Faecal cytokine profiling as a marker of intestinal inflammation in acutely decompensated cirrhosis

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Graphical abstract
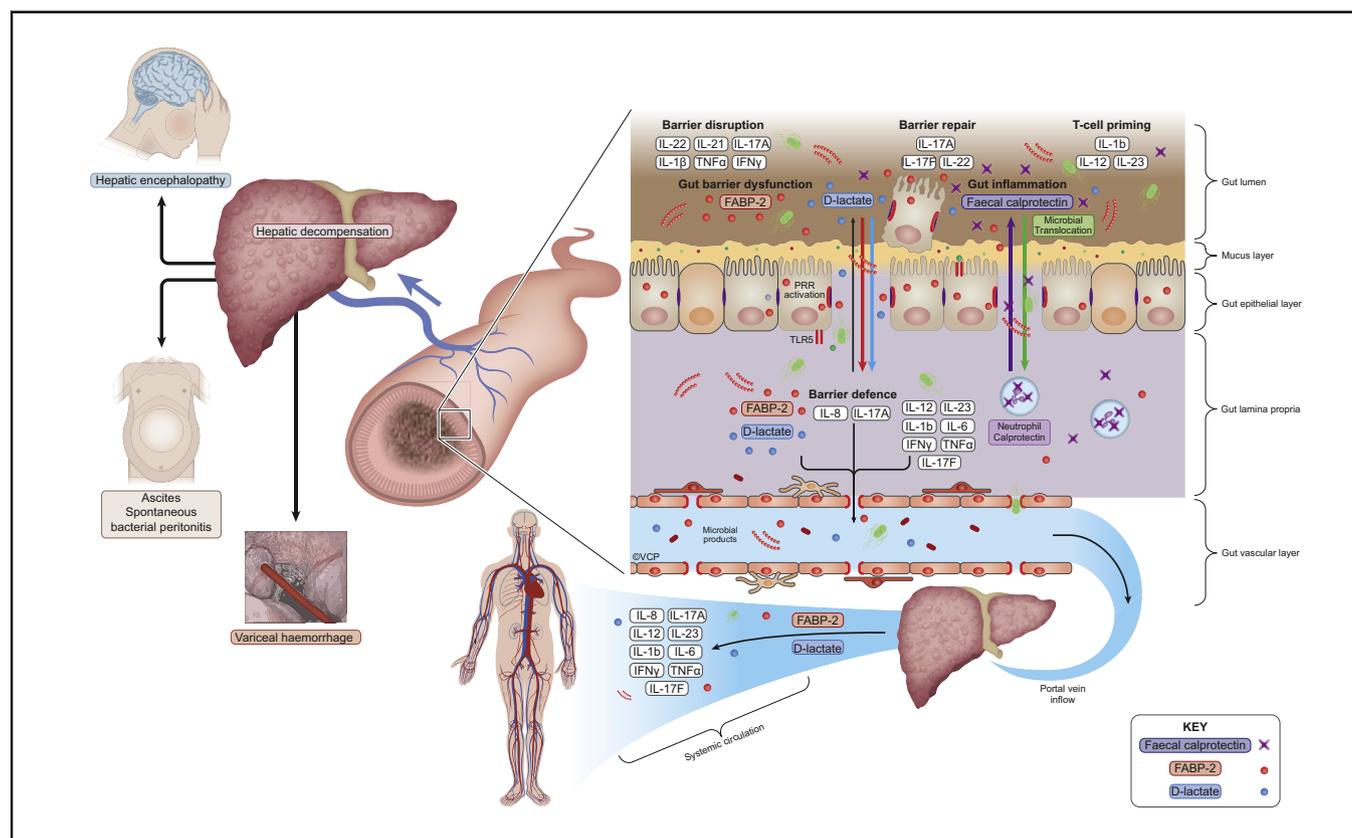
Highlights

- The gut micro-environment in cirrhosis primes mucosal and systemic immune responses.

- Gut inflammation is challenging to study in cirrhosis with a paucity of targeted assays.

- Exacerbated gut immune responses and barrier damage occur in acutely decompensated cirrhosis.

- Gut cytokine profiles in acute decompensation have very different patterns to innate-like systemic inflammation.

Lay summary

The gut barrier is crucial in cirrhosis in preventing infection-causing bacteria that normally live in the gut from accessing the liver and other organs via the bloodstream. Herein, we characterised gut inflammation by measuring different markers in stool samples from patients at different stages of cirrhosis and comparing this to healthy people. These markers, when compared with equivalent markers usually measured in blood, were found to be very different in pattern and absolute levels, suggesting that there is significant gut inflammation in cirrhosis related to

- Upregulation of faecal T cell-mediated type 1 and type 17 effector cytokines occurs in AD.

- Faecal cytokines and gut barrier markers accurately differentiate between stable cirrhosis and AD.

different immune system pathways to that seen outside of the gut. This provides new insights into gut-specific immune disturbances that predispose to complications of cirrhosis, and emphasises that a better understanding of the gut-liver axis is necessary to develop better targeted therapies.

Faecal cytokine profiling as a marker of intestinal inflammation in acutely decompensated cirrhosis

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Background & Aims: Gut dysbiosis and inflammation perpetuate loss of gut barrier integrity (GBI) and pathological bacterial translocation (BT) in cirrhosis, contributing to infection risk. Little is known about gut inflammation in cirrhosis and how this differs in acute decompensation (AD). We developed a novel approach to characterise intestinal immunopathology by quantifying faecal cytokines (FCs) and GBI markers.

Methods: Faeces and plasma were obtained from patients with stable cirrhosis (SC; n = 16), AD (n = 47), and healthy controls (HCs; n = 31). A panel of 15 cytokines and GBI markers, including intestinal fatty-acid-binding protein-2 (FABP2), D-lactate, and faecal calprotectin (FCAL), were quantified by electrochemiluminescence/ELISA. Correlations between analytes and clinical metadata with univariate and multivariate analyses were performed.

Results: Faecal (F) IL-1β, interferon gamma, tumour necrosis factor alpha, IL-21, IL-17A/F, and IL-22 were significantly elevated in AD *vs.* SC (q <0.01). F-IL-23 was significantly elevated in AD *vs.* HC (p = 0.0007). FABP2/D-lactate were significantly increased in faeces in AD *vs.* SC and AD *vs.* HC (p <0.0001) and in plasma (p = 0.0004; p = 0.011). F-FABP2 correlated most strongly with disease severity (Spearman's rho: Child-Pugh 0.466; p <0.0001; model for end-stage liver disease 0.488; p <0.0001). FCAL correlated with plasma IL-21, IL-1β, and IL-17F only and none of the faecal analytes. F-cytokines and F-GBI markers were more accurate than plasma in discriminating AD from SC.

Conclusions: FC profiling represents an innovative approach to investigating the localised intestinal cytokine micro-environment in cirrhosis. These data reveal that AD is associated with a highly inflamed and permeable gut barrier. FC profiles are very different from the classical innate-like features of systemic inflammation. There is non-specific upregulation of T_H1/T_H17 effector cytokines and those known to mediate intestinal barrier damage. This prevents mucosal healing in AD and further propagates BT and systemic inflammation.

Introduction (Box 1)

In health, the gut barrier is crucial in the defence against the extensive and continuous exposure of the liver to the intestinal microbiota, their immunogenic products (pathogen-associated molecular patterns [PAMPs]), and microbial metabolites. However, in cirrhosis, there is increasing evidence that this barrier is dysfunctional, more permeable, and highly inflamed.[1] The gut barrier consists of several layers, which determine the extent to which microbes and their PAMPs can access the host circulation. The first line of defence is the mucus layer,[2] which physically separates the microbiota from the next layer consisting of intestinal epithelial cells (IECs) that are bound by tight junctions.[3] Below this is the lamina propria, which, in addition to consisting of non-cellular connective tissue elements, is an immune dense layer, where several types of innate and adaptive immune cells are concentrated and where aggregations of lymphoid nodules give rise to the specialised areas known as Peyer's patches.[4] The gut vascular barrier (GVB) represents the final layer controlling the entry of microbes and PAMPs into the portal circulation, and therefore the liver.[5] IEC and GVB disruption has been shown to be crucial in the development of non-alcoholic fatty steatohepatitis.[6]

This dysfunctional 'gut-liver axis' is driven by intestinal microbial dysbiosis, translocation of pathogenic gut microbes and their PAMPS, a process termed bacterial translocation (BT), which initiates both intestinal mucosal dysfunction and systemic immune paresis.[7–9] The relative contribution of each component is, however, not well understood.[10,11] Increased BT has been shown to be a key process that contributes to acute

Box 1. Short summary.

1. <u>What is already known about this subject?</u>
Gut dysbiosis and microbial overgrowth contribute to increased intestinal inflammation and loss of barrier integrity in cirrhosis.

Gut barrier dysfunction enables pathological bacterial translocation, which contributes to cirrhosis-associated immune dysfunction and heightened susceptibility to infection, and predisposes to hepatic decompensation.

Little is known about gut-specific inflammation in cirrhosis and how this differs in patients with and without acutely decompensated cirrhosis.

2. <u>What are the new findings?</u>
Intestinal cytokine profiles are different in pattern and absolute quantity from the classical features of systemic inflammation in acute decompensation (AD), with no upregulation of either faecal IL-8 or faecal IL-10, involved in pro- or anti-inflammatory pathways, respectively, in comparison with stable cirrhosis (SC).

Multiple faecal cytokines are elevated in AD in keeping with a generalised and non-specific upregulation of type 1 and type 17 effector cytokines and those known to be drivers of innate immune-mediated inflammation in the gut.

Faecal D-lactate and fatty-acid-binding protein-2—markers of gut barrier damage and intestinal inflammation—accurately differentiate between patients with AD and SC, with greater sensitivity and specificity than equivalent plasma measurements.

3. <u>How might it impact on clinical practice in the foreseeable future?</u>
These data highlight the importance of the role of intestinal inflammation and barrier dysfunction, which affect the gut-liver axis in cirrhosis.

Gut-specific targets may better enable non-antibiotic-based therapies to be developed that prevent progression to AD, particularly in an era of ever-increasing antimicrobial multidrug resistance.

This technique can be applied to other disease paradigms, where gut inflammation and epithelial barrier integrity are thought to play an important role.

decompensation (AD) and acute-on-chronic liver failure (ACLF), the latter being associated with extra-hepatic organ failure(s) and very high short-term mortality.[12,13] How gut barrier dysfunction and inflammation affect the clinico-pathological transition from stable cirrhosis (SC) to AD and/or ACLF is unknown.

Pathogenic gut bacterial and fungal species, such as those observed in cirrhosis, are able to intimately adhere to the intestinal mucosa, induce barrier disruption, and alter host mucosal immune responses.[14–17] In particular, interferon gamma (IFNγ), IL-22, IL-17A, and IL-17F play a critical role in establishing host antimicrobial immunity.[18] How intestinal inflammation promotes systemic inflammation in relation to gut-derived BT in cirrhosis remains unclear and requires elucidation. Limited data exist on faecal calprotectin (FCAL) levels in cirrhosis as a surrogate marker of gut inflammation. Calprotectin is a calcium- and zinc-binding protein of granulocytes, accounting for over 60% the soluble cytosolic proteins found in human neutrophil granulocytes.[19] FCAL correlates positively with the degree of intestinal neutrophil migration,[20] and as such has become established as a diagnostic and prognostic marker for the assessment of localised intestinal inflammation[21] in inflammatory bowel disease (IBD),[22,23] as it is resistant to degradation during intestinal transit.[24] Very limited data are available in cirrhosis, where FCAL is reported to be elevated in patients with cirrhosis (some with hepatic encephalopathy) when compared with healthy controls (HCs).[25]

The intestinal immune system is regionally specialised because of conditioning by the gut micro-environment and inhabiting microbiome. Adaptations are reflected in its complex gut-associated lymphoid tissues and isolated immune cells, including an almost exclusive population of antigen-experienced T cells scattered throughout the intestinal epithelial compartment. A further layer of specialisation is local priming of these effector lymphocytes attributable to the complex cytokine milieu generated as a result of pattern recognition receptor activation. IECs or antigen-presenting cells of the gut lamina propria help prime T cell differentiation into protective T cell subsets, which, together with the innate immune system, form the first line of defence against invading pathogens[26–28] and play a crucial role in maintaining gut barrier integrity.[29] The healthy gut microbiome contributes to the constitutive development of T_H17 cells in the intestinal lamina propria.[30,31] T_H17 cells induce the recruitment of neutrophils and activation of IECs, enhancing the clearance of extracellular pathogens in concert with other immune cells, such as IgA-secreting plasma cells and group 3 innate lymphoid cells.[15]

Peripheral circulating cytokine profiling has been shown to be related to prognosis at different stages of cirrhosis,[32] and differentiates patients with and without AD and ACLF, and clinical outcomes, including short-term mortality.[33] However, relatively little is known about localised gut inflammation and the key immunological events that mediate barrier disruption in cirrhosis using similar cytokine markers, and how this relates to BT and clinically relevant outcomes. This lack of knowledge stems primarily from difficulty in obtaining intestinal tissue in patients with cirrhosis and a paucity of non-invasive techniques. The aim of the current investigation was to develop a method of characterising and differentiating gut mucosal inflammation and injury in patients with SC and AD by utilising faeces as a biological matrix. A panel of cytokines and markers of gut barrier integrity and inflammation were measured and compared in both faeces and plasma, which, in combination, are herein referred to as analytes.

Materials and methods

Study participants and biological sampling
Patients were consecutively recruited at King's College Hospital after admission to the ward or when reviewed in the hepatology outpatient clinic. The study was granted ethics approval by the national research ethics committee (12/LO/1417) and local research and development department (KCH12-126), and performed conforming to the Declaration of Helsinki. Patient participants, or their nominee in the case of incapacitation of a potential participant, provided written informed consent within 48 h of presentation. Patients were managed according to standard evidence-based protocols and guidelines.[34] Patient and public involvement and engagement were undertaken with a patient advisory group who partnered with us to determine the acceptability of the study, and provided their perspective on study design, informational material, and measures to minimise participation burden, and agreeing on a dissemination plan of the findings.

AD was defined by the acute development of 1 or more major complications of cirrhosis, including ascites, hepatic encephalopathy, variceal haemorrhage, and bacterial infection.[13] Main exclusion criteria included pregnancy, hepatic or non-hepatic malignancy, pre-existing immunosuppressive states, active

HBV/HCV/HIV infection, and known IBD. Demographic, clinical, and biochemical metadata were collected at the time of sample. Standard clinical composite scores used for risk stratification and prognostication included the Child-Pugh score,[35] model for end-stage liver disease (MELD),[36] United Kingdom model for end-stage liver disease (UKELD),[37] and Chronic Liver Failure Consortium-acute decompensation (CLIF-C AD).[38]

Faecal and plasma collection and sample preparation

Faecal lysates (FLs) were produced from frozen faecal samples by combined chemical and mechanical homogenisation using an optimised extraction method. Sample collection and preparation, including FL generation, are described in Supplementary methods.

Faecal and plasma cytokine analyses

The following cytokines were quantified in paired faecal and plasma samples to enable a comparison between the gut and the systemic compartments:

- mucosal-associated cytokines involved in local immune modulation and barrier repair: IL-21, IL-22, IL-17E (IL-25), and IL-10[39–44];
- innate/adaptive cytokines belonging to the type 1/type 17 antimicrobial axis: IL-12p70, IL-23, IFNγ, IL-17A, IL-17F[39,40,42,43,45]; and
- cytokines conventionally associated with systemic inflammatory responses to infection: IL-1β, IL-6, IL-8, and tumour necrosis factor alpha (TNFα).[39,40,46,47]

Cytokines were measured in plasma or neat FL using an electro-chemiluminescence platform or by ELISA as per manufacturer's instructions, as is described in detail in Supplementary methods.

Fatty-acid-binding protein-2 and D-lactate quantification

Intestinal fatty-acid-binding protein-2 (FABP2)[48] and the microbial metabolite D-lactate[49–51] were quantified to serve as gut-specific markers of intestinal barrier integrity and BT, to assess whether these differentiated AD from SC, and in the HC cohort to define whether 'physiological' or basal levels were detectable. FABP2 was quantified using the human FABP2/I-FABP Quantikine ELISA Kit (R&D Systems). Plasma D-lactate was measured using a colourimetric assay (Abcam, Cambridge, UK). All assays were conducted according to the manufacturers' instructions. Optical densities were measured with a FLUOstar® Omega Absorbance Microplate Reader.

FCAL quantification

FCAL was measured from frozen faecal samples using a commercially available ELISA (BÜHLMANN Laboratories AG, Schönenbuch, Switzerland) that measures calprotectin in a quantitative manner. This is described in further detail in the Supplementary methods.

Statistical and bioinformatic analyses

Analyte values were obtained using 4-/5-parameter logistic regression standard curves as appropriate. The AD, SC, and HC groups were compared using the Mann-Whitney U test or the Kruskal-Wallis test with Dunn's correction for *post hoc* multiple comparisons for independent continuous variables, the Wilcoxon signed-rank test for paired continuous variables, and the Chi-square test for categorical variables.

Multiple hypothesis testing for group comparisons was controlled using the Benjamini-Hochberg false discovery rate (FDR) algorithm. Mann-Whitney U or Kruskal-Wallis p values are indicated in text, figures, and tables as 'MWp' or 'KWp', respectively, whilst FDR-adjusted q values are indicated as 'BHq'. Correlations were evaluated using the Pearson's or Spearman's correlation coefficients as appropriate, and multiple correlation testing was controlled using the Bonferroni family-wise error rate (FWER) correction. Hierarchical clustering of cytokine and intestinal analytes was based on complete linkage by Pearson's correlation distance metric. Multivariate analysis was performed using (unsupervised) principal component analysis (PCA) and orthogonal projection to latent structures discriminant analysis (OPLS-DA) (supervised). These are described in detail in Supplementary methods.

Results

Participant characteristics

Table 1 summarises the demographic, clinical, and biochemical characteristics of the recruited patients. The study included 63 patients with cirrhosis (18–75 yr of age) classified according to the European Association for the Study of the Liver- Chronic Liver Failure Consortium criteria[34] as SC (n = 16) or acutely decompensated cirrhosis (AD; n = 47) and a cohort of 31 gender-matched HCs. Aetiology of cirrhosis included alcohol (AD/SC: 66%/38%), non-alcohol-related fatty liver disease (AD/SC: 17%/6%), and previously treated hepatitis C infection (SC: 31%).

The patients with AD and SC were older than the HCs (median [inter-quartile range] 54 [44–59] and 61 [54–68] *vs.* 31 [28–37] yr, respectively); the patients with SC were marginally older than the patients with AD (61 [54–68] *vs.* 54 [44–59] yr; *p* = 1.5E-2). No patients with AD had experienced a variceal haemorrhage in the 7 days before recruitment nor developed spontaneous bacterial peritonitis. The AD group (28/47 [58.3%]) compared with the SC group (1/16 [6.3%]) was more frequently receiving any antimicrobial therapy at the time of sampling, although there were no significant differences in the rates of administration of either parenteral antibiotics, rifaximin-α, or antifungal therapies between the 2 groups. Similarly, a higher proportion of patients with AD (29/47 [61.70%]) with alcohol-related cirrhosis were actively drinking compared with the SC group (5/16 [31.25%]).

Compared with the patients with SC who were all Child-Pugh A with preserved hepatic synthetic function, the patients with AD had higher bilirubin, international normalised ratio, total leucocyte/neutrophil/monocyte counts, venous ammonia, blood lactate, and C-reactive protein, together with lower serum albumin, sodium, and platelet/lymphocyte counts. Child-Pugh, MELD, UKELD, and CLIF-C AD scores were significantly higher in AD relative to SC.

Faecal and plasma cytokines and gut barrier integrity markers across patient and control groups

A summary of our findings for all faecal and plasma analytes is given (Figs 1 and 2; Table S1). When comparing across all 3 groups (AD *vs.* SC *vs.* HCs) by *post hoc* comparisons, faecal FABP2 (Fig. 1A) was significantly different across groups (q = 0.000025), with higher levels in the AD group compared with the SC (Dunn's *p* = 0.000092) and HC groups (Dunn's *p* = 0.000088). Faecal D-lactate (Fig. 1A) was significantly different across groups

Table 1. Summary of clinical characteristics of study groups (all values given as median [IQR]).

Parameter	SC	AD	p value AD vs. SC (Mann-Whitney U or Chi-square tests)
Number per group	16	47	
Age (yr)	61.0 (53.8–67.6)	54.0 (44.0–59.0)	0.015
Gender (M:F)	(0/1) = 4/12	(0/1) = 17/30	0.410
BMI (kg/m^2)	26.33 (23.78–27.70)	25.76 (24.11–29.36)	0.430
Aetiology of cirrhosis, n/N (%)			
Alcohol actively drinking	5/16 (31.25)	29/47 (61.70)	0.035
Alcohol abstinent	1/16 (6.25)	2/47 (4.26)	0.750
NAFLD	1/16 (6.25)	8/47 (17.02)	0.290
Primary sclerosing cholangitis	2/16 (12.50)	2/47 (4.26)	0.240
Autoimmune hepatitis-related cirrhosis	0/16 (0.00)	2/47 (4.26)	0.660
Cryptogenic cirrhosis	1/16 (6.25)	1/47 (2.13)	0.420
Wilson's disease	0/16 (0.00)	1/47 (2.13)	0.760
Hepatitis C-related cirrhosis with SVR	5/16 (31.25)	0/47 (0.00)	<0.001
Clinical features at enrolment			
Temperature (°C)	36.50 (36.00–36.70)	36.70 (36.50–36.90)	0.018
Ascites (%)	0	14.9	0.34
Ascites grade (none/moderate/severe) (%)	100/0/0	31.9/44.7/23.4	<0.001
Hepatic encephalopathy (%)	(0/1) = 16/0 (0)	(0/1) = 43/4 (8.5)	0.510
Mean arterial pressure (mmHg)	92.50 (88.50–99.08)	78.33 (73.83–88.83)	<0.001
Heart rate (beats/min)	69.50 [63.75–79.25]	76.00 (66.50–88.00)	0.073
Antimicrobial therapy at enrolment, n/N (%)			
Antibiotics (any)	1/16 (6.3)	28/47 (58.3)	<0.001
Antibiotics (parenteral)	0	11/47 (23.4)	0.20
Antibiotics (oral)	1/16 (6.3)	18/47 (38.3)	0.018
Rifaximin	1/16 (6.3)	13/47 (27.7)	0.081
Antifungal therapy	0	1/47 (2.1)	0.77
Haematology			
Haemoglobin (g/dl)	134.00 (113.75–147.25)	105.00 (93.00–118.00)	0.004
Leucocyte count (×10^9/L)	4.72 (4.33–6.95)	5.07 (3.37–6.70)	0.680
Neutrophils (×10^9/L)	2.73 (2.41–4.27)	3.06 (2.13–4.62)	0.920
Lymphocytes (×10^9/L)	1.42 (1.14–2.02)	1.09 (0.79–1.69)	0.021
Monocytes (×10^9/L)	0.35 (0.23–0.48)	0.43 (0.28–0.58)	0.570
Eosinophils (×10^9/L)	0.15 (0.10–0.18)	0.15 (0.09–0.22)	0.690
Basophils (×10^9/L)	0.03 (0.02–0.04)	0.03 (0.02–0.05)	0.440
Platelet count (×10^9/L)	179.00 (110.50–236.50)	99.00 (68.00–144.00)	0.006
INR	1.12 (1.05–1.16)	1.49 (1.31–1.81)	<0.001
Biochemistry			
Serum sodium (mmol/L)	140.00 (139.00–141.25)	136.00 (130.50–138.50)	<0.001
Urea (mmol/L)	4.80 (4.15–6.50)	5.00 (4.00–6.75)	0.890
Serum creatinine (mmol/L)	74.50 (62.50–90.25)	67.00 (58.00–79.00)	0.270
Serum total bilirubin (mmol/L)	12.50 (9.25–15.25)	51.00 (29.00–135.00)	<0.001
AST (IU/L)	30.00 (24.50–37.00)	61.00 (45.50–85.00)	<0.001
Gamma-GT (IU/L)	85.00 (30.50–149.50)	90.00 (64.00–152.50)	0.440
Albumin (g/L)	41.00 (39.00–44.25)	32.00 (27.50–35.50)	<0.001
Total protein (g/L)	73.00 (70.00–75.00)	67.00 (62.50–72.50)	0.003
Venous ammonia (μmol/L)	36.00 (26.75–59.00)	50.00 (38.00–74.00)	0.033
C-reactive protein (mg/L)	2.00 (2.00–3.45)	7.00 (2.15–21.85)	0.001
Faecal calprotectin (mg/g)	20.00 (11.00–26.00)	74.00 (34.00–147.00)	0.0028
Blood lactate (mmol/L)	1.35 (1.13–1.83)	1.60 (1.35–2.10)	0.260
Disease severity and prognostic scores			
Child-Pugh score	5.00 (5.00–5.00)	8.00 (7.00–10.00)	<0.001
MELD score	8.00 (7.00–8.50)	18.00 (13.00–26.00)	<0.001
UKELD score	47.09 (44.94–49.03)	54.31 (52.11–61.53)	<0.001
CLIF-C AD score	43.32 (41.22–47.88)	47.63 (41.26–55.29)	0.047

AD, acute decompensation; AST, aspartate aminotransferase; BMI, body mass index; CLIF-C AD, Chronic Liver Failure Consortium-acute decompensation; GT, glutamyl transferase; INR, international normalised ratio; IQR, inter-quartile range; MELD, model for end-stage liver disease; NAFLD, non-alcoholic fatty liver disease; SC, stable cirrhosis; SVR, sustained virologic response; UKELD, United Kingdom model for end-stage liver disease.

(q = 7.2E-3), with lower levels in AD compared with SC (Dunn's p = 0.004). Plasma D-lactate was comparable across all 3 groups, whereas plasma FABP2 was different (q = 0.0039), with higher levels in the AD group compared with the HC group (Dunn's p = 0.0017). FCAL was significantly higher in AD compared with the SC (Dunn's p = 0.0027) and HC groups (Dunn's p <0.0001). Notably, both faecal and plasma FABP2 and

D-lactate as well as FCAL were always comparable between the SC and HC groups.

Elevated levels of multiple faecal cytokines were detected in AD when compared across all 3 groups, with more IL-17E, IL-21, IL-22, IL-12p70, IL-23, IFNγ, IL-17A, IL-17F, IL-1β, IL-6, and TNFα (Fig. 1B–E). Faecal IL-8 and IL-10 were unchanged across groups (Fig. 1E). When compared across the same patients and controls,
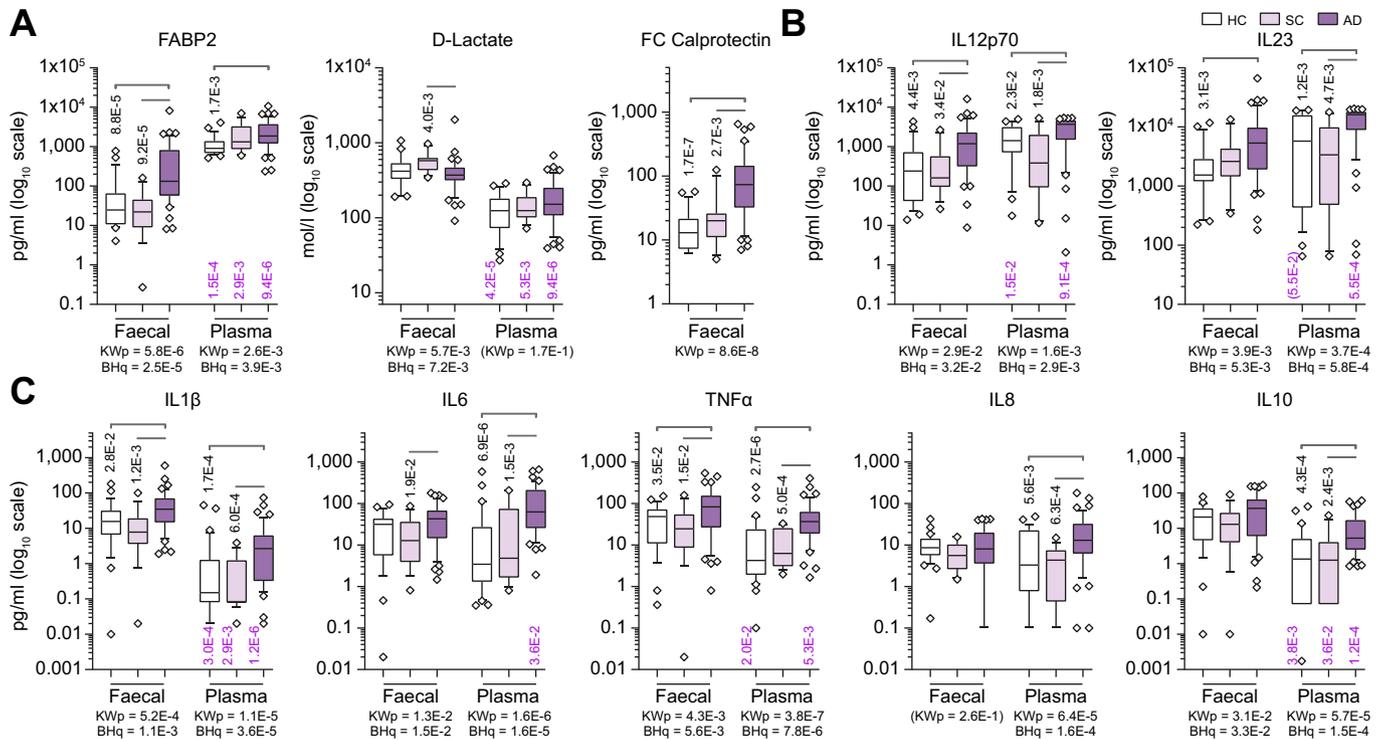
Fig. 1. Faecal and plasma cytokine, FABP2, and D-lactate concentrations comparing acutely decompensated and stable cirrhosis to healthy controls. KW*p* and BH*q* values: Kruskal-Wallis *p* values and BH adjusted *q* values for overall intergroup comparisons. Bracketed high values: Dunn's corrected *p* values for multiple comparisons, when KW*p* is significant. Base purple values: BH adjusted *q* values for paired faeces *vs.* plasma (Wilcoxon) comparisons for each group. (A) Surrogate markers of intestinal barrier damage, gut inflammation, and bacterial translocation. (B) Type 1/type 17 balance, effector cytokines. (C) Conventional markers of inflammation. AD, acute decompensation; BH, Benjamini-Hochberg; FABP2, fatty-acid-binding protein-2; FC, faecal; FCAL, faecal calprotectin; HC, healthy control; PL, plasma; SC, stable cirrhosis.

the plasma levels of all 13 cytokines measured were elevated in the AD group (Fig. 1B–E). All plasma and faecal analytes were comparable in patients with different aetiologies. Notably, no differences were detected in any of the faecal and plasma cytokines when compared between the SC and HC groups. Similarly, an analysis of the presence or absence of antibiotic therapy at the time of biological sampling on the various analytes measured in the AD group was undertaken. This was not possible in the SC group because of low proportion of those on antibiotics in the group (1/16). The analysis on the AD group of on *vs.* no antibiotics showed that all faecal and plasma analyte levels were comparable regardless of antibiotic use. Only faecal FABP2 was marginally higher in patients with AD treated with antibiotics compared with those who were not treated (*q* = 0.078), but this is only a trend.

Inter-compartmental comparison of faecal and plasma analytes

We next compared paired faecal and plasma analytes in the same patients and controls, reflective of differences between the gut micro-environment and the systemic circulation, respectively (Figs 1 and 2; Table S1). FABP2 was consistently higher, whilst D-lactate was consistently lower in plasma in all 3 groups when compared with faecal levels (Fig. 1A). Mucosal-associated IL-17E and IL-21 were higher in faeces in all 3 groups, whilst IL-22 was comparable between the 2 compartments (Fig. 2A). Cytokines belonging to the type 1/type 17 antimicrobial axis (IL-12p70 and IL-23) had elevated plasma levels in the HC and AD groups

(Fig. 1B). Conversely, levels of effector cytokines IL-17A and IL-17F (and IFNγ to a lesser extent) were elevated in faeces compared with plasma in all 3 groups (Fig. 2B). Amongst typical pro- and anti-inflammatory cytokines, IL-1β, TNFα, and IL-10 were overall higher in faeces compared with plasma in all groups, but IL-6 and IL-8 levels were comparable between the 2 compartments (Fig. 1C); only the AD group had moderately more plasma than faecal IL-6 in comparison with the HC group.

Independent regulation of faecal and plasma analytes in patients

The production of cytokines and the release of intestinal integrity markers may be correlated in inflammatory states. To investigate this, we performed Pearson's correlation-based hierarchical clustering of all faecal and plasma analytes in patients and controls (Fig. 3). We identified 3 significant distinct and independent coregulation clusters. Clusters 1 and 2 contained cytokines (IL-21, IL-22, IL-17E, IFNγ, IL-17A, IL-17F, IL-1β, IL-6, TNFα, and IL-10) that separated based on either faecal or plasma origin, respectively, indicating strong colinearity within each compartment, and in addition suggesting a complete lack of association of these various cytokines between the 2 matrices. Cluster 3 contained IL-12p70, IL-23, and D-lactate regardless of anatomical origin, suggestive of a role for this microbial metabolite in the regulation of the type 1/type 17 antimicrobial axis. The remaining analytes (IL-8, FABP2, and FCAL) did not cluster, suggesting independent regulation compared with the aforementioned clustered analytes.

A

B
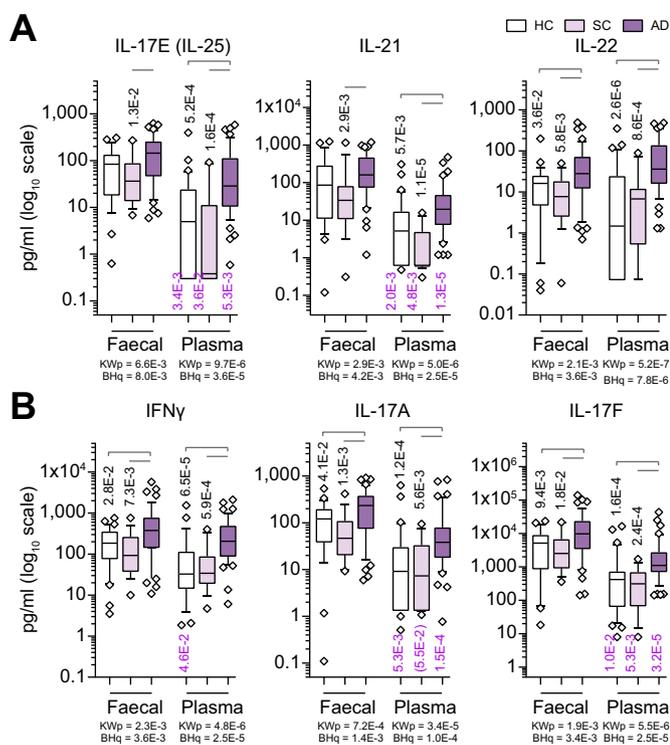
Fig. 2. Faecal and plasma cytokine, FABP2, and D-lactate concentrations comparing acutely decompensated and stable cirrhosis to healthy controls. KW*p* and BH*q* values: Kruskal-Wallis *p* values and BH adjusted *q* values for overall intergroup comparisons. Bracketed high values: Dunn's corrected *p* values for multiple comparisons, when KW*p* is significant. Base purple values: BH adjusted *q* values for paired faeces *vs.* plasma (Wilcoxon) comparisons for each group. (A) Cytokines relevant to mucosal immune modulation. (B) IL-12/23 balance, master regulation of type 1 *vs.* type 17 responses. AD, acute decompensation; BH, Benjamini-Hochberg; FABP2, fatty-acid-binding protein-2; FC, faecal; HC, healthy control; IFNγ, interferon gamma; PL, plasma; SC, stable cirrhosis.

Correlation between faecal and plasma analytes with clinical disease severity and prognostication scores

When correlated with clinical characteristics, multiple positive trends were found between plasma and faecal cytokines, FABP2, and D-lactate with Child-Pugh, MELD, and UKELD scores, but not with the CLIF-C AD score (Fig. 4). Significant correlations, based on a Bonferroni-corrected *p* ≤1.28E-04 threshold, were found only when comparing faecal FABP2 to Child-Pugh, MELD, and UKELD scores, and plasma IL-8 to MELD and UKELD scores. Plasma D-lactate, whilst not significantly correlated to any disease score, did correlate with serum bilirubin in isolation. FCAL, which is conventionally used as a diagnostic marker of gut inflammation, was therefore utilised here as a clinical parameter. In doing so, FCAL did not correlate with any of the faecal analytes measured. FCAL did, however, show positive correlations with plasma cytokines (more strongly with IL-21, IL-1β, and IL-17F).

Discrimination between AD and SC using faecal and plasma analytes

Having identified significant correlations within faecal or plasma analytes, we sought to investigate latent discrimination potential by multivariate methods. Supplemental Figures 1 and 3 illustrate the results for faecal and plasma analytes, respectively.

When using faecal analytes, a clinical distinction between the SC and AD groups mapped with PCA clusters (PC$_{1+2}$ = 72.1% variance explained) (Fig. S1A), and OPLS-DA analysis identified faecal FABP2 and D-lactate as the main contributors to this discrimination (Fig. S1B). The OPLS-DA model was significant by OPLS-DA diagnostics (Fig. S1B). When examining plasma analytes, the separation between AD and SC was still present (PCA variance explained; PC$_{1+2}$ = 69.3%) (Fig. S3A), but in contrast to faecal analytes, the plasma-based OPLS-DA identified plasma cytokines as the main discriminating factors, whilst plasma FABP2 and D-lactate were found to have no discriminatory ability (Fig. S3B). This plasma-based model was also significant by OPLS-DA diagnostics (Fig. S3B). This highlights a critical difference between the 2 anatomical compartments and provides further support for the independent faecal and plasma coregulation clusters discussed previously.

The separation between the SC and AD groups was next investigated by receiver operating characteristic (ROC) curve analysis (Fig. S2). Faecal FABP2 and D-lactate were identified as first- and second-best discriminators with areas under the ROC (AUROC) curve of 0.886 ± 0.059 and 0.875 ± 0.059, respectively (*q* = 0.004 for both) and cut-offs with sensitivities ≥84% and specificities ≥78% (Table S2A). Linear combinations of the 11 significant faecal analytes (FABP2, D-lactate, IL-21, IL-1β, IL-6, IL-17F, IL-12p70, IL-22, IL-17E, IFNγ, and IL-17A) were then used to create discriminant scores (DSs), whose performance was assessed by AUROC analyses (Fig. S2; Table S2B; individual DS equations in Table S3). The DS model based on faecal FABP2 plus faecal D-lactate improved the AUROC to 0.940 ± 0.035 (*q* = 0.00084) with higher sensitivity (89%) and specificity (100%) compared with FABP2 alone. Sequential addition of other faecal cytokines (IL-21, IL-1β, and IL-6) diluted this effect, leading to less powerful models. Thus, faecal FABP2 and faecal D-lactate as markers of gut barrier integrity and intestinal inflammation appear to be highly effective discriminators of the SC and AD groups.

We also performed the same analysis with plasma analytes (Fig. S4), but in contrast to the faecal findings, plasma-based AUROC analysis identified plasma FABP2 and plasma D-lactate as the only 2 analytes lacking SC/AD discrimination. Plasma IL-21 was the strongest discriminator (AUROC 0.860 ± 0.057; *q* = 0.0038; sensitivity/specificity = 77%/82%), followed by all the other 12 plasma cytokines (TNFα, IL-23, IL-17F, IL-1β, IL-8, IL-12p70, IL-22, IFNγ, IL-17A, IL-17E, IL-10, and IL-6) (Table S4A). DSs built sequentially combining all 13 significant plasma cytokines showed that all the top 5 plasma parameters (IL-21, TNFα, IL-23, IL-17F, and IL-1β) were necessary to achieve the best AUROC (0.922 ± 0.037; *q* = 0.000016; sensitivity/specificity = 82%/92%) compared with all other models (Fig. S4; Table S4B; individual DS equations in Table S5). Notably, the DS model using only faecal FABP2 and D-lactate achieved better discrimination than the DS model using the top 5 plasma parameters.

Discussion

In this study, we describe for the first time profiling of faecal cytokines and faecal markers of gut barrier integrity in cirrhosis. We demonstrate that intestinal inflammation involving the gut-liver axis is strongly associated with AD and more so than with equivalent plasma markers. In fact, we found that faecal cytokines and gut barrier integrity markers discriminated between the SC and AD groups with superior sensitivity (95%) and
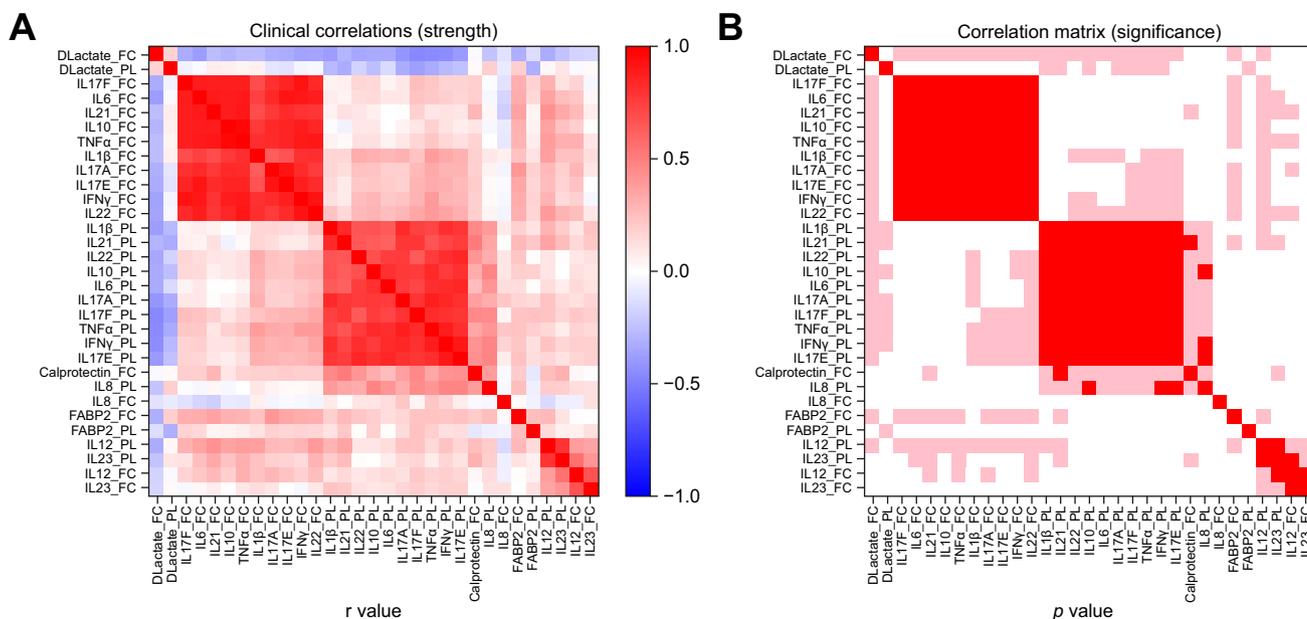
Fig. 3. Intercorrelations between all the analytes, faecal and plasma cytokines, FABP2, D-lactate, and faecal calprotectin. (A) Intercorrelation matrix obtained by hierarchical clustering, using Pearson's correlation and 'complete' method as clustering parameters. (B) Significance plot representing the *p* values associated with the correlation levels from (A), using the following thresholds: white, non-significant; pink, *p* ≤0.05 (trend); red, *p* ≤0.00012 (significant). The significance threshold was determined by Bonferroni FWER correction. FABP2, fatty-acid-binding protein-2; FWER, family-wise error rate; IFNγ, interferon gamma; TNFα, tumour necrosis factor alpha.

extremely high specificity (100%) when compared with the same circulating plasma analytes.

Faecal cytokine measurements have been previously reported for IL-2 and IFNγ in diarrhoea caused by noroviruses[52] and TNFα in Crohn's disease,[53] but not in the context of cirrhosis. Other conventional markers of gut inflammation, such as FCAL,[20] have also been shown to be non-specifically elevated in decompensated cirrhosis.[25,54,55] A relative limitation of FCAL, however, is that it is representative of mainly neutrophil activity and not of the other critical innate (*e.g.* innate lymphoid cells) and adaptive (*e.g.* T-regulatory and T-helper) immune cell subsets that are increasingly recognised as involved in gut mucosal homeostasis and dysregulation.

Increased levels of mucosal cytokines in the IL-23-T_H17 axis were detected in the faeces of the AD group. Mucosal IL-12p70-T_H1 axis was also upregulated in AD, but to a lesser extent, with IL-12p70 and IL-23 representing 2 intimately related master regulators of T cell-mediated type 1/type 17 effector balance.[45] Our results suggest a generalised and non-specific upregulation of type 1 and type 17 effector cytokines, which may be more detrimental in propagating non-specific gut mucosal inflammation.[42,45] In this context, faecal IL-1β, IL-6, and TNFα were also elevated in the AD group compared with the SC and HC groups; these cytokines are considered promiscuous innate drivers of inflammation throughout the T_H1–T_H17 spectrum, with increased tissue concentrations observed in chronic inflammatory pathologies affecting the gut.[42,45] Dual T_H1/T_H17 induction is reported in animal models during infection with *Citrobacter rodentium*, when the epithelial layer is severely disrupted and bacterial invasion had occurred,[15] providing context to our findings for evidence of similar dual T_H1/T_H17 pathway induction in patients with cirrhosis and detrimental effect on the gut barrier.

Even under physiological conditions, the gut has a basal level of inflammation as evidenced by our HC data, requiring finely tuned interactions between the different cytokines and their receptors to support intestinal mucosal homeostasis. In addition, many of the cytokines measured have dichotomous pro- and anti-inflammatory roles in mucosal immunity.[56] For example, IL-1β, IL-6, TNFα, and IL-17A are cytokines with well-known pro-inflammatory roles, but are also involved in promoting epithelial proliferation, crucial for both wound closure and replacing cells lost through homeostatic and likely pathological shedding.[57–59] IL-1β levels correlate with the severity of intestinal inflammation in Crohn's disease as a result of increases in IEC tight junction permeability.[60] IL-22 is involved in repair and protection of barrier surfaces, especially in conjunction with IL-17A/F, IL-36γ, and IL-23, which, during intestinal injury, collectively drive antimicrobial peptide secretion, recruitment and activation of immune cells, and barrier protection.[42,43,61] However, IL-22 can also increase IEC tight junction permeability and enhance the pro-inflammatory capacity of TNFα depending on the micro-environment, whilst IL-17A can be destructive by promoting neutrophilic inflammation.[62,63]

IL-21 has also been found to beneficially control inflammatory pathways in the intestine, yet is upregulated in IBD, stimulating the secretion of extracellular-matrix-degrading enzymes by fibroblasts and enhancing T cell recruitment by IECs.[43,44] Prolonged combined IFNγ and TNFα expression has been shown to contribute to an impairment of barrier function of IECs,[43,64] with the latter inducing IEC damage by excessive neutrophil adhesion and degranulation.[43] Similarly, IL-1β and TNFα activate immune responses suppressing intestinal pathogens, but excessive levels exacerbate inflammation.[56,65] Collectively, in AD, we observed a complex cytokine micro-environment that can drive intestinal inflammation and perpetuate intestinal barrier disruption that is
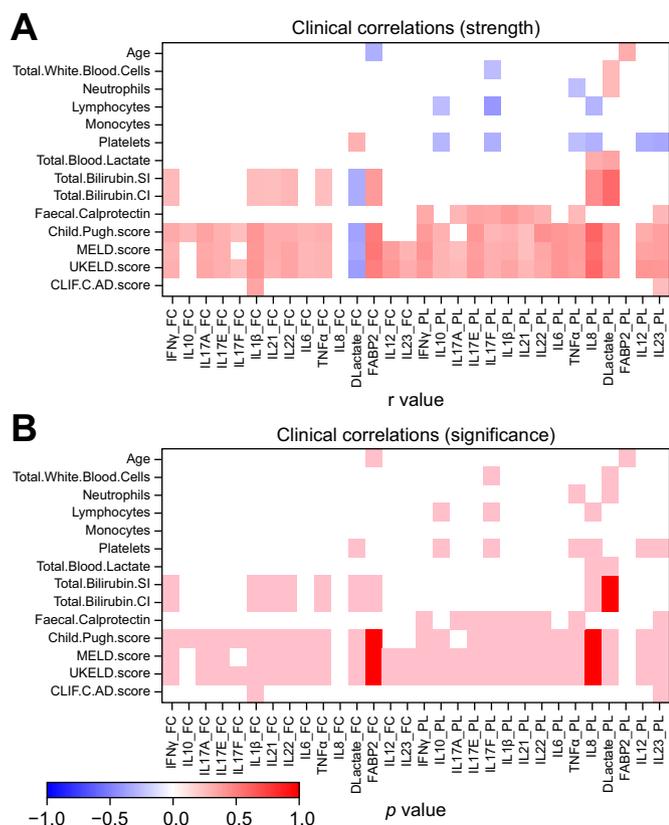
Fig. 4. Correlation between faecal/plasma analytes and clinical parameters. (A) Correlation level plot of faecal and plasma cytokines, FABP2, and D-lactate with individual clinical parameters, including faecal calprotectin and disease composite severity and prognostication scores. Pearson's correlation or Spearman's correlation coefficients were calculated as appropriate, and only correlations with $p \leq 0.05$ are represented. (B) Significance plot representing the p values associated with the correlation levels using the following thresholds: white, non-significant; pink, $p \leq 0.05$ (trend); red, $p \leq 0.00013$ (significant). The significance threshold was determined by Bonferroni FWER correction. CLIF-C AD, Chronic Liver Failure Consortium-acute decompensation; FABP2, fatty-acid-binding protein-2; FWER, family-wise error rate; IFNγ, interferon gamma; MELD, model for end-stage liver disease; TNFα, tumour necrosis factor alpha; UKELD, United Kingdom model for end-stage liver disease.

evident in these patients given the panel of gut barrier integrity markers measured in tandem. This combination, if left unchecked, can lead to pathological BT, hepatic inflammation, and fibrotic transformation, and may even predispose to the development of hepatocellular carcinoma.[66,67] The findings in this study strongly support the role of these faecal cytokines in driving disease progression in cirrhosis, which, given their pattern, appear to be derived mainly from T cell adaptive immune-mediated processes. The plasma cytokine profiles in AD, conversely, appear to be driven more by innate immune responses, which are well characterised.[68] These data highlight the need for more focused studies to elucidate the precise origins, effects, and roles of these cytokines at the gut interface. There may be a need to therapeutically rebalance these dichotomously acting cytokines in the gut mucosa, and equally, targeting these cytokines in the systemic circulation may not be the way forward.[69] For example, patients with alcoholic hepatitis and underlying cirrhosis who were treated with systemic TNFα

inhibitors experienced a higher rate of adverse events, including serious infections and overall mortality.[70]

Faecal IL-8 and IL-10 were not found to be elevated in the AD group compared with the SC or HC group in contrast to what was observed in matched plasma samples. This may be indicative of a defective AD-specific response in keeping with cirrhosis-associated immune dysfunction, and which may be associated with deleterious downstream consequences such as hampering differentiation of anti-inflammatory T-regulatory cells[45]; limiting IL-8-mediated recruitment of neutrophils to the gut epithelium[71]; and an inability to recover from gut barrier injury,[72] in combination perpetuating intestinal injury and inflammation, and propagating BT.

D-Lactate and FABP2 were quantified to reflect gut barrier damage and intestinal inflammation when conventionally measured in plasma.[49] Measurement of faecal FABP2 is novel and may represent IEC shedding from the epithelial monolayer into the lumen, causing transient gaps or micro-erosions in the gut barrier, resulting in increased intestinal permeability[73] and contributing to pathological BT. A 6-fold increase in faecal FABP2 levels was found in the AD group when compared with the SC and HC groups, suggesting that gut barrier injury is involved in the progression from stable to decompensated cirrhosis.

FABP2, when elevated in plasma, conventionally indicates enterocyte damage,[74–76] was overall higher than that measured in faeces, but failed to significantly distinguish between AD and SC despite a trend towards higher levels in AD. Faecal and not plasma measurement of FABP2 provided a more sensitive assessment of gut mucosal injury in cirrhosis and the ability to differentiate between AD and SC. FABP2 was also the only faecal analyte to correlate positively with liver disease composite scores, such as Child-Pugh, MELD, and UKELD, consistent with faecal FABP2 measurement being representative of the intestinal niche. These cross-sectional single time point data suggest a biological relevance of faecal FABP2 to the severity of cirrhosis and hepatic decompensation, and along with the other analytes measured should be studied in greater detail in patients longitudinally given the need to establish causality as well as their biomarker potential.

D-Lactate is found in high levels in pathological states as a result of increased gut microbial production,[51,77] and elevated levels in plasma are considered to be an indicator of BT[50] attributable to a breach in the gut epithelial barrier. This metabolite has been found elevated in the plasma of patients with gut ischaemia[78] and alcohol-related liver disease.[49] Faecal D-lactate measurement in this study is new, and levels were significantly different across all 3 groups, but unlike FABP2, lower levels were detected in the AD group when compared with the SC and HC groups. In contrast, plasma D-lactate levels were comparable across all groups with a trend towards higher levels in the AD group. The lower faecal D-lactate levels in the AD group are previously unreported and may reflect several mechanisms:

- increased translocation from the intestinal lumen into the systemic circulation *via* an impaired gut barrier;
- enrichment of D-lactate-metabolising gut microbial species[79] as a result of gut dysbiosis in AD patients, such that faecal levels of this bacterial metabolic substrate fall; and
- loss of D-lactate-producing gut bacterial species (such as *Lactobacillus* spp.[80]), which has been reported to occur in alcohol-induced liver injury.[81]

FCAL was measured as a conventional marker of gut epithelial inflammation,[20] and in this context was used as a clinical measure with which to compare the faecal and plasma analytes. FCAL has been shown to be elevated in decompensated cirrhosis in this study and in previous works.[25,54] FCAL broadly and non-specifically quantifies intestinal inflammation and has been shown in other conditions, such as Crohn's disease, where gut inflammation is the pathologically defining hallmark to positively correlate with plasma cytokines, such as IFNγ, IL-6, TNFβ, and IL-17A.[82] Recently, patients with severe acute respiratory syndrome coronavirus 2 causing coronavirus disease 2019 were reported to have elevated FCAL levels depending on the presence of diarrhoeal symptoms and where FCAL positively correlated with plasma IL-6 levels.[83] Given that FCAL is derived mainly from neutrophils and relates to the presence of neutrophils in the epithelium and intestinal erosions or ulcers,[84] it cannot provide insights into the other increasingly recognised and important inflammatory pathways linked to the various aforementioned innate and in particular adaptive immune cell subtypes, especially of the T_H1/T_H17 axis. That FCAL was significantly higher in the AD group *vs.* the SC group and correlated positively with conventional markers of liver disease severity (Child-Pugh and MELD) and with some plasma cytokines, but did not with any of the faecal analytes, suggests that the pathways driving intestinal inflammation in AD are T cell mediated and likely more biologically relevant to those related to neutrophil activity.

What is striking is that no significant differences were found in any of the analytes by way of cytokines, D-lactate, FABP2, and FCAL when comparing between the SC and HC groups, regardless of the faecal or blood matrix in which they were measured. This is suggestive of there being relative biological equipoise in compensated cirrhosis in gut barrier integrity and systemic pro- and compensatory anti-inflammatory processes, which once disturbed are associated with progression to hepatic decompensation and cirrhosis-associated immune dysfunction.[85] These findings differ from existing reports, where concentrations of IL-6, IL-10, and IL-17A were higher in the plasma of the SC group than in the HC group[32]; notably, in the same study, only these 3 cytokines were measured, and no faecal analysis was undertaken.

As to the origin of these faecal cytokines, there are several mechanisms that are likely to be contributory and require further investigation:

- leakage across the epithelium caused by gut barrier disruption[53];
- vectorial (apical) secretion, previously reported for IL-1β, IL-6, and IL-8, which mediate autocrine epithelial restitution[86];
- immune cells, such as neutrophils and macrophages, passing into the gut lumen[87]; and/or
- IEC present in the faeces, which are shed because of mucosal injury in AD, as evidenced by elevated faecal FABP2 levels.

Equilibrium between the rate of epithelial shedding at the villus tip and generation of new cells in the crypt is key to maintaining intestinal tissue homeostasis. However, in intestinal inflammatory states, pathological IEC shedding causes micro-erosions in the epithelial barrier, resulting in increased intestinal permeability.[73] Enhanced mucosal expression of IL-23, IL-1β, IL-21, IFNγ, TNFα, IL-17E, and IL-17F in conjunction with reduced IL-8 and IL-10 expression, as detected in the faeces of the AD group, would provide a micro-environment propagating gut barrier disruption.[39,42,43,72] The levels and combinations of cytokines detected in the faeces of the AD group may therefore contribute to increased pathological IEC shedding and enhanced BT, as evidenced by elevated faecal FABP2 and plasma D-lactate levels, respectively.

Confounding factors that may have impacted on the findings reported are the higher proportion of patients in the AD group that were more likely to be actively drinking with alcohol as the primary cause of cirrhosis and also more likely to be treated with antibiotics. Existing data show deleterious changes in the gut microbiome, bile acid, and other metabolic profiling in actively drinking patients with cirrhosis,[88] and that alcohol directly increases gut permeability and mediates barrier disruption,[49,89,90] as well as the more widespread deleterious impact of antibiotics on the gut microbiome.[91] Alcohol ingestion and antibiotic treatment play important roles in both gut epithelial barrier function and systemic inflammation. Whilst the impact of these factors requires further investigation, it is important to note for the latter that analyses of the impact of antibiotic therapy on the various analytes measured in the AD group did not reveal any significant effect on any of the faecal or plasma markers.

In conclusion, profiling of cytokines and gut barrier integrity markers in faeces as a biological matrix represents an innovative approach to the localised assessment of the intestinal cytokine micro-environment in cirrhosis with simultaneous evaluation of gut mucosal inflammation and barrier dysfunction. Our data demonstrate that AD is associated with a highly inflamed and damaged gut barrier, and that faecal cytokine and gut barrier integrity marker profiles, which appear to be T cell driven, are very different from the classical and innate-like features of systemic inflammation in cirrhosis, as determined by plasma-based assays. This study begins to delineate the complex mechanisms governing intestinal inflammation in cirrhosis, which are increasingly recognised as a major driver in disease progression and hepatic decompensation, and which have been elusive and challenging to study to date. This is an important area that warrants immediate attention and further study focusing on the underlying mechanisms at a cellular level. A more complete understanding of how cytokine biology promotes intestinal mucosal homeostasis and damage at different stages of cirrhosis also presents an opportunity for developing treatments. Similarly, pharmacological modulation of faecal cytokine production by gut-targeting therapies[56] needs further exploration.

Abbreviations

ACLF, acute-on-chronic liver failure; AD, acute decompensation; AUROC, area under the receiver operating characteristic; BT, bacterial translocation; CLIF-C AD, Chronic Liver Failure Consortium-acute decompensation; DS, discriminant score; FABP2, fatty-acid-binding protein-2; FCAL, faecal calprotectin; FDR, false discovery rate; FL, faecal lysate; FWER, family-wise error rate; GVB, gut vascular barrier; HC, healthy control; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; MELD, model for end-stage liver disease; OPLS-DA, orthogonal projection to latent structures discriminant analysis; PAMP, pathogen-associated molecular

pattern; PCA, principal component analysis; ROC, receiver operating characteristic; SC, stable cirrhosis; UKELD, United Kingdom model for end-stage liver disease.

Conflicts of interest

None of the authors have any conflicts of interest to declare.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

AR performed all data analyses, generated all data tables and figures, participated in data interpretation, and edited the paper. EHG performed laboratory-based analyte measurements. SA participated in sample processing and clinical metadata collation. AZ recruited study participants, and undertook sampling and clinical metadata collation. MJWM provided insights for statistical data and multivariate analyses. RPV assisted with specific laboratory assays. RW and SC provided intellectual content and participated in editing of paper. VCP recruited study participants, designed the study, participated in data interpretation, and wrote and revised the paper. LAE designed the study, developed the faecal assay protocol, participated in data interpretation, and edited the paper. All authors have read and approved the final version of the paper.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2020.100151.

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