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The Serum Proteome and Ursodeoxycholic Acid Response in Primary Biliary Cholangitis

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BACKGROUND AND AIMS: Stratified therapy has entered clinical practice in primary biliary cholangitis (PBC), with routine use of second-line therapy in nonresponders to first-line therapy with ursodeoxycholic acid (UDCA). The mechanism for nonresponse to UDCA remains, however, unclear and we lack mechanistic serum markers. The UK-PBC study was established to explore the biological basis of UDCA nonresponse in PBC and identify markers to enhance treatment.

APPROACH AND RESULTS: Discovery serum proteomics (Olink) with targeted multiplex validation were carried out in 526 subjects from the UK-PBC cohort and 97 healthy controls. In the discovery phase, untreated PBC patients (n = 68) exhibited an inflammatory proteome that is typically reduced in scale, but not resolved, with UDCA therapy (n = 416 treated patients). Nineteen proteins remained at a significant expression level (defined using stringent criteria) in UDCA-treated patients, six of them representing a tightly linked profile of chemokines (including CCL20, known to be released by biliary epithelial cells (BECs) undergoing senescence in PBC). All showed significant differential expression between UDCA responders and nonresponders in both the discovery and validation cohorts. A linear discriminant analysis, using serum levels of C-X-C motif chemokine ligand 11 and C-C motif chemokine ligand 20 as markers of responder status, indicated a high level of discrimination with an AUC of 0.91 (CI, 0.83-0.91).

CONCLUSIONS: UDCA under-response in PBC is characterized by elevation of serum chemokines potentially related to cellular senescence and was previously shown to be released by BECs in PBC, suggesting a potential role in the pathogenesis of high-risk disease. These also have potential for development as biomarkers for identification of high-risk disease, and their clinical utility as biomarkers should be evaluated further in prospective studies. (Hepatology 2021;0:1-15).

Primary biliary cholangitis (PBC) is a chronic cholestatic liver disease with a significant inflammatory/immune component.[1] The condition is characterized by damage to, and eventual destruction of, the small intrahepatic bile ducts. Duct

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; BECs, biliary epithelial cells; CCL19, C-C motif chemokine ligand 19; CCL20, C-C motif chemokine ligand 20; CXCL9, C-X-C motif chemokine ligand 9; CXCL10, C-X-C motif chemokine ligand 10; CXCL11, C-X-C motif chemokine ligand 11; CXCL13, C-X-C motif chemokine ligand 13; EpCAM, epithelial cell adhesion molecule; GO, Gene Ontology; NPX, normalized protein expression; PBC, primary biliary cholangitis; PPI, protein-protein interaction; ROC, receiver operating characteristic; UDCA, ursodeoxycholic acid.

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**These authors made an equal contribution.*

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damage is accompanied by portal inflammation, and interface hepatitis is frequently observed in patients with more-aggressive disease.[1] The disease is thought to arise because of a complex interplay of the cholestatic and immune processes. Standard therapy in PBC now uses a stratified approach.[2] Cholestatic injury, with damage to the biliary epithelial cells (BECs) lining the small intrahepatic bile ducts and eventual ductopenia, are the hallmarks of PBC and are thought to be mediated, at least in part, by hydrophobic bile acids. BEC apoptosis and senescence can both be observed within the affected liver.[3,4] Immune response in PBC is characterized by the almost universal presence of autoantibody and -reactive T-cell responses.[5] Autoreactive B- and T-cell responses in PBC have been well characterized, with autoreactivity against the highly conserved mitochondrial self-antigen, pyruvate dehydrogenase, being almost universal in patients.[6,7] Localization of $CD8^+$ T-cell responses directed at mitochondrial antigens around affected ducts,[8] and the particular enrichment observed in early stages of the disease, point to a key role for the autoreactive immune response in PBC pathogenesis and a potential inter-relationship with bile duct injury processes. Understanding the inter-relationship between cholestatic and immune elements of the disease will be key as we move forward in terms of disease therapy.

First-line therapy for all patients is with ursodeoxycholic acid (UDCA),[9] with second-line agents, such as obeticholic acid (OCA) and bezafibrate, being introduced in patients showing an inadequate response to UDCA.[10,11] Despite the apparent role played by immune processes in PBC pathogenesis, both first- and second-line treatment approaches currently recommended for PBC principally target the cholestasis component of the disease. To date, immunotherapy trials have proved disappointing.[12-14] At present, adequacy of response to UDCA, and thus the need for second-line therapy, is assessed using blood biochemical markers of ongoing liver injury, typically alkaline phosphatase (ALP) and bilirubin. The UK-PBC program was established to recruit a large cohort of fully characterized PBC patients in the UK in order to study disease mechanisms, with a particular focus on why an important subgroup of patients do not respond to UDCA. One of the primary aims was to develop better tools for early identification of this subgroup.

In this two-stage study, we explored the serum inflammatory/immune proteome in PBC and its relationship to UDCA treatment response and nonresponse. There were three groups of participants: treatment-naïve, newly presenting patients; patients established on UDCA therapy, stratified into UDCA responders and nonresponders; and healthy, age-matched controls. Evaluating PBC from the beginning of its development, we aimed to identify the roles of key analytes in the different cohorts, differences between the cohorts, and what this could mean for downstream therapies.

Participants and Methods

STUDY DESIGN AND SUBJECTS

The aim of the study was to explore the serum proteome of untreated and UDCA-treated PBC patients

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to inform our understanding of the biology of the disease. In the discovery phase of the study, the serum proteomic profiles of informative subject groups from the UK-PBC patient cohort were assessed. These groups were treatment-naïve PBC patients (i.e., newly presenting patients before commencement of UDCA therapy; n = 68), PBC patients treated for a minimum of 12 months with UDCA therapy at an optimal dose and stratified for UDCA response (n = 416), and healthy community controls (n = 97). In the validation stage, putative UDCA-response chemokine markers identified in the discovery phase were validated in a second, fully independent cohort of PBC patients stratified for UDCA response status recruited from the Newcastle clinical cohort (n = 46).

PBC study participants in the discovery phase were all members of the UK-PBC Cohort (www.UK-PBC.com). This cohort was established to undertake studies of treatment efficacy in PBC to understand, in particular, the biological basis of high-risk disease. The details of the UK-PBC patient cohort have been described in detail.[15-21] This is a large, prospective, national, cross-sectional cohort of PBC patients with detailed clinical data collection. Within the cohort is a nested subcohort with additional biofluid sampling and banking to accompany the clinical data collection.[22] The current study used samples from this subcohort. The validation cohort were patients under clinical follow-up in Newcastle for PBC who were not participants in the UK-PBC nested cohort study. All diagnoses of PBC were made on the basis of the standard criteria used in clinical practice, namely the presence of at least two of the three key PBC characteristics of cholestatic liver biochemistry, positive serum antimitochondrial or PBC-specific antinuclear antibodies at a titer of >1 in 40, and compatible or diagnostic features on liver biopsy.[2] Subjects were classified as being patients with cirrhosis if they had biopsy confirmation of cirrhosis, varices, ascites, or a FibroScan value >17.3 KPa.[23]

UDCA-Treated PBC

These were steady-state PBC patients who had been taking UDCA at a therapeutic dose (13-15 mg/kg/d) for >12 months. Clinical parameters, including serum biochemistry, were available both pretreatment and at the study point. This allowed all UDCA-treated patients to be assigned to responder and nonresponder groups using the POISE (Phase 3 Study of Obeticholic Acid in Patients With Primary Biliary Cirrhosis) criteria (ALP <1.67 × upper limit of normal [ULN] and bilirubin <1 × ULN).[10] UK-PBC risk score 10-year predicted survival was also calculated for all UDCA-treated patients after therapy.[19] Patients taking fibrates and or OCA were not included in the current study.

Treatment-Naïve PBC

These were newly presenting PBC patients sampled before the commencement of UDCA or any other PBC-related therapy.

Healthy Controls

These were healthy community controls, matched at the group level for age and sex to the treatment-naïve PBC patients. This cohort was recruited by the NIHR Cambridge BioResource specifically for comparison with the UK-PBC Nested Cohort and was used in the discovery phase of the study.

METHODS

Blood was collected under the approval of the Human Tissue Authority by the UK-PBC tissue bioresource with written informed patient consent obtained in accordance with research and ethics committee approval (14/NW/1146). Serum samples, once collected, were stored at −80°C until assayed using the Olink proteomics platform (www.olink.com). Serum (4 μL) was analyzed for 368 analytes from four distinct and complementary panels, including Cardiovascular II & III, Inflammation and Oncology II (www.olink.com/downloads). Overall, 356 of 368 analytes passed a quality-control threshold of >25% detectability. Using Proximity Extension Assay technology, antibodies labeled with oligonucleotides were hybridized to sample antigens, generating a barcode. In turn, this was amplified by microfluidic qPCR with normalized protein expression (NPX) data generated by a delta delta cycle threshold calculation method.

For the validation study, we measured levels of informative chemokines identified in the discovery stage as being associated with UDCA nonresponse (C-X-C motif chemokine ligand 9 [CXCL9; monokine induced by gamma interferon], C-X-C motif chemokine ligand 10 [CXCL10; interferon

gamma-induced protein 10], C-X-C motif chemokine ligand 11 [CXCL11; interferon-inducible T-cell alpha chemoattractant], C-X-C motif chemokine ligand 13 [CXCL13; B-cell-attracting chemokine 1], C-C motif chemokine ligand 19 [CCL19; macrophage inflammatory protein-3-beta], and C-C motif chemokine ligand 20 [CCL20; macrophage inflammatory protein-3-alpha]) using the Meso Scale Discovery (MSD, Rockville, MD) electrochemiluminescence assay platform. Specifically, we created a custom multi-PLEX panel for detecting the above analytes using the U-PLEX Custom Biomarker format. In order to retain uniformity and validity, assays were performed exactly as per the manufacturer's instructions. Briefly, each of the biotinylated capture antibodies were covalently linked to specific spots within each well through use of unique linkers. Following a quenching step, plates were ready for sample application. Sera were thawed on ice and clarified by centrifugation at 18,000*g* for 10 minutes at 4°C. Sera were diluted 1:1 with supplied diluent and 25 μL per well added in duplicate. A standard analyte cocktail dose-calibration curve was prepared and 25 μL per well added in duplicate. Plates were incubated for 2 hours at room temperature with shaking (200 RPM). Following a wash step, SULFO-TAG conjugated detection antibody cocktail (50 μL) was added to all wells and plates incubated as before. After washing, read buffer was added (150 μL) and the plates were read on the MSD Sector Imager 6000 instrument. Data were analyzed using MSD Discovery Workbench (v4.0). This software uses four-parameter logistic (FourPL) calibration curve fitting from which sample values were derived. Each analyte had a lower limit of detection below all the sample values so all samples were in range and measurable. All sample values for all analytes are given as pg/mL.

STATISTICAL ANALYSIS

In the discovery study, NPX data were analyzed in R statistical programming software (version 3.5; R Foundation for Statistical Computing, Vienna, Austria), and differential expression between patient cohorts (UDCA-treated PBC, treatment-naïve PBC, and healthy controls [age-matched[) was calculated using the limma package (version 3.36.2; R Foundation for Statistical Computing). This is displayed as log fold change versus $-\log^{10}$ *P* value (adjusted for multiple comparisons); all values were thresholded at $P < 0.05$ and 1.5-fold change. *P* values were adjusted for multiple comparisons using the Benjamini-Hochberg *post hoc* method. Direct comparison of individual analyte NPX data was performed with an unpaired *t* test using Welch's correction (GraphPad Prism software 7.0; GraphPad Software Inc., La Jolla, CA). Multiple linear regression was performed on the NPX data for correlations with ALP and log-transformed UK-PBC 10-year mortality/transplant risk score in the different PBC cohorts, again using the limma package in R. *P* values were adjusted for multiple comparisons as before. Protein-protein interaction (PPI) was analyzed using the STRING platform and the Gene Ontology (GO) classification.[24] Chemokines identified as disease associated in the discovery phase were used as descriptors of UDCA responder status in linear discriminant analysis to identify those chemokines that could be used to optimally discriminate between patient responder status. We used a step-wise forward variable selection method that started by identifying the markers that separated the responders and nonresponders most accurately. The model was then extended by including further variables depending on Wilk's lambda. Wilk's lambda tests how well each marker contributes to the model's ability to discriminate between groups. Each marker was tested by putting it into the model and then taking it out again, generating a Wilks Λ statistic. The significance of the change in Λ was measured with an *F* test. If the *F* value was greater than the critical value for *F* with the sample sizes, then the marker was included in the model and the analysis repeated with the remaining cytokines. Selected markers (all chemokines) were then incorporated into a linear discriminant analysis so that discriminant functions could be abstracted and used to reclassify the original data into responder and nonresponder. Error rate of classification was calculated (the proportion of cases incorrectly assigned to responder and nonresponder groups). Because the data set had comparatively few cases, we used a bootstrapping procedure to estimate the robustness of the final model. For this, we randomly selected 90% of the case data and undertook the discriminant analysis on the subset and reclassified cases within it to responder and nonresponder groups. We repeated this 500 times and calculated the proportion of cases correctly classified. Analyses were repeated with log-transformed data, because the data were skewed with a few patients

with elevated levels of either chemokine. There was an assumption that the data were multivariate normality in using linear discriminant analysis, so we used logistic regression to assess the validity of the variables in the final model as predictors of responder/nonresponder status. After having identified the key discriminators for responder and nonresponder, we used ROC plot analysis to assess the diagnostic potential of the classification because the discrimination threshold was varied. We calculated the AUC for the receiver operating characteristic (ROC) plot as a further measure of the discriminative power of the model. AUC is scaled between 0 and 1; at 0 all cases are incorrectly classified, whereas at 1 all cases are correctly classified. Values between 0.8 and 0.9 are considered excellent. All analyses were undertaken in the R statistical programming language using the libraries KlaR and MASS.

Results

Characteristics of the study cohorts are outlined in Table 1A (discovery cohort) and Table 1B (confirmatory cohort). Of the 416 subjects in the UDCA-treated group in the discovery cohort, 406 had full data allowing UDCA response status to be assessed (97.5%). Of these, 127 (31%) were nonresponders using the POISE criteria. In the validation cohort, all the patients had response data and 41% were UDCA nonresponders. Liver function tests were all normal in healthy controls.

DISCOVERY PROTEOMICS STUDY

Initially, we explored and compared the inflammatory proteome in treatment-naïve PBC patients, UDCA-treated patients, and healthy controls in the discovery study (Fig. 1A-D). A consistent PBC disease profile, in terms of the protein signature in serum, was noted when treatment-naïve PBC patients were compared to healthy controls. Proteins significantly overexpressed in PBC are detailed in Table 2. The inflammatory proteome in UDCA-treated patients was almost identical to treatment-naïve patients, with only a single protein with a different expression pattern between UDCA-treated and treatment-naïve patients (epithelial cell adhesion molecule; EpCAM).

TABLE 1. Clinical Characteristics of the Study Cohorts for (A) the Proteomics Discovery Stage and (B) the Chemokine Confirmatory Stage of the Study

	UDCA-Treated PBC	Treatment-Naïve PBC	Healthy Controls
	(n = 416)	(n = 68)	(n = 97)
(A)			
Median age (years)	63	58	64
Female (%)	89	82	76
With cirrhosis, n (%)	28 (7)	1 (1.5)	na
UDCA treatment (%)	100	0	na
ALP at 1 year (SD)	178.2 (121.5)	226.5 (193.1)	70.9 (22.2)
ALT at 1 year (SD)	37.6 (28.9)	53.1 (34.7)	19.7 (8.6)
Bilirubin at 1 year (SD)	11.4 (10)	11.1 (8.4)	11.2 (5.5)
	UDCA Responder PBC	UDCA Nonresponder	
	(n = 27)	(n = 19)	
(B)			
Median age (years)	65	56	
Female (%)	91	89	
UDCA treatment (%)	100	100	
ALP at 1 year (SD)	80.9 (6.7)	342.3 (22.1)	
ALT at 1 year (SD)	24.3 (4.0)	81.7 (11.9)	
Bilirubin at 1 year (SD)	6.6 (0.5)	15.6 (4.4)	

Abbreviation: na, not applicable.

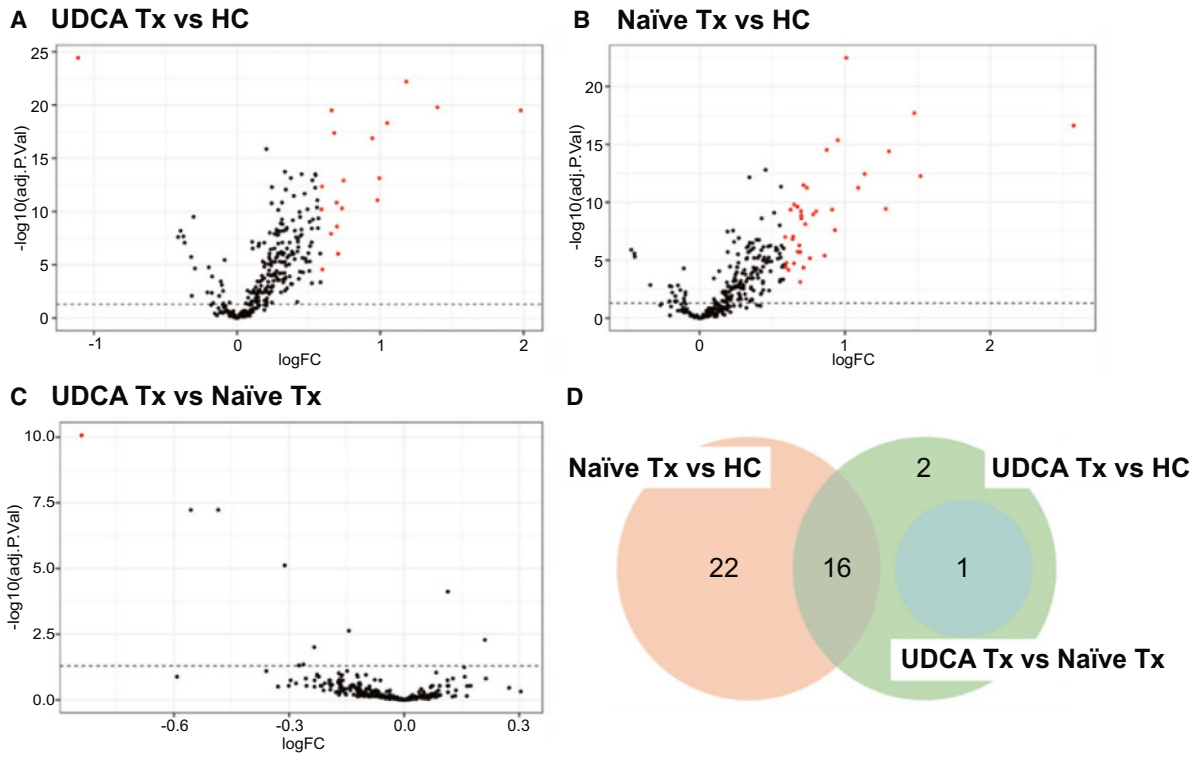

FIG. 1. Differential expression of serum proteins between UDCA-treated PBC, treatment-naïve PBC, and healthy control groups. (A) UDCA Tx (treated; n = 416) versus HC (n = 97), (B) naïve Tx (n = 68) versus HC, and (C) UDCA Tx versus naïve Tx. (D) Venn diagram summary of comparison between different cohorts. All analytes were thresholded for $P < 0.05$ and 1.5-fold change and are presented on a log scale. Red dots are significantly changed analytes >1.5-fold change (FC). *P* values were adjusted for multiple comparisons according to the Benjamini-Hochberg method. Abbreviations: HC, healthy controls; Tx, treatment.

The extent to which analytes were differentially regulated in a similar manner in both groups of PBC patients (Fig. 1D) would suggest that although UDCA treatment reduces expression levels of inflammatory proteins in PBC, it does not change the expression pattern or normalize expression.

We went on to explore the relationship between the PBC proteome and both ALP levels in UDCA-naïve and UDCA-treated patients (Fig. 2A,B; Supporting Table S1) and the UK-PBC Risk Score 10-year predicted risk of death or transplant (Fig. 2C; Supporting Table S2). Ninety-two analytes in the treatment-naïve cohort and 184 analytes in the UDCA-treated cohort showed a significant correlation with ALP levels. This finding suggests that ALP levels are associated with disease mechanistic markers in both the treatment-naïve and UDCA-treated states. Similarly, in the UDCA-treated patient cohort, the UK-PBC Risk Score 10-year risk correlated significantly with 138 analytes, validating the score as a mechanistically relevant prognostic tool in PBC. A similar pattern of associations was also observed for the UK-PBC score 5- and 15-year predicted mortality/transplant risk (data not shown).

Significant PPI was observed among the proteins significantly overexpressed in treatment-naïve patients compared to healthy controls (PPI enrichment *P* value, $<10^{-16}$), suggesting a functional expression pattern. The greatest enrichment was observed for cytokine-mediated signalling pathways, inflammatory response, and leucocyte chemotaxis GO terms (Table 3A). Comparison of UDCA-treated PBC patients and healthy controls demonstrated 19 proteins that remained significantly elevated despite UDCA therapy (Supporting Fig. S1), with significant pathway enrichment particularly around chemotaxis where six inter-related chemokines were significantly elevated despite UDCA treatment (CCL19, CCL20, CXCL9, CXCL10, CXCL11, and CXCL13; Table 3B). All six chemokines showed significantly higher expression

TABLE 2. Proteins Showing Significant Differential Expression Between Study Groups in the Discovery Proteomics Stage of the Study

Naïve Tx vs. HC		UDCA Tx vs. Naïve Tx	
Protein Analyte	Log Fold Change	Protein Analyte	Log Fold Change
HAOX1	2.57	EpCAM	–0.84
CCL20	1.52		
CCL19	1.48		
CXCL9	1.30		
CA5A	1.28		
CXCL10	1.14		
CXCL11	1.09		
CD40	1.01		
5′-NT	0.95		
SCAMP3	0.93		
DECR1	0.91		
CXCL13	0.87		
ACE2	0.86		
TNFRSF6B	0.80		
VIM	0.78		
IL8	0.76		
TNFRSF4	0.74		
CXCL6	0.72		
EN-RAGE	0.71		
MetAP2	0.71		
XCL1	0.70		
IL-18R1	0.70		
IL-1ra	0.70		
AP-N	0.70		
AZU1	0.69		
NEMO	0.69		
GDF-15	0.69		
IL-4RA	0.68		
IL16	0.67		
TNFRSF9	0.65		
CCL3	0.65		
IL-12B	0.64		
CDCP1	0.64		
CD163	0.63		
IL6	0.60		
TXLNA	0.59		

Abbreviations: 5′-NT, 5′-nucleotidase; ACE2, angiotensin-converting enzyme 2; AP-N, aminopeptidase N; AZU1, azurocidin 1; CA5A, carbonic anhydrase 5A; CCL3, C-C motif chemokine ligand 3; CDCP1, CUB domain-containing protein 1; CXCL6, C-X-C motif chemokine ligand 6; DECR1, 2,4-dienoyl-CoA reductase; EN-RAGE, extracellular newly identified receptor for advanced glycation end products binding protein; GDF-15, growth/differentiation factor-15; HAOX1, hydroxyacid oxidase 1; IL-1ra, interleukin-1 receptor antagonist; IL-4RA, Interleukin 4 receptor alpha; IL-18R1, interleukin-18 receptor 1; MetAP2, methionyl aminopeptidase 2; NEMO, NF-kappa-B essential modulator; SCAMP3, secretory carrier-associated membrane protein 3; TNFRSF4, TNF receptor superfamily member 4; TNFRSF6B, TNF receptor superfamily member 6b; TNFRSF9, TNF receptor superfamily member 9; Tx, treatment; TXLNA, taxilin alpha; VIM, vimentin; XCL1, X-C motif chemokine ligand 1.

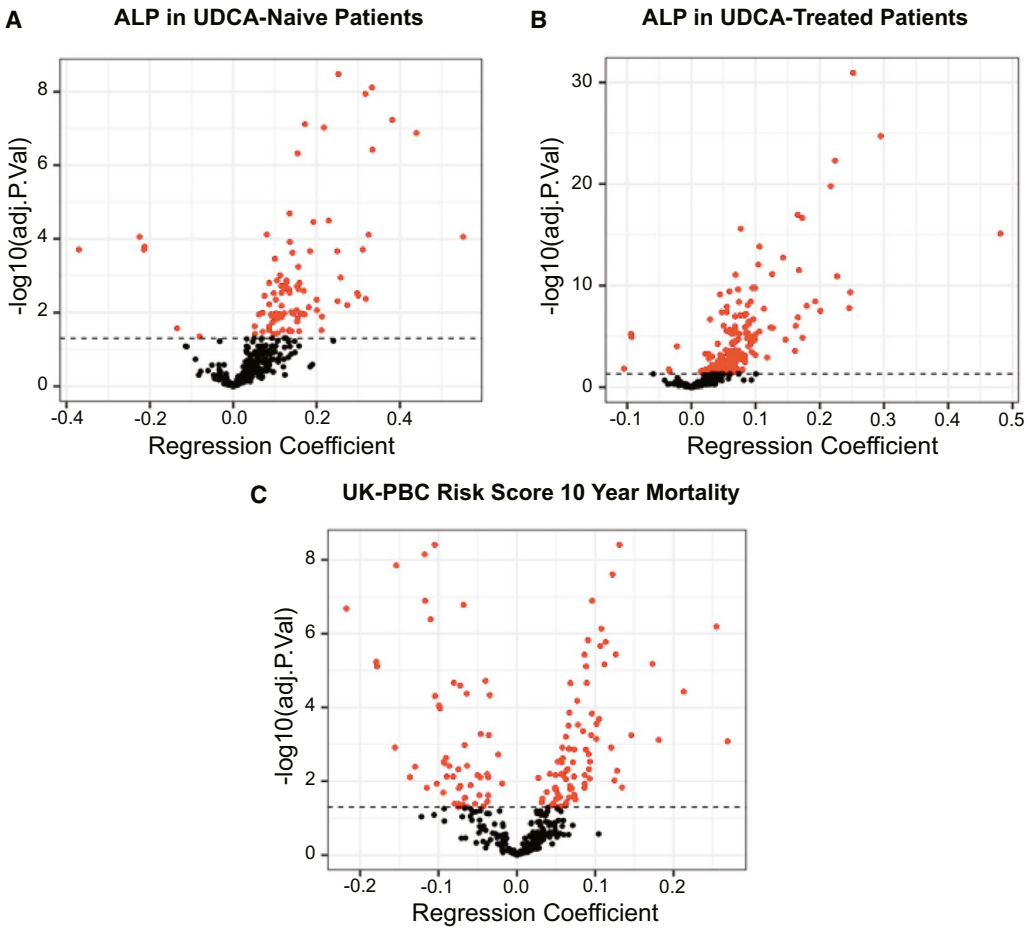

FIG. 2. Correlation of the PBC proteome with (A) ALP in UDCA-naïve patients, (B) ALP in UDCA-treated patients, and (C) log-transformed UK-PBC 10-year mortality/transplant risk in UDCA-treated patients. Serum ALP significantly correlates with a high number of analytes in both treatment-naïve cohorts and UDCA-treated patients, and UK-PBC risk score 10-year mortality risk correlates with analytes in the UDCA-treated group. These findings support a link between these biochemical/clinical markers and the inflammatory process in the disease. *P* values were adjusted for multiple comparisons according to the Benjamini-Hochberg method. Abbreviation: adj.P.Val, adjusted *P* value.

levels in UDCA nonresponders using the POISE criteria than responders (Fig. 3). Significant elevation of all six chemokines was still observed when this analysis was restricted to patients without cirrhosis. This is important given previous reports that cirrhosis *per se* can elevate related chemokine levels (Supporting Table S3). No proteins were significantly elevated in UDCA responders compared to nonresponders.

Correlations were significantly stronger for the six chemokines with ALP than with UK-PBC risk score (Supporting Tables S1 and S2). Exploration of the components of the UK-PBC risk score showed, in addition to the strong association with ALP levels described above, strong associations with alanine aminotransferase (ALT) levels (Supporting Table S3). In contrast, only limited relationships to bilirubin, albumin, and platelet count were observed.

VALIDATION CHEMOKINE STUDY

We next studied the panel of six chemokines identified in the discovery phase of the study as being associated with UDCA nonresponse in a second PBC patient cohort stratified for UDCA response and nonresponse using a multiplex assay system. All six chemokines again had significantly higher levels in UDCA nonresponders compared to responders (Supporting Fig. S2), confirming the original

TABLE 3. Functional Enrichments in the (A) Treatment-Naïve PBC Protein Network and (B) the UDCA-Treated Protein Network in the Discovery Proteomics Stage of the Study

GO Term	Description	False Discovery Rate
(A)		
0019221	Cytokine-mediated signaling pathway	3.6×10^{-22}
0006954	Inflammatory response	7.9×10^{-20}
0030595	Leucocyte chemotaxis	9.1×10^{-18}
0006952	Defense response	4.2×10^{-17}
0006955	Immune response	1.9×10^{-16}
0051707	Response to other organism	4.0×10^{-16}
0050921	Positive regulation of chemotaxis	6.8×10^{-16}
0032496	Response to lps	2.2×10^{-15}
0070098	Chemokine-mediated signaling pathway	8.4×10^{-15}
0002687	Regulation of signaling receptor activity	1.5×10^{-14}
(B)		
0050900	Leucocyte migration	3.4×10^{-9}
0048247	Lymphocyte chemotaxis	3.4×10^{-9}
0031640	Killing of other organism	3.4×10^{-9}
0006954	Inflammatory response	3.4×10^{-9}
0030595	Leucocyte chemotaxis	6.2×10^{-9}
0019730	Antimicrobial humoral response	$9.1 \times \times 10^{-9}$
0070098	Chemokine-mediated signaling pathway	1.4×10^{-8}
0019221	Cytokine-mediated signaling pathway	1.6×10^{-8}
0002687	Positive regulation of leucocyte migration	1.6×10^{-7}
0002690	Positive regulation of leucocyte chemotaxis	1.3×10^{-6}

Data are shown for the 10 GO biological processes showing the greatest enrichment for each.
Abbreviation: lps, lipopolysaccharide.

observation. Furthermore, the level of each of the six chemokines was, individually, highly predictive of UDCA nonresponse/response status (Fig. 4).

Finally, we went on to explore the capacity of chemokine elevation to act as a predictive mechanistic marker for identification of UDCA nonresponse status using linear discriminant analysis in the confirmatory study cohort. Wilks' lambda values for each of the chemokines, and the significance of their discriminating power for separating responders and nonresponders, are shown in Table 4. Only four of the chemokines were good discriminators, and two of these, CXCL9 and CXCL13, did not lead to a significant increase in discrimination when added sequentially to a model that included CCL20 and CXCL11. Reclassification of the original confirmatory study data set led to 40 patients (87% of the cases) being correctly classified, with 2 patients predicted to be nonresponders who were responders and 4 predicted to be responders who were not. A biplot of the nonresponder and responder levels of the two chemokines is shown in Fig. 5A. The classification status is based on use of discriminant functions that effectively find a plane in multivariate space that separates the groups. In the two groups, case values on this line below zero constitute membership of the nonresponder group, and values above zero predicted membership of the responder group. The bootstrapped discriminant analyses, based on 500 repeat random samples of 90% of the data, gave a mean percentage correctly classified as 85.1% (SD = 2.3), suggesting that this is a robust classifier even when the sample size was reduced to 90% of the data. Results of the analyses for the log-transformed data were very similar and are not presented further. A model for predicting the probability of a person being a responder or nonresponder on the basis of their serum levels of CXCL11 and CCL20 can be accessed at https://naturalandenvironmentalscience.shinyapps.io/PBC_responder/. The AUC derived from the ROC plot analysis for the optimal predictive model was 0.91 (CI, 0.83-0.99), indicating discriminatory power of these two chemokines to differentiate between responders and nonresponders (Fig. 5B).

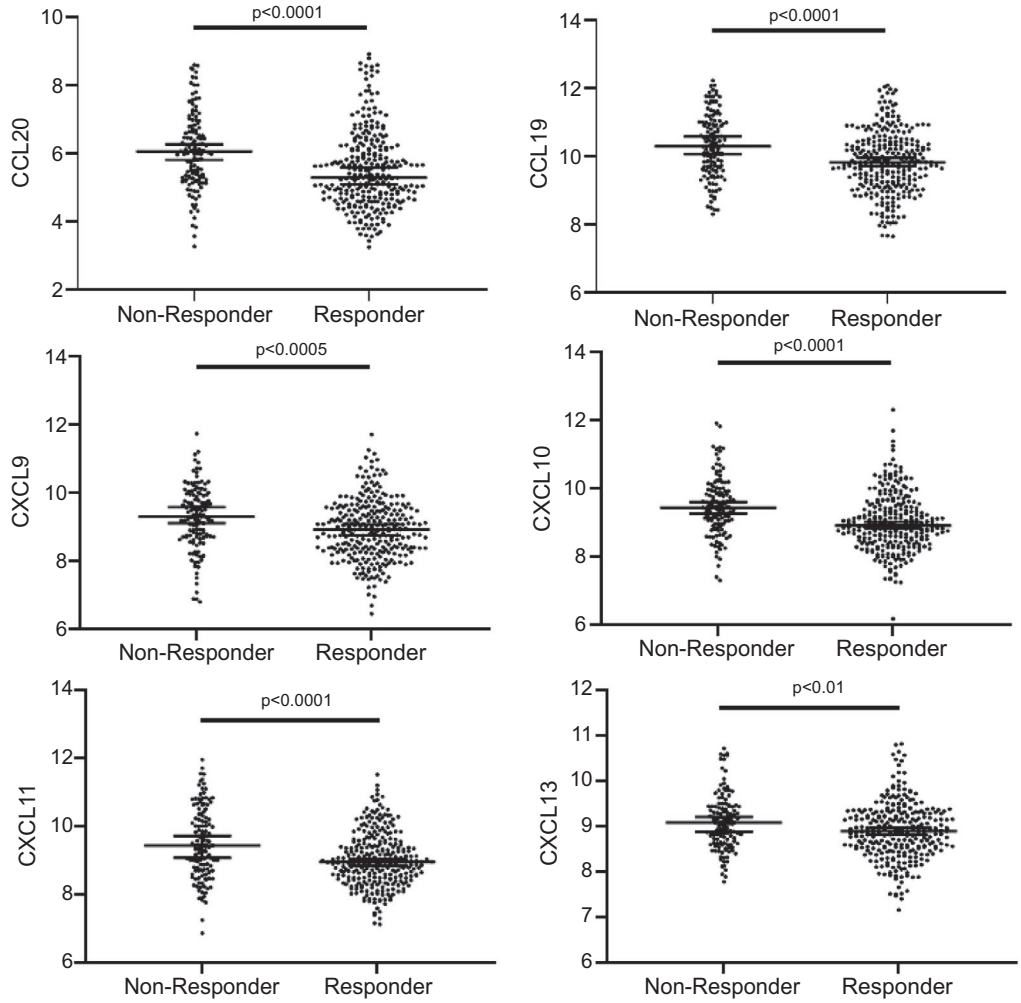

FIG. 3. Up-regulated chemokines in serum of UDCA nonresponders in the discovery proteomics study. Response criterion used was Paris 1 (although the effect was the same for the example chemokine, CCL20, regardless of the response criterion applied; Supporting Fig. S1). All analytes were assayed by the Olink platform and generated NPX. *P* values were calculated using an unpaired *t* test with Welch's correction. $^{****}P < 0.0001$; $^{**}P < 0.001$.

Discussion

In this study, we used two independent cohorts of phenotyped PBC patients, including treatment-naïve and UDCA-treated patients stratified by their therapy response status, to characterize the serum proteome in the disease. Our aim was to explore the biological basis of nonresponse to UDCA in order to better understand how to identify and treat higher-risk, therapy nonresponding patients. The findings begin to shed light on the mechanisms underpinning incomplete response to UDCA in PBC.

Our first observation was that, unsurprisingly, treatment-naïve PBC patients show elevation of a series of inflammatory protein markers in their serum when compared to healthy community controls. This supports the idea that there is a significant inflammatory/immune component to PBC pathogenesis. Elevations of these proteins correlate with both ALP levels, pre-UDCA and post-UDCA therapy, and with the UK-PBC risk score predicted 10-year mortality post-UDCA therapy.[19] To date, approval of second-line therapy in PBC has been on the basis of significant improvement in the biochemical marker, ALP.[1] Although ALP is predictive of disease risk in clinical practice,[25] it is a surrogate marker at best and the lack of an apparent mechanistic link to the disease has weakened its value in the eyes of regulatory bodies.

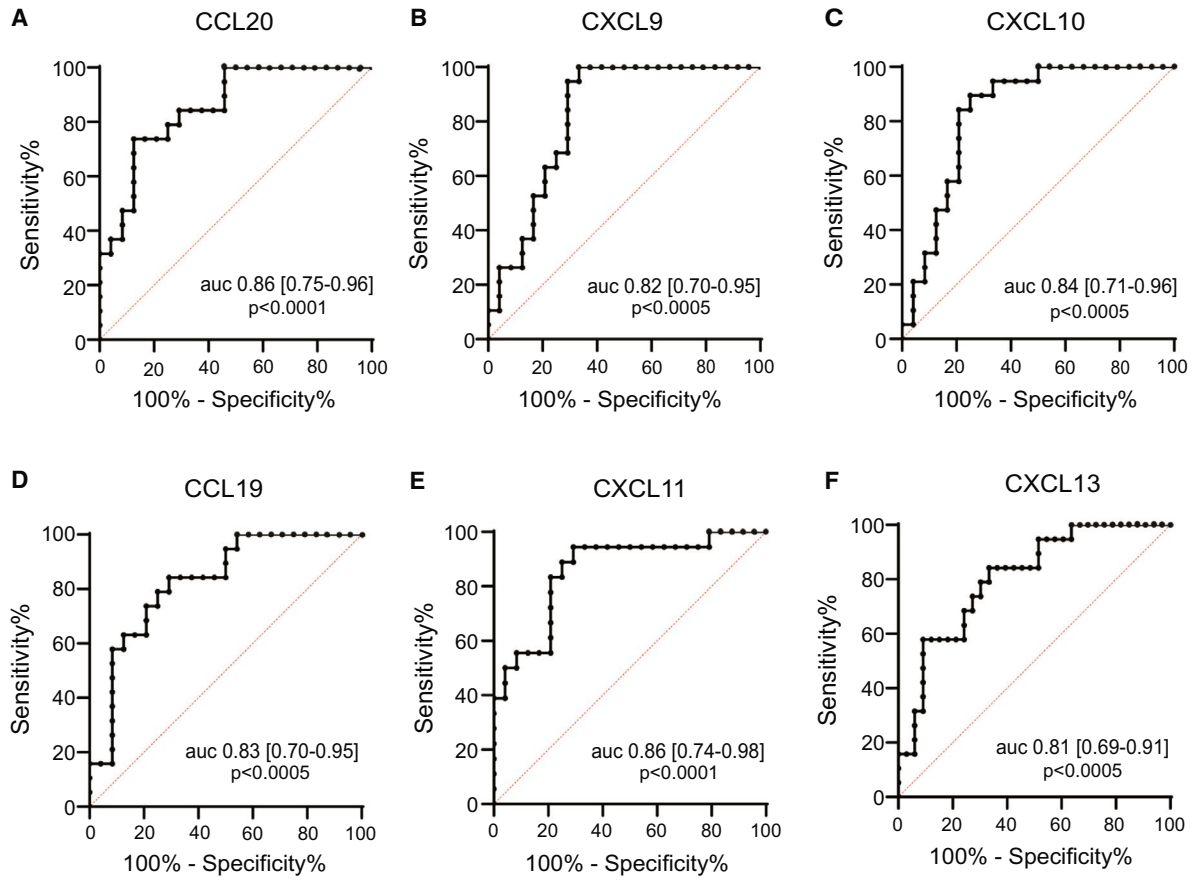

FIG. 4. Predictive value of serum chemokine levels for UDCA response status in the confirmatory study. All six chemokines identified in the discovery study as remaining elevated in PBC patients despite UDCA therapy, with significantly higher levels in UDCA nonresponders than responders, were also highly predictive of UDCA response status in the confirmatory study using an independent study cohort. (A) CCL20, (B) CXCL9, (C) CXCL10, (D) CCL19, (E) CXCL11, (F) CXCL13.

TABLE 4. Wilks' Lambda and Discrimination Between Responder and Nonresponder Status for Chemokines in the Confirmatory Chemokine Study

Chemokine	Wilks' Lambda	*F* Statistic	*P* Value	*F* Difference	*P* Difference
CCL20	0.689	19.85	5.68E-0.5	19.85	5.68E-0.5
CXCL11	0.518	19.99	7.29E-07	14.17	4.91E-0.4
CXCL9	0.498	14.13	1.64E-0.6	1.74	1.94E-0.1
CXCL13	0.476	11.26	2.94E-0.6	1.82	1.84E-0.1

CXCL9 and CXCL13 were nonsignificant at $P > 0.05$ and were hence excluded from the final linear discriminant analysis model.

Our observation of a clear correlation between components of the PBC proteome and levels of serum ALP (as well as with the composite UK-PBC risk score[19]) supports a direct link between ALP and underpinning immune and inflammatory processes and gives additional confidence in its use as a surrogate marker in clinical trials.

Comparison of the proteome in treatment-naïve and UDCA-treated patients in the discovery phase identified only a single protein (EpCAM) that was normalized by UDCA treatment. The other components of the PBC proteome were reduced in expression level, but remained present at significantly elevated levels after UDCA therapy. This finding

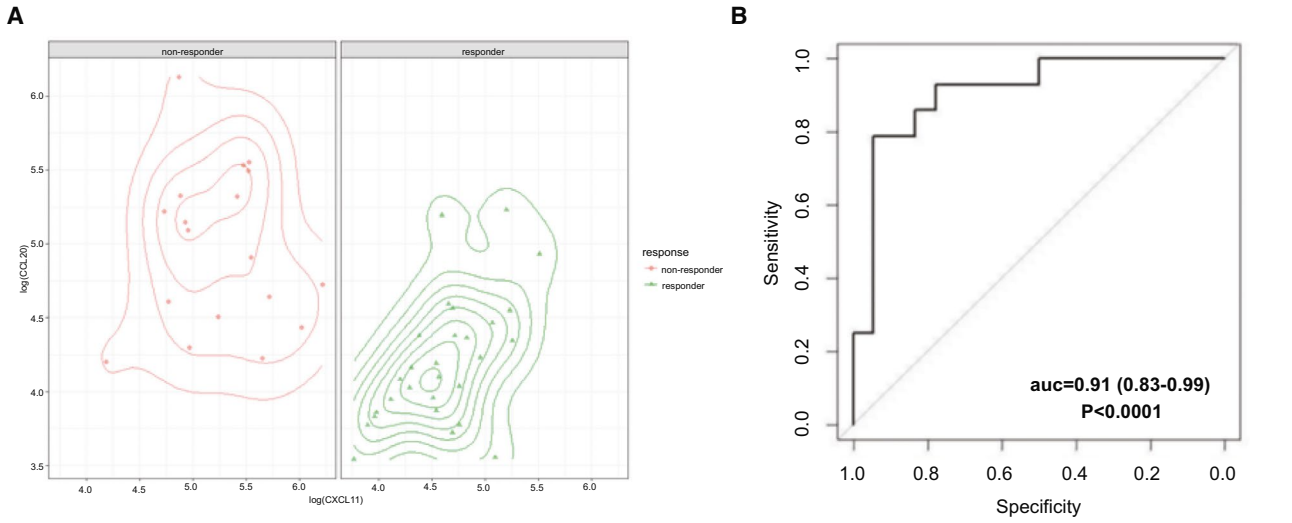

FIG. 5. Predictive value of the integrated predictive model incorporating CXCL11 and CCL20 derived using linear discriminant modeling. (A) Density plot showing log-transformed individual levels of CCL20 and CXCL11 in nonresponders and responders. Highest density occurs where the contours are closest together. Note that while the contour lines in both plots would overlap if superimposed on the same graph, the centers for each group are different, responders generally having lower levels of both CCL20 and CXCL11 than nonresponders. (B) ROC analysis. The integrated model outperforms any individual chemokine and is the optimal tool for further evaluation in prospective studies.

suggests that UDCA treatment modifies, but typically does not resolve, disease-associated inflammatory processes in PBC.

The normalization of elevated serum EpCAM levels in PBC with UDCA is open for study. EpCAM is an adhesion molecule expressed in the liver both by a mature cholangiocyte and a precursor cell population.[26,27] Serum EpCAM has also recently been identified as a serum risk marker in inflammatory bowel disease.[28] Our interpretation of the findings in relation to EpCAM in PBC is that it is acting as a blood biomarker of cholangiocyte injury/functional cholangiocyte stem cell proliferation. In this model, normalization with UDCA reflects reducing biliary injury and subsequent precursor cell proliferation. This finding sheds further light on the actions of UDCA in the setting of PBC.

A significant element of the untreated PBC proteome, persisting even after UDCA, is a network of chemokines (CCL20, CCL19, CXCL9, CXCL10, CXCL11, and CXCL13). CXCL9, -10, and -13 have been shown to be elevated in PBC patients, and not normalized by UDCA therapy; our findings confirm these earlier observations.[29-31] In particular, levels of the individual CXCL chemokines were strongly correlated in the current study (data not shown), probably reflecting a shared regulation in the form of IL-27.[32] Interestingly, the recent international meta-analysis of the genetic basis of PBC has identified the IL-27 pathway as a PBC susceptibility pathway.[33] Significant up-regulation of CXCL9, CXCL10, and CCL19 has also been described in a murine PBC model, with levels being reduced by IL-22 therapy.[34] CCL20, the gene encoding which was also identified as a susceptibility locus in PBC in the recent meta-analysis,[33] is released by injured BECs in PBC.[35,36]

In our study, we demonstrate, and then confirm in a second population, that levels of the six chemokines are significantly higher in UDCA nonresponders than UDCA responders. This effect was not a reflection of surrogate effects of cirrhosis given that the same association with UDCA nonresponse was observed when the analysis was restricted to patients without cirrhosis. Elevation of two chemokines, CCL20 and CXCL11, was sufficient to identify high-risk patients with a high degree of accuracy. Elevation in serum CCL20 and CXCL11 in high-risk PBC may reflect the process of BEC senescence. We have previously demonstrated that senescent BECs directly release CCL20.[37] Furthermore, although not studied as yet in the liver, CXCL11 release by endothelial cells

undergoing senescence has been reported.[38] BEC senescence has been shown to be a key adverse event in PBC pathogenesis, linked to both UDCA nonresponse and high risk of disease progression.[39] In a transcriptomic and immunohistochemical study of the baseline liver biopsies of patients who went on to be nonresponders to UDCA and show disease progression, duct injury, including senescence as denoted by p21 expression, was present at disease outset, with CCL20 transcription up-regulated.[39] In contrast, CCL20 transcription was not upregulated in liver biopsies of low-risk patients at presentation and BEC senescence was absent. CCL20 appears to play a key role in the epithelial targeting of C-C motif chemokine receptor 6 expressing T helper 17 and IL-17-producing $CD8^+$ T cells, strongly implicated in the immune pathogenesis of PBC.[40] Therefore, there appears to be an important, bidirectional crosstalk between immune cells and senescent BECs, with CCL20 playing a key role.[41] Far from being passive targets of immune injury in PBC, the BECs (especially in the context of cholestasis-induced senescence) appear to be complicit in shaping and localizing the immune response that contributes to their injury. The apparent role of BECs in modifying and localizing T-cell reactivity may help to explain the importance of interface hepatitis as an adverse marker in high-risk, UDCA nonresponsive disease.[42]

The apparent ongoing chemokine response in UDCA-treated PBC raises the question as to whether directly targeting the chemokine network itself may be a treatment option. A single study, targeting CXCL10, has explored this approach.[14] This trial was negative, potentially because of the pleomorphic nature of the IL-27-induced chemokine response. If the chemokine response is indeed a consequence of BEC injury, driving the augmented immune response resulting from senescence that drives inflammation, it may be more productive to target the upstream processes resulting in BEC senescence, such as BEC oxidative stress in the context of cholestasis, rather than individual chemokines. In this context, our observation that measurement of just CCL20 and CXCL11 is sufficient to identify UDCA nonresponders with a high degree of accuracy (addition of the other chemokines to a predictive model does not further improve accuracy) points to a potentially useful mechanistic biomarker for high-risk PBC.

Our study has important limitations, and further work is needed in three particular areas. The first is a need to study a larger number of informative UDCA nonresponders. The UK-PBC study cohort used in the study had 31% UDCA nonresponders. This figure is typical for the UK experience for UDCA nonresponse, but does mean that less-informative responders formed the majority of the participants. The second is a need for longitudinal sampling to explore within persons the change with UDCA, rather than relying on the current cross-sectional data. Finally, it will be important to explore changes in our CCL20/CXCL11 marker with licensed and emerging second-line therapies. Our replication of the chemokine association with UDCA nonresponse in a second cohort does suggest, however, that the key finding is robust. A further limitation relates to the proteomics approach used. We selected four complementary marker panels based on earlier understanding of the biology of PBC (details of the markers can be found at www.olink.com/downloads). Clearly, the study cannot provide data in relation to any marker not included in these panels. Finally, the predictive value of the chemokine markers demonstrated in our study is for surrogate markers of disease-associated risk rather than direct clinical endpoints. Interestingly, the association is strongest with ALP and ALT than it is with bilirubin, albumin, and platelet counts (as well as the UK-PBC risk score integrating all five parameters). One interpretation of this would be that chemokine elevation relates more closely to current disease activity than it does to severity in terms of progression to cirrhosis.

In conclusion, we have demonstrated that PBC has a characteristic inflammatory proteome. This is only modified to a modest degree by standard therapy with UDCA with, in particular, a prominent ongoing chemokine response even on a therapeutic dose. This suggests a mechanism for UDCA nonresponse, potentially invoking ongoing BEC stress/senescence despite UDCA therapy. CCL20 and CXCL11 should be evaluated as potential mechanistic disease biomarkers in the context of clinical trials.

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