# Cross talk between the liver microbiome and epigenome in patients with metabolic dysfunction-associated steatotic liver disease

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## Summary

**Background** The pathogenesis of MASLD (metabolic dysfunction-associated steatotic liver disease), including its severe clinical forms, involves complex processes at all levels of biological organization. This study examined the potential link between the liver microbiome profile and epigenetic factors.

**Methods** Liver microbial DNA composition was analysed using high throughput 16S rRNA gene sequencing in 116 individuals, with 55% being female, across the spectrum of liver disease severity. Total activity of histone deacetylases (HDACs) and acetyltransferases (HATs) was assayed in nuclear extracts from fresh liver samples. In addition, we measured the global 5-hydroxymethylcytosine (5-hmC) levels of liver DNA.

**Findings** Patients with MASLD showed a 2.07-fold increase ($p = 0.013$) in liver total HAT activity. Moreover, a correlation was observed between liver total HAT activity and the score for histological steatosis (Spearman's R = 0.60, $p = 1.0E-3$) and disease severity (R = 0.40, $p = 2.0E-2$). Liver HAT and HDAC activities also showed associations with the abundance of several liver bacterial DNAs. Additionally, liver global levels of 5-hmC showed negative correlation with the read number of *Bacteroidetes* (R = –0.62, $p = 9.3E-4$) and *Gammaproteobacteria* (R = –0.43, $p = 3.2E-2$), while it was positively correlated with the abundance of *Acidobacteria* (R = 0.42, $p = 4.1E-2$) and *Actinobacteria* (R = 0.47, $p = 1.8E-2$).

**Interpretation** The host liver epigenome, including the activity of enzymes involved in maintaining the balance between protein acetylation and deacetylation and the global DNA hydroxy-methylation status, may be the target of microbial signals.

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**Keywords:** NAFLD; MASLD; Microbiota; Epigenetics; HDAC; HAT; 5-Hydroxymethylcytosine

## Introduction

Non-alcoholic fatty liver disease (NAFLD), now known as metabolic dysfunction-associated steatotic liver disease (MASLD),[1] is a prevalent condition that affects up to 25% of the global population.[2–6] The development of MASLD, including its severe clinical manifestations,

*Abbreviations:* MASLD, metabolic dysfunction-associated steatotic liver disease; MASL, metabolic dysfunction-associated steatotic liver; MASH, metabolic dysfunction-associated steatohepatitis

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## Research in context

### Evidence before this study

Non-alcoholic fatty liver disease (NAFLD), currently known as metabolic dysfunction-associated steatotic liver disease (MASLD), is a widespread condition that affects up to a quarter of the global population. The pathogenesis of the disease, along with its severe clinical manifestations, comprises intricate and multi-scale processes at all levels of biological organization, ranging from DNA to RNA and their epigenetic modifications, proteins, metabolites, and the microbiome. Indeed, evidence exists that links epigenetic changes to the development of MASLD. Furthermore, the gut microbiota serves as an environmental signal that can initiate epigenetic alterations in the host. Pioneering investigations into the liver tissue metataxonomic profile of patients with MASLD have unveiled an extensive spectrum of bacterial DNA in the liver that may contribute to the severity of the disease. However, no research has yet explored the relationship between the liver microbiome and liver epigenome in patients with MASLD.

### Added value of this study

Here, we examined the potential correlation between the liver microbiome and epigenetic factors in patients with MASLD across the range of histological disease severity. The findings of this pilot study suggest a multifaceted interplay between the microbiome composition of liver tissue and tissue-specific epigenetic alterations, encompassing total HAT and HDAC enzymatic activity, alongside hepatic global 5-hmC levels. We conducted a causal mediation analysis to examine the extent to which bacterial DNA might affect histological features through changes in the liver acetylome. The results suggest that the effects of *Firmicutes* (*Clostridiales*) and *α-Proteobacteria* on the histological severity of steatosis are likely to be influenced by changes in HAT activity. Liver total HAT activity may contribute up to 43% and 38% of the overall impact on histological liver steatosis of the total effect of *Firmicutes* (*Clostridiales*) and *α-Proteobacteria* (*Sphingomonadales*), respectively.

### Implications of all the available evidence

Our study suggests that there is a complex relationship between the tissue microbiome composition and tissue epigenetic modifications in patients with MASLD. Microbial signals can affect DNA hydroxymethylation and the activity of enzymes related to histone acetylation. Manipulating the microbiome could be a potential intervention avenue for remodelling the liver epigenome in the context of the disease. However, the extent to which these modifications are reversible ought to be further examined.

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Previous studies have provided insight into the role of the faecal microbiome in the pathogenesis of MASLD.[7–9] However, the composition of the gut microbiome does not entirely elucidate tissue-specific mechanisms linked to disease severity such as liver inflammation, ballooning of hepatocytes, and fibrosis.

A recent study from our group suggested that the metataxonomic signature of the liver may be responsible for variations in both the pathological mechanisms of MASLD and the physiological functions of the host.[10] In addition, we investigated the association between the metataxonomic profile of the liver in patients with MASLD and genetic factors. Our findings suggested that the host's genetic makeup partially affects the liver microbiota.[11] By combining risk alleles associated with severe histological traits (PNPLA3-rs738409, TM6SF2-rs58542926, MBOAT7-rs641738, and HSD17B13-rs72613567) with a variant influencing macronutrient intake (*FGF21*-rs838133) in a polygenic risk score, we have demonstrated that this score explains approximately 7.4% of the variation in taxa at the genus level.[11]

Moreover, there is evidence linking epigenetic changes to the development of MASLD.[12–17] However, the connection between the liver microbiome and the liver epigenomic factors in patients who have MASLD has yet to be studied.

The gut microbiota acts as an environmental signal that can trigger epigenetic modifications in the host.[18–21] It produces various bioactive compounds including choline metabolites, lipids, and short-chain fatty acids, all of which have considerable impacts on host physiology.[22] Specifically, hosts can respond to environmental stimuli, such as gut microbiota, by modifying DNA methylation and histones. These two epigenetic modifications are relevant to MASLD since they are reversible and responsive to metabolic changes.[12,23]

Histone acetylation may be considered a switch that enables the conversion between domains and structures of permissive and repressive chromatin.[24] Acetylation of the epsilon amino group of specific lysine residues found in core histones is an essential epigenetic mark for regulating multiple cellular processes. Histone acetylation by histone acetyltransferases (HATs) is particularly significant, as it is associated with active regions of the genome.[24] Conversely, histone deacetylation by histone deacetylases (HDACs) results in transcriptional repression.[24]

5-hydroxymethylcytosine (5-hmC) modulates gene transcription by influencing DNA demethylation and chromatin structure remodelling.[25] Likewise, 5-hmc controls epigenetic reprogramming and the regulation of tissue-specific gene expression.[25] For example, we

found that patients with MASLD had significantly lower non-nuclear 5-hmC staining compared to controls, and a variant (rs3998860) of the ten-eleven translocation (TET) 1 protein, which is responsible for catalysing the conversion of methylcytosine to 5-hmC, was associated with MASLD severity, suggesting that 5-hmC may be involved in the disease pathogenesis.[13] Here, we examine the association between the microbiome and epigenomic factors of the liver in isolated nuclear extracts of fresh liver biopsies from patients representing the full range of liver disease severity of MASLD. Our analysis revealed connections between liver total enzymatic activity of HATs and HDACs and global liver 5-hmC methylation levels with the liver abundance of specific bacterial DNAs.

We also used causal mediation models as an additional statistical approach to investigate the extent to which bacterial DNA affects histological features by modifying the liver acetylome. The analysis suggests that bacterial DNA may influence the degree of steatosis through the modification of HAT activity.

## Methods

### Studied population

The current study consists of a secondary analysis of bacterial 16S rRNA reads obtained from the livers of 116 individuals. Specifically, we re-used microbiome data of our previously published studies.[10,11]

Although we are using the current accepted disease terminology (MASLD), it is important to note that patients were identified as having NAFLD according to the previous definition of the condition.[5] These individuals are categorized as either patients without MASLD (n = 19) or patients with MASL (metabolic dysfunction-associated steatotic liver n = 44) and MASH (metabolic dysfunction-associated steatohepatitis n = 53), as previously described.[10] A matched cohort study design was employed to guarantee that the non-MASLD liver samples had similar patient characteristics, including demographics and anthropometrics. The sex of participants was self-reported. There was no gender bias in the study design, and biological samples were chosen based on the availability, quality, and integrity of the DNA. Patients were included if they had histopathologic evidence of steatotic liver disease, specifically MASL or MASH, on a liver biopsy conducted during the study period. Selecting biological samples for the current study depended on having adequate, high-quality nucleic acids (DNA) to perform metagenomic analyses. Exclusion criteria were applied. Secondary causes of steatosis, including alcohol abuse (≥30 g for men and ≥20 g for women, of alcohol daily), total parenteral nutrition, hepatitis B and hepatitis C virus infection, and the use of drugs known to trigger steatosis, as well as antibiotics, immunosuppressive medication, and proton-pump inhibitor use, were also reasons for exclusion. In addition, the study excluded patients with the following diseases: autoimmune liver disease, monogenic forms of metabolic liver disease, Wilson's disease, and alpha-1-antitrypsin deficiency. We selected non-MASLD liver tissue samples from patients with no evidence of MASLD or metabolic syndrome, whose age and sex matched the patients with MASLD, from the Liver Unit. These patients had near-normal liver histology in specimens obtained by percutaneous liver biopsy. The subjects' liver biopsy was performed due to persistently mildly elevated serum liver enzyme activity. In all non-MASLD liver samples, potential causes of common liver disease were excluded, and only those without evidence of steatotic change on histological examination were included in the study. Non-MASLD livers were histologically diagnosed with minimal changes. Biological samples were selected consecutively from participants with MASLD and without MASLD during the same study period and from the same population of patients, attending participating institutions located in Argentina. This ensured that the matched patients shared the same demographic characteristics (occupation, educational level, place of residence, and ethnicity). Arterial hypertension was defined as a systolic blood pressure of at least 140 mm Hg and/or diastolic blood pressure of at least 90 mm Hg after multiple examinations and taking the average of two or more blood pressure readings on a minimum of two subsequent visits. All liver specimens were obtained via liver biopsy that was performed utilizing a modified 1.4-mm-diameter Menghini needle (Hepafix, Braun, Germany), under local anaesthesia, with ultrasound guidance either on an outpatient basis or during bariatric surgery. Samples surgically excised from the left lobe were immediately collected after the abdomen was opened but before organs were manipulated. A section of every liver biopsy sample was routinely immersed in 40 g/l formaldehyde (pH 7.4), embedded in paraffin, and stained with haematoxylin and eosin, Masson trichrome, as well as silver impregnation for reticular fibres. Each biopsy was at least 3 cm long and had a minimum of eight portal tracts.[10]

### HAT and HDAC activities measurements

Histone acetylation levels are regulated by the opposing actions of HATs and HDACs. Despite the wealth of information on gene-specific histone acetylation regulations, there is limited knowledge regarding the reciprocal relationship between total hepatic HAT and HDAC levels and the liver microbiome. HDACs remove acetyl groups from lysine residues on a range of substrate proteins, including transcription factors and other nuclear and cytoplasmic proteins, as well as histones. HDACs are classified into four classes according to phylogenetic sequence analysis: class I (HDAC1–3 and –8), class II (HDAC4–7 and –9), class III, also known as sirtuins (SIRT1–7), and class IV (HDAC11),

each with a distinct set of characteristics.[23,24] As part of our human study exploring the relationship between the liver epigenome and the liver microbiome, we decided to investigate the total activity of HAT and HDAC in liver tissue as an approximation of the potential perturbed changes in the liver acetylome in the presence of some bacterial DNAs or vice versa. Histone acetylation is a dynamic process in which even slight modifications to acetylases or deacetylases can significantly impact the overall HAT activity linked to target genes. The alteration in whole-cell histone acetylation levels can indicate a change in HAT activity itself or a decline in HDAC activity. HAT and HDAC activities were evaluated on liver cell nuclear extracts using the EpiQuick HAT Activity/Inhibition Assay Kit (P-4003) and the EpiQuick HDAC Activity/Inhibition Assay Kit (P-4034) from Epigentek, Inc. (Farmingdale, NY 11735, USA) in accordance with the manufacturer's protocol. The level of acetylated/deacetylated products, which is indicative of enzyme activity, was quantified via colorimetric analysis using a microplate spectrophotometer at 450 nm. The OD intensity measured is proportionate to the activity of the HAT/HDAC enzyme, with detectable levels as low as 0.5 ng of enzyme. A HDAC assay standard of deacetylated histones is provided within the kit for quantifying HDAC enzyme activity. Nuclear proteins were extracted from the nucleus of liver cells using the EpiQuik Nuclear Extraction Kit II (Nucleic Acid-Free) from Epigentek, Inc OP-0022 (Farmingdale, NY 11735, USA), following the manufacturer's protocol.

### Quantification of liver global 5-hmC in DNA

We measured global levels of 5-hydroxymethylcytosine (5-hmC) in DNA isolated from fresh liver specimens of both patients and controls using the Quest 5-hmC™ DNA ELISA Kit (Zymo Research, Irvine, CA 92614, USA) (D5425 & D5426) in accordance with the manufacturer's guidelines. The detection threshold of this system is 0.02% per 100 ng input DNA. The detection of 5-hmC was achieved using a sandwich-based ELISA kit that includes a control DNA set, which was calibrated to accurately quantify the percent 5-hmC in the sample DNA using a standard curve. All samples were measured in triplicate, enabling the estimation of the mean 5-hydroxymethylcytosine (5-hmC) level. The 5-hmC levels did not follow a normal distribution and were thus log-transformed for statistical analyses. We investigated the liver global 5hmC levels because it could act as an independent epigenetic marker with unique signalling properties and has been previously linked to disease pathophysiology.[13]

### Microbial DNA data collection

We collected liver metataxonomic information from the tissue samples of the 116 individuals as previously described.[10] Briefly, microbial DNA was obtained from fresh liver specimens using a manual protocol which includes bacterial wall disruption by agitation with spheres. Barcoded primers were used to amplify bulk DNA samples, and DNA libraries were constructed. High throughput 16S rRNA sequencing was later conducted on an IlluminaMiSeq platform by Macrogen Inc. (NGS Division, Seoul, South Korea). A negative control was employed to assess the potential existence of contaminant DNA and/or cross-contamination; the sample exhibited no product and did not pass the quality assurance analysis.[10] The amplicons of the hypervariable regions 3 (V3) and 4 (V4) of the 16S rRNA gene were produced via PCR amplification utilizing primers referenced elsewhere.[10] The sequencing data for the 16S rRNA gene were filtered, denoised, and processed utilising the QIIME2 (version 2018.11) platform (http://qiime2.org/index.html). To address the non-normal distribution of microbial community composition data, the 16S rRNA abundances were normalised by total sum combined with squared root transformation. The default transformation offered by the MicrobiomeAnalyst platform (https://www.microbiomeanalyst.ca/) was utilised. Moreover, high-quality sequences were assigned to operational taxonomic units (OTUs) using the QIIME pipeline.[26] Default parameters were employed to select the OTUs for constructing the OTU table. High-quality amplicon sequence variants were classified utilising the vsearch algorithm with default parameters and SILVA 16S-only 99% identity database (release 137) to construct our BIOM feature table of OTUs. An independent molecular approach, as previously explained,[10] further confirmed the evaluation of variations in liver bacterial DNA composition across the entire disease severity spectrum.

### Statistical analysis

We determined the fold change in the abundance of the taxon of interest by calculating the ratio of the mean of sequencing reads assigned to the taxon normalized by the total number of sequencing reads, using a square root transformation in each group. To compare clinical, biochemical, and histological characteristics, we employed the Mann–Whitney U test, except for the female/male ratio between the studied groups, which was assessed with a Chi-square test. As an initial screening in univariate analyses, the relationship between taxa abundance and HAT and HDAT activities was assessed using Spearman's rank correlation coefficient.

Data are presented as mean ± standard deviation. Statistical analysis was conducted using the nonparametric Kruskal–Wallis test followed by Mann–Whitney U test. A $p$-value less than 0.05 was considered statistically significant.

Mediation analyses were conducted to determine if bacterial DNAs influenced histological features or other traits through epigenetic modifications as a mediator, such as liver total HAT or HDAC enzymatic activity. We tested the indirect effect of the log-transformed

independent variable (bacterial DNA abundance) through the mediator (HAT activity) on the steatosis score. In the generalized structural equation method (gsem), as the disease severity (control-MASL-MASH) is an ordinal categoric variable, we used ologit distribution in the model. Otherwise, when the dependent variables were continuous variables, a linear regression was used in the structural equation method (sem).

In STATA, nonlinear combinations (nlcom) commands were used to estimate total, direct, indirect, and partial pathway coefficients, standard errors (SE), and *p*-values. The path from exposure to the outcome via the mediator represents an indirect "mediation" effect. It expresses the fraction of the exposure total effect that is mediated through a specific mediator. The direct and indirect effects in mediation analysis, along with their implications, are explained in Supplementary Fig. S1 using a causal diagram. To avoid over-attributing a complete biological effect of X on Y to M mediation, we combined the direct and indirect effects, irrespective of their sign (absolute values, |x|) to calculate the magnitude of the total effect. We then determined the percentage of that magnitude attributable to the indirect effect.

### Functional prediction profiling

We used PICRUST (Phylogenetic investigation of communities by reconstruction of unobserved states) for functional prediction profiling. This computational technique uses OTU abundance tables to predict gene abundance and relies on reference OTUs from Greengenes. The STAMP software package (version 2.1.3), which uses biological relevance in the form of confidence intervals, was used to determine variations in pathways that were differentially enriched ($p < 0.05$) and their effect size (η2) (http://kiwi.cs.dal.ca/Software/STAMP). We applied the Welch test for two groups with a Benjamini-Hochberg false discovery rate of 0.001 to decrease the quantity of features in the figure.

### Sample-size estimation

Sample size and power calculations indicate that a sample with ∼40 participants per disease severity group (MASL, formerly NAFL and MASH, formerly NASH) has a power of ∼0.80 to detect media differences representing ∼2.0 fold-changes with a probability of <0.05 for type I error.

### Blinding

For all the experimenters, authors were blind to group assignment and outcome assessment.

### Ethics

Biological specimens, including blood samples and liver biopsies, were collected from all subjects with written, informed consent under Institutional Review Board-approved protocols with protocol numbers: 104/HGAZ/09, 89/100, 1204/2012, and updated DI-2019-376-GCABA-HGAZ. The protocol DI-2019-376-GCABA-HGAZ, entitled "Genetics of steatohepatitis and its association with the liver tissue microbiome," was followed. All data were de-identified prior to use in the study. All investigations carried out in this study were performed in accordance with the guidelines set forth in the 1975 Declaration of Helsinki, as revised in 1993.

### Role of funders

The funders had no role in the conceptualization, study design, data collection, analysis, interpretation of data, in writing the paper, or in the decision to submit the paper for publication.

## Results

The demographic, clinical, biochemical, and histological characteristics of the patients are shown in Table 1. Both sexes were equally represented in our sample; self-reported by study participants sex disaggregated features are disclosed in Supplementary Table S1. Liver total HAT activity exhibited a significant association with MASLD as a dichotomous trait. As a result, patients with MASLD showed a 2.07-fold increase ($p = 0.013$) in liver HAT activity. Additionally, a notable correlation was found between total HAT activity and the score of histologic steatosis (Spearman R = 0.60, $p = 1.0E-3$) as well as between HAT activity and the spectrum of disease severity (control, MASL, and MASH, R = 0.40, $p = 2.0E-2$). No associations were found between total HDAC activity levels and MASLD. However, liver total HDAC activity was correlated with serum glucose levels (R = 0.36, $p = 3.1E-2$). When evaluating the composition of the liver tissue microbiome and its association with the liver acetylome, on the one hand, we identified a positive correlation between the total HAT enzyme activity in the liver and the quantity of bacterial DNA from actinobacteria, certain *Proteobacteria* (alpha and gamma-proteobacteria), and *Spirochaetes* (Fig. 1a and b). Conversely, liver total HAT enzyme activity was negatively correlated with bacterial DNA abundance from *Bacteroidetes*, *Firmicutes* and some *Verrucomicrobia* (Fig. 1a and b). On the other hand, a positive correlation was found between liver HDAC enzyme activity and the amount of bacterial DNA from *Planctomycetes*, alpha and gamma *Proteobacteria*, *Spirochaetes* and *Actinobacteria* (Fig. 2a and b). Conversely, a negative correlation was observed with some *Bacteroidetes*, *Firmicutes* and *Verrucomicrobia* (Fig. 2a and b).

Furthermore, the bacterial DNA, which was described to be more abundant in MASH and disease severity, was negatively correlated with global 5-hmC levels, including Bacteroidetes (R = –0.62, $p = 9.3E-4$), Gammaproteobacteria (R = –0.43, $p = 0.032$), Acidobacteria (R = –0.42, $p = 0.041$), and *Actinobacteria* (R = –0.47, $p = 0.018$). The abundance of *Rhodovibrionales* (Alphaproteobacteria) was positively correlated with total liver 5-hmC levels (R = 0.45, $p = 0.024$).

| | Control group | | Patients with biopsy-confirmed diagnosis of MASLD | | | |
|---|---|---|---|---|---|---|
| | Subjects without morbid obesity | Subjects with morbid obesity | Patients without morbid obesity | | Patients with morbid obesity | |
| | | | MASL | MASH | MASL | MASH |
| Demographic, clinical, and biochemical variables | | | | | | |
| Number of subjects | 9 | 10 | 21 | 26 | 23 | 27 |
| Female/Male (n) | 5/4 | 6/4 | 10/11 | 16/10 | 12/11 | 15/12 |
| Age, years | 43.8 ± 8 | 44.8 ± 8 | 49.4 ± 11 | 46.7 ± 13 | 43 ± 9 | 48 ± 10 |
| BMI, kg/m$^2$ | 24 ± 3 | 55 ± 14 | 30 ± 5[a] | 34 ± 6[c,b] | 53 ± 13 | 49 ± 10 |
| Type 2 diabetes (n) | 0 | 4 | 6 | 15[c,b] | 8 | 18[b] |
| Fasting plasma glucose, mg/dL | 87 ± 10 | 105 ± 23 | 105 ± 26[a] | 124 ± 39[c,b] | 101 ± 22 | 138 ± 63[b] |
| Fasting plasma insulin, µU/mL | 5 ± 2.5 | 11 ± 8.4 | 14 ± 7[a] | 19.6 ± 12.4[c] | 13 ± 7 | 35 ± 45[c,b] |
| HOMA-IR index | 1.02 ± 0.5 | 2 ± 1.2 | 3.5 ± 1.7[a] | 6.1 ± 6[c] | 3.1 ± 1.7 | 16 ± 40[c,b] |
| Total cholesterol, mg/dL | 188 ± 38 | 190 ± 36 | 203 ± 39 | 196 ± 42 | 180 ± 37 | 179 ± 49 |
| HDL-cholesterol, mg/dL | 60 ± 15 | 40 ± 10 | 58 ± 16 | 51.2 ± 15 | 45 ± 10 | 37 ± 6[b] |
| LDL-cholesterol, mg/dL | 110 ± 42 | 121 ± 36 | 124 ± 35 | 120 ± 36 | 122 ± 28 | 126 ± 43 |
| Triglycerides, mg/dL | 101 ± 24 | 128 ± 65 | 143 ± 94 | 148 ± 67 | 154 ± 57 | 191 ± 102 |
| ALT, U/L | 42 ± 26 | 19.7 ± 8 | 56 ± 42 | 52 ± 31[c,b] | 28 ± 27[a] | 43 ± 20[c] |
| AST, U/L | 32 ± 11 | 19.6 ± 8 | 40 ± 23 | 87 ± 59 | 29 ± 20 | 31 ± 14[c] |
| Histological features | | | | | | |
| Degree of steatosis (0–3) | 0 | 0 | 1.5 ± 0.7[a] | 2.2 ± 0.44[c] | 1.73 ± 0.81[a] | 2.19 ± 0.8[c] |
| Lobular inflammation (0–3) | 0 | 0 | 0.7 ± 0.73[a] | 1.24 ± 0.8[c,b] | 0.35 ± 0.6[a] | 1.42 ± 0.8[c,b] |
| Hepatocellular ballooning (0–2) | 0 | 0 | 0 ± 0[a] | 0.8 ± 0.6[c,b] | 0.18 ± 0.4[a] | 1.07 ± 0.62[c,b] |
| Fibrosis stage | 0 | 0 | 0 ± 0[a] | 1.61 ± 0.6[c,b] | 0.04 ± 0.2[a] | 1.7 ± 0.6[c,b] |
| NAFLD activity score (NAS) | 0 | 0 | 2.5 ± 1.19[a] | 4.3 ± 1[c,b] | 2.26 ± 1.44[a] | 4.7 ± 2[c,b] |

MASLD: Metabolic dysfunction-associated steatotic liver disease; MASL: Metabolic dysfunction-associated steatotic liver; MASH: Metabolic dysfunction-associated steatohepatitis; BMI: body mass index; HOMA: homeostatic model assessment; ALT and AST: Serum alanine and aspartate aminotransferase. Results are expressed as mean ± SD except indicated otherwise. [a]$p < 0.001$ Indicates NAFL vs. controls. [b]$p < 0.001$ indicates comparisons between NAFL and NASH. [c]$p < 0.001$ denotes comparisons between NASH and control subjects. $p$ value stands for statistical significance using Mann–Whitney $U$ test, except for female/male proportion that $p$ value stands for statistical significance using Chi-square test.

***Table 1:*** **Baseline clinical, biochemical, and histological characteristics of the studied cohort.**

## Causal mediation analysis

It is important to note that correlation or association does not imply causation. Therefore, we used causal mediation analysis to understand how much of the effect of bacterial DNA on MASLD and relevant histological or other features could be mediated by changes

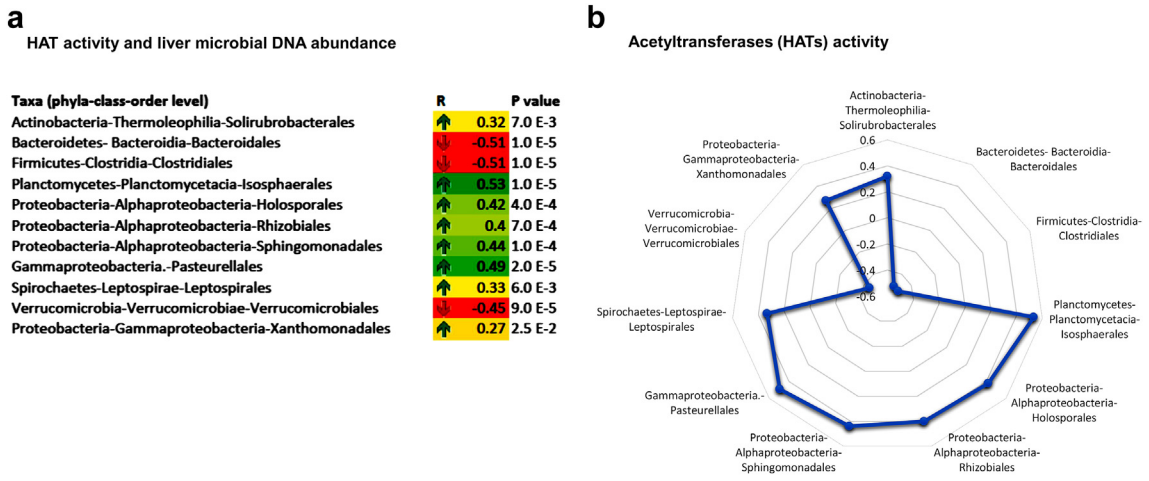


***Fig. 1:*** Total HAT activity of the liver and the liver microbiome. The figure depicts the correlation analysis, using the Spearman rank correlation, between the activity of the total HATs enzymatic activity and the abundance of microbial DNA in the liver (panel **a**). Additionally, a radar chart is presented in panel **b** that illustrates all significant correlation coefficients, both negative and positive, mapped onto an axis. Sample size: 116 fresh liver samples were obtained from patients and controls.

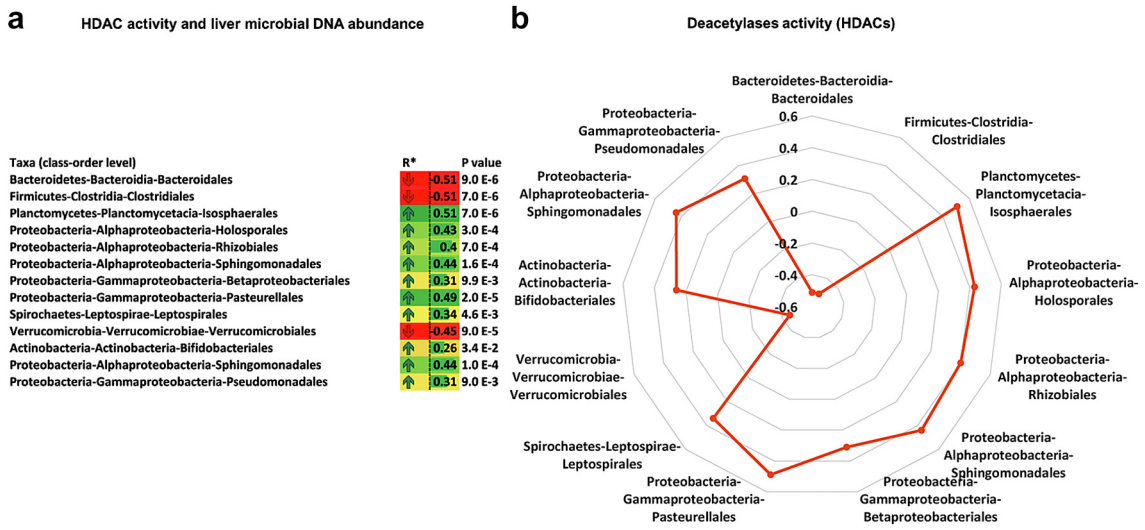


***Fig. 2:*** Total HDAC activity of the liver and the liver microbiome. The figure depicts the correlation analysis, using the Spearman rank correlation, between the activity of the total HDACs enzymatic activity and the abundance of microbial DNA in the liver (panel **a**). Additionally, a radar chart is presented in panel **b** that illustrates all significant correlation coefficients, both negative and positive, mapped onto an axis. Sample size: 116 fresh liver samples were obtained from patients and controls.

in the liver acetylome. The purpose of utilising this resource was to analyse and quantify the direct and indirect effects in the observed correlations.

Causal mediation analysis suggests that *Firmicutes*/*Clostridiales* and *Proteobacteria* (both at the class/order level) likely influence the degree of histological steatosis through the modification of HAT activity. The analysis indicated that the activity of total HAT in the liver could possibly mediate the impact of *Firmicutes* (*Clostridiales*) as there is an indirect effect of bacteria on the degree of steatosis through HAT activity, with a value of B: −0.003 ± 0.001, $p$ = 4.3 E-02 (Fig. 3). Then, bacterial-induced total HAT activity changes contributes to 43% of the total effect. Additionally, the analysis indicates that *α-Proteobacteria* (*Sphingomonadales*), which indirectly affects the degree of steatosis through HAT activity (B: 0.042 ± 0.021 $p$ = 4.7 E-2), may contribute up to 38% of the overall impact on histological liver steatosis (Fig. 3).

Nevertheless, there was no direct or indirect effect mediated by HAT of bacterial DNA on disease severity (control liver-MASL-MASH). Similarly, there was no direct or indirect effect mediated by HDAC of the selected bacteria on glucose levels (Supplementary Fig. S2).

### Analysis of the predicted functional profile offers information on potential metabolic outcomes in the host

The functional potential of microbial communities was assessed using the PICRUSt approach.

Significant predictions show that changes in hepatic total HAT activity correspond to consequential alterations in bacterial metabolic pathways (see Fig. 4a and b). L-lysine fermentation to acetate and butanoate is the most relevant pathway, which is linked to lower levels of hepatic total HAT enzymatic activity.

Another important pathway is the super pathway of biosynthesis of geranylgeranyldiphosphate I (via mevalonate), in which geranylgeranyl diphosphate (also known as geranylgeranyl pyrophosphate or GGPP) plays a crucial role in the biosynthesis of various terpenes and terpenoids, including pivotal compounds like ubiquinones and menaquinones. Additionally, GGPP is employed in posttranslational modifications of proteins, specifically geranylgeranylation, which is vital for membrane adhesion and certain functions. The predicted functionality of HAT activity collectively supports the mediation analysis, indicating an indirect effect of bacteria on the degree of steatosis through HAT activity.

## Discussion

Here, we investigated the possible connection between the microbiome of the liver and epigenetic factors in patients with MASLD throughout the spectrum of the histological disease severity. The results of this human study indicate a complex interplay between the microbiome composition of liver tissue and tissue-specific epigenetic modifications, including the total enzymatic activity of HAT and HDAC, as well as hepatic global 5-hmC levels.

We observed that bacterial DNA as a representative of the impact of bacterial products including lipopolysaccharides (LPS), previously found to be significantly enriched in NASH and disease severity,[10] positively correlated with liver total HAT and HDAC levels. The

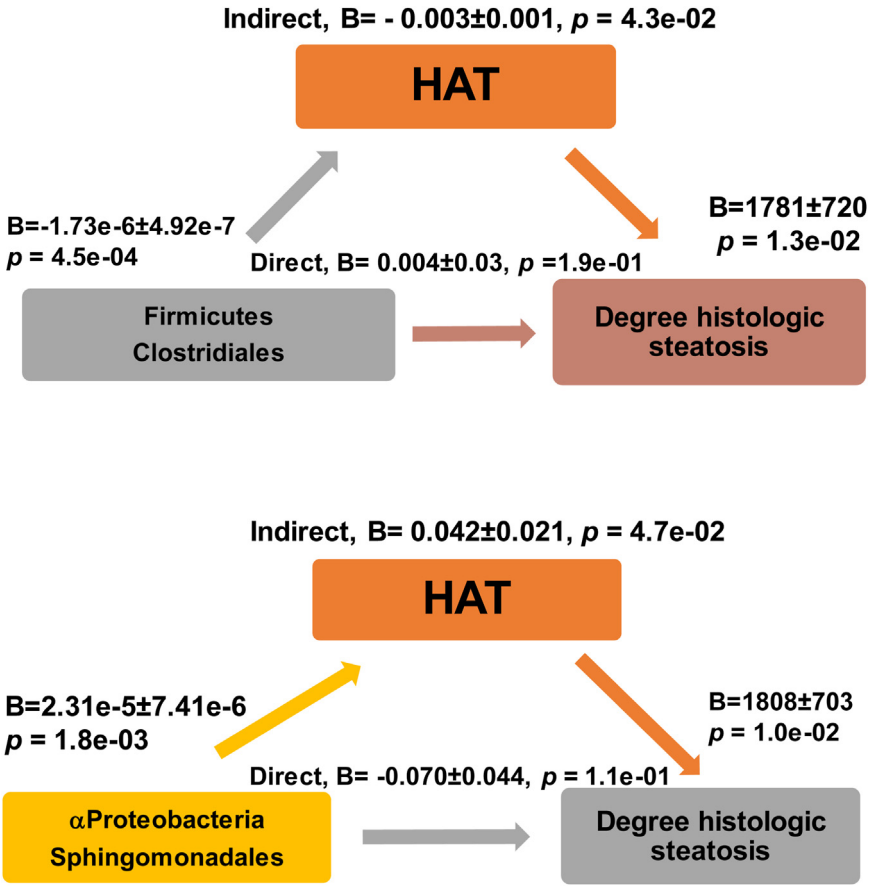


***Fig. 3:*** Causal mediation analysis on the degree of steatosis through HAT activity. Depicted is the path diagram, which includes unstandardized regression coefficients (B) ± standard error and p values for each path, of the mediation analysis showing that liver DNA from specific bacteria may exert part of the effect on the degree of histologic steatosis through alterations in liver total HAT levels.

observed bacterial taxa included *α-Proteobacteria*, *γ-Proteobacteria*, *Actinobacteria*, and *Bifidobacteriales*. Conversely, bacterial DNA, which was reported to be significantly reduced in MASLD severity,[10] including *Bacteroidales* and *Firmicutes/Clostridiales*, is negatively correlated to the liver total HAT and HDAC levels. Finally, bacterial DNA enriched in MASH and the disease severity, including *γ-Proteobacteria*, *Actinobacteria*, *Acidobacteria*, and *Bacteoidetes*, were found to have a negative correlation with the total liver 5-hmC levels.

To provide clues on the potential explanations underlying these observations, we conducted a causal mediation analysis, indicating that the impact of *Firmicutes* (*Clostridiales*) and *α-Proteobacteria* on the histological severity of steatosis is probably mediated through alterations in HAT activity. It should be noted that causal mediation analysis does not necessarily imply causality.

The role of microbiome-related factors, in addition to the significant impact of genetic factors in controlling the human epigenome, is increasingly recognized.[19,27] These factors can be classified into three main groups: metabolites originating from the microbiota, components originating from the microbiota such as bacterial DNA or LPS, and proteins secreted by the microbiota.[28]

Several mechanisms may potentially account for our findings. Initially, microbiota-generated short-chain fatty acids (SCFAs) may affect cellular functions via the inhibition of HDACs and/or alterations to the epigenome. Different intestinal bacteria produce varying levels of SCFAs. In the human colon, *Bacteroidetes* members predominantly synthesize acetate and propionate, while *Firmicutes* members primarily produce butyrate.[29–31] Butyrate is a potent inhibitor of HDACs and it also lowers LPS levels, especially associated with the taxa *Firmicutes/Clostridia*.[32,33] However, we found that dietary-derived butyric ingestion may increase the risk of MASLD in the general population.[34]

The liver plays a crucial role in filtering blood from both the hepatic artery and portal vein, making it an integral part of the body's filtration system. Its complex structure captures not only microbes but also bacterial products, which have the potential to trigger

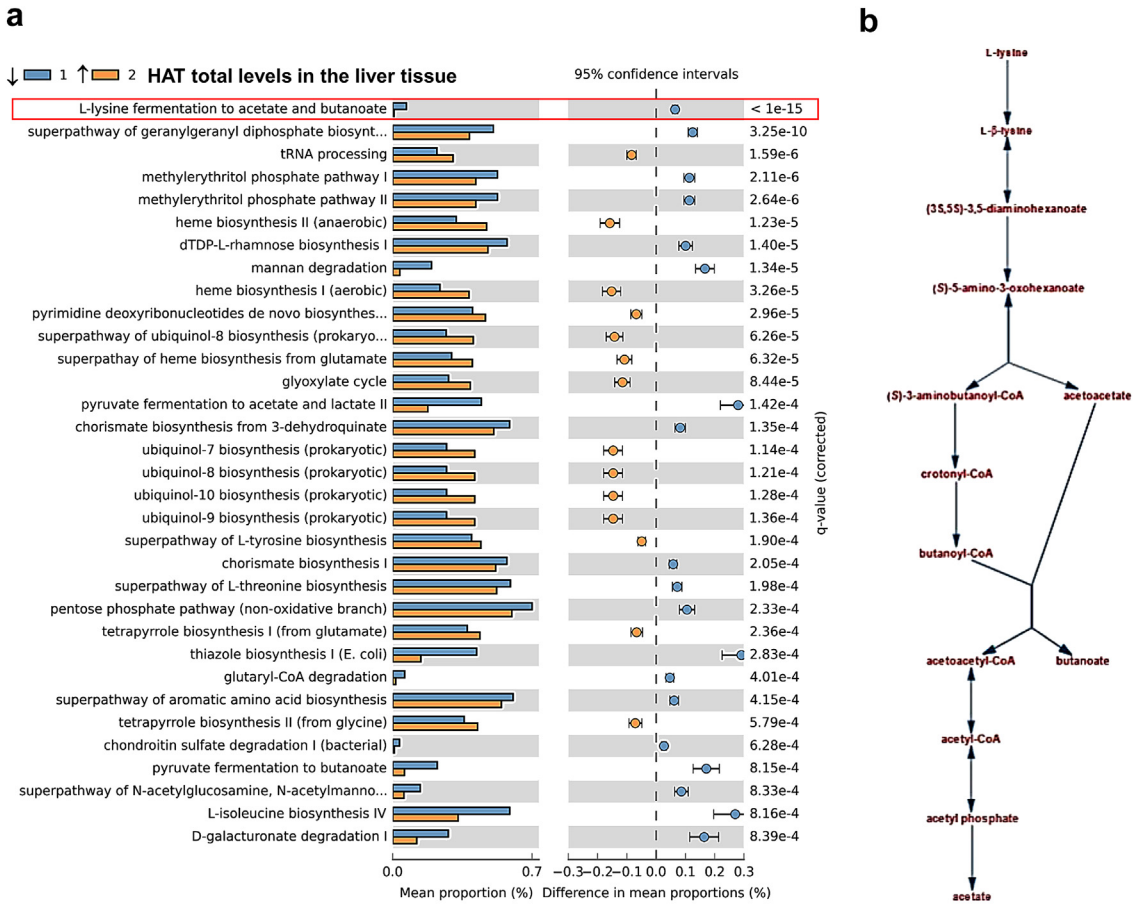


***Fig. 4:*** Functional predicted potential of microbial communities based on hepatic total HAT enzymatic activity. **a**. The functional potential of microbial communities, with specific emphasis on metabolic pathways, was evaluated using the PICRUSt approach. A bar plot was employed to depict the differences in the metabolic pathway enrichment between groups, utilizing Welch's t-test for two groups. Only predicted functions exhibiting a Benjamini-Hochberg false discovery rate of 0.001 are displayed. **b**. BioCyc ID: P163-PWY: L-lysine is converted to L-β-lysine (β-lysine) which is then converted to (3S, 5S)-3,5-diaminohexanoate, followed by deamination to a keto acid and reaction with acetyl-CoA to form (S)-3-aminobutanoyl-CoA and acetoacetate. (S)-3-aminobutanoyl-CoA is deaminated to crotonyl-CoA, which is reduced to butanoyl-CoA.

an inflammatory response. Previous studies have revealed a distinct association between free fatty acids and HAT activity in liver cells. For instance, free fatty acids directly trigger HAT activity, causing faster intracellular lipid build-up.[35] In addition, HAT activity inhibition has been found to affect lipogenesis-related gene expression and decrease lipid accumulation in both mouse steatosis and HepG2 hepatoma cells.[36] Our current research aligns with these findings. In detail, a decrease in enzymatic activity of HAT within the liver seems to be associated with increased production of bacterial-derived butanoate and GGPP. This increase is biologically associated with both anti-inflammatory effects for a healthy colon and antioxidant activity.

In this regard, it has been demonstrated that the concentration of SCFAs is roughly five times greater in the portal vein than in the peripheral venous blood, indicating that the primary source of these compounds is the gut.[37]

In addition, LPS, a prominent constituent of the outer membrane of Gram-negative bacteria, enhances transcription of proinflammatory cytokine genes, such as interleukin-12 (IL-12), IL-6, and tumour-necrosis factor (TNF), through chromatin remodelling and histone acetylation. It has been demonstrated that genes that are still responsive remain extensively acetylated after prolonged exposure to LPS derived from bacteria. Thus, LPS leads to the transcriptional modulation of multiple HDACs through signalling cascades, implying the involvement of numerous genes.[38,39] Interestingly, liver positive staining for LPS was found significantly increased in MASLD.[10]

It is important to note certain study limitations when interpreting these findings. Specifically, our results were obtained through examining the bacterial DNA profile

of liver specimens whilst measuring the total HAT/HDAC enzymatic activity and global levels of 5-hmC. Therefore, one possible criticism could be the necessity to demonstrate a functional and mechanistic correlation between bacterial communities and specific HAT/HDAC enzymes within the liver tissue. Furthermore, it is possible that the selection of patients solely from hospitals in Argentina may limit the scope of the findings. As a result, a broader biological sample of patients from different global regions would be required to conduct a comprehensive investigation of the effect of the liver epigenome on liver microbial DNA composition.

In conclusion, the results of this descriptive study suggest a complex interplay between the tissue microbiome composition and tissue epigenetic modifications. Specifically, we have shown that microbial signals can alter DNA hydroxy-methylation and histone acetylation-associated enzyme activities, likely resulting in modifications of the liver epigenome. Therefore, manipulating the microbiome presents a potential intervention target for the liver epigenome but at what extent these changes are reversible must be explored.

#### Contributors

All authors (CJP; AS; TFG; GOC; MG; and SS) read and approved the final version of the manuscript. CJP: study concept and design; biological material collection, data acquisition; data analysis and interpretation; molecular studies; general study supervision; statistical analysis; functional prediction; manuscript drafting; and securing funding. AS: bioinformatics analysis of bacterial sequencing data and data processing and presentation. TGF: performed molecular studies, nuclear cell extraction; enzymatic tests; GOC and MG: performed liver biopsies and collected biological samples. SS: study concept and design; data acquisition; performed liver biopsies and collected biological material; histological evaluation; molecular studies; data analysis and interpretation; general study supervision; manuscript drafting; securing funding. In addition, CJP, GOC, and SS have verified the underlying data.

#### Data sharing statement

Data supporting the observations of this study, including the methodology, are available upon reasonable request from the corresponding authors. Access to patient data may be restricted as some participants in the study did not consent to publicly sharing their information.

#### Declaration of interests

Nothing to declare.

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#### Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.ebiom.2024.104996.

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