Hepatic Medicine: Evidence and Research

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ORIGINAL RESEARCH

Linking Brain and Immune Transcriptomes to Gut-Derived Metabolites in Hepatic Encephalopathy: An Explorative Integrative Multi-Omics Approach

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Purpose: Hepatic encephalopathy (HE), one of the more important cerebral complications attributable to acute liver failure and more severe forms, has traditionally been associated with hyperammonemia. However, recent studies implicate gut microbiota-derived metabolites in HE pathogenesis through systemic inflammation and neurotoxicity. Despite this, the integrated molecular mechanisms linking these metabolites to HE remains poorly described. This study addresses this gap by employing a bioinformatics-based systems biology approach to identify interactions between gut metabolites and host genes, thereby identifying novel diagnostic and therapeutic targets.

Methods: Differentially expressed genes (DEGs) from peripheral (GSE184200: $CD4^+$ T lymphocytes) and central (GSE57193: fusiform gyrus) tissues of HE patients were analyzed using GEO2R. Genes associated with five gut microbiota-derived metabolites including choline metabolites, lipid metabolites, short-chain fatty acids, tryptophan catabolites, and secondary bile acids were extracted from GeneCards. Overlapping genes between HE-related genes and gut-derived metabolites were then subjected to multi-level enrichment analyses (pathway, phenotype, cell type, and cellular component), and miRNA/drug prediction using Enrichr and ToppGene.

Results: We identified nine hub genes related to gut-systemic interactions and 29 hub genes associated with gut-brain interactions in the context of HE. The most significantly impaired pathways were signaling by interleukin 24 and TP53, as well as oxidative stress, which were affected by gut metabolites and peripheral HE-related genes. Similarly, mitochondrial fatty acid β-oxidation, neuroinflammation, and glutamate tone were significantly altered by gut metabolites and central HE-related genes. Additionally, we predicted 18 miRNAs and 13 potential drugs that target both gut microbiota metabolites and HE.

Conclusion: This study offers the first systems-level framework connecting gut-derived metabolites to HE through gene networks, challenging the ammonia-centric viewpoint. The predicted miRNAs and drugs offer translational potential for precision medicine in HE. Experimental validation of these targets is required to advance therapeutic strategies.

Keywords: hepatic encephalopathy, gut-liver-brain axis, microbiome metabolites, bioinformatics, drug repurposing, oxidative stress, neuroinflammation

Introduction

Hepatic encephalopathy (HE) is a spectrum of neuropsychiatric complications that arise from acute liver failure, portosystemic shunting, or advanced liver disease. These complications significantly impact health related quality of life and increase the rates of hospitalization.[1] HE is classified into two distinct categories-covert HE and overt HE-based

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on the severity of mental alterations.[2] The occurrence of covert HE has been reported in a significant percentage of patients with cirrhosis, ranging from 20% to 80%.[3–5] Individuals with covert HE typically show no obvious clinical signs and are usually identified through abnormalities in psychometric tests.[6] Covert HE is associated with lower health-related quality of life, reduced survival rates, and is a strong risk factor for hospitalization in cirrhotic patients.[6–8] In contrast, overt HE presents a broad range of symptoms, including lethargy, disorientation, asterixis, apathy, bradykinesia, poor short-term memory, somnolence, and coma.[9] Approximately 30% to 45% of patients with cirrhosis and 10% to 50% of patients with a transjugular intrahepatic portosystemic shunt experience overt HE.[10–15] Overt HE is associated with a high mortality rate and prolonged hospital stays in cirrhotic patients.[16–19]

The pathogenesis of HE is complex and not yet fully understood. It is believed to be multifactorial, involving hyperammonemia, systemic inflammation, oxidative stress, manganese deposition, endotoxins, and various precipitating factors such as hyponatremia, hypokalemia, infections, hypovolemia, constipation, gastrointestinal bleeding, and metabolic alkalosis. These factors disrupt the blood-brain barrier (BBB) integrity, and inducing neuroinflammatory responses including microglial activation and astrocyte hypertrophy.[1,20–26]

Emerging evidence implicates gut-derived metabolites as key mediators in HE pathophysiology;[27–32]. Studies in cirrhotic patients and experimental animal models have highlighted the importance of the gut-brain axis in HE.[33–43] Intestinal dysbiosis in cirrhosis alters the production of microbial metabolites. These molecules may cross the compromised intestinal and BBB, directly modulating neuronal and glial cells.[20,33,34] These findings suggest that gut-derived metabolites, as neuroactive substances, are key molecules linking the intestinal tract to the central nervous system (CNS). Gut microbiota-derived metabolites represent a heterogeneous group of bioactive molecules that have particular relevance to liver and brain function. Choline metabolites (eg trimethylamine and trimethylamine N-oxide) regulate lipid metabolism and vascular health, but the disturbance in their metabolism has been associated with liver injury, atherosclerosis, and neuroinflammation.[44,45] Lipid metabolites such as lipopolysaccharide (LPS) and sphingolipids, regulate immune system activity and maintain gut barrier integrity, but their disturbance has been identified to trigger systemic inflammation and potentially lead to CNS problems.[34,46,47] Short-chain fatty acids (SCFAs, eg acetate, butyrate, propionate) regulate intestinal barrier function, immune activity, and neural signaling and can influence BBB permeability.[30,48–50] Catabolites of tryptophan (eg indole, kynurenine, serotonin) maintain neurotransmitter balance, regulate immune activity, and trigger neuroinflammatory pathways.[51–54] Secondary bile acids have also been identified as facilitating lipid digestion, intestinal permeability, and inflammatory signaling, and both liver injury and neurologically driven dysfunction have been linked to negative regulation in bile acid metabolism and their imbalance has been implicated in both liver injury and neurological dysfunction.[55–57] Although each metabolite group has been individually linked to disease processes, the integrated molecular mechanisms connecting these gut-derived molecules to HE remain poorly defined. The present study applied an integrative bioinformatics approach to investigate these connections at a systems level.

Using publicly available transcriptomic datasets of peripheral and brain tissues from HE patients, we identified differentially expressed genes (DEGs) and cross-referenced them with metabolite-associated genes from curated databases. This strategy enables the identification of overlapping molecular signatures, the addressing of key signaling pathways, and the prediction of regulatory miRNAs and potential drug candidates. By integrating multi-omic and pathway analyses, we aim to provide a comprehensive molecular framework of the gut-liver-brain axis in HE, offering novel insights and therapeutic opportunities beyond the scope of traditional experimental studies.

Methods

Data Acquisition and Processing of Target Genes

The Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/geo/) was utilized to identify genes associated with HE. DEGs were extracted from two datasets: GSE184200 (representing periphery-related genes) and GSE57193 (representing centrally related genes) using the GEO2R web tool, as described previously.[58] GEO2R applies the limma package for R, and statistical significance was determined using an adjusted p-value (Benjamini-Hochberg false discovery rate, FDR) of <0.05 to account for multiple testing between the HE and healthy groups. The GSE184200

dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE184200) consisted of RNA-seq data from circulating $CD4^+$ T lymphocytes, with six cirrhosis patients with minimal HE and eight healthy controls. The GSE57193 dataset (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57193) contained RNA-seq data from fusiform gyrus (post-mortem brain samples) from four cirrhotic patients with HE and four healthy controls. These datasets were chosen for tissue relevance and availability of matched controls. We note that both datasets have relatively small sample sizes, which may limit statistical power and increase the influence of inter-individual variability. However, they were included because they had high relevance and well-annotated transcriptomic data. Covariates such as age and sex were considered qualitatively but were not incorporated into the DEG analysis due to the small sample sizes and lack of complete covariate balance. GEO2R analysis determined that target genes were upregulated when the log fold change (log FC) was positive and downregulated when the log FC was negative, relative to the healthy controls. Subsequently, the

GeneCards database (https://www.genecards.org/) was used to identify genes associated with gut-derived metabolites using metabolite names as keywords. Five categories of gut metabolites were retrieved from GeneCards: choline metabolites, lipid metabolites, SCFAs, tryptophan catabolites, and secondary bile acids. Each category included at least two major metabolites. The choline metabolites category included methylamine and trimethylamine (TMA), while lipid metabolites included lipopolysaccharide and sphingolipids. The SCFAs category featured acetate, butyrate, hexanoate, propionate, and SCFA. Indole, kynurenine, and serotonin represented the tryptophan catabolites group, whereas bile acid, deoxycholate, deoxycholic acid, lithocholate, and lithocholic acid were key metabolites in the secondary bile acids category. Within each metabolite category, we first identified the genes shared among its constituent metabolites, defining these as gut-derived metabolite-associated genes for that category. We then combined the resulting category, specific gene sets into a final comprehensive set, representing the union of genes across all five categories, which we refer to as the key gut-derived metabolite-associated genes. Finally, we identified the genes common to this comprehensive set and the HE-related gene sets from GSE184200 and GSE57193, which were subsequently used to explore potential peripheral and central pathways through enrichment analyses (Figure 1).

Functional Enrichment and Tissue Expression Analyses

The Enrichr database (https://maayanlab.cloud/Enrichr/) was used to perform enrichment analyses on the target genes.[59] To identify the most relevant signaling pathways and phenotypes linked to these genes, we applied the WikiPathways and Human Phenotype Ontology databases in Enrichr. Furthermore, the CellMarker Augmented and Jensen modules in Enrichr were utilized to predict the cell types most affected by gut metabolites and HE-associated genes, as well as to identify the primary cell components involved. A p-value of less than 0.05 was set as the cutoff for statistical significance in all analyses.

For tissue-specific gene expression analysis, we uploaded the target genes to the Genotype-Tissue Expression (GTEx) portal (https://www.gtexportal.org/home/), as described previously.[60] Using the GTEx Multi Gene Query tool, we visualized the expression profiles of the target genes across various organs, expressed as transcripts per million (TPM).

miRNA Target Prediction, miRNA-Protein Interaction Network Construction, and Drug Repurposing

To identify the most relevant miRNAs and potential drugs for the target genes, we used ToppGene. We uploaded the target genes into the ToppFun panel of ToppGene and utilized TargetScan and DrugBank for miRNA prediction and drug repurposing, respectively. miRNAs and drugs with a p-value less than 0.05 were deemed statistically significant. Additionally, we employed Cytoscape v3.10.1 to build a miRNA-gene interaction network, illustrating the relationship between each target gene and its corresponding miRNAs.

Results

Identification of Genes Associated with HE and Gut-Derived Metabolites

Following the analysis of two HE-related datasets, we identified 99 DEGs (FDR < 0.05) in the GSE184200 dataset (blood $CD4^+$ T lymphocytes). Among these, 58 genes were upregulated and 41 genes were downregulated (Figure 2A).

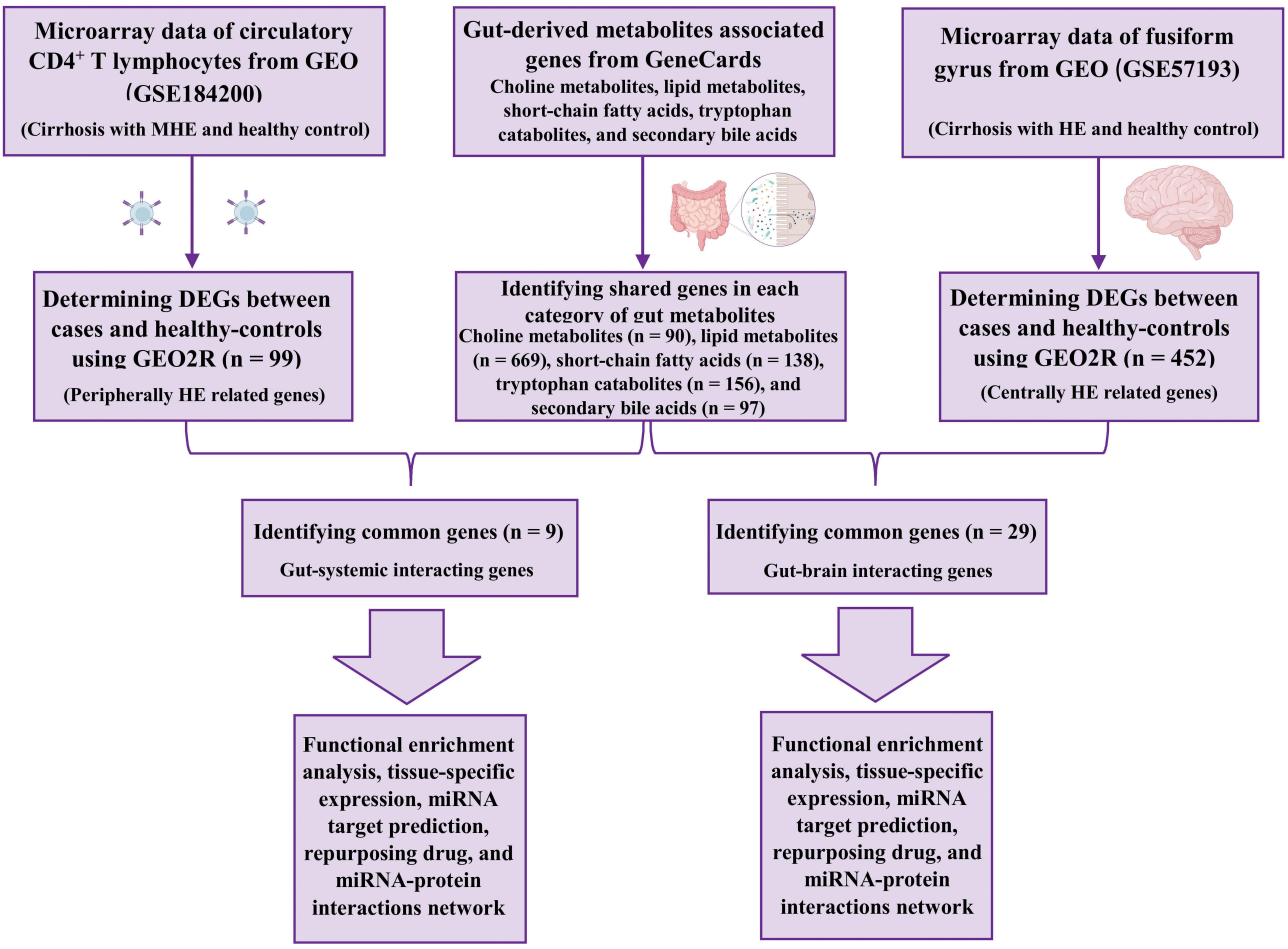

Figure 1 Flowchart of the research process.

For the GSE57193 dataset (fusiform gyrus), we identified 452 DEGs (FDR < 0.05), with 268 upregulated and 184 downregulated genes (Figure 2B).

After searching GeneCards for genes associated with gut-derived metabolites, we identified a large number of genes linked to the five planned categories of metabolites (Figure 3A). A total of 13 genes were common between the gut metabolite-related genes and HE-related peripheral genes (Figure 3B). After removing duplicates, 9 unique genes remained, including *GADD45A, ABCG2, ODC1, FTH1, PDGFRB, CDKN1A, BCAT1, ACAA2*, and *SLC25A20* (Figure 3C).

In addition, 31 genes were found to be common between gut-derived metabolite-associated genes and HE-related central genes (Figure 3D). After eliminating duplicates, the final list contained 29 genes: *GLUL, TXNIP, CASP6, CNR2, MSN, PLD2, NRP1, BCL2L11, EGFR, S1PR1, BDNF, PLIN2, GFAP, MR1, PXN, MAP2K4, IL18R1, TRPV1, CCNB1, SLC25A20, HADHA, ACAA2, CHKA, ACADS, CPT2, GCDH, GRIN2C, ECE1*, and *SLC1A2* (Figure 3E). We then performed separate enrichment analyses for the 9 genes shared by gut metabolites and peripheral HE, and the 29 genes shared by gut metabolites and central HE.

Pathway, Human Phenotype, Cell Type, and Cellular Component Enrichment Analysis for Peripheral Target Genes (Gut-Systemic Interactions in HE)

WikiPathway enrichment analysis was conducted to identify the main activated signaling pathways associated with both gut-derived metabolites and peripheral HE-related genes. Several significant pathways were identified, including imatinib and chronic myeloid leukemia, interleukin-24 (IL-24) signaling, TP53 network, oxidative damage response, and ATM signaling pathway for gut metabolites and peripheral HE-related genes (Figure 4A).

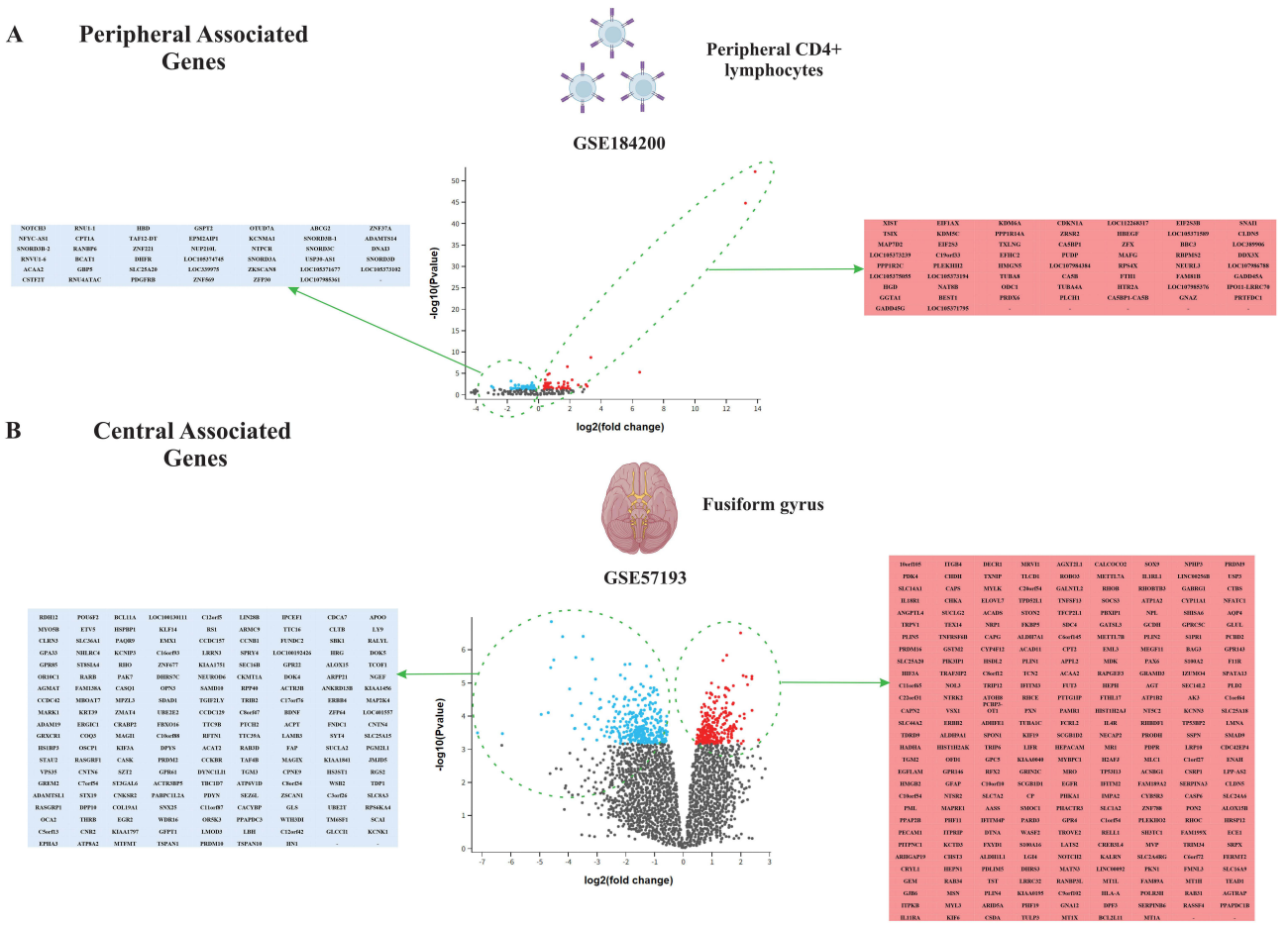

Figure 2 The differentially expressed genes between cirrhotic patients with HE and healthy controls. (**A**) Volcano plot of DEGs from circulatory CD4$^+$ T lymphocytes, and (**B**) volcano plot of DEGs from the fusiform gyrus. The blue tables represent downregulated genes, while the red tables represent upregulated genes in each dataset.

The Human Phenotype Ontology analysis revealed key phenotypic abnormalities linked to our peripheral target genes, including hypercalcemia, atrioventricular block, hypercortisolism, ventricular tachycardia, increased serum ferritin, rhabdomyolysis, hyperparathyroidism, basal ganglia calcification, bradycardia, hepatomegaly, abnormalities in iron homeostasis and gastric mucosa, hyperammonemia, and hypotension (Figure 4B).

Additionally, CellMarker Augmented analysis was used to predict the cell types potentially affected by both gut-derived metabolites and peripheral HE-related genes. The results indicated that the primary affected cell types include hematopoietic stem cells (bone marrow), hepatoblasts (liver), mesangial cells (kidney), embryonic stem cells (germ), cardiac progenitor cells (heart), cancer stem cells (colon), glial cells (undefined), pericytes (undefined), and neural stem cells (brain) (Figure 4C).

Jensen Compartments analysis highlighted several key cellular components involved in the interaction with gut-derived metabolites and peripheral HE-related genes, including the PCNA complex, type III intermediate filament, Bcl-2 family protein complex, BAX complex, caspase complex, COMA complex, B cell receptor complex, mitochondrial membrane, mitochondrial envelope, integrin alphav-beta8 complex, and NF-kappa β complex (Figure 4D).

Pathway, Human Phenotype, Cell Type, and Cellular Component Enrichment Analysis for Central Target Genes (Gut-Brain Interactions in HE)

In a similar manner, we performed all the previously mentioned enrichment analyses for gut-derived metabolites and centrally related HE genes. The results of WikiPathway enrichment revealed several key signaling pathways implicated in both gut metabolites and centrally associated HE genes, including mitochondrial long-chain fatty acid beta-oxidation,

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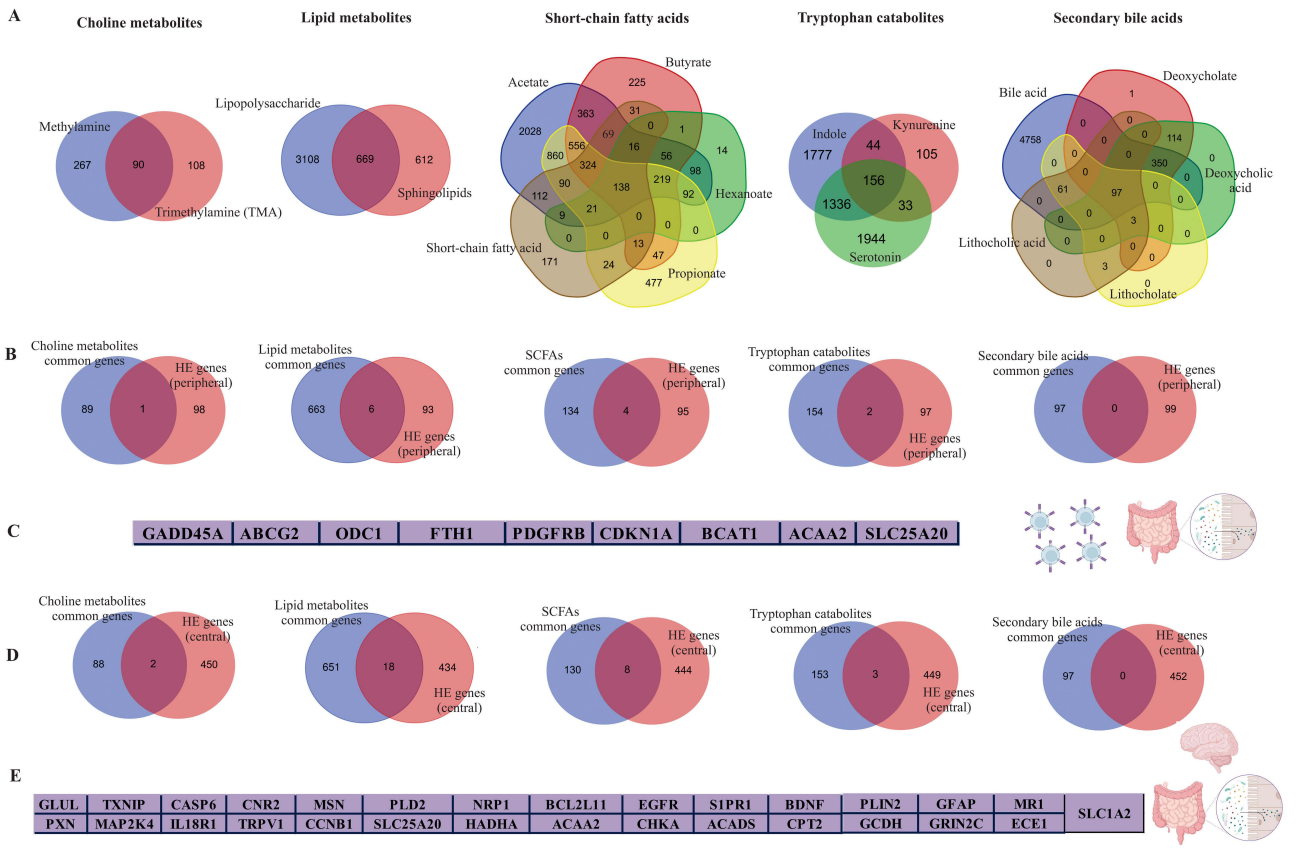

Figure 3 Genes associated with gut microbiota-derived metabolites and shared with he-related genes. (**A**) Venn diagram of genes related to intestinal bacterial metabolites across five categories.(**B**) Venn diagram of common genes between gut-derived metabolites and peripheral HE-related genes. (**C**) Seven common genes shared between gut-derived metabolite-related genes and peripheral HE-associated genes. (**D**) Venn diagram of common genes between gut-derived metabolites and central HE-related genes. (**E**) Twenty-seven common genes shared between gut-derived metabolite-related genes and central HE-associated genes.

neuroinflammation and glutamatergic signaling, apoptosis, ErbB signaling, Fas ligand pathway, stress-induced heat shock protein responses, chronic hyperglycemia and neuron function impairment, VEGFA-VEGFR2 signaling, physico-chemical features and toxicity-related pathways, IL-24 signaling, MAPK signaling, and EGFR tyrosine kinase inhibitor resistance (Figure 5A).

Human Phenotype Ontology analysis indicated that major phenotypic anomalies associated with gut metabolite-related genes and centrally HE-related genes included abnormalities in dicarboxylic acid metabolism, hyperammonemia, rhabdomyolysis, abnormalities in long-chain fatty acid metabolism, lethargy, myoglobinuria, bradycardia, elevated hepatic transaminases, and hepatomegaly (Figure 5B).

To assess the most affected cells by both gut-derived metabolites and HE-related central genes, we performed a cell-type specific analysis using CellMarker Augmented. The results revealed that the most impacted cell types were astrocytes (brain and embryonic prefrontal cortex), CD1C-CD141- dendritic cells (blood), effector $CD8^+$ memory T cells (peripheral blood), glutamatergic neurons (undefined and dorsolateral prefrontal cortex), $CD4^+$ cytotoxic T cells (liver), glial cells (brain), astrocytes (spinal cord), interstitial cells (dorsolateral prefrontal cortex), oligodendrocyte precursor cells (brain), dendritic cells (peripheral blood), and plasmacytoid dendritic cells (bone marrow) (Figure 5C).

Jensen Compartments analysis highlighted several key cellular components involved in both gut metabolites and HE, including type III intermediate filaments, Bcl-2 family protein complexes, tubulin complexes, fatty acid synthase complexes, mitochondrial permeability transition pore complexes, BAX complexes, caspase complexes, COMA complexes, growth cone filopodia, electron transfer flavoprotein complexes, glial cell projections, dendritic filopodia, and neuronal cell bodies (Figure 5D).

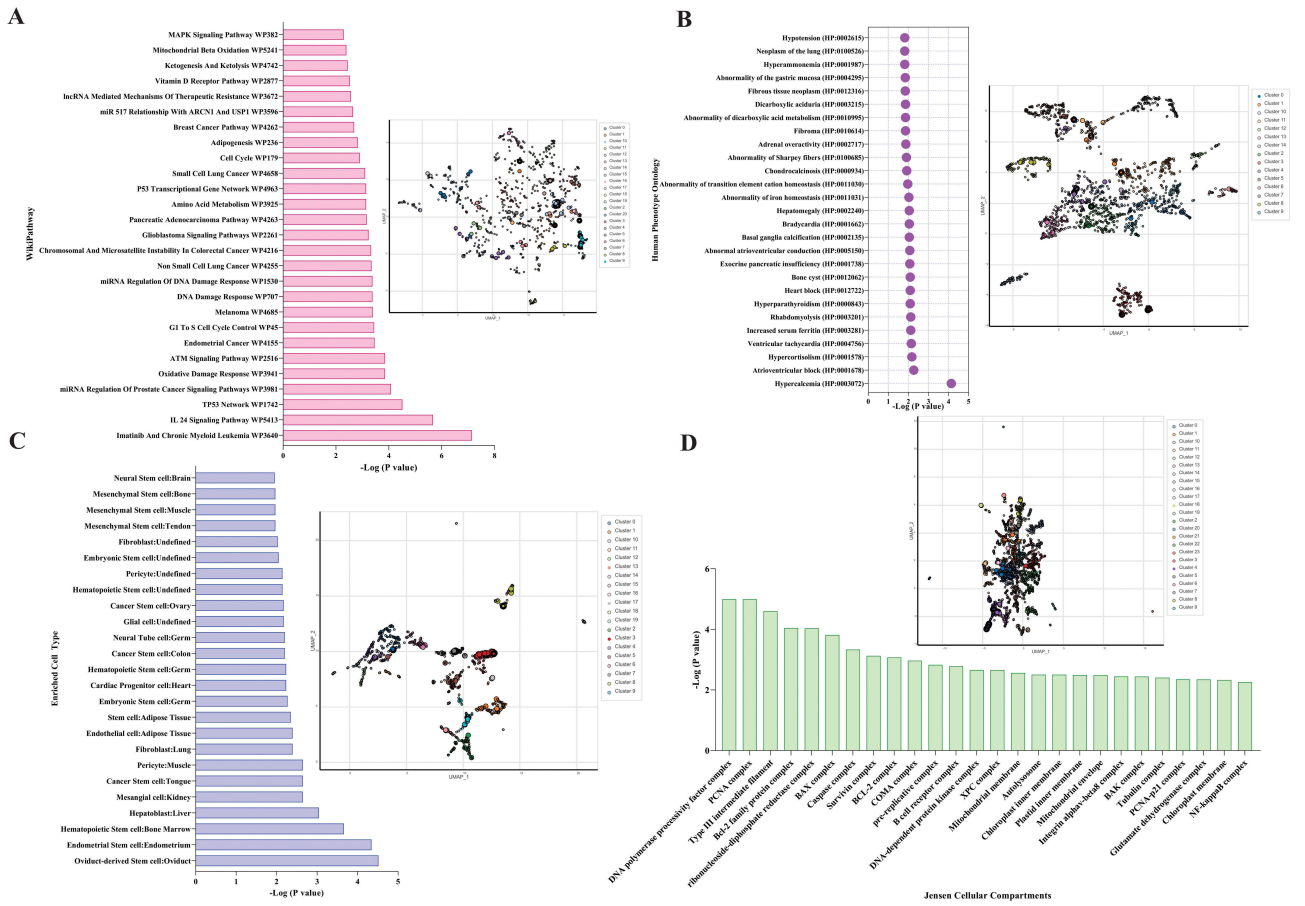

Figure 4 Results of the functional enrichment analysis for common genes shared by gut-derived metabolites and peripheral HE-related genes using Enrichr. (**A**) Pathway enrichment, (**B**) Human Phenotype Ontology, (**C**) cell-type specific analysis, (**D**) and cellular component enrichment analysis for shared genes between gut-derived metabolites and peripheral HE-related genes. All statistically significant results are shown as -log(p-value). Scatterplots of all terms in each category's gene set library are displayed next to each graph.

miRNA Target Prediction, Tissue Expression Analysis, and Drug Repurposing for Target Genes

TargetScan predicted eight primary miRNAs for gut-derived metabolites and peripheral HE-related genes, including hsa-miR-374b-5p, hsa-miR-374a-5p, hsa-miR-301b-3p, hsa-miR-454-3p, hsa-miR-130b-3p, hsa-miR-130a-3p, hsa-miR-301a-3p, and hsa-miR-1193 (Figure 6A). Similarly, we identified ten potential miRNAs for gut-derived metabolites and central HE-related genes, such as hsa-miR-124-3p.1, hsa-miR-506-3p, hsa-miR-124-3p.2, hsa-miR-653-5p, hsa-miR-137, hsa-miR-27b-3p, hsa-miR-27a-3p, hsa-miR-325-3p, hsa-miR-148a-3p, and hsa-miR-148b-3p (Figure 6B).

Additionally, tissue-specific expression analysis visualized the expression patterns of our target peripheral (Figure 6C) and central (Figure 6D) genes across various tissues, including the liver, whole blood, colon, and small intestine, as well as in specific regions of the brain, such as the spinal cord, cerebellum, cerebellar hemisphere, pituitary, substantia nigra, hypothalamus, hippocampus, basal ganglia, cerebral cortex, and amygdala.

Notably, we used the DrugBank database to predict potential drugs for both categories of target genes. Six potential drugs- G418, alpha-difluoromethylornithine, pyridoxine-5'-phosphate, L-valine, spermine, and becaplermin- were proposed for both gut-derived metabolites and peripheral HE-related genes (Figure 6E). Similarly, we predicted seven potential drugs for gut-derived metabolites and central HE-related genes, including L-carnitine, L-glutamic acid, flavin-adenine dinucleotide, pegaptanib, panitumumab, perhexiline, and nabilone (Figure 6F).

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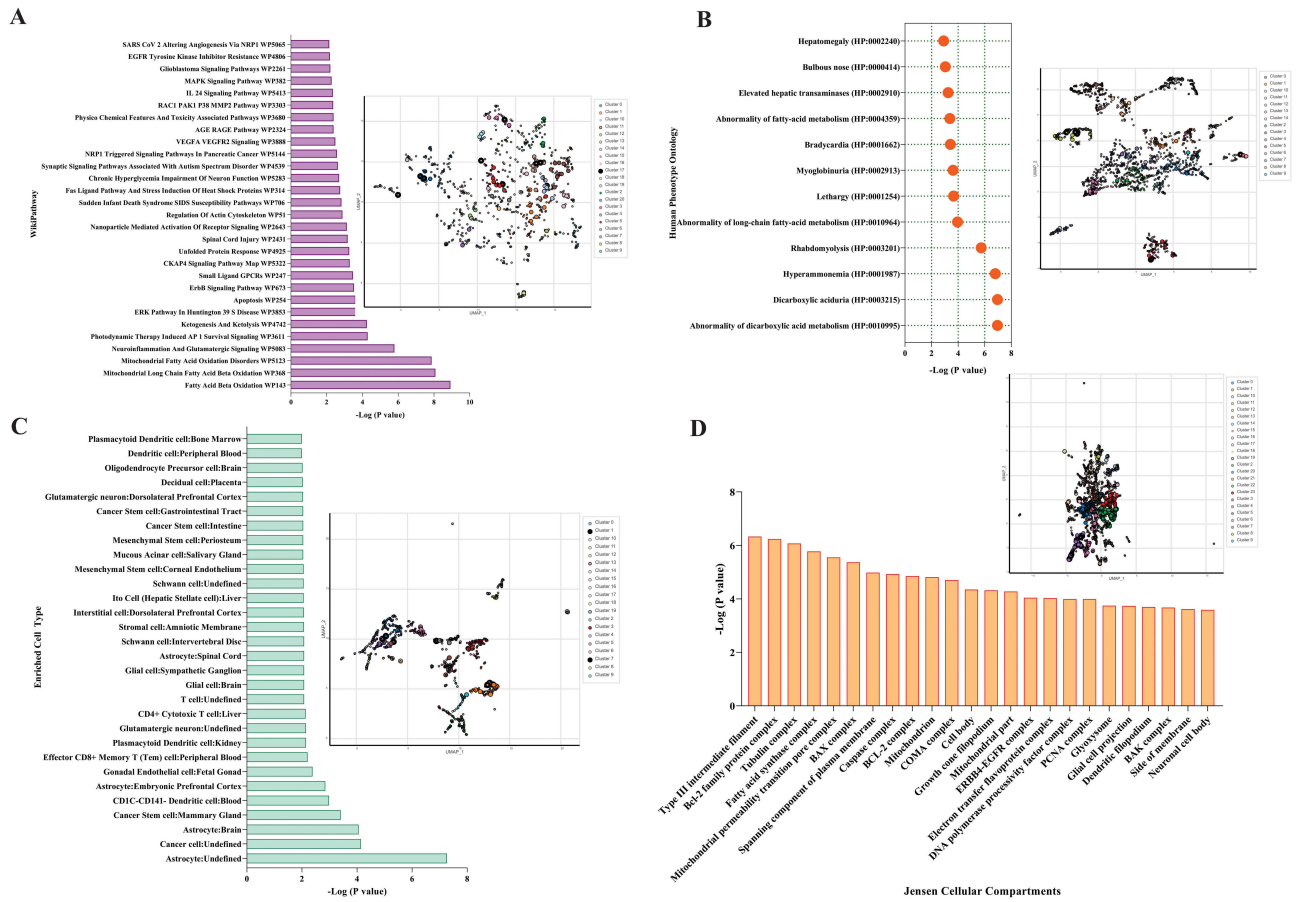

Figure 5 Results of functional enrichment analysis for common genes between gut-derived metabolites and central HE-related genes using Enrichr. (**A**) Pathway enrichment, (**B**) Human Phenotype Ontology, (**C**) Cell-Type Specific Analysis, (**D**) and Cellular Component Enrichment Analysis for shared genes between gut-derived metabolites and central HE-related genes. All statistically significant results are shown as -log(p-value). Scatterplots for all terms in each category's gene set library are displayed next to each graph.

Discussion

Ammonia as Key Contributing Factor in HE

The pathophysiology of HE, as a serious cerebral complication of both acute and chronic liver diseases, remains unclear to this day. Nevertheless, a few theories are emphasized, including those about ammonia, systemic inflammation, oxidative stress, manganese deposition, and endotoxemia. A central factor historically emphasized in HE research is ammonia, whose accumulation in the systemic circulation and CNS has long been considered a primary driver of the disease.[1,21,61] The primary source of ammonia is the breakdown of nitrogen-containing substances by gut bacteria that produce urease. Under normal conditions, the majority of ammonia in the intestines is converted into urea by healthy hepatocytes and subsequently eliminated by the kidneys. When hepatocytes are damaged during liver disease, their ability to synthesize urea is reduced. This causes elevated ammonia levels to reach the CNS. This condition is known as hyperammonemia.[62,63] Ammonia causes dysfunction in multiple cell types in the CNS, including endothelial cell damage, astrocyte swelling, and microglial activation. These dysfunctions result in pathological changes such as disruption of the BBB (a functional barrier between blood and brain tissue), impairment of the glymphatic system (a cellular pathway for clearing neurotoxic substances), and neuroinflammation, which have been seen in HE patients.[64–68] These pathological phenomena are key findings in hyperammonemic animal models and patients with HE.[38,69–80] Studies demonstrated that circulatory levels of ammonia are correlated with the severity and mortality of HE in patients with cirrhosis.[81–83] Since ammonia plays a role in these pathogenic changes in the CNS microenvironment, ammonia-lowering techniques are considered the mainstay of pharmacological treatment for HE.[84–86] However, some cirrhosis patients

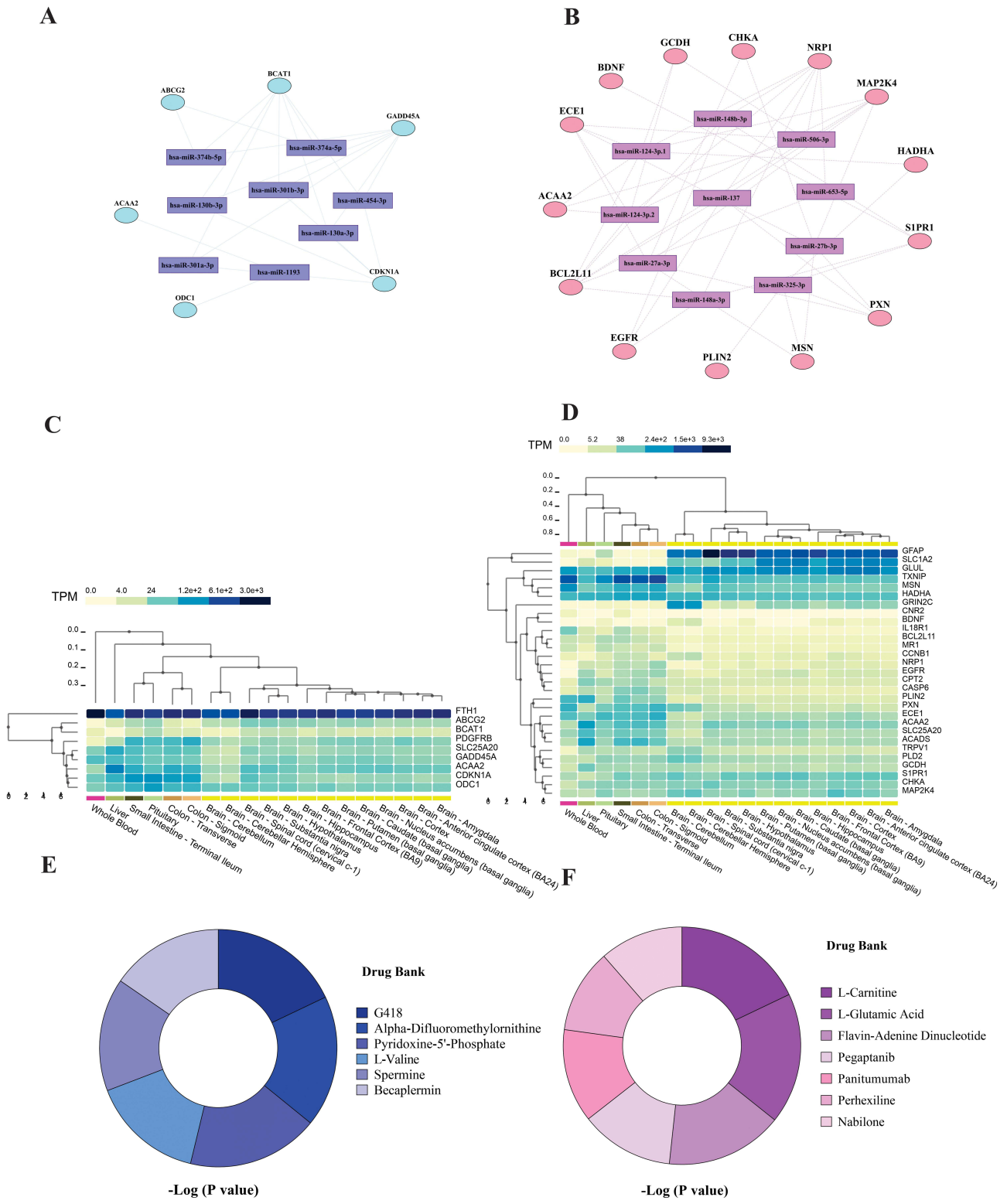

Figure 6 miRNA prediction, tissue-specific enrichment analysis, and drug repurposing for gut-derived metabolites-related and HE-related genes. Reconstructed miRNA-gene interaction networks for shared genes between gut metabolites-associated genes and peripheral HE-related genes (**A**) or central HE-related genes (**B**). Results of tissue-specific expression analysis for common genes between gut metabolites-associated genes and peripheral HE-related genes (**C**) or central HE-related genes (**D**). Predicted potential drugs for shared genes between gut metabolites-associated genes and peripheral HE-related genes (**E**) or central HE-related genes (**F**).

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without HE have been shown to have elevated ammonia levels in certain investigations.[87] On the other hand, some HE patients lacked hyperammonemia.[83,88] These results suggest that ammonia is not the only factor that contributes to the development of HE. Additionally, new research indicates that HE is no longer just a disease of the liver and brain, but is caused by multi-organ dysfunction, including the muscles, kidneys, and particularly the intestinal tract.

Role of Gut Microbiota and Its Metabolites in HE

Beyond ammonia, increasing evidence points to the gut microbiota and its metabolites as critical contributors to HE pathogenesis, influencing systemic inflammation, neurotoxicity, and gut-liver-brain communication. There is evidence linking the gut microbiota to host metabolism; however, precise processes are still unknown. Evidence has indicated that alteration of gut microbiota and its metabolites are associated with HE in cirrhotic patients.[89,90] In cirrhosis and HE, gut dysbiosis and alteration of gut-derived metabolites have increased the permeability of intestinal barrier, resulting in bacterial translocation, hyperammonemia, endotoxemia, and an increase in the levels of inflammatory mediators in the circulation. These neurotoxic agents through portosystemic shunt affect the permeability of the BBB and reach the CNS, where astrocytes, microglia and neurons are in direct contact with them.[20,27,33,91–95] Trimethylamine is a gut-derived metabolite that is produced from dietary quaternary amines, particularly choline. TMA is associated with neurodegenerative diseases, chronic kidney disease, atherosclerosis, and severe cardiovascular disease through the induction of inflammatory pathways.[96–100] Current studies demonstrated that circulatory levels of TMA are associated with liver steatosis, development of alcoholic liver disease, and cirrhosis.[101–103] Other metabolites originating from the gut bacteria called sphingolipids regulate host lipid and sphingolipid metabolisms, preserve intestinal barrier integrity, and modify the immune system.[104–108] Deficiency of gut-derived sphingolipids is associated with increased ceramide levels in host organs.[105,106] LPS is a structural component of Gram-negative bacteria that changes the integrity of the intestinal barrier, triggers gut inflammatory responses, induces endotoxemia, and produces systemic inflammation as well as increases the permeability of the BBB in animal models of HE.[109–112] The SCFAs are other gut-derived metabolites that are mainly produced by anaerobic bacteria through fermentation of dietary fibers. SCFAs modulate immune responses, decrease inflammatory conditions, maintain intestinal barrier integrity, regulate gut microbiome environment, protect cardiovascular system, regulate the permeabilization of the BBB, and have beneficial effects in neurodegenerative disorders.[48,113–116] Gut dysbiosis and a reduced population of SCFA-producing bacterial species were associated with the severity of disease in cirrhotic patients. Also, the levels of circulatory SCFAs were decreased in cirrhotic patients with a previous history of overt HE.[49] Likewise, serum SCFAs were inversely associated with model for end-stage liver disease (MELD) score, systemic inflammation and previous history of HE in patients with cirrhosis.[117] Gut bacteria also play an important role in the metabolism of tryptophan which produces other types of gut-derived metabolites, including indole, serotonin, and kynurenine.[118,119] Plasma levels of indole were significantly correlated with ammonia levels and severity of HE in cirrhotic patients.[120] Alterations of serotonergic tone and kynurenine pathway were also associated with encephalopathy in liver diseases.[121–127] Furthermore, circulatory levels of tryptophan metabolisms were associated with systemic inflammation in cirrhotic patients with HE.[30] Another type of metabolite derived from intestinal bacteria is secondary bile salts, which are formed from the hydrolysis of primary bile acids in colon by 7-alpha-dehydroxylase. High levels of these bile acids in the intestine are associated with inflammation, and oxidative stress as well as impaired the integrity of intestinal barrier and altered mucus secretion.[128–130]

Peripheral Mechanisms in HE: Gut-Systemic Interactions

To explore how gut-derived metabolites influence the systemic compartment in HE, we examined peripheral hub genes linking intestinal signals to immune and metabolic pathways. The gut-liver axis plays a pivotal role in HE pathogenesis, with our study identifying nine key hub genes (*GADD45A, ABCG2, ODC1, FTH1, PDGFRB, CDKN1A, BCAT1, ACAA2*, and *SLC25A20*) that connect gut-derived metabolites to peripheral immune and metabolic disturbances. These genes are functionally enriched in critical pathways including IL-24 signaling, TP53 network activation, and oxidative stress responses, which provide a mechanistic understanding of how intestinal dysbiosis could contribute to HE progression through systemic effects.

The beneficial effect of IL-24 as an anti-tumor, anti-oxidant and anti-proliferative cytokine on liver injury has been shown in thioacetamide-induced HE and carbon tetrachloride-induced cirrhosis mice.[131,132] The IL-24 signaling pathway emerges as particularly significant, with this cytokine demonstrating both anti-fibrotic and anti-oxidant properties that have been shown to mitigate liver injury in experimental models.[131–133] The findings of the study suggest that gut microbial metabolites may modulate IL-24-associated pathways. This cytokine potentially influencing hepatic stellate cell activation and systemic inflammatory responses.[131,134,135] This interaction could represent either a protective mechanism or a maladaptive response in HE, highlighting the need for further investigation into the precise role of IL-24 in this context.

Similarly, the TP53 network appears prominently in our analysis, reflecting its central role in regulating cell cycle arrest and apoptosis. In the context of chronic liver disease, gut metabolites, such as LPS and trimethylamine-N-oxide (TMAO), may dysregulate TP53 signaling contributing to hepatocyte dysfunction and promoting Kupffer activation.[136–139] Activated Kupffer cells produced pro-inflammatory cytokines and activated hepatic stellate cells, which progress liver fibrosis through a P53 proteins signaling-dependent manner.[139–141] Consequently, this may exacerbate systemic inflammation and contribute to the progression of HE, suggesting that gut metabolites capable of modulating TP53 pathways warrant further investigation.

Oxidative stress represents another critical pathway linking gut metabolites to peripheral HE manifestations.[21,142] An increase in the production of reactive oxygen species and reduced antioxidant capacity have been reported in the circulation of bile duct ligation rats as a model of type C HE.[143,144] Genes such as *FTH1* that function in iron homeostasis demonstrate the potential effects of gut-derived metabolites on redox balance.[145,146] Secondary bile acids and TMAO may further exacerbate oxidative damage, creating a vicious cycle of liver injury and systemic inflammation[32,147–149] that promotes HE development.

The influence of specific gut metabolites on these peripheral pathways is particularly noteworthy. Impaired TMAO levels, derived from choline metabolism, have been associated with hepatic steatosis and endothelial dysfunction, potentially worsening systemic inflammation and HE progression.[101,150,151] Similarly, bacterial LPS can trigger toll-like receptor 4 (TLR4)-mediated inflammation through endotoxemia, while alterations in sphingolipid metabolism may impact hepatocyte survival.[152–155] The reduction of beneficial SCFAs like butyrate and propionate in cirrhosis may further compromise gut barrier integrity, facilitating bacterial translocation and systemic inflammation.[117,156–159]

Clinically, the results suggest the possibility of diagnostic markers, such as *PDGFRB* with vascular impairment, and treatment options, such as modulation of antioxidant systems to reverse oxidative stress. It is important to note, however, that the links described are currently based on bioinformatic analyses and have not been verified experimentally. Future studies that demonstrate causality are necessary, which can be achieved in a preclinical setting through the use of germ-free mouse models or fecal microbiota transplantation. In addition, studies in human cohorts that compare circulating metabolite profiles with gene expression data in patients with HE could help substantiate these findings.

In the future, research should focus on several important directions. Mechanistic work is needed to clarify how individual metabolites influence key signaling pathways, such as TP53 and IL-24, within hepatocytes and immune cells. On the therapeutic side, strategies aimed at reshaping the gut microbiome, could be explored to determine whether they can beneficially modify peripheral gene expression patterns in HE. Advancing these lines of inquiry will be critical for moving from bioinformatic predictions to practical tools for diagnosing and treating HE.

Central Mechanisms in HE: Gut-Brain Axis Dysregulation

Shifting focus to the CNS, our analysis identified 29 hub genes that connect gut-derived metabolites to brain-specific processes. These molecular interactions ultimately converge at three interconnected pathological mechanisms that promote HE progression: impaired astrocyte metabolism, sustained neuroinflammation, and disturbances in excitatory neurotransmission, all mediated through the actions of microbial metabolites on susceptible brain cell populations.

Fatty acid beta-oxidation as a mitochondrial aerobic cycle, provides another source of energy (beyond glucose) for brain tissue by hydrolyzing the fatty acid into acetyl-CoA which mainly happens in astrocytes.[160,161] There is still no data on the possible link between HE and impaired brain fatty acid beta-oxidation. However, mitochondrial fatty acid beta-oxidation capacity is impaired in animal models of hepatic steatosis and patients with chronic hepatitis C.[162–164]

Furthermore, studies have demonstrated that impaired hepatocyte fatty acid beta-oxidation is associated with liver disease.[165,166]

At the same time, a strong neuroinflammatory cascade seems to be triggered through multiple gut-mediated pathways. The translocation of bacterial LPS across the compromised gut and BBB activates TLR4 on microglia, initiating a pro-inflammatory cytokine storm.[167–170] This process is further enhanced by tryptophan metabolites such as kynurenine, which trigger neurotoxic quinolinic acid production, and TMAO, which potentiates NLRP3 inflammasome activation.[171–174] Identification of critical neuroinflammation markers such as TXNIP, IL18R1, and GFAP within the central gene network aligns with emerging concepts of microglial priming in HE, where chronic low-grade inflammation creates a sensitized neural environment vulnerable to secondary insults.[175–178] The inflammatory conditions in brain tissue are correlated with cognitive impairments and motor dysfunction following liver diseases.[20,175,179,180]

Alteration of neurotransmitter levels, especially glutamate (ie increased extracellular concentration, over-activation of glutamate receptors, glutamate toxicity) has also been shown in the brain tissues of hyperammonemic animal models of HE.[21,181–184] One of the most significant observations is the marked disturbance in glutamatergic neurotransmission, increased by the involvement of several key hub genes such as *GLUL, SLC1A2*, and *GRIN2C*, that play essential roles in glutamate recycling and signaling. The gut microbiome seems to impact this system through three interconnected pathways: ammonia-induced impairment of astrocytic glutamate uptake,[185,186] SCFA altering glutamate decarboxylase activity,[187–189] and bile acids interfering with NMDA receptor function.[190,191] This combined assault on excitatory neurotransmission offers a convincing explanation for the excitotoxic damage and neurological symptoms seen in HE, bridging the gap between intestinal dysbiosis and cerebral dysfunction.

The growing understanding of these mechanisms reveals several potential treatment strategies, such as using ketogenic interventions to support astrocyte metabolism, anti-inflammatory approaches for targeting gut microbiota to reduce inflammation, and stabilizing glutamatergic signaling. Yet key uncertainties persist about how these processes interact and evolve over time (temporal dynamics). Future research should combine longitudinal studies with advanced molecular profiling in animal models to map out the progression of events and pinpoint the best timing for interventions. Moreover, the interplay between central mechanisms and peripheral inflammation needs deeper investigation to create holistic therapies for HE that effectively address disruptions across the gut-liver-brain axis.

Implications for Diagnostics and Therapeutics

The finding of peripheral and central hub genes that link gut-derived metabolites to HE pathophysiology indicates promising opportunities for biomarker discovery and potential therapeutic targets. For diagnostics, genes such as PDGFRB, involved in vascular integrity, and *GFAP* (indicative of astrocytic activation) could be used as measurable indicators of gut-liver-brain axis disruption in clinical settings. Incorporating these into specific miRNA signature panels to provide sensitive and specific diagnostics for HE, including in the differential diagnosis between covert and overt stages.

Hub genes identified within the framework of oxidative stress (eg *FTH1*), neuroinflammatory (eg *IL18R1*), and glutamatergic signaling (eg *SLC1A2*) represent rational targets from a therapeutic standpoint. Burn more directly, microbiome-directed therapies, bile acid modulators, or SCFA supplementation could be thought of as approaches that could modify upstream microbial or metabolic pathways and indirectly normalize the expression of these hub genes and their downstream effects.

Limitations of the Study

While this study's bioinformatics approach uses multiple datasets to disclose potential relationships, these findings should be considered as preliminary. Several limitations should be acknowledged for the study. First of all, the analyses were based solely on publicly available transcriptomic datasets and curated metabolite-gene associations, which depend on the quality, sample size, and heterogeneity of the source studies. The two HE datasets used, had relatively small cohorts, enhancing the risk of inter-individual variability and limiting statistical power. Second, the bioinformatic approach identifies associations, not causality; the predicted molecular interactions, miRNAs, and drug candidates remain theoretical until validated through in vitro, in vivo, and clinical studies. Third, the metabolite-gene relationships were

inferred from database annotations and not by measurement of metabolomic data from the same patient samples, which can diminish the inference of context-dependent metabolic changes. Fourth, the investigation did not consider confounding variables such as etiology of liver disease, comorbidities, medication use, or nutritional status, each of which has potential to impact both the microbiome and gene expression in the host. Fifth, all data analyzed in this study were cross-sectional, meaning that temporal dynamics or disease progression cannot be evaluated; each of which are important for understanding mechanistic sequences in HE. Finally, the heterogeneity underlying covert and overt HE was not specifically mentioned, and while the underlying molecular signatures identified in our study may or may not differ between clinical subtypes, this topic must be addressed in future research. The limitations we encountered illustrate the importance of integrative, longitudinal, multi-omic studies in bigger and more phenotypically diverse patient populations, all together with targeted experimental validation to confirm and alter the proposed molecular targets presented.

Conclusion Remarks and Future Directions

This study significantly advances our understanding of HE by mapping the gut-liver-brain axis through an integrated bioinformatics analysis, identifying 38 hub genes that connect gut microbial metabolites to systemic and central manifestations of disease. This research challenges the long-held focus on ammonia as the primary driver of HE, revealing instead three critical pathophysiological pathways: (I) metabolic dysfunction via impaired β-oxidation, (II) neuroinflammation from microbial products, and (III) SCFA/bile acid-mediated glutamatergic excitotoxicity. The study identified 18 miRNA biomarkers for improved HE diagnosis and 13 promising drug candidates to enable precision medicine approaches which targeted combined microbiome modulation, anti-inflammatory strategies and neurometabolic support in the future. Clinically, the findings advocate for a shift in the management of HE toward a multidimensional approach targeting gut barrier function, systemic inflammation, and brain homeostasis simultaneously. Critical next steps in this research area include validating mechanisms in tissue derived from patient models and germ-free animals, initial phases of prospective Phase I–II clinical trials for lead compounds, and validating miRNA-based diagnostic panels. Other nascent horizons for this field of research may include a process to assess gut-vascular barrier integrity, bacterial translocation in liver diseases, and fecal microbiota transplantation. Future biotechnological convergence of single-cell omics technologies with machine learning and newly designed organ-on-chip approaches will accelerate discovery and reveal previously underappreciated molecular mechanisms. This study provides a mechanistic foundation and therapeutic pathway by redefining HE as an inter-organ crosstalk disorder mediated by gut metabolites. Changing focus from managing symptoms to targeting the root cause of interorgan syndromes is transformative; This enables personalized strategies based on individual microbiome-metabolic profiles, offering transformative potential for this devastating liver complication. Next steps efforts must bridge these insights to clinical applications while further elucidating gut-liver-brain axis dynamics to optimize patient outcomes.

Data Sharing Statement

The datasets analyzed in this study are publicly available from the Gene Expression Omnibus (GEO) and GeneCards databases. Further details and analysis files are available from the corresponding author, Ali Sepehrinezhad, upon reasonable request.

Ethical Approval

This study submitted to the Ethical Committee of Mashhad University of Medical Sciences. The Committee did not encounter any deviation from ethical principals in this research. As the research involved only secondary analysis of publicly available and anonymized datasets (GEO and GeneCards), the Committee deemed it exempt from additional approval or informed consent requirements.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically

reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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