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SHORT REPORT

Serological Markers of Intestinal Barrier Function and Inflammation as Potential Predictors of Recurrent Primary Sclerosing Cholangitis

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Abstract: The impairment of intestinal barrier function is implicated in primary sclerosing cholangitis, but the clinical evidence is scarce. Therefore, we performed a cross-sectional study to evaluate serological markers of inflammation and intestinal permeability (Reg3a, iFABP, Zonulin, Calprotectin) in patients after liver transplantation (LT) for PSC. The cohort included 26 subjects with PSC recurrence (rPSC), 87 subjects without PSC recurrence (non-rPSC), and a unique control group consisting of post-LT patients (n = 113) transplanted due to alcohol cirrhosis. Generalized Linear Models were calculated to assess the association between serological markers of intestinal barrier function or inflammation (IP_Models) and PSC diagnosis per se (IP_Model_1), non-rPSC (IP_Model_2) or rPSC incidence (IP_Model_3) and compared with models (ST_Models) based on validated PSC markers (ALP, GGT, bilirubin). The increased probability of PSC occurrence (IP_Model_1, $p < 0.001$, AIC = 182) was associated with higher serum Reg3a concentration, while a negative association was found for iFABP, BMI, and age. The probability of non-recurrence (IP_Model_2, $p < 0.001$, AIC = 167) was associated with lower Reg3a concentration, older age, and BMI. The performance of IP_Models_1,2 and ST_models_1,2 was comparable. rPSC prediction was less precise by both models (IP_Model_3 $p = 0.063$, AIC = 92; ST_Model_3 $p < 0.001$, AIC = 108). rPSC incidence was positively associated with fecal calprotectin and serum zonulin concentrations, while it was independent of Reg3a, iFABP, age or BMI. In conclusion, this pilot study suggests that impaired intestinal permeability is associated with the pathophysiology of rPSC. Our data could serve as a basis for testing in a larger independent validation cohort and, if confirmed, help to explain the mechanisms underlying the pathophysiology of PSC and the recurrence of this disease after transplantation.

Keywords: zonulin, iFABP, Reg3a, recurrence of primary sclerosing cholangitis, liver transplantation

Introduction

Primary sclerosing cholangitis (PSC) is an idiopathic, progressive cholestatic liver disease, associated with inflammatory bowel disease (IBD).[1] Despite conservative treatment, the disease usually progresses, and ultimately, a liver transplantation (LT) is the only curative solution for more than 50% of patients.[2] Nevertheless, recurrence of PSC (rPSC) in liver graft is reported in 17–25% of patients, which significantly worsens outcomes after liver transplantation,[3] and the pathogenesis of recurrent disease is still not entirely understood.

The gold standard for assessing PSC severity, progression, and complications is serum concentrations of liver enzymes, alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and total bilirubin, in combination with magnetic resonance cholangiopancreatography (MRCP) and histology.[1]

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Nevertheless, there are several lines of evidence that the gut–liver axis, altered immune function, intestinal barrier dysfunction, and microbial dysbiosis have a significant impact on the pathogenesis of PSC.[1] The different microbiota composition in patients with PSC compared with controls was reported.[4,5] In regard to rPSC, active colonic inflammation has been identified as one of the risk factors for rPSC,[6] as it contributes to the impairment of intestinal barrier function and promotes translocation of bacterial endotoxins, metabolites, and other bacterial products via the portal vein into the liver,[7] increasing inflammation and fibrogenesis. Several studies have proven an association of serologic markers of intestinal barrier dysfunction with worse outcomes of PSC,[8,9] but no studies are reporting intestinal permeability markers in the post-transplant population.

Our study focused on three serologic markers (zonulin, Reg3a, and iFABP) that are considered good markers of impaired intestinal barrier function, each reflecting different aspects of gut epithelial integrity and permeability. The regenerating family member 3 alfa (Reg3a) is a multifunctional protein with antimicrobial and antiapoptotic function, primarily secreted by Paneth cells in the intestine and in the pancreas.[10] Circulating levels of REG3α increase in response to gut epithelial damage and correlate with disease severity in inflammatory gut conditions such as ulcerative colitis. REG3α can translocate into the blood through increased intestinal permeability, making it a soluble marker of gut epithelial integrity and damage.[11] The intestinal fatty acid binding protein (iFABP) is found in enterocytes, and contributes to fatty acid transport and metabolism.[12] Elevated serum iFABP levels reflect early epithelial injury and correlate with increased intestinal permeability determined by functional measures of permeability (eg, lactulose/mannitol ratio).[13,14] Zonulin is the only known physiological regulator of intercellular tight junctions and has been shown to correlate with intestinal permeability as well as intestinal inflammation in IBD and could thus be used as a measurement of impaired gut barrier function.[15,16] Fecal calprotectin is a neutrophil-derived protein that indicates the migration of neutrophils to the intestinal mucosa, which occurs during intestinal inflammation. It helps differentiate inflammatory bowel disease from non-inflammatory conditions like irritable bowel syndrome.

The presented study aimed to investigate the association between impaired intestinal permeability and the pathophysiology of rPSC in patients following liver transplantation. To achieve this, we compared serological markers of intestinal permeability in post-LT PSC patients with those in a suitable control group consisting of patients who underwent transplantation for alcoholic liver disease (ALD).

Methods

Patients

The patients who underwent orthotopic liver transplantation (LT) for PSC at the Institute for Clinical and Experimental Medicine in Prague between 1995 and 2021 were included in the study. Exclusion criteria for PSC patients were history of colectomy or other intestinal surgery, active malignancy, active alcohol abuse, BMI >35, pregnancy, or lactation. The control group consisted of patients who underwent LT for ALD with or without hepatocellular carcinoma (HCC). The exclusion criteria for the control group were BMI >35, age >65, active alcohol abuse, active malignancy, pregnancy or lactation, history of HBV or HCV infection, or detectable viremia at the time of sampling, history of colectomy or IBD.

Study Design

We have performed a cross-sectional single-center study. PSC patients were divided based on the presence of rPSC. Demographic data, including age, sex, IBD status, rPSC status, type of immunosuppression, IBD treatment, and retransplantation history, were collected. Based on current guidelines, all PSC patients underwent coloscopy with biopsies. The endoscopic Mayo score (eMayo) was evaluated, and all the biopsies were evaluated for Nancy histological index (NHI). Control group patients underwent coloscopy with no biopsies. Therefore, the Mayo and Nancy indices, as well as fecal calprotectin, were not evaluated in this group. Blood and stool samples were collected and stored at −80°C until analysis. rPSC was diagnosed based on the Mayo criteria proposed by Graziadei et al,[17] and patients have undergone MRCP to evaluate their rPSC status. The authors are accountable for all aspects of the work and ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted following the Declaration of Helsinki (as revised in 2013). The study was approved by the

Institutional Ethics committee of the Institute for Clinical and Experimental Medicine and Thomayer University Hospital, Prague, Czech Republic (n. 31869/20, 10.6.2020). Written informed consent was obtained from each study participant. All organs were donated voluntarily with written informed consent and were conducted in accordance with the Declaration of Istanbul.

Biochemical Analyses

Serum concentrations of intestinal permeability markers were assessed using commercially available kits (Human I-FABP ELISA Kit, MyBiosource, USA; IDK Zonulin Serum ELISA, Immunodiagnostik ®, Germany; Human Reg3a ELISA, ThermoFisher Scientific, USA), following the manufacturer's instructions.

Statistics

Associations between serological markers and PSC incidence were determined with a multivariable binary logistic regression model. The data was divided into a train/test dataset to evaluate the accuracy of the multivariate models from their confusion matrix. Baseline characteristics between PSC and control were compared using Wilcoxon rank test. The statistical analyses were performed using R software packages (version 4.2.2) and in-house scripts. Power analysis was performed based on the formula described in Hsieh et al.[18]

Results and Discussion

Characteristics of the Group

The clinical characteristics of the study participants are listed in Table 1. A total of 182 liver transplant recipients were enrolled in the study, 113 patients for PSC and 69 patients for ALD with or without HCC. rPSC was diagnosed in 23% of patients (n = 26) and PSC-IBD in 89% of patients (n = 100) in the PSC group. Most of the IBD patients were diagnosed

Table 1 Cohort Characteristics. The Values are Given as Median (1st, 3rd Quartile) When Appropriate. For Continuous Variables, the Statistical Significance of the Median Difference Was Calculated by the Kruskal–Wallis Test, and if Significant, Pairwise Post-Hoc Mann–Whitney *U*-Test Was Performed. For Categorical Variables, the Fisher Exact Test Was Used

	Control	Non-rPSC	rPSC	Pairwise Test Statistics		
	(A)	(B)	(C)	A vs B	A vs C	B vs C
Sex (F/M)	26/43	26/61	4/22	n.s.	n.s.	n.s.
Age (years)	61 (56; 65)	50 (44; 62.5)	50 (43; 60)	<0.001	0.002	0.69
Post_LTx (month)	58 (6; 305)	86 (47; 176)	78 (49; 151)	n.s.	n.s.	n.s.
BMI ($kg.m^{-2}$)	27.5 (24.6; 30.9)	24.8 (22.5; 27.3)	23.7 (21.0; 25.4)	<0.001	<0.001	0.051
s_ALT (μkat/mL)	0.40 (0.33; 0.59)	0.41 (0.33; 0.55)	0.54 (0.36; 0.96)	n.s.	n.s.	n.s.
s_AST (μkat/mL)	0.45 (0.36; 0.70)	0.46 (0.38; 0.59)	0.53 (0.42; 1.02)	n.s.	n.s.	n.s.
s_ALP (μkat/mL)	1.55 (1.18; 2.23)	1.38 (1.12; 1.87)	2.02 (1.91; 4.06)	0.142	0.001	<0.001
s_GGT (μkat/mL)	0.52 (0.32; 1.05)	0.37 (0.26; 0.66)	1.36 (0.76; 2.80)	0.017	<0.001	<0.001
s_bilirubin (μmol/mL)	14.8 (10.8; 20.5)	18.3 (13.7; 26.1)	24.1 (16.3; 34.0)	0.009	0.006	0.196
IBD present/absent (n)	0/69	76/11	24/2	<0.001	<0.001	n.s.
CD/UC (n)	N/A	2/74	1/23			
Nancy 0/1/2/3/4 (n)	N/A	31/8/27/10/11	10/2/6/5/3	n.s.	n.s.	n.s.

(*Continued*)

Table 1 (Continued).

	Control (A)	Non-rPSC (B)	rPSC (C)	Pairwise Test Statistics		
				A vs B	A vs C	B vs C
eMayo 0/1/2/3 (n)	N/A	45/30/11/1	10/8/8/0	n.s.	n.s.	n.s.
DM present/absent (n)	28/41	16/71	6/20	0.004	0.178	0.805
TAC/CYA (n)	68/1	80/7	25/1			

Abbreviations: BMI, Body mass index; CD, Crohn disease; CYA, cyclosporine; DM, diabetes mellitus; eMayo, clinical assessment of disease activity (4-level classification); F, Female; IBD, inflammatory bowel disease; LTx, Liver Transplantation; M, Male; Nancy index, histological assessment of intestinal inflammation (5-level classification); s_ALP, serum alkaline phosphatase; s_ALT, serum alanine transaminase; s_AST, serum aspartate transaminase; s_GGT, serum gamma-glutamyl transferase; UC, ulcerative colitis; TAC, tacrolimus.

with ulcerative colitis (UC, n = 97) while only three suffered from Crohn's disease. At the time of sample collection, the majority of the UC patients were in endoscopic remission (eMayo 0, n = 45) or had mild disease activity (eMayo 1, n = 30), fewer had moderate (eMayo 2, n = 11) and severe disease activity (eMayo 3, n = 1). In CD patients, one patient had endoscopic remission, one patient had mild, and one severe disease activity (Table 1). The median age was lower in both PSC groups, reflecting the earlier onset of this disease compared to the manifestation of alcohol cirrhosis. The gender distribution was approximately 1:2 (F/M) in all groups, reflecting the male predominance in PSC. Nine patients in the rPSC group and one patient in the control group had undergone liver retransplantation. We did not observe any elevation of the markers of hepatocellular injury (ALT, AST) in any groups. In contrast, the markers of cholestasis (ALP, GGT, bilirubin) were significantly higher in both PSC groups than in controls. The former two parameters also distinguished between the non-rPSC and rPSC groups. The standard immunosuppression regimen was tacrolimus-based. However, there were some exceptions as displayed in Table 1.

Serological Markers of Intestinal Permeability and Inflammation as Predictors of PSC and rPSC

As mentioned in the introduction, all three molecules (Reg3a, iFABP, zonulin) are being investigated as potential markers of intestinal permeability, but they may reflect different aspects of intestinal barrier dysfunction. Therefore, their combined use may provide more comprehensive information. We built a multivariable binary logistic regression models for the prediction of PSC diagnosis independent of recurrence (IP_Model_1), for the prediction of non-rPSC (IP_Model_2), and for the prediction of rPSC (IP_Model_3) using all three permeability markers as predictors. We further included BMI and age as covariates, as the relationship between these variables and intestinal permeability markers has been described.[19,20] The intestinal inflammatory marker calprotectin, a known risk factor for PSC,[6] was only included among the predictors in Model 3, as it was unavailable in the controls. Model significance was assessed using a log-likelihood test, comparing the full model to a null model that included only the covariates, and the Akaike Information Criterion (AIC). The results of the models are shown in Table 2.

The increased probability of PSC occurrence estimated by IP_Model_1 was associated with higher Reg3a serum concentration (OR = 1.01, 95% CI: 1.00–1.02, p = 0.031), while a negative relationship was found for iFABP (OR = 0.998, 95% CI: 0.998–0.9997, p = 0.009), BMI (OR = 0.85, 95% CI: 0.77–0.93, p < 0.001) and age (OR = 0.96, 95% CI: 0.92–1.00, p = 0.035). The effect of serum levels of zonulin was not significant (OR = 0.97, 95% CI: 0.94–1.01, p = 0.123).

In the IP_Model_2, prediction of PSC without recurrence (non-rPSC), the probability of non-recurrence status was associated with lower Reg3a serum concentration (OR = 0.99, 95% CI: 0.99–1.00, p = 0.039), higher age (OR = 1.05, 95% CI: 1.00–1.09, p = 0.036), and higher BMI (OR = 1.15, 95% CI: 1.04–1.27, p = 0.005). All these conditions may reflect a more beneficial health status of non-rPSC subjects. BMI in this group fell within the normal range (median 24.8 kg m-2), while lower Reg3a concentration indicates less inflammatory status.

For IP_Model_3, in contrast to previous models, BMI, age, Reg3A, and iFABP were not significantly associated with rPSC, while both higher serum concentrations of zonulin (OR = 1.06, 95% CI: 1.00–1.14, p = 0.050) and calprotectin

Table 2 GLM Linear Models Based on Intestinal Permeability and Inflammation Markers

Dependent Variable			IP_Model_1 all_PSC	IP_Model_2 non_rPSC	IP_Model_3 rPSC
Variable predictors	BMI	β_{BMI}	−0.877	0.163	−0.139
		p-value$_{\beta}$	< 0.001	< 0.001	n.s.
		OR_{BMI}	0.845	1.147	0.87
	Age	β_{Age}	−0.04	0.045	0.002
		p-value$_{\beta}$	0.035	0.035	n.s.
		OR_{Age}	0.961	1.046	1.002
	Zonulin	$\beta_{zonulin}$	−0.027	0.03	0.063
		p-value$_{\beta}$	n.s.	n.s.	0.050
		$OR_{zonulin}$	0.972	1.029	1.064
	Reg3a	β_{Reg3a}	0.008	−0.007	−0.002
		p-value$_{\beta}$	0.031	0.038	n.s.
		OR_{Reg3a}	1.007	0.993	0.997
	iFABP	β_{iFABP}	−0.001	0.001	0.0002
		p-value$_{\beta}$	0.009	n.s.	n.s.
		OR_{iFABP}	0.998	1.001	1
	Calprotectin	β_{calp}	N/A	N/A	0.001
		p-value$_{\beta}$	N/A	N/A	< 0.01
		OR_{calp}	N/A	N/A	1.001
	Model significance		<0.001	<0.001	0.063
	Log Likelihood		−85.24	−77.48	−39.07
	AIC		182.48	166.964	92.134

Notes: *(IP_Models)*: Model_1 – predictors for PSC; Model_2 – predictors for PSC after LT without rPSC; Model_3 predictors for rPSC.

Abbreviations: AIC, Akaike Information Criterion; BMI, Body mass index; iFABP, intestinal fatty acid binding protein; OR, odds ratio; N/A, not applicable; Reg3a, regenerating family member 3 alpha.

(OR = 1.001, 95% CI: 1.000–1.001, p = 0.033) increased the probability of PSC recurrence. In contrast to the non-rPSC subjects, where we found a significant correlation between BMI and serum zonulin (r = 0.23, p = 0.003), no relationship between these two variables was observed in the rPSC subgroup. The association between BMI and zonulin concentrations in serum was described in various pathological conditions, like obesity and obesity-related insulin resistance[20] or IBD.[19] Therefore, the independence of BMI and zonulin as predictors in this model suggests that rPSC differs from the non-rPSC in terms of intestinal permeability parameters. As shown previously, LTx does not restore gut microbiota composition[5] and PSC patients often retain gut dysbiosis, characterized by reduced microbial diversity and increased abundance of pathogenic bacteria, even in a recurrence-free state. The altered microbiota composition may prevent the full restoration of intestinal barrier function and contribute to intestinal inflammation.[21–23]

Validated Serological Markers as Predictors of PSC and rPSC

Cholestasis, a typical pathophysiology related to PSC, is associated with elevated serum concentrations of ALP, GGT, and bilirubin. Therefore, we built three multivariable binary logistic regression models to explore the association of these markers with the diagnosis of PSC (ST_Models) (Table 3). ST_Model_1, predicting PSC diagnosis irrespective of recurrence, showed that higher ALP levels were associated with increased odds of PSC (OR = 1.47, 95% CI: 0.99–2.36), although this association was only marginally significant (p = 0.081). GGT levels were significantly inversely associated with PSC (OR = 0.79, 95% CI: 0.61–0.96, p = 0.038), and higher bilirubin was positively associated with PSC

Table 3 GLM Linear Models Based on Validated Serological PSC Markers

Dependent Variable			ST_Model_1 all_PSC	ST_Model_2 non_rPSC	ST_Model_3 rPSC
Variable predictors	ALP	β_{ALP}	0.383	0.043	0.763
		p-value$_{\beta}$	n.s.	n.s.	0.044
		OR_{ALP}	1.47	1.04	2.14
	GGT	β_{GGT}	−0.237	0.387	0.197
		p-value$_{\beta}$	0.038	0.033	n.s.
		OR_{GGT}	0.79	1.47	1.22
	BMI	β_{BMI}	−0.136	0.095	−0.153
		p-value$_{\beta}$	0.001	0.032	0.040
		OR_{BMI}	0.87	1.1	0.86
	Bilirubin	$\beta_{bilirubin}$	0.048	−0.050	−0.023
		p-value$_{\beta}$	0.014	0.011	n.s.
		$OR_{bilirubin}$	1.05	0.95	0.98
	Age	β_{Age}	−0.059	0.06	0.001
		p-value$_{\beta}$	< 0.001	0.002	n.s.
		OR_{Age}	0.94	1.06	1
	Model significance		0.003	0.005	<0.001
	Log Likelihood		−95.57	−85.98	−48.21
	AIC		203	183	108

Notes: *(ST_Models)*: Model_1 – predictors for PSC; Model_2 – predictors for PSC after LT without rPSC; Model_3 predictors for rPSC.

Abbreviations: AIC, Akaike Information Criterion; ALP, Alkaline Phosphatase; BMI, Body mass index; GGT, Gamma Glutamyl Transferase; OR, odds ratio.

(OR = 1.05, 95% CI: 1.01–1.09, p = 0.014). Both lower BMI (OR = 0.87, 95% CI: 0.80–0.95, p = 0.001) and younger age (OR = 0.94, 95% CI: 0.91–0.97, p < 0.001) were significantly associated with PSC diagnosis.

ST_Model_2, predicting of PSC without recurrence, revealed that GGT (OR = 1.47, 95% CI: 1.09–2.18, p = 0.033), BMI (OR = 1.10, 95% CI: 1.01–1.20, p = 0.032), and age (OR = 1.06, 95% CI: 1.02–1.10, p = 0.002) were significantly positively associated with non-rPSC diagnosis, whereas bilirubin was inversely associated (OR = 0.95, 95% CI: 0.91–0.99, p = 0.011). ALP was not significantly associated with non-rPSC (OR = 1.04, 95% CI: 0.59–1.85, p = 0.881).

In contrast, ST_Model_3, focused exclusively on rPSC patients, showed that ALP was strongly associated with rPSC (OR = 2.15, 95% CI: 1.04–4.69, p = 0.044), while GGT and bilirubin were not statistically significant. Lower BMI remained significantly associated with rPSC (OR = 0.86, 95% CI: 0.73–0.98, p = 0.040). Age was not associated with rPSC (OR = 1.00, 95% CI: 0.96–1.04, p = 0.956).

Comparison of Predictive Models Based on Validated Serological Markers and Intestinal Permeability Measures

Based on AIC or Log Likelihood criteria of model validity, the performance of each of the three models is comparable when using validated serum markers (ALP, GGT, bilirubin) or intestinal permeability measures (zonulin, Reg3a, iFABP), though the outcomes obtained when using intestinal permeability measures were lower. The best performance was reached for the prediction of PSC per se (ST_Model_1 AIC = 203, IP_Model_1 AIC = 182), followed by models predicting non-rPSC (ST_Model_2 AIC = 183, IP_Model_2 AIC = 167). rPSC prediction was less precise for both datasets (ST_Model_3 AIC = 108, IP_Model_3 AIC = 92).

Limitations of the Study

We are aware of the fact that the observed effects are rather subtle. The size of the cohort, especially the rPSC group, was rather small, which is the limitation given by the rare nature of the disease and the rate of the recurrence.

Conclusion

Our data suggest different intestinal permeability marker signatures associated with uncomplicated and recurrent PSC. These data could serve as a basis for testing on a larger independent validation cohort and, if confirmed, could contribute to the explanation of the mechanisms underlying the PSC pathophysiology as well as post transplantation recurrence of this disease.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

All authors declare no conflicts of interest in this work.

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