

# Anti-GP2 IgA Autoantibodies are Associated with Poor Survival and Cholangiocarcinoma in Primary Sclerosing Cholangitis

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**Objective:** Pancreatic autoantibodies (PABs), comprising antibodies against glycoprotein 2 (anti-GP2), are specifically associated with complicated phenotypes in Crohn's disease (CD), but have also been observed with variable frequencies in patients with ulcerative colitis (UC).

Interestingly, we previously identified primary sclerosing cholangitis (PSC) as a common comorbidity in anti-GP2 positive UC patients. We therefore aimed to characterize the role of anti-GP2 in PSC.

**Design:** In an evaluation phase, sera from 138 well-characterized Norwegian PSC patients together with healthy controls (n=52), and UC patients without PSC (n=62) were assayed for the presence of PABs by indirect immunofluorescence. 180 PSC patients from Germany served as a validation cohort together with 56 cases of cholangiocarcinoma without PSC, 20 cases of secondary sclerosing cholangitis and 18 autoimmune hepatitis.

**Results:** In both PSC cohorts, anti-GP2 IgA positivity was widespread (52% and 47%) and consistently identified patients with poor survival during follow-up (Norwegian/German cohort: p Log Rank=0.016/0.018). Particularly, high anti-GP2-IgA titers were predictive for poor survival (p=0.006/p=0.004).

In PSC, a meta-analysis across the cohorts, though, yielded an odds ratio (OR) of cholangiocarcinoma in anti-GP2 IgA<sup>+</sup> PSC patients of 5.0 (p=0.001).

Importantly, this association remained independent of disease duration, bilirubin level and age.

**Conclusion:** Anti-GP2 IgA consistently identifies PSC patients with severe phenotypes, being associated with poor survival due to cholangiocarcinoma. Therefore anti-GP2 IgA yields promising characteristics as a novel prognostic tool in PSC patients' risk stratification.

**Significance of this study****What is already known on subject?**

- Pancreatic autoantibodies (anti-GP2/anti-CUZD1) specifically identify CD patients with distinct complicated phenotypes. However they display variable – yet unsolved - presence in UC patients.
- Preliminary data suggest PSC to be a common comorbidity among anti-GP2 positive UC patients.
- Several autoantibodies are described in PSC. However, their correlations to clinical parameters remain inconsistent.
- Particularly, no single prognostic tool, biomarker or other sparsely present risk factors in the general PSC population are capable of estimating the risk for PSC-associated malignancies.

**What are the new findings?**

- The pancreatic autoantibody anti-GP2 IgA was frequently observed in about 50% of PSC patients Independent of associated IBD and identified distinct severe phenotypes in PSC.
- A meta-analysis across the cohorts yielded an OR of 5.0 of cholangiocarcinoma in anti-GP2 IgA<sup>+</sup> PSC patients.
- Anti-GP2 IgA identified patients with poor survival and harbored predictive potential for early death or liver transplantation (LTX) in two independent cohorts from Norway and Germany. This effect was primarily based on PSC-associated biliary tract cancer.

**How might it impact on clinical practice in the foreseeable future?**

- Anti-GP2 IgA has to be considered as a novel prognostic/diagnostic tool in PSC and PSC-associated cholangiocarcinoma. Prospectively, it might serve as a complementary tool in established clinical scores (MELD) with the aim of early prioritized LTX.
- The presence of a secretory IgA antibody against the bacteria-binding antigen GP2 opens new avenues for further studies on bacterial (infectious) triggers in PSC-etiology.

## Introduction

Primary sclerosing cholangitis (PSC) constitutes an unsolved medical burden with high mortality, which is primarily based on the premalignant character of PSC[1]. Particularly, PSC-related cancer of the biliary tree is an important complication, among other, causing high mortality[2]. The progressive and destructive inflammation of the bile ducts in PSC requires liver transplantation in the majority of the patients after a median of 12 to 18 years[3].

In addition, PSC is in up to 80% of patients accompanied by inflammatory bowel disease (IBD) and vice versa, 4.0 to 7.5% of patients with ulcerative colitis (UC) suffer from PSC[4], conferring additionally an increased risk for developing colorectal carcinoma[5].

However, the lifetime risk of PSC patients for developing biliary tract cancer (cholangiocarcinoma, gall bladder cancer) is estimated much higher with approximately 13-14 % or even up to 33% in some cohorts[1, 6, 7]. The current knowledge concerning risk factors for cholangiocarcinoma (CCA) in PSC is limited[1,3,5] and the evidence is insufficient to use these factors for stratifying the risk of PSC patients, who are more likely to benefit from a screening program.

Despite the combined use of modern diagnostic tools, many PSC patients with biliary tract cancer are not diagnosed until an advanced stage[1, 8]. Moreover, nearly 50% of patients with biliary tract cancer are diagnosed simultaneously with their diagnosis of PSC or within the first year thereafter[9]. Identifying patients at risk for biliary tract cancer thus remains one of the major challenges with the surveillance program.

Currently, no single prognostic tool is capable of estimating the risk in the individual patient[5, 10].

Thus there is a clear need for new biomarkers, providing information about the cancer risk and prognostic information for the decision of early and prioritized liver transplantation in PSC.

The etiology of PSC as well as IBD remains elusive, though increasing evidence suggests an autoimmune-mediated inflammation in both diseases[11-13]. In PSC, the presence of several autoantibodies is one of the features indicative of an autoimmune etiology; however, no clear

conclusions can be drawn from the literature regarding their correlation to clinical parameters or etiological implications[13].

The co-occurrence and partly overlapping genetics of PSC and IBD, as well as frequently observed autoantibodies in both conditions (p-ANCA), suggest that common pathophysiological motifs could be relevant in PSC and IBD. Among others, we recently observed an association of the pancreatic autoantibody (PAB) against glycoprotein 2 (anti-GP2) with distinct phenotypes in Crohn's disease (CD) but with a low frequency in ulcerative colitis[14, 15]. PABs, which additionally comprise antibodies against CUB and zona pellucida-like domains 1 (anti-CUZD1), yielded also prognostic information in IBD (CD respectively)[14]. The use of PABs as additional biomarkers in IBD is supported by the circumstance that they not only in some cases precede specific CD-phenotypes (stricturing/perianal disease) but also appear to be stable disease markers[14, 15].

Nevertheless, the functional implication of anti-GP2 positivity and the association with complicated disease phenotypes in CD remains elusive[16].

PABs are directed against proteins predominantly expressed in the exocrine pancreas[17, 18], the intestinal epithelium[19] but – as GP2 – are additionally present in different concentrations in the bile juice of PSC patients with and without CCA[20]. Despite the interpretation of PABs as Crohn's disease specific markers, some studies also reported anti-GP2 sero-positivity with variable prevalence in UC patients[21]. Interestingly, we formerly identified a remarkable occurrence of anti-GP2 IgA in IBD patients with concomitant PSC (3 out of 9 positive, 33%, data not published). Therefore, we now investigated an association of anti-GP2 with PSC in two larger and independent cohorts with a focus on concomitant IBD, clinical parameters and outcomes like liver transplantation.

## Patients and Methods

**Study population:** In the evaluation phase, 138 serum samples of patients with well-characterized large duct PSC (78% male) from the biobank of the Norwegian PSC Research Centre (Oslo University Hospital, Rikshospitalet) were assayed for pancreatic autoantibodies (anti-GP2 IgA and IgG/anti-CUZD1 IgA and IgG), see Table 1. In addition, clinical biochemistry including liver enzymes was retrieved from clinical databases (Suppl. Table 1). Revised Mayo risk score (MRS)[22] and APRI (AST/platelet relation index) were determined for each Norwegian PSC patient.

Norwegian PSC samples had been collected between the years 2008 and 2012 (median follow-up [range] 2.2 years [0.0-4.3] from serum sampling). In Heidelberg the collection of PSC samples took place in the period from 2006 to 2015 (median follow-up [range] 3.5 years [0.0-8.83] from serum sampling)[23].

During the observation period, dates of certain censor events like liver transplantation or occurrence of biliary tract cancer like cholangiocarcinoma (CCA) and gall bladder cancer (GBC) were well documented. All PSC patients were screened clinically and endoscopically for concomitant inflammatory bowel disease.

Inflammatory bowel disease was defined according to accepted criteria[24].

Subsequently, significant associations were validated in an independent German cohort (n=180, 68% male) at the Centre for Liver Transplantation in Heidelberg (University Hospital Heidelberg, Germany). In addition, we refer to a small cohort from Lübeck in the introduction (data not shown), which consisted of 9 patients with IBD-related PSC. This cohort comprised 77.8% UC patients and 22.2% CD patients. One third of this cohort was male and mean age was 30.6 years (*SD*: 14) with a mean disease duration of 13.7 years (*SD*: 9).

The control groups consisted of 52 gender- and age-matched healthy blood donors from the University Hospital of Oslo (Norway) as well as 62 patients with well-defined diagnosis of ulcerative colitis recruited at the University Hospital of Lübeck (Germany), see Table 1. These UC patients were

included with respect to age- and gender matching and subsequently analysed for the prevalence of pancreatic autoantibodies (anti-GP2, anti-CUZD1).

Additional 56 patients with CCA without PSC, 20 patients with secondary sclerosing cholangitis (SSC) and 18 with autoimmune hepatitis (AIH) were included from the University hospitals Heidelberg and Oslo (Table 1). The protocol was in accordance with the Declaration of Helsinki.

**Antibody testing:** Analysis of anti-GP2 and anti-CUZD1 was performed by indirect immunofluorescence (IIF) (Euroimmun, Lübeck, Germany). A detailed description has been given previously[15]. For further information please see supplementary file.

**Statistical analysis:** We performed statistical analysis using IBM SPSS Statistics, version 22 (SPSS Inc. Chicago, IL, USA) and GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego CA, USA.

To test for normal distribution, the Kolmogorov-Smirnov test was used. Non-normally distributed data are indicated with median and quartiles as measures of central tendencies. For normally distributed data mean and standard deviation are used. Relative frequencies (in percent) refer to the number of cases without missing data.

For contingency analysis the Chi-square-independence-test was applied. Comparison of continuous variables was performed using the Mann-Whitney-U-test or t-test, depending on data distribution. The level of significance was set at 0.05. Logistic and linear regression models were performed to test for independency of the detected associations from potential clinical confounders (e.g. disease duration, gender, age, concomitant IBD). Survival curves were created according to Kaplan-Meier method. Log-Rank test was applied for comparison of survival distribution. In order to further evaluate a combination of potential prognostic factors a Cox Proportional Hazard model was given in a last step. The Cox model was used with a force inclusion strategy as well as with the forward inclusion and backward exclusion methods.

For meta-analysis of both cohorts in terms of biliary tract cancer the Cochran-Mantel-Haenzel test was used. Heterogeneity of the odds ratios was assessed with Breslow-Day test.



## Results

### **High prevalence of anti-GP2 in PSC and significant occurrence in other biliary large duct diseases**

As shown in Table 1, the Norwegian and German PSC patients were similar as evaluated by age at serum sampling (41.4 vs 40.7 years), gender (77.5 vs 67.4% males) and prevalence of concomitant IBD (74.5% vs. 69.4%, respectively) and subtypes of IBD. We observed a high prevalence of anti-GP2 IgA in Norwegian PSC patients (n=71, 52%) compared to HC (n=1, 1.9%) ( $p<0.001$ , OR=55.7, Table 1). Prevalent anti-GP2 IgA positivity (n=84, 46.7%) was confirmed in an independent German PSC cohort. As depicted in Table 1, the anti-GP2 IgA prevalence in UC without PSC was at the level of HC ( $p=0.9$ ). A significant anti-GP2 IgA occurrence was also detected in both benign and malignant biliary large duct disease controls but not in autoimmune hepatitis (Table 1).

**Table 1.** Clinical and serological characteristics of PSC patients as well as healthy controls and disease controls (UC, CCA, SSC).

	PSC(n=138) Norwegian cohort		PSC(n=180) German cohort		HC (n=52)		UC (n=62)		CCA (n=56)		SSC (n=20)		AIH (n=18)	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
<b>Age at the time of blood sample</b> [years]	41.4	13.5	40.7	13.0	41.4	6.6	41	14.3	64.5	9.4	53.5	10.4	46.0	18.0
<b>Age at diagnosis</b>	34.2	13.3	33.7	13.4	-	-	-	-	63.7	9.4	50.7	14.5	35.2	19.4
<b>Disease duration</b> [years]	4.3	6.2	7.5	6.7	-	-	6	7.6	0.8	1.0	3.0	4.6	10.7	10.1
	n	%	n	%	n	%	n	%	n	%	n	%	n	%
<b>Male gender</b>	107	77.5	141	67.4	40	76.9	48	77.4	30	53.6	16	80.0	8	44.4
<b>Anti-GP2 IgA</b>	71	52.2	84	46.7	1	1.9	1	1.6	20	35.7	11	55.0	1	5.6
<b>Anti-GP2 IgG</b>	2	1.4	2	1.4	0	0	0	0.0	1	1.8	0	0.0	0	0.0
<b>Anti-CUZD1 IgA</b>	15	11	15	10.6	0	0	5	8.1	-	-	-	-	0	0.0
<b>Anti-CUZD1 IgG</b>	14	10.1	6	4.2	1	1.9	3	4.8	-	-	-	-	0	0.0
<b>Inflammatory bowel disease</b>	102	74.5	125	69.4	-	-	62	100	-	-	0	0.0	0	0.0
<b>Ulcerative colitis</b>	81	59.1	99	55	-	-	62	100	-	-	-	-	-	-
<b>Crohn´s disease</b>	11	8.0	18	10	-	-	-	-	-	-	-	-	-	-
<b>Indeterminate colitis</b>	10	7.3	4	2.2	-	-	-	-	-	-	-	-	-	-
<b>Biliary tract cancer (CCA or GBC)</b>	19	13.8	20	11.1	0	0	0	0	56	100	0	0		
<b>Liver transplantation</b>	33	23.9	50	27.8	0	0	0	0	0	0.0	4	20.0	8	44.4

[GP2=glycoprotein 2, CUZD1=CUB and zona pellucid-like domains 1, UC=ulcerative colitis, HC=healthy controls, SD=standard deviation, CCA=cholangiocarcinoma, GBC=gall bladder cancer, SSC=secondary sclerosing cholangitis; AIH=Autoimmune hepatitis].

### **Clinical parameters of anti-GP2 IgA positive PSC patients differed significantly from anti-GP2 IgA negative patients**

Clinical characteristics of the Norwegian and German PSC patients were then analyzed according to their anti-GP2 IgA status (Table 2). Median disease duration at the time of blood collection was numerically longer in anti-GP2 IgA positive patients in both cohorts (Norway: 0.94 vs. 2.78 years, respectively and Germany: 5.33 vs. 7.04 years, respectively), but the differences were only statistically significant in the Norwegian cohorts (Table 2), and a marked prevalence (Norwegian cohort: 46.9% and German cohort: 34.2%, respectively) of anti-GP2 IgA positivity was already present in patients with short disease duration ( $\leq 1$  year).

Age at diagnosis was similar in anti-GP2 IgA positive and negative patients in the Norwegian cohort, while anti-GP2 IgA positive patients were younger in the German cohort (Table 2).

In both cohorts, the prevalence of anti-GP2 IgA was similar irrespective of the presence of IBD or not, 52% vs. 54% in the Norwegians and 49% vs. 42% in the Germans, respectively (Table 2). There were also no significant differences regarding the IBD subtypes observed. In contrast, multiple measures indicated more severe disease in anti-GP2 IgA positive than negative PSC patients (Table 2 and Supplementary Table 1). In particular, increased Mayo risk score (MRS) was observed in anti-GP2 IgA positive compared with negative PSC patients in both the Norwegian and German cohort ( $p < 0.001$ ). Additionally, anti-GP2 IgA titers correlated with disease severity (Suppl. Figures 2+3) as indicated by correlation analysis for anti-GP2 IgA with serum-bilirubin (Spearman's  $r_{(\text{bilirubin})} = 0.493$ ,  $p < 0.001$ ) and the MRS (Spearman's  $r_{(\text{MRS})} = 0.499$ ,  $p < 0.001$ ).

Similar associations between anti-GP2 IgA positivity and biochemical markers of a more pronounced biliary duct inflammation were observed in CCA as well as SSC (Suppl. Table 2+3).

**Table 2.** PSC patients' characteristics according to anti-GP2 IgA seropositivity.

	<b>Norwegian cohort</b>			<b>German cohort</b>		
	Anti-GP2 IgA negative (n=65)	Anti-GP2 IgA positive (n=71)	p:	Anti-GP2 IgA negative (n=96)	Anti-GP2 IgA positive (n=84)	p:
<b>Characteristics:</b>	<b>Median (IQR):</b>	<b>Median (IQR):</b>		<b>Median (IQR):</b>	<b>Median (IQR):</b>	
<b>Age at time of blood samples (years)</b>	40.4 (33.57-49.29)	41.1 (34.87-52.10)	0.341	42.5 (34.04-51.38)	39.0 (29.63-45.46)	<b>0.015</b>
<b>Age at diagnosis (years)</b>	34.7 (26.17-49.34)	34.0 (27.46-45.71)	0.88	34.46(27.17-46.13)	27.54(20.96-35.79)	<b>&lt;0.001</b>
<b>PSC duration at serum extraction (years)</b>	.94 (.00-4.49)	2.78 (.05-9.62)	<b>0.046</b>	5.33 (0.96-11.08)	7.04 (3.33-11.88)	0.170
<b>Mayo risk score</b>	-.35 (-.35-.98)	.80 (-.07-2.20)	<b>0.001</b>	-0.64 (-1.16 - -0.07)	-0.12(-0.63-0.90)	<b>&lt;0.001</b>
<b>Bilirubin [<math>\mu</math>mol/l]</b>	12.4 (8.0-23.9)	29.3 (15.1-81.8)	<b>&lt;0.001</b>	12.83(8.55-22.23)	22.23(13.68-51.30)	<b>&lt;0.001</b>
<b>Male Gender</b>	<b>n (%):</b> 47 (72.3%)	<b>n (%):</b> 58 (81.7%)	0.22	<b>n (%):</b> 66 (68.8%)	<b>n (%):</b> 57 (67.9%)	1.0
<b>Presence of IBD (UC/CD/IC)</b>	48 (75%)	52 (73.2%)	0.85	64 (66.7%)	61 (72.6%)	0.42
No IBD+PSC	16 (25.0%)	19 (26.8%)		32 (33.3%)	23 (27.4%)	
UC +PSC	36 (56.3%)	43 (60.6)		53 (55.2%)	46 (54.8%)	
CD+PSC	6 (9.4%)	5 (7.0%)		7 (7.3%)	11(13.1%)	
IC+PSC	6 (9.4%)	4 (5.6%)		4 (4.2%)	4(4.8%)	
<b>Cholangiocarcinoma</b>	2 (3.1%)	14 (19.7%)	<b>&lt;0.001</b>	4 (4.2%)	11 (13.1%)	<b>0.031</b>
<b>Gall bladder cancer</b>	0 (0%)	3 (4.2%)	<b>&lt;0.001</b>	3 (3.1%)	2 (2.4%)	0.762
<b>Liver transplantation</b>	13 (20.0%)	20 (28.2%)	0.32	18 (18.8%)	32 (38.1%)	<b>0.005</b>

**Table 2.** [IQR=interquartile range, IBD=inflammatory bowel disease, UC=ulcerative colitis, CD=Crohn's disease, IC=indeterminate colitis]. Two Norwegian PSC patients out of 138 were excluded from analysis due to inconclusive results in PAB testing.

### **Association of anti-GP2 IgA with cholangiocarcinoma**

A strong association was observed between anti-GP2 IgA positivity and biliary tract cancer (CCA and GBC) in the Norwegian cohort; 19.7% of the anti-GP2 IgA positive patients developed CCA and 4.2% developed GBC, compared with 3.1% (CCA) and 0% (GBC) of the anti-GP2 IgA negative patients, respectively ( $p < 0.001$  for both comparisons, Table 1). When analysing the German cohort, only the association with CCA was validated; 13.1% of the anti-GP2 IgA positive patients developed CCA compared with 4.2% ( $p = 0.031$ ) (Table 2).

Overall, more than 86% of the PSC-associated cholangiocarcinoma's were anti-GP2 IgA<sup>+</sup> (Table 2) compared to 35.7% anti-GP2 IgA positivity in CCA without PSC ( $p < 0.001$ ).

Notably, multivariate logistic regression analyses demonstrated an independent association of anti-GP2 IgA ( $p = 0.045/p = 0.042$ ) with CCA irrespective of bilirubin, age and PSC duration in both cohorts (Table 3).

Differences in anti-GP2 IgA titers between PSC patients with or without biliary tract cancer were not detected (Norwegian cohort:  $p = 0.55$ ; German cohort:  $p = 0.95$ ).

A meta-analysis across the cohorts yielded an odds ratio of cholangiocarcinoma in anti-GP2 IgA positive of 5.0 in all PSC patients (95% CI=2.0-12.7,  $p < 0.001$ ), with no heterogeneity of the odds ratios observed between the cohorts ( $p = 0.378$ ).

With respect to the correlation between anti-GP2 IgA and markers of disease severity, the association of anti-GP2 IgA and biliary tract cancer in both cohorts was retested under exclusion of all patients with more than just a slight increase in bilirubin ( $> 51.3 \mu\text{mol/l}$ ).

Though the cases of CCA, which were included for analysis, consequently more than halved in both cohorts a meta-analysis still revealed an odds ratio of CCA in anti-GP2 IgA positive of 2.0 in all PSC patients (95% CI=0.7-5.8,  $p = 0.225$ ).

**Table 3.** Multivariate logistic regression analysis for the associations of anti-GP2 IgA with cholangiocarcinoma (CCA).

Association of anti-GP2 IgA with CCA					
	<i>B</i>	<i>p-value</i>	<i>Exp (B)</i>	<i>95%-CI for Exp (B)</i>	
Norwegian cohort					
<b>anti-GP2 (IgA)</b>	-1.967	<b>.045</b>	.140	.020	.960
<b>bilirubin</b>	-.017	<b>.004</b>	.984	.973	.995
<b>Age at diagnosis</b>	-.150	.002	.860	.783	.945
<b>Disease duration</b>	-.043	.545	.958	.833	1.101
German cohort					
<b>anti-GP2 (IgA)</b>	-1.447	<b>.042</b>	.235	.058	.946
<b>Bilirubin</b>	-.009	.053	.991	.982	1.00
<b>Age at diagnosis</b>	-.076	.002	.927	.883	.973
<b>Disease duration</b>	-.095	.045	.909	.828	.998

**Table 3.** The positive association of anti-GP2 IgA with CCA was significant in a multivariate logistic regression model in both cohorts, demonstrating its independency of serum-bilirubin, disease duration and age at diagnosis.

[*B*=regression coefficient; *Exp (B)*=predicted shift in hazard for each unit increase in the variable. *CI*=confidence interval; GP2=glycoprotein 2; CCA=cholangiocarcinoma].

**Anti-GP2 IgA positive PSC patients exhibited poorer cumulative survival rates**

Next, the influence of anti-GP2 IgA on liver transplantation-free survival in PSC was analyzed during a four-year, respectively ten-year, observation period. Anti-GP2 IgA positivity was associated with shorter liver transplantation-free survival in both the Norwegian and German cohort, as analyzed by Kaplan-Meier plot ( $p=0.016$  and  $p=0.018$ , respectively), see Figure 1a/b.

When categorizing the PSC patients according to anti-GP2 IgA titer: negative ( $<1:10$ ), low (titers from 1:10 to 1:99) and high (1:100 or above), the survival was reduced in the groups with high anti-GP2 IgA titer (Figure 1c/d).

Consequently, univariate Cox regressions revealed high anti-GP2 IgA titer as a predictor for poor survival rates in both cohorts (hazard ratio=2.4, (95%-CI: 1.3-4.6),  $p=0.006$  in the Norwegian cohort and hazard ratio=2.6 (95%-CI: 1.37-5.02),  $p=0.004$  in the German cohort).

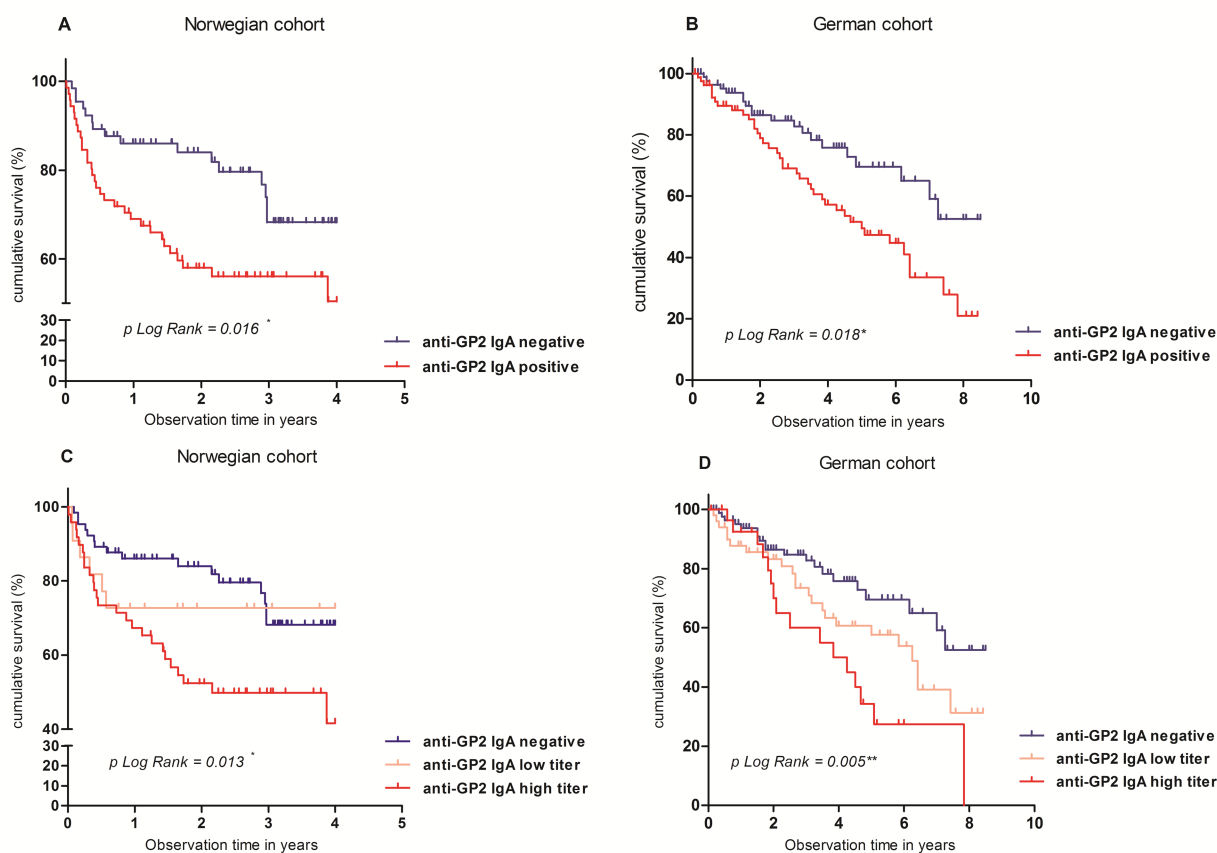
The poor survival in the anti-GP2 IgA positive Norwegian and German patients could in part be explained by an increased frequency of biliary tract cancer. However, in both cohorts were trends towards reduced liver transplantation free survival in anti-GP2 IgA positive individuals also when patients developing biliary tract cancer (CCA or GBC) were removed from the analysis ( $p=0.34$  and  $p=0.055$ , respectively), see Figure 2a/b. When analyzing only patients with death as an endpoint (i.e. removing patients being liver transplanted) there was a similar reduced survival in anti-GP2 IgA positive patients ( $p=0.021$  in the Norwegian cohort,  $p=0.069$  in the German cohort, Suppl. Figure 4).

To test the predictive potential of anti-GP2 IgA on liver transplantation free survival in PSC, a multivariate Cox regression analysis was performed, including anti-GP2 IgA, MRS and disease duration. In this multivariate Cox regression only the MRS remained independently associated with outcome, whereas anti-GP2 IgA was not significant (Norwegian cohort:  $p=0.353$  [anti-GP2 IgA];  $p<0.001$  [MRS];  $p=0.877$  [disease duration]), with comparable results in the German cohort (German cohort:  $p=0.258$  [anti-GP2 IgA];  $p<0.001$  [MRS];  $p=0.339$  [disease duration]).

Further exploration of the data showed that anti-GP2 IgA positivity remained a significant predictor for poor survival ( $p=0.021$ ) in multivariate Cox regression in the German cohort, when MRS was

subdivided into three categories with p-values of <0.001 (low MRS),  $p=0.304$  (intermediate MRS) and <0.001 (high MRS), while this was not the case in the Norwegian cohort, subdividing the MRS into three categories had no effect on the significance of anti-GP2 IgA ( $p=0.94$ ).

**Figure 1.** Kaplan-Meier survival curves for PSC patients, depending on anti-GP2 IgA status or titer during a 4-year, respectively 10-year, observation period.



**(A)** Survival curves significantly differed between anti-GP2 IgA positive and negative Norwegian PSC patients during a 4-year observation period ( $p=0.016$ , Log Rank). In detail: Mean survival of anti-GP2 IgA positive patients was 2.5 years ( $SEM=0.20$ ; [95%-CI=2.1; 2.9]). Mean survival of anti-GP2 IgA negative patients was 3.2 years ( $SEM=0.17$ ; [95%-CI=2.9; 3.6]).

**(B)** Survival curves significantly differed between anti-GP2 IgA positive and negative German PSC patients during a 10-year observation period ( $p=0.039$ , Log Rank). In detail: Mean survival of anti-GP2 IgA positive

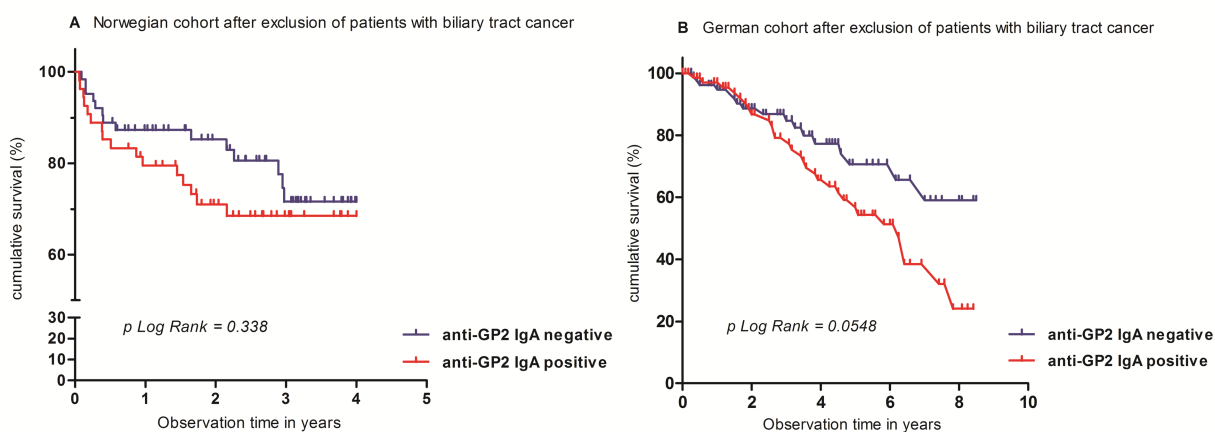


patients was 5.2 years ( $SEM=0.36$ ;  $[95\%-CI=4.5; 5.9]$ ). Mean survival of anti-GP2 IgA negative patients was 6.4 years ( $SEM=0.4$ ;  $[95\%-CI=5.6; 7.2]$ ).

**(C + D)** PSC patients were categorized in three groups: anti-GP2 IgA negative ( $<1:10$ ), low (titers from  $1:10$  to  $1:99$ ) and high ( $1:100$  or above). Survival curves significantly differed ( $p=0.013$ , Log Rank and  $p=0.005$ , Log Rank). Moreover, high anti-GP2 IgA titer was associated with reduced survival in a univariate Cox regression model for both cohorts ( $p=0.006$  and  $p=0.004$ ).

[GP2=glycoprotein 2].

**Figure 2.** Kaplan-Meier survival curves for PSC patients after exclusion of the portion, who develop biliary tract cancer, depending on anti-GP2 IgA status.



**(A)** Survival curves of Norwegian PSC patients did not differ significantly after exclusion of patients, developing biliary tract cancer ( $p=0.338$ , Log Rank).

**(B)** Survival curves of German PSC patients did not differ significantly after exclusion of patients, developing biliary tract cancer ( $p=0.0548$ , Log Rank).

[GP2=glycoprotein 2].

## Discussion

In this first study of anti-GP2 autoantibodies in PSC, anti-GP2 IgA was detected in about half the patients in two independent cohorts. Anti-GP2 IgA was consistently associated with reduced transplantation-free survival and a high frequency of CCA. Moreover, the association of anti-GP2 IgA with CCA in PSC was independent of other CCA risk factors in PSC such as duration of disease, higher age at diagnosis and serum-bilirubin, indicating that anti-GP2 identified patients with a distinct, more severe, disease phenotype.

The only serological marker in PSC with a similar prevalence as anti-GP2 IgA is p-ANCA, which has been observed in the range of 26 to 94% of PSC patients[13]. Although some studies have reported associations between p-ANCA and IBD status, biliary tract complications and disease stage[25-28], no consistent clinical correlations have been observed with ANCA across the more than 20 studies performed[13]. In the present study, anti-GP2 IgA not only correlated with biochemical markers of disease activity but also was associated with reduced transplantation-free survival based on increased prevalence of CCA in anti-GP2 IgA positives, irrespective of the CCA risk factors mentioned above[1-3,5] and irrespective of the Mayo risk score. Notably, anti-GP2 IgA seems to be present early in the disease, as shown by a high frequency also in patients with short duration ( $\leq 1$  year) of PSC (46.9%).

CCA is a major challenge in PSC with limited diagnostic and therapeutic options and although more than half of the cases occur in the first year after diagnosis of PSC [9] the subsequent risk increases with disease duration[2]. Therefore better opportunities for an early diagnosis and new prognostic tools for risk stratification are extremely important. Still, CCA in PSC is often diagnosed in advanced stages[1, 8].

In this regard, the optimal time for liver transplantation is intensively discussed[29]. Anti-GP2 IgA may therefore be additionally useful as a tool to identify patients with increased risk for biliary cancer warranting intensified follow-up. Although anti-GP2 IgA was not an independent predictor of

transplantation-free survival when including MRS in multivariate Cox regression analysis of the Norwegian cohort, anti-GP2 IgA was independently associated with CCA in both cohorts.

Hence, further prospective studies should re-evaluate anti-GP2 IgA as an independent predictor for CCA and survival in PSC. Such studies may also clarify, whether anti-GP2 IgA is not only associated with CCA but also with the observed poor non-cancer-related survival in PSC.

Anti-GP2 cannot be considered specific for PSC since anti-GP2 autoantibodies also occur in other diseases such as Crohn's disease[17]. Notably, the presence of anti-GP2 of the IgA type in about 50% of the PSC patients is higher than that observed in Crohn's disease (3.4% - 24%), in which IgG subclasses regularly predominate, and a lot higher than in UC and healthy controls, where it is virtually absent (0% - 2.9%)[14-16, 30].

Regarding IBD in PSC, this is most often classified as UC, although certain clinical characteristics (including right-sided dominance, backwash ileitis, rectal sparing) suggest that UC-related PSC could represent a third IBD type[31, 32]. This may explain the similar frequency of anti-GP2 IgA in PSC patients with UC and CD observed in both the patient panels in the present study. The observation that anti-GP2 IgA also had a similar prevalence in patients without concomitant IBD suggests that anti-GP2 is primarily related to the manifestation of the disease process in the bile ducts and not the intestine. Indeed, we also observed anti-GP2 IgA positivity in other large duct diseases such as CCA without PSC and SSC but not in AIH, and in particular in association with elevated liver biochemistry, suggesting that it may be a marker of severe cholestatic liver disease. Consequently, reservation is justified whether anti-GP2 IgA may be implicated in the aetiopathogenesis of PSC, although it is so far unknown to what degree sclerosing cholangitis is a disease specific phenomenon or a final common pathway of different aetiologies.

Nevertheless, the consistent identification of a distinct severe phenotype in PSC based on an autoantibody, which is even present in half of patients with short duration PSC and which is, at least in IBD, known to be very stable over the disease course[14,15] points up towards a novel, urgently needed, diagnostic potential in PSC.

Furthermore, the independent association of anti-GP2 IgA with CCA and the consistent association with poor survival together with the observation that GP2 protein levels are reduced in the bile of PSC patients with CCA[20] suggests a pathophysiological function of these anti-GP2 antibodies with biliary GP2 function. Substantial evidence demonstrates that the antigen GP2 exerts bacteria-binding properties in the intestinal lumen[33, 34] and it was suggested that GP2 is centrally involved in the development of antigen-specific mucosal immune responses against gut bacteria[33]. Considering that key elements of PSC etiology include infectious triggers[11] or toxic/immunological effects of bile acids[35], the autoantibody anti-GP2 could reflect a shared autoimmune mechanism of PSC and IBD.

The major strength of the present study is the inclusion of two large and well-characterized PSC cohorts from Norway and Germany to validate a preliminary observation in a small number of UC patients. It should be kept in mind that the cohorts were recruited from tertiary care centers, although PSC patients at all stages were included. The retrospective character of the study restricts the predictive significance of the survival analysis depending on anti-GP2 IgA. Therefore, further prospective studies with distinct clinical endpoints are warranted to decipher the exact predictive potential of anti-GP2 IgA in PSC.

In conclusion, anti-GP2 IgA identifies PSC patients with severe phenotypes independent from concomitant IBD. The association with poor survival in two independent European cohorts due to CCA highlights promising characteristics of anti-GP2 IgA as a novel prognostic tool in PSC patients' risk stratification. Further studies are warranted to accurately define the clinical utility of anti-GP2 IgA as a predictive biomarker in PSC. Moreover, the association of anti-GP2 IgA with PSC and PSC-related cancer opens new avenues for further studies of GP2 function in the biliary tree.

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**Conflict of interest** L. Komorowski, S. Mindorf, B. Teegen and T. Nitzsche are employees of Euroimmun AG, Lübeck, Germany. For all other authors no conflict of interest was declared.

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