Elevated serum levels of diamine oxidase, D-lactate and lipopolysaccharides are associated with metabolic-associated fatty liver disease

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text

Introduction

Metabolic-associated fatty liver disease (MAFLD), a new definition of fatty liver, has been proposed for the diagnosis of fatty liver disease with metabolic dysfunction [1]. Unlike the diagnostic criteria for nonalcoholic fatty liver disease (NAFLD), those for MAFLD are based on the evidence of fatty liver with overweight/obesity, metabolic dysregulation and the presence of type II diabetes mellitus (T2DM) instead of a series of exclusion criteria [1]. This new definition has been confirmed to be critical for diagnosis, drug discovery and treatment.

Intestinal barrier function plays a crucial role in the genesis and development of MAFLD [2]. The theory of the gut–liver axis, which highlights the communication between the gut and liver, suggests that intestinal barrier dysfunction is associated with the pathogenesis of chronic liver diseases [3]. Increased permeability of the intestinal barrier permits the gut microbiota and its metabolites to enter the blood circulation and travel to the liver via the portal vein [4]. Translocation of bacteria and bacterial products can trigger chronic inflammation, hepatocyte injury and metabolic disorders [5]. Hence, for the prevention and promising treatment of MAFLD, it is necessary to verify the relationship between MAFLD and intestinal barrier integrity and explore the effect of intestinal barrier impairment on the metabolic characteristics of MAFLD patients.

Emerging evidence has shown that as convenient and accessible biomarkers, the serum levels of diamine oxidase [6], D-lactate [7] and lipopolysaccharide [8] can reflect the integrity of the intestinal barrier [9]. Diamine oxidase is an intracellular cytoplasmic protein in intestinal epithelial cells. When the intestinal epithelial barrier is disturbed, diamine oxidase is released into the bloodstream, and increased serum levels of diamine oxidase are associated with intestinal barrier impairment [6]. Increased serum concentrations of D-lactate and lipopolysaccharide, which are metabolites of intestinal flora, can be used to verify the translocation of intestinal flora and their related metabolites into the bloodstream as biomarkers [10]. Therefore,
serum levels of diamine oxidase, D-lactate and lipopolysaccharide can be used to assess intestinal barrier dysfunction quickly and with minimal invasiveness.

In this work, we investigated the role of serum biomarkers of intestinal barrier integrity in MAFLD patients to characterize potential risk factors and provide new targets and strategies for the prevention and treatment of MAFLD.

Patients and methods

Study population

This study was designed as a single-centre, retrospective study in Wuhan, China. The present study was performed in accordance with relevant guidelines and regulations and was approved by the ethics committee of the Zhongnan Hospital of Wuhan University. Informed consent was obtained from the research subjects, and we protected personal information during data collection.

We reviewed the medical data of all inpatients treated in the Department of Gastroenterology at Zhongnan Hospital of Wuhan University from January 2017 to January 2022. The inclusion criteria were as follows: patients (a) between 18 and 75 years old; (b) who had undergone abdominal ultrasonography; and (c) who had undergone measurement of the three serum biomarkers. Participants were excluded if they met the following criteria: (a) carcinoma; (b) severe heart, lung, liver, or kidney disease; (c) nonsteroidal anti-inflammatory drug use in the previous month; (d) intestinal diseases; (e) history of gastrointestinal surgery; and (f) bacterial infection, except Helicobacter pylori. Patients were recorded only once during the study period. A total of 523 participants who had abdominal ultrasonography and measurements of serum levels of diamine oxidase, D-lactate and lipopolysaccharide as part of their clinical review were enrolled. Patients lacking biochemical data (n = 41) were then excluded. Finally, 491 patients were included in the present study. In this study, 197 participants without MAFLD and 294 participants with MAFLD were included.

Data collection

All data were collected at the time of hospitalization. Patients with several hospitalizations during the study were documented only once. We recorded the following clinical information in the computerized hospital database (HIS): age, sex, height, weight, blood pressure, comorbidities, medication and current alcohol intake. BMI and the presence/absence of T2DM, hypertension and dyslipidaemia were obtained by clinical review according to standard criteria [1,11,12]. We collected the following laboratory parameters from the computerized hospital database at Zhongnan Hospital: full blood count, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase, γ-glutamyl transpeptidase (GGT), total protein, albumin, globulin, total bilirubin, direct bilirubin, total bile acid (TBA), total cholesterol, triglycerides, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein cholesterol (LDL), lipoprotein(a) [Lp(a)], nonesterified fatty acid (NEFA), blood urea nitrogen, creatinine, uric acid, electrolytes, fasting glucose, diamine oxidase, D-lactate and lipopolysaccharide.

Metabolic-associated fatty liver disease definition

MAFLD was diagnosed according to the criteria including evidence of fatty liver (hereby, ultrasonography and computed tomography), in addition to one of the following: overweight/obesity defined as BMI ≥ 23 kg/m² in this Asian cohort, presence of T2DM, or lean/normal weight with evidence of metabolic dysregulation [1]. Metabolic dysregulation was defined as the presence of at least two metabolic risk abnormalities: (a) waist circumference ≥ 90 cm in men and ≥ 80 cm in women, (b) blood pressure ≥ 130 mm Hg or specific drug treatment, (c) plasma triglycerides ≥ 150 mg/dL or specific drug treatment, (d) plasma HDL-cholesterol < 40 mg/dL for men and < 50 mg/dL for women or specific drug treatment, (e) fasting glucose ≥ 100 mg/dL, (f) homeostasis model assessment-insulin resistance score ≥ 2.5, and (g) high-sensitivity C-reactive protein level > 2 ml/L (1). Although the homeostasis model assessment-insulin resistance score and plasma high-sensitivity C-reactive protein level are metabolic risk abnormalities, these were not available in our dataset.

Qualitative ultrasonographic evaluations of metabolic-associated fatty liver disease patients

Skilled technicians, who were unaware of the objective of this study, graded each US examination based on the presence and severity of liver steatosis. Then, 294 patients with MAFLD were classified into the following three classes: mild steatosis, moderate steatosis and severe steatosis. The classification criteria were based on [13,14].

Measurement of gut barrier biomarkers

All blood collection was performed after patients were hospitalized for 12 h. Serum was isolated by centrifugation at 2500 x g for 10 min. The serum levels of gut mucosal barrier function parameters [endotoxin (lipopolysaccharide), D-lactate and diamine oxidase] were measured by a dry chemical method using the Intestinal Mucosal Barrier Biochemical Index Analysis System (JY-DLT; Beijing Zhongsheng Jinyu Diagnostic Technology Co., Ltd., Beijing, China) according to the manufacturer’s instructions. The experiments were performed within 4 h after blood collection.

Statistical analysis

The data were analysed using IBM SPSS 26.0 and tabulated with Microsoft Office software. Categorical data were compared by the chi-square test. Continuous variables were examined by the Mann–Whitney U test. Frequencies and proportions are used to represent categorical variables. Medians and interquartile ranges are used to represent continuous values. The correlations were performed by Spearman’s rank test and Pearson’s correlation analysis. We analysed 28 potentially related factors using univariate analysis. Those risk factors with P values less than 0.05 in the univariate analysis were substituted into the binary logistic regression model to verify important risk factors for MAFLD. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated. The results were expressed as ORs with corresponding 95% CIs.
Results

Patient characteristics

The study group comprised 231 (47.0%) females and 260 (53.0%) males. The clinical and biochemical characteristics of patients without and with MAFLD are depicted in Table 1. There were no significant differences in the age distribution; however, MAFLD patients tended to be male and had a higher BMI. There were higher frequencies of hypertension and T2DM and a higher serum level of uric acid in MAFLD patients than in those without MAFLD. Moreover, patients with MAFLD had higher serum liver enzyme (AST, ALT and GGT) levels and higher serum levels of total cholesterol, triglycerides, HDL cholesterol, LDL and NEFAs (P<0.001) (Table 1).

Increased serum levels of diamine oxidase, D-lactate and lipopolysaccharide were associated with metabolic-associated fatty liver disease

The serum levels of D-lactate, diamine oxidase and lipopolysaccharide were significantly higher in patients than in those without MAFLD. Moreover, patients with MAFLD had higher serum liver enzyme (AST, ALT and GGT) levels and higher serum levels of total cholesterol, triglycerides, HDL cholesterol, LDL and NEFAs (P<0.001) (Table 1).

Serum levels of intestinal barrier biomarkers in patients with different grades of liver steatosis

Fatty infiltration of the liver in patients with MAFLD was assessed and diagnosed by ultrasonography. Patients with MAFLD were divided into three groups: mild steatosis (n=154), moderate steatosis (n=110) and severe steatosis (n=30). The serum levels of diamine oxidase, D-lactate and lipopolysaccharide in these three groups were analysed (Fig. 2). The results showed that the serum level of diamine oxidase was significantly higher in the moderate

Table 1. Comparisons of clinical and biochemical characteristics between patients without and with metabolic-associated fatty liver disease

|                      | Patients without MAFLD (n=197) | Patients with MAFLD (n=294) | P value |
|----------------------|---------------------------------|-----------------------------|---------|
| Age                  | 55 (45–64)                      | 53 (44–60)                  | 0.068   |
| Sex (female/male)    | 56.9%/43.1% (112/85)            | 40.3%/59.7% (119/175)       | <0.001  |
| BMI (kg/m²)          | 22.21 (19.60–23.70)             | 25.95 (23.94–28.09)         | <0.001  |
| Type 2 diabetes mellitus (presence/absence) | 4.1% (8/189)              | 15.6% (46/248)              | <0.001  |
| Hypertension (presence/absence) | 18.8% (37/196)           | 32.3% (95/99)               | 0.001   |
| Alcohol intake habit (yes/no) | 6.6% (13/184)              | 11.9% (35/259)              | 0.063   |
| White blood cell count (10⁹/L) | 5.10 (4.40–6.10)         | 6.21 (5.15–7.36)            | <0.001  |
| Red blood cell count (×10¹²/L) | 4.30 (4.00–4.62)          | 4.57 (4.22–4.92)            | <0.001  |
| Hemoglobin (g/L)     | 131.9 (123.0–141.5)            | 140.8 (130.1–151.4)         | <0.001  |
| Platelet count (×10⁹/L) | 195 (166–236)               | 213 (175–255)               | 0.005   |
| AST (U/L)            | 221 (190–263)                  | 28 (19–47)                  | <0.001  |
| ALT (U/L)            | 4.30 (4.00–4.62)               | 4.57 (4.22–4.92)            | <0.001  |
| GGT (U/L)            | 9.70 (7.53–12.21)              | 15.00 (11.02–20.81)         | <0.001  |
| D-lactate (mg/L)     | 13.72 (7.88–17.15)             | 23.16 (17.87–35.37)         | <0.001  |
| Lipopolysaccharide (U/L) | 13.20 (5.69–15.77)         | 14.04 (9.105–17.16)         | 0.006   |

Multivariate logistic regression analysis revealed that BMI (OR=1.324; 95% CI, 1.156–1.517; P<0.001) and tri-glycerides (OR=2.649; 95% CI, 1.437–4.931; P=0.002), NEFA (OR 1.002; 95% CI, 1.000–1.004; P=0.011), diamine oxidase (OR 1.149; 95% CI, 1.055–1.251; P=0.011) and D-lactate (OR 1.221; 95% CI, 1.139–1.308; P<0.001) levels were independently associated with the presence of MAFLD (Fig. 1).
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Steatosis group (\(P=0.005\)) and the severe steatosis group (\(P=0.009\)) than in the mild steatosis group (\(P=0.002\)). The serum D-lactate level was also higher in the moderate steatosis group (\(P<0.001\)) and severe steatosis group (\(P=0.003\)) than in the mild steatosis group. Finally, the serum level of lipopolysaccharide was significantly higher in the moderate steatosis group than in the mild steatosis group (\(P=0.022\)).

Correlations between laboratory parameters and serum levels of intestinal barrier biomarkers

We analysed the correlations between the three biomarkers and laboratory parameters to verify the association between intestinal barrier impairment and MAFLD. The serum level of diamine oxidase was positively correlated with ALT and GGT (\(r=0.303, P<0.0001; r=0.266, P<0.001\); Fig. 3a and b) levels. Serum ALT, GGT, total cholesterol and triglycerides levels were positively correlated with D-lactate levels (\(r=0.366, P<0.001; r=0.348, P=0.031; r=0.238, P<0.001; r=0.373, P<0.001\); Fig. 3c–f). Furthermore, no association was found between serum lipopolysaccharide levels and laboratory parameters.

Increased serum lipopolysaccharide levels in metabolic-associated fatty liver disease patients with multiple metabolic abnormalities

According to the increase in the number of metabolic abnormalities, patients were classified into two groups: <2 metabolic abnormalities and ≥2 metabolic abnormalities [15]. The serum levels of D-lactate, diamine oxidase and lipopolysaccharide in patients with different numbers of metabolic abnormalities are shown in Table 3. The serum level of lipopolysaccharide was higher in MAFLD patients with ≥2 metabolic abnormalities than in those with <2 metabolic abnormalities (\(P=0.034\)). However, there was no significant difference in the serum levels of diamine oxidase and D-lactate in patients with different numbers of metabolic abnormalities.

Discussion

Our research illustrates that MAFLD patients had significantly higher serum levels of diamine oxidase, D-lactate and lipopolysaccharide than those without MAFLD, indicating the association between MAFLD and intestinal barrier dysfunction and translocation of gut bacterial...
metabolites. The serum diamine oxidase, D-lactate and lipopolysaccharide levels increased as the degree of fat infiltration detected by ultrasonography increased. Diamine oxidase and D-lactate are independent risk factors for MAFLD and might be used to improve diagnosis, prevention and identification of potential therapeutic targets for impaired intestinal barrier function in patients with MAFLD. Moreover, our results suggested that diamine oxidase, D-lactate and lipopolysaccharide were related to multiple metabolic abnormalities in MAFLD patients.

Recent studies have evaluated intestinal barrier integrity with several biomarkers, such as 51Cr-ethylene diamine tetraacetate (51Cr-EDTA) and zona occludens-1 (ZO-1) [16]. However, detection of the urinary excretion of 51Cr-EDTA is time-consuming, and duodenal biopsy to obtain specimens for immunohistochemical expression is not easy to perform for the majority of patients with MAFLD [17]. Serum diamine oxidase, D-lactate and lipopolysaccharide are more convenient and better-accepted biomarkers for intestinal barrier impairment [6,9]. Diamine oxidase, a type of oxidative deaminase, is especially active in the intestinal mucosa. Normally, there is a small amount of diamine oxidase in circulation, and its plasma levels are positively correlated with intestinal barrier impairment [18]. D-lactate is produced mostly by intestinal bacteria through the glycolysis pathway, and humans express only L-lactate dehydrogenase but not D-lactate dehydrogenase [19]. D-lactate is released into the circulation when the intestinal barrier is damaged and intestinal mucosal permeability is increased. Therefore, an increased serum level of D-lactate is usually associated with abnormal intestinal permeability [20]. Lipopolysaccharide, a component of the cell wall of gram-negative bacteria, is transferred into the blood when there is gut microbiota dysbiosis and altered intestinal barrier permeability [21]. These three serum biomarkers are used to evaluate intestinal barrier permeability and assess the efficacy of gastrointestinal disease treatment in clinical practice [22]. Hence, it was reasonable to select serum biomarkers to conduct the present study.

In our present study, ultrasonography was used for the diagnosis and qualitative assessment of liver steatosis. We discovered that circulating diamine oxidase, D-lactate and lipopolysaccharide levels showed a corresponding increase with the severity of steatosis, implying that increased intestinal barrier permeability may be linked to deterioration of MAFLD. In addition, our analysis of clinical characteristics showed that elevated diamine oxidase and D-lactate levels are related to ALT and GGT concentrations, while serum D-lactate is correlated with total cholesterol and triglycerides. Several mechanisms could explain why patients with MAFLD have a higher serum level of D-lactate. Recent evidence has shown that microbial d-lactate can promote Kupffer cells to catch and eliminate enterogenic flora from the bloodstream in the portal vein [23]. Activated Kupffer cells play a crucial role in the progression of NAFLD/NASH, as they can cause inflammation and regulate the lipid metabolism of liver cells by secreting cytokines [24]. An elevated level of D-lactate may be related to metabolic dysregulation and may take part in lipid metabolism by activating liver-resident Kupffer cells. On the other hand, metabolites of the intestinal microbiome, including butyrate, bile acids and lipopolysaccharide, have multiple effects on liver cells or macrophages, leading to increased cytokine release and hepatic steatosis [25–27]. Recent evidence has also shown that dysregulation of bile acid homeostasis caused by the gut microbiome is associated with NAFLD severity [26,28]. These studies suggested that metabolites of the intestinal microbiome could have a mutual and complex role in the development of MAFLD and be associated with a worse metabolic state in patients with MAFLD. More research and analyses on gut flora products are required to identify the pathogenesis of and develop treatments for MAFLD.

Intestinal barrier impairment could be caused by many factors, including diet, alcohol intake, medication and dysbiosis of the gut microbiome [29,30]. Accumulating evidence has shown that excessive food intake is significantly associated with alterations in the intestinal barrier [31]. Both a high-fat diet and a fructose-rich diet contribute to dysbiosis of the gut microbiome and increased intestinal permeability [32,33]. Alteration of the gut microbiome has been discovered in patients with metabolic dysregulation. Diminished abundances of Ruminococcaceae, Fusobacterium, Bifidobacterium, Faecalibacterium...
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P. prausnitzii and Bacteroidetes and higher abundances of Enterobacteriaceae, Porphyromas and Fusobacterium have been detected in patients with NAFLD [34,35]. Xia et al. found that Lactobacillus, Bifidobacterium and Clostridium cluster I negatively correlated with D-lactate and endotoxin, while Lactobacillus negatively correlated with diamine oxidase. Moreover, Fusobacterium nucleatum and Enterobacteriaceae correlated positively with D-lactate, diamine oxidase and endotoxin [36]. Hence, intestinal dysbacteriosis may increase the risk of MAFLD as a result of increased intestinal barrier permeability.

Serum lipopolysaccharide elevation in NALFD/NASH has been reported in several studies [37]. Alteration of intestinal flora and increased intestinal mucosal permeability lead to a higher level of lipopolysaccharide in circulation and favour a proinflammatory state [38]. Lipopolysaccharide can stimulate TLR4 expression in the membranes of hepatocytes and Kupffer cells, leading to the secretion of inflammatory cytokines [39]. In the subgroup study, patients with MAFLD were classified into two groups, those with <2 metabolic abnormalities and those with ≥2 metabolic abnormalities, and serum levels of intestinal barrier biomarkers were compared. The serum lipopolysaccharide levels were higher in MAFLD patients with multiple metabolic abnormalities, indicating

### Table 3. Comparisons of serum levels of diamine oxidase, D-lactate and lipopolysaccharide between metabolic-associated fatty liver disease patients with <2 metabolic abnormalities and those with ≥2 metabolic abnormalities

|                     | MAFLD patients with <2 metabolic abnormalities (n = 151) | MAFLD patients with ≥2 metabolic abnormalities (n = 143) | P value |
|---------------------|----------------------------------------------------------|----------------------------------------------------------|---------|
| Diamine oxidase (U/L)| 14.30 (10.34–20.22)                                       | 15.19 (11.76–20.84)                                       | 0.420   |
| D-lactate (mg/L)    | 23.36 (17.20–34.87)                                       | 23.15 (18.53–35.780)                                      | 0.323   |
| Lipopolysaccharide (U/L) | 13.40 (8.14–16.27)                                        | 14.61 (10.02–17.60)                                       | 0.034   |

Quantitative variables were presented median (interquartile range). The Wilcoxon–Mann–Whitney test was utilized to compare data between MAFLD patients with <2 metabolic abnormalities and those with ≥2 metabolic abnormalities.

MAFLD, metabolic-associated fatty liver disease.
that endotoxin translocation was related to complex metabolic disorders in MAFLD. On the other hand, low or normal lipopolysaccharide levels were observed in MAFLD patients who had endotoxin hyperresponsiveness, resulting in a state similar to that of elevated lipopolysaccharide levels in this population [37]. The status of endotoxin hyperresponsiveness can also explain the normal lipopolysaccharide levels of some MAFLD patients in this study. There is an interaction between metabolic dysregulation and intestinal barrier function. Disrupted intestinal homeostasis can lead to metabolic abnormalities and subsequent metabolic illnesses, such as hypertension, T2DM and MAFLD [40]. In return, intestinal epithelial integrity is affected by hyperglycaemia and hyperlipidaemia, causing an abnormal influx of gut microbial products into the circulation and chronic inflammation [41]. Thus, the restoration of intestinal barrier homeostasis and metabolic regulation will require more attention in future studies and therapy.

While these results are novel, there are several limitations to our current investigation. First, our data were gathered retrospectively from a single centre. More participants need to be enrolled for us to create subgroups and confirm our conclusion. Second, more biomarkers are needed in the evaluation of intestinal barrier impairment and translocation of bacterial metabolites. Finally, the cause of intestinal barrier impairment was not clear in our study, and the association of MAFLD with the intestinal microbiome and their related metabolites requires further studies in vivo and in vitro.

In summary, our study was designed to verify the potential risk factors based on the association between MAFLD and increased intestinal barrier permeability. Overweight/obesity and higher plasma levels of diamine oxidase, D-lactate, triglycerides and NEFA were associated with a higher risk of MAFLD. Additionally, the study suggested that the translocation of endotoxins and damage to the intestinal barrier were related to multiple metabolic abnormalities. More research in vivo is required to confirm the potential mechanism underlying the increase in intestinal barrier permeability, the gut microbiota and their products in MAFLD.

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R.Z., Y.-n.C. and J.Z. designed the study and carried out the data collection. J.L. and Q.Z. designed the study. All authors wrote the article, read and approved the final article.

All datasets generated for this study are included in the article/Supplementary Material.

This study was performed in accordance with the Declaration of Helsinki and with the approval of the Ethical Committee of the Zhongnan Hospital of Wuhan University.

Conflicts of interest

There are no conflicts of interest.

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