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Metabolic profiling of tryptophan pathways: Implications for obesity and metabolic dysfunction-associated steatotic liver disease

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Abstract

Background and Aims: The rise in obesity highlights the need for improved therapeutic strategies, particularly in addressing metabolic dysfunction-associated steatotic liver disease (MASLD). We aim to assess the role of tryptophan metabolic pathways in the pathogenesis of obesity and in the different histological stages of MASLD.

Materials and Methods: We used ultra-high performance liquid chromatography to quantify circulating levels of 15 tryptophan-related metabolites from the kynurenine, indole and serotonin pathways. A cohort of 76 subjects was analysed, comprising 18 subjects with normal weight and 58 with morbid obesity, these last being subclassified into normal liver (NL), simple steatosis (SS) and metabolic dysfunction-associated steatohepatitis (MASH). Then, we conducted gene expression analysis of hepatic IDO-1 and kynyrenine-3-monooxygenase (KMO).

Results: Key findings in obesity revealed a distinct metabolic signature characterized by a higher concentration of different kynurenine-related metabolites, a decrease in indole-3-acetic acid and indole-3-propionic acid, and an alteration in the serotonin pathway. Elevated tryptophan levels were associated with MASLD presence (37.659 (32.577–39.823) μM of tryptophan in NL subjects; 41.522 (38.803–45.276) μM in patients with MASLD). Overall, pathway fluxes demonstrated an induction of tryptophan catabolism via the serotonin pathway in SS subjects and into the kynurenine pathway in MASH. We found decreased IDO-1 and KMO hepatic expression in NL compared to SS.

Conclusions: We identified a distinctive metabolic signature in obesity marked by changes in tryptophan catabolic pathways, discernible through altered metabolite profiles. We observed stage-specific alterations in tryptophan catabolism

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fluxes in MASLD, highlighting the potential utility of targeting these pathways in therapeutic interventions.

KEYWORDS
kynurenine, metabolic dysfunction-associated steatotic liver disease, serotonin, tryptophan

1 | INTRODUCTION

Obesity is a severe medical condition characterized by excessive body fat accumulation that significantly impacts health.[1] In the last years, there has been a substantial increase in the incidence of obesity and related diseases.[2] Studies have shown that obesity is associated with a dysregulation of amino acid metabolism, among other metabolic alterations.[3,4] Specifically, increased levels of kynurenine, an intermediate metabolite of the tryptophan pathway, have been found in the plasma of patients with obesity.[5,6]

Kynurenine is considered an immunosuppressive factor. In this sense, Teng Huang et al. postulate that the increase in its levels may occur as a compensatory effect against the low-grade chronic inflammation, common in people with obesity.[7] Various authors suggested that the increase in kynurenine levels may be due to the overexpression of indoleamine 2,3-dioxygenase 1 (IDO1),[8] the enzyme responsible for catalysing the conversion of tryptophan to kynurenine.[8]

Moreover, obesity is a risk factor highly implicated in various metabolic pathologies, including the severe condition of metabolic dysfunction-associated steatotic liver disease (MASLD).[9] The incidence of MASLD is in approximately one-third of the worldwide population, making it the most common chronic liver disease.[10] MASLD includes a wide variety of liver pathological lesions, from hepatic simple steatosis (SS), defined by the accumulation of more than 5% of fat in the hepatocytes, to metabolic dysfunction-associated steatohepatitis (MASH).[10,11] MASH is characterized by a ballooning of the hepatocytes and, above all, by lobular inflammation with or without fibrosis, in addition to hepatic steatosis. This severe stage can progress into liver cirrhosis and hepatocellular carcinoma.[12,13] Given that the pathogenesis of MASLD is complex and there is still no specific treatment for MASH approved by regulatory agencies.[14]

The metabolism of tryptophan is divided into three main pathways leading to serotonin, kynurenine and indole derivatives, as shown in Figure 1; and the fluxes between these pathways seems to be affected by metabolic alterations.[16] Kynurenine is a central molecule of the tryptophan pathway, which is metabolized into three derivatives: quinolinic acid (QA), kynurenic acid (KYNA) and anthranilic acid. QA and anthranilic acid have been identified as pro-inflammatory metabolites, whereas KYNA seems to have an anti-inflammatory role.[17,18]

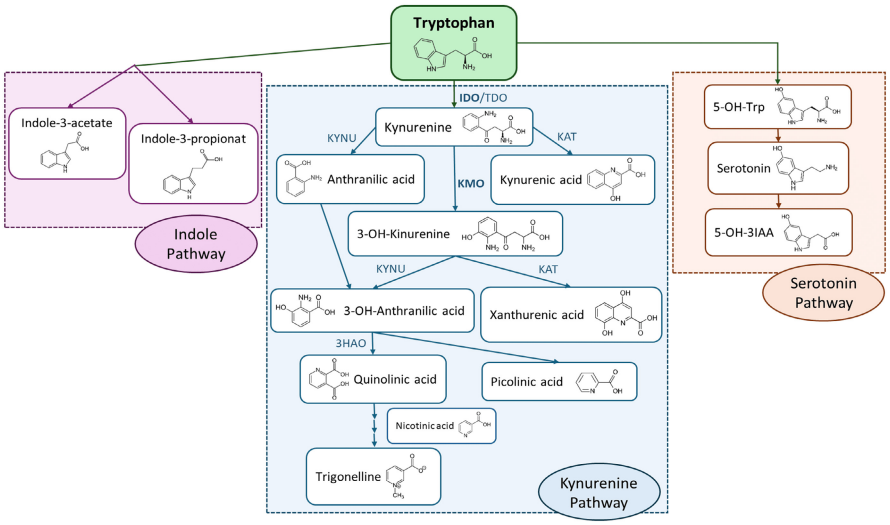

FIGURE 1 Complete tryptophan metabolic pathway is composed by the indole pathway, mainly developed in the gut microbiota; the kynurenine pathway, usually taking place in the liver; and the serotonin pathway, mostly in the enterochromaffin-like cells from the intestinal lumen.[15] 3HAO, 3-hydroxyanthranilate 3,4-dioxygenase; IDO, indoleamine 2, 3-dioxygenase; KAT, kynurenine aminotransferase; KMO, kynurenine monooxygenase; KYNU, L-kynureninase; TDO, tryptophan 2,3-dioxygenase.

Regarding MASLD, high levels of kynurenine have been found to induce metabolic inflammation and liver fibrosis. Hence, it seems that a link exists between tryptophan metabolism and MASLD pathogenesis.[19] On the other hand, lower tryptophan levels and increased expression of tryptophan-related enzymes (IDO 1 and TRP 2–3 dioxygenase 2 (TDO)), and its intermediate metabolites, such as kynurenine and serotonin, have been linked to increased inflammation and hepatic fibrosis.[20]

There are several potential approaches to modulate the tryptophan pathway, which may improve MASH consequences, such as supplementation of tryptophan, indole and indole derivatives, inhibiting the metabolic way of tryptophan to serotonin, and altering the kynurenine pathway.[15] Limited studies have reported the levels of tryptophan metabolites in the context of the pathogenesis of MASH, including the assessment of circulating tryptophan levels by Zhou et al.[21] and Chen et al.[22] Puyn et al. reported high levels of KYNA to be beneficial in MASLD pathogenesis, decreasing lipogenic gene expression and lipid accumulation.[23]

Therefore, our aim is to assess the main tryptophan catabolic pathways within the context of obesity, subsequently focusing on individuals with morbid obesity (MO) and varying histological stages of MASLD or normal liver (NL). Employing ultra-high-performance liquid chromatography (UHPLC), we quantify the 15 circulating levels of the tryptophan-related metabolites depicted in Figure 1. We also determined the hepatic expression of key enzymes in different MASLD stages. We aim to contribute valuable knowledge to the fields of both obesity and MASLD, offering potential targets for therapeutic interventions and advancing our understanding of the molecular states governing obesity-related disorders.

2 | MATERIALS AND METHODS

2.1 | Subjects

The cohort that was included in this study was composed of 76 patients, divided according to their BMI, into a control group with normal weight (NW, BMI 19–25 kg/m^2, $n = 18$) and a group with MO (BMI ≥40 kg/m^2, $n = 58$).

The research received approval from the institutional review board (Institut Investigació Sanitària Pere Virgili CEIm; 23c/2015). Written informed consent was obtained from all participants. All individuals with MO underwent planned laparoscopic bariatric surgery, with a blood sample drawn preoperatively and a liver biopsy taken in cases of suspected MASLD at the time of surgery.

The exclusion conditions were[1]: an intake of ethanol greater than 10 g/day or other toxins[2]; menopausal women or women taking contraceptives to prevent interference; and[3] patients with diseases other than MASLD, such as infectious, neoplastic, or acute or chronic liver diseases. In subjects taking other medications, blood was sampled in the morning just before taking medicines.

2.2 | Liver pathology

Liver samples collected during bariatric surgery from the MO cohort were stained with Masson's trichrome and haematoxylin-eosinase stain and evaluated by an experienced pathologist. The degree of steatosis, inflammation, ballooning and the presence of fibrosis of the patients was analysed using the Kleiner criteria.[24]

2.3 | Anthropometric evaluation and biochemical analyses

We perform physical, anthropometric and biochemical evaluations on all group members. Blood samples were collected preoperatively by nursing staff using BD Vacutainer® tubes. By centrifugation at 3500 rpm, at 4°C for 15 min, aliquots of serum and plasma were obtained from empty and ethylenediaminetetraacetic acid (EDTA) tubes. Biochemical parameters were evaluated using a conventional automated analyser, and insulin resistance was estimated using HOMA2-IR. We used EDTA tubes for blood collection to analyse biochemical and tryptophan pathway-related parameters. The NAFLD fibrosis score (NFS) was calculated according to the formula described by Angulo and colleagues.[25]

2.4 | Tryptophan-related mediators' analysis by UHPLC

The serum samples were analysed by UHPLC to obtain the concentrations of tryptophan metabolites. This analysis was performed in the EURECAT Company. Serum samples (100 µL) were placed in a centrifuge tube and mixed with 10 µL of internal standard (Table S1) and 500 µL of 10 mM ammonium formate in methanol. Samples were vortexed and centrifuged for 5 min at 15000 rpm and 4°C. Supernatants were purified with Phree phospholipid removal plates (Phenomenex), evaporated and resuspended in 100 µL of 0.1% formic acid in methanol: water (2:8, v/v) and transferred to glass vials. The chromatographic separation was performed with the gradient detailed in Table S2. Mobile phase A was 0,1% formic acid and B was

0,1% formic acid in methanol. The column temperature was set at 50°C and the injection volume was 1 μL. The source parameters applied operating in positive electrospray ionization (ESI) are detailed in Table S3.

2.5 | Gene expression analysis of tryptophan metabolism-involved enzymes

In the MO cohort, a liver biopsy was collected during laparoscopic bariatric surgery, always after liver pathology suspicion, conserved in fresh and immediately stored at −80°C. The RNeasy mini kit (Qiagen, Barcelona, Spain) was used to extract total RNA from liver samples. Reverse transcription to cDNA was performed with the High-Capacity RNA-to-cDNA Kit (Applied Biosystems, Madrid, Spain). Real time quantitative polymerase chain reaction (PCR) was carried out with the TaqMan Assay predesigned by Applied Biosystems for the detection of IDO1 (Hs00984148_m1), the Trp pathway-limiting enzyme, and kynyrenine-3-monooxygenase (KMO) (Hs00175738_m1), the enzyme that induces kynurenine conversion into QA. The expression of each gene was calculated and standardized to the expression of the housekeeping gene 18S (Hs4333760f). Then, relative expression was normalized using the control group (NL) as a reference. All reactions were duplicated in 96-well plates, using the QuantStudio™ 7 Pro Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Determination of IDO-1 and KMO mRNA expression was analysed in a subset of liver samples: $n = 32$ (10 NL, 11 SS and 10 MASH).

2.6 | Statistical analysis

All the data obtained in this study was analysed using python and several statistical packages (see Table S4 for specifics on packages and versions).

Two individuals were found to have outlier values exceeding 10 times the interquartile range and were therefore removed from the analysis (neither listed in Table 1 nor Table 2).

Comparative analyses utilized the non-parametric Mann–Whitney *U*-test, with *p*-values adjusted for multiple comparisons via the Benjamini-Hochberg method indicated. Significance was determined for adjusted $p < 0.05$, and variables were expressed as the median and interquartile range (25th-75th). Supplementary material includes data listed both as medians and interquartile ranges, as well as means and standard deviations (Tables S7–S9). Ratios of pathway fluxes were calculated to assess the overall metabolic activity by employing min-max normalization for all metabolite concentrations and grouping them per individual based on the pathways to which the metabolites belonged, as shown in Figure 1.

3 | RESULTS

3.1 | Baseline characteristics of the cohort

The study cohort ($n = 76$) was classified based on their body mass index (BMI) into two groups: those with NW (BMI 19–25 kg/m^2, $n = 18$) and another group with MO (BMI > 40 kg/m^2, $n = 58$). The anthropometric and biochemical parameters of patients with NW and MO are detailed in Table 1. The MO group presented a higher weight and BMI in comparison to the NW group, with significantly higher levels of glucose metabolism-related variables such as glucose, insulin and glycosylated haemoglobin (HbA1c), and elevated levels of triglycerides and liver-associated enzymes, including aspartate aminotransferase (AST), alanine aminotransferase (ALT) and gamma-glutamyltransferase (GGT). In contrast, high-density lipoprotein cholesterol (HDL-C) and albumin levels were significantly decreased in patients with MO.

Then, the group with MO was subclassified according to their liver histopathological features into NL ($n = 20$), SS ($n = 19$) and MASH ($n = 19$). The anthropometric and biochemical parameters of these subgroups can be found in Table 2.

In the cohort with MO, based on liver categorization, glucose, HbA1c, cholesterol, triglyceride levels and the APRI score were found to be significantly elevated in MASH subjects compared to NL. A significant elevation in the levels of HbA1c was also reported in SS subjects compared to NL individuals. The only parameter significantly altered between SS and MASH subjects was the APRI score.

Sex is a variable that we could not balance in our cohort. Our MO patients undergo bariatric surgery, which is a much more common procedure for women than for men[26] and it is at that time that we can collect a liver biopsy. Given the impact this can have on our subsequent analysis, we assessed the difference in the metabolites analysed (detailed in further sections) in both men and women. Data is provided in Table S5 for the whole cohort and in Table S6 for the MO cohort. Only one metabolite was significantly different between men and women when assessing the whole cohort (5-hydroxy-L-tryptophan, $p = 0.0473$), and this difference was not reported in the MO group.

TABLE 1 Biochemical and anthropometric variables of patients with NW or MO of the study.

Variable	NW ($n=18$)	MO ($n=58$)
Age (years)	40 (34–48)	47 (41–51)
Sex (% women)	100	82.76
Weight (kg)	59.50 (58.00–63.00)	116.00 (107.00–128.30)*
BMI (kg/m^2)	22.90 (21.53–24.16)	43.43 (41.68–46.42)*
DBP (mmHg)	65.00 (64.50–67.25)	71.00 (62.50–85.50)
SBP (mmHg)	120.00 (106.75–122.25)	125.00 (113.50–137.00)
Glucose (mg/dL)	82.00 (77.50–86.50)	101.00 (79.00–128.00)*
Insulin (mUI/L)	5.59 (5.28–7.34)	9.60 (5.98–15.68)*
HbA1c (%)	5.20 (4.90–5.20)	5.70 (5.40–6.90)*
Cholesterol (mg/dL)	177.50 (157.50–204.50)	175.50 (155.75–191.00)
HDL-C (mg/dL)	65.00 (56.25–79.75)	34.00 (30.00–45.00)*
LDL-C (mg/dL)	97.00 (80.00–108.00)	107.50 (90.72–122.98)
TG (mg/dL)	60.50 (48.25–64.75)	135.00 (113.00–162.75)*
AST (UI/L)	16.00 (14.00–22.75)	32.00 (21.00–44.00)*
ALT (UI/L)	14.50 (12.00–20.00)	34.00 (22.50–50.00)*
GGT (UI/L)	12.00 (11.00–17.00)	23.00 (18.00–39.50)*
ALP (Ul/L)	55.00 (48.00–68.00)	61.00 (54.50–78.00)
Bilirubin (mg/dL)	0.44 (0.36–0.72)	0.43 (0.32–0.64)
Albumin (g/dL)	4.39 (4.30–4.62)	4.00 (3.80–4.12)*

Note: Data are expressed as the median (interquartile range).

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; GGT, gamma-glutamyltransferase; HbA1c, glycosylated haemoglobin; HDL-C, high density lipoprotein cholesterol; HOMA2-IR, homeostatic model assessment method of insulin resistance; LDL-C, low density lipoprotein cholesterol; MO, morbid obesity; NW, normal weight; SBP, systolic blood pressure; TG, triglycerides.

*Significant differences between NW and MO ($p<0.05$).

3.2 | Correlation between tryptophan metabolites and cohort characteristics

First, we investigated the possible correlations between tryptophan metabolites and the anthropometric and biochemical parameters presented in Table 1, as illustrated in Figure 2A. Regarding BMI, it was significantly associated with QA (Figure 2B), KYNA (Figure 2A), and 5-hydroxy-L-tryptophan (Figure 2A). Conversely, a negative association between BMI and trigonelline (Figure 2C) as well as with tryptophan (Figure 2D), serotonin, indole-3-acetic acid, and indole-3-propionic acid is reported (Figure 2A).

Triglyceride levels (TG) showed a similar trend to BMI regarding correlations. The strongest correlations observed for TG in absolute values are with QA (Figure 2E), 3-hydroxianthranilic acid (Figure 2F) and 5-hydroxy-L-tryptophan (Figure 2G).

Then, we focused on the levels of the aminotransferases AST and ALT, given the relationship of these enzyme levels with liver status.[27] AST and ALT levels presented a high correlation (data not shown), so only correlations with ALT are reported. Our analysis revealed ALT was positively correlated with QA levels (Figure 2H). Additionally, ALT levels were positively associated with 5-hydroxy-L-tryptophan (Figure 2J), kynurenic acid, 3-hydroxy-anthranilic acid and 2-picolinic acid (Figure 2A). In contrast, ALT levels showed a negative association with trigonelline (Figure 2I) and indole-3-propionic acid levels (Figure 2A).

Finally, regarding histological parameters, hepatic steatosis grade was positively associated to tryptophan and kynurenine levels. On the other hand, hepatic lobular inflammation and ballooning were negatively correlated to indole-3-propionic acid, while fibrosis was positively associated to 3-hydroxyanthranilic acid.

3.3 | Tryptophan metabolites in accordance with obesity

Upon observing that certain metabolites from the tryptophan pathway are associated with the BMI levels of the patients in the cohort (Figure 2B–D), we wanted to compare the levels of these metabolites in relation

TABLE 2 Biochemical and anthropometric variables of MO patients of the study subclassified according to liver histology.

Variable	NL ($n=20$)	SS ($n=19$)	MASH ($n=19$)
Age (years)	44 (41–47)	48 (43–54)	48 (41–51)
Sex (% women)	95	63.2	88.3
Weight (kg)	113.50 (106.25–126.65)	125.00 (113.00–129.50)	113.50 (105.00–123.70)
BMI (kg/m^2)	43.61 (41.54–46.79)	43.27 (41.80–46.38)	43.01 (41.88–45.70)
DBP (mmHg)	71.00 (61.50–83.00)	74.00 (65.00–86.00)	65.00 (59.50–84.50)
SBP (mmHg)	127.00 (111.00–134.50)	120.00 (112.00–138.00)	128.00 (120.00–136.00)
Glucose (mg/dL)	86.50 (63.50–106.00)	112.00 (83.50–140.00)	103.50 (90.00–125.00)[c]
Insulin (mUI/L)	7.24 (5.82–11.33)	11.61 (5.48–14.36)	12.18 (6.93–17.85)
HbA1c (%)	5.40 (5.18–5.65)[a]	6.30 (5.70–7.50)	6.45 (5.57–7.88)[c]
Cholesterol (mg/dL)	169.50 (154.00–177.50)	160.00 (150.50–174.50)	184.00 (181.00–196.50)[c]
HDL-C (mg/dL)	42.00 (32.80–46.25)	39.00 (31.00–44.00)	30.10 (27.75–38.25)
LDL-C (mg/dL)	101.00 (81.85–115.75)	97.00 (90.22–107.75)	116.55 (106.00–126.65)
TG (mg/dL)	121.00 (103.75–133.00)	137.50 (117.00–155.25)	170.00 (136.25–208.25)[c]
AST (UI/L)	27.00 (21.00–41.00)	32.00 (24.00–38.00)	39.00 (22.00–60.00)
ALT (UI/L)	25.00 (20.50–44.50)	37.00 (28.00–49.00)	43.50 (25.75–69.50)
GGT (UI/L)	19.00 (17.50–30.00)	25.00 (19.00–39.00)	30.00 (20.00–62.00)
ALP (Ul/L)	64.00 (51.50–71.50)	59.00 (56.75–73.50)	64.00 (57.00–81.25)
Bilirubin (mg/dL)	0.40 (0.30–0.51)	0.50 (0.39–0.66)	0.43 (0.32–0.60)
Albumin (g/dL)	4.00 (3.83–4.10)	4.05 (3.90–4.10)	3.90 (3.68–4.30)
APRI score	0.26 (0.23–0.35)	0.27 (0.21–0.34)[b]	0.56 (0.41–0.64)[c]
FIB-4 index	1.00 (0.71–1.25)	0.77 (0.66–0.83)	0.93 (0.91–1.35)
NFS	−0.46 (−1.30–0.23)	−0.69 (−1.07–0.07)	−1.35 (−1.48 to −0.01)
Liver histology			
Steatosis Grade 0/1/2/3	20/0/0/0	0/10/9/0	0/6/7/6
Lobular inflammation Grade 0/1/2/3	20/0/0/0	17/2/0/0	3/11/5/0
Ballooning 0/1/2	18/2/0	15/4/0	2/15/2
Fibrosis stage 0/1/2/3/4	19/1/0/0/0	19/0/0/0/0	12/4/1/1/1

Note: Data are expressed as the median (interquartile range). $p<0.05$ were considered significant.

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DBP, diastolic blood pressure; GGT, gamma-glutamyltransferase; HbA1c, glycosylated haemoglobin; HDL-C, high density lipoprotein cholesterol; HOMA2-IR, homeostatic model assessment method of insulin resistance; LDL-C, low density lipoprotein cholesterol; MO, morbid obesity; SBP, systolic blood pressure; TG, triglycerides.

[a]Significant differences between the NL and SS groups.

[b]Significant differences between SS and MASH groups.

[c]Significant differences between NL and MASH groups.

to the presence or absence of obesity (Figure 3A). We found a significant increase in the levels of kynurenic acid, 3-hydroxyanthranilic acid, QA, 2-picolinic acid and 5-hydroxy-L-tryptophan in MO cohort. On the other hand, we observed a decrease in tryptophan, trigonelline, serotonin, indole-3-acetic acid, and indole-3-propionic acid levels in patients with MO.

We further employed Principal Component Analysis (PCA) to differentiate individuals based on their weight categories. In Figure 3B, the PCA plot distinctly segregates individuals into NW and MO groups.

3.4 | Tryptophan metabolites according to liver histology

Given the identified connections between metabolites of the tryptophan pathway and MASLD-related parameters, we aimed to examine the levels of each metabolite in relation to the liver histology. Taking into account the literature linking IDO activity and inflammation, we also calculated it as the ratio between kynurenine and tryptophan.

Focusing on MO cohort, we first analysed the levels of tryptophan pathway metabolites in the presence or

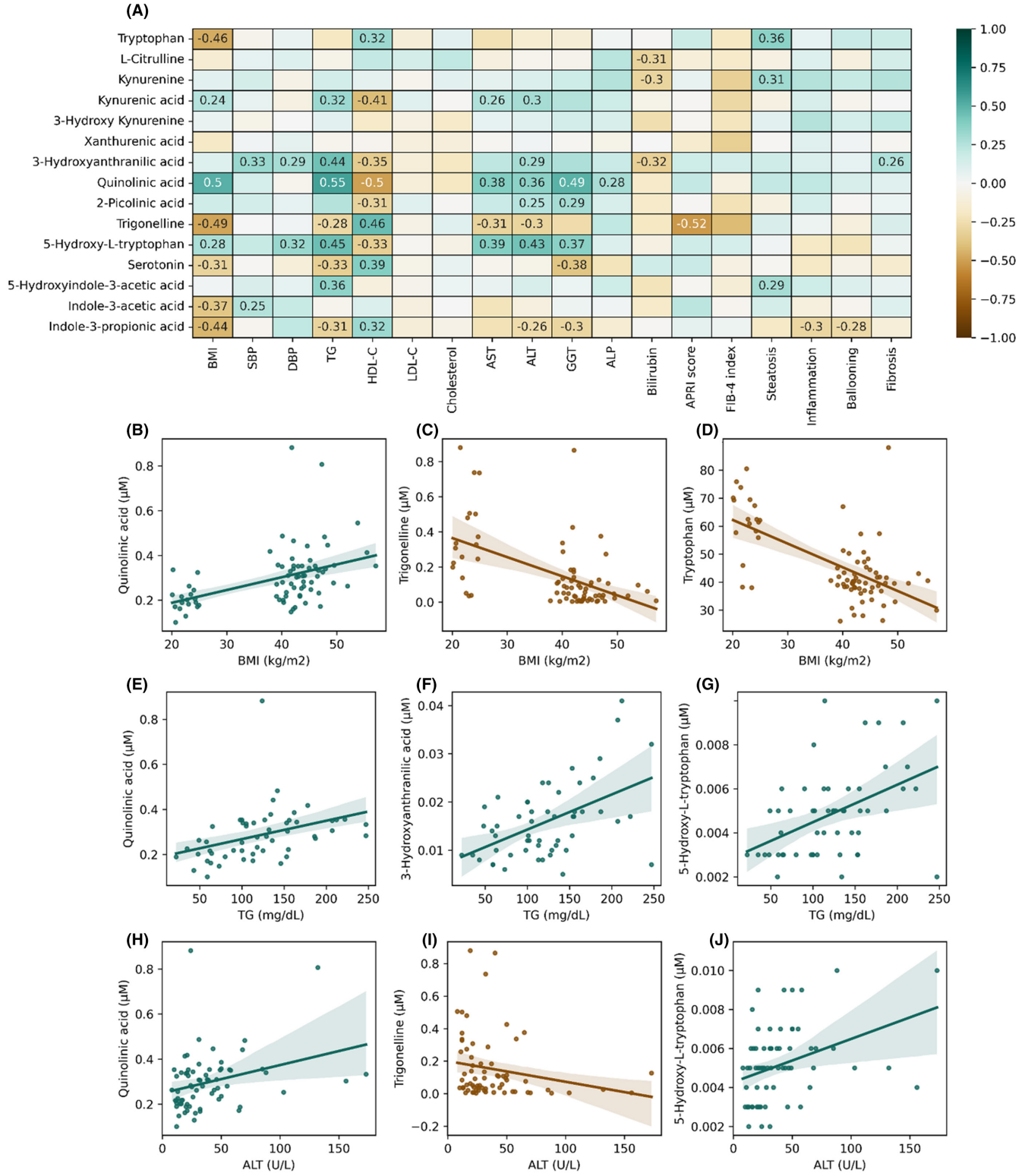

FIGURE 2 Correlation between tryptophan metabolites, biochemical, anthropometric variables in the whole cohort, and histological variables in the MO cohort. (A) Correlation plot between anthropometric and biochemical parameters against tryptophan metabolites. Spearman's ρ are displayed only for significant correlations ($p < 0.05$). Scatter plots with regression lines illustrating the relationship between BMI (kg/m^2) and (B) circulating levels of quinolinic acid (μM), (C) trigonelline (μM), and (D) tryptophan (μM). Scatter plots showing the correlation between triglycerides (TG) (mg/dL) and (E) quinolinic acid, (F) 3-hydroxyanthranilic acid (μM) and (G) 5-hydroxy-L-tryptophgan. Scatter plots showing the relationship between ALT (U/L) and (H) quinolinic acid, (I) trigonelline and (J) 5-hydroxy-L-tryptophan. Regression line with the shaded area representing the 95% confidence interval.

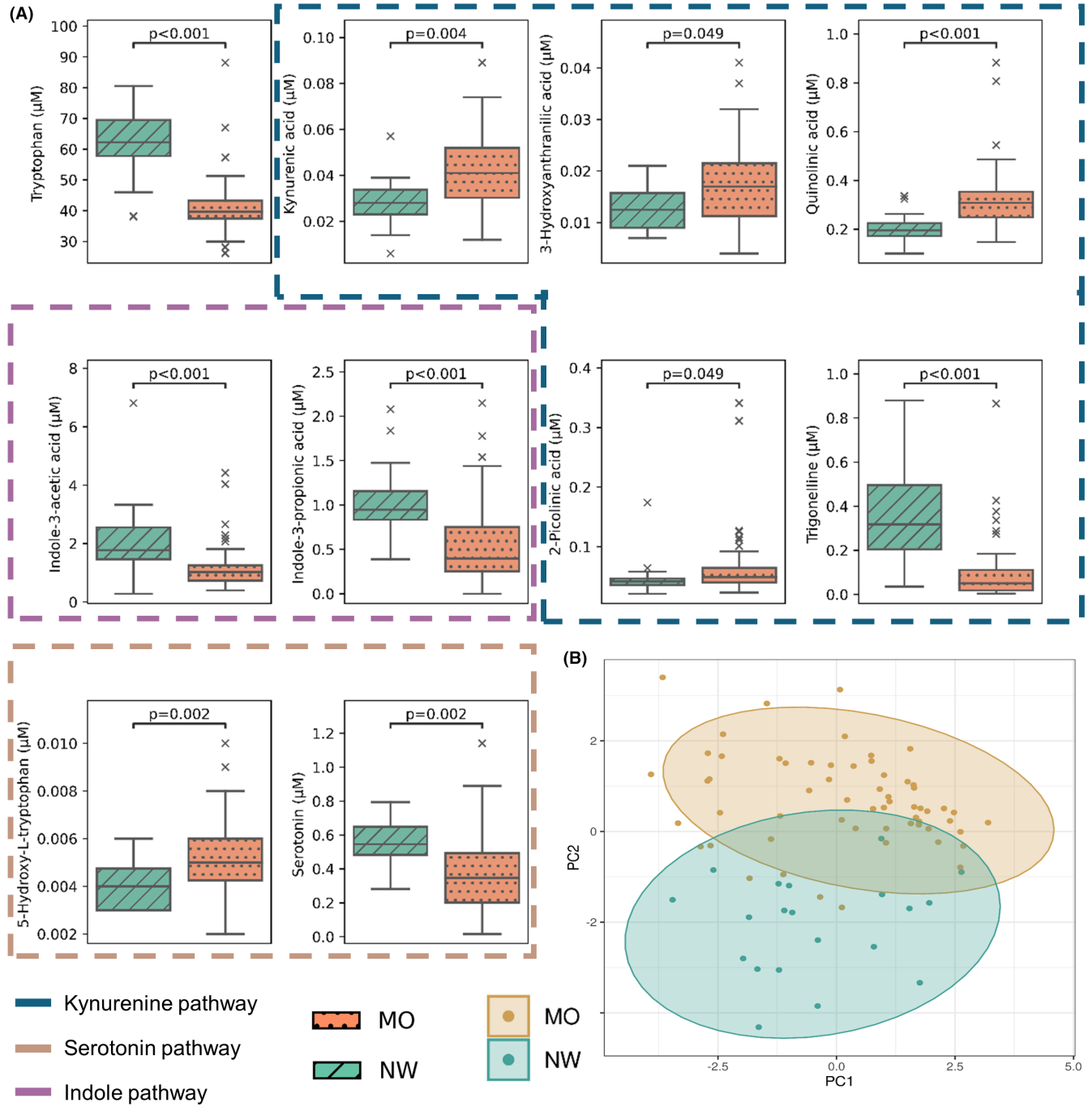

FIGURE 3 Levels of tryptophan and related metabolites according to BMI. (A) Concentration of metabolites in NW ($n = 18$) and MO ($n = 58$); adjusted $p < 0.05$ were considered significant. Complete data on means and standard deviations provided in Table S6. (B) Principal Component Analysis (PCA) of tryptophan metabolites in relation to obesity. Each point represents an individual's metabolite profile, with NW individuals depicted in blue and MO individuals in orange. The axes represent the principal components that capture the maximum variance in the dataset, with PC1 on the horizontal axis and PC2 on the vertical axis.

absence of MASLD. We obtained significantly higher tryptophan levels in MASLD presence compared to NL subjects (subjects with MASLD; 41.522 (38.803–45.276) μM of tryptophan; subjects with NL: 37.659 (32.577–39.823) μM, adjusted $p = 0.0235$). Subsequent exploration of metabolite levels in relation to MASH, after adjusting p-values for multiple comparisons, did not yield statistically significant differences. Similar observations persisted when stratifying patients by different MASLD stages (SS, MASH, or NL, PCA detailed in Figure S1). Although not statistically significant following correction, both kynurenine and QA levels exhibited an increasing trend, while indole-3-propionic acid showed a decreasing trend (Table S10).

3.5 | Tryptophan metabolism pathways: Kynurenine, serotonin and indole, in relation to hepatic histology

We analysed the fluxes of these three pathways comprising the tryptophan cascade in accordance with liver histological features, as depicted in Figure 4A. Our findings revealed an increased flux in the kynurenine pathway among MASH subjects compared to NL individuals. We also identified an induction of the serotonin pathway in subjects with SS compared to NL individuals.

Subsequently, we evaluated the ratio between these pathways at each hepatic histological stage, as outlined in Figure 4B. Specifically, we observed an induction of the kynurenine pathway relative to the indole pathway in the MASH stage compared to the NL stage. Additionally, a positive balance ratio for the kynurenine pathway over the serotonin pathway was found in the MASH stage compared to SS.

3.6 | Gene expression analysis of hepatic IDO1 and KMO

For a better understanding of the findings regarding the metabolites of the tryptophan pathway and the flow of its cascades, we aimed to analyse the hepatic expression of IDO, the rate-limiting enzyme of the pathway. Despite its low expression in the liver, its catalytic activity in this tissue is high.[28] Additionally, we sought to study the expression of KMO as it triggers the proinflammatory feature of this pathway and may be more closely related to a chronic inflammatory disease like MASH.[29]

In this regard, we conducted analyses on a subset of patients and found that the expression of both enzymes is decreased in NL compared to SS, but there are no significant differences in enzyme expression between NL and MASH, nor between SS and MASH (Figure 5).

4 | DISCUSSION

In this study, our aim was to assess the specific role of tryptophan metabolic pathways in the pathogenesis of obesity and MASLD. With respect to obesity, our key findings indicate a notable shift in tryptophan catabolic pathways, characterized by several altered metabolites. These alterations create a distinct metabolic signature in terms of the tryptophan cascade, differentiating MO individuals from those with NW. In the context of MASLD, we observed that elevated tryptophan levels are associated with

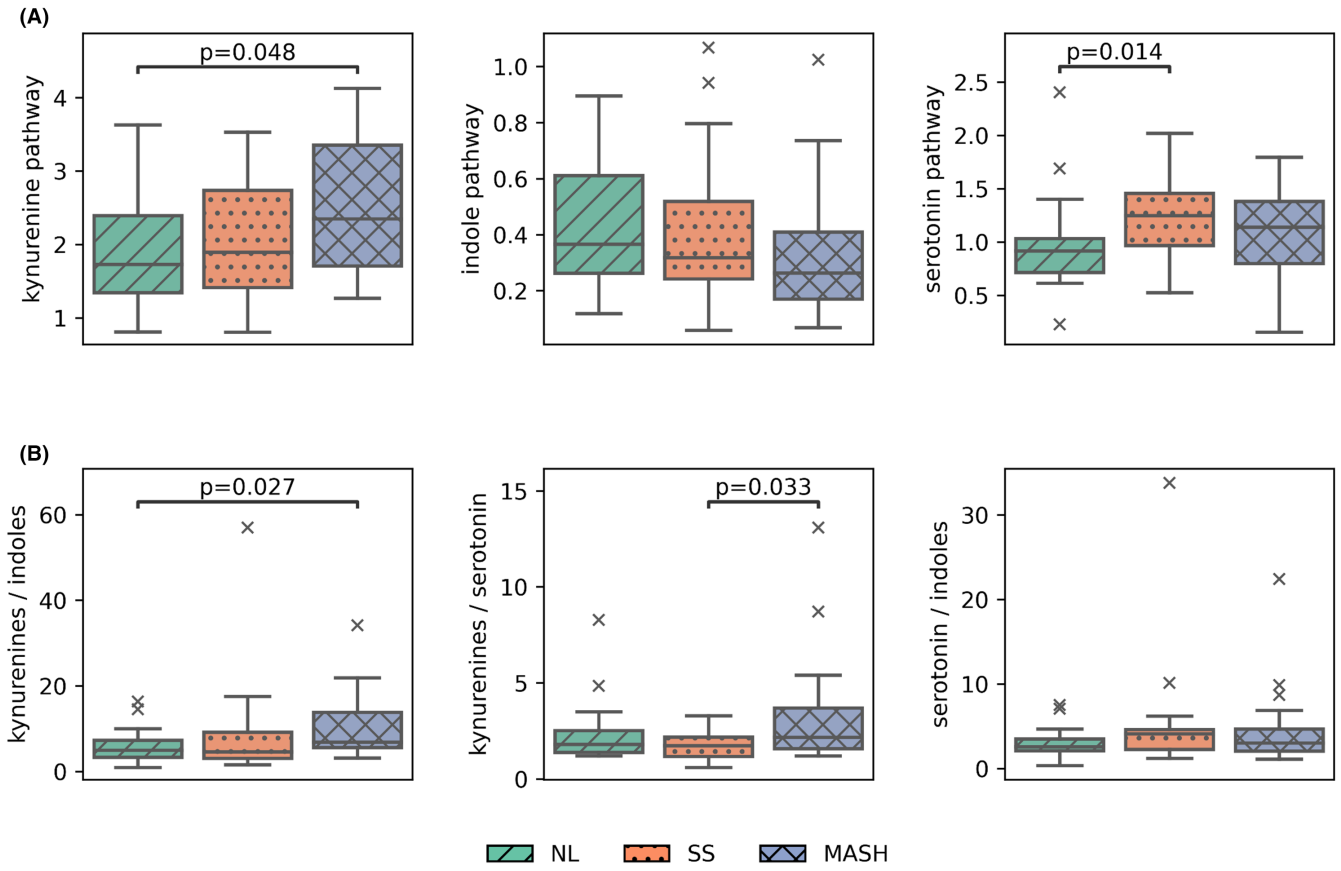

FIGURE 4 Fluxes (A) and ratios (B) of the three tryptophan metabolism pathways: kynurenine, serotonin and indole, depending on liver histology in the MO cohort; NL ($n = 20$), SS ($n = 19$) and MASH ($n = 19$); $p < 0.05$ were considered significant.

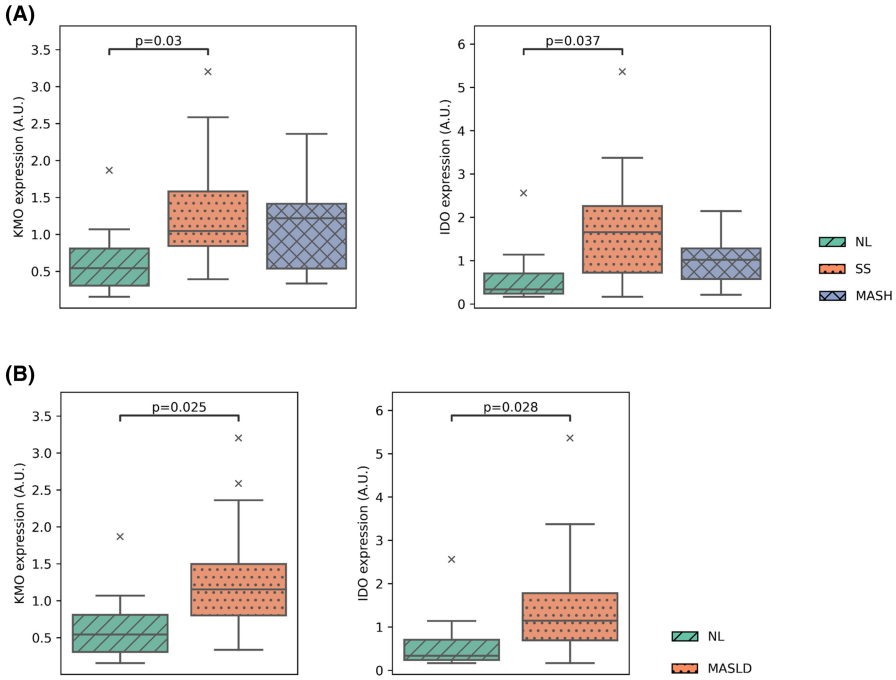

FIGURE 5 Hepatic relative expression of IDO1 and KMO enzymes. Hepatic mRNA expression of IDO1 and KMO (arbitrary units), (A) depending on liver histology in a subset of the MO cohort; NL ($n=9$), SS ($n=10$) and MASH ($n=10$), and (B) depending on the presence of MASLD (NL $n=9$, MASLD $n=20$); $p<0.05$ were considered significant.

the presence of MASLD. Additionally, we found that the alterations in tryptophan catabolism fluxes appear to be specific to each stage of MASLD.

Regarding obesity, we found a distinct metabolic shift in tryptophan metabolism, characterized by a decrease in tryptophan levels. Specifically, there is an increase in catabolism through the kynurenine pathway, accompanied by a decrease via the indole pathway. Decreased tryptophan levels in obese subjects have been reported previously,[30–32] although some authors have described the contrary finding[33] or no association.[34,35] The subjects from our MO cohort were preparing to undergo bariatric surgery and were thus on a very low-calorie diet (VLCD) before the sample extraction. In this sense, Strasser et al. have reported a decrease in tryptophan levels attributed to a VLCD.[36] The literature is significantly more consistent regarding the shift in tryptophan catabolism due to obesity. Elevated plasma concentrations of kynurenine metabolites in obese subjects have been reported in both children[37] and adults,[6,34,35] attributed to the increased expression of IDO1 and other enzymes from the kynurenine pathway in primary adipocytes, induced by proinflammatory cytokines.[6,7] A decrease in the circulating gut-derived indole metabolites has also been documented in obese individuals,[31,38] raising the question of a bidirectional association, where indoles regulate inflammatory processes and gut inflammation alters the synthesis of microbiota-driven indoles.[31,39] The negative association between trigonelline and BMI is also consistent with recent literature.[40–42]

On the other hand, we report an increase in 5-hydroxy-L-tryptophan in obesity, along with a positive association with triglyceride levels and a concurrent decrease in serotonin levels. Peripheral serotonin is synthetized from tryptophan by the sequential action of two enzymes: tryptophan hydroxylase isoform 1 (Tph1), which converts tryptophan to 5-hydroxytryptophan, and aromatic L-amino acid decarboxylase (AADC), which catalyses the conversion of 5-hydroxy-L-tryptophan to serotonin.[43] Considering the lower levels of circulating tryptophan in obese subjects, the activity of Tph1 appears to be increased. Some studies have shown that inhibition of Tph1 in the adipose tissue of mice leads to a reduction in body weight, and that a high-fat diet induces *Tph1* mRNA expression in adipose tissue.[43,44] Consequently, recent research has explored the use of Tph1 inhibitors that do not cross the blood–brain barrier as potential obesity treatments.[45] Additionally, in a previous study we also reported decreased circulating levels of serotonin in a MO cohort compared to NW subjects.[46]

Focusing on liver parameters, we first noted a positive correlation between AST and ALT levels, which are typically elevated in hepatocellular lesions,[27] and QA and KYNA levels. Furthermore, both aminotransferases showed a negative correlation with indole-3-propionic acid, which was also negatively correlated to hepatic lobular inflammation and ballooning. A closer examination of metabolite levels according to liver histology in the MO cohort revealed a consistent trend: tryptophan levels were significantly increased in MASLD presence, and correlated with hepatic steatosis grade. Then, from individuals with NL to those with SS and MASH, there was an increasing trend in kynurenine and QA serum levels, accompanied by a decreasing trend in indole-3-propionic acid levels. Further analysis of overall pathway

fluxes revealed a significant increase in the kynurenine pathway in MASH subjects compared to NL subjects. Additionally, the serotonin pathway exhibited a significant increase in SS subjects compared to NL subjects. An increase in L-tryptophan serum levels was also reported by de Mello et al. in the different MASLD stages,[47] and this amino acid has also been shown to cause fatty liver by injection in rats.[48] Conversely, Celinski et al. found that tryptophan attenuates the levels of proinflammatory cytokines and improves aminotransferase levels and lipid profiles in MASLD patients.[49] Lower levels of tryptophan, but increased levels of downstream metabolites, have been related to metabolic inflammation and fibrosis.[21,22] On the other hand, QA has been reported to be a pro-inflammatory molecule.[50] In MASH conditions, intestinal dysbiosis leads to increased lipopolysaccharides (LPS) and inflammatory cytokines, which have been reported to activate IDO1, the regulatory enzyme of the kynurenine pathway.[22] Furthermore, LPS increases KMO activity, the enzyme responsible for converting L-kynurenine into 3-hydroxy-L-kynurenine, which consequently alters the kynurenic pathway toward QA production.[51] The deregulation of these pathways, induced by intestinal dysbiosis, causes a decrease in the production of indole and its derivatives at the intestinal level.[52,53] However, the fact that liver transaminases and tryptophan downstream metabolites followed the same trend is consistent with an increase of both in the disease. These findings are also consistent with the negative correlation of the indole derivative previously commented on.

Tryptophan fluxes in different MASLD histological stages reinforce the idea that the kynurenine pathway plays a key role in MASLD pathogenesis and, probably, in MASH development.[15] At the same time, the serotonin pathway seems to be involved in the steatosis process in MASLD, a fact also highlighted in other studies.[46,54] Therefore, both pathways could be interesting therapeutic targets for MASLD pharmacological strategies.

A key finding of the present study is that under MASLD conditions, there seems to be a tendency for tryptophan to be metabolized toward the kynurenine pathway due to the activation of IDO1, a key regulator of the pathway. We report a significant increase in the hepatic expression of IDO1 in MASLD patients. An increase in the expression of this enzyme is typically associated with obesity.[8,35] Regarding its involvement with the liver, Mo and colleagues found an increase in hepatic protein levels and expression of IDO1 in mice after bile duct ligation, an intervention that leads to liver fibrosis,[55] and an IDO1 inhibitor has been shown to decrease liver fibrosis in rats.[56] However, other studies have reported a protective effect of IDO1 against liver inflammation and fibrosis in mice on a high-fat diet.[57] Overall, different models seem to lead to different results, and research regarding its possible involvement with MASLD in humans is scarce.

Another key finding regarding MASLD is the increase in QA levels, generated by an elevated KMO activity. We additionally report an increased hepatic expression of KMO in MASLD patients. Increased KMO levels in several inflammatory diseases and cancers[58,59] and KMO inhibition, a strategy that has been explored for several neurological disorders,[58,60,61] has already been proposed as a potential therapy for MASH patients elsewhere.[22]

A significant strength of our study lies in the comprehensive analysis of tryptophan metabolism, where we detected levels of 15 different metabolites in serum. Additionally, the diagnosis of MASLD stages was conducted using liver biopsies, the gold-standard technique. However, there are limitations to consider. First, our cohort composition, encompassing only NW and MO individuals, resulted in a gap in our study population, as it excluded individuals with BMI ranges between 25 and 40 kg/m^2. Second, while liver biopsies offer the most accurate diagnosis of MASLD stages, they may not be representative of the entire liver. All correlations between aminotransferase levels and these metabolites were heteroscedastic, indicating increasing variance at higher enzyme levels and suggesting the involvement of additional, complex factors in these relationships.

In conclusion, our study provides valuable insights into the landscape of tryptophan metabolism in the context of obesity and MASLD. Regarding obesity, we uncovered a distinctive metabolic signature characterized by an increase in catabolism via the kynurenine pathway, a decrease via the indole route, and a down-regulation in Tph1 activity. Regarding MASLD, our exploration unveiled a nuanced association between tryptophan and liver pathology. The observed alterations in tryptophan catabolism fluxes were specific to different histological stages of MASLD, emphasizing the potential utility of targeting these pathways (for instance, via the inhibition of IDO1 or KMO) in therapeutic interventions. Future research should explore our limitations to provide a more comprehensive understanding of the metabolic intricacies associated with obesity and MASLD.

AUTHOR CONTRIBUTIONS

Carmen Arto: Writing—original draft; review and editing; formal analysis; conceptualization. **Elena Cristina Rusu**: Writing—original draft; review and editing; methodology; visualization; formal analysis. **Helena Clavero-Mestres**: Writing—review and editing; methodology; formal analysis. **Andrea Barrientos-Riosalido**: Writing—original draft; methodology; conceptualization. **Laia Bertran**: Writing—original draft; review

and editing; conceptualization. **Razieh Mahmoudian**: Writing—original draft; data curation. **Carmen Aguilar**: Writing—original draft; data curation. **David Riesco**: Writing—review and editing; data curation. **Javier Ugarte Chicote**: Writing—review and editing; Data curation. **David Parada**: Writing—review and editing; data curation. **Salomé Martínez**: Writing—review and editing; Data curation. **Fàtima Sabench**: Writing—review and editing; data curation. **Cristóbal Richart**: Writing—review and editing; data curation. **Teresa Auguet**: Writing—original draft; review and editing; formal analysis; supervision; conceptualization.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All relevant data supporting the findings of this study are included within the paper and the accompanying supplementary material. Any additional information or data can be obtained upon reasonable request to the corresponding author.

INFORMED CONSENT STATEMENT

Informed consent was obtained from all subjects involved in the study.

INSTITUTIONAL REVIEW BOARD STATEMENT

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of the Pere Virgili Health Research Institute (IISPV) (CEIm; 23c/2015; 11 May 2015).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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