes of bile microbiota in the disease course. However, our results imply that calprotectin increase in bile may be caused by infection of the bile duct mucosa independent of dominant stenosis. As infections go along with neutrophil infiltration of the mucosa, the finding of higher calprotectin concentrations in infected bile seems plausible. In clinical practice, it is challenging to decide whether and which kinds of microbes in bile require antibiotic treatment. Our data encourage further studies on this subject, as biliary calprotectin may be a helpful tool in making this decision.

The outcome of our cohort was assessed based on transplantation-free survival. Forty of the included 106 PSC patients reached the combined endpoint of death or liver transplantation after a median of 10.6 years from the first diagnosis of the disease. This interval corresponds to the ones described by other authors.^{9–12} Our data show that biliary calprotectin concentrations are inversely related to transplantation-free survival. We tried to define a cut-off biliary calprotectin concentration to best predict transplantation-free survival and optimized it for

the lowest possible *P* value if patients with biliary calprotectin concentrations above and below that value were compared by log-rank test. The defined cut-off value of 11,610 ng/mL differentiated very well between the 2 subgroups; yet, only 17% of our cohort displayed biliary calprotectin concentrations >11,610 ng/mL. Also, sensitivity of this cut-off concentration to predict transplantation-free survival was very low, and AUC of biliary calprotectin as a predictor of survival rate was also poor. For validation of our data, we also analyzed other parameters influencing transplantation-free survival in our cohort. Although univariate analysis revealed nonsterility of bile, high biliary calprotectin concentration, high plasma ALP concentration, and high MRS as risk factors of short transplantation-free survival, only plasma ALP and MRS withstood multivariate analysis and can thus be defined as independent risk factors in our cohort. It has to be noted that there may be a statistical explanation why biliary calprotectin did not reach significance in multivariate analysis, although its *P* value was even smaller than that for ALP in univariate analysis. The number of patients who could be included into multivariate analysis (*n* = 47) was only less than half of that available for univariate analysis, and most patients had to be excluded due to missing MRSs. Smaller case numbers generally imply larger *P* values. At that, patients responsible for the high hazard ratio of biliary calprotectin in univariate analysis may have dropped out in multivariate analysis due to missing MRS or ALP values.

Does biliary calprotectin relate to other potential disease markers in PSC? In our study, like in that by Voigtländer et al,³⁶ a weak positive correlation was established between plasma ALP concentrations and biliary calprotectin concentrations. We found an additional weak positive correlation between plasma CRP concentrations and biliary calprotectin concentrations. In contrast to Voigtländer et al,³⁶ we detected no correlation between MRS and biliary calprotectin concentrations. Unfortunately, MRS determination was only possible retrospectively in 61 patients of our PSC cohort. A reason for the discrepancy between the 2 studies might be that we had fewer PSC patients in the “MRS high” group than Voigtländer et al, at least if only the patients with known MRS were considered. In all, we suggest that the correlation between MRS and biliary calprotectin concentrations might be only weak, if existent at all. This finding may not be surprising, as MRS mainly reflects end-stage liver disease, and biliary calprotectin may rather be a marker of the earlier disease course of PSC. The association between the presence of dominant bile duct stenoses and biliary calprotectin concentration suggests that biliary calprotectin concentration is influenced by the pattern and extent of bile duct affection. Unfortunately, no cholangiographic classification score—as described by Ponsioen et al^{43,44}—was prospectively determined for this study, so that we could not reasonably evaluate for a relation between biliary calprotectin concentrations and an endoscopic score. A clear disadvantage of biliary calprotectin as a marker of disease activity in PSC is that sample collection requires an invasive procedure, other than the use of fecal calprotectin in IBD. Another approach is to determine calprotectin in serum. Yet, so far, the only data on serum calprotectin in PSC have not been promising.³⁶

The main limitation of this study is its retrospective design, implying a considerable number of missing data, especially for the calculation of MRS. Thus especially the number of patients who could be introduced into multivariate Cox regression analyses was relatively small, even though the number of patients who was included into the study is respectable for a

rare disease as PSC. A selection bias may arise from the fact that bile samples for calprotectin determination were chosen according to their remnant volumes. Another limitation of this study is that we analyzed biliary calprotectin concentrations in only 1 bile specimen per patient, and that we did not include samples from different time points. It cannot be denied that the determination of biliary calprotectin in 1 sample from a single time point is just a snapshot and may not be representative, so that more data should be acquired to address this topic.

CONCLUSION

In conclusion, biliary calprotectin is a parameter that, in combination with other parameters like serum ALP and MRS, may be an important additional factor determining disease activity and predicting the outcome of PSC. It may help to define intervals between ERCs in these patients and to decide on the necessity of antibiotic treatment. Biliary calprotectin could also serve as an outcome measure to assess the anti-inflammatory potential of novel drugs in the treatment of PSC. Therefore, we suggest that prospective studies, preferably in multicenter settings, should be performed to further evaluate biliary calprotectin as an independent disease marker or component of a novel prognostic model in PSC.

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