

RESEARCH ARTICLE

Open Access



Evaluation of circulating cell-free DNA in cholestatic liver disease using liver-specific methylation markers

Sohan Punia¹, Brian D. Juran¹, Ahmad H. Ali¹, Erik M. Schlicht¹, Raymond M. Moore², Zhifu Sun² and Konstantinos N. Lazaridis^{1*}

Abstract

Background: Quantification of circulating organ-specific cell-free DNA (cfDNA) provides a sensitive measure of ongoing cell death that could benefit evaluation of the cholestatic liver diseases primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), which lack reliable non-invasive biomarkers. Our goal in this pilot study was to determine whether liver-specific cfDNA levels are increased in PBC and PSC patients relative to controls and in advanced versus early disease, to evaluate their potential as novel disease biomarkers.

Methods: Peripheral blood derived bisulfite-treated DNA was PCR amplified from patients with PBC (n = 48), PSC (n = 48) and controls (n = 96) to evaluate methylation status at 16 CpG sites reported to be specifically unmethylated in liver tissue near the genes IGF2R, ITIH4 and VTN. Amplicons were used to prepare paired end libraries which were sequenced on a MiSeq sequencer. Trimmed reads were aligned and used to determine unmethylation ratios and to calculate concentration of liver-specific cfDNA. Comparisons between groups were performed using the two-tailed Mann–Whitney Test and relationships between variables were evaluated using Pearson's Correlation.

Results: Levels of liver-specific cfDNA, as measured at the 3 genetic loci, were increased in PBC and PSC patients relative to controls and in late-stage relative to early-stage patients. As well, cfDNA levels were correlated with levels of alkaline phosphatase, a commonly used biochemical test to evaluate disease severity in liver disease, in patients, but not in controls.

Conclusions: cfDNA offers promise as a non-invasive liquid-biopsy to evaluate liver-specific cell-death in patients with cholestatic liver diseases.

Keywords: PBC, PSC, CfDNA, Liver, Cholestasis

Background

Primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC) are rare, cholestatic liver diseases of unknown etiology. Disease pathogenesis in PBC and PSC is complex, involving genetic and environmental

factors, and despite both being immune-mediated cholestatic liver diseases, there are significant differences. For instance, PSC involves inflammation and fibrosis in both the intra- and extra-hepatic bile ducts, while in PBC only the intra-hepatic ducts are affected [1]. PSC primarily affects males (~60%) while PBC affects mainly females (~90%) [1]. The majority of PSC patients (~80%) have co-existing inflammatory bowel disease (IBD), whereas PBC patients only rarely have IBD [1]. Similarly, PSC patients have a highly elevated risk of liver and colon

*Correspondence: Lazaridis.Konstantinos@mayo.edu

¹ Division of Gastroenterology and Hepatology, College of Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA

Full list of author information is available at the end of the article



cancers while PBC patients do not [1]. Finally, there are approved medications to treat PBC, ursodeoxycholic acid and obeticholic acid, neither of which has FDA approval for use in PSC, which currently lacks therapeutic options [2]. Regardless of the differences, both PBC and PSC are progressive diseases and orthotopic liver transplantation (OLT) is eventually required in many patients [3, 4]. Despite some recent progress, PSC and PBC still lack reliable non-invasive prognostic biomarkers [5], hampering the prediction of disease outcomes and assessment of the effect of therapy [6]. To address this unmet need, we have utilized an assay designed to detect liver-specific circulating cell-free DNA (cfDNA) in plasma as a potential prognostic biomarker for PBC and PSC.

Apoptotic and injured dying cells are constantly releasing DNA into the blood and levels of this cfDNA have been shown to increase in cancer, cardiovascular disease, sepsis, autoimmune diseases and following intensive exercise [7–11]. Detection of cfDNA coming from particular organs relies on DNA methylation signatures that are organ specific. Such signatures have recently been reported for a wide range of tissues and cell types including the liver [12–14]. For instance, a recent study reported CpGs near the genes IGF2R, VTN and ITIH4 to be specifically unmethylated in the liver and showed these marks to be detectable in plasma of normal controls and increased following liver transplantation and in the context of liver damage in the setting of sepsis [12]. However, other liver pathologies were not assessed in this report. Our goal in this pilot study was to determine whether the levels of these liver-specific unmethylated CpGs are increased in the plasma cfDNA of PBC and PSC patients relative to controls and in late-stage versus early-stage disease, as a means to evaluate their potential utility as novel disease biomarkers.

Methods

Study subjects

The study was approved by the Mayo Clinic Institutional Review Board and conforms to standards laid out in the Declaration of Helsinki. All participants provided written informed consent. Patients with PSC were selected from the PSC Resource of Genetic Risk, Environment and Synergy Studies (PROGRESS) [15] and patients with PBC were participants of the Mayo Clinic PBC Genetic Epidemiology Registry and Biospecimen repository [16]. As age and sex distributions differ between PBC and PSC, separate control populations with no history of liver disease were selected for each disease from the aforementioned resources. The diagnosis of PSC and PBC was based on standard clinical, biochemical, cholangiographic and histological criteria [17, 18]. PBC and PSC patients were selected to equally represent early and

late disease stages. For PSC, late disease was defined as having serum alkaline phosphatase (ALP) greater than 3 times the upper limit of normal (ULN) and/or bilirubin greater than 2.5 mg/dL at time of sample collection or progression to OLT within 4 years of follow up. Late PBC was defined similarly, although bilirubin values were not available. Early PSC and PBC was defined as having ALP less than 1.1 times the ULN at sample collection with no evidence of elevated bilirubin, cirrhosis or OLT in follow up.

Plasma and cfDNA preparation

Plasma samples were collected in EDTA-containing tubes and stored at -80°C prior to use. Thawed samples were centrifuged two times for 10 min at 1500 rpm at 4°C to remove cellular debris and the supernatant was stored at -80°C prior to further processing. cfDNA was extracted from 2 ml of plasma using the Qiagen Cell-Free DNA (cfDNA) Purification Kit (Qiagen) and cfDNA concentration was measured using Qubit (Thermo Scientific). The cfDNA was then treated with bisulfite using the Zymo Research- EZ DNA Methylation-Gold™ Kit (Zymo Research) following the manufactures recommended protocol.

Next generation sequencing

Bisulfite-treated DNA was PCR (multiplex) amplified using the Qiagen multiplex PCR kit (Qiagen) using primers specific for bisulfite-treated DNA but independent of methylation status at 16 monitored CpG sites in the vicinity of IGF2R (6 CpGs), VTN (5 CpGs) and ITIH4 (4 CpGs), which are specifically unmethylated in liver tissue, as described previously [12]. Primer sequences were, IGF2R: L: TGGGTGTTGTTATTTTGTGTTGA and R: CTACAAAATACACACCCCAA (94 bp); ITIH4: L: ATAGTGAAGATGTTAGTTTGTGTTTTT and R: AACACACTTACCTAATAACCAAAC (137 bp); VTN: L: GGTATTTTGAAGAGGTAGGTTT and R: ACCTAAATACCCCAAACACTCAT (108 bp) and CpG locations are provided in Table 1. PCR products were cleaned with ExoSap-IT (Thermo Scientific) and sent to the genome analysis core at Mayo Clinic for library preparation and sequencing. Quality and quantity of amplicon DNA were analyzed by Qubit (Thermo Scientific) and bioanalyzer (Agilent). Individual paired end libraries were prepared using the NEBUltra II kit (New England Biolabs) without DNA fragmentation. As the combined read length of the 3 multiplex amplicons were only 339 bp, each disease/control group of 96 samples were barcoded and sequenced on a single lane of a MiSeq sequencer (Illumina).

Table 1 CpGs targeted in the NGS assay

CpG name	Chromosome	Location ^a	Read count median (IQR)	Unmethylated ratio median (IQR)
ITI4-1	3	52,830,996	36,680 (31,875–44,483)	0.280 (0.216–0.346)
ITI4-2	3	52,831,009	36,718 (31,854–44,520)	0.035 (0.012–0.067)
ITI4-3	3	52,831,051	36,624 (31,843–44,426)	0.089 (0.064–0.129)
ITI4-4	3	52,831,059	36,420 (31,637–44,149)	0.099 (0.075–0.137)
IGF2R-1	6	160,079,556	38,527 (31,251–44,719)	0.049 (0.025–0.077)
IGF2R-2	6	160,079,562	38,498 (31,238–44,699)	0.041 (0.023–0.066)
IGF2R-3	6	160,079,586	38,584 (31,288–44,758)	0.033 (0.015–0.062)
IGF2R-4	6	160,079,588	38,574 (31,292–44,757)	0.038 (0.019–0.066)
IGF2R-5	6	160,079,593	38,564 (31,255–44,715)	0.030 (0.015–0.056)
IGF2R-6	6	160,079,595	37,314 (30,258–43,314)	0.035 (0.020–0.069)
IGF2R-7	6	160,079,606	36,947 (30,090–43,003)	0.033 (0.021–0.067)
VTN-1	17	28,369,323	10,402 (8209–13,100)	0.064 (0.030–0.108)
VTN-2	17	28,369,333	10,365 (8187–13,077)	0.045 (0.014–0.086)
VTN-3	17	28,369,339	10,646 (8210–13,097)	0.049 (0.019–0.095)
VTN-4	17	28,369,351	10,379 (8195–13,060)	0.052 (0.023–0.092)
VTN-5	17	28,369,368	9058 (6937–11,259)	0.062 (0.030–0.109)

^a HG38 human genome coordinates

Bioinformatics and statistical data analysis

Adapter sequences were trimmed from the de-multiplexed raw sequence data in fastq format using Trim Galore [Trim Galore v0.4.4, https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/]. Paired-end reads greater than 20 bases long after trimming and low quality base removal were aligned to human reference genome hg38 using BSMAP (v2.73) [19] with default parameters, followed by sorting and indexing the aligned BAM files. Methylation data was extracted for uniquely mapped read pairs from aligned bam files by a BSMAP script and the data was merged by CpG position across all samples. Off-target CpG sites were excluded and only the 16 targeted CpGs were analyzed further. CpGs were considered unmethylated if “TG” was read and methylated if “CG” was read. We determined absolute levels of cfDNA in genome equivalents per ml (Geg/ml) as previously described [14]. Briefly, we calculated the unmethylation ratio for each locus by dividing the number of unmethylated reads by the total number of reads for all included CpGs. Then, we multiplied this ratio by the total concentration of cfDNA isolated from the 2 ml plasma sample. Finally, we converted from units of ng/ml to genomic equivalents per ml by multiplying by a factor of 303, assuming the mass of a single haploid genome to be 3.3 picograms. The values obtained represent the amount of liver-specific cfDNA in circulation, as measured for each locus, and were used in downstream analyses. Categorical variables were compared using chi-square or Fisher's exact test and continuous variables were compared using

the Mann–Whitney test whereby values were expressed as median and inter-quartile range (IQR). Correlation between variables was determined by calculating the Pearson correlation coefficient. P-values of 0.05 or less were considered significant.

Results

Patient characteristics

A total of 48 PBC patients and 48 PSC patients were selected and matched to separate groups of 48 unaffected controls based on sex, reported race and age at sample collection. Following data generation, one of the PSC patients was found to be an outlier, having liver-specific DNA levels greater than twofold higher at each locus than all other patients, and was removed from the study, leaving 47 PSC patients. The characteristics of these patient-control groups are presented in Table 2 (PBC) and Table 3 (PSC). The patient groups were further separated into two groups of 24 patients with early- or late-stage disease based on biochemical and clinical data. These groups were well-matched for most parameters, but PBC patients with late-stage disease were younger at diagnosis than those with early disease, median 42.9 years versus 52.1 years, respectively, $p = 0.0185$ (Table 2). In PSC this trend was opposite, with advanced disease patients being diagnosed later than patients in the early disease group, median 46.1 years versus 36.2 years, respectively. However, this difference was not statistically significant, $p = 0.0693$ (Table 3).

Table 2 Characteristics of PBC patients and controls

	Controls (n = 48)	PBC (all) (n = 48)	p-value ^a	PBC (early) (n = 24)	PBC (late) (n = 24)	p-value ^b
Sex, % male	12.5	12.5	> 0.9999	8.3	16.7	0.6662
Race, % Caucasian	98	91.7	0.3616	95.8	87.5	0.6085
Age at sample collection (yrs), median (IQR)	56.3 (48.2–61.0)	56.3 (41.3–56.5)	0.9927	57.8 (53.3–62.2)	51.7 (46.5–61.0)	0.1071
ALP (xULN), median (IQR)	0.60 (0.48–0.73)	1.17 (0.83–4.02)	< 0.0001	0.83 (0.71–0.94)	3.98 (3.17–5.33)	< 0.0001
AMA, (% positive)	0	81.3	< 0.0001	79.2	83.3	> 0.9999
Age at Dx (yrs), median (IQR)	na	49.4 (41.3–56.5)	–	52.1 (46.3–57.4)	42.9 (39.9–51.9)	0.0185
Disease duration (yrs), median (IQR)	na	5.0 (2.0–9.0)	–	4.5 (2.0–9.0)	5.5 (2.0–10.8)	0.6116
Clin. FU (yrs), median (IQR)	na	6.0 (4.0–10.0)	–	8.5 (5.3–10.0)	5.0 (2.0–8.8)	0.0133
UDCA treatment, (%)	na	97.9	–	100	95.8	> 0.9999

^a p-value for control versus PBC (all) comparison

^b p-value for PBC (early) versus PBC (late) comparison; ALP (xULN): alkaline phosphatase expressed as times the upper limit of normal, AMA: anti-mitochondrial antibodies, Clinical FU: clinical follow-up after sample collection, UDCA: ursodeoxycholic acid

Table 3 Characteristics of PSC patients and controls

	Controls (n = 48)	PSC (all) (n = 47)	p-value ^a	PSC (early) (n = 24)	PSC (late) (n = 23)	p-value ^b
Sex, % male	58.3	59.6	> 0.9999	62.5	56.5	0.4158
Race, %Caucasian	100	98.0	0.4947	95.8	100	0.6085
Age at sample collection (yrs), median (IQR)	52.3 (44.1–62.1)	54.3 (32.8–62.1)	0.4847	47.0 (29.8–58.7)	57.4 (42.5–63.5)	0.1599
ALP (xULN), median (IQR)	0.59 (0.47–0.72)	1.03 (0.86–3.65)	< 0.0001	0.86 (0.74–0.95)	3.65 (2.96–5.36)	< 0.0001
Total bilirubin, median (IQR)	na	0.8 (0.5–1.8)	–	0.6 (0.5–0.9)	1.6 (0.8–3.4)	< 0.0001
Age at Dx (yrs), median (IQR)	na	42.4 (28.2–53.9)	–	36.2 (22.0–48.5)	46.1 (34.9–57.5)	0.0693
IBD type	na		–			0.3947
UC, n		36		18	18	
CD, n		2		2	0	
Ind. IBD, n		3		2	1	
None, n		6		2	4	
Disease duration (yrs), median (IQR)	na	6.0 (3.0–12.0)	–	5.0 (2.0–12.5)	6.0 (3.0–12.0)	0.8035
Clin. FU (yrs), median (IQR)	na	5.0 (3.0–6.0)	–	5.0 (4.0–6.8)	4.0 (1.0–6.0)	0.0699
UDCA treatment, (%)	na	70.2	–	69.6	77.3	0.7381

^a p-value for control versus PSC (all) comparison

^b p-value for PSC (early) versus PSC (late) comparison; ALP (xULN): alkaline phosphatase expressed as times the upper limit of normal, IBD: inflammatory bowel disease, UC: ulcerative colitis, CD: Crohn's disease, Ind. IBD: Indeterminate IBD, Clin. FU: clinical follow-up after sample collection, UDCA: ursodeoxycholic acid

Assay performance

We found that the multiplex amplicon-based method provides for very high read counts at each CpG site, with median counts in the range of 10,000 for CpGs in the VTN amplicon and over 30,000 for CpGs in the ITIH4 and IGF2R amplicons (Table 1). The ratios of unmethylated to methylated CpGs were relatively consistent in the CpG sites at IGF2R and VTN across the study population, with median values ranging from 0.030–0.041 to 0.045–0.064, respectively (Table 1). However, the unmethylated ratios of CpGs in ITIH4 were more variable, with one of the CpGs, ITIH4-1, being significantly higher than other evaluated CpGs with a median unmethylation ratio value of 0.280

(Table 1). This suggests either an assay-based artifact or that ITIH4-1 unmethylation may not truly be liver-specific, and thus, it was removed from the analysis. Liver-specific DNA concentrations in our controls seemed to be higher than those in the original report [12], possibly due to minor technical differences in the assay used. Consistent with the previous report [12], we did not detect an influence of age on liver-specific DNA concentration in controls (Fig. 1a). Likewise, age did not influence liver-specific DNA concentration in PBC (Fig. 1b) or PSC (Fig. 1c) patients. Finally, we found that sex did not influence liver-specific DNA levels as measured by all 3 genes: IGF2R (Fig. 2a), ITIH4 (Fig. 2b) and VTN (Fig. 2c).

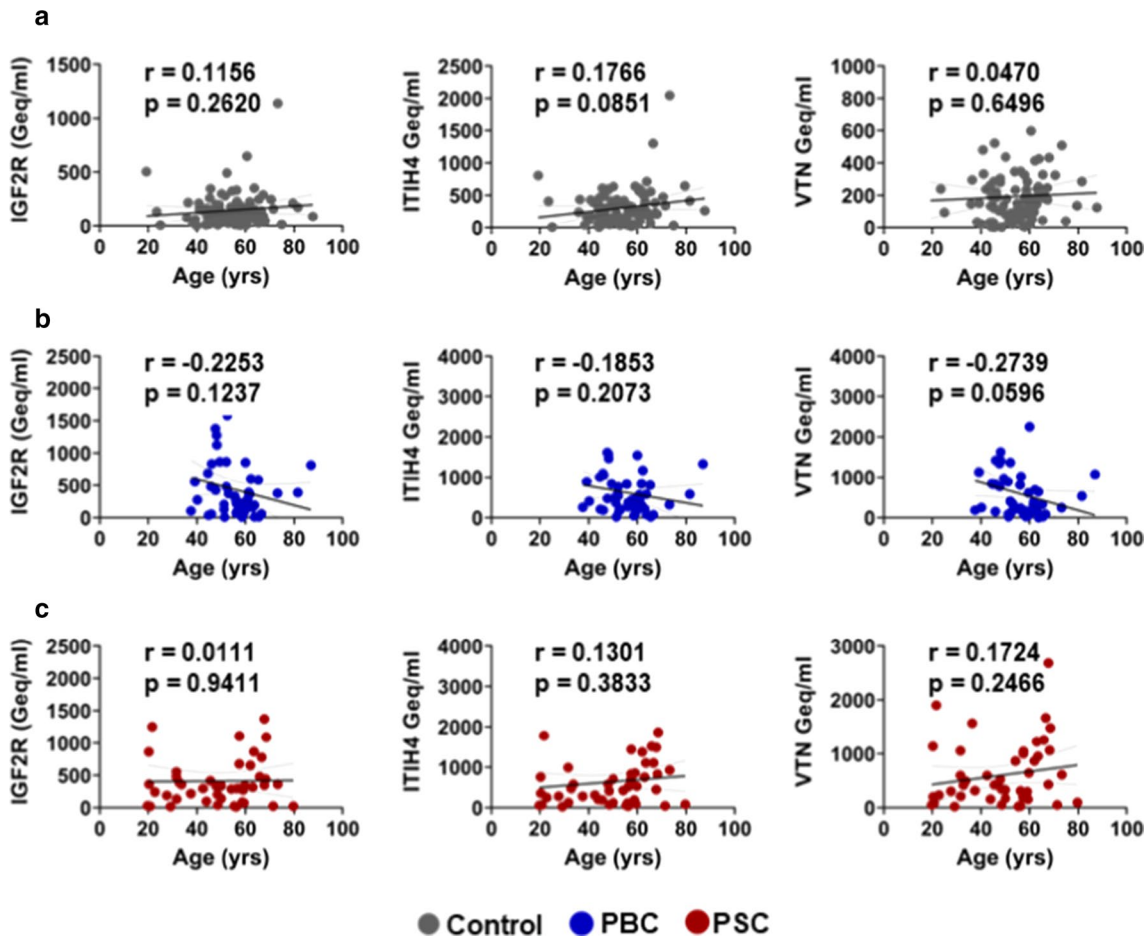


Fig. 1 Lack of correlation between liver-specific cfDNA levels and participant age. Our study did not identify correlation between age and liver-specific DNA levels as measured at all 3 genes: IGF2R, ITIH4 and VTN in **a** Controls, **b** PBC patients or **c** PSC patients. Data presented as a plot of age in years versus cfDNA values expressed as genomic equivalents per ml (Geq/ml), with linear regression line and 95% confidence interval shown. Correlation was evaluated using the Pearson correlation coefficient (*r*)

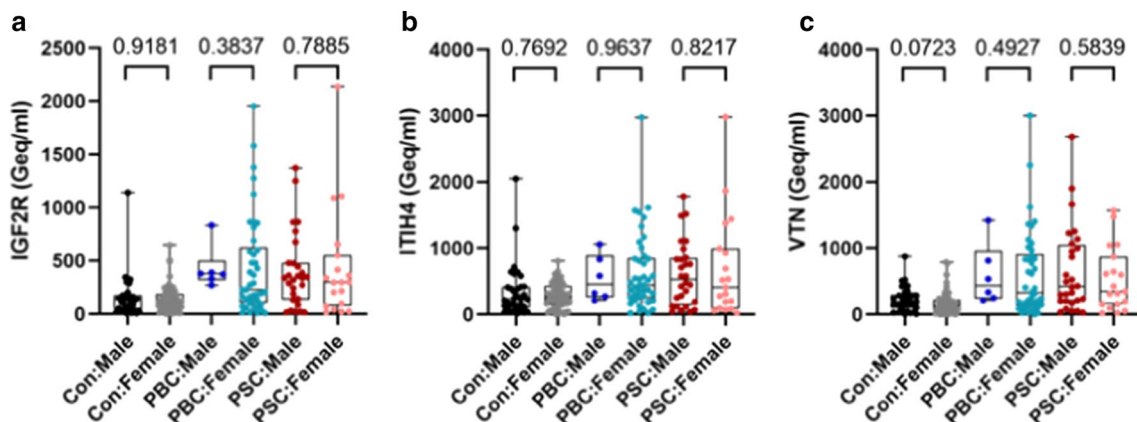


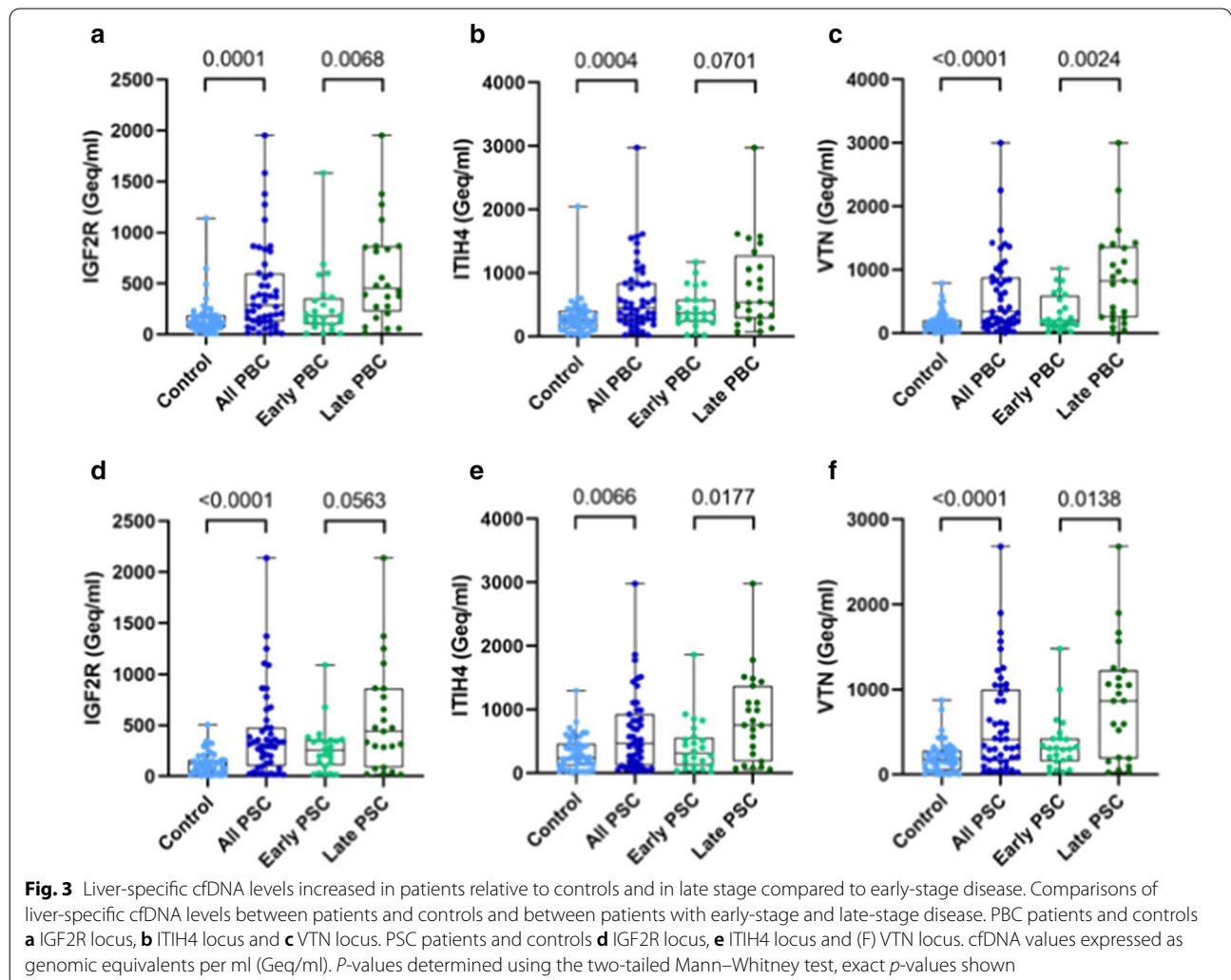
Fig. 2 Lack of correlation between liver-specific cfDNA levels and participant sex. Our study did not identify correlation between sex and liver-specific DNA levels as measured at all 3 genes: IGF2R, ITIH4 and VTN in **a** Controls, **b** PBC patients or **c** PSC patients. cfDNA values expressed as genomic equivalents per ml (Geq/ml). *P*-values determined using the two-tailed Mann–Whitney test, exact *p*-values shown

Liver-specific circulating cfDNA is increased in PBC and PSC patients compared to controls and in late-stage compared to early-stage disease

The liver-specific circulating cfDNA (Geq/ml) values were used to make comparisons between patient and control groups and between patients with early- and late-stage disease. Results of these analyses are shown in Fig. 3. PBC patients demonstrated significantly increased liver-specific DNA compared to controls as measured by all 3 genes, IGF2R: (median (IQR)) 281.93 (126.24–597.61) Geq/ml versus 112.85 (66.71–184.19) Geq/ml, $p=0.0001$ (Fig. 3a), ITIH4: 441.79 (256.40–837.79) Geq/ml versus 256.83 (122.99–399.69) Geq/ml, $p=0.0004$ (Fig. 3b), and VTN: 334.73 (149.76–879.57) Geq/ml versus 133.13 (72.03–202.66), $p<0.0001$ (Fig. 3c). These levels were also significantly increased in PBC patients with late-stage compared to early-stage disease when measured by IGF2R: (median (IQR)) 453.79 (221.47–862.67) Geq/ml versus

180.20 (95.97–355.83) Geq/ml, $p=0.0068$ (Fig. 3a) and VTN: 822.01 (248.81–1359.55) Geq/ml versus 198.29 (114.34–595.37), $p=0.0024$ Geq/ml (Fig. 3c). However, the increase when measured using ITIH4 was not statistically significant (median (IQR)) 539.26 (281.77–1273.28) Geq/ml versus 364.84 (225.98–581.50), $p=0.0701$ (Fig. 3b).

PSC patients also demonstrated significantly increased liver specific DNA compared to controls as measured by all 3 genes, IGF2R: (median (IQR)) 317.46 (96.93–479.11) Geq/ml versus 122.10 (35.65–165.06) Geq/ml, $p<0.0001$ (Fig. 3d), ITIH4 475.76 (124.81–929.76) Geq/ml versus 247.23 (117.87–470.36) Geq/ml, $p=0.0066$ (Fig. 3e), and VTN: 415.12 (170.68–1000.30) Geq/ml versus 184.02 (53.83–285.65) Geq/ml, $p<0.0001$ (Fig. 3f). These levels were also significantly increased in PSC patients with late-stage compared to early stage disease when measured by ITIH4 (median (IQR)): 759.85 (189.59–1377.80) Geq/ml versus 318.34



(121.44–566.00) Geq/ml, $p=0.0177$ (Fig. 3e) and VTN: 867.24 (194.71–1227.01) Geq/ml versus 307.94 (159.98–427.80) Geq/ml, $p=0.0138$ (Fig. 3f). However, the increase when measured using IGF2R was not quite statistically significant (median (IQR)) 443.17 (86.90–862.92) Geq/ml versus 257.40 (105.79–360.62), $p=0.0563$ (Fig. 3d).

Liver-specific circulating cfDNA levels are correlated with alkaline phosphatase levels in PBC and PSC patients but not in controls

Liver function tests, particularly ALP, are often used to evaluate liver damage and disease severity in cholestatic liver diseases such as PBC and PSC [20, 21]. Thus, we evaluated the potential correlation between liver-specific circulating cfDNA and ALP (expressed as times the ULN) using the Pearson correlation coefficient. The results of these analyses are presented in Fig. 4 and show significant correlation between ALP and cfDNA levels as measured by all 3 genes in PBC (Fig. 4a) and PSC (Fig. 4b) but not in controls (Fig. 4c). We also had data available for Total bilirubin, another commonly used liver function test, in the PSC patients and found that those values did not correlate with liver-specific DNA levels as measured by any of the 3 genes (Fig. 4d).

Discussion

Interrogation of organ-specific methylation patterns in circulating cfDNA is an emerging approach with great clinical potential, especially in the setting where traditional means of evaluation require invasive techniques such as biopsy. Such an approach would be particularly valuable for evaluating cholestatic liver diseases such as PBC and PSC as clinical guidelines do not recommend routine use of biopsy in these conditions due to risk of complications related to this invasive procedure. Here we demonstrate that liver-specific circulating cfDNA methylation patterns are increased in PBC and PSC patients relative to control groups and in late-stage compared to early-stage disease. As well, we demonstrate that the cfDNA levels correlate with ALP, a commonly used biochemical test to evaluate disease severity in PBC and PSC. Together, these findings suggest cfDNA assays may have potential clinical utility in cholestatic liver disease.

The bulk of research into the use of circulating cfDNA to evaluate disease has focused on noninvasive tumor evaluation [7], prenatal testing [22] and solid organ transplantation [23]; primarily exploiting differences in DNA sequence. Studies relying on organ-specific DNA methylation patterns have recently become more practical and are showing promise in a wide range of diseases including diabetes [24], cardiovascular disease [25] and neurodegenerative disorders [26]. Utility of cfDNA in the

context of liver transplantation [27, 28] and other liver diseases including Hepatitis B [29], nonalcoholic fatty liver disease [30] and hepatocellular carcinoma [31] has been reported. However, to our knowledge, there has not been another study looking at the potential of cfDNA as a biomarker in PSC and PBC.

In our study we focus on an assay that interrogates CpGs at 3 genetic loci that were previously reported to be specifically unmethylated in the liver. For the genes IGF2R and ITIH4 the unmethylated state was described to be specific to hepatocytes, while VTN was unmethylated in both hepatocytes and cholangiocytes (i.e., biliary epithelial cells) [12]. However, methylation state of the CpGs in other major liver-resident cell-types such as Kupffer cells, liver sinusoid epithelial cells and hepatic stellate cells was not reported, and thus, a small proportion of the signal could be coming from these cells. Bile acid induced hepatocellular injury due to ongoing cholestasis has been long appreciated as a pathological feature of PBC and PSC [32] and the precise mechanisms of how this occurs are becoming more clear [33]. Thus, monitoring hepatocyte death as a proxy for ongoing disease activity is a valid approach, which our data supports. However, the use of cholangiocyte-specific epigenetic marks may prove more beneficial, particularly for PBC, in which cholangiocyte apoptosis plays a pivotal role in pathogenesis [34]. Indeed, discovery of cell-type specific epigenetic modifications in cholangiocytes and other liver-resident cells should be a priority for future studies seeking to utilize cfDNA to monitor cholestatic and other liver diseases.

While our study was designed to be able to detect the differences in cfDNA that we describe, there are limitations to our approach. First, we used stored plasma samples collected under variable conditions and thus, there could be the contribution of additional DNA from leukocytes that underwent cell death after sample collection in the cfDNA, potentially diluting the liver-specific signal. To avoid this, future studies should use samples that were purpose-collected using up-to-date methods and appropriate sampling tubes designed for collection of cfDNA. Second, we rely on amplicon-based next-generation sequencing, which is a time-consuming process. Future studies should focus on using emerging approaches such as digital droplet PCR [35], which once optimized can be performed quickly and reproducibly. Finally, there is significant inter-individual variability present in the data. Most notably, we find that some patients with early, and even late stage disease, have liver-specific cfDNA levels at the low end of what is observed in the controls. Whether this was due to variation in sample handling or is influenced by other

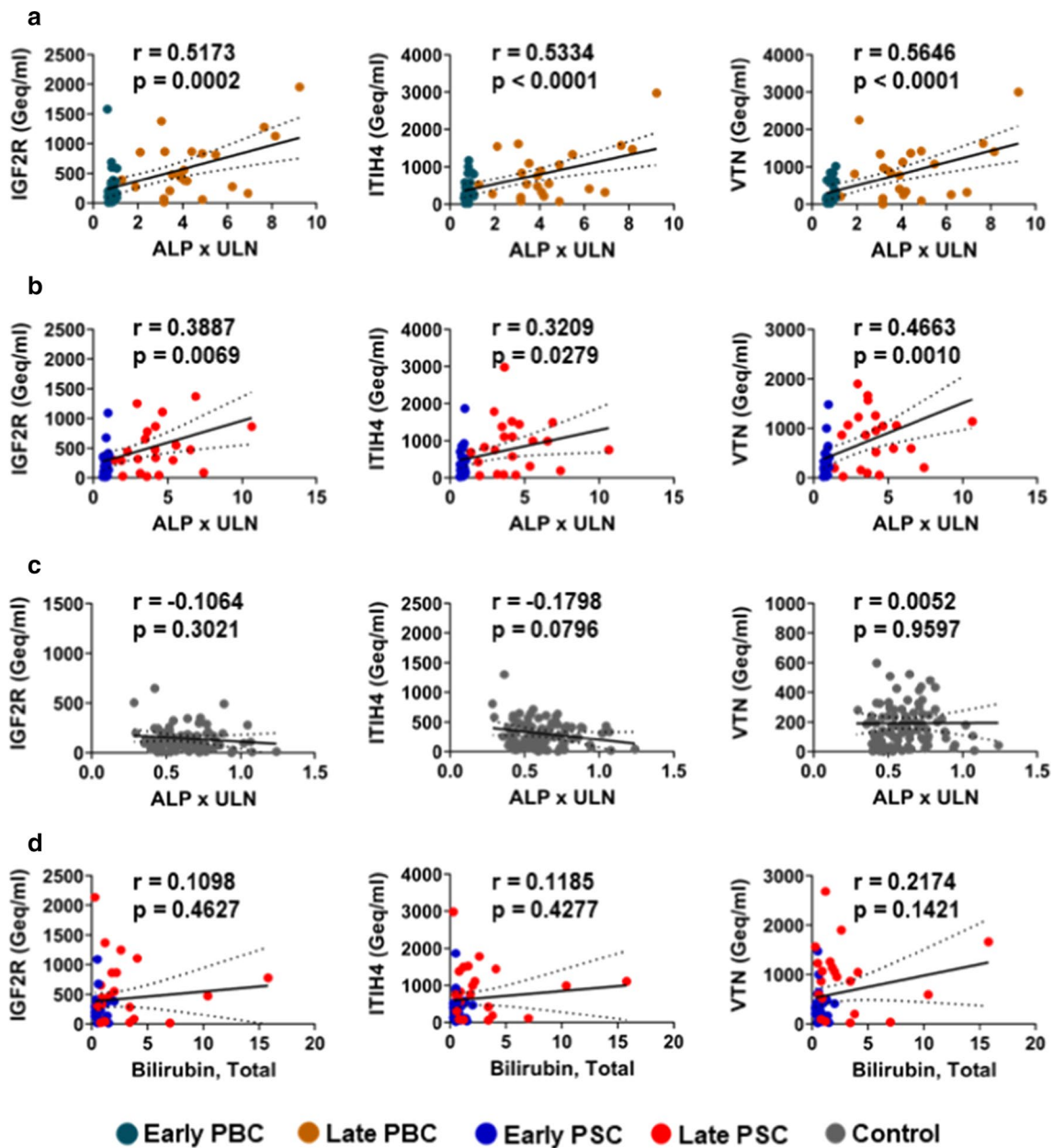


Fig. 4 Correlation between liver-specific cfDNA levels and alkaline phosphatase in PBC and PSC patients. Alkaline phosphatase levels were correlated with liver-specific cfDNA as measured at all 3 genes: IGF2R, ITIH4 and VTN in **a** PBC patients and **b** PSC patients but not in **c** controls. **d** Total bilirubin levels were not correlated with liver-specific cfDNA levels in PSC patients. Data presented as a plot of alkaline phosphatase expressed as times the upper limit of normal (ALP x ULN) or Bilirubin, Total versus cfDNA values expressed as genomic equivalents per ml (Geq/ml), with linear regression line and 95% confidence interval shown. Correlation was evaluated using the Pearson correlation coefficient (r)

factors such as ursodeoxycholic acid treatment remains to be determined. Larger studies, purpose-designed to

evaluate such effects and the extent of intra-individual variability in cfDNA measurements over time will be needed to inaugurate clinical utility of cfDNA in PBC and PSC.

Conclusions

In conclusion, cfDNA offers promise to become a non-invasive liquid-biopsy to evaluate liver-specific cell-death in patients with cholestatic and possibly other liver diseases. However, several challenges need to be overcome before this technology is ready for routine clinical use.

Acknowledgements

We are indebted to the patients and controls that provided biological samples for this study.

Authors' contributions

SP, BDJ and KNL conceived the study and prepared the manuscript. SP performed the wet-lab bench work. SP, BDJ, RMM and ZS analyzed the data and prepared figures. EMS recruited and consented participants. AHA and KNL compiled and reviewed clinical data. All authors read and approved the final manuscript.

Funding

This study was supported by the NIH (RC2 DK118619 to K.N.L.) and the Chris M. Carlos and Catharine Nicole Jockisch Carlos Endowment Fund in Primary Sclerosing Cholangitis (PSC) (K.N.L.).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

All participants provided written informed consent. The study was approved by the Mayo Clinic Institutional Review Board (IRB protocol 18-010735) and conforms to standards laid out in the Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹ Division of Gastroenterology and Hepatology, College of Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55905, USA. ² Division of Biomedical Statistics and Informatics, Mayo Clinic, Rochester, MN 55905, USA.

Received: 4 February 2021 Accepted: 25 March 2021

Published online: 01 April 2021

References

- Boonstra K, Beuers J, Ponsioen CY. Epidemiology of primary sclerosing cholangitis and primary biliary cirrhosis: a systematic review. *J Hepatol*. 2012;56:1181–8. <https://doi.org/10.1016/j.jhep.2011.10.025>.
- Ali AH, Tabibian JH, Lindor KD. Update on pharmacotherapies for cholestatic liver disease. *Hepatol Commun*. 2017;1:7–17. <https://doi.org/10.1002/hep4.1013>.
- Lazaridis KN, LaRusso NF. Primary sclerosing cholangitis. *N Engl J Med*. 2016;375:1161–70. <https://doi.org/10.1056/NEJMra1506330>.
- Leung KK, Deeb M, Hirschfeld GM. Review article: pathophysiology and management of primary biliary cholangitis. *Aliment Pharmacol Ther*. 2020. <https://doi.org/10.1111/apt.16023>.
- Kouroumalis E, Samonakis D, Voumvouraki A. Biomarkers for primary biliary cholangitis: current perspectives. *Hepat Med*. 2018;10:43–53. <https://doi.org/10.2147/HMER.S135337>.
- Bakhshi Z, et al. An update on primary sclerosing cholangitis epidemiology, outcomes and quantification of alkaline phosphatase variability in a population-based cohort. *J Gastroenterol*. 2020;55:523–32. <https://doi.org/10.1007/s00535-020-01663-1>.
- Heitzer E, Haque IS, Roberts CES, Speicher MR. Current and future perspectives of liquid biopsies in genomics-driven oncology. *Nat Rev Genet*. 2019;20:71–88. <https://doi.org/10.1038/s41576-018-0071-5>.
- Baker H, DeAngelis B, Frank O. Vitamins and other metabolites in various sera commonly used for cell culturing. *Experientia*. 1988;44:1007–10. <https://doi.org/10.1007/BF01939904>.
- Dwivedi DJ, et al. Prognostic utility and characterization of cell-free DNA in patients with severe sepsis. *Crit Care*. 2012;16:R151. <https://doi.org/10.1186/cc11466>.
- Duvvuri B, Lood C. Cell-free DNA as a biomarker in autoimmune rheumatic diseases. *Front Immunol*. 2019;10:502. <https://doi.org/10.3389/fimmu.2019.00502>.
- Stawski R, et al. Decreased integrity of exercise-induced plasma cell free nuclear DNA—negative association with the increased oxidants production by circulating phagocytes. *Sci Rep*. 2019;9:15970. <https://doi.org/10.1038/s41598-019-52409-w>.
- Lehmann-Werman R, et al. Monitoring liver damage using hepatocyte-specific methylation markers in cell-free circulating DNA. *JCI Insight*. 2018. <https://doi.org/10.1172/jci.insight.120687>.
- Liu X, et al. Comprehensive DNA methylation analysis of tissue of origin of plasma cell-free DNA by methylated CpG tandem amplification and sequencing (MCTA-Seq). *Clin Epigenetics*. 2019;11:93. <https://doi.org/10.1186/s13148-019-0689-y>.
- Moss J, et al. Comprehensive human cell-type methylation atlas reveals origins of circulating cell-free DNA in health and disease. *Nat Commun*. 2018;9:5068. <https://doi.org/10.1038/s41467-018-07466-6>.
- Ji SG, et al. Genome-wide association study of primary sclerosing cholangitis identifies new risk loci and quantifies the genetic relationship with inflammatory bowel disease. *Nat Genet*. 2017;49:269–73. <https://doi.org/10.1038/ng.3745>.
- Juran BD, et al. ImmunoChip analyses identify a novel risk locus for primary biliary cirrhosis at 13q14, multiple independent associations at four established risk loci and epistasis between 1p31 and 7q32 risk variants. *Hum Mol Genet*. 2012;21:5209–21. <https://doi.org/10.1093/hmg/dds359>.
- Chapman R, et al. Diagnosis and management of primary sclerosing cholangitis. *Hepatology*. 2010;51:660–78. <https://doi.org/10.1002/hep.23294>.
- Lindor KD, Bowlus CL, Boyer J, Levy C, Mayo M. Primary biliary cholangitis: 2018 practice guidance from the American Association for the Study of Liver Diseases. *Hepatology*. 2019;69:394–419. <https://doi.org/10.1002/hep.30145>.
- Xi Y, Li W. BSMAP: whole genome bisulfite sequence MAPPING program. *BMC Bioinform*. 2009;10:232. <https://doi.org/10.1186/1471-2105-10-232>.
- Lammers, W. J. et al. Levels of alkaline phosphatase and bilirubin are surrogate end points of outcomes of patients with primary biliary cirrhosis: an international follow-up study. *Gastroenterology*. **147**, 1338–1349 e1335; quiz e1315. <https://doi.org/10.1053/j.gastro.2014.08.029> (2014).
- Ponsioen CY, et al. Surrogate endpoints for clinical trials in primary sclerosing cholangitis: Review and results from an International PSC Study Group consensus process. *Hepatology*. 2016;63:1357–67. <https://doi.org/10.1002/hep.28256>.
- Rafi I, Hill M, Hayward J, Chitty LS. Non-invasive prenatal testing: use of cell-free fetal DNA in Down syndrome screening. *Br J Gen Pract*. 2017;67:298–9. <https://doi.org/10.3399/bjgp17X691625>.
- Knight, S. R., Thorne, A. & Lo Faro, M. L., Donor-specific cell-free DNA as a biomarker in solid organ transplantation. A systematic review. *Transplantation*. **103**, 273–283, <https://doi.org/10.1097/TP.0000000000002482> (2019).
- Akirav EM, et al. Detection of beta cell death in diabetes using differentially methylated circulating DNA. *Proc Natl Acad Sci USA*. 2011;108:19018–23. <https://doi.org/10.1073/pnas.1111008108>.
- Polina IA, Ilatovskaya DV, DeLeon-Pennell KY. Cell free DNA as a diagnostic and prognostic marker for cardiovascular diseases. *Clin Chim Acta*. 2020;503:145–50. <https://doi.org/10.1016/j.cca.2020.01.013>.
- Lehmann-Werman R, et al. Identification of tissue-specific cell death using methylation patterns of circulating DNA. *Proc Natl Acad Sci USA*. 2016;113:E1826–1834. <https://doi.org/10.1073/pnas.1519286113>.
- Goh SK, et al. The measurement of donor-specific cell-free DNA identifies recipients with biopsy-proven acute rejection requiring treatment after

- liver transplantation. *Transpl Direct*. 2019;5:e462. <https://doi.org/10.1097/TXD.0000000000000902>.
28. Krenzien F, et al. Increased cell-free DNA plasma concentration following liver transplantation is linked to portal hepatitis and inferior survival. *J Clin Med*. 2020. <https://doi.org/10.3390/jcm9051543>.
 29. Xia WY, et al. Liquid biopsy for non-invasive assessment of liver injury in hepatitis B patients. *World J Gastroenterol*. 2019;25:3985–95. <https://doi.org/10.3748/wjg.v25.i29.3985>.
 30. Hardy T, et al. Plasma DNA methylation: a potential biomarker for stratification of liver fibrosis in non-alcoholic fatty liver disease. *Gut*. 2017;66:1321–8. <https://doi.org/10.1136/gutjnl-2016-311526>.
 31. Hlady RA, et al. Genome-wide discovery and validation of diagnostic DNA methylation-based biomarkers for hepatocellular cancer detection in circulating cell free DNA. *Theranostics*. 2019;9:7239–50. <https://doi.org/10.7150/thno.35573>.
 32. Guicciardi ME, Malhi H, Mott JL, Gores GJ. Apoptosis and necrosis in the liver. *Compr Physiol*. 2013;3:977–1010. <https://doi.org/10.1002/cphy.c120020>.
 33. Cai SY, et al. Bile acids initiate cholestatic liver injury by triggering a hepatocyte-specific inflammatory response. *JCI Insight*. 2017;2:e90780. <https://doi.org/10.1172/jci.insight.90780>.
 34. Lleo A, Maroni L, Glaser S, Alpini G, Marziani M. Role of cholangiocytes in primary biliary cirrhosis. *Semin Liver Dis*. 2014;34:273–84. <https://doi.org/10.1055/s-0034-1383727>.
 35. Shemer R, Magenheim J, Dor Y. Digital droplet PCR for monitoring tissue-specific cell death using DNA methylation patterns of circulating cell-free DNA. *Curr Protoc Mol Biol*. 2019;127:e90. <https://doi.org/10.1002/cpmb.90>.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:

- fast, convenient online submission
- thorough peer review by experienced researchers in your field
- rapid publication on acceptance
- support for research data, including large and complex data types
- gold Open Access which fosters wider collaboration and increased citations
- maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.

Learn more biomedcentral.com/submissions

