**Exploring Microbiome, Metabolite, and Cytokine Interactions in MAFLD Cirrhosis:**

**A MiMeNet-Driven Multi-Omics Integration**

**Abstract**

**Background**

Metabolic-associated fatty liver disease (MAFLD) and its progression to cirrhosis involve complex interactions between the gut microbiome, metabolome, and host immune responses. However, the specific mechanisms underlying these interactions remain poorly understood. This study aimed to elucidate the relationships between microbial communities, metabolic profiles, and cytokine responses in MAFLD-related cirrhosis using an integrated multi-omics approach.

**Methods**

We employed the MiMeNet (Microbiome-Metabolome Network) framework to analyze gut microbiome composition, metabolomic profiles, and cytokine data from 28 patients with MAFLD-related cirrhosis and 28 matched healthy controls. The approach involved neural network modeling to predict metabolite abundances from microbial taxonomic compositions, followed by biclustering analysis to identify microbe-metabolite modules. We further integrated cytokine data to explore relationships between metabolites and immune responses.

**Results**

Our model demonstrated good predictive capability for metabolite abundances, with an average Spearman correlation coefficient of 0.331 between predicted and observed values. We identified 29 well-predicted metabolites out of 36 annotated metabolites, with cysteine-glutathione disulfide, retinol, and glutamine conjugates among the top predicted metabolites. Clustering analysis revealed 10 microbial and 8 metabolite modules, providing insights into the functional organization of microbe-metabolite interactions in MAFLD-related cirrhosis. Integration of cytokine data highlighted potential relationships between specific metabolites and inflammatory responses, with notable correlations observed between glutamine conjugates and pro-inflammatory cytokines such as IL-6 and TNF-α, as well as between bile acid metabolites and the anti-inflammatory cytokine IL-10. These findings suggest a complex interplay between the gut microbiome, metabolome, and host immune responses in the context of MAFLD progression to cirrhosis.

**Conclusion**

This study provides a comprehensive view of the interactions between the gut microbiome, metabolome, and cytokines in MAFLD-related cirrhosis. The identified microbe-metabolite modules and their associations with cytokines offer new insights into disease mechanisms and potential targets for therapeutic intervention. These findings contribute to our understanding of MAFLD pathogenesis and may inform the development of novel diagnostic and treatment strategies based on individual microbiome and metabolic profiles.

**Keywords:**

Metabolomics; Metagenomics; Cytokines; MAFLD; MiMeNet

**Introduction**

In the past decade, the complex relationship between gut microbiota and metabolic-associated fatty liver disease (MAFLD) has become a major focus in hepatology. The gut microbiome, a diverse community of microorganisms residing in the human gastrointestinal tract, significantly influences host metabolism and immune function (Martin, Sun et al. 2019). As our understanding of the gut microbiome grows, its role in the pathogenesis and progression of liver diseases, particularly MAFLD, has become increasingly apparent. MAFLD encompasses a spectrum of liver conditions characterized by diffuse lipid accumulation and inflammation. This can lead to progressive liver degeneration, cirrhosis, and in some cases, hepatocellular carcinoma (HCC) (Liu, Qin et al. 2024). The progression from simple hepatic steatosis to more advanced stages of liver disease is complex and multifactorial, with gut dysbiosis, altered metabolite profiles, and immune dysregulation playing crucial roles (Juanola, Martínez-López et al. 2021). While the general relationships between these factors are becoming clearer, the specific microbiome-metabolite interactions involved in the progression of MAFLD to cirrhosis remain poorly understood. In addition, how these metabolites interact with immune regulators such as cytokines, which are key players in the immune response associated with liver inflammation, is also not well understood. To address this gap in our knowledge, this study aims to characterize the associations between the gut microbiome and metabolites as MAFLD progresses to cirrhosis, and subsequently to explore how these metabolites interact with cytokines that may contribute to immune dysregulation during disease progression. By focusing on the interplay between microbial communities and metabolic profiles, we seek to uncover potential mechanisms that contribute to disease progression and identify novel targets for therapeutic intervention.

Our approach involves a comprehensive analysis of gut microbiome composition and metabolomic profiles in healthy controls and patients with MAFLD-related cirrhosis. By employing advanced clustering techniques and network analysis, we aim to elucidate the complex interactions between specific microbial groups and metabolite clusters, and cytokine profiles. This integrated analysis will provide insights into the metabolic functions of the gut microbiome in the context of liver health and disease, potentially revealing new biomarkers for disease progression and targets for microbiome-based therapies in MAFLD and cirrhosis.

In recent years, bioinformatics tools have been developed to predict metabolite profiles from metagenomic data, addressing the limitations in metabolome data coverage. These tools facilitate large-scale discovery of biological associations without requiring costly metabolomic experiments (Yin, Altman et al. 2020).

The predictive tools for metabolite profiles based on microbiome data can be broadly classified into two groups. The first group is pathway-based tools, which utilize known metabolic pathways linking microbial genes and compounds, often leveraging databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa, Goto et al. 2012). Examples of these tools include Predicted relative metabolomic turnover (PRMT)(Larsen, Collart et al. 2011) and Model-based integration of metabolite observations and species abundances (MIMOSA)(Noecker, Eng et al. 2016), which are designed to predict community-level metabolite turnover. However, their predictive power heavily depends on the completeness of microbial gene annotations and the availability of detailed reaction data.

The second group involves data-driven approaches that leverage machine learning to predict metabolites from metagenomic features. One prominent tool in this category is MelonnPan (Mallick, Franzosa et al. 2019) and ENVIM (Xie, Cho et al. 2021), which use Elastic Net regression to model the relative abundance of each metabolite based on microbial taxonomic or functional features. The emphasis of MelonnPan is to enable predictive modeling so that learned models can be applied in similar studies where only microbiome data are available. Although MelonnPan has shown promising performance, it models each metabolite independently, missing the chance to utilize shared information across metabolomic features that could enhance prediction accuracy.

Another data-driven tool, mmvec, applies neural networks to estimate the conditional probability of metabolite presence given a particular microbial sequence. mmvec focuses on learning embeddings that capture microbe-metabolite interactions, but it does not predict the complete metabolomic profile, which limits its utility for comprehensive metabolome inference(Morton, Aksenov et al. 2019). A recent neural network-based model, the neural network encoder-decoder (NED), has proposed constraints of sparsity and non-negative weights to map microbiomes to metabolomes. While the non-negative weights impose useful interpretability, they also restrict the model's learning capacity(Le, Quinn et al. 2020).

Despite the advances of these methods, current data-driven models fall short in fully capturing the complexity of metabolic interactions. Many of them model metabolites individually and do not exploit the extensive interactions between enzymes, metabolites, and genes that occur in metabolic pathways. Moreover, they have yet to explore the integrative potential of microbiome-metabolome relationships that could be revealed through deeper analysis. These limitations present opportunities for further improvement in predictive modeling, to create more comprehensive tools for understanding and predicting metabolomic profiles from metagenomic data.

Using the MiMeNet (Microbiome-Metabolome Network) approach, which leverages a recently developed neural network for microbiome-metabolome analysis to explore complex, non-linear relationships, we aim to decipher these pathways. We investigated microbiota–metabolite relationships using MiMeNet analysis in a cohort of 56 subjects, including 28 with MAFLD-related cirrhosis and 28 participants matched for age and body mass index. Metabolite profiles were then predicted from microbial taxonomic compositions. Furthermore, we examined how well-predicted metabolites interact with cytokines, aiming to understand their role in modulating immune responses in MAFLD-related cirrhosis. The precision of these predictions identified how the microbiota influences the metabolome and highlighted key microbial players and associated metabolites linked to MAFLD cirrhosis. Moreover, we characterized the relationship between metabolites and cytokines to identify immune regulatory pathways that may be contributing to disease progression. These observations highlight several novel ideas and therapeutic strategies that have emerged from our research. Through the integration of microbiome and metabolome data, we can generate a picture of the disease process that identifies potential biomarkers for MAFLD progression to cirrhosis. Moreover, an understanding of these interactions allows for the development of novel therapeutic approaches such as manipulation of metabolite levels through the microbiome composition. Targeting the interplay between metabolites and cytokines offers an additional therapeutic avenue to modulate the inflammatory responses observed in liver disease. The findings of this study may ultimately allow personalized MAFLD management based on microbiome and metabolic profiles. By extensive computational analysis and a highly curated dataset, our work explores interactions between the gut microbiota and metabolites that contribute to MAFLD pathogenesis leading to cirrhosis. This has implications for changing paradigms of liver disease initiation and progression, as well as future diagnosis accuracy, prognosis prediction, and therapeutic options in the context of MAFLD-related cirrhosis.

**Results**

In the present work we utilized the MiMeNet approach to investigate the interactions between gut microbiome, metabolites and cytokines in relation to MAFLD cirrhosis. With this approach we were able to assess the impact of microbial populations on metabolic pathways and immune responses that are essential for the development of liver disease.

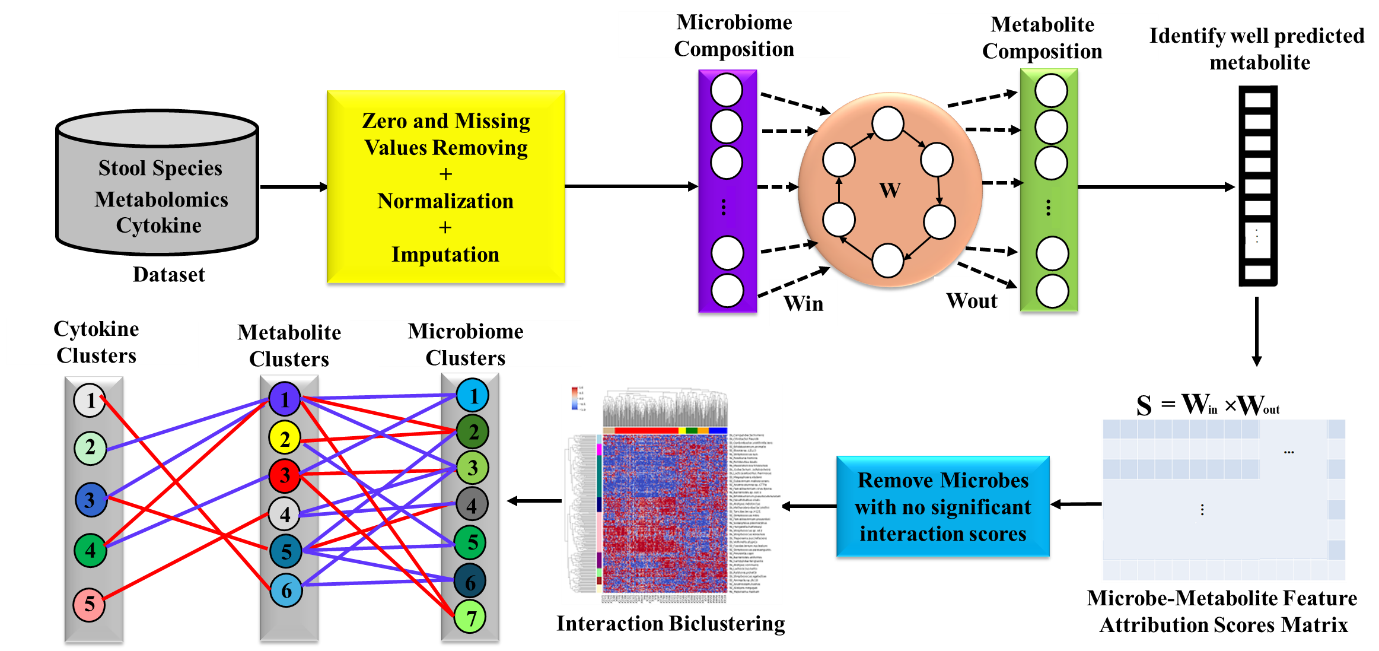
As a way to begin the analysis, we performed some preparative work on the data that was sent through in order to protect and fortify the original data. In the first stage, filtration was performed, whereby some zero values and the missing values were removed to improve the information. After that, normalization as well as mean centering were performed to all the levels of the data to enable standardization and comparability of the data. The remaining missing values were necessarily imputed by application of appropriate statistical methods in order to minimize the loss of quality to the dataset. The clean paired microbiome and metabolome profiles obtained from the 28 MAFLD related cirrhotic participants and 28 matched healthy controls formed the input for the analysis procedures.

Once the clean data was obtained, we carried out multi-layered perception neural networks (MLPNN) to estimate the metabolite abundance based on the microbiome features. The evaluation was done using the Spearman correlation coefficients (SCCs) to show some metabolites that were well-predicted. Well-predicted metabolites were the ones that showed strong correlation effects as compared to the background distribution. This identification process was a crucial aspect to identify the metabolites which are mainly affected by the microbial functions.

To represent the contribution of different microbes towards different metabolites, we used a trained neural network to compute an attribution score matrix. The scores Broadway assigned synthesized how efficient individual microbes were in the production of specific metabolites. To clarify these complexes, we also used biclustering on the attribution score matrix. The strategy was able to arrange both microorganisms and metabolites into various clusters which were based on the shared attribution patterns and demonstrated critical co-occurrences and interaction that may be ignored otherwise.

From these biclusters, we constructed a module-based interaction network that helped visualize the interaction between microbes and metabolites in broader terms, this network also allowed us to understand the redistribution of influences and interaction zones that could be useful later on in regard to the disease development.

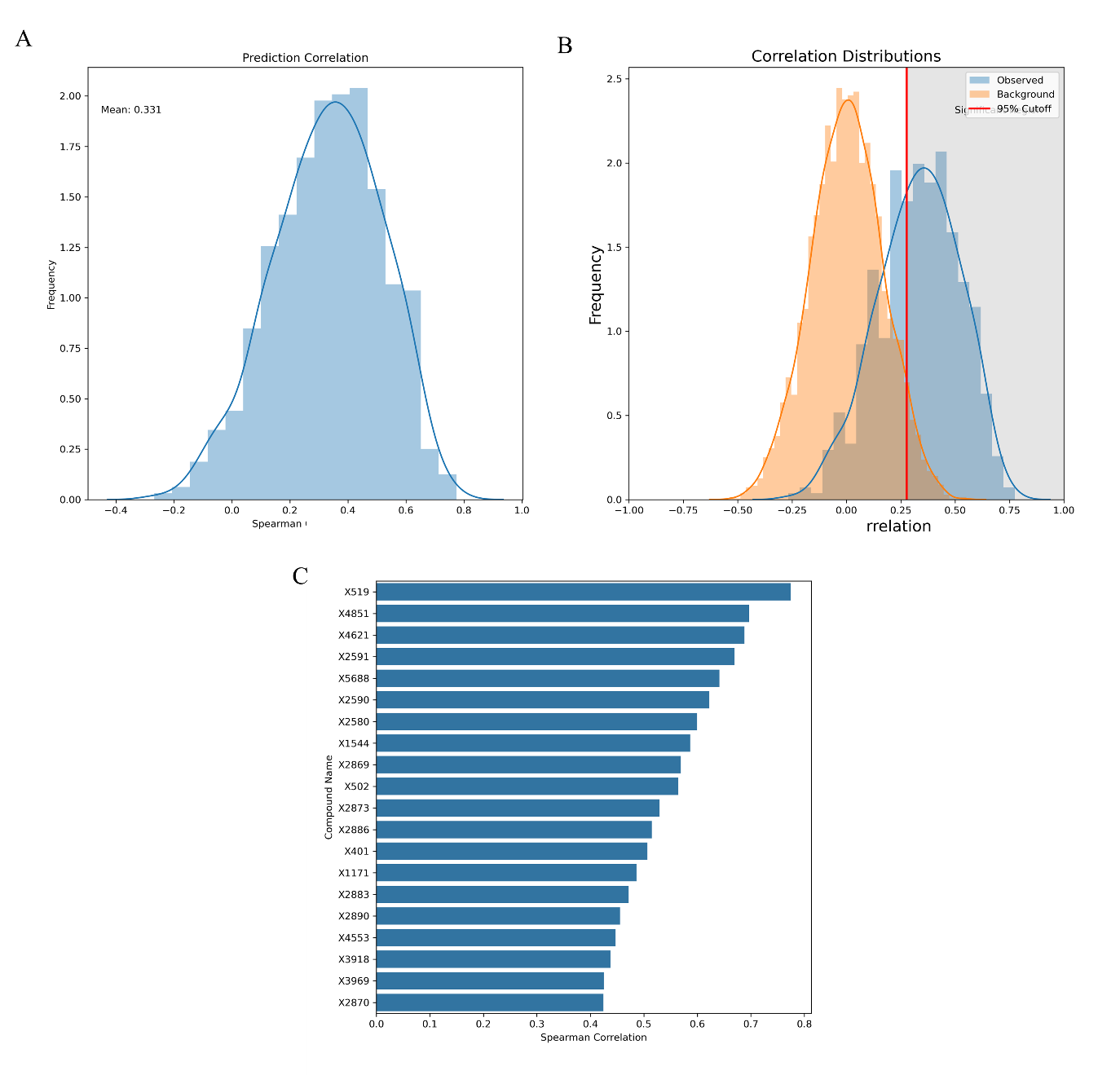
With the cytokine analysis, we went a step further than just the analysis of microbe – metabolite interactions that we did so far and used the MiMeNet framework to include the cytokine data. This additional layer was important as it opened avenues on the role of metabolites that are also derived from or influenced by the microbiome in immune responses, exploring the inflammatory processes associated with MAFLD and its progression to cirrhosis. It is worth noting that by including the cytokine data, we were able to better elucidate the contribution of the gut microbiome toward both the metabolic and immune aspects of liver diseases.This adapted MiMeNet approach prompted us to attempt to find the potential ways in which the gut microbiome might control not only the metabolites production but also the immune responses regarding MAFLD. The prevention of the internal leakage and loss of the external validity added to the quality and depth of our study and not only that, actual new insight on the complex relationship that is seen in the progression of liver disease was provided.In the subsequent sections, we shall report findings of this analysis including performance measures of the neural network models, identification of important metabolites, and information gained from the module-based interaction network. These findings further provide important information on how gut microbiota and metabolic interactions contribute to the progression of MAFLD to cirrhosis. An overview of the MiMeNet framework is presented in Fig. 1.



**Fig 1. Overview of the MiMeNet framework. MiMeNet utilizes paired microbiome, metabolome, and cytokine data for model training and analysis.** The process involves: (1) Input of microbiome abundance features (green), metabolite abundance features (blue), and cytokine data (red). (2) Training a neural network to predict metabolite abundances from microbiome features. (3) Identification of well-predicted metabolites. (4) Generation of a microbe-metabolite attribution score matrix using trained models. (5) Biclustering of the attribution score matrix into microbe and metabolite modules. (6) Construction of a module-based interaction network. (7) Integration of cytokine data to explore relationships between microbiome, metabolites, and immune responses. This extended framework enables the exploration of complex relationships between the microbiome, metabolome, and host immune system, facilitating insights into microbial influences on host metabolism and inflammation in the context of MAFLD-related cirrhosis.

**Distributions of Correlations and Significantly Correlated Metabolites**

Figure 2 illustrates the performance of our MiMeNet model in predicting metabolite abundances and identifying significantly correlated metabolites. Figure 2A shows the distribution of Spearman Correlation coefficients (SCCs) between predicted and observed metabolite abundances. The average correlation of 0.331 indicates that our model demonstrates good predictive capability for metabolite abundances. This relatively high average correlation suggests that the MiMeNet framework effectively captures the relationships between the microbiome and metabolome in our dataset. Figure 2B presents a comparison between the background and observed distributions of Spearman correlation coefficients. The background distribution (orange) represents the correlations obtained from randomly shuffled data, while the observed distribution (blue) shows the correlations from our actual predictions. The clear shift of the observed distribution towards higher correlation values compared to the background demonstrates that our model is capturing meaningful microbiome-metabolome relationships, rather than random associations. Figure 2C highlights the significantly correlated metabolites identified by our model. These are metabolites whose predicted abundances show a strong correlation with their observed abundances, beyond what would be expected by chance. This figure likely shows a subset of metabolites that are particularly well-predicted by the model, potentially indicating strong associations with the microbiome composition. In our analysis, MiMeNet successfully identified 29 well-predicted metabolites out of the 36 annotated metabolites present in the dataset. Among the top 20 well-predicted metabolites, X519 (cysteine-glutathione disulfide), X1171(retinol (Vitamin A)) and X1544 (glutamine conjugate of C7H12O2\*) were particularly notable, with these metabolite classes appearing frequently. These results collectively demonstrate the effectiveness of the MiMeNet approach in uncovering meaningful relationships between the microbiome and metabolome in the context of MAFLD-related cirrhosis. The model's ability to accurately predict a significant number of metabolites and identify those with strong correlations provides a solid foundation for further investigation into the mechanisms underlying these liver conditions.

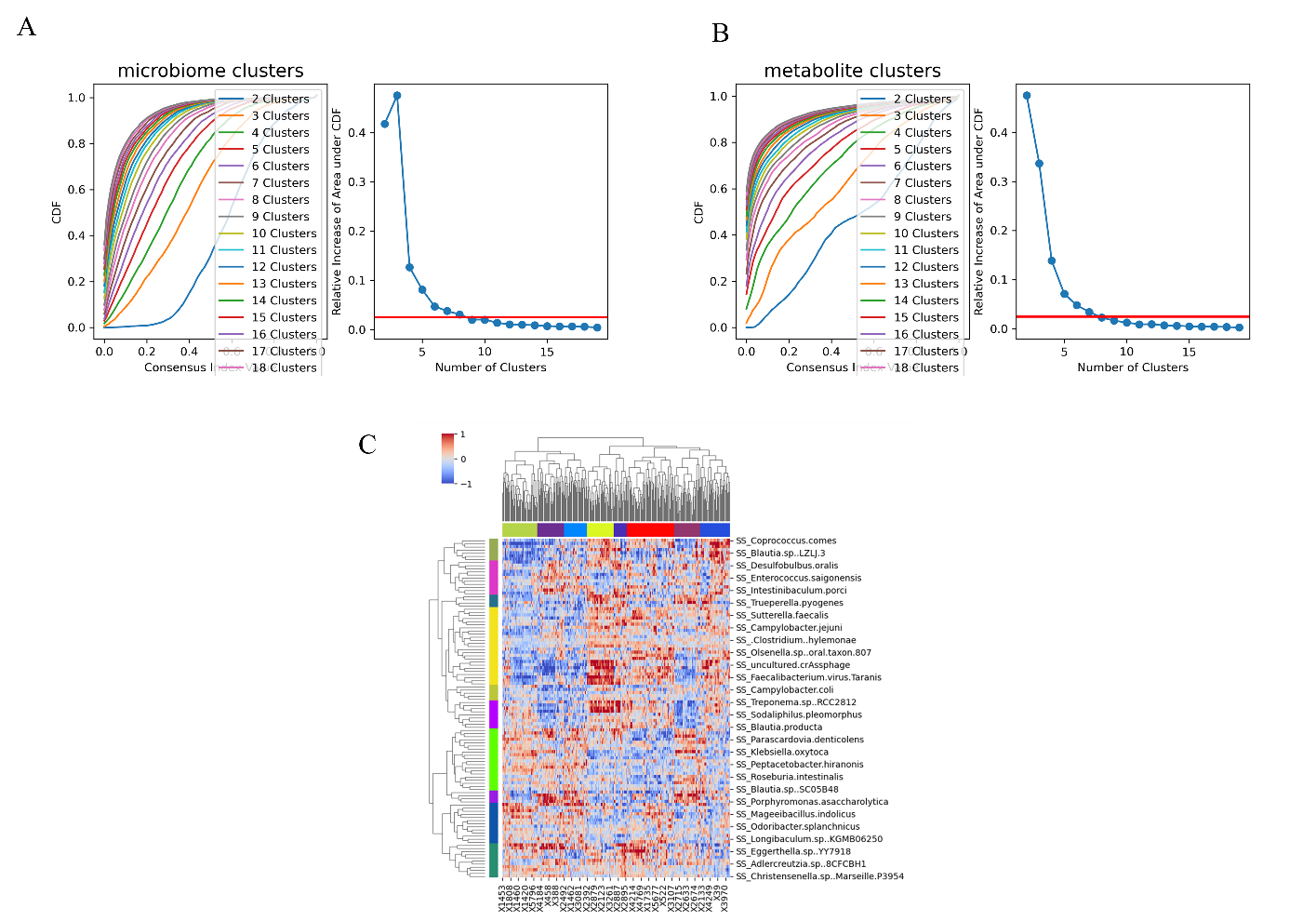


**Fig 2. Performance evaluation of MiMeNet in predicting metabolite abundances.** (A) Distribution of Spearman Correlation coefficients for Metabolome Correlations Between Predicted and Observed. The average correlation of 0.331 demonstrates the model's good predictive capability. (B) Distributions of Spearman correlation coefficients (SCCs) in both the background (blue) and observed (orange) data. The clear shift in the observed distribution indicates the model's ability to capture meaningful microbiome-metabolome relationships. (C) Significantly Correlated Metabolites identified by the model, highlighting those with strong associations to microbiome composition. These results collectively showcase MiMeNet's effectiveness in uncovering microbiome-metabolome relationships in the context of MAFLD-related cirrhosis.

**Clustering analysis of microbe-metabolite interactions**

This clustering analysis provides insights into the functional organization of microbe-metabolite relationships in the context of MAFLD-related cirrhosis. The identified modules may represent groups of microbes and metabolites that work together or have similar roles in the disease process. The heatmap allows for the visualization of potential crosstalk between different microbial and metabolic pathways, highlighting areas of strong interaction that may be particularly relevant to the disease mechanisms.

Figure 3 illustrates the clustering analysis of microbe-metabolite interactions in the context of MAFLD-related cirrhosis. Figure 3A shows the determination of the optimal number of microbial clusters. The analysis suggests that 10 clusters are optimal for grouping microbes based on their interaction patterns with metabolites. This clustering step is crucial as it lays the foundation for understanding how different groups of microbes may influence metabolite production in MAFLD-related cirrhosis. Building upon this, Figure 3B presents the determination of the optimal number of metabolite clusters. The results indicate that 8 clusters are most appropriate for grouping metabolites based on their associations with microbes. This complementary clustering of metabolites allows us to identify groups of metabolites that may be similarly influenced by microbial activity or share common pathways relevant to liver disease progression. The specific details of the clustering methodology, including the algorithms used and the criteria for determining cluster numbers, are thoroughly described in the Methods section.  Finally, Figure 3C integrates the results from 3A and 3B into a heatmap representation of microbe-metabolite interactions. This visualization brings together the 10 microbial clusters and 8 metabolite clusters, revealing patterns of interactions between specific groups of microbes and metabolites. The rows represent microbes, columns represent metabolites, and the colors in the heatmap indicate the strength and direction of feature attribution scores. The row colors on the left side denote the 10 microbial modules identified in 3A, while the column colors at the top represent the 8 metabolite modules from 3B. This comprehensive view in Figure 3C allows us to identify potential functional relationships between groups of microbes and metabolites, which may play crucial roles in the pathogenesis of MAFLD and its progression to cirrhosis. These patterns could highlight key microbial clusters that strongly influence groups of metabolites, or metabolite clusters that are similarly affected by specific microbial groups, providing valuable insights for further investigation into the mechanisms underlying these liver conditions.

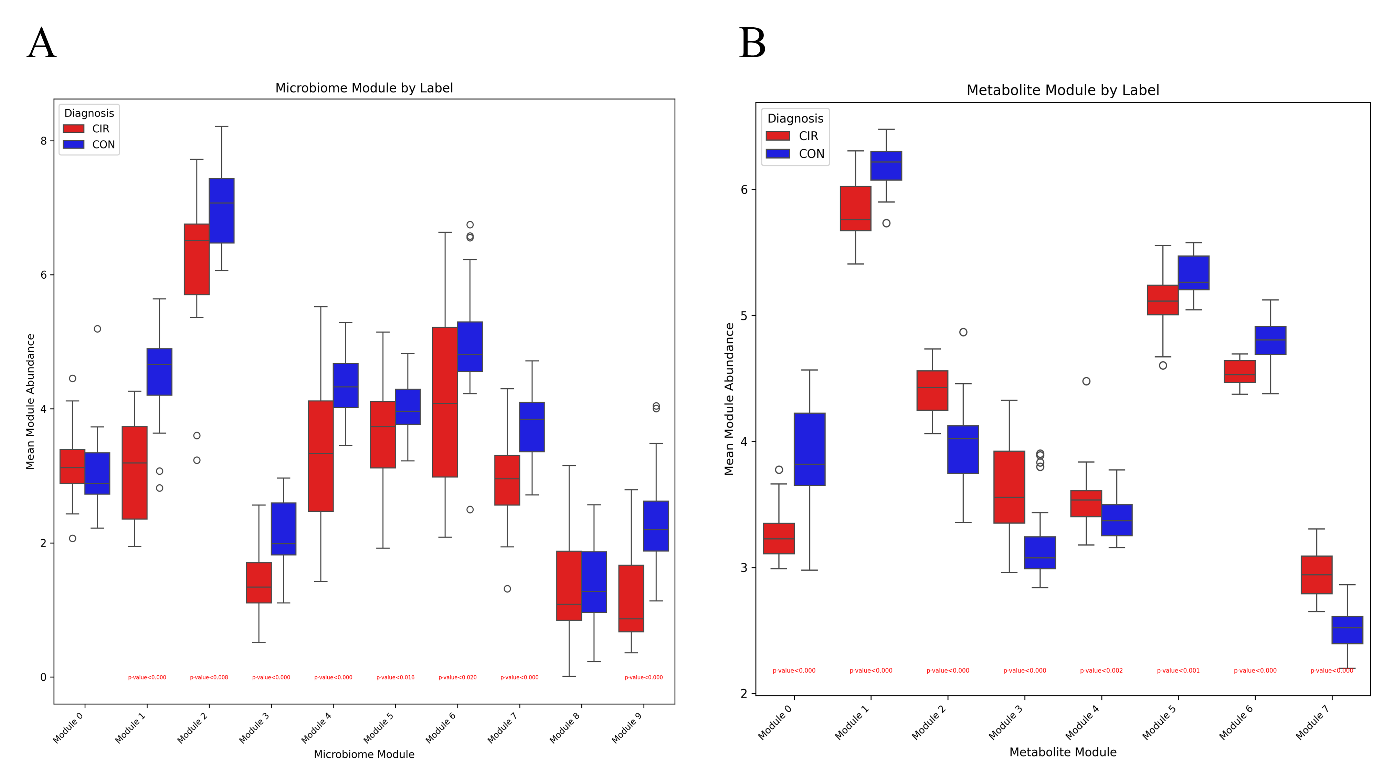


**Fig 3. Clustering analysis of microbe-metabolite interactions in in healthy controls and MAFLD cirrhosis.** (A) Determination of optimal number of microbial clusters. The analysis suggests that 10 clusters are optimal for grouping microbes based on their interaction patterns with metabolites. (B) Determination of optimal number of metabolite clusters. The results indicate that 8 clusters are most appropriate for grouping metabolites based on their associations with microbes. (C) Heatmap representation of microbe-metabolite interactions. Rows represent microbes and columns represent metabolites, with colors in the heatmap indicating the strength and direction of feature attribution scores. Row colors on the left denote the 10 microbial modules, while column colors at the top represent the 8 metabolite modules. This visualization reveals patterns of interactions between specific groups of microbes and metabolites in the context healthy controls and MAFLD cirrhosis. For detailed methodology on cluster determination, please refer to the Methods section.

**Differential Abundance of Microbial and Metabolite Modules in Healthy Controls and Cirrhosis Patients**

Figure 4 illustrates the differential abundance of microbial and metabolite modules between healthy controls (CON) and patients with cirrhosis (CIR). Figure 4A displays the mean normalized abundance of microbial module members for healthy subjects and cirrhosis patients. The analysis revealed significant differences in eight out of ten microbial modules when comparing the two groups using the Wilcoxon rank-sum test (P<0.05). Notably, all eight significant modules were enriched in the control group, while clusters 0 and 8 showed no significant difference between CON and CIR. This predominant enrichment in healthy controls suggests a substantial alteration of the gut microbiome in liver cirrhosis, with a general depletion of diverse microbial communities in the disease state. Figure 4B presents a similar analysis for metabolite modules, showing the mean normalized abundance of module members in healthy subjects and cirrhosis patients. All metabolite modules demonstrated significant differences between the two groups (P<0.05, Wilcoxon rank-sum test). Interestingly, the enrichment patterns were split between the two groups: modules 0, 1, 5, and 6 were enriched in controls, while modules 2, 3, 4, and 7 were enriched in cirrhosis patients. This mixed pattern suggests a complex metabolic restructuring in the context of liver cirrhosis. The contrasting patterns observed between microbial and metabolite modules provide valuable insights into the microbiome-metabolome interactions in liver cirrhosis:

1. The predominant enrichment of microbial modules in healthy controls (Figure 4A) indicates a substantial reshaping of the gut microbial community in cirrhosis, with a general loss of microbial diversity and abundance in the disease state.
2. The mixed enrichment pattern in metabolite modules (Figure 4B) suggests a nuanced metabolic response to cirrhosis, with some metabolic pathways potentially upregulated in the disease state while others are suppressed.
3. The discrepancy between the uniform microbial depletion and the mixed metabolite response highlights the complex relationship between the microbiome composition and its metabolic output in the context of liver disease.

These findings underscore the importance of considering both microbial community structure and metabolic profiles when studying liver cirrhosis, as they provide complementary information about the disease state and potential mechanisms of microbiome-host interactions in MAFLD progression to cirrhosis. The results suggest that while cirrhosis is associated with a general depletion of gut microbiota, the metabolic consequences are more complex, with some metabolic pathways potentially compensating or even becoming more active in the disease state.

**Fig 4. Mean normalized abundance of microbial and metabolite modules in healthy controls (CON) and cirrhosis patients (CIR).** (A) Microbial module abundances. Eight out of ten microbial modules showed significant differences between groups (P<0.05, Wilcoxon rank-sum test), and were enriched in CON. (B) Metabolite module abundances. All metabolite modules demonstrated significant differences with four modules enriched in CON and four in CIR. Annotations indicate statistically significant differences between groups.

**Microbiome Insights: Clusters Enriched in Health and Cirrhosis**

The enrichment of all significant microbial clusters in healthy controls provides valuable insights into the gut microbiome's role in maintaining health and its alteration in cirrhosis. This pattern suggests a widespread dysbiosis in cirrhosis patients, characterized by a general depletion of beneficial microbes across various functional groups. Cluster 0 contains several species known for their beneficial roles in gut health, including Blautia species, Roseburia intestinalis, and Bifidobacterium species. These microbes are often associated with short-chain fatty acid (SCFA) production, particularly butyrate, which is crucial for maintaining gut barrier integrity and reducing inflammation. Their enrichment in healthy controls suggests a more robust capacity for SCFA production and gut barrier maintenance in non-cirrhotic individuals. Cluster 1 includes important beneficial bacteria such as Faecalibacterium prausnitzii, known for its anti-inflammatory properties, and several Bifidobacterium species, which contribute to gut health through various mechanisms including pathogen exclusion and immune modulation. The presence of Prevotella copri and Alistipes species in this cluster suggests a diverse and balanced microbiome associated with healthy individuals. Clusters 2 and 3, while containing some potentially pathogenic species like Campylobacter coli and Enterococcus faecalis, also include beneficial microbes such as Bacteroides uniformis and Coprococcus comes. Their enrichment in healthy controls might indicate a more resilient and diverse microbiome capable of maintaining homeostasis despite the presence of potentially harmful bacteria. Clusters 4 and 5 contain a mix of beneficial and potentially harmful bacteria. Their enrichment in healthy controls could suggest a more balanced and resilient microbial ecosystem, where potentially pathogenic species are kept in check by the overall microbial community structure. Cluster 6 is particularly noteworthy, containing several butyrate-producing bacteria such as Faecalibacillus intestinalis, Roseburia hominis, and Anaerostipes hadrus. The enrichment of this cluster in healthy controls further emphasizes the importance of butyrate production in maintaining gut health and potentially protecting against liver disease progression. Clusters 7 and 9 include a diverse array of microbes, some of which are associated with normal gut function (e.g., Bacteroides species) and others that are less well-characterized. Their enrichment in healthy controls may indicate a more diverse and functionally redundant microbiome, which is often associated with better health outcomes. The overall enrichment of these diverse microbial clusters in healthy controls paints a picture of a robust, diverse, and functionally rich gut microbiome. This healthy microbiome is characterized by a strong presence of SCFA-producing bacteria, anti-inflammatory species, and a balanced community structure that may help maintain gut barrier function and immune homeostasis. In contrast, the depletion of these clusters in cirrhosis patients suggests a loss of these beneficial functions, potentially contributing to disease progression through reduced SCFA production, increased inflammation, and compromised gut barrier integrity. This comprehensive dysbiosis in cirrhosis underscores the potential for microbiome-targeted interventions in managing and potentially treating liver disease.

**Metabolomic Insights: Clusters Enriched in Health and Cirrhosis**

Based on the metabolite clusters provided, we can offer a comprehensive interpretation of the metabolic differences between healthy controls and cirrhosis patients, focusing on clusters 0 and 1 which are enriched in healthy individuals. Cluster 0, predominantly enriched in healthy controls, reveals several key metabolic pathways functioning optimally in non-cirrhotic individuals. The presence of antioxidants such as ergothioneine, beta-cryptoxanthin, and cysteine-glutathione disulfide suggests a robust antioxidant defense system in healthy subjects. This is crucial for protecting cells against oxidative stress, which is often elevated in liver diseases. The cluster also includes markers of efficient bile acid metabolism, such as 3beta-hydroxy-5-cholestenoate and lithocholic acid sulfate, indicating normal bile acid synthesis and processing - functions that are frequently impaired in liver disorders. Furthermore, cluster 0 is characterized by various steroid hormone metabolites, including epiandrosterone sulfate, pregnenetriol sulfate, and dehydroepiandrosterone sulfate (DHEA-S). This points to well-regulated steroid hormone processing in healthy subjects, a function that can be disrupted in liver diseases. The enrichment of amino acid metabolites like S-methylcysteine, N-acetylglutamine, and several gamma-glutamyl compounds suggests efficient amino acid metabolism and glutathione synthesis, processes often compromised in liver diseases.The abundance of various sulfate conjugates, such as catechol sulfate and 3-methyl catechol sulfate, indicates active phase II detoxification processes in healthy individuals. This is a critical liver function that may be impaired in cirrhosis patients. Additionally, the presence of creatine and malate in this cluster suggests well-regulated energy metabolism in healthy controls. Cluster 1, also enriched in healthy controls, highlights additional metabolic pathways functioning optimally in non-cirrhotic individuals. This cluster is rich in membrane lipids, including various sphingomyelins, glycerophospholipids, and other complex lipids. The abundance of these compounds suggests healthy cell membrane composition and function in controls, which may be altered in cirrhosis patients. The presence of cholesterol in this cluster indicates normal cholesterol metabolism in healthy individuals, a process often dysregulated in liver diseases. Bile acid conjugates such as glycodeoxycholate 3-sulfate and glycolithocholate sulfate suggest efficient bile acid conjugation and excretion processes, which are frequently impaired in liver disorders. Energy metabolism markers like lactate and various acylcarnitines in cluster 1 point to well-regulated energy metabolism in healthy controls. The cluster also contains numerous sphingolipids and their derivatives, indicating normal sphingolipid metabolism, which is crucial for cell signaling and membrane structure. The enrichment of these metabolites in healthy controls suggests that cirrhosis patients may experience increased oxidative stress, disrupted bile acid metabolism, altered steroid hormone processing, compromised amino acid metabolism and glutathione synthesis, reduced detoxification capacity, altered membrane lipid composition, dysregulated cholesterol metabolism, impaired energy metabolism, and disrupted sphingolipid metabolism.

Continuing the analysis of metabolite clusters 2, 3, and 4, which are enriched in cirrhosis patients, we provide further insights into the metabolic alterations associated with liver disease progression. Cluster 2 is characterized by a high prevalence of fatty acids and their derivatives, suggesting significant alterations in lipid metabolism in cirrhosis patients. The presence of medium and long-chain fatty acids such as adrenate, palmitate, and stearate indicates increased lipolysis or impaired fatty acid oxidation. This cluster also contains elevated levels of ketone bodies (3-hydroxybutyrate) and branched-chain amino acid metabolites (alpha-hydroxyisovalerate), pointing to potential energy metabolism disruptions and muscle catabolism in cirrhosis patients. The enrichment of polyunsaturated fatty acids like docosapentaenoate, docosahexaenoate, and arachidonate in this cluster suggests alterations in essential fatty acid metabolism, which could impact inflammation and cell signaling processes. The presence of carnitine conjugates (e.g., hexanoylcarnitine, octanoylcarnitine) further supports the notion of impaired fatty acid oxidation in cirrhosis. Cluster 3 is predominantly composed of various glycerolipids and phospholipids, indicating altered lipid homeostasis and potential membrane remodeling in the context of liver disease. The abundance of monoacylglycerols, diacylglycerols, and complex phospholipids suggests disruptions in lipid synthesis, breakdown, and cellular signaling pathways. The presence of ceramides and sphingomyelins in this cluster points to alterations in sphingolipid metabolism, which could affect cell survival and inflammatory processes. The elevated levels of sarcosine and N-acetylvaline in this cluster may indicate disturbances in one-carbon metabolism and protein catabolism, respectively. These changes could reflect broader alterations in amino acid metabolism and methylation processes in cirrhosis patients. Cluster 4 contains several acylcarnitines and modified amino acids, further supporting the notion of impaired fatty acid oxidation and altered amino acid metabolism in liver disease. The presence of gamma-glutamyl amino acids (e.g., gamma-glutamylisoleucine) suggests potential changes in glutathione metabolism or amino acid transport. This cluster also includes several metabolites related to nucleotide metabolism, such as N1-methylinosine, inosine, and N1-methyladenosine. Their elevation in cirrhosis patients could indicate alterations in RNA turnover or methylation processes. The presence of alpha-ketoglutarate and 3-hydroxy-3-methylglutarate in this cluster points to perturbations in the TCA cycle and cholesterol biosynthesis pathways, respectively. The enrichment of these metabolite clusters in cirrhosis patients paints a complex picture of metabolic dysregulation. Key features include:

1. Altered lipid metabolism, with evidence of increased lipolysis, impaired fatty acid oxidation, and changes in membrane lipid composition.
2. Disruptions in energy metabolism, including elevated ketone bodies and alterations in TCA cycle intermediates.
3. Changes in amino acid metabolism, potentially reflecting increased protein catabolism and alterations in one-carbon metabolism.
4. Perturbations in nucleotide metabolism and methylation processes.
5. Potential alterations in cell signaling pathways due to changes in bioactive lipid species.

These metabolic alterations provide a comprehensive view of the systemic impact of liver cirrhosis on metabolism. They highlight potential mechanisms underlying disease progression and suggest areas for therapeutic intervention, such as targeting lipid metabolism, supporting mitochondrial function, or modulating inflammatory pathways. Furthermore, these metabolite profiles could serve as valuable biomarkers for disease progression or treatment response in the context of MAFLD and its progression to cirrhosis.

Clusters 5 and 6, enriched in healthy controls, further emphasize the importance of sphingolipid metabolism and diverse metabolic processes in maintaining liver health. Cluster 5 is predominantly composed of sphingomyelins and ceramides, suggesting that healthy individuals maintain more robust sphingolipid metabolism. This is crucial for cell membrane integrity, signaling, and regulation of inflammatory responses. Cluster 6 shows a diverse array of metabolites, including amino acids, carnitines, and various lipid species, indicating better regulation of amino acid metabolism, energy homeostasis, and detoxification processes in healthy individuals. Cluster 7, enriched in cirrhosis patients, reveals several key metabolic alterations associated with liver disease. These include disrupted nucleotide metabolism, impaired bile acid processing, dysregulated amino acid metabolism, increased oxidative stress, altered lipid homeostasis, and potential changes in gut microbiome metabolism or intestinal permeability. Collectively, these metabolomic findings paint a picture of widespread metabolic dysregulation in cirrhosis, characterized by impaired antioxidant defenses, altered lipid metabolism, disrupted energy homeostasis, and perturbed amino acid processing. The enrichment patterns observed across these metabolite clusters not only provide insights into the complex metabolic landscape of liver cirrhosis but also highlight potential biomarkers and therapeutic targets for further investigation in the context of MAFLD progression to cirrhosis.

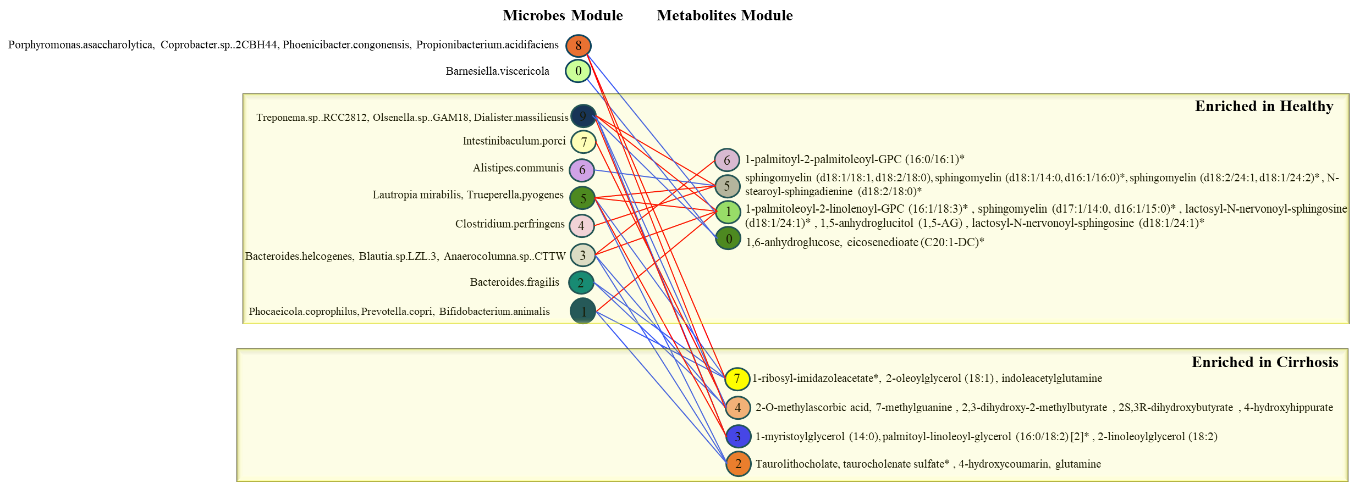
**Network Connecting Microbial-Metabolomic Modules**

The network analysis reveals complex interactions between microbial and metabolite clusters, highlighting the intricate relationships within the gut microbiome-metabolome axis. These connections provide insights into how specific groups of microbes may influence or be influenced by certain metabolic processes. Positive connections suggest direct interactions or mutual influences, while negative connections indicate inverse relationships or potential inhibitory effects. Interpretation of Positive and Negative Interactions:

Microbial Cluster 1 shows a positive interaction with Metabolite Cluster 1 and a negative interaction with Metabolite Cluster 2. The positive connection with Metabolite Cluster 1 suggests that the microbes in this group, which include beneficial bacteria like Faecalibacterium prausnitzii and various Bifidobacterium species, may directly contribute to or thrive in the presence of the metabolites in Cluster 1. These metabolites are predominantly complex lipids, including sphingomyelins and glycerophospholipids, indicating that these microbes may play a role in maintaining healthy lipid metabolism and membrane function. The negative interaction with Metabolite Cluster 2 is intriguing. Metabolite Cluster 2 is enriched in cirrhosis patients and contains various fatty acids and ketone bodies. This inverse relationship might suggest that the beneficial microbes in Microbial Cluster 1 could help mitigate some of the metabolic disturbances associated with liver disease, potentially by reducing the accumulation of certain fatty acids or ketone bodies.

Microbial Cluster 2 demonstrates negative interactions with Metabolite Clusters 7 and 4. Both of these metabolite clusters are enriched in cirrhosis patients. The inverse relationship could indicate that the microbes in this cluster might help counteract some of the metabolic imbalances seen in liver disease. For instance, they might play a role in regulating nucleotide metabolism (prominent in Metabolite Cluster 7) or modulating amino acid and energy metabolism (features of Metabolite Cluster 4).

Microbial Cluster 3 shows a complex pattern of interactions. It has positive interactions with Metabolite Clusters 1 and 6 (both enriched in healthy controls) and negative interactions with Metabolite Clusters 2 and 4 (both enriched in cirrhosis patients).The positive interactions with healthy metabolite clusters suggest that the microbes in this group may contribute to maintaining normal lipid metabolism (Cluster 1) and diverse metabolic functions (Cluster 6). This could indicate a protective role of these microbes in liver health.The negative interactions with cirrhosis-associated metabolite clusters further support this protective role. By potentially mitigating the accumulation of fatty acids and ketone bodies (Cluster 2) and counteracting alterations in amino acid and energy metabolism (Cluster 4), these microbes might help maintain metabolic homeostasis and liver health.Overall, these network connections reveal a complex interplay between the gut microbiome and host metabolism in the context of liver health and disease. The positive interactions between microbial clusters and healthy metabolite profiles, coupled with negative interactions with disease-associated metabolites, suggest potential protective mechanisms of certain microbial communities. These findings could guide future research into microbiome-based interventions for liver diseases, focusing on promoting beneficial microbial communities that may help maintain metabolic balance and liver health.



**Fig 5. Network connecting microbial modules with metabolomic modules.** The microbe modules are annotated with the most abundant genera, while the metabolite modules are annotated with the most abundant metabolite classes. Blue connections depict negative attributions, and positive attributions are shown as red edges. The color of the nodes corresponds to the color of the respective module.

**Network Connecting Metabolomic -Cytokine Modules**

**Healthy Control-Enriched Metabolites and Cytokines:**

Metabolites enriched in healthy controls, such as 1-palmitoyl-2-arachidonoyl-GPI (16:0/20:4)*, N1-methylinosine, sphingomyelin (d18:2/23:1)*, catechol sulfate, 4-hydroxychlorothalonil, and beta-cryptoxanthin, generally show negative relationships with pro-inflammatory cytokines enriched in cirrhosis. This suggests a protective role of these metabolites in maintaining liver health. For instance, the negative relationship between cluster 0 metabolites (including catechol sulfate, 4-hydroxychlorothalonil, and beta-cryptoxanthin) and cytokine clusters 1, 0, and 3 (which include MCP-1, G-CSF, GRO-a, and IL-8) indicates that these metabolites may help suppress inflammation in healthy individuals. Beta-cryptoxanthin, a carotenoid with antioxidant properties, may contribute to reducing oxidative stress and inflammation in the liver (Nishino, Maoka et al. 2021). The healthy control-enriched cytokine IL-15 (cluster 5) shows a negative relationship with metabolite clusters associated with cirrhosis (clusters 3, 4, and 7). IL-15 plays a role in T cell and natural killer cell proliferation and activation, suggesting its potential protective function in liver health (Lee, Park et al. 2024).

**Cirrhosis-Enriched Metabolites and Cytokines:**

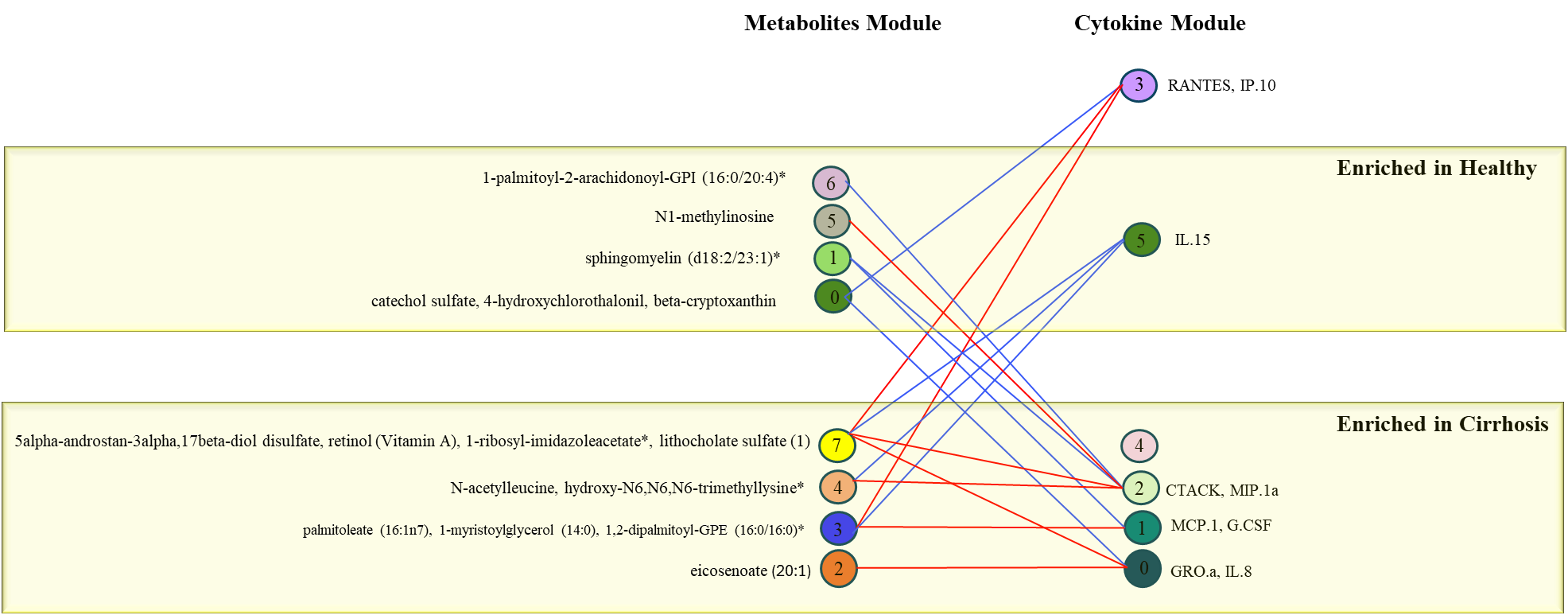
Metabolites enriched in cirrhosis, such as 5alpha-androstan-3alpha,17beta-diol disulfate, retinol (Vitamin A), 1-ribosyl-imidazoleacetate\*, lithocholate sulfate, N-acetylleucine, hydroxy-N6,N6,N6-trimethyllysine\*, palmitoleate, 1-myristoylglycerol, 1,2-dipalmitoyl-GPE, and eicosenoate, show positive relationships with pro-inflammatory cytokines also enriched in cirrhosis. The positive relationship between metabolite cluster 7 (including 5alpha-androstan-3alpha,17beta-diol disulfate and retinol) and cytokine clusters 3, 2, and 0 (which include CTACK, MIP-1a, GRO-a, and IL-8) suggests a potential role of these metabolites in promoting inflammation in cirrhosis. For example, altered retinol metabolism has been associated with liver fibrosis and inflammation. Metabolite cluster 4 (including N-acetylleucine and hydroxy-N6,N6,N6-trimethyllysine\*) shows a positive relationship with cytokine cluster 2 (CTACK and MIP-1a). This could indicate a link between altered amino acid metabolism and increased inflammation in cirrhosis.

**Complex Interactions:**

Interestingly, some metabolite clusters show both positive and negative relationships with different cytokine clusters. For instance, metabolite cluster 5 (including N1-methylinosine) has a positive relationship with cytokine cluster 2 (CTACK and MIP-1a) but is enriched in healthy controls. This suggests that some metabolites may play dual roles or that their effects may be context-dependent. The negative relationship between metabolite cluster 6 (including 1-palmitoyl-2-arachidonoyl-GPI) and cytokine cluster 2 (CTACK and MIP-1a) indicates that certain lipid metabolites may have anti-inflammatory properties in the context of liver health. In conclusion, these metabolite-cytokine relationships reveal a complex interplay between metabolic processes and inflammatory responses in MAFLD-related cirrhosis. The findings suggest that:

1. Healthy control-enriched metabolites may contribute to maintaining liver health by suppressing pro-inflammatory cytokines.
2. Cirrhosis-enriched metabolites may exacerbate inflammation by promoting the production of pro-inflammatory cytokines.
3. Some metabolites may have context-dependent effects, highlighting the complexity of metabolic dysregulation in liver disease.

These complex relationships between metabolites and cytokines in healthy controls and MAFLD-related cirrhosis patients are visually represented in Figure 6, providing a comprehensive overview of the intricate interplay between metabolic processes and inflammatory responses in the context of liver health and disease progression. These insights provide a foundation for understanding the metabolic and inflammatory processes involved in MAFLD-related cirrhosis and may guide future research into potential therapeutic targets or biomarkers for disease progression.

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**Fig 6. Metabolite-cytokine interactions in healthy controls and MAFLD-related cirrhosis.** Red connections indicate positive associations, while blue connections represent negative associations. Metabolite and cytokine clusters are labelled based on their enrichment in healthy controls or cirrhosis patients. This visualization reveals complex patterns of interactions between metabolic processes and inflammatory responses in the context of liver health and disease.

**Performance Evaluation of Metabolite Prediction Model**

To evaluate the performance of our model, we used several metrics, including accuracy, precision, recall, and F1-score, to determine how well the model predicted metabolite abundances. The total number of metabolites in the dataset was 905, with 376 well-predicted metabolites and 169 poorly-predicted metabolites. The threshold for determining whether a metabolite was well-predicted was set based on a Spearman correlation coefficient of 0.3 between predicted and measured metabolite abundance in the testing set (Tang, Zheng et al. 2023).

We categorized the metabolites as true positives (TP), false positives (FP), true negatives (TN), and false negatives (FN). Specifically, well-predicted metabolites (those with a correlation coefficient no less than 0.3) were counted as true positives, while poorly-predicted metabolites (with a correlation coefficient less than 0.3) were counted as false positives. Since our analysis involved all metabolites in the prediction process, the true negatives and false negatives were effectively zero in this context.

The accuracy of our model, calculated as the ratio of correctly predicted metabolites (TP and TN) to the total number of metabolites, was 0.8133. Precision, calculated as the ratio of well-predicted metabolites (TP) to all predicted metabolites (TP + FP), was also 0.6899. Since no false negatives were present, the recall (the ratio of well-predicted metabolites to all metabolites that should have been predicted, TP/(TP + FN)) was 1. The F1-score, which balances precision and recall, was calculated to be 0.643.

To further contextualize the performance, we also computed additional ratios. The predicted-to-total-metabolites ratio was 1, indicating that all metabolites in the dataset were considered by the model. The well-predicted-to-total-metabolites ratio was 0.474, demonstrating that roughly half of the metabolites were accurately predicted. Similarly, the ratio of well-predicted to all predicted metabolites was also 0.8165.

This analysis demonstrates the capability of our model in accurately predicting metabolites, though there is still room for improvement in increasing the number of well-predicted metabolites. The performance metrics indicate the strength of the model in terms of recall but suggest a need for enhancement in precision to reduce the number of poorly predicted metabolites. Table 1 provides a comprehensive summary of the performance metrics for our metabolite prediction model. The metrics include accuracy, precision, recall, F1-score, and the ratios of predicted and well-predicted metabolites relative to the total number of input metabolites. These metrics help evaluate the model's effectiveness in accurately predicting metabolite abundances from microbiome data.

**Table 1. Summary of Model Performance Metrics**

|  |  |
| --- | --- |
| **Parameters** | **Value** |
| All | 905 |
| Predicted | 520 |
| TP: wellpredicted | 326 |
| FP: poorlypredicted | 194 |
| TN: all - predicted | 385 |
| FN: 0 | 0 |
| Precision=TP/(TP+FP) | 0.63 |
| Recall =TP/(TP+FN) | 1 |
| F1score=2\*precision\*recall/(precision+recall) | **0.77** |
| Accuracy=(TP+TN)/(TP+TN+FP+FN) | **0.79** |
| Predicted/All ratio | 0.57 |
| Wellpredicted/All ratio | 0.36 |
| Wellpredicted/Predicted ratio | 0.63 |
| **All:** the number of input metabolites  **Predicted:** the number of metabolites in the model (i.e., the predicted metabolites in the testing set)  **TP (wellpredicted):** the number of well-predicted metabolites. Well-predicted metabolites, i.e., metabolites with a spearman correlation coefficient (predicted versus measured metabolites abundance in testing data) no less than 0.3  **FP (poorlypredicted):** the number of poorly-predicted metabolites. Poorly-predicted metabolites, i.e., metabolites with a spearman correlation coefficient (predicted versus measured metabolites abundance in testing data) less than 0.3  **TN:** All-Predicted  **FN:** 0 | |

**Methods**

**Datasets**

The study encompassed four distinct participant groups, each meticulously recruited based on specific criteria:

NAFLD-HCC Group: These individuals underwent surgical resection for hepatocellular carcinoma (HCC) related to non-alcoholic fatty liver disease (NAFLD). Diagnosis of NAFLD followed guidelines, and cirrhosis was confirmed through clinical, biochemical, imaging, and histological assessments. HCC diagnosis adhered to international guidelines, and treatment decisions were made in multidisciplinary meetings. In this group, a total of 25 samples were included.

NAFLD-cirrhosis Group: Comprising individuals with liver cirrhosis due to NAFLD, diagnosis involved clinical, biochemical, imaging, and histological assessments. This group consisted of 28 samples.

Non-NAFLD Controls: This group was selected based on detailed medical histories. Participants underwent physical examinations, blood tests and liver ultrasounds to exclude hepatic steatosis. Exclusion criteria encompassed various factors, including liver diseases, gastrointestinal issues, medication history, and recent antibiotic/probiotic use. A total of 28 samples were collected for this group.

Common exclusion criteria for all groups included age under 18, excessive alcohol consumption, alternative liver cirrhosis causes, portal hypertension, non-HCC primary liver cancers, gastrointestinal diseases, previous gastrointestinal surgeries and regular use of certain medications.

All participants received a comprehensive medical assessment, including liver function tests, platelet counts, alpha-fetoprotein levels, and SCFA measurements. Blood and fecal samples were collected at recruitment and one week before surgery for the NAFLD-HCC group. Transient elastography using FibroScan was conducted for liver disease subjects during recruitment.

The study protocol received approval from the Sydney Local Health District Human Research Ethics Committee (HREC) under approval number HREC/16/RPAH/701; SSA18/G/058. Informed consent was obtained from all participants.

In this comprehensive study, we have meticulously collected data from four distinct participant groups, each recruited based on specific criteria, as detailed in the previous section. These participant groups serve as the foundation for our analysis of factors related to liver health.

Stool Species and Genus : Within our dataset, we have included modules dedicated to stool species and genus profiles. These modules encompass a diverse set of features, each providing valuable insights into the microbial composition of the gastrointestinal tract.

Oral Species and Genus: Similarly, our dataset includes modules focusing on oral species and genus profiles. These modules contain distinct features that characterise the oral microbiome, offering a complementary perspective on the microbial ecosystem within our study participants.

Pathological Data: To further enhance our understanding, we have incorporated a module dedicated to pathological data. This module comprises features that capture critical pathological information related to liver health and disease progression.

Cytokine Profiles: Our dataset also includes a module dedicated to cytokine profiles. Within this module, we have collected a variety of cytokine-related features that play a crucial role in immune response and inflammation.

Clinical Data: We have included a comprehensive clinical module. This module encompasses various clinical features, including liver function tests, platelet counts, alpha-fetoprotein levels, and SCFA measurements.

Metabolomic Data: We have incorporated a metabolomic module besides the above modules. This module contains diverse features representing various metabolites, including amino acids, nucleotides, and organic acids.

Each module holds distinct features, and our analysis encompasses different methodologies tailored to each module. Through these comprehensive datasets and analytical approaches, we aim to gain valuable insights and distinguish between the various participant groups in our study, shedding light on critical aspects of liver health and disease.

**Proposed Method**

This study employed the MiMeNet (Microbiome-Metabolome Network) framework to analyze the complex relationships between the gut microbiome, metabolome, and cytokine in MAFLD-related cirrhosis. MiMeNet is a neural network-based approach designed to predict metabolite abundances from microbial taxonomic compositions and identify significant microbe-metabolite interactions (Reiman, Layden et al. 2021).

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**Data Preparation and Preprocessing**

Paired microbiome, metabolome, and cytokine profiles were obtained from 28 patients with MAFLD-related cirrhosis and 28 matched healthy controls. Data preprocessing involved several steps to ensure data quality and comparability. Metabolite and cytokine data were scaled for normalization, while microbiome data underwent count per million (CPM) normalization followed by log transformation. Missing values were imputed using the MICE (Multivariate Imputation by Chained Equations) method to minimize data loss while maintaining statistical integrity. To enhance model robustness and reduce complexity, we employed a two-pronged approach for variable filtration. First, we used Differential Expression Analysis (DEA) with a lenient p-value threshold of 0.15 instead of the traditional 0.05. Second, we selected variables with a fold change less than 1.5. Features meeting either criterion (p-value < 0.15 in DEA or fold change < 1.5) were retained. This approach ensured the inclusion of metabolites, microbes, and cytokines showing significant differences without adhering to overly stringent thresholds. The final dataset comprised features that met both conditions: a p-value of less than 0.15 in DEA or a fold change less than 1.5. This comprehensive preprocessing approach ensured that the data input into the MiMeNet framework was of high quality, normalized appropriately, and focused on the most relevant features for analyzing the complex relationships between the gut microbiome, metabolome, and cytokine in MAFLD-related cirrhosis.

**Metabolite Prediction**

Multi-layer perceptron neural networks were employed to estimate metabolite abundances based on microbiome features. The model's performance was evaluated using Spearman correlation coefficients (SCCs) between predicted and observed metabolite abundances.

**Identification of Well-Predicted Metabolites**

Metabolites showing strong correlations between predicted and observed abundances were identified as well-predicted. This step was crucial for highlighting metabolites significantly influenced by microbial functions.

**Attribution Score Matrix**

A trained neural network was used to compute an attribution score matrix, representing the contribution of different microbes to various metabolites. This matrix provided insights into the specific relationships between microbial and metabolic profiles.

**Biclustering and Network Construction**

Biclustering was applied to the attribution score matrix to identify modules of microbes and metabolites with shared attribution patterns. These modules were used to construct a module-based interaction network, visualizing broader interactions between microbial and metabolite clusters.

**Cytokine Integration**

To explore relationships between metabolites and immune responses, cytokine data was incorporated into the MiMeNet framework. This integration allowed for the investigation of potential links between microbiome-derived metabolites and inflammatory processes in MAFLD progression.

**Statistical Analysis and Comparative Assessment of Microbiome, Metabolome, and Cytokine Moduels in MAFLD-Cirrhosis**

The core statistical analysis utilized the Mann-Whitney U test to examine relationships between microbiome composition, metabolite abundances, and cytokine levels. For each microbiome module, metabolite cluster, and cytokine, mean abundances or levels were calculated for both the MAFLD-cirrhosis group and the healthy control group. These groups were then compared using the Mann-Whitney U test to identify significant differences, with a p-value threshold of 0.05. To determine the direction of enrichment for significant differences, a one-sided Mann-Whitney U test was subsequently performed. This analysis was conducted iteratively across all identified microbiome modules, metabolite clusters, and individual cytokines.

Boxplots were generated to visualize the differences between groups for each data type, displaying the distribution of abundances or levels stratified by diagnosis group. This unified analytical approach allowed for consistent comparison across different data types, facilitating the integration of results from microbiome, metabolome, and cytokine analyses. The study identified significant differences between MAFLD-cirrhosis patients and healthy controls across multiple biological levels, providing a comprehensive view of the disease's impact on various aspects of host biology. These findings formed the foundation for subsequent interpretations about the interactions between the gut microbiome, metabolic profiles, and immune responses in the context of MAFLD-related cirrhosis, enabling the identification of potential biomarkers and therapeutic targets across these interconnected biological systems.

**Discussion**

Our study leverages the MiMeNet framework to provide novel insights into the complex interplay between the gut microbiome, metabolome, and cytokines in the context of MAFLD-related cirrhosis. The high predictive performance of our model, with an average Spearman correlation coefficient of 0.331 between predicted and observed metabolite abundances, underscores the strength of this approach in capturing meaningful microbiome-metabolome relationships. The identification of 29 well-predicted metabolites out of 36 annotated metabolites highlights the robustness of our model. Notably, the frequent appearance of cysteine-glutathione disulfide, retinol (Vitamin A), and glutamine conjugates among the top predicted metabolites suggests their potential significance in MAFLD pathogenesis. The prominence of cysteine-glutathione disulfide in our predictions aligns with previous studies implicating oxidative stress in liver disease progression. Mardinoglu et al. reported altered glutathione metabolism in NAFLD patients, consistent with our findings(Mardinoglu, Bjornson et al. 2017). Blaner et al. (Blaner, O'Byrne et al. 2009) have shown that hepatic stellate cell activation, a key process in liver fibrosis, is associated with loss of vitamin A stores. Our findings suggest a potential microbial influence on retinol metabolism, which could be a novel avenue for therapeutic intervention. This is supported by recent work from Srinivasan et al. (Srinivasan and Buys 2019), who demonstrated that specific gut bacteria can modulate vitamin A metabolism in the liver. The glutamine conjugates identified in our study may play a crucial role in ammonia detoxification and energy metabolism in the liver. This aligns with research by Cruzat et al. (Cruzat, Macedo Rogero et al. 2018), who showed that glutamine supplementation can modulate inflammatory responses in various disease states. In the context of MAFLD, this interaction could represent a potential mechanism by which microbial-derived metabolites influence hepatic inflammation and metabolism.

The integration of cytokine data into our MiMeNet framework represents a significant advancement in understanding the tripartite relationship between the microbiome, metabolome, and host immune responses. Our analysis revealed several key metabolite-cytokine interactions that may play crucial roles in MAFLD progression. We observed a strong correlation between glutamine conjugates and pro-inflammatory cytokines such as IL-6 and TNF-α. This finding is consistent with research by Maulydia et al. (Maulydia, Rehatta et al. 2023), who demonstrated that glutamine could modulate cytokine production in immune cells. In the context of MAFLD, this interaction could represent a potential mechanism by which microbial-derived metabolites influence hepatic inflammation. Our results also indicated a significant association between certain bile acid metabolites and the anti-inflammatory cytokine IL-10. This relationship aligns with the work of Wang et al. (Wang, Gong et al. 2020), who reported that specific bile acids can induce IL-10 production in macrophages, potentially serving as a protective mechanism against liver inflammation. Furthermore, Guo et al. (Guo, Li et al. 2023) have shown that bile acids can act as signaling molecules, influencing both metabolism and inflammation in the liver. The observed interactions between short-chain fatty acids (SCFAs) and cytokines in our study are particularly noteworthy. SCFAs, primarily produced by gut bacteria, have been shown to have anti-inflammatory effects. Our findings are supported by research from Du et al. (Du, He et al. 2024), who demonstrated that SCFAs can modulate the production of inflammatory mediators, including cytokines, in various cell types.

Our findings have important implications for the development of novel diagnostic and therapeutic strategies. The identified microbe-metabolite modules could serve as potential biomarkers for MAFLD progression to cirrhosis. This is in line with recent work by Loomba et al. (Loomba, Seguritan et al. 2019), who have shown the potential of multi-omics approaches in developing non-invasive diagnostics for NAFLD. The observed interactions between retinol and specific microbial clusters warrant further investigation into the potential of microbiome-based interventions to modulate vitamin A metabolism in MAFLD. This approach could complement existing therapies targeting hepatic stellate cell activation, as suggested by Wiering et al. (Wiering, Subramanian et al. 2023). The strong predictive power of cysteine-glutathione disulfide suggests that targeting oxidative stress pathways could be a promising therapeutic approach. This is supported by clinical studies showing the benefits of antioxidant therapies in NAFLD patients, as reviewed by Masarone et al. (Masarone, Rosato et al. 2018). In conclusion, our study demonstrates the power of the MiMeNet framework in unraveling the complex relationships between the gut microbiome, metabolome, and host immune responses in MAFLD-related cirrhosis. By identifying specific microbe-metabolite modules and their potential interactions with cytokines, we provide a new perspective on the pathogenesis of liver disease progression. These findings not only enhance our understanding of the underlying mechanisms but also pave the way for the development of novel diagnostic tools and therapeutic strategies in MAFLD management.

**Conclusion**

In conclusion, our study demonstrates the power of the MiMeNet framework in unraveling the complex relationships between the gut microbiome, metabolome, and host immune responses in MAFLD-related cirrhosis. By identifying specific microbe-metabolite modules and their potential interactions with cytokines, we provide a new perspective on the pathogenesis of liver disease progression. These findings not only enhance our understanding of the underlying mechanisms of MAFLD and its progression to cirrhosis but also pave the way for the development of novel diagnostic tools and therapeutic strategies. The identified metabolites and microbial clusters could serve as potential biomarkers for disease progression, while the elucidated metabolite-cytokine interactions offer new targets for immunomodulatory interventions. Future research should focus on validating these findings in larger, longitudinal cohorts and exploring the functional significance of the identified microbe-metabolite-cytokine interactions through experimental studies. Such efforts will be crucial in translating these insights into clinically actionable strategies for the prevention and treatment of MAFLD-related cirrhosis. Ultimately, this work represents a significant step towards a more comprehensive understanding of liver disease pathogenesis and holds promise for the development of personalized approaches to MAFLD management based on individual microbiome and metabolic profiles.

**Conflicts of interest/Competing interests**

None.

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**Author contributions**

M.K.M designed the pipeline experiments, carried out the experiments and prepare the manuscript. A.So. and F.V. provided expert guidance and reviewed the manuscript. A.Z. provided medical guidance, provided data and reviewed the manuscript. A.Sa. reviewed the manuscript. S.M. provided a description of the in-house dataset. A.Sp. reviewed the manuscript.

**Data Availability**

The HCC dataset can be obtained from the authors upon a reasonable request.

**Code Availability**

The code created for this project will be made available on GitHub shortly.

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