

# Identification of apoptosis as key biochemical mechanism in nonalcoholic fatty liver disease after hepatic steatosis through bioinformatics and functional analyses

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## ABSTRACT

**Background:** Non-alcoholic fatty liver disease (NAFLD) represents a growing global health crisis and is closely associated with increases in obesity and type 2 diabetes. Despite its ubiquity, its underlying biochemical mechanisms and effective therapeutic strategies remain poorly defined, hampering the development of targeted interventions. **Methods:** Candidate genes were obtained from the GEO database, and Kyoto Encyclopedia of Genes and Genomes enrichment analysis and gene set enrichment analysis were used to identify pathways involved in NAFLD-related pathways. The top genes with higher degree in the protein-protein interaction network were crossed with the top genes enriched in key pathways, and then the correlation analysis between key genes and chemotherapy response was performed. **Result:** Apoptosis, oxidative stress, NF- $\kappa$ B pathway, etc. are key pathways related to NAFLD. Lcn2, Mt1, Egr1, Jun, Nqo1, Btg2, Foxq1 and Hyou1 were enriched in key pathways of apoptosis. **Conclusion:** Apoptosis, oxidative stress, NF- $\kappa$ B pathway are key pathways related to NAFLD.

## KEYWORDS

NAFLD; Apoptosis; GEO; KEGG; GSEA

## 1. INTRODUCTION

Non-Alcoholic Fatty Liver Disease (NAFLD) encompasses a spectrum of liver conditions not attributable to alcohol consumption, ranging from simple steatosis (nonalcoholic fatty liver, NAFL) to nonalcoholic steatohepatitis (NASH), leading to cirrhosis and potentially hepatocellular carcinoma (HCC). As the most common liver disorder in the Western world, NAFLD is closely associated with metabolic syndrome, characterized by insulin resistance, obesity, dyslipidemia, and hypertension, mirroring the global epidemic of these conditions [1, 2].

The pathogenesis of NAFLD is multifactorial, involving genetic predisposition, dietary habits, and lifestyle factors, encapsulated in the "multiple-hit hypothesis". This theory suggests that liver fat accumulation, or hepatic steatosis, represents the "first hit", making the liver more susceptible to various "second hits" such as lipotoxicity, oxidative stress, mitochondrial dysfunction, and inflammation, culminating in cell injury, apoptosis, and fibrosis. Despite its prevalence and significant morbidity, the molecular mechanisms driving the progression from simple steatosis to more severe forms of NAFLD remain incompletely understood [3, 4].

Apoptosis, a form of programmed cell death, has emerged as a key player in this progression. Distinct from necrosis, apoptosis is a tightly regulated process critical for maintaining cellular homeostasis and organ function. In the context of NAFLD, aberrant activation of apoptotic pathways contributes to hepatocyte death, inflammation, and fibrogenesis. Furthermore, the role of apoptosis in NAFLD is

underscored by its potential as a therapeutic target. Modulating apoptosis in hepatic cells offers a promising avenue for preventing or halting the disease's progression [5-7].

The complexity of NAFLD, characterized by its silent nature in early stages and the lack of reliable non-invasive diagnostic markers for NASH, poses significant challenges for its management and treatment. Understanding the biochemical pathways, including apoptosis that underpins NAFLD progression, is crucial for developing targeted therapies and improving patient outcomes. This study aims to dissect the role of apoptosis in NAFLD after hepatic steatosis, leveraging bioinformatics and functional analyses to unveil the molecular underpinnings of this disease. Our findings promise to shed light on novel biomarkers and therapeutic targets, paving the way for precision medicine in NAFLD.

## 2. MATERIALS AND METHODS

### 2.1. Section Headings

In order to explore the key pathways affecting NAFLD, samples were screened from the NAFLD-related microarray retrieved from the GEO database, NAFLD-related expression microarray GSE24031, downloaded from the GEO database (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24031>). GSE24031 is included in 4 subgroups: LF-low (LFL) responders showing normal liver morphology, LF-high (LFH) responders showing benign hepatic steatosis, and HF-low (HFL) responders showing liver morphology with macrovesicles Pre-NASH of lipid droplets, as well as HF-high (HFH) responders exhibiting overt NASH, characterized by hepatocyte expansion, presence of Mallory bodies, and activated inflammatory cells, with 4 samples each, we screened for LF-low (LFL) and HF high (HFH) 4 samples each, 8 samples in total

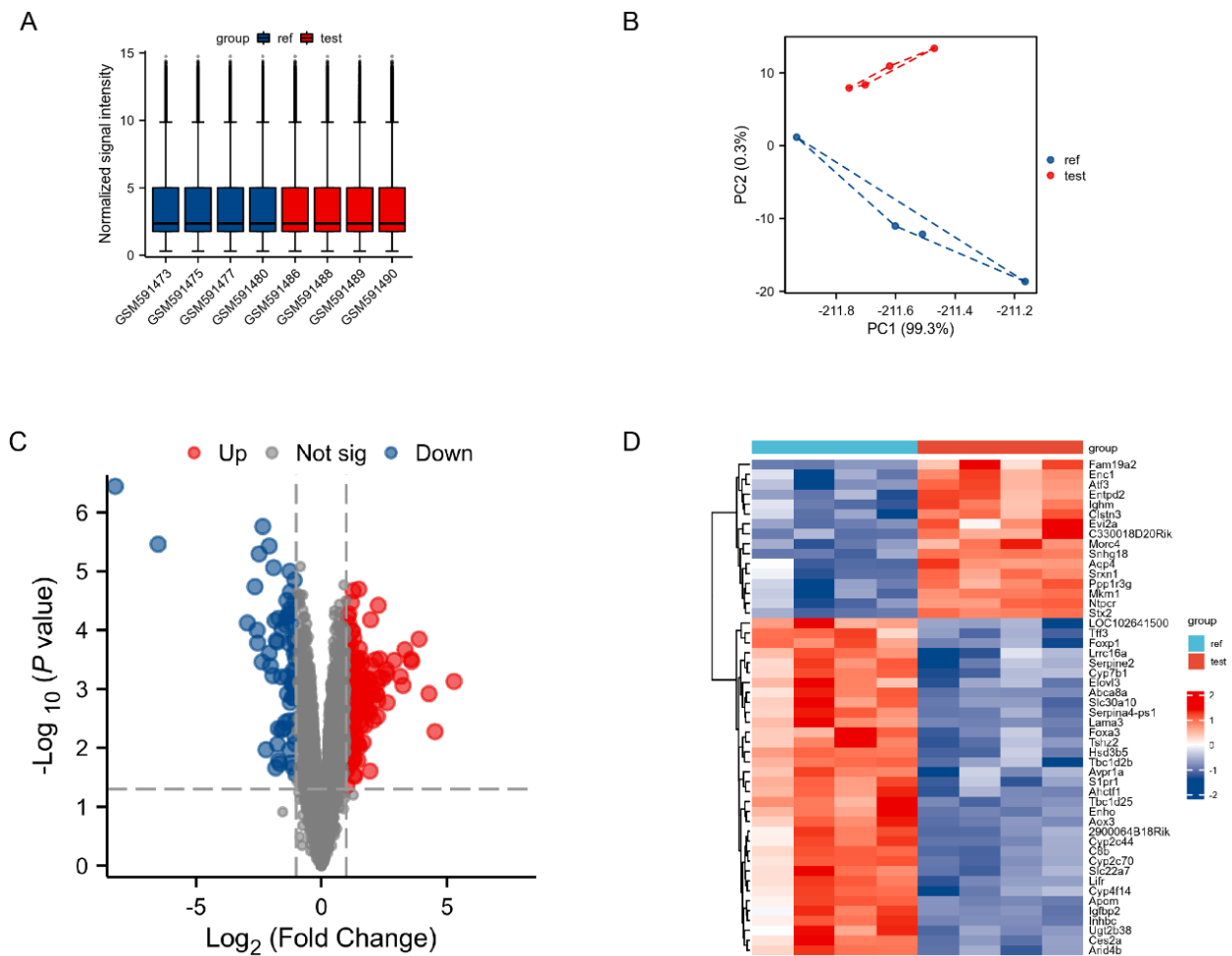
The R language “limma” package (<http://www.bioconductor.org/packages/release/bioc/html/limma.html>) was applied to identify differentially expressed genes (DEGs), where  $|\log_{2}FC| > 1$  and  $p < 0.05$  was used as the screening threshold. Furthermore,  $|\log_{2}FC| > 1$  and  $p < 0.05$  were set as thresholds for identifying DEGs in GSE24031. Heatmaps were drawn using the “pheatmap” package (<http://www.bioconductor.org/packages/release/bioc/html/pheatmap.html>). We then used NAFLD-related microarrays from the GEO database GSE24031. In order to determine the pathways related to NAFLD, GSEA was performed on the NAFLD-related microarray GSE24031 using GSEA software (v4.0.0) and HALLMARK database,  $|NES| > 1$ ,  $p\text{-val} < 0.05$ ,  $q\text{-val} < 0.25$  as the threshold, and rich Collection of pictures.

### 2.2. Gene functional enrichment analysis and gene set enrichment analysis (GSEA)

Carry out gene function enrichment analysis to find the key signaling pathways involved in NAFLD. The Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis of NAFLD-related key genes was performed using the R language "clusterProfiler" package (<http://www.bioconductor.org/packages/release/bioc/html/clusterProfiler.html>), thresholded at  $p < 0.05$ , and bubble plots drawn. In addition, Gene Ontology (GO) and KEGG enrichment analysis were performed on NAFLD-related candidate genes, and bubble charts were drawn.

### 3. RESULTS

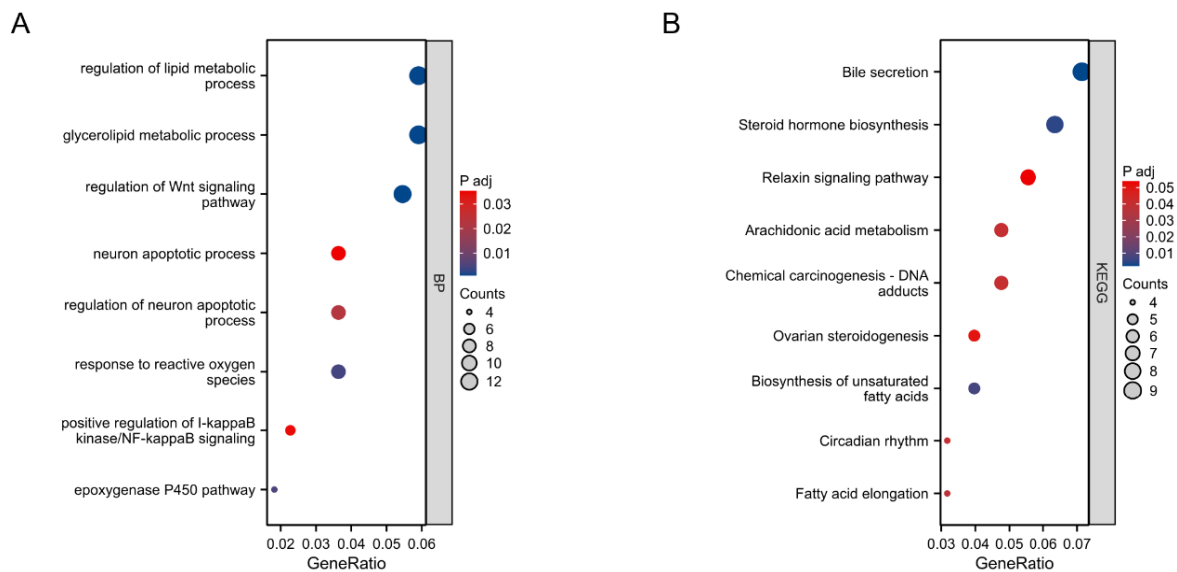
#### 3.1. Special Signs



**Figure 1.** A, Box plot of genes in NAFLD key gene screening GSE46169 (normal group, n = 4; model group, n = 4). B, PCA plot. C, Volcano plot The red dots in Figures A and B represent significantly up-regulated genes, the green dots represent significantly down-regulated genes, and the black dots represent genes with insignificant expression differences. D cluster heat map

The NAFLD related microarray GSE24031 was retrieved through the GEO database. As shown in Figure A, check the sample correction situation through the box plot. The horizontal line in the middle of the box represents the median, the top of the box represents the upper quartile, and the bottom of the box represents the lower quartile. If there are black dots above and below the box, it means there are outliers in this sample. Generally, we only need to pay attention to whether the median line of each sample is on the same horizontal line. We find that the sample has been corrected. The PCA plot shows the details of the differences between the groups, indicating that the modeling was successful. The volcano plot shows up-regulated and down-regulated genes, and the heat map shows that differential genes present obvious cluster distribution according to groups.

### 3.2. Gene Ontology and Pathway Enrichment Analysis Illuminate Key Biological Processes and Pathways



**Figure 2.** A, Box plot of genes in NAFLD key gene screening GSE46169 (normal group, n = 4; model group, n = 4).

#### Gene Ontology and Pathway Enrichment Analysis Illuminate Key Biological Processes and Pathways

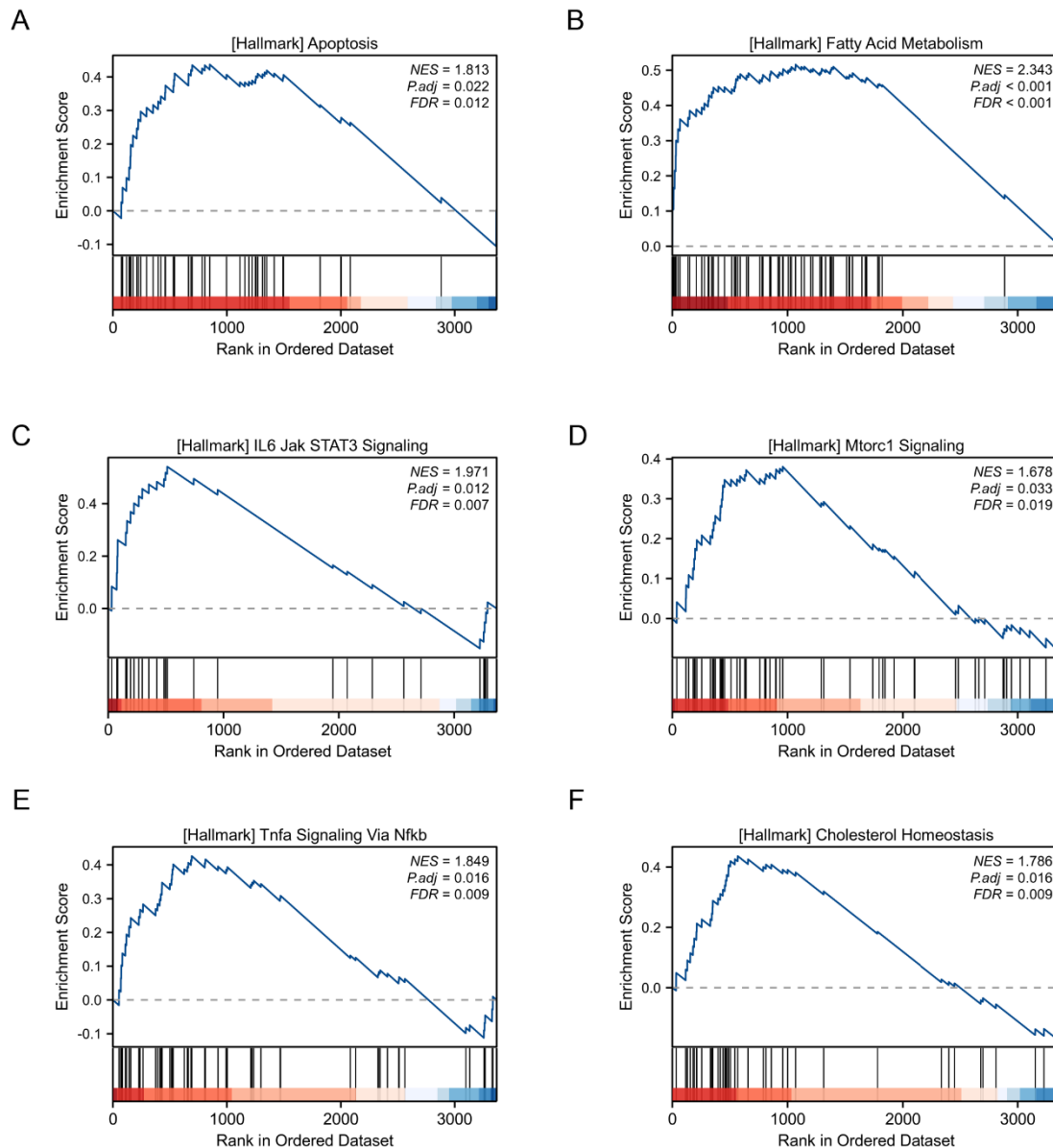
The gene ontology (GO) enrichment analysis yielded a focused view of the biological processes significantly affected in NAFLD. As depicted in Figure A, processes such as the regulation of lipid metabolic process, glycerolipid metabolic process, and response to reactive oxygen species were prominently altered, which is consistent with the known dysregulation of lipid metabolism and oxidative stress in NAFLD. Notably, the neuron apoptotic process and its regulatory mechanisms were also significantly enriched, highlighting the potential involvement of apoptotic pathways in NAFLD pathogenesis beyond the commonly affected hepatic cells. These findings align with emerging evidence suggesting NAFLD's systemic implications, including its neurological impact.

The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis, shown in Figure B, further reinforced the role of metabolic processes, with pathways like bile secretion and steroid hormone biosynthesis being highlighted. Intriguingly, pathways typically associated with carcinogenesis, such as the DNA adducts pathway, were also enriched, which may suggest a link between NAFLD progression and an increased risk of hepatic malignancies. Pathways related to circadian rhythm and fatty acid elongation were identified as well, underscoring the complex interplay between metabolism and cellular processes in the liver.

Both analyses indicated that adjusted P-values (P adj) were significantly low for the identified processes and pathways (P adj < 0.05), reinforcing the robustness of our findings. The GeneRatio, an indication of the proportion of differentially expressed genes found within a particular category, showed substantial representation across the enriched processes and pathways, suggesting their prominent roles in the disease mechanism.

The bubble charts facilitated the visualization of the enriched GO terms and KEGG pathways, with the size of each bubble corresponding to the count of genes involved, and the color intensity reflecting the level of significance. The neuron apoptotic process, in particular, demonstrated both a high gene count and significance level, emphasizing its potential importance in NAFLD's progression.

### 3.3. Gene Ontology and Pathway Enrichment Analysis Illuminate Key Biological Processes and Pathways



**Figure 3.** GSEA enrichment and NAFLD-related pathways.

As shown in Figures A-F, in order to explore the specific mechanisms affecting NAFLD, we used GSEA to identify the main enrichment pathways of NAFLD. Based on microarray GSE24031, the results showed that differential genes were mainly enriched in fatty acid metabolism, lipogenesis, cholesterol metabolism, apoptosis, and MTORC1 signaling pathway.

## 4. DISCUSSION

The current investigation into the molecular underpinnings of NAFLD following hepatic steatosis has identified apoptosis as a pivotal biochemical pathway implicated in disease progression. The bioinformatics approach employed allowed for a high-throughput analysis of differentially expressed genes (DEGs), highlighting several candidate genes and pathways with potential roles in NAFLD pathogenesis. Enrichment analyses further emphasized the dysregulation of lipid metabolism and oxidative stress, aligning with existing literature that associates these processes with the disease's onset and progression [8-10].

Notably, the enrichment of neuronal apoptotic processes invites reconsideration of NAFLD as a disease with potentially wide-ranging systemic effects, extending beyond liver pathology. This observation aligns with reports of NAFLD's extrahepatic manifestations and underscores the need for a holistic view of the disease that considers its multifaceted nature. The neuron apoptotic process, along with other identified pathways, may serve as a nexus for understanding the broader implications of NAFLD, including neurological impacts.

The association between pathways typically involved in carcinogenesis, such as DNA adduct formation, and NAFLD progression provides a putative link to the increased risk of hepatic malignancies observed in advanced NAFLD cases. This association with chemical carcinogenesis pathways suggests that chronic liver disease, particularly NAFLD, may create a hepatic environment conducive to oncogenic transformations. These findings advocate for heightened vigilance in the clinical follow-up of patients with NAFLD for potential malignant transformations.

Moreover, the KEGG pathway analysis shed light on the roles of circadian rhythm and fatty acid elongation, pathways not traditionally at the forefront of NAFLD research. These insights suggest a potential influence of circadian biology on lipid homeostasis and energy metabolism in the liver, which could inform the timing of interventions or the consideration of lifestyle factors that may influence disease outcomes [11-16].

The gene set enrichment analysis (GSEA) reinforced these findings by demonstrating significant enrichment in pathways relating to fatty acid metabolism, lipogenesis, cholesterol metabolism, apoptosis, and MTORC1 signaling pathway. Such convergence of bioinformatics approaches substantiates the involvement of these pathways in NAFLD and illustrates the disease's complexity. These pathways, especially those related to apoptosis and the MTORC1 signaling pathway, provide a compelling argument for targeted therapeutic strategies that may mitigate the apoptotic drive in NAFLD and potentially disrupt the progression to more severe disease states [17].

The identification of genes such as *Lcn2*, *Mt1*, *Egr1*, *Jun*, *Nqo1*, *Btg2*, *Foxq1*, and *Hyou1* enriched in key pathways of apoptosis also opens avenues for future research, including the development of novel biomarkers for NAFLD progression. The robustness of the dataset, characterized by low adjusted P-values and significant GeneRatio, lends credibility to these genes as potential candidates for further functional studies [18, 19].

In conclusion, our study has established a solid foundation that apoptosis and related pathways are integral to the pathogenesis of NAFLD. The potential targets identified here not only provide a deeper understanding of NAFLD at the molecular level but also pave the way for the development of new diagnostic and therapeutic approaches. However, experimental validation in cell models and clinical settings is necessary to confirm the bioinformatics predictions and to explore the causative relationship between these pathways and NAFLD progression [20].

Future studies are warranted to explore the mechanistic roles of the identified genes in NAFLD and to validate the therapeutic potential of modulating these pathways. Longitudinal studies examining the expression of these genes and the activity of the pathways they influence over the course of NAFLD progression could provide invaluable insights into disease dynamics and intervention points. Moreover, the exploration of non-invasive biomarkers for the early detection of NAFLD progression remains a priority, which could greatly enhance patient outcomes through timely intervention [21,22].

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