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RESEARCH ARTICLE

Intestinal permeability and immune-inflammatory markers in patients with idiosyncratic drug-induced liver injury, drug-induced steatosis and metabolic dysfunction–associated steatotic liver disease (MASLD)

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Background and Purpose: Adverse immuno-inflammatory responses possibly influenced by bacterial compounds reaching the liver as a consequence of altered intestinal permeability appear to be crucial in the pathogenesis of drug-induced liver injury and steatotic liver diseases. This study aimed to assess intestinal permeability and immuno-inflammatory status in patients by measuring indirect biomarkers.

Experimental Approach: Circulating marker levels were measured in serum and plasma samples of 36 healthy controls, 32 patients with drug-induced liver injury, 14 with autoimmune hepatitis, 13 with viral hepatitis, 40 with metabolic dysfunction–associated steatotic liver disease (MASLD) and 16 with drug-induced steatosis. All patients with acute liver injury were identified (visit 1) and followed for >30 days (visit 2). Correlation analyses were performed to determine potential associations.

Key Results: Drug-induced liver injury, autoimmune hepatitis and viral hepatitis patients had higher levels of LBP, CD14, CD163, MCSF-1R (CSFR) and ICAM-1 and significantly lower levels of MAdCAM-1 and zonulin at detection of liver injury compared with healthy controls or the second visit. Drug-induced steatosis and MASLD patients had increased levels of S100A9, S100A12 and zonulin. MASLD patients with significant fibrosis (F2–F4) also had higher levels of CD163 and MCSF-1R. No difference was found between drug-induced steatosis and MASLD with no or low fibrosis.

Conclusion and Implications: Our results highlight similarities in macrophage activation, intestinal barrier dysfunction and translocation of bacterial products in liver

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma glutamyl transpeptidase; MASLD, metabolic dysfunction–associated steatotic liver disease; PAMPs, pathogen-associated molecular patterns; SLD, steatotic liver disease; TBL, total bilirubin; ULN, upper limit of normal.

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injury of various aetiologies. A better understanding of the pathophysiological mechanisms may aid the development of targeted therapies for liver inflammation and fibrosis.

KEYWORDS
drug-induced steatosis (DIS), hepatotoxicity, idiosyncratic drug-induced liver injury (DILI), immune response, inflammation, intestinal permeability, steatosis

1 | INTRODUCTION

Many drugs or dietary and herbal products are, albeit infrequently, associated with cases of acute or chronic liver injury. Despite its relatively low incidence, idiosyncratic drug-induced liver injury (DILI) is an important public health problem due to being the reason for a significant fraction of acute liver failure cases (Reuben et al., 2016). Drug-induced liver injury is also a concern for the pharmaceutical industry, being a major cause of drug withdrawal both during drug development and post-marketing (Kullak-Ublick et al., 2017). The drug-induced liver injury pathophysiology is unclear and its diagnosis remains challenging, as it may mimic other acute or chronic liver diseases, such as viral hepatitis (VH), autoimmune hepatitis (AIH) or metabolic dysfunction-associated steatotic liver disease (MASLD).

The relationship between drug-induced liver injury and MASLD can be bidirectional (Lammert et al., 2019). On the one hand, MASLD may increase the risk of drug-induced liver injury to certain drugs. On the other hand, there are some drugs that can induce or worsen hepatic steatosis. This phenotype of drug-induced liver injury, named drug-induced steatosis (DIS), is scarcely reported in drug-induced liver injury registries and poorly understood in terms of pathophysiology and mechanistic differences from other steatotic liver diseases (SLDs) (Di Pasqua et al., 2022; Satapathy et al., 2015).

Recent studies highlight the role of immune responses in drug-induced liver injury development, noting increased activated T-cells (Cueto-Sanchez et al., 2021) and specific human leukocyte antigen (HLA) associations (Lucena et al., 2011; Nicoletti et al., 2017; Urban et al., 2017). Additionally, damage caused by local inflammatory processes seems to be crucial for drug-induced liver injury onset (Sernoskie et al., 2021) and significantly influences the progression of MASLD, and drug-induced steatosis to severe complications like steatohepatitis and liver fibrosis (Dong et al., 2024).

Evidence suggests that changes in the intestinal barrier are a contributing factor to an exacerbated immune response in many liver disorders (Albillos et al., 2020). During increased intestinal permeability, metabolites or pathogen-associated molecular patterns (PAMPs), such as **lipopolysaccharide (LPS)**, can reach the liver via portal circulation and interact with pattern recognition receptors on the liver resident cells and potentially induce or aggravate a harmful immune response (Chopyk & Grakoui, 2020).

Experimental animal models have shown that LPS signalling pathways are required to reproduce drug-induced liver injury by some drugs (Deng et al., 2006; Gong et al., 2022). Intestinal dysbiosis, which can lead to increased permeability and endotoxemia (Chu et al., 2023), has been reported in both drug-induced liver injury and MASLD (Roman-Saguillo et al., 2024; Zazueta et al., 2024).

Intestinal integrity relies on a multilayered barrier, whose permeability can be assessed indirectly by measuring peripheral markers related to its structure or homeostasis (Schoultz & Keita, 2020). For instance, zonulin has been correlated with increased intestinal permeability as it reversibly disrupts tight junctions in the intestine (De Munck et al., 2020; Fasano, 2012). The intestinal **fatty acids binding protein 2 (FABP2/I-FABP)** is an enterocyte-specific protein, whose concentration in circulation increases after intestinal epithelium injury, informing on the integrity of the intestinal barrier (Voth et al., 2017; Yuan et al., 2021). The presence of LPS in blood is highly indicative of bacterial translocation from the gut (Koutsounas et al., 2015). Additional valid markers of gut permeability are the **LPS-binding protein (LBP)** and **CD14** (Reiberger et al., 2013; Tabung et al., 2017), which participate in the interaction and clearance of LPS, leading to production of proinflammatory molecules

What is already known

- Increased intestinal permeability and immune-inflammatory processes contribute to drug-induced liver injury and steatotic liver diseases.

What does this study add

- Macrophage activation is essential in the development of liver diseases.
- The liver-gut axis plays a common role in various acute and steatotic liver diseases.

What is the clinical significance

- Circulating biomarkers help assessing intestinal permeability and immunomodulatory status during liver injury.
- New mechanistic information supports therapeutic target discovery.

essential for T cell activation through **TLR4** signalling (Muta & Takeshige, 2001).

Immune-inflammatory processes can both induce and be triggered by intestinal barrier dysfunction, contributing to liver disease progression (Fukui, 2016; Hietbrink et al., 2009). Circulating markers may reflect different aspects of these interactions, including bacterial translocation, macrophage and neutrophils activation, and leukocyte recruitment. For instance, CD163, primarily expressed in M2-like macrophages, plays a role in tissue repair and bacterial clearance but is shed from the cell surface and released into circulation upon activation by proinflammatory stimuli, including LPS (Fabriek et al., 2009). Increased serum levels of CD163 have been widely associated with hepatic inflammation and fibrosis, reflecting macrophage activation in acute and chronic liver diseases (Aprilia et al., 2024; Etzerodt & Moestrup, 2013; Gantzel et al., 2020; Gronbaek et al., 2016; Hintz et al., 2002; Moller et al., 2007; Nielsen et al., 2020). Similarly, **colony stimulating factor 1 receptor/macrophage colony-stimulating factor receptor (CSFR/MCSF-1R)**, a key regulator of macrophage proliferation and differentiation, plays a role in immune cell recruitment and may participate in immune-inflammatory processes in liver diseases (Xiang et al., 2023). Microbial translocation can also lead to up-regulation of the **intracellular adhesion molecule 1 (ICAM-1)** and **mucosal vascular addressin cell adhesion molecule-1 (MAdCAM-1)**, which also play important roles in liver inflammation and may reflect transmigration of leukocytes into injured liver (Blobaum et al., 2023; Sole et al., 2016; Zhong et al., 2021). Particularly, MAdCAM-1 is a key regulator of gut-liver immune crosstalk, mediating enterohepatic circulation of lymphocytes in liver diseases (Schippers et al., 2021). In addition, there is evidence that calcium-binding proteins S100A9 and S100A12 may be relevant in certain types of liver injury by mediating neutrophil chemotaxis activation. These proteins are elevated during inflammatory processes, including those triggered by bacterial components (Moles et al., 2014; Vandal et al., 2003).

To our knowledge, the status of intestinal integrity and its relationship with immune-inflammatory markers has not yet been investigated in drug-induced liver injury and drug-induced steatosis. In this study, we aimed to investigate the relationship between markers of intestinal barrier function and immuno-inflammatory status in drug-induced liver injury and other acute liver diseases, as well as in conditions such as drug-induced steatosis and metabolic dysfunction–associated steatotic liver disease (MASLD), to better understand common pathophysiological mechanisms between these pathologies.

2 | METHODS

2.1 | Study population and sample collection

The study population included patients with acute liver injury (ALI) and steatotic liver diseases (SLDs). The acute liver injury group included 32 patients with drug-induced liver injury, 13 with acute viral hepatitis and 14 with acute presentation of autoimmune hepatitis. The SLD group included 16 patients with drug-induced steatosis and 40 patients with MASLD. Patients were compared with a cohort of 36 healthy controls (HC) without any known liver condition. All patients were prospectively recruited and enrolled into the Spanish drug-induced liver injury Registry. Serum and plasma samples were collected from all donors and stored at −80°C until analysis. To assess changes in disease progression, drug-induced liver injury, viral hepatitis and autoimmune hepatitis patients were followed from the time of detection (visit 1, v1) until >30 days later (visit 2, v2). Visit 1 (v1) was chosen to analyse the status of biomarkers at the acute phase of liver injury, while visit 2 (v2) aimed to assess the initial recovery phase when significant clinical and biochemical improvements are typically observed. For drug-induced steatosis, MASLD and healthy controls groups, a single sample was collected at the time of enrolment. The use of samples and clinical data for research purposes was approved by the local Ethics Committee of the coordinating centre at the Virgen de la Victoria University Hospital in Malaga. All patients gave written informed consent prior to the collection of biological samples. At visit 1, none of the patients had received treatment for their liver condition. During the follow-up period, at visit 2, patients with drug-induced liver injury and viral hepatitis received nonspecific supportive care, while autoimmune hepatitis patients received standard corticosteroid therapy according to current guidelines.

2.2 | Inclusion and exclusion criteria

The biochemical criteria for inclusion of drug-induced liver injury patients were as follows: ALT ≥ 5 × upper limit of normal (ULN) or ALT ≥ 3 × ULN + total bilirubin (TBL) > 2 × ULN or alkaline phosphatase (ALP) ≥ 2 × ULN. The diagnosis of drug-induced liver injury was based on the presence of a compatible temporal relationship between drug exposure and liver injury as well as exclusion of alternative causes, such as viral hepatitis (hepatitis A virus, hepatitis B virus, hepatitis C virus, hepatitis E virus, cytomegalovirus and Epstein–Barr virus), autoimmune hepatitis, biliary disorders, vascular diseases or hepatic tumours. Patients with no temporal relationship between the drug and the liver damage were also excluded. All cases were adjudicated by an expert panel consisting of at least three hepatologists with expertise in drug-induced liver injury. The viral hepatitis and autoimmune hepatitis patients met the same biochemical criteria as drug-induced liver injury. Type of injury, defined by the R value (R = ALT/ULN/ALP/ULN), and severity of the acute episode were determined as described elsewhere (Aithal et al., 2011).

The MASLD group included patients diagnosed with steatosis by one or more of the following factors (Rinella et al., 2023): fatty liver index (FLI) > 60, controlled attenuation parameter (CAP) > 248 db·m^{-1}, the presence of steatosis on abdominal ultrasound or liver biopsy and the presence of metabolic syndrome. The exclusion criteria for the MASLD group were alcohol intake >20 g·day^{-1} (men) and >10 g·day^{-1} (women), other causes of chronic liver disease,

steatogenic drug treatment or pregnancy. MASLD patients were divided into two groups according to the degree of liver fibrosis: no significant liver fibrosis (F0/F1) and significant fibrosis (F2/F3/F4) measured by transient elastography (FibroScan®). Drug-induced steatosis in the drug-induced steatosis group was defined as the presence of fat on liver biopsy or imaging in patients on long-term steatogenic drugs with no prior evidence of hepatic steatosis. The healthy controls presented normal liver tests, on the day of inclusion into the study.

2.3 | Circulating intestinal permeability and inflammation marker analyses

Serum LBP, CD14, CD163, MCSF-1R (CSFR), I-CAM, MAdCAM-1, S100A9 and S100A12 levels were determined using the Luminex platform with custom-made panels (R&D Systems, Minneapolis, MN, USA) following the manufacturer instructions. I-FABP and zonulin levels were measured in serum using the Human FABP2/I-FABP Quantikine ELISA Kit (DFBP20; R&D systems) and zonulin ELISA kit (Immunodiagnostik AG, Bensheim, Germany), respectively. LPS was quantified in plasma using an ELISA kit (#EKC34448, Biomatik, Wilmington, DE, USA), following the manufacturer instruction. Circulating LPS levels were only quantified in healthy controls ($n = 21$), drug-induced liver injury ($n = 19$) and autoimmune hepatitis ($n = 6$) due to limited sample availability.

2.4 | Data and statistical analysis

The data and statistical analysis adhere to the recommendations for experimental design and analysis outlined by the *British Journal of Pharmacology* (Curtis et al., 2025). Differences between the groups were compared using Kruskal–Wallis tests followed by post hoc analyses using Mann–Whitney test only if Kruskal-Wallis test statistic (H) achieved $P < 0.05$. The Wilcoxon test was used for paired sample comparisons. All statistical analyses were performed only when the sample size of each study group was at least $n = 5$; all groups met this requirement. No outliers were excluded from the data analysis based on statistical criteria; however, individual values falling outside the detection limits of the assays were excluded. The reported group size corresponds to the number of biologically independent patient samples and statistical analyses were performed using these independent values. Technical replicates were not treated as independent measurements. Group data are plotted as mean ± standard deviation (SD), with individual data points shown as spots. Associations between the candidate biomarkers and conventional biochemical parameters were determined using a Spearman correlation analysis. All p values were considered statistically significant when $P < 0.05$. The data were analysed using GraphPad Software (Prism 9) (GraphPad Software, San Diego, CA) and RStudio (4.4.0). Post hoc power analysis for all statistically significant comparisons was conducted using G*Power (version 3.1.9.6).

2.5 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/24 (Alexander, Fabbro, et al., 2023; Alexander, Kelly, et al., 2023).

3 | RESULTS

3.1 | Characteristics of the study population

Demographic and clinical characteristics of the study cohorts are shown in Table 1. The mean age was similar between groups. Women predominated among healthy controls and patients with autoimmune hepatitis and drug-induced steatosis, while men were more frequent among patients with viral hepatitis and MASLD F0–F1. Patients with MASLD had higher BMI values. Regarding liver biochemical parameters, patients in the acute liver injury groups had higher ALT, AST, gamma glutamyl transpeptidase (GGT), ALP and TBL than those in the SLD groups. Patients with autoimmune hepatitis and viral hepatitis also had higher levels of AST, ALT and TBL than those diagnosed with drug-induced liver injury. The values of these parameters decreased at visit 2 and approached those of the healthy controls group. Hepatocellular pattern of injury and moderate severity predominated in the acute liver injury groups.

In drug-induced liver injury patients, the most frequent causative agent class was anti-infectives (53.3%), with amoxicillin-clavulanate being most prevalent drug (17.8%). The next most frequent causative agent class was herbal products (8.9%), followed by lipid modifying agents (6.7%). The full list of causative agents for each drug-induced liver injury patient is shown in Table S1. The drugs responsible for causing steatosis in the drug-induced steatosis group were **tamoxifen** (56%), followed by **methotrexate** (38%) and **amiodarone** (6%); 88% of drug-induced steatosis patients did not have significant fibrosis (Table S2).

3.2 | Intestinal and immune-inflammatory markers during acute drug-induced liver injury and acute non-drug-induced liver injury liver disease

Drug-induced liver injury, autoimmune hepatitis and viral hepatitis had higher CD163, MCSF-1R and ICAM-1 and lower MAdCAM-1 and zonulin serum concentrations compared with healthy controls at visit 1. Drug-induced liver injury and viral hepatitis had also higher levels of LBP and CD14 than healthy controls at visit 1 (Figure 1). Differences were observed between drug-induced liver injury, autoimmune hepatitis and viral hepatitis patients for MCSF-1R, with the highest level found in autoimmune hepatitis and the lowest level in drug-induced liver injury (Figure 1d). In relation to plasma LPS levels, at visit 1 the greatest differences were seen between autoimmune hepatitis

TABLE 1 Demographics and clinical characteristics of the study cohorts.

Parameter	HC	DILI	AIH	VH	DIS	F1 ≤ MASLD	F2 ≥ MASLD
n	36	32	14	13	16	20	20
Female, n (%)	23 (64)	16 (50)	11 (79)	3 (23)	12 (75)	6 (30)	10 (50)
Age (years), mean ± SD	44 ± 14	49 ± 16	48 ± 17	47 ± 20	51 ± 8	50 ± 12	60 ± 9
BMI ($kg \cdot m^{-2}$), mean ± SD	26 ± 5	25 ± 5	23 ± 2	25 ± 4	27 ± 4	28 ± 4	32 ± 4
Diabetes, n (%)	1 (3)	2 (7)	0 (0)	3 (23)	2 (20)	4 (20)	13 (65)
Dyslipidaemia, n (%)	0 (0)	6 (26)	1 (8)	2 (22)	3 (30)	7 (35)	10 (50)
Hypertension, n (%)	4 (12)	7 (30)	1 (8)	2 (20)	4 (40)	5 (25)	16 (80)
Severity, %							
Mild	NA	14 (44)	1 (7)	2 (15)	NA	NA	NA
Moderate	NA	17 (53)	12 (86)	10 (77)	NA	NA	NA
Severe	NA	1 (3)	1 (7)	0 (0)	NA	NA	NA
Death/liver transplantation	NA	0 (0)	0 (0)	1 (8)	NA	NA	NA
Type of injury, %							
Hepatocellular	NA	20 (65)	12 (92)	9 (69)	NA	NA	NA
Cholestatic	NA	6 (19)	1 (8)	1 (8)	NA	NA	NA
Mixed	NA	5 (16)	0 (0)	3 (23)	NA	NA	NA

Laboratory parameters		Visit 1	Visit 2	Visit 1	Visit 2	Visit 1	Visit 2			
AST, $IU \cdot L^{-1}$	23 ± 7	414 ± 560	31 ± 17	1,061 ± 455	122 ± 197	765 ± 730	157 ± 214	29 ± 10	31 ± 15	46 ± 22
ALT, $IU \cdot L^{-1}$	21 ± 12	638 ± 591	56 ± 38	1,281 ± 569	220 ± 331	1,056 ± 1,272	103 ± 129	36 ± 19	56 ± 42	68 ± 32
GGT, $IU \cdot L^{-1}$	18 ± 13	493 ± 859	147 ± 257	340 ± 254	167 ± 145	346 ± 284	111 ± 125	35 ± 24	79 ± 93	138 ± 124
ALP, $IU \cdot L^{-1}$	67 ± 19	309 ± 296	112 ± 87	227 ± 123	114 ± 51.3	264 ± 192	162 ± 273	69 ± 18	80 ± 41	93 ± 51
TBL, $mg \cdot dl^{-1}$	0.6 ± 0.2	5.2 ± 5.5	0.9 ± 0.7	11.7 ± 7.4	1.5 ± 1	7.2 ± 7.5	4.6 ± 12.7	0.6 ± 0.2	0.8 ± 0.5	0.6 ± 0.3

Note: All percentages were calculated based on available clinical data.

Abbreviations: AIH, autoimmune hepatitis; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DILI, drug-induced liver injury; DIS, drug-induced liver steatosis; GGT, gamma glutamyl transpeptidase; HC, healthy controls; MASLD, metabolic dysfunction–associated steatotic liver disease; TBL, total bilirubin; VH, viral hepatitis.

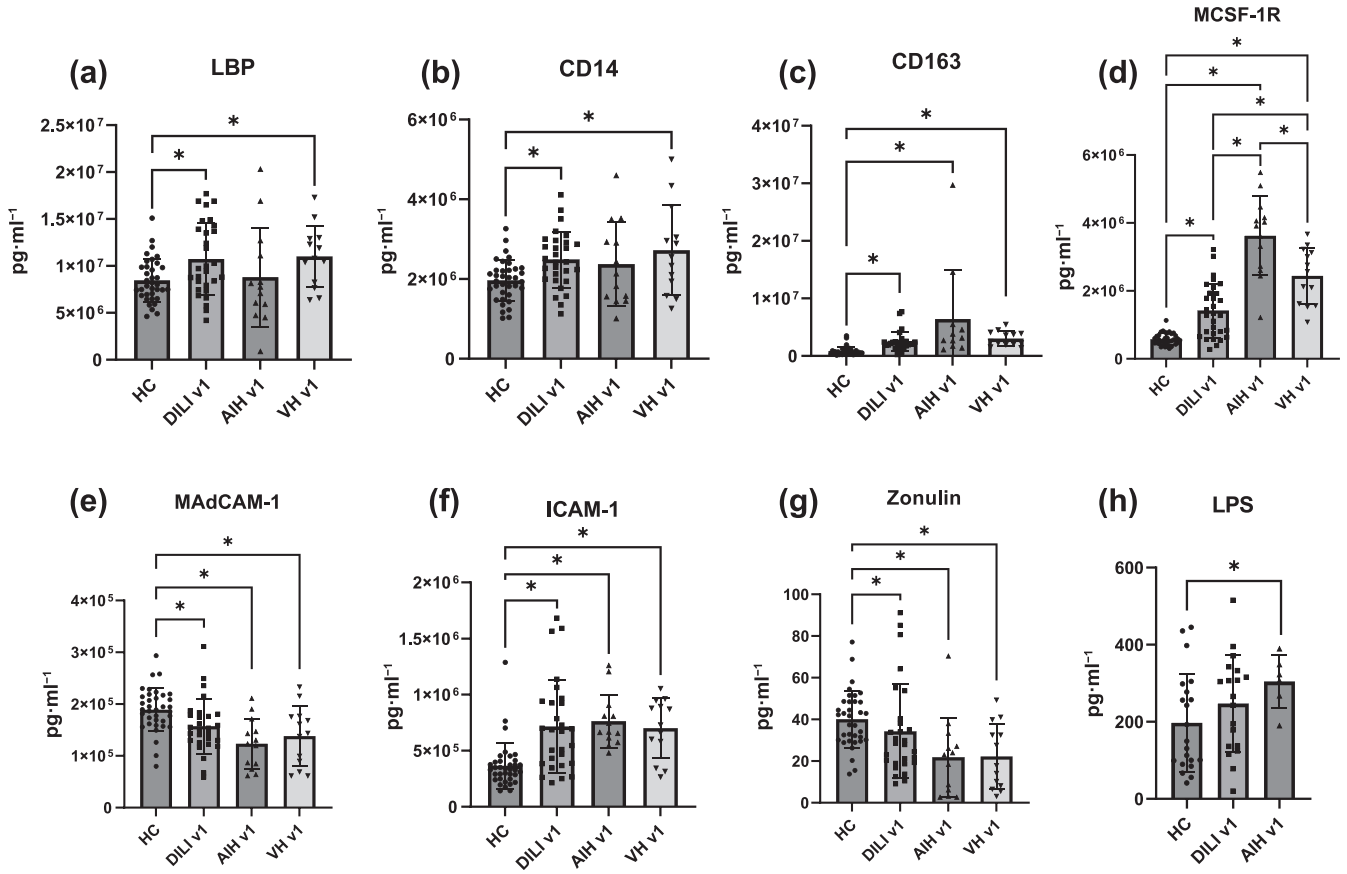

FIGURE 1 Circulating levels of (a) LBP, (b) CD14, (c) CD163, (d) MCSF-1R, (e) MAdCAM-1, (f) ICAM-1, (g) zonulin and (h) LPS for healthy controls (HC, n = 36), patients with drug-induced liver injury (DILI, n = 32), autoimmune hepatitis with acute onset (AIH, n = 14) and viral hepatitis (VH, n = 13) at visit 1 (v1). Circulating LPS levels were only quantified in HC (n = 21), DILI (n = 19) and AIH (n = 6). Data are presented as mean ± SD; *P < 0.05.

and healthy controls. Drug-induced liver injury patients also showed a trend towards increased LPS levels, although this was not significant (Figure 1h). No significant differences were found between groups for S100A9, S100A12 and I-FABP, although drug-induced liver injury showed a tendency towards having higher levels of S100A9, S10012 and lower levels of I-FABP than healthy controls. Detailed p values and post hoc statistical power analyses are shown in Tables S3 and S4, respectively.

3.3 | Intestinal and immune-inflammatory markers >30 days after the acute episode

At visit 2, >30 days after liver injury detection, all acute liver injury groups showed decreased levels of LBP, CD14, CD163, MCSF-1R and ICAM-1 compared with visit 1 (Figure 2). In contrast, MAdCAM-1 levels increased in all groups at visit 2, approaching the levels observed in the healthy controls group. Although no significant changes in circulating levels of I-FABP were found in drug-induced liver injury and viral hepatitis compared with healthy controls at visit 1, levels were higher at the follow-up visit (Figure 2h). Notably, drug-induced liver injury patients had higher plasma LPS levels at visit 1 compared with visit 2 (Figure 2i). Detailed p values and post hoc statistical power analyses are shown in Tables S5 and S4, respectively.

3.4 | Intestinal and immune-inflammatory markers in drug-induced steatosis and MASLD patients

All three groups of patients with SLD showed increased levels of S100A9, S100A12 and zonulin compared with healthy controls. When stratifying MASLD patients according to liver fibrosis, several differences were found between MASLD F0–F1 and drug-induced steatosis versus MASLD F2–F4 patients. The MASLD F2–F4 group had higher levels of LBP, CD163 and MCSF-1R, while no differences were found between the drug-induced steatosis and MASLD F0–F1 groups (Figure 3). No significant differences were found when comparing CD14, ICAM-1 and I-FABP levels between groups, although MASLD F2–F4 appeared to have higher I-FABP levels than healthy controls. Detailed P values and post hoc statistical power analyses are shown in Tables S6 and S4, respectively.

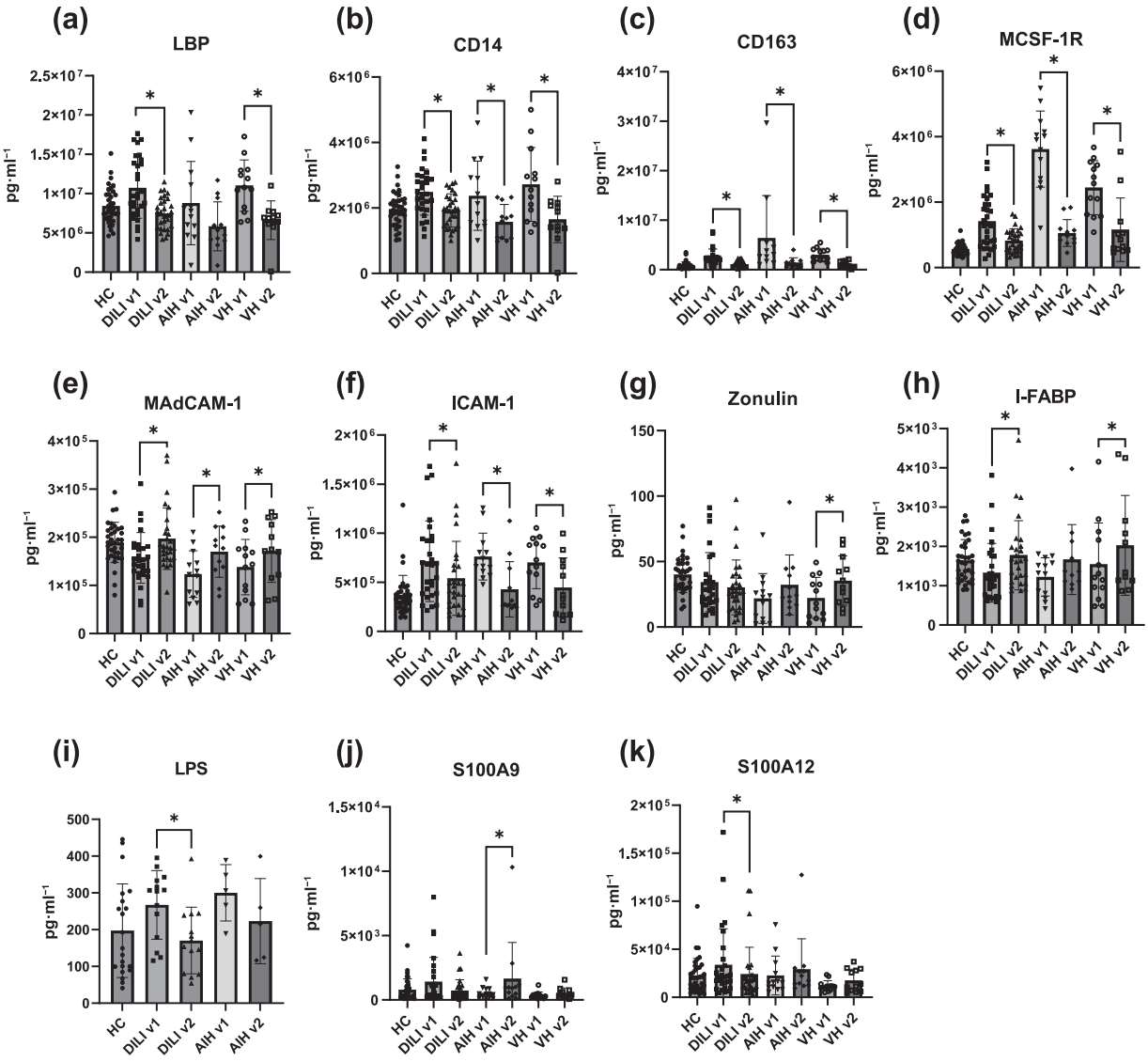

FIGURE 2 Circulating levels of (a) LBP, (b) CD14, (c) CD163, (d) MCSF-1R, (e) MAdCAM-1, (f) ICAM-1, (g) Zonulin, (h) zonulin and (i) LPS, (j) S100A9 and (k) S100A12 for healthy controls (HC, n = 36), patients with drug-induced liver injury (DILI, n = 32), autoimmune hepatitis (AIH, n = 14) and viral hepatitis (VH, n = 13) at visit 1 (v1) and 2 (v2). Circulating LPS levels were only quantified in HC (n = 21), DILI (n = 19) and AIH (n = 6). HC values are displayed as a visual guide but were not included in the statistical analysis. Data are presented as mean ± SD; *$P < 0.05$.

3.5 | Correlations between intestinal permeability, immune-inflammatory and traditional biochemical markers

Correlation analyses between intestinal permeability and immune-inflammatory biomarkers and traditional biochemical parameters (AST, ALT, GGT, ALP, TBL and INR) in patients with acute liver injury were performed using Spearman's rank correlation coefficient (Figure 4). CD163 and MCSF-1R showed a moderate correlation with AST ($\rho = 0.62$ and 0.74, respectively, $P < 0.0001$) and ALT ($\rho = 0.63$ and 0.65, respectively, $P < 0.0001$). MCSF-1R was also significantly correlated with TBL ($\rho = 0.69$, $P < 0.0001$). Moderate correlations were found between LBP and CD14 ($\rho = 0.62$, $P < 0.0001$), CD163 and MCSF-1R ($\rho = 0.68$, $P < 0.0001$) and S100A9 and S100A12 ($\rho = 0.74$, $P < 0.05$). ICAM-1, MCSF-1R and CD163 were correlated weakly with ALP, GGT and INR.

4 | DISCUSSION

In this study, we evaluated a variety of markers indirectly related to intestinal barrier function and immuno-inflammatory status in patients with acute liver injury (drug-induced liver injury, autoimmune hepatitis and viral hepatitis) and SLD (drug-induced steatosis) and metabolic dysfunction–associated steatotic liver disease (MASLD). The inclusion of different markers allowed the assessment of intestinal barrier dysfunction from multiple perspectives, considering alterations in tight junction regulation (zonulin), enterocyte damage (I-FABP) and

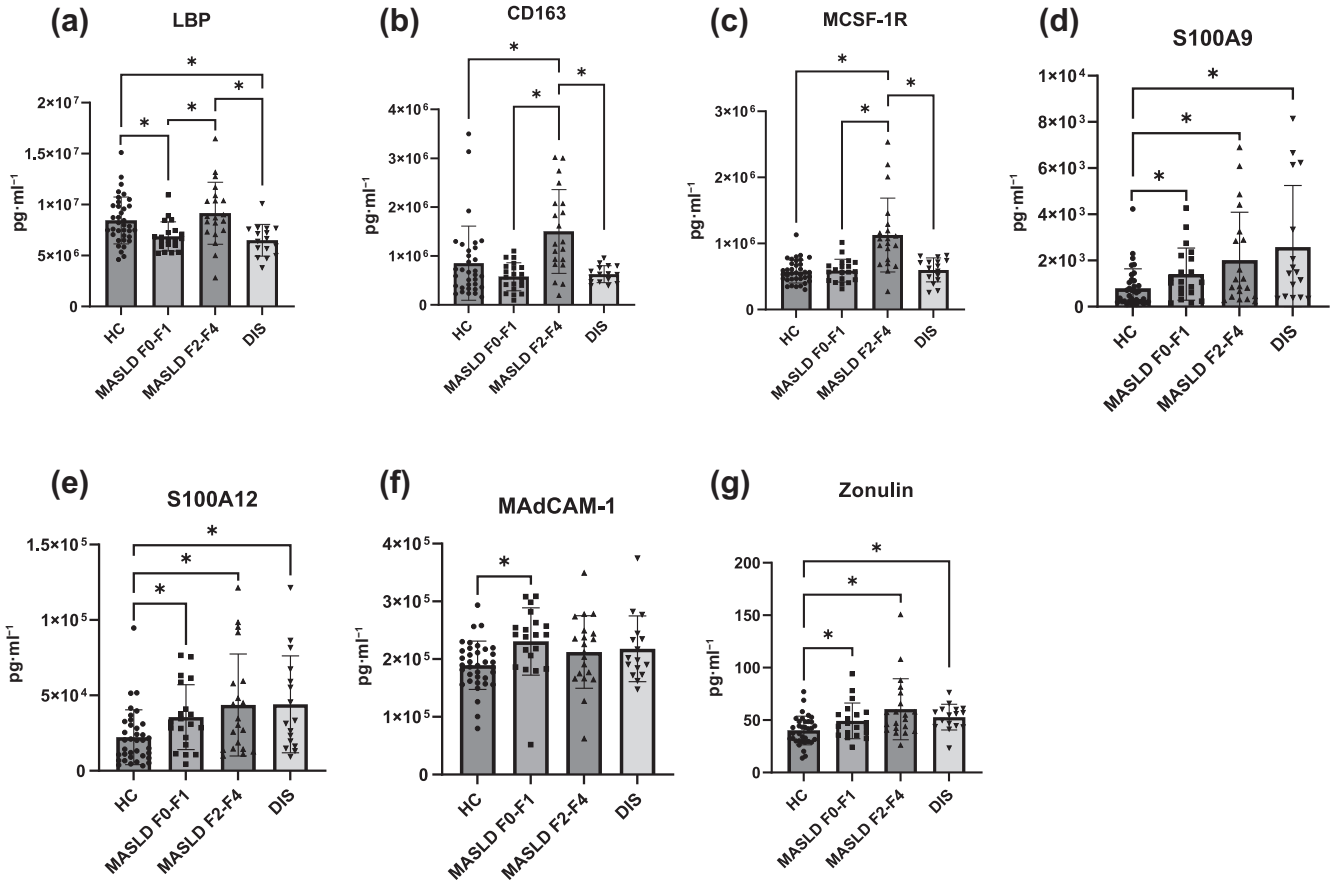

FIGURE 3 Circulating levels of (a) LBP, (b) CD163, (c) MCSF-1R, (d) S100A9, (e) S100A12, (f) MAdCAM-1 and (g) zonulin for healthy controls (HC, n = 36), patients with metabolic dysfunction-associated steatotic liver disease (MASLD) F0–F1 (n = 20), MASLD F2–F4 (n = 20) and drug-induced steatosis (DIS, n = 16). Data are presented as mean ± SD; *$P < 0.05$.

bacterial translocation (LPS, LBP, CD14) as complementary endpoints. The analysis of MCSF-1R (CSFR), CD163, S100A9, S100A12, MAdCAM-1 and ICAM-1 as immuno-inflammatory markers aimed to assess innate immune responses, focusing on macrophage and neutrophil activation, as well as immune cell migration.

Acute liver injury patients had increased levels of LBP and CD14. This suggests that intestinal barrier dysfunction and PAMPs may partly contribute to acute liver injury pathogenesis, supporting the gut-liver axis hypothesis. Although autoimmune hepatitis patients had no significant differences in LBP and CD14 at visit 1, there was an upward trend that was confirmed when comparing visit 1 with visit 2. In addition, autoimmune hepatitis patients showed the highest levels of LPS. Kupffer cells and macrophages in the liver may respond to LPS, triggering a TLR4 signalling pathway that releases proinflammatory factors and chemokines, thereby worsening liver inflammation (Nicoletti et al., 2019).

The involvement of macrophages during the inflammatory damage in acute liver injury patients is supported by elevated CD163 levels. Our results are in line with the findings of Du et al., who detected elevated levels of CD163, LPS and LBP in drug-induced liver injury patients (Du et al., 2021). High levels of soluble CD163 are found in various acute and chronic inflammatory diseases, serving as a reliable marker of macrophage activation and positively correlating with the severity of liver injury (Aprilia et al., 2024; Du et al., 2021; Gronbaek et al., 2016; Moller et al., 2007). Interestingly, although the exact biological role is unknown, soluble CD163 has demonstrated immunomodulatory functions by reducing T-lymphocyte activation and proliferation *in vitro* (Frings et al., 2002). It has been reported that LPS can highly influence CD163 shedding (Hintz et al., 2002). Inflammation-induced shedding of CD163 is followed by a subsequent increase in surface CD163 expression (Van Gorp et al., 2010), suggesting a regulatory mechanism to prevent excessive immune activation. Therefore, apart from reflecting the degree of active inflammation, elevated CD163 levels may also indicate a proportional immunomodulatory response.

The importance of macrophages during acute liver injury is also supported by elevated levels of MCSF-1R, possibly indicating increased macrophage proliferation. However, the role of the MCSF-1R signalling pathway in inflammatory liver diseases is still unclear (Xiang et al., 2023). Damaged hepatocytes can release MCSF-1, one of MCSF-1R ligands, which promotes Kupffer cells proliferation and the infiltration of blood monocytes into the liver. MCSF-1 contributes to the generation of M2 macrophage phenotype that promotes hepatocyte proliferation and liver recovery (Tacke & Wynn, 2015). In

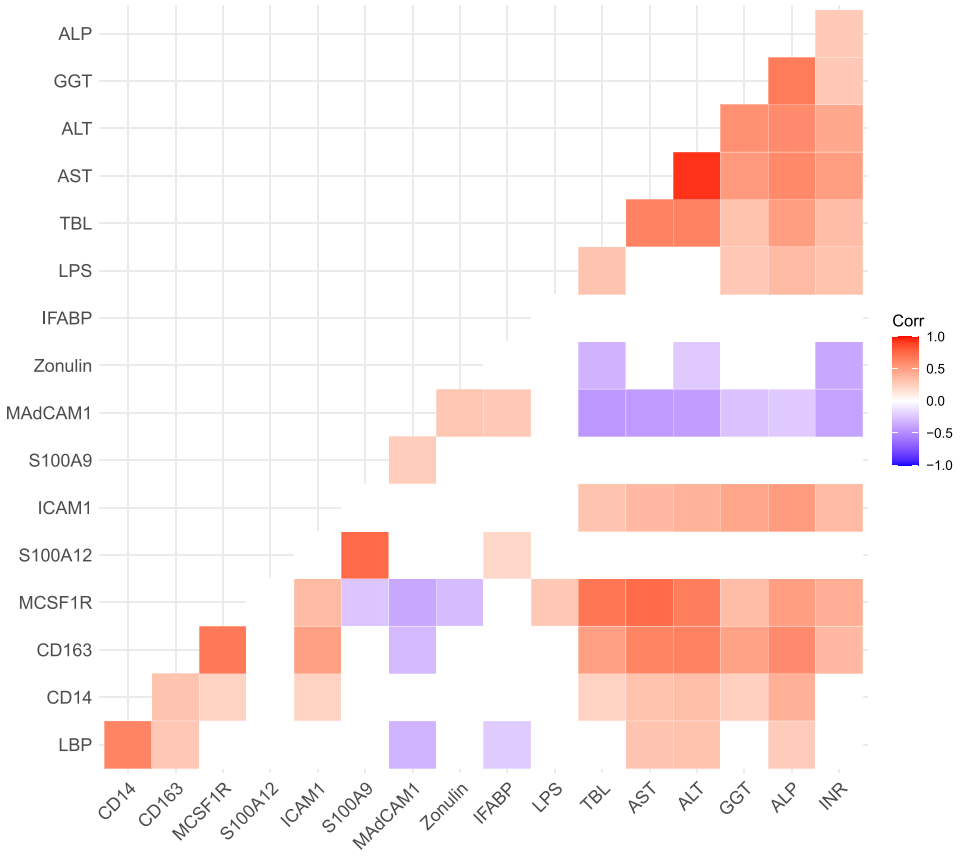

FIGURE 4 Spearman correlation heatmap of intestinal permeability and immune-inflammatory biomarkers and traditional biochemical parameters, including aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), total bilirubin (TBL), gamma-glutamyl transferase (GGT) and international normalized ratio (INR) for acute liver injury (ALI) patients at visit 1. Red indicates positive correlation, blue negative correlation. Nonsignificant correlations are left blank.

addition, MCSF-1R was described as a promising diagnostic and prognostic marker in liver diseases (Church et al., 2019). This may explain why drug-induced liver injury patients had lower increases in both CD163 and MCSF-1R compared with autoimmune hepatitis and viral hepatitis, which had a higher proportion of moderate and severe cases. The lack of significant changes in S100A9 and S100A12 levels in drug-induced liver injury, autoimmune hepatitis and viral hepatitis patients may indicate that neutrophil-driven inflammation is not the predominant mechanism in these forms of acute liver injury. Interestingly, M2 macrophages have shown to protect against liver injury through inhibiting S100A9-mediated inflammation during acute on chronic liver failure (Bai et al., 2021).

The simultaneous role of CD163 and MCSF-1R in acute liver damage is further supported by the positive correlation between these proteins and AST and ALT. Their correlation with AST and TBL also supports their prognostic relevance. Interestingly, it has been described that MCSF-1 signalling strongly induces CD163 expression in macrophages (Buechler et al., 2000).

Patients with acute liver injury had decreased zonulin and showed no changes in I-FABP. These findings suggest that increased gut permeability in acute liver injury is unlikely due to direct intestinal epithelial cell death or impairment of tight junction proteins via zonulin signalling. It has been shown that proinflammatory cytokines, which are known to be increased in acute liver injury (Bonkovsky et al., 2018), can rapidly induce paracellular intestinal permeability without affecting tight junction expression (Wang et al., 2005). Lower zonulin levels in acute liver injury patients could also reflect a protective mechanism to restore intestinal barrier integrity. Calgin and Cetinkol found reduced circulating zonulin in hepatitis B patients, suggesting a compensatory response to prevent PAMPs from entering the bloodstream (Calgin & Cetinkol, 2019).

Acute liver injury patients had alterations in ICAM-1 and MAdCAM-1. Both proteins have been associated with inflammatory processes, signalling between the gut and the liver and leukocyte migration (Hintermann & Christen, 2019). High levels of ICAM-1 in acute liver injury patients may indicate systemic inflammation or an ongoing reparatory process, as ICAM-1 plays both proinflammatory and anti-inflammatory roles (Bui et al., 2020). This elevation has also been observed in other immune-mediated liver diseases (Denk et al., 2014). In contrast, MAdCAM-1 levels decreased in acute liver injury patients, possibly indicating endothelial dysfunction or regulatory mechanisms that decrease MAdCAM-1 shedding to modulate lymphocyte migration to the liver. In line with this, blocking MAdCAM-1 has shown to reduce liver inflammation by preventing the recruitment of pro-inflammatory cells to the liver (Gupta et al., 2024; Schippers et al., 2021). Interestingly, low MAdCAM-1 levels have recently been associated with intestinal dysbiosis (Fidelle et al., 2023).

The few differences in biomarkers levels between drug-induced liver injury, autoimmune hepatitis and viral hepatitis patients suggest common immune-mediated mechanisms driving liver injury and intestinal permeability alterations. Macrophage-driven inflammation and immune cell migration to the liver are hallmarks of liver disease,

contributing to both the initiation of inflammatory responses and the activation of tissue repair mechanisms. The inflammatory state likely results in increased intestinal permeability in these pathologies. The shared changes in immunomodulatory markers may also reflect the initiation of reparative anti-inflammatory responses. The consistent decrease in zonulin levels suggests a compensatory mechanism to restore intestinal barrier integrity and reduce bacterial translocation as part of a protective response. While the exact mechanisms remain unclear, these findings suggest interrelated roles of immune dysregulation, bacterial translocation and repair in the pathophysiology of liver disease.

Contrary to what was observed in the acute liver injury group, patients in the SLD group had increased zonulin levels compared with healthy controls. This aligns with findings from MASLD cohorts reporting elevated serum zonulin levels and increased intestinal permeability (De Munck et al., 2020). It is important to note that dyslipidaemia, obesity and insulin resistance have been described as important risk factors for altered intestinal integrity (Leech et al., 2019). Although CD14 levels remained unchanged in patients with SLD, higher LBP levels were observed in MASLD F2–F4 compared with MASLD F0–F1 and drug-induced steatosis. These differences support the hypothesis that increased intestinal permeability aggravates hepatic inflammation and fibrosis through the action of PAMPs (Albillos et al., 2020). However, the relationship between endotoxemia and the degree of fibrosis needs further study (De Munck et al., 2020).

The SLD groups showed increased serum concentrations of the immuno-inflammatory molecules S100A9 and S100A12. Inflammation is closely related to SLD development and is associated with progression to fibrosis, potentially leading to cirrhosis and hepatocellular carcinoma (Dong et al., 2024). Evidence from animal models suggests that S100A9 up-regulation plays a role in MASLD development and contributes to fibrosis by enhancing myofibroblast migration (Cai et al., 2022; Chang et al., 2024). In addition, MASLD patients with significant fibrosis had increased levels of CD163 and MCSF-1R. Consistent with our findings, Kazankov et al. also reported that CD163 concentrations are associated with severity of MASLD (Kazankov et al., 2016). Additionally, other studies have identified associations between CD163 levels and fibrosis progression and have found correlations between gut permeability and CD163 in cirrhosis (Kawanaka et al., 2023; Rainer et al., 2018). This relationship may be influenced by the involvement of proliferative M2 macrophages, which contribute to fibrogenic processes following inflammation (Xi et al., 2021). No differences were found in any of the markers between drug-induced steatosis and MASLD F0–F1 patients, suggesting that these conditions may share pathological mechanisms involving intestinal permeability and inflammation, regardless of whether the origin of steatosis is pharmacological or not.

Consistent with the potential macrophage phenotype switch indicated by high CD163 and MSCF-1R, a previous immunophenotyping study led by our group demonstrated increased expression of the immunoregulatory molecule PD-L1 in monocytes from patients with drug-induced liver injury, autoimmune hepatitis, viral hepatitis and MASLD (Cueto-Sanchez et al., 2021). Interestingly, PD-L1 signalling has been associated with macrophage polarization towards an M2-like phenotype (Wei et al., 2021).

Our study analyses a broad panel of markers that provide new insights into liver diseases of diverse aetiologies, but it is not without limitations. As noted by Schoultz and Keita, there is no gold standard for measuring intestinal barrier function and proposed markers of intestinal permeability may be influenced by other pathological processes (Schoultz & Keita, 2020). However, we observed a trend towards healthy controls values in most markers as recovery from the acute condition progressed. It is also important to note that absence of LPS data in the viral hepatitis and SLD groups prevents confirmation of endotoxaemia in these patients, although other indirect markers of permeability suggest this. In addition, further studies are needed to determine if exacerbated inflammation is a cause or result of increased intestinal permeability. Finally, the sample sizes in certain patient groups, such as autoimmune hepatitis, viral hepatitis and drug-induced steatosis, were relatively small, reflecting the rarity of these conditions. Nevertheless, the data were derived from a prospectively enrolled and well-characterized cohort, ensuring robustness of the findings and providing valuable insights that warrant validation in larger, multicentre studies.

The results of this study provide useful information on disease mechanisms. A better understanding of the pathophysiology of liver injury may help to establish future therapeutic strategies. For example, MCSF-1R has been proposed as a therapeutic target for the treatment of inflammatory liver diseases, by reducing macrophages proliferation and recruitment (Xiang et al., 2023). Targeting glucocorticoids to CD163 macrophages has been effective in limiting steatohepatitis progression in *in vivo* models (Skytthe et al., 2020). In cases where dysbiosis is behind increased intestinal permeability, therapies aimed at restoring microbiota homeostasis may be useful strategies to mitigate the adverse effects of PAMPs translocation. For example, treatments aimed at restoring gut integrity and probiotic levels have been shown to inhibit LPS signalling pathways and alleviate drug-induced liver injury caused by antituberculosis drugs (Gong et al., 2022). Furthermore, the role of intestinal permeability changes and macrophage activation in liver disease progression suggests that these markers may be relevant for monitoring disease progression and treatment response in future clinical studies. Notably, the particularly elevated levels of MCSF-1R and CD163 in MASLD F2–F4 patients support the relevance of these markers for disease stratification and as potential therapeutic targets. For instance, circulating CD163 has already shown promise as a marker for monitoring treatment response in autoimmune hepatitis and MASLD (Gronbaek et al., 2016; Rodgaard-Hansen et al., 2017).

In conclusion, this study highlights the pivotal role of macrophage activation and intestinal barrier dysfunction in the pathogenesis of liver injury across various aetiologies. Elevated intestinal permeability markers support the gut-liver axis hypothesis, suggesting that microbial translocation contributes to liver inflammation. The differences found in immunological markers highlight the complexity of immune-inflammatory responses and regulations during liver injury. Understanding these mechanisms provides valuable information that could guide the development of targeted therapies to modulate liver inflammation or fibrosis.

AUTHOR CONTRIBUTIONS

D. E. Di Zeo-Sánchez: Conceptualization (lead); data curation (equal); formal analysis (equal); investigation (lead); methodology (equal); software (equal); visualization (equal); writing—original draft (lead). **I. Díaz-Alberola**: Investigation (equal); writing—original draft (equal). **J. M. Pinazo-Bandera**: Resources (equal). **M. Garcia-Cortés**: Funding acquisition (equal), resources (equal). **J. Sanabria Cabrera**: Resources (equal). **M. Robles-Díaz**: Resources (equal). **I. Álvarez-Álvarez**: Data curation (equal); methodology (equal). **M. I. Lucena**: Conceptualization (equal); funding acquisition (equal); project administration (equal); supervision (equal); writing—review and editing (equal). **R. J. Andrade**: Funding acquisition (equal); project administration (equal); supervision (equal); writing—review and editing (equal). **M. Villanueva-Paz**: Investigation (equal); methodology (equal); supervision (lead); visualization (equal); writing—review and editing (equal). **C. Stephens**: Conceptualization (equal); funding acquisition (equal); methodology (equal); supervision (lead); writing—review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. Some data may not be made available because of privacy or ethical restrictions.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design and Analysis as recommended by funding agencies, publishers and other organizations engaged with supporting research.

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