

Regulation of Inflammation, Lipid Metabolism, and Liver Fibrosis by Core Genes of M1 Macrophages in NASH

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Background: Although immune cells play a critical role in lipid metabolism and inflammation regulation in patients with non-alcoholic steatohepatitis (NASH), the specific immune cells involved and associated genes remain unclear.

Methods: We identified differential immune cell profiles between normal liver and NASH specimens using the CIBERSORT algorithm. Next, we conducted a weighted gene co-expression network analysis (WGCNA) to identify genes highly correlated with these immune cells in NASH. Subsequently, core genes of immune cells were identified using machine learning algorithms.

Results: The abundance of M1 macrophages significantly increased in patients with NASH. The Random Forest (RF) algorithm identified six M1 macrophage-related genes (*COL10A1*, *FAP*, *IL32*, *STMN2*, *SUSD2*, and *THY1*) crucial in NASH. These six genes positively correlated with five inflammatory genes (*CCL2*, *IL1B*, *TNF*, *CSF1*, and *IL15*), lipid synthesis gene (*FAS*), collagen synthesis genes (*COL1A1* and *COL3A1*), liver fibrosis stage, NASH activity score (NAS), and aspartate aminotransferase (AST) levels. These were negatively correlated with the lipid transport gene (*CD36*), beta fatty acid oxidation gene (*PPARA*), and M2 macrophage abundance. Moreover, a predictive model based on these six genes achieved a C-index of 0.902 for diagnosing NASH across four cohorts. The expression of these six genes accurately stratified patients with NASH into low disease activity cluster 1 and high disease activity cluster 2.

Conclusion: These six core genes of M1 macrophages contribute to NASH progression by regulating inflammation, lipid metabolism, and liver fibrosis.

Keywords: NASH, M1 macrophages, machine learning, diagnosis, cluster

Background

Non-alcoholic steatohepatitis (NASH) refers to a progressive manifestation of non-alcoholic fatty liver disease (NAFLD). It is characterized by hepatic steatosis, inflammation, and hepatocellular injury, and exerts a significant global public health burden.¹⁻⁴ The prevalence of NASH escalates concomitantly with an increase in obesity, type 2 diabetes mellitus (T2DM), and metabolic syndrome.⁵⁻⁷ The increased prevalence of NASH in non-obese individuals underscores the intricate interplay of genetic, environmental, and lifestyle factors.⁸ The multifaceted pathogenesis of NASH involves genetic susceptibility, insulin resistance, oxidative stress, dysregulated lipid metabolism, gut microbiota dysbiosis, and interactions among pro-inflammatory cytokines, culminating in hepatic lipid accumulation, hepatocellular injury, and inflammation.^{9,10} In addition, the recruitment of immune cells such as macrophages and lymphocytes, alongside hepatic stellate cell activation, eventually causes liver fibrosis.^{11,12}

Hepatic macrophages, particularly M1 macrophages, have been implicated in the pathogenesis of NASH by inducing inflammatory signaling, promoting the expression of pro-inflammatory genes, and activating hepatic stellate cells, thus exacerbating liver fibrosis and cirrhosis.¹³ Although liver biopsy remains the gold standard for diagnosing NASH, one of

its major shortcomings is its invasiveness, which necessitates urgent exploration of non-invasive biomarkers.¹⁴ Several studies are being conducted worldwide to elucidate the pathophysiology of NASH and identify therapeutic targets.

In conclusion, NASH is a serious public health challenge that requires an understanding of its pathogenesis and provides effective therapeutic strategies. In previous studies, we have demonstrated that AKR1B10, TYMS and TREM2 are key mitochondrial genes in the development of NASH.¹⁵ However, it is still necessary to reveal the immune cells that play a key role in NASH at the immune level and thus identify the key immune genes. This study may provide potential immunotherapeutic targets and innovative diagnostic methods for NASH.

Methods

The study design is visually represented in Figure 1.

Processing of Raw Data

Four datasets (GSE126848, GSE135251, GSE89632, and GSE48452) were retrieved from the GEO database, each processed with its respective platform file. After addressing batch effects, GSE126848, GSE135251, and GSE89632 were merged into a new dataset, Merge-Cohort, using the ComBat method. Samples in the Merge-Cohort were randomly split into training and internal testing sets in a 7:3 ratio, and GSE48452 served as the external validation set. Differential expression analysis between normal liver and NASH samples in the training set was conducted using the “limma” package.

Identifying Genes Related to M1 Macrophages Among DEGs

The CIBERSORT algorithm¹⁶ was applied to analyze immune cell types and quantities across the training set, internal testing set, Merge-Cohort, and external validation set. Subsequently, M1 macrophages exhibiting discrepant quantities between normal and NASH groups were selected for comprehensive examination. The WGCNA algorithm¹⁷ was applied

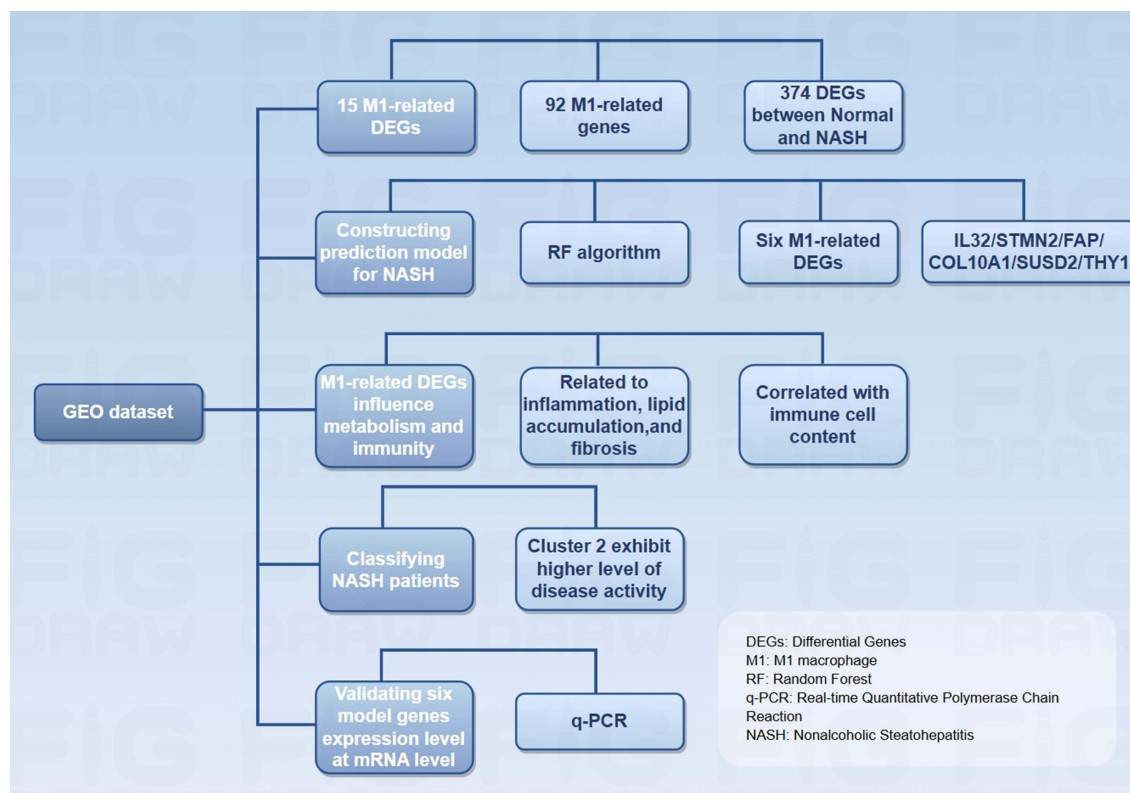


Figure 1 Flow chart of study design.

to divide the genes in the training set into different gene modules based on their correlation with immune cells. Genes extracted from the gene modules most significantly correlated with M1 macrophages and intersected with DEGs, yielding DEGs associated with M1 macrophages. Subsequently, the biological pathways involving the aforementioned genes were investigated using the bioinformatics database Metascape.¹⁸

Developing a NASH Prediction Model

A new set of 113 machine learning algorithms, comprising 12 algorithm combinations (Lasso, Ridge, Enet, StepAIC, SVM, glmBoost, LDA, plsRglm, RandomForest, GBM, XGBoost, and NaiveBayes), was utilized to further narrow down the number of core genes associated with M1 macrophages and construct a classification prediction model. The area under the ROC curve (AUC) was computed to assess the predictive accuracy of the model in predicting NASH in the training set, internal testing set, Merge-Cohort, and GSE48452. Finally, the evaluation results of the model were visualized using a heatmap. In addition, the AUC values for individual model genes in diagnosing NASH were calculated.

Biological Pathway Enrichment Analysis

Differential expression of model genes between normal and NASH groups was explored in four cohorts. Based on the expression of model genes, gene set variation analysis (GSVA) enrichment analysis¹⁹ was conducted to investigate the involvement of biological pathways in the development of NASH.

Correlation Analysis Between Model Genes and NASH-Related Biomarkers

A protein–protein interaction network of model genes is depicted in the GeneMINA database,²⁰ and the biological pathways involving the interacting genes were analyzed using the Metascape database. Furthermore, we conducted a comprehensive analysis of the associations between the model genes and inflammation-related genes, lipid synthesis genes, and fibrosis-related genes. Next, the relationship between model genes and the content of immune cells was investigated. The associations between the model genes and AST, alanine aminotransferase (ALT), ballooning degeneration, steatosis, liver fibrosis, and NAS score were analyzed at both metabolic and pathological levels.

Non-Negative Matrix Factorization (NMF) Clustering Analysis

Non-negative matrix factorization (NMF) clustering analysis was employed to group the NASH samples in the training set based on the expression of six model genes.²¹ Further, the optimal value of K was selected according to the clustering parameters to divide the samples into different subtypes. Differential expression of model genes, inflammatory genes, lipid metabolism genes, fibrosis genes, macrophage content, ALT and AST levels, and NAS scores were compared among different subtypes. In addition, biological pathways involved in different subtypes were analyzed. Finally, differential genes among different subtypes were analyzed using the WGCNA algorithm, followed by biological pathway enrichment analysis and disease type enrichment analysis using the Metascape database.

Validation of Differential Gene Expression in Normal and NASH Liver Tissues

Ten liver tissue samples were obtained from patients with normal body weight, and an additional ten samples were collected from obese patients. All specimens underwent fixation, embedding, and sectioning procedures. Samples from patients with normal body weight did not exhibit any pathological changes, whereas all samples from obese patients were diagnosed with non-alcoholic steatohepatitis (NASH). RNA extraction and cDNA synthesis were performed using kits from the Jiangsu Nearshore Protein Company. Following RNA extraction, cDNA was synthesized and quantified, with β -actin serving as the housekeeping gene. The expression levels of the target genes were compared using a *t*-test. Primer sequences are detailed in [Supplementary Table 1](#).

Furthermore, protein extraction and Western blot (WB) analysis were conducted on three normal liver tissue samples and three NASH tissue samples. Antibodies targeting THY1 and the internal control β -actin were purchased from Sanying Biotechnology Company in Wuhan, China. For the experiment, 20 mg of frozen liver tissue from each sample was mixed with pre-cooled steel beads and lysis buffer, then homogenized using a tissue homogenizer at a frequency of

60Hz for 120 seconds to ensure thorough grinding. After homogenization, the steel beads were removed, and the protein lysate was transferred to a centrifuge tube and placed on ice for 30 minutes to allow for complete tissue lysis. After lysis, the supernatant was extracted by ultracentrifugation, and protein concentrations were assessed. Based on the molecular size of the target protein, a 10% separating gel was used for gel electrophoresis, followed by wet transfer of proteins onto a membrane. The membrane was then washed and blocked with Tris-buffered saline containing Tween and 5% skim milk. Subsequently, the samples were incubated with the primary antibody overnight at 4°C, rinsed, and incubated with the secondary antibody before development.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (version 9.0). A *t*-test was used to compare the data from two groups showing a normal distribution and homogeneity of variance. Otherwise, non-parametric tests were utilized. Statistical significance was determined based on the *P*-values. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Results

Raw Data Were Divided into Four Groups

Four cohorts containing NASH sample information were obtained, namely GSE126848, GSE135251, GSE89632, and GSE48452, were obtained from the GEO database. The first three cohorts, after successful batch effect removal (Figure 2A and B), were merged into Merge-Cohort. Subsequently, Merge-Cohort was randomly divided into training and internal testing sets at a ratio of 7:3. GSE48452 was designated as the external validation set. The numbers of normal liver samples in the training set, testing set, Merge-Cohort, and GSE48452 cohorts were 41, 19, 60, and 41, respectively (Figure 2C). The numbers of NASH samples were 122, 68, 190, and 18, respectively (Figure 2D). Based on the filtering

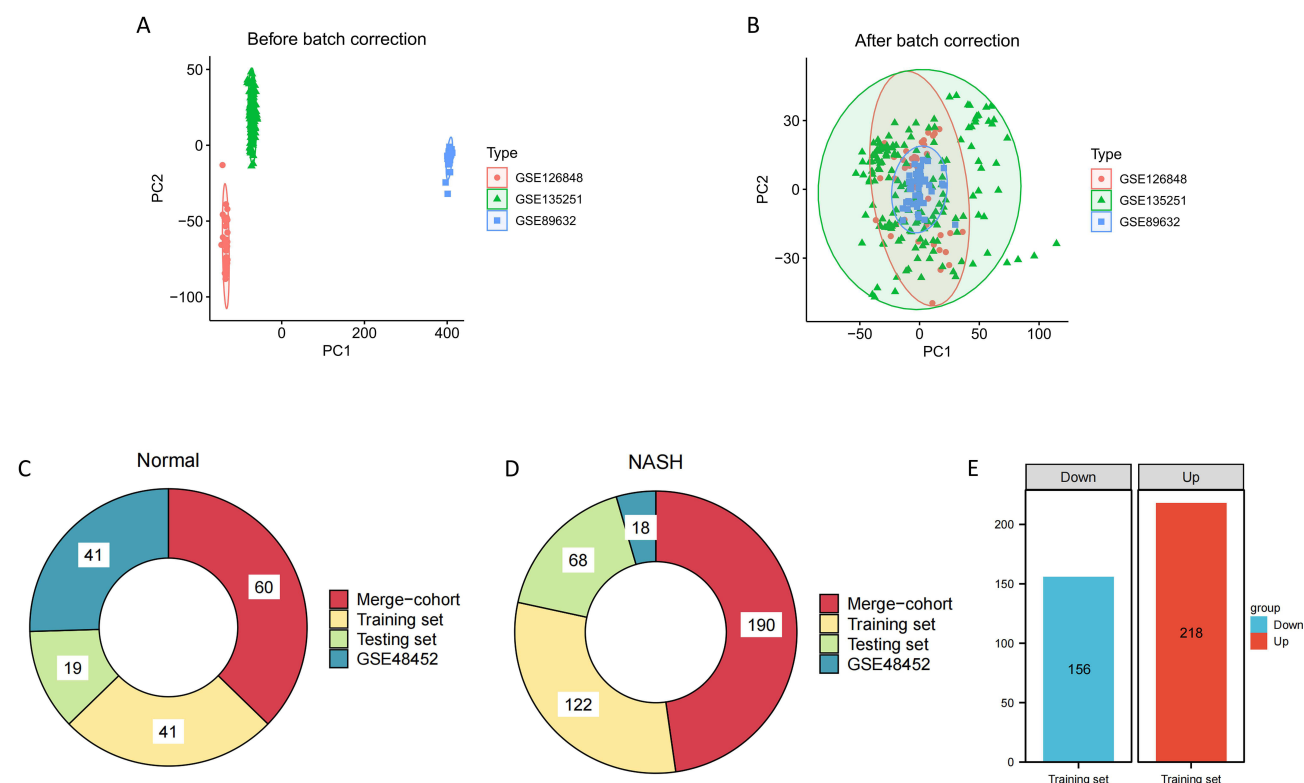


Figure 2 The raw data were subjected to grouping. (A) Before batch effect removal, PCA plots of the three cohorts showed distinct separation of samples. (B) After batch effect removal, PCA plots of the three cohorts exhibited a mixing of samples. (C) The number of normal liver samples in the four cohorts. (D) The number of NASH samples in the four cohorts. (E) DEGs between normal and NASH liver specimens in the training set.

criteria ($\log_{2}FC > 0.585$ and adjusted P -value < 0.05), 374 DEGs were identified between normal liver samples and NASH samples in the training set (Figure 2E).

Identification of 15 Key Genes

The CIBERSORT algorithm was used to partition the data into 22 types of immune cells, with M1 macrophages being the only immune cell significantly upregulated in the NASH groups across four cohorts (training set, testing set, Merge-Cohort, and GSE48452) (Figures 3A–D). This suggests the crucial role of M1 macrophages in the occurrence and progression of NASH. Next, we conducted an in-depth analysis to explore M1 macrophage-associated genes using WGCNA. The soft threshold was set to 3, and genes in the training set were divided into seven gene modules, with the yellow module being the most significantly correlated with M1 macrophages (Figure 3E). The intersection of genes in this module with previously identified intergroup DEGs (Figure 3F) yielded 15 significantly upregulated M1 macrophage-associated genes in NASH (Figure 3G). The top enriched biological pathway for these 15 genes was the extracellular matrix organization associated with fibrotic processes within the liver microenvironment (Figure 3H).

Classification Prediction Model Constructed Using RF Algorithm

The 15 genes were screened using 113 machine-learning algorithms and were further identified as playing crucial roles in NASH. These were used to construct a diagnostic model for NASH. Among all algorithms, the C-index value of the RF algorithm was the highest, at 0.902. Its diagnostic accuracies for NASH in the training set, testing set, Merge-Cohort, and GSE48452 were 1.000, 0.868, 0.950, and 0.789, respectively (Figure 4A). In addition, six genes were identified by the

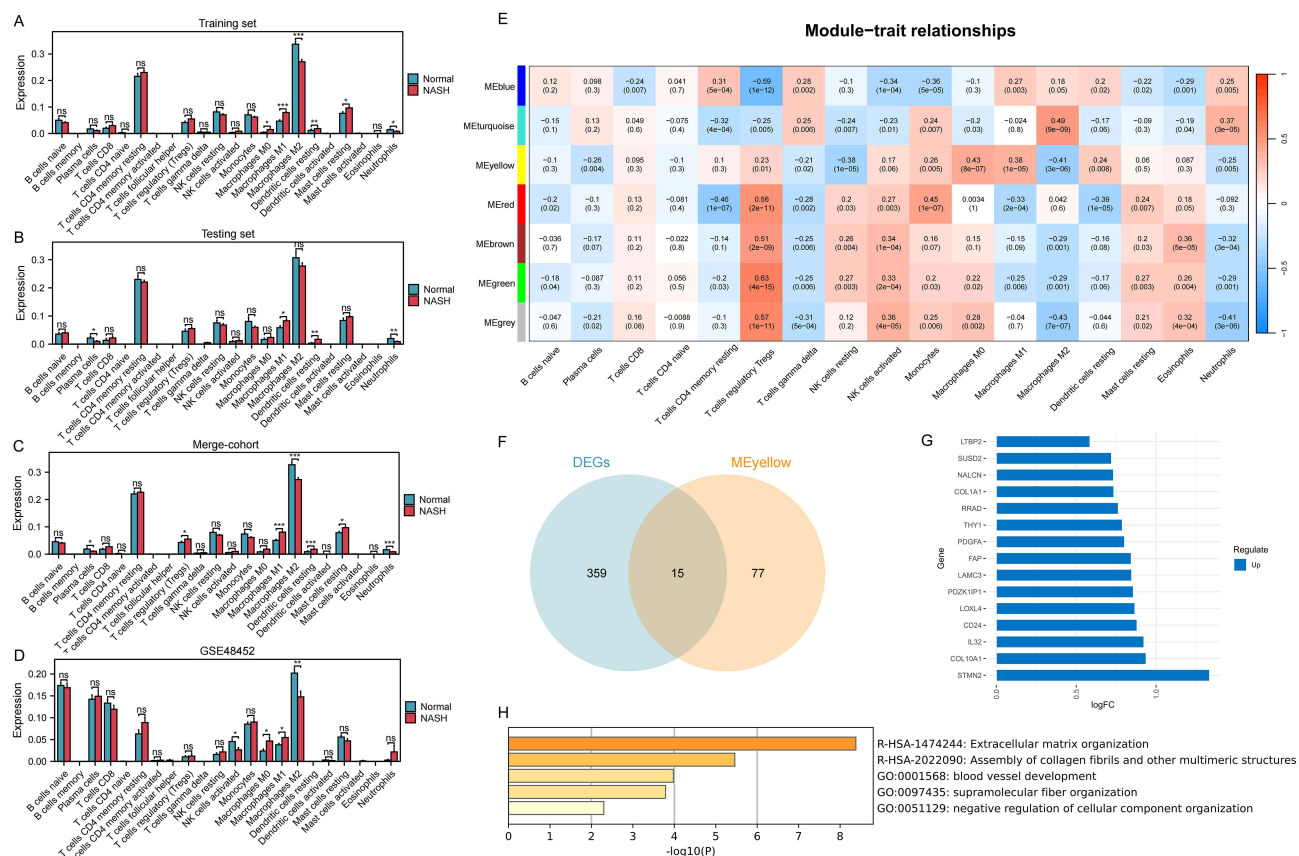


Figure 3 Selection of core genes related to M1 macrophages. (A–D) Samples from the training set, testing set, Merge-Cohort, and GSE48452 were classified into 22 types of immune cells. (E) Genes in the training set were partitioned into seven gene modules. (F, G) The intersection of differentially expressed genes with the yellow gene module yielded 15 upregulated genes in NASH. (H) The top five enriched biological pathways for these 15 genes. ns: no statistical difference, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

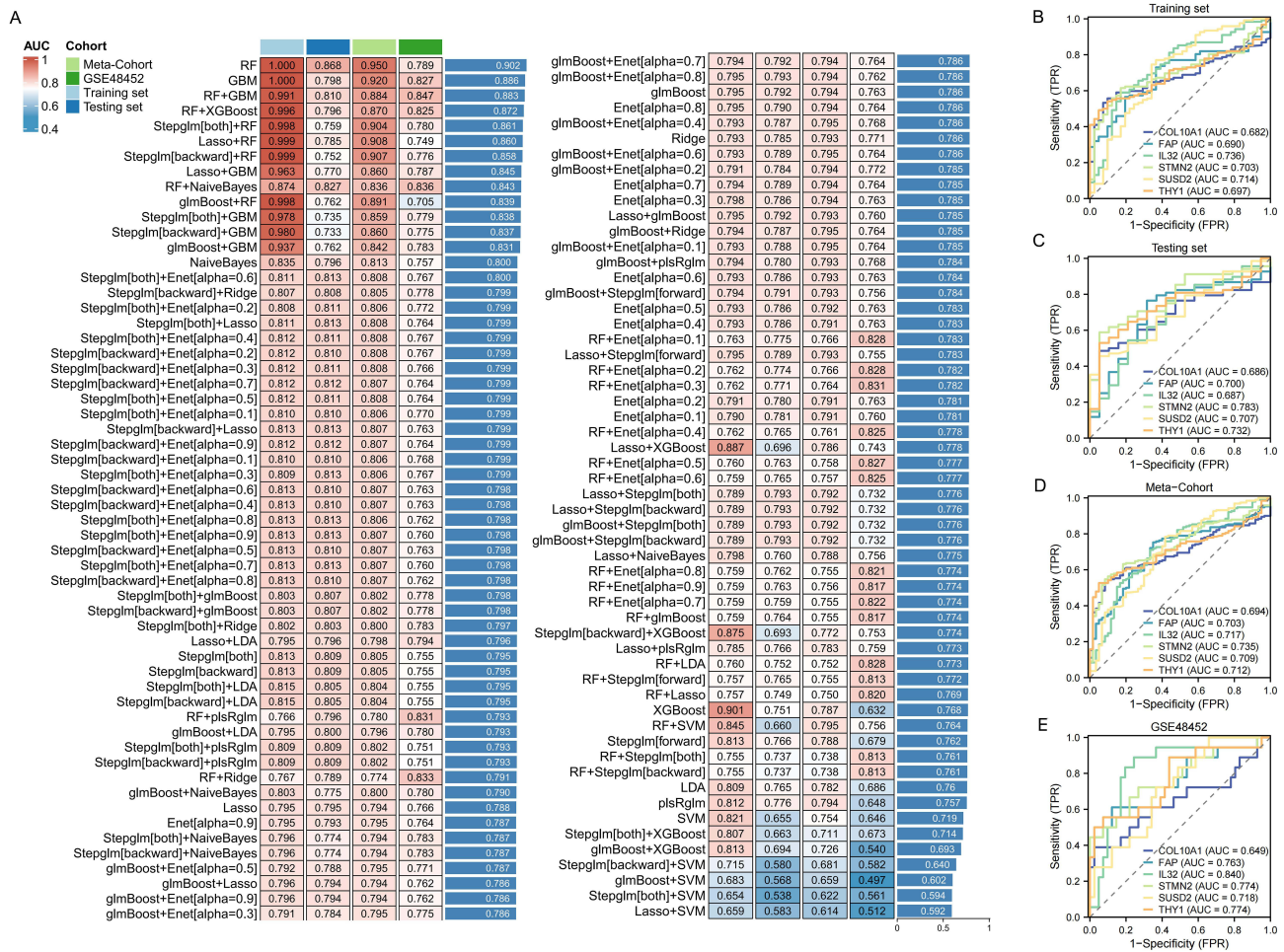


Figure 4 Core genes screened by machine learning algorithms. **(A)** The heatmap depicting the AUC values for the accuracy of NASH identification in the four cohorts by the NASH diagnostic model was constructed using 113 algorithms. **(B-E)** The AUC values for the accuracy of NASH diagnosis individually using the six genes in the four cohorts.

RF algorithm: *COL10A1*, *FAP*, *IL32*, *STMN2*, *SUSD2*, and *THY1*. The individual AUC values for diagnosing NASH using these six genes in the four cohorts were lower than those in the diagnostic model (Figure 4B–E).

Upregulation of Six Model Genes Involved Biological Pathways Relevant to NASH Progression

Subsequently, we studied the biological significance of these six genes. They were significantly upregulated not only in the NASH group of the training set (Figure 5A) but also in the three validation cohorts (Figure 5B–D). Their upregulation was associated with biological pathways relevant to the progression of NASH. The upregulation of *COL10A1* is involved not only in the “Notch signaling pathway” implicated in liver inflammation and fibrosis but also in the “diabetes pathway” leading to hepatic lipid accumulation and inflammatory responses (Figure 5E). The upregulation of *FAP* is associated with the “tryptophan metabolism pathway”, which contributes to increased oxidative stress and inflammation (Figure 5F). The elevated expression of *IL32* and *STMN2* is linked to the “mTOR signaling pathway” involved in regulating lipid synthesis, inflammation, and fibrosis (Figure 5G and H). The upregulation of *SUSD2* is associated with the “type II diabetes pathway” (Figure 5I), whereas the upregulation of *THY1* involves the “bile acid biosynthesis pathway”, leading to bile stasis and liver inflammation, as well as the “Hedgehog signaling pathway” involved in regulating hepatic lipid metabolism and fibrosis processes (Figure 5J).

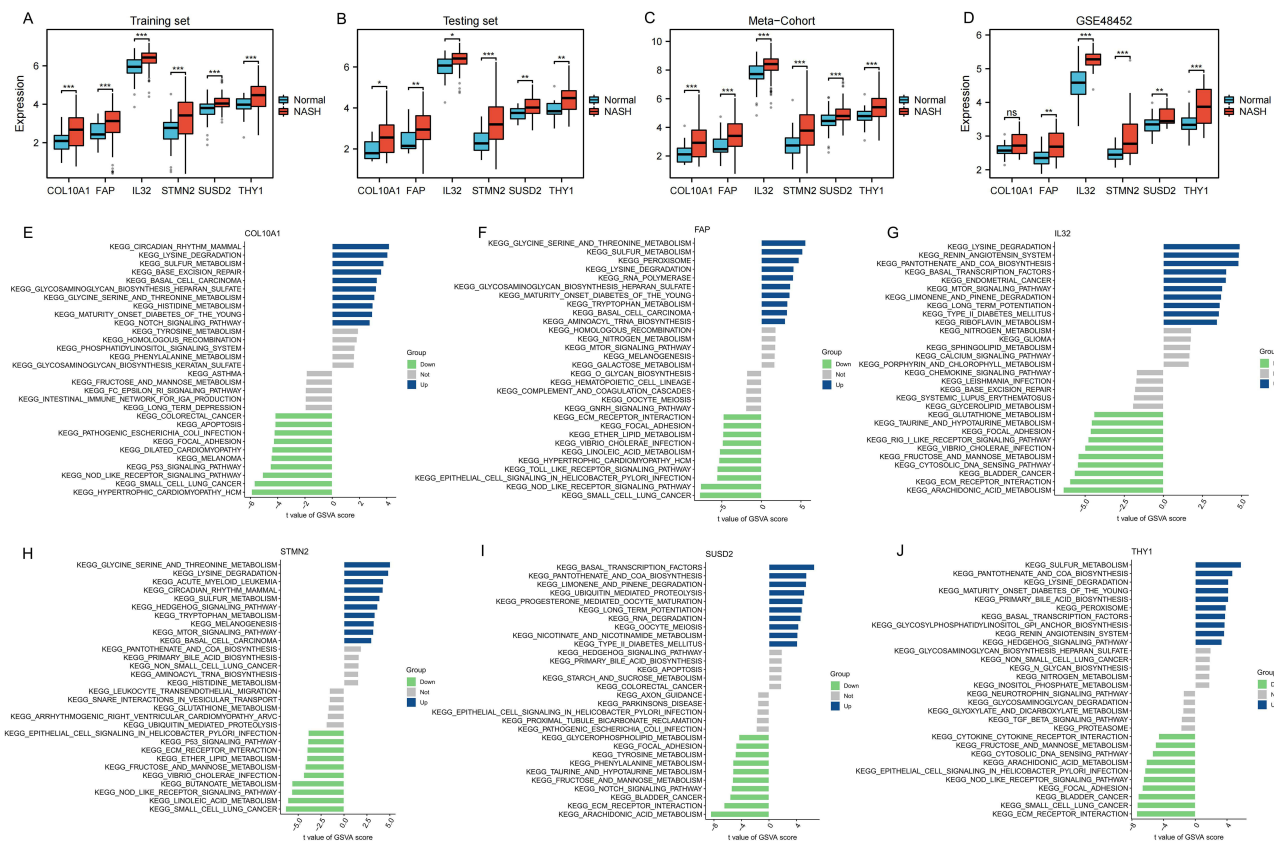


Figure 5 Enrichment landscape of model genes is depicted. (A–D) The six genes are significantly upregulated in all four cohorts. (E–J) The biological pathway enrichment analysis diagram of six genes. ns: no statistical difference, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Model Genes Promote the Progression of Inflammation, Lipid Accumulation, Macrophage Proliferation, and Elevated AST Levels

The GeneMINA database was used to identify 20 proteins interacting with the model genes (Figure 6A). They were enriched in biological processes such as “cell adhesion” and regulation of “G protein-coupled receptor signaling pathways” (Figure 6B). Moreover, the model genes were significantly positively correlated with pro-inflammatory genes (*CCL2*, *IL1B*, *TNF*, *CSF1*, *IL15*, *PDGFA*, *TGFB1*, *TGFB2*, and *TGFB3*), and significantly negatively correlated with the anti-inflammatory gene *IL10* (Figure 6C). Furthermore, they were significantly positively correlated with lipid synthesis genes (*FAS*), hepatic fibrosis genes (*COL1A1* and *COL3A1*), and significantly negatively correlated with the β -fatty acid oxidation gene (*PPARA*) (Figure 6C). A significant negative correlation was observed between the model genes and anti-inflammatory M2 macrophages at the level of immune infiltration (Figure 6D). At the metabolic level, *COL10A1* and *FAP* were significantly positively correlated with the liver injury marker AST (Figure 6E). Regarding disease activity, *COL10A1* and *SUSD2* were significantly positively associated with liver fibrosis, and all six model genes displayed significant positive correlations with the NAS score (Figure 6E and F).

Classification of NASH Samples

The clustering was optimal at a clustering parameter K of 2. Consequently, the NASH samples in the training set were divided into two clusters, Cluster 1 and Cluster 2, based on the expression of six model genes (Figure 7A). The results of principal component analysis demonstrated a clear separation between the two groups (Figure 7B). Compared to Cluster 1, the expression of six model genes was significantly upregulated in Cluster 2 (Figure 7C). Furthermore, in Cluster 2, pro-inflammatory genes (*CCL2*, *TNF*, *IFNG*, *IL15*, *IL7*, *PDGFA*, *TGFB2*, and *TGFB3*), lipid synthesis genes (*FAS*), and liver fibrosis genes (*COL1A1* and *COL3A1*) were significantly upregulated, whereas the β -fatty acid oxidation gene

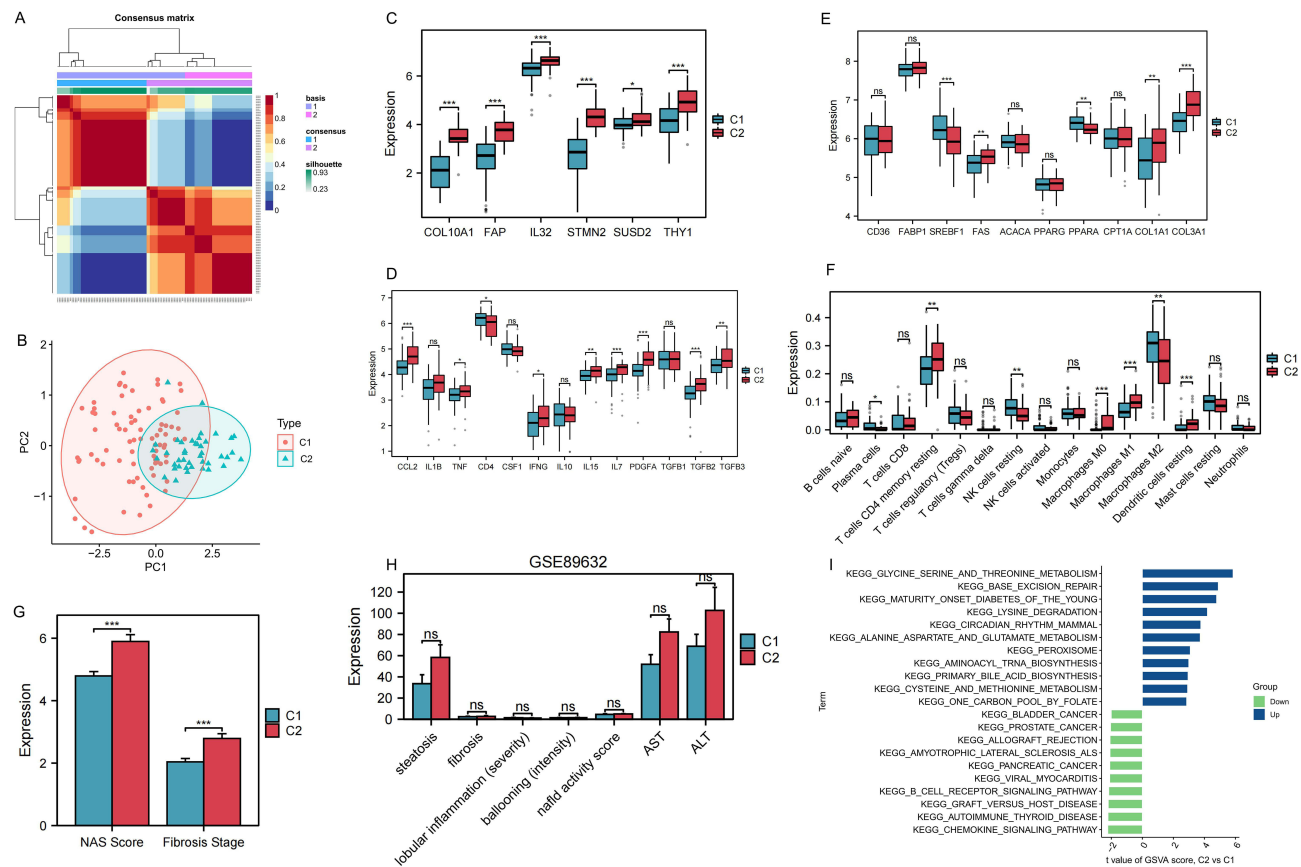


Figure 7 The severity of disease activity was higher in Cluster 2. (A) NMF clustering analysis was utilized to partition NASH samples into two groups. (B) PCA plot demonstrated a clear demarcation between samples in Cluster 1 and Cluster 2. (C) In Cluster 2, six model genes were significantly upregulated. (D) The expression of inflammation-related genes was higher in Cluster 2 compared to Cluster 1. (E) Differences in the expression of lipid metabolism genes and fibrosis genes were observed between the two clusters. (F) Variations in the content of immune cells between the two clusters were evident. (G) Cluster 2 exhibited higher NAS scores and liver fibrosis stages. (H) In the GSE89632 cohort, higher levels of ALT and AST were observed in Cluster 2, along with increased severity of hepatic steatosis, ballooning degeneration, and liver fibrosis. (I) GSEA plot revealed significantly enriched biological pathways in Cluster 1 and Cluster 2. ns: no statistical difference, *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

(Figure 9B), aligning with the outcomes of the previous bioinformatics analysis. Given the limited research on THY1 protein in non-alcoholic steatohepatitis (NASH), its selection for further validation was strategic. Delightfully, the results aligned with expectations, demonstrating a significant increase in THY1 protein levels in NASH (Figure 9C).

Discussion

Non-alcoholic steatohepatitis (NASH) is an increasingly prevalent chronic liver disease, whose pathogenesis remains incompletely understood. We studied the underlying genetic mechanisms of NASH development through a comprehensive analysis of patient samples, shedding light on the intrinsic nature of NASH progression and its clinical significance. Furthermore, we established a predictive model for NASH based on key genes associated with M1 macrophages, offering new perspectives and possibilities for the diagnosis and treatment of NASH.

Firstly, systematic data mining and bioinformatics analysis were used to extract extensive NASH sample information from the GEO database. Following data grouping and batch effect removal, the reliability and stability of subsequent analyses were ensured. The analysis identified 374 DEGs, providing important clues for further investigation into the pathogenesis of NASH. Secondly, the CIBERSORT algorithm and WGCNA were applied to study the changes in immune cells and the expression patterns of related genes during NASH pathogenesis. Significant upregulation of M1 macrophages in NASH was identified, and 15 key genes intricately associated with M1 macrophages were further characterized. These genes primarily participated in extracellular matrix remodeling and fibrosis processes, revealing the crucial function of M1 macrophages in inflammation progression and fibrosis formation during NASH pathogenesis.

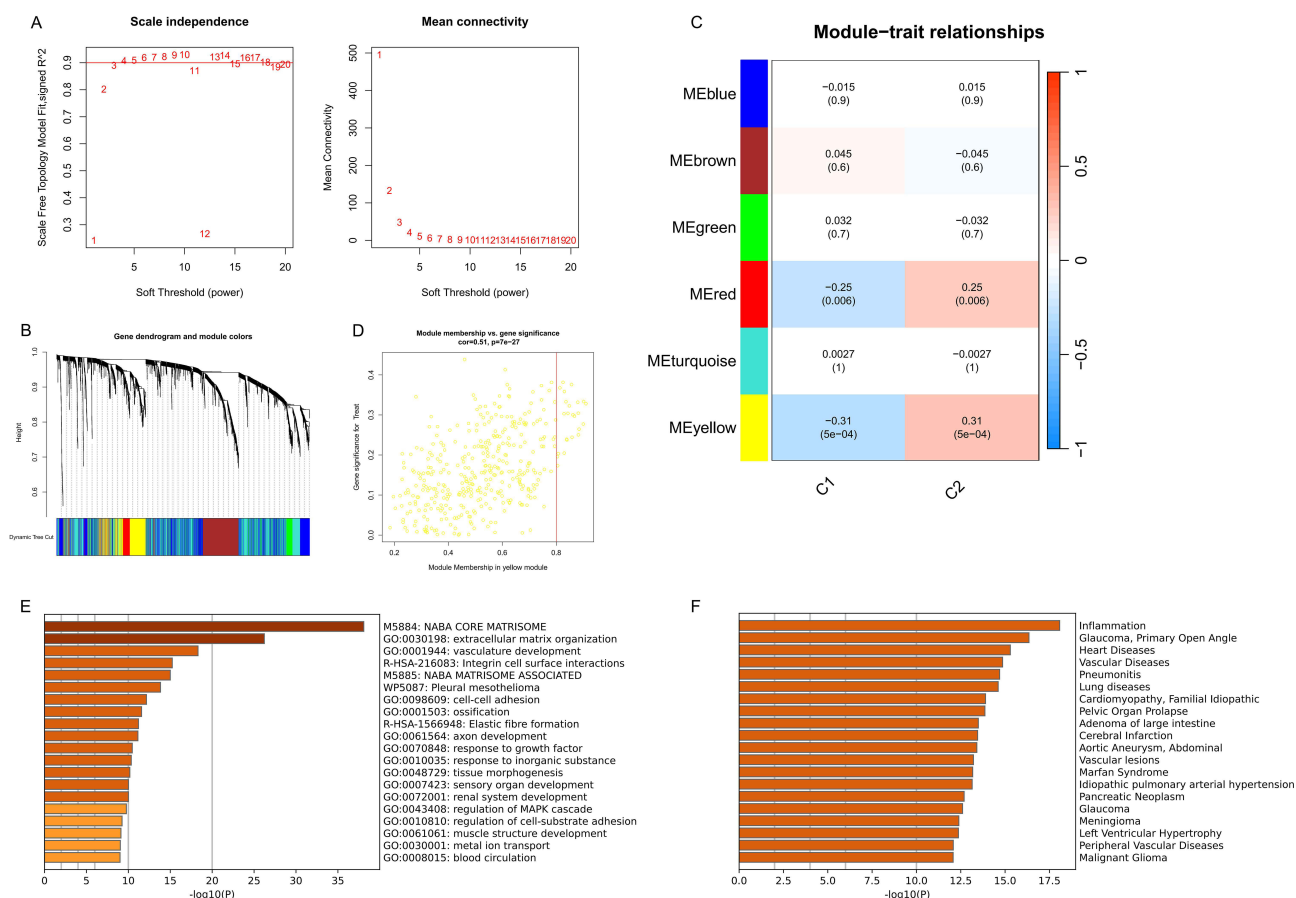


Figure 8 A total of 358 differential genes between the two clusters were identified through the WGCNA analysis. **(A)** The soft threshold was set to 3. **(B)** Six gene modules were identified. **(C)** Genes in the yellow module exhibited the most significant differences between the two clusters. **(D)** The 358 genes in the yellow module were significantly upregulated in Cluster 2. **(E)** The biological pathways associated with these 358 differential genes were examined. **(F)** The disease types enriched by these 358 differential genes were investigated.

Subsequently, the RF algorithm was used to select *COL10A1*, *FAP*, *IL32*, *STMN2*, *SUSD2*, and *THY1* from these 15 genes. A highly accurate NASH diagnostic model was constructed based on these six genes. The model exhibited good predictive performance across training, testing, merged, and external validation cohorts, providing clinicians with a simple yet effective tool for early diagnosis and monitoring of NASH progression. Furthermore, we conducted a comprehensive study of the roles and potential clinical significance of these six model genes in NASH pathogenesis. We found that these genes participate in multiple biological pathways, such as the Notch signaling pathway, diabetes pathway, and mTOR signaling pathway. The Notch signaling pathway is a crucial cellular signaling cascade with pivotal regulatory roles in biological processes such as proliferation, differentiation, and apoptosis. Studies have reported its positive correlation with insulin resistance, hepatic steatosis, and liver fibrosis in non-alcoholic steatohepatitis (NASH).^{22,23} The diabetes pathway is intricately involved in the pathogenesis of NASH through its effects on insulin resistance, dyslipidemia, oxidative stress, and inflammation.²⁴ The mTOR signaling pathway is a crucial cellular signaling cascade regulating several metabolic pathways, including glucose metabolism, lipid metabolism, and amino acid metabolism, by modulating autophagy processes.²⁵ The activation of this pathway has been known to promote hepatic steatosis.²⁶ Moreover, they were significantly correlated with pro-inflammatory genes (*IL1B*, *TNF*, and *IL15*), lipid synthesis genes (*FAS*),²⁷ hepatic fibrosis genes (*COL1A1* and *COL3A1*), AST levels, NAS scores, and M1 macrophage content, whereas exhibiting significant negative correlations with β -fatty acid oxidation gene (*PPARA*)²⁸ and M2 macrophage content. Thus, these six genes promote inflammation progression, lipid accumulation, and exacerbation of liver fibrosis in NASH.

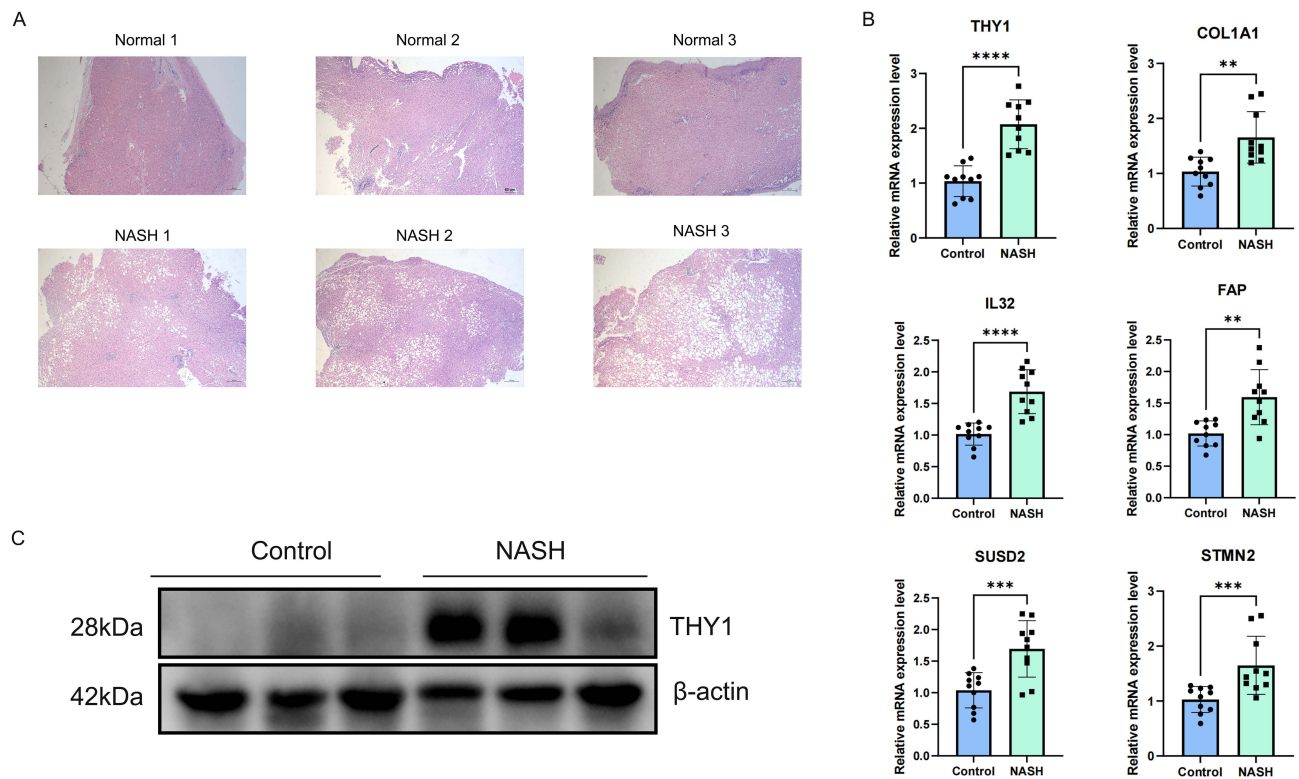


Figure 9 Significant Upregulation of Six Key Genes in Non-alcoholic Steatohepatitis (NASH). **(A)** Hematoxylin and eosin (H&E) stained images of liver tissues from normal individuals and those with NASH. **(B)** At the mRNA level, there is a pronounced increase in the expression of six key genes in NASH. **(C)** At the protein level, a significant upregulation of THY1 protein is observed in NASH. **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$.

Finally, based on these six genes, NASH samples were divided into two distinct phenotypic clusters. Patients in Cluster 2 exhibited higher disease activity and more severe liver fibrosis. This finding provides new insights for designing personalized therapy for NASH and important clues for further elucidating the pathogenesis of NASH.

Conclusion

The in-depth analysis of NASH patient samples revealed key mechanisms and biomarkers of NASH development. In addition, it provided new insights and theoretical foundations for the diagnosis and treatment of NASH. However, the study had certain limitations such as a small sample size and limited experimental data, indicating the findings require further validation and refinement through additional research.

Ethical Statement

This study was conducted in accordance with the ethical standards of the Ethics Committee of The First Affiliated Hospital of Anhui Medical University (approval no. 2023497) and with the 1964 helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

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Disclosure

The authors report no conflicts of interest in this work.

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