

## ORIGINAL ARTICLE

# Early and accurate detection of cholangiocarcinoma in patients with primary sclerosing cholangitis by methylation markers in bile

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## Abstract

**Background and Aims:** Primary sclerosing cholangitis (PSC) is associated with increased risk of cholangiocarcinoma (CCA). Early and accurate CCA detection represents an unmet clinical need as the majority of patients with PSC are diagnosed at an advanced stage of malignancy. In the present study, we aimed at establishing robust DNA methylation biomarkers in bile for early and accurate diagnosis of CCA in PSC.

**Approach and Results:** Droplet digital PCR (ddPCR) was used to analyze 344 bile samples from 273 patients with sporadic and PSC-associated CCA, PSC, and other nonmalignant liver diseases for promoter methylation of cysteine dioxygenase type 1, cannabinoid receptor interacting protein 1, septin 9, and vimentin. Receiver operating characteristic (ROC) curve analyses revealed high AUCs for all four markers (0.77–0.87) for CCA detection among patients with PSC. Including only samples from patients with PSC diagnosed with CCA  $\leq$  12 months following bile collection increased the accuracy for cancer detection, with a combined sensitivity of 100% (28/28) and a specificity of 90% (20/203). The specificity increased to 93% when only including patients with PSC with longtime follow-up (> 36 months) as controls, and remained high (83%) when only including patients with PSC and dysplasia as controls ( $n = 23$ ). Importantly, the bile samples from the CCA-PSC

**Abbreviations:** CA 19-9, carbohydrate antigen 19-9; CCA, cholangiocarcinoma; *CDO1*, cysteine dioxygenase type 1; *CNRIP1*, cannabinoid receptor interacting protein 1; dCCA, extrahepatic CCA; ddPCR, droplet digital PCR; ERCP, endoscopic retrograde cholangiopancreatography; FISH, fluorescence in situ hybridization; iCCA, intrahepatic CCA; NM, nonmalignant; pCCA, perihilar CCA; PSC, primary sclerosing cholangitis; ROC, receiver operating characteristic; *SEPT9*, septin 9; *VIM*, vimentin.

Hege Marie Vedeld and Marit Mæhle Grimsrud shared co-first authorship.

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≤ 12 patients, all positive for the biomarkers, included both early-stage and late-stage CCA, different tumor growth patterns, anatomical locations, and carbohydrate antigen 19-9 levels.

**Conclusions:** Using highly sensitive ddPCR to analyze robust epigenetic biomarkers, CCA in PSC was accurately detected in bile, irrespective of clinical and molecular features, up to 12 months before CCA diagnosis. The findings suggest a potential for these biomarkers to complement current detection and screening methods for CCA in patients with PSC.

## INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease, characterized by progressive inflammation and fibrosis of the biliary tree.<sup>[1–3]</sup> In the absence of any effective medical treatment, the majority of patients with PSC develop end-stage liver disease, with liver transplantation representing the only curative option.<sup>[1]</sup> Although 5-year survival after liver transplantation is good (exceeds 80%), development of cholangiocarcinoma (CCA) prior to transplantation represents a feared complication of PSC, with a major negative impact on life expectancy.<sup>[3,4]</sup>

Patients with PSC have a 160-fold to 600-fold increased lifetime risk of developing CCA compared to the general population, amounting to a 20% reported lifetime incidence of CCA.<sup>[4–7]</sup> CCA in PSC is commonly diagnosed at a young age (40–50 years), in contrast to sporadic CCA that often develops at a more advanced age (70–80 years).<sup>[5]</sup> Detecting CCA in PSC is challenging, partly due to nonspecific presenting features and overlapping findings in malignant and benign disease progression in PSC. Even with combined use of multiple detection modalities, including tumor serum marker carbohydrate antigen 19-9 (CA 19-9), imaging, biliary brushing for cytological and fluorescence in situ hybridization (FISH) evaluation, and intraductal biopsies, the diagnostic accuracy for CCA in PSC is low.<sup>[8–10]</sup> Consequently, CCA is commonly detected at an advanced stage. Owing to late diagnosis, CCA carries a dismal prognosis among patients with PSC. The median survival is < 12 months in patients with unresectable disease, and CCA currently represents the most frequent cause of PSC-associated deaths.<sup>[5]</sup> If CCA is detected at an early stage, curative surgical resection or liver transplantation is possible, with reported 5-year survival rates up to 35% and 74%, respectively.<sup>[11,12]</sup> As earlier tumor detection may significantly improve survival among patients with PSC and CCA, establishment of accurate detection methods for early-stage CCA, which can be implemented in surveillance algorithms, represents a clear unmet need.

Aberrant DNA methylation alterations are valuable as cancer biomarkers, including early detection markers.

They are highly prevalent in most cancer types, may be found in precancerous lesions or early-stage cancers, and may further be detected in various body fluids that can be obtained by noninvasive or minimally invasive procedures.<sup>[13]</sup> In patients with CCA, aberrant DNA methylation has been observed in both blood and bile, emphasizing the potential of such markers for early detection of CCA.<sup>[13,14]</sup> We have previously identified a DNA methylation biomarker panel suitable for detecting CCA, with high sensitivity (85%) and specificity (98%), using biliary brushes.<sup>[15]</sup> In the current study we aimed at validating these markers in small volumes of bile for early and accurate detection of CCA in patients with PSC, using droplet digital PCR (ddPCR).

## MATERIALS AND METHODS

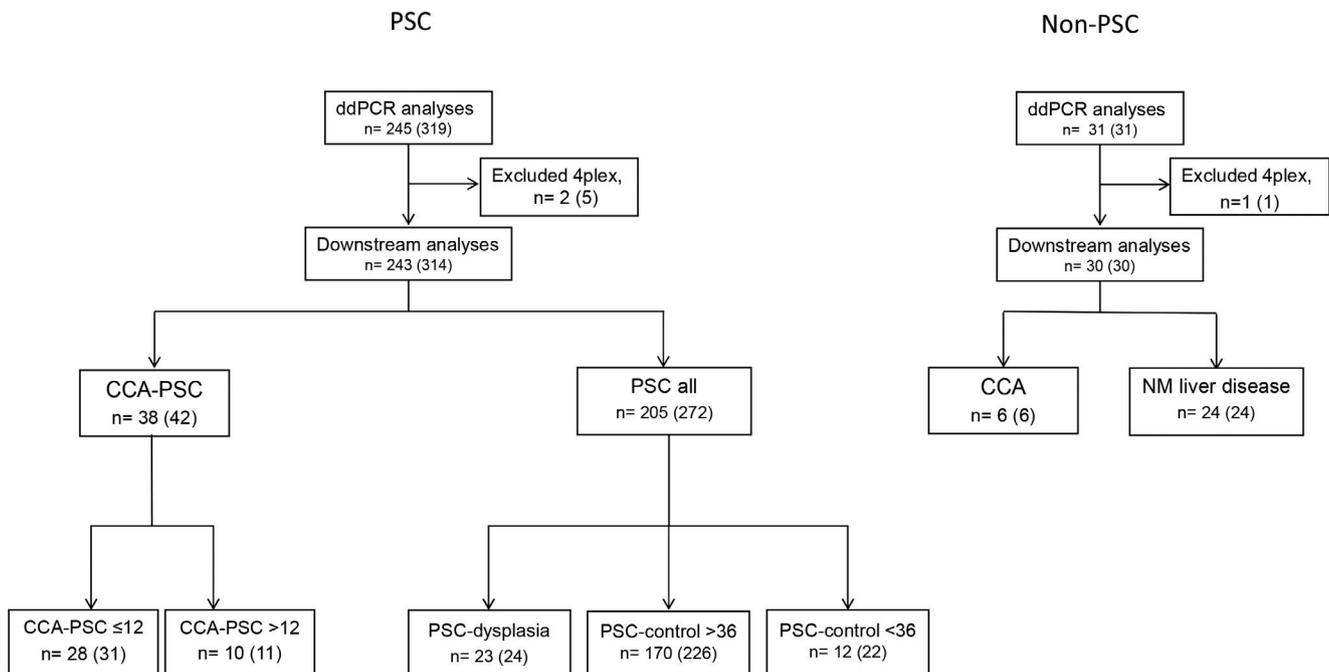
### Patient samples and clinical data

A total of 344 bile samples from 273 patients diagnosed with CCA (with or without underlying PSC), PSC with biliary dysplasia, nonmalignant (NM) PSC, and other benign liver diseases were included in the study. Patients were recruited at Oslo University Hospital, Rikshospitalet (Oslo, Norway;  $n = 219$ ), Karolinska University Hospital (Stockholm, Sweden;  $n = 12$ ), and Helsinki University Hospital (Helsinki, Finland;  $n = 42$ ) between 2008 and 2019. Diagnosis of PSC was based on typical findings on magnetic resonance cholangiography and/or endoscopic retrograde cholangiopancreatography (ERCP) according to established criteria.<sup>[3,16]</sup> CCA diagnosis was established by histopathological analysis of tissue samples or through combined clinical, biochemical, radiological, and cytological assessments.<sup>[3,17]</sup> Diagnosis of low-grade or high-grade biliary dysplasia was based on evaluation of biliary brush specimens using standard cytological criteria or, in patients undergoing liver transplantation, by histopathological examination of the explanted liver.<sup>[18,19]</sup> In addition, all obtained brush cytologies were classified into the following categories: 0, acellular; 1, normal; 2, atypia; 3, suspicious of carcinoma; 4, diagnostic of carcinoma (Table S1). Patients with CCA receiving

chemotherapy at the time of bile sampling were not included in the study.

Samples were split based on main disease category, including (1) patients with underlying PSC and (2) patients with non-PSC-related diseases (Figure 1). The PSC group was divided into two main groups: (1) CCA-PSC ( $n = 38$  [42 samples], including patients with CCA and concomitant PSC) and (2) PSC all ( $n = 205$  [272 samples], including benign PSC without CCA). The CCA-PSC group was further divided into subgroups based on time from bile sampling to a confirmed CCA diagnosis using standard diagnostic criteria, including (1) CCA-PSC  $\leq 12$  ( $n = 28$  [31 samples],  $\leq 12$  months from bile sampling to a confirmed CCA diagnosis) and (2) CCA-PSC  $> 12$  ( $n = 10$  [11 samples],  $> 12$  months from bile sampling to CCA diagnosis). We chose a threshold of 12 months from bile sampling to an established CCA diagnosis to increase the likelihood of CCA actually being present (CCA-PSC  $\leq 12$ ) and simultaneously to see if the markers could detect CCA earlier (up to 12 months) than currently used detection methods. The benign PSC group was further divided into

(1) PSC-dysplasia ( $n = 23$  [24 samples], patients with PSC and evidence of biliary dysplasia based on assessment of biliary brush cytology specimens or histological assessment of explant liver  $\pm 2$  months from bile collection but with no evidence of CCA development), (2) PSC-control  $> 36$  ( $n = 170$  [226 samples], patients with PSC with no evidence of CCA or biliary dysplasia based on histological assessment of explanted liver at or after bile collection or  $> 36$  months of follow-up [median 105 months] without established CCA or biliary dysplasia for nontransplanted patients), and (3) PSC-control  $< 36$  ( $n = 12$  [22 samples], patients with PSC with no evidence of CCA or biliary dysplasia after bile collection but only including nontransplanted patients with  $< 36$  months of follow-up [median 26 months]). We chose a threshold of 36 months of follow-up from bile sampling to divide the benign PSC group in two, to minimize the probability of presence of CCA in the strictest control group (PSC-control  $> 36$ ). We further included PSC-dysplasia as a separate group to evaluate the methylation pattern in this potentially premalignant group. The non-PSC group was divided into (1)



**FIGURE 1** Flowchart of bile samples included in the study. Number of patients, followed by number of samples in parentheses. Samples are split based on main disease category, including (1) patients with underlying PSC (left panel) and (2) patients with non-PSC-related diseases (right panel). PSC samples (left panel) are divided into the following groups: (1) CCA-PSC ( $n = 38$ , 42 samples), including CCA-PSC  $\leq 12$  ( $n = 28$ , 31 samples;  $\leq 12$  months from bile sampling to CCA diagnosis) and CCA-PSC  $> 12$  ( $n = 10$ , 11 samples;  $> 12$  months from bile sampling to CCA diagnosis), and (2) all PSC samples (without CCA,  $n = 205$ , 272 samples), including PSC-dysplasia ( $n = 23$ , 24 samples; patients with PSC with evidence of biliary dysplasia based on assessment of biliary brush cytology specimens or histological assessment of explant liver  $\pm 2$  months from bile collection), PSC-control  $> 36$  ( $n = 170$ , 226 samples; patients with PSC with no evidence of CCA or biliary dysplasia based on histological assessment of explanted liver at or after bile collection or  $> 36$  months of follow-up without established CCA or biliary dysplasia for nontransplanted patients), and PSC-control  $< 36$  ( $n = 12$ , 22 samples; patients with PSC with no evidence of CCA or biliary dysplasia after bile collection but only including nontransplanted patients with  $< 36$  months of follow-up). Non-PSC samples (right panel) are divided into (1) CCA ( $n = 6$ , 6 samples, of which five were collected at the same time as a confirmed CCA diagnosis and one was collected 3 months prior to diagnosis) and (2) NM liver disease ( $n = 24$ , 24 samples; hereditary, idiopathic nonalcoholic fatty liver, biliary stone, and autoimmune liver disease other than PSC)

CCA ( $n = 6$  [6 samples]) and (2) other NM liver diseases ( $n = 24$  [24 samples], including hereditary, idiopathic nonalcoholic fatty liver, biliary stone, and autoimmune liver disease other than PSC). The samples in the non-PSC group were included to explore the methylation levels of the markers in non-PSC-related CCA and other NM liver diseases.

Bile was collected during ERCP, if possible before injecting contrast medium; at liver transplantation; or in one case at liver resection. All samples were stored at  $-80^{\circ}\text{C}$  or  $-20^{\circ}\text{C}$  (Karolinska University Hospital) prior to DNA extraction.

For summarized clinical and histopathological information of the included samples, see Tables 1 and 2; and for detailed information about each bile sample, see Table S1.

## DNA extraction

For DNA extraction, using aliquots of 100–200  $\mu\text{l}$  of bile per sample, three methods were tested: High Pure Viral (Roche Diagnostics, Basel, Switzerland), PrepFiler (Thermo Fisher Scientific, MA), and a standard phenol chloroform procedure. The phenol chloroform procedure was performed as described,<sup>[15]</sup> while

the manufacturers' protocols were followed for the Pure Viral and the PrepFiler kits. Resulting DNA yields were measured using NanoDrop ND-1000. The High Pure Viral kit provided the highest amount of DNA (mean output of four samples, 532 ng), followed by phenol chloroform (mean output of three samples, 72 ng) and PrepFiler (mean output of four samples, 22.5 ng) (Table S2).

To compare the amount of amplifiable DNA from the three extraction methods, samples were bisulfite-treated (described in the following section), and 32 ng of bisulfite-treated DNA (from each sample) was amplified by quantitative methylation specific PCR (qMSP) as described,<sup>[20]</sup> using the ALU primer and probe set. Following bisulfite treatment and qMSP amplification, phenol chloroform outperformed the two kits, with lower ALU cycle threshold values, indicating more amplifiable DNA (Table S2). For the 350 bile samples reported in the following, DNA was extracted from 100 to 200  $\mu\text{l}$  bile using the phenol chloroform procedure.

## Bisulfite treatment

Five-hundred nanograms of DNA was bisulfite-converted using the EpiTect bisulfite kit (Qiagen, Hilden,

**TABLE 1** Clinical characteristics separated by groups

	CCA-PSC ( $n = 38$ )	PSC ( $n = 205$ )
Gender (male)	76%	76%
Median age CCA diagnosis, years (range)	53.9 (46.0)	NA
Median age benign liver diagnosis, years (range)	49.4 (51.1)	31.0 (59.1)
IBD, number (%)		
Crohn's	5 (13)	29 (14)
UC	22 (58)	115 (56)
Indet. colitis	0 (0)	3 (1)
No	9 (24)	50 (24)
NA	2 (5)	8 (4)
Cirrhosis, number (%)		
Yes	5 (13)	39 (19)
No	20 (53)	152 (74)
NA	13 (34)	14 (7)
Acute cholangitis, number (%)		
Yes	2 (5)	1 (0)
No	36 (95)	204 (100)
NA	0 (0)	0 (0)
Median CA19-9 level, U/ml (range)	47 (8,638)	19 (384)
Missing	3	10
Dead	74%	3%

Abbreviations: IBD, inflammatory bowel disease; Indet., indeterminate; NA, not available; UC, ulcerative colitis.

**TABLE 2** Histopathological data for patients with CCA-PSC

<i>n</i> = 38	Number (%)
Tumor location	
iCCA	14 (37)
pCCA	15 (39)
dCCA	7 (18)
xCCA	2 (5)
AJCC (8th edition)	
AJCC 0	1 (3)
AJCC 1	1 (3)
AJCC 2	6 (16)
AJCC 3	15 (39)
AJCC 4	9 (24)
NA	6 (16)
T	
Tis	1 (3)
T1	5 (13)
T2	12 (32)
T3	5 (13)
T4	6 (16)
Tx	9 (24)
N	
N0	5 (13)
N1	20 (53)
Nx	13 (34)
M	
M0	29 (76)
M1	9 (24)
Resection margin	
R0	9 (24)
R1	6 (16)
NA	23 (61)
Perineural growth	
Yes	12 (32)
No	2 (5)
NA	24 (63)
Vascular encasement	
Yes	6 (16)
No	10 (26)
NA	22 (58)
Differentiation	
Low (G3)	4 (11)
Medium (G2)	11 (29)
High (G1)	1 (3)
NA	22 (58)
Growth pattern	
Mass-forming	10 (26)
Periductal-infiltrating	6 (16)

(Continues)

**TABLE 2** (Continued)

<i>n</i> = 38	Number (%)
Intraductal-growing	1 (3)
NA	21 (55)

Note: The stage is from time of diagnosis.

Abbreviations: AJCC 0–4, American Joint Committee on Cancer classification stages 0–4; G, grade of differentiation; M, distant metastases; N, lymph node metastases; NA, not available; T, tumor stage evaluation; xCCA, unclassifiable CCA subtype.

Germany) according to the manufacturer's protocol. Desulfonation of the samples was performed using a QIAcube (Qiagen) with elution in 40 µl buffer.

## DNA methylation analyses

Primer and probe sequences for the target genes cysteine dioxygenase type 1 (*CDO1*), cannabinoid receptor interacting protein 1 (*CNRIP1*), septin 9 (*SEPT9*), and vimentin (*VIM*) and the 4Plex control comprising ephrin type A receptor 3, kelch repeat and BTB domain containing 4, pleckstrin homology and FYVE domain containing 1, and synaptotagmin 10 have been published.<sup>[15,21]</sup>

The QX200 Droplet Digital PCR System (BioRad) was used for DNA methylation analyses. The ddPCR consisted of 1 × ddPCR Supermix for Probes (BioRad), 818 nM of each primer, 182 nM of each probe, and 30 ng bisulfite-converted DNA template, in a final volume of 22 µl. Droplet generation was performed in the QX200 Droplet Generator, using 20 µl of the ddPCR mixture and 70 µl droplet generation oil (BioRad). Samples were transferred to a 96-well PCR plate (BioRad) and sealed in the PX1 PCR Plate Sealer (BioRad). The PCR was performed in a T100 Thermal Cycler (BioRad), using the manufacturer's recommended cycling conditions. Using a ramp rate of 2°C/s, amplification was performed at 95°C for 10 min, before 40 cycles of 30 s at 94°C and 1 min at 60°C, followed by enzyme deactivation at 98°C for 10 min. The QX200 Droplet Reader (BioRad) was applied for detection of fluorescence signals.

Data analysis was performed using QuantaSoft, version 1.7.4.0917 (BioRad). An in-house developed algorithm, PoDCall (<https://bioconductor.org/packages/release/bioc/html/PoDCall.html>), was applied for positive droplet calling as described.<sup>[21]</sup> Normalized DNA methylation levels were calculated by dividing the concentration (copies per microliter) of the target gene by the concentration (copies per microliter) of the 4Plex and multiplying by 400. For each target gene, samples with low DNA amount (4Plex concentration < 10 copies/µl) were excluded. Six samples had a 4Plex concentration < 10 for all the target genes (and were excluded altogether), and six samples had a 4Plex concentration < 10 for one to three genes and were excluded for the specific gene(s).

## Statistical analysis

A Mann-Whitney *U* test was used to test for differences in DNA methylation levels between the defined sample groups, and Fisher's exact test was used to examine the relation between methylation status (methylated or unmethylated for one or more of four markers) and sample group, as well as clinical and pathological data. Receiver operating characteristic (ROC) curve analysis was performed to evaluate the diagnostic biomarker potential of the individual markers. In addition, combined ROC curves were generated by summarizing the normalized methylation values for the four markers. The AUC, sensitivities, specificities, and 95% CIs were calculated. The cutoff providing the highest possible specificity (> 90%) without compromising the sensitivity was chosen for dichotomizing the samples. In cases where multiple samples from the same patient were analyzed, only one sample was selected for biomarker analysis. Samples in the group PSC-dysplasia were selected over PSC-control > 36, which were selected over PSC-control < 36. For the remaining samples, random selection was performed, to select only one sample from each patient. Samples lacking methylation values (*CDO1*, *n* = 1 [0.3%]; *CNRIP1*, *n* = 1 [0.3%]; *SEPT9*, *n* = 1 [0.3%]; *VIM*, *n* = 4 [1.2%]) were excluded. In the analyses of the biomarker panel, only samples with information for all four markers were included. Four samples were excluded because of missing values. Overall survival was calculated from time of bile sampling until death from any cause, and cases were censored at last follow-up. The effect of the four methylation markers was independently evaluated in a univariate approach, using the log rank method. The Kaplan-Meier method was used to estimate the survival curve. The analyses were performed using GraphPad Prism 8.1.2 and IBM SPSS Statistics 25.

Written informed consent was obtained from all study subjects. For long-archived samples, where this was not possible, an exception from informed consent was obtained by the local ethics committee to allow use of the samples. Study protocols were approved by the ethics committees of all the recruiting centers, as well as the Regional Committee for Medical and Health Research Ethics South Eastern Norway (6.2008.1723), the Swedish Ethical Review Authority (2013/2084-31/2 with amendment 2016/1023, 2018/1786-32), and Helsinki University Hospital (278/13/03/01/2009).

## RESULTS

A total of 344 samples from 273 patients passed the inclusion criteria after ddPCR, including 44 patients (48 samples) with CCA, of whom 38 (42 samples) had concomitant PSC, and 229 (296 samples) patients with NM liver diseases, of whom 205 (272 samples) had PSC. All of these samples were subjected to downstream analyses, with the most detailed analyses performed among patients with CCA complicated with PSC and PSC controls (Figure 1; Table S1).

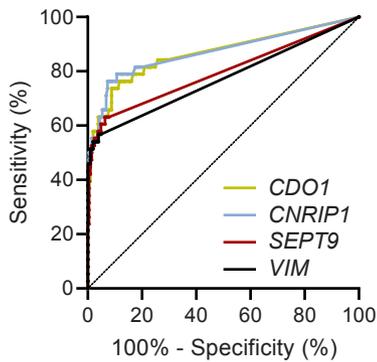
### CCA-PSC versus PSC-controls

High accuracy for CCA detection in patients with underlying PSC up to 12 months prior to CCA diagnosis established by standard diagnostic modalities

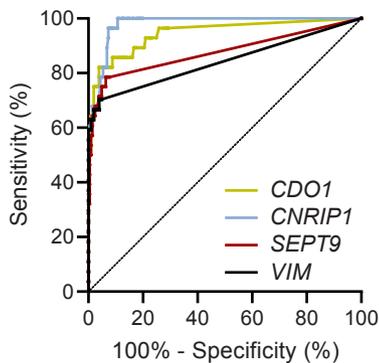
To investigate the potential of the four DNA methylation markers to detect CCA in patients with underlying PSC, we included all CCA samples derived from patients with concomitant PSC (*n* = 38), and as controls we included all patients with PSC (*n* = 205), irrespective of the presence of dysplasia and length of follow-up. First, we compared the normalized methylation levels of all four markers in the two groups, showing that samples from patients with CCA-PSC displayed significantly higher methylation levels than samples from patients with PSC for all four markers (Mann-Whitney *U*, *p* < 0.0001; Figure S1). Second, we did a ROC curve analysis to see how well the markers could differentiate between CCA-PSC and PSC. Plotting the normalized DNA methylation values from the CCA-PSCs against the normalized DNA methylation values from PSCs demonstrated that the individual biomarkers had high accuracy in distinguishing the two groups, with AUCs, sensitivities, and specificities ranging 0.77–0.87, 54%–76%, and 93%–98%, respectively (Figure 2A). Thirty of the 38 CCA samples had at least one methylated marker (79% sensitivity for the biomarker panel), while only 20 of the 203 samples (with valid DNA biomarker results) from patients with PSC were positive for the methylation panel (90% specificity). Combining the markers resulted in an AUC of 0.88 (Figure S2A).

Further, to see if time from bile sampling to a CCA diagnosis could affect the sensitivity of the DNA methylation markers, we included only CCA samples from patients diagnosed with CCA-PSC ≤ 12 months from

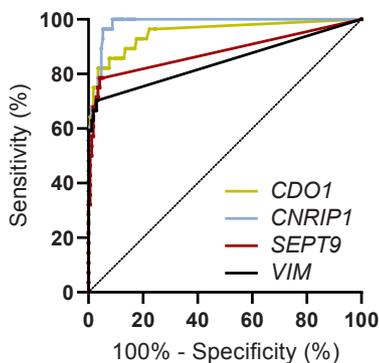
**FIGURE 2** ROC curves, calculated AUCs, and sensitivity and specificity values for the four individual DNA methylation biomarkers in bile. (A) Samples from patients with CCA-PSC (*n* = 38) versus all PSC (*n* = 205), (B) samples from patients with PSC diagnosed with CCA ≤ 12 months after bile sampling (CCA-PSC ≤ 12, *n* = 28) versus PSC all (*n* = 205), (C) CCA-PSC ≤ 12 (*n* = 28) versus PSC-control > 36 (*n* = 170, including samples from patients with PSC showing no evidence of biliary dysplasia or CCA in explanted liver or with > 36 months of follow-up), and (D) CCA-PSC ≤ 12 (*n* = 28) versus PSC-dysplasia (*n* = 23, including patients with PSC with evidence of biliary dysplasia based on assessment of biliary brush cytology specimens or histological assessment of explant liver ±2 months from bile collection)

**(A) CCA-PSC vs PSC all**

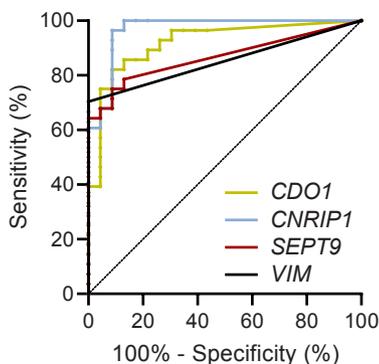
	AUC (95% CI)	P-value	Cutoff	Sensitivity	Specificity
<b>CDO1</b>	0.86 (0.79-0.94)	<0.0001	1.9	63 %	96 %
<b>CNRIP1</b>	0.87 (0.79-0.95)	<0.0001	0.58	76 %	93 %
<b>SEPT9</b>	0.79 (0.70-0.89)	<0.0001	0.605	55 %	98 %
<b>VIM</b>	0.77 (0.67-0.87)	<0.0001	0.705	54 %	98 %
<b>Methylation panel (<math>\geq 1/4</math>)</b>				79 %	90 %

**(B) CCA-PSC  $\leq 12$  vs PSC all**

	AUC (95% CI)	P-value	Cutoff	Sensitivity	Specificity
<b>CDO1</b>	0.94 (0.89-1.00)	<0.0001	1.9	82 %	96 %
<b>CNRIP1</b>	0.98 (0.96-0.99)	<0.0001	0.58	96 %	93 %
<b>SEPT9</b>	0.88 (0.78-0.97)	<0.0001	0.605	68 %	98 %
<b>VIM</b>	0.84 (0.74-0.95)	<0.0001	0.705	67 %	98 %
<b>Methylation panel (<math>\geq 1/4</math>)</b>				100 %	90 %

**(C) CCA-PSC  $\leq 12$  vs PSC-control  $>36$** 

	AUC (95% CI)	P-value	Cutoff	Sensitivity	Specificity
<b>CDO1</b>	0.95 (0.90-1.00)	<0.0001	1.9	82 %	96 %
<b>CNRIP1</b>	0.98 (0.97-1.00)	<0.0001	0.58	96 %	95 %
<b>SEPT9</b>	0.88 (0.79-0.97)	<0.0001	0.605	68 %	98 %
<b>VIM</b>	0.84 (0.74-0.95)	<0.0001	0.705	67 %	98 %
<b>Methylation panel (<math>\geq 1/4</math>)</b>				100 %	93 %

**(D) CCA-PSC  $\leq 12$  vs PSC-dysplasia**

	AUC (95% CI)	P-value	Cutoff	Sensitivity	Specificity
<b>CDO1</b>	0.92 (0.84-1.00)	<0.0001	1.9	82 %	91 %
<b>CNRIP1</b>	0.97 (0.92-1.00)	<0.0001	0.58	96 %	91 %
<b>SEPT9</b>	0.87 (0.76-0.97)	<0.0001	0.605	68 %	96 %
<b>VIM</b>	0.85 (0.74-0.96)	<0.0001	0.705	67 %	100 %
<b>Methylation panel (<math>\geq 1/4</math>)</b>				100 %	83 %

bile sampling ( $n = 28$ ). As controls, we included (1) all PSC samples ( $n = 205$ ), (2) samples from the PSC-control > 36 group ( $n = 170$ ; no evidence of CCA or biliary dysplasia based on histological assessment of explanted liver or > 36 months of follow-up in non-transplanted patients), and (3) PSC-dysplasia ( $n = 23$ ; patients with PSC with evidence of biliary dysplasia based on assessment of biliary brush cytology specimens or histological assessment of explanted liver  $\pm 2$  months from bile collection but with no evidence of CCA development). ROC curve analysis, plotting the normalized DNA methylation values for CCA-PSC  $\leq 12$  against the normalized values for all PSC samples showed that the ability to separate the two groups was high, with AUCs of 0.98 (*CNRIP1*), 0.94 (*CDO1*), 0.88 (*SEPT9*), and 0.84 (*VIM*). The sensitivities and specificities of the individual markers ranged 67%–96% and 93%–98%, respectively (Figure 2B). Interestingly, the sensitivity for the biomarker panel (defined by one or more of four methylation positive markers) was 100% with a specificity of 90%. Including only the PSC-control > 36 as controls increased the specificity for the biomarker panel to 93% (Figure 2C). Finally, we included only the PSC-dysplasia samples as controls. The ability to distinguish the two groups remained high with AUCs ranging from 0.85 to 0.97 for the individual markers and a sensitivity and specificity for the biomarker panel of 100% and 83%, respectively (Figure 2D). AUCs for the combined biomarkers can be found in Figure S2B–D, including all PSC, PSC-control > 36, and PSC-dysplasia as controls, respectively.

Of further interest, the bile samples from the CCA-PSC  $\leq 12$  patients, all positive for the methylation panel, were collected from patients with both early-stage and late-stage cancer at diagnosis (0–2 and 3–4), different tumor growth patterns (mass-forming, periductal-infiltrating, and intraductal-growing), tumor size (< 3, 3–5, and > 5 cm), and anatomical location (intrahepatic CCA [iCCA], perihilar CCA [pCCA], and extrahepatic CCA [dCCA]) (Tables 2 and 3). Thus, the methylation panel achieved a sensitivity of 100% and a specificity of 90% for detecting CCA in patients with underlying PSC, including dysplasia, up to 12 months prior to a confirmed CCA diagnosis and irrespective of clinical and molecular features (Table 3). All CCA samples negative for methylation were analyzed > 12 months prior to CCA diagnosis (Table S1). Of note, no correlation was observed between age and methylation among CCA-PSC for any of the four genes. For *CNRIP1* and *VIM* a weak correlation was observed among patients with PSC (Table S3).

### DNA methylation in patients with PSC and underlying biliary dysplasia

To investigate if methylation levels of one or more markers in the four-gene panel increased stepwise

in bile derived from benign, dysplastic, and malignant stages of disease, we compared the methylation levels of the four genes in the PSC-dysplasia group ( $n = 23$ ) with the benign PSC-control > 36 group ( $n = 170$ ) and the CCA-PSC  $\leq 12$  group ( $n = 28$ ). The normalized methylation levels in the benign PSC and the PSC-dysplasia groups compared with the CCA group were significantly different for all four markers (Figure S3). Although no significant difference in methylation levels for any of the four markers was observed between the PSC-dysplasia group and the PSC-control > 36 group, an increase in methylation status was observed from the PSC-control > 36 group (7%) to the PSC-dysplasia group (17%) and to CCA (100%; Fisher's exact,  $p < 0.001$ ) (Table S4).

### Comparison of the methylation panel with CA 19-9 levels and brush cytology in indicating CCA in PSC

We also wanted to compare the results of the methylation panel in bile with the informativeness of CA 19-9 levels and conventional brush cytology assessments in detecting CCA in PSC. Because FISH analyses were not available for the majority of the samples, no comparisons were made between FISH and the methylation panel.

Of the 28 CCA-PSC  $\leq 12$  patients, CA 19-9 levels were obtained from 25 patients, of whom 14 (56%) had a CA 19-9 level > 100 U/ml. The biomarker panel was positive in all of these 14 patients (100% sensitivity). In addition, the biomarker panel was positive in all of the 11 samples with a CA 19-9 level < 100 U/ml (Table 4 and Figure 3). Among the 203 patients with PSC with valid DNA biomarker results, CA 19-9 measurements were available for 193 patients, including 13 (7%) with a CA 19-9 level > 100 U/ml and 180 (93%) with a CA 19-9 level < 100 U/ml. The biomarker panel was negative in 92% of the patients with CA 19-9 > 100 U/ml ( $n = 13$ ) and in 90% of the patients with CA 19-9 < 100 U/ml ( $n = 180$ ) (Table 4). Including only PSC-control > 36, the biomarker panel was negative in 100% of the patients with CA 19-9 > 100 U/ml. Thus, the high sensitivity (100%) of the biomarker panel for CCA diagnosis was independent of CA 19-9 level, and the specificity remained high (> 90%) in patients with CA 19-9 level above and below 100 U/ml.

In the CCA-PSC  $\leq 12$  group parallel cytology specimens from biliary brushings had been obtained for 22 patients (24 samples). Among these, brush cytology samples were not conclusive (acellular or nondiagnostic/scarcely material) in seven samples (29%), of which all (100%) were positive for the methylation panel (Figure 3). Of the remaining 17 samples with conclusive results, 12 (71%) were scored as positive for low-grade or high-grade dysplasia, while the combined

**TABLE 3** Associations between methylation of the biomarker panel and clinical and molecular features

	CCA-PSC ≤ 12			CCA-PSC > 12		
	Total <i>n</i>	Pos. <i>n</i> (%)	Neg. <i>n</i> (%)	Total <i>n</i>	Pos. <i>n</i> (%)	Neg. <i>n</i> (%)
No. of patients	28	28 (100)	0 (0)	10	2 (20)	8 (80)
Gender						
Male	20	20 (100)	0 (0)	9	1 (11)	8 (89)
Female	8	8 (100)	0 (0)	1	1 (100)	0 (0)
AJCC (8th edition)						
0–2	7	7 (100)	0 (0)	1	0 (0)	1 (100)
3–4	19	19 (100)	0 (0)	5	1 (20)	4 (80)
NA	2	2 (100)	0 (0)	4	1 (25)	3 (75)
Tumor location						
iCCA	8	8 (100)	0 (0)	6	1 (17)	5 (83)
pCCA	13	13 (100)	0 (0)	2	0 (0)	2 (100)
dCCA	5	5 (100)	0 (0)	2	1 (50)	1 (50)
xCCA	2	2 (100)	0 (0)	–	–	–
Perineural growth						
Yes	11	11 (100)	0 (0)	1	0 (0)	1 (100)
No	2	2 (100)	0 (0)	–	–	–
NA	15	15 (100)	0 (0)	9	2 (22)	7 (78)
Vascular encasement						
Yes	5	5 (100)	0 (0)	1	0 (0)	1 (100)
No	9	9 (100)	0 (0)	1	0 (0)	1 (100)
NA	14	14 (100)	0 (0)	8	2 (25)	6 (75)
Differentiation						
Low	3	3 (100)	0 (0)	1	0 (0)	1 (100)
Medium	10	10 (100)	0 (0)	1	0 (0)	1 (100)
High	1	1 (100)	0 (0)	–	–	–
NA	14	24 (100)	0 (0)	8	2 (25)	6 (75)
Growth pattern						
Mass-forming	8	8 (100)	0 (0)	2	0 (0)	2 (100)
Periductal-infiltrating	5	5 (100)	0 (0)	1	1 (100)	0 (0)
Intraductal-growing	1	1 (100)	0 (0)	–	–	–
NA	14	14 (100)	0 (0)	7	1 (14)	6 (86)
Tumor diameter						
<3 cm	7	7 (100)	0 (0)	–	–	–
3–5 cm	8	8 (100)	0 (0)	1	0 (0)	1 (100)
>5 cm	6	6 (100)	0 (0)	4	1 (25)	3 (75)
NA	7	7 (100)	0 (0)	5	1 (20)	4 (80)
Resection margins						
R0	8	8 (100)	0 (0)	1	0 (0)	1 (100)
R1	5	5 (100)	0 (0)	1	0 (0)	1 (100)
NA	15	15 (100)	0 (0)	8	2 (25)	6 (75)
Age at bile sampling, years						
10–29	1	1 (100)	0 (0)	–	–	–
30–39	4	4 (100)	0 (0)	2	0 (0)	2 (100)
40–49	6	6 (100)	0 (0)	2	1 (50)	1 (50)

(Continues)

TABLE 3 (Continued)

	CCA-PSC ≤ 12			CCA-PSC > 12		
	Total <i>n</i>	Pos. <i>n</i> (%)	Neg. <i>n</i> (%)	Total <i>n</i>	Pos. <i>n</i> (%)	Neg. <i>n</i> (%)
50–59	9	9 (100)	0 (0)	5	1 (20)	4 (80)
60–79	8	8 (100)	0 (0)	1	0 (0)	1 (100)
IBD						
UC	19	19 (100)	0 (0)	3	0	3 (100)
Crohn's disease	4	4 (100)	0 (0)	1	1 (100)	0 (0)
None	4	4 (100)	0 (0)	5	1 (20)	4 (80)
NA	1	1 (100)	0 (0)	1	0 (0)	1 (0)
CA 19-9						
>100 U/ml	14	14 (100)	0 (0)	0	–	–
<100 U/ml	11	11 (100)	0 (0)	10	2 (20)	8 (80)
NA	3	3 (100)	0 (0)			
Bile origin						
ERCP	26	26 (100)	0 (0)	10	2 (20)	8 (80)
Resection	1	1 (100)	0 (0)	–	–	–
Transplantation	1	1 (100)	0 (0)	–	–	–
Alive						
Yes	9	9 (100)	0 (0)	1	1 (100)	0 (0)
No	19	19 (100)	0 (0)	9	1 (11)	8 (89)

Abbreviations: AJCC 0–4, American Joint Committee on Cancer classification stages 0–4; IBD, inflammatory bowel disease; NA, not available; Neg., negative for the biomarker panel (none of the four markers methylated); Pos., positive for the biomarker panel (one or more of the four markers methylated); UC, ulcerative colitis.

methylation panel was scored as positive in 100% of the samples (17/17) (Figure 3).

Among the PSC-control > 36 group ( $n = 170$ ), parallel bile (methylation) and brush cytology results were available for 127 patients. Among these patients brush cytology was not conclusive (acellular or nondiagnostic material) in 42 patients (33%). Of the remaining 85 patients, all scored as negative for dysplasia; 80 (94%) were also negative for the methylation panel.

### High concordance in methylation status between multiple samples from the same patient

For 52 patients, including four with CCA-PSC and 48 with PSC, multiple samples (two to four) were analyzed. For the majority of the patients where longitudinal sampling was present, disease category did not change during the sampling period (e.g., all samples from the same patient belonged to the CCA-PSC ≤ 12 group or to the PSC-control > 36 group). For the four patients with CCA (eight samples), a 100% concordance in methylation status for the biomarker panel was observed, while for the patients with PSC, 41 of 48 (85%) had concordant methylation results (Figure S4). None of the PSC samples with deviating results (one

sample scored as negative for the biomarker panel and one sample scored as positive for the biomarker panel) were collected during ongoing acute cholangitis. However, the majority of the samples with deviating results had been collected > 2 years apart.

In addition, prior to this effort we analyzed the biomarkers in biliary brushes and tissue samples.<sup>[15,22]</sup> Figure S5 provides a direct comparison of methylation in bile, brush, and tissue of the four biomarkers in a subset of the samples (54 patients) included in previous efforts. In general, there was a high concordance in methylation status between the different materials for the same patients.

### DNA methylation level and association with survival

To investigate if the level of methylation in bile could have an effect on survival, we did a log rank test. Dividing the CCA-PSC ≤ 12 samples into high (normalized DNA methylation level > 10) and 0/low (normalized DNA methylation level < 10) level of methylation showed that patients with a high level of methylation had a tendency for shorter overall survival compared to patients with no or a low level of methylation (*CDO1*,  $p = 0.059$ ; *CNRIP1*,  $p = 0.016$ ; *SEPT9*,  $p = 0.078$ ; and *VIM*  $p = 0.203$ ) (Figure S6).

**TABLE 4** Sensitivity and specificity of the biomarker panel in patients with CA 19-9 level above and below 100 U/ml

	CCA-PSC ≤ 12		CCA-PSC > 12		PSC all		PSC-control > 36		PSC-dysplasia						
	Total n	Pos. (%)	Neg. (%)	Total n	Pos. (%)	Neg. (%)	Total n	Pos. (%)	Total n	Pos. (%)	Neg. (%)				
No. of patients	28	28 (100)	0 (0)	10	2 (20)	8 (80)	203	20 (10)	183 (90)	168	12 (7)	156 (93)	23	4 (17)	19 (83)
CA 19-9															
> 100 U/ml	14	14 (100)	0 (0)	0	0	0	13	1 (8)	12 (92)	11	0 (0)	11 (100)	2	1 (50)	1 (50)
< 100 U/ml	11	11 (100)	0 (0)	10	2 (20)	8 (80)	180	18 (10)	162 (90)	150	12 (8)	138 (92)	21	3 (14)	18 (86)
NA	3	3 (100)	0 (0)	—	—	—	10	1 (10)	9 (90)	7	0 (0)	7 (100)	—	—	—

Note: Group definitions: CCA-PSC ≤ 12, patients with CCA and concomitant PSC and ≤ 12 months from bile sampling to a confirmed CCA diagnosis; CCA-PSC > 12, patients with CCA and concomitant PSC and > 12 months from bile sampling to CCA diagnosis; PSC-dysplasia, patients with PSC and evidence of biliary dysplasia based on assessment of biliary brush cytology specimens or histological assessment of explant liver ±2 months from bile collection; PSC-control > 36, patients with PSC and no evidence of CCA or biliary dysplasia based on histological assessment of explanted liver at or after bile collection or > 36 months of follow-up without established CCA or biliary dysplasia for nontransplanted patients; PSC all, benign PSC without CCA.

Abbreviations: NA, not available; Neg., negative for the biomarker panel (none of the four markers methylated); Pos., positive for the biomarker panel (one or more of the four markers methylated).

## CCA without underlying PSC versus other NM liver disease

### The methylation panel has potential to detect CCA in a non-PSC setting

Finally, to investigate the potential of the biomarkers to also detect CCA in a non-PSC setting, we compared the CCA samples derived from patients without underlying PSC ( $n = 6$ ) with samples from patients with other NM liver diseases ( $n = 24$ ). Results for the individual biomarkers are summarized in Figure S7, showing AUCs of 0.78 (*CDO1*), 1.00 (*CNRIP*), 0.75 (*SEPT9*), and 0.90 (*VIM*). Combined, the biomarker panel had a sensitivity of 100% and a specificity of 100% for distinguishing the two groups. Although we had few samples, these data show that the biomarkers have the potential to also differentiate CCA without underlying PSC from benign non-PSC liver diseases. For clinical and histopathological data on CCA and other NM liver diseases, see Table S5A,B.

## DISCUSSION

By analyzing an exceptionally large series of bile samples from well-characterized patients, we demonstrate that four DNA methylation biomarkers accurately differentiate patients with CCA from patients with NM liver disease. The diagnostic accuracy for CCA was high both in sporadic and in PSC-associated cancer, underscored by high specificity and the finding of positive methylation markers in all bile samples obtained up to 12 months prior to a confirmed CCA diagnosis. Furthermore, we have prior to this effort shown that the markers were highly methylated in CCA tumor tissue,<sup>[22]</sup> then in biliary brushes.<sup>[15]</sup> In the current study the utility of the methylation panel for early CCA diagnosis was validated using ddPCR in a limited amount of bile, emphasizing the robustness of the biomarkers. As such, this work represents a major advance in the field. The promising results suggest a potential for using these DNA methylation markers in bile (1) to complement current detection methods for CCA and (2) for CCA surveillance in patients with PSC. This also represents one of the largest liquid biopsy-derived biomarker studies in PSC-CCA to date.<sup>[14]</sup>

Two previous studies have also suggested DNA methylation analyses of bile as a possible modality to detect CCA. Klump et al.<sup>[23]</sup> reported that cyclin-dependent kinase inhibitor 2A (*CDKN2A*) was methylated in 52% (p16) and 48% (p14) of bile samples from patients with CCA and in only 6% of healthy individuals. Although promising, as a proof of concept for CCA detection using bile, *CDKN2A* methylation was also present in 46% of patients with PSC without an established CCA diagnosis, making this marker unsuitable for early

ID	Group	Methylation panel	Brush cytology	CA 19-9 level
1	CCA-PSC ≤12	●	●	○
2	CCA-PSC ≤12	●	NA	NA
3	CCA-PSC ≤12	●	NA	NA
4	CCA-PSC ≤12	●	●	○
5	CCA-PSC ≤12	●	NA	NA
6	CCA-PSC ≤12	●	●	●
7	CCA-PSC ≤12	●	●	○
8a	CCA-PSC ≤12	●	○	●
8b	CCA-PSC ≤12	●	○	●
9	CCA-PSC ≤12	●	NC	●
10	CCA-PSC ≤12	●	NC	●
11	CCA-PSC ≤12	●	NA	●
12	CCA-PSC ≤12	●	NA	●
13a	CCA-PSC ≤12	●	NC	○
13b	CCA-PSC ≤12	●	NC	○
14	CCA-PSC ≤12	●	NC	○
15	CCA-PSC ≤12	●	●	○
16	CCA-PSC ≤12	●	○	●
17a	CCA-PSC ≤12	●	○	●
17b	CCA-PSC ≤12	●	NA	●
18	CCA-PSC ≤12	●	○	○
19	CCA-PSC ≤12	●	NC	○
20	CCA-PSC ≤12	●	●	○
21	CCA-PSC ≤12	●	NC	●
22	CCA-PSC ≤12	●	●	●
23	CCA-PSC ≤12	●	●	●
24	CCA-PSC ≤12	●	●	○
25	CCA-PSC ≤12	●	NA	●
26	CCA-PSC ≤12	●	●	○
27	CCA-PSC ≤12	●	●	●
28	CCA-PSC ≤12	●	●	●
29	CCA-PSC >12	○	NC	○
30	CCA-PSC >12	○	NC	○
31	CCA-PSC >12	○	NC	○
32a	CCA-PSC >12	○	○	○
32b	CCA-PSC >12	○	NC	○
33	CCA-PSC >12	●	○	○
34	CCA-PSC >12	○	○	○
35	CCA-PSC >12	○	○	○
36	CCA-PSC >12	●	●	○
37	CCA-PSC >12	○	○	○
38	CCA-PSC >12	○	NA	○

**FIGURE 3** Comparison of DNA methylation in bile with CA 19-9 levels and biliary brush cytology. Red circle, sample scored as positive for methylation/cytology/CA 19-9; white circle, sample scored as negative for methylation/cytology/CA 19-9. The methylation panel was considered positive if one or more of the four biomarkers were methylated. Biliary brush cytology was scored positive if low/moderate-grade or high-grade dysplasia was identified and negative if only normal cells were identified. If more than one brush sample was taken, the case was scored based on the highest grade of dysplasia found. In cases where the sample was scored between two grades of dysplasia, the highest grade was used. For CA 19-9 levels, 100 U/ml was used as the threshold: a CA 19-9 level > 100 U/ml was considered positive, while a CA 19-9 level < 100 U/ml was considered negative. The “b” samples are not included in the biomarker analysis. NA, not available; NC, acellular or nondiagnostic/scarcely material not sufficient for further scoring

detection of CCA in patients with PSC.<sup>[23]</sup> Shin et al.<sup>[24]</sup> analyzed 17 aberrantly methylated biomarkers in dCCA in a training and a validation set of bile specimens (53 CCA and 32 NM controls). The four best-performing panels from these two sets were then analyzed in an independent test set (24 CCA and 16 NM controls). With 100% specificity and sensitivities of 70%, 74%, and 83% for the training, validation, and test sets, respectively, the best-performing marker panel showed potential for accurate dCCA detection.<sup>[24]</sup> However, no PSC controls were included in the study. Because inflammation, as observed in PSC, is known to affect the methylation pattern,<sup>[25]</sup> the lack of PSC controls could affect the specificity of a potential detection test aimed at patients with PSC. In our study, the few false positives detected were all among the PSC controls; and if only including other NM liver diseases excluding PSC, we obtained a specificity of 100%. In general, we further observed higher methylation levels in patients with CCA and underlying PSC compared to those with CCA but without PSC (not shown). This has also been reported by others, using peptide markers in urine<sup>[26]</sup> and bile.<sup>[27]</sup>

Using blood to identify biomarkers is also attractive for cancer surveillance, considering both compliance and availability. Two genes, short stature homeobox 2 and *SEPT9*, have been reported to be methylated in 35% and 25%, respectively, of plasma samples from patients with CCA, with 99% specificity.<sup>[28]</sup> Although these findings are interesting and the specificity is good, the low sensitivity limits the usefulness of the markers as surveillance tools in a clinical setting. The performance in blood was furthermore poorer than that in tissue, leading one to question whether the amount of circulating cell free tumor DNA in blood is high enough for early and robust detection of CCA.<sup>[28]</sup> Because bile is drained directly from the biliary tract where the primary tumor resides, it is reasonable to believe that bile contains higher concentrations of tumor DNA from CCA compared to blood, and therefore may prove superior to blood for early and accurate CCA diagnosis.

So far, no evidence-based surveillance strategy exists for CCA in patients with PSC. Current practice includes interval screening with serum-based CA 19-9 and ultrasound or MR imaging/cholangiopancreatography. If results are positive, ERCP with biliary brush cytology is typically performed,<sup>[8,29–32]</sup> often in combination with FISH.<sup>[31]</sup> Other surveillance strategies also exist, including using ERCP and brush cytology as primary surveillance tools.<sup>[33,34]</sup> Both CA 19-9<sup>[31]</sup> and imaging<sup>[32]</sup> display suboptimal accuracy for CCA detection, although MRI was recently reported to be superior to ultrasound for detection of early-stage CCA in asymptomatic patients with PSC.<sup>[35]</sup> The specificity of biliary brush for cytology and FISH is reported to be high (pooled specificity 97% and 96%, respectively), but the sensitivity is variable and often low (pooled sensitivity 47% and 50%, respectively).<sup>[36,37]</sup> False-negative

results may occur when the tumor resides in areas not accessible to or not targeted by brush cytology sampling. It can further be challenging to obtain adequate cytological material for evaluation due to the fibrotic and desmoplastic process in PSC and CCA.<sup>[19]</sup> Our findings demonstrate the challenge of obtaining appropriate biliary brush material for diagnostic purposes, with a large proportion of the brush cytologies in the CCA group and in the benign PSC group not being informative (~30%). The performance of biliary brush cytology is also largely dependent on the individual endoscopists and cytopathologists. This may lead to considerable variability in the diagnostic informativeness of biliary brush cytology between centers.<sup>[38]</sup> In contrast, it is easier to standardize methodologies for bile sampling and methylation profiling. Also, using bile as diagnostic material for CCA, the chance of obtaining representative material increases because cells and DNA are shed into bile independently of tumor localization. Indeed, using highly sensitive ddPCR methodology in limited amounts of bile, we successfully detected alterations in methylation levels that robustly differentiated patients with CCA from those without CCA, including in patients with underlying PSC. The high sensitivity for CCA detection was irrespective of clinical and molecular features, including cancer stage at diagnosis, tumor growth pattern, tumor size, anatomical location, and CA 19-9 levels. The few samples from patients with PSC with a later confirmed CCA diagnosis not detected by the biomarkers included bile samples collected > 12 months prior to a clinical diagnosis. Hence, there is a high chance that these patients had not developed CCA at the time of bile sampling.

The current study included CCA-PSC samples from three independent centers from three different countries. Unfortunately, due to the rarity of the disease, there were too few samples to include them as three independent series. We do, however, observe that the markers show 100% sensitivity among the CCA-PSC ≤ 12 group, irrespective of center/country, with similar high specificities in the PSC-control > 36 group (Sweden, 100%; Finland, 90%; Norway, 93%). Despite our promising findings, validation in larger multicenter sample series is warranted. Ideally, such analyses should be performed long-term, longitudinally, in a large prospective cohort of bile samples from patients with PSC, to see whether patients with PSC who later develop CCA show early signs of methylation, and further to systematically compare the accuracy of the methylation panel with conventional diagnostic measures for CCA. The current study demonstrates that methylation changes may be detected up to 12 months prior to standard CCA detection methods. Of further interest, patients with biliary dysplasia, who are at higher risk of developing CCA,<sup>[39]</sup> displayed a higher methylation frequency (17%) compared to patients with PSC without findings

of dysplasia (7%). Still, including longitudinal data and consecutive bile samples from patients with PSC could give a better indication of how long prior to malignancy these methylation changes are expected to occur. It might also give an indication of whether these changes are associated with biliary dysplasia or predominantly represent markers for CCA development that are independent of the dysplasia–carcinoma sequence. Four of 12 (33%) PSC samples from patients without an established CCA diagnosis but with < 36 months of follow-up were methylated for the biomarkers. We cannot rule out that some of these might be false positives, caused by severe inflammation, cholestasis, or other benign factors in the bile ducts. Another possibility is that these patients represent a high-risk group for CCA development. The PSC samples included in the study are derived from tertiary center referrals of patients undergoing ERCP, surgical resection, or liver transplantation. As such, the PSC samples included are enriched for advanced PSC cases, and it is not unlikely that a subgroup of these patients is at higher risk for CCA development. However, we observed no association between concurrent bacterial acute cholangitis or cirrhosis and DNA methylation level of any of the individual markers or with the methylation panel.

Interestingly, the individual DNA methylation markers analyzed in the current study for their diagnostic potential have also been evaluated by others for their prognostic value. Nakamoto et al. reported that the overall survival in patients with dCCA was poorer in patients with hypermethylated *CDO1*,<sup>[40]</sup> while a study by Chen et al. showed that low expression of *CNRIP1*, caused by DNA methylation, was associated with poor prognosis in patients with iCCA.<sup>[41]</sup> DNA methylation level of *SEPT9* in tissue showed, on the other hand, no correlation with overall survival.<sup>[28]</sup> Although an in-depth analysis of the prognostic value of the four biomarkers was outside the scope of the current study, we did a log rank test to investigate if the methylation level in bile could have an effect on survival, observing that our results were in agreement with what was reported by others.

In conclusion, by analyzing an unprecedented large series of bile samples predominantly derived from patients with PSC, we have shown that testing for DNA methylation biomarkers in bile detects CCAs in patients with PSC with high accuracy and at an earlier stage than conventional modalities. The findings suggest that these biomarkers have potential to complement standard modalities for improved CCA detection and potentially for CCA surveillance in patients with PSC. Prospective validation of the markers in a clinical setting is warranted.

#### CONFLICT OF INTEREST

Dr. Bergquist received grants from Gilead.

## AUTHOR CONTRIBUTIONS

Hege Marie Vedeld: data acquisition and analysis, interpretation of data, manuscript drafting  
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 Guro E. Lind: study concept and design, data acquisition and analysis, interpretation of data, manuscript revision and approval, study supervision.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website. Supplementary Material

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