

Bioinformatics analysis of potential ferroptosis and non-alcoholic fatty liver disease biomarkers

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Abstract. Ferroptosis plays a crucial role in the development of non-alcoholic fatty liver disease (NAFLD). In this study, we aimed to use a comprehensive bioinformatics approach and experimental validation to identify and verify potential ferroptosis-related genes in NAFLD. We downloaded the microarray datasets for screening differentially expressed genes (DEGs) and identified the intersection of these datasets with ferroptosis-related DEGs from the Ferroptosis database. Subsequently, ferroptosis-related DEGs were obtained using SVM analysis; the LASSO algorithm was then used to identify six marker genes. Furthermore, the CIBERSORT algorithm was used to estimate the proportion of different types of immune cells. Subsequently, we constructed drug regulatory networks and ceRNA regulatory networks. We identified six genes as marker genes for NAFLD, demonstrating their robust diagnostic abilities. Subsequent functional enrichment analysis results revealed that these marker genes were associated with multiple diseases and play a key role in NAFLD *via* the regulation of immune response and amino acid metabolism, among other pathways. The expression of hepatic EGR1, IL-6, SOCS1, and NR4A1 was significantly downregulated in the NAFLD model. Our findings provide new insights and molecular clues for understanding and treating NAFLD. Further studies are needed to assess the diagnostic potential of these markers for NAFLD.

Key words: Bioinformatics analysis — Non-alcoholic fatty liver disease — Ferroptosis — Biomarkers

Introduction

Non-alcoholic fatty liver disease (NAFLD) is usually diagnosed through exclusion criteria, including alcohol use, viral infections, and other liver-damaging factors. It manifests as an accumulation of excessive fat and is often associated with metabolic dysfunctions, including insulin resistance

and hyperlipidemia (Targher et al. 2021). The majority of hepatic lipid metabolism abnormalities stem from imbalances in lipid synthesis and metabolism, resulting in the excessive accumulation of lipids and subsequent dysfunction of hepatocytes. NAFLD comprises two distinct conditions: simple hepatic steatosis and non-alcoholic steatohepatitis (NASH). Left untreated, these conditions may advance to cirrhosis and liver cancer, underscoring NAFLD's significance as a critical public health issue. This prevalent chronic liver disorder affects a quarter of the global population, with its incidence escalating annually (Younossi et al. 2018). According to a mathematical model, the epidemic of obesity and diabetes is expected to increase the incidence of NAFLD, and the death rates from associated illnesses will double (Estes et al. 2018). Two concepts, namely “second hit” and “multiple hits,” elucidate the pathophysiology of NAFLD.

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Current comprehension indicates that the onset of NAFLD is influenced by genetic and epigenetic factors, mitochondrial dysfunction, endoplasmic reticulum stress, and insulin resistance (Chen et al. 2020). Nevertheless, the absence of sensitive and specific biomarkers for early clinical detection and treatment of NAFLD presents a significant challenge. Consequently, investigating the mechanisms underlying NAFLD is imperative for identifying prospective therapeutic targets and devising innovative treatment approaches.

Ferroptosis is a recently discovered form of programmed cell death triggered by iron-mediated lipid peroxidation (Li J et al. 2020). Hepatocyte lipid accumulation-induced programmed cell death can lead to liver tissue damage and inflammation (Manne et al. 2018). Ferroptosis could significantly activate the inflammatory response in NASH. Iron overload is prevalent among NAFLD patients, with iron-induced lipid peroxidation playing a substantial role in NAFLD pathogenesis (Gao et al. 2021). Malondialdehyde and 4-hydroxynonenal, the secondary products of lipid peroxidation, have been demonstrated in multiple studies to serve as markers of oxidative stress in individuals with NAFLD (He et al. 2023). Antioxidants that inhibit lipid peroxidation effectively reduce serum aminotransferases in patients with NAFLD (Violi and Cangemi, 2010). Furthermore, regulators of iron metabolism show a substantial elevation in NAFLD mice induced by the methionine-choline deficient (MCD) diet. Administration of ferroptosis inhibitors (Ferrostatin-1 and Liproxstain-1) effectively attenuated liver injury, inflammation, and fibrosis in MCD diet-fed mice (Li X et al. 2020). Hepatic ferroptosis was shown to occur before apoptosis in hepatocytes (Tsurusaki et al. 2019; Zhang et al. 2021), suggesting that it may be a promising therapeutic target for treating or preventing NAFLD. Hence, we employed bioinformatics analysis to investigate and validate the effectiveness of genes linked to ferroptosis as biomarkers for NAFLD, along with their roles in the hepatic immune system.

Material and Methods

Data sources

In this study, the microarray datasets of NAFLD and normal samples were downloaded from the GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). The GSE89632 and GSE63067 datasets, which contained 31 healthy control liver samples and 50 NAFLD liver samples, were obtained from the GPL14951 and GPL570 platforms (Arendt et al. 2015; Frades et al. 2015), ferroptosis-related genes (FRGs), which include 369 driver genes, 348 suppressor genes, and 11 marker genes, were discovered in the FerrDb database (<http://www.zhounan.org/ferrdb>) and the detailed genes were shown in Sup-

plementary material (Table S1) (Zhou and Bao 2020). The drug-gene interaction database (DGIdb) (<https://dgidb.org/>) was employed for drug-targeting marker gene prediction (Wagner et al. 2016).

Data processing and analysis of expression differences

Initially, the probes undergo annotation using the dataset's annotation file. In cases where multiple queries correspond to the same gene, the average expression value is adopted as the gene expression value following ID transformation. GSE89632 and GSE63067 were amalgamated as the training set for analysis in the main body of this study, and batch correction of the dataset was performed using the "SVA" package in R software (Leek et al. 2012). PCA plots were used to illustrate the batch effect of the normalized dataset. In addition, the limma package was used for differential analysis to identify differentially expressed genes (DEGs) (Table S2) based on the screening criteria $|\log FC| > 1$ and $p\text{-adj} < 0.05$ (Colaprico et al. 2016), and intersected with FRGs to obtain differentially expressed ferroptosis genes (DE-FRGs) for subsequent analysis.

Functional analyses for the DE-FRGs

Gene ontology (GO) functional enrichment analysis comprises three components: biological process (BP), cellular component (CC), and molecular function (MF). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis is widely employed to elucidate biological mechanisms and pathways. The "clusterProfiler" R package (version 3.16.1) was utilized to conduct GO and KEGG pathway enrichment analyses (Yu et al. 2012). Results with significant differential enrichment were filtered out using $p\text{-value} < 0.05$ and $p\text{-adj} < 0.01$ as a criterion.

Identification of optimal diagnostic gene biomarkers for NAFLD

The least absolute shrinkage and selection operator (LASSO) with 10-fold cross-validation and support vector machine-recursive feature elimination (SVM-RFE) were employed to discover supplementary gene markers for diagnosing NAFLD. The LASSO regression algorithm, a renowned linear prediction technique, predicts outcomes by utilizing regression coefficients (Motamedi et al. 2022). SVM-RFE is a machine learning method rooted in support vector machines, which identifies optimal core genes through iterative elimination of feature vectors generated by the support vector machine (Zhao et al. 2022). The SVM algorithm projects input data into a higher-dimensional feature space by mapping a kernel function, thus facilitating classification compared with the original feature space (Uddin et al.

2019). Our objective was to ascertain the optimal diagnostic gene markers for NAFLD by amalgamating the biomarkers acquired from both algorithms. Additionally, we conducted receiver operating characteristic (ROC) curve analysis on the training set utilizing the “pROC” package (Chai et al. 2023). The area under the curve (AUC) was calculated to validate the diagnostic effectiveness of the primary gene marker. Furthermore, our objective was to build a logistic regression model leveraging the predictive capabilities of the GLM package in R, incorporating six marker genes. This model was utilized to predict sample types within the training set and evaluate diagnostic performance using the ROC curve.

Single-gene Gene Set Enrichment Analysis (GSEA) enrichment analysis

The R package GSEA tool was utilized to calculate correlations between the six marker genes and all other genes in the training set, enabling further exploration of the pathways correlated with the marker genes (Subramanian et al. 2005). The KEGG signaling pathway set was used as a preset set to find its abundance in the gene set. Each marker gene's specific enrichment findings were merged into Table S3.

Single-gene Gene Set Variation Analysis (GSVA) enrichment analysis

GSVA represents an unsupervised and nonparametric approach to gene set enrichment analysis that estimates the score attributed to a particular pathway or signature based on transcriptomic data (Hänzelmann et al. 2013). In the current study, we conducted GSVA analysis for each marker gene utilizing the KEGG pathway set as the background gene set. We downloaded the “c2.cp.kegg.symbols” file from GSVA's MSigDB database (Qin et al. 2023). Additionally, the R package “limma” was employed to assess the differences in GSVA scores among samples categorized by high and low expression levels of the marker genes. For cases where $t > 0$, activation of the pathway was assumed in the high-expression group and conversely, if $t < 0$, initiation of the pathway was presumed in the low-expression group.

Immune infiltration analysis

As previous studies have done, the CIBERSORT algorithm calculates the proportion of different immune cell types based on the expression levels of immune cell-related genes (Yu et al. 2023; Zhao et al. 2023). The output of the 22 infiltrated immune cells was integrated to generate a matrix of immune cell fractions for analysis (Liu et al. 2024). The CIBERSORT program package was employed to assess the proportion of 22 immune cell types in liver samples from the training set in this study. Each sample's total proportion

of the 22 immune cell types equaled 1, with $p < 0.05$ indicating a significant correlation (Table S4). Bar graphs illustrate the distribution of the 22 immune cell types across different samples, and the vioplot was utilized to visualize differences between NAFLD and normal immune cell groups. A heat map depicting the correlation between marker genes and the 22 immune cell types was generated using the “corrplot” R package (Hu 2020).

Construction of ceRNA network

Targetscan, MiRanda, and MiRDB databases were used to predict mRNA-miRNA couples based on six marker genes, and only miRNAs predicted by all three databases concurrently were kept (John et al. 2004; McGeary et al. 2019; Chen and Wang 2020). A ceRNA network of mRNA-miRNA-lncRNA was created by searching the anticipated miRNAs in the spongeScan database and screening the miRNA-lncRNA pairings (Furió-Tarí et al. 2016). Finally, we used Cytoscape software to visualize the ceRNA network (Doncheva et al. 2019).

Establishment of the mouse NAFLD model

A total of twenty C57BL/6 mice (males, six weeks old) were purchased from Liaoning Changsheng biotechnology Co. Ltd (Benxi, China). The mice were housed in a temperature-controlled chamber ($22 \pm 2^\circ\text{C}$) with a 12-hour light/dark cycle. Twenty male C57BL/6 mice, aged six weeks, were randomly assigned to either a high-fat diet (HFD) group or a control (CON) group, each consisting of 10 mice. Mice in the CON group were fed a standard chow diet for approximately 20 weeks, with 12% of calories from fat, 29% from protein, and 59% from carbohydrates. Mice in the HFD group were fed a diet containing 60% fat (d12492, mediceience, Jiangsu, China) for about 20 weeks before euthanasia and sampling. After 20 weeks, all mice were anesthetized with 2% isoflurane, followed by euthanasia *via* cervical dislocation, with efforts made to minimize pain. Tissue samples were collected, fixed in 10% formalin for 24 hours, dehydrated, and embedded in paraffin. Tissue sections, 4 μm thick, were prepared, and hematoxylin and eosin (H&E) staining was performed. All methods were carried out in accordance with the Animal Care Guidelines of Harbin Medical University (2022-DWSYLLCZ-20), and all methods are reported in accordance with ARRIVE guidelines for the reporting of animal experiments.

Quantitative RT-PCR analysis

Total RNA was extracted from homogenized tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Subsequently, 1 μg of total RNA was reverse transcribed using

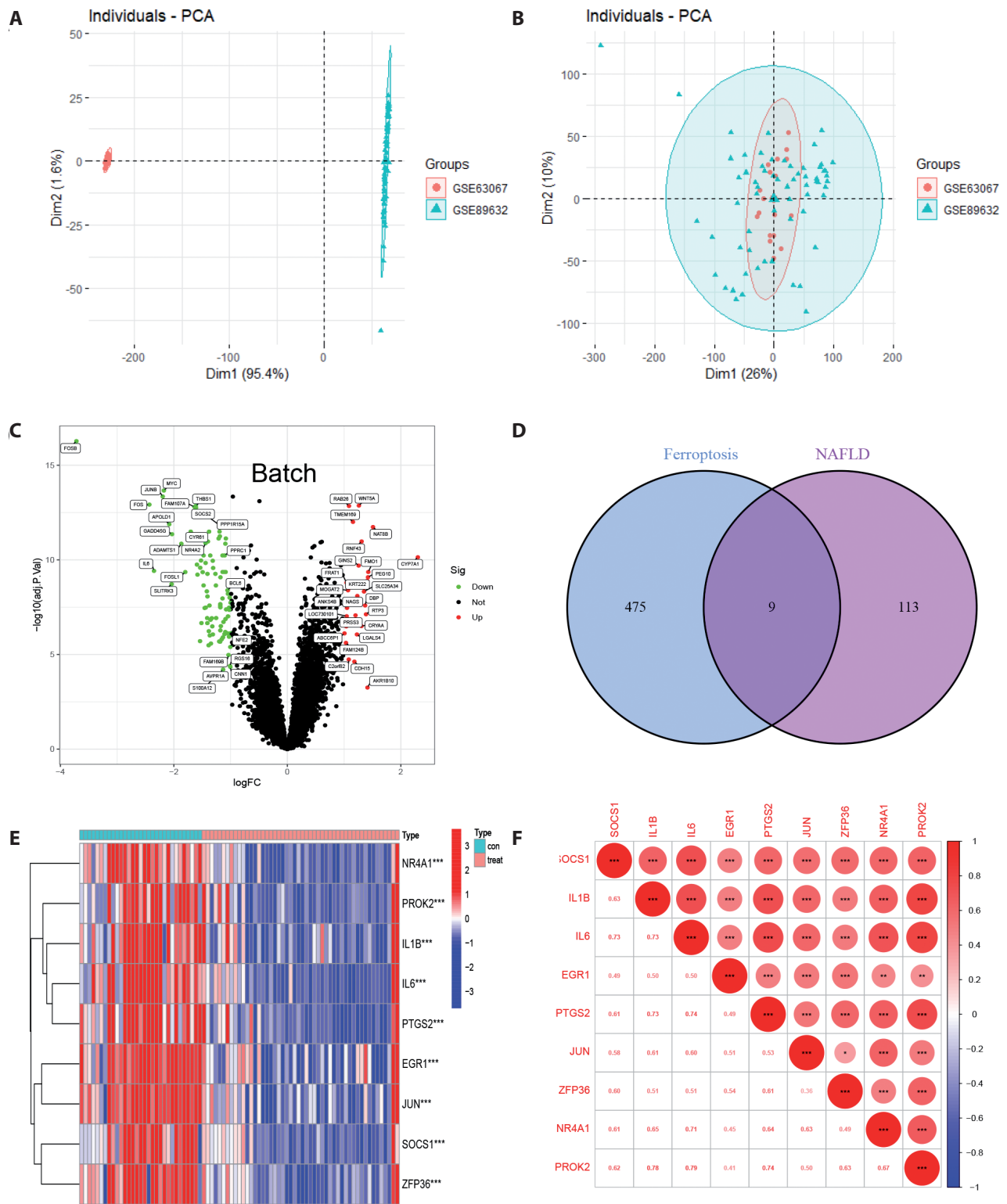


Figure 1. PCA plots illustrating batch effects between the data sets before (A) and after (B) normalization. C. After batch correction, we identified the DEGs in the training set, and in the volcano plot, up-regulated genes are shown in red and down-regulated genes are shown in green. D. DEGs were intersected with ferroptosis-related genes to obtain 9 ferroptosis-related differential genes (DE-FRGs). E. Heat map showing the expression pattern of NAFLD DE-FRGs in the sample. F. The correlation of 9 NAFLD DE-FRGs. They are all positively correlated with each other. (See online version for color figure.)

PrimeScript reverse transcriptase (Takara, Kusatsu, Japan), and 2*SYBR Green qPCR (Vazyme, Nanjing, China) was then performed for gene expression analysis. The reaction system was 10 μ l, with 2* SYBR Green qPCR 5 μ l and primers (each) 0.2 μ l. The PCR conditions were 95°C for 30 s, followed by 45 cycles of 95°C for 10 s, 60°C for 30 s. The primer sequences are listed in Table S5.

Statistical analysis

The experimental data were analyzed using GraphPad Prism 9.0. Results are expressed as mean $\pm$ standard deviation (SD). Student's *t*-test was employed to compare differences between the two groups, with significance set at $p < 0.05$.

Results

Identification of potential DE-FRGs in NAFLD

The GSE89632 and GSE63067 datasets were merged, normalized, and subjected to batch correction. In Figure 1A, principal component analysis (PCA) results before batch correction are presented for the two datasets, each represented by different colors. The plot indicates complete separation between the two datasets without overlap. Figure 1B displays PCA results after batch correction, revealing the successful elimination of the batch effect, thus enabling the combination of the two datasets for further analysis. According to the screening conditions of $|\log_{2}FC| > 1$ and $p_{adj} < 0.05$, 122 DEGs were significantly differentially expressed in NAFLD tissues compared to normal tissues, which were 28 up-regulated genes and 94 down-regulated genes. Among them, nine genes exhibited differential expression in NAFLD tissues compared to normal tissues and were identified as ferroptosis-related genes, all of which were downregulated (Fig. 1D). The expression patterns of DEGs and DE-FRGs in the samples were illustrated in the volcano plot and the clustering heatmap, respectively (Fig. 1C,E). Figure 1F displays the interactions between these genes. Strong positive correlations were observed between IL-6 and PTGS2, NR4A1, PROK2, IL-1B, and SOCS1. Furthermore, PTGS2 and PROK2 exhibited a significant positive correlation with IL-1B. Although the associations between EGR1, JUN, ZFP36, and other DE-FRGs were notable, the correlations were not particularly high.

Functional analyses for the DE-FRGs

GO and KEGG enrichment studies clarified the biological processes and pathways connected to NAFLD DE-FRGs. The results showed that BP enrichment analysis indicated that DE-FRGs was significantly associated with “response

to lipopolysaccharide,” “response to molecule of bacterial origin,” and “fat cell differentiation.” CC enrichment was associated with “cytoplasmic ribonucleoprotein granule,” “ribonucleoprotein granule.” Moreover, MF was associated with “DNA-binding transcription activator activity, RNA polymerase II-specific” and “DNA-binding transcription activator activity” (Fig. 2A,B). In particular, KEGG analysis showed significant enrichment in several immune-related pathways (IL-17 signaling pathway, C-type lectin receptor signaling pathway, TNF signaling pathway) (Fig. 2C,D).

Six DE-FRGs were identified as diagnostic genes for NAFLD

To evaluate the diagnostic potential of DE-FRGs and distinguish between individuals with NAFLD and healthy controls, the machine learning algorithms LASSO and SVM-RFE were employed to identify significant DE-FRGs. Following core gene filtration, 10-fold cross-validation of LASSO identified six genetic markers, as shown in Figures 3A and B. Subsequently, after identifying nine DE-FRGs, SVM-RFE determined the optimal combination of feature genes for NAFLD diagnosis (Fig. 3C). The intersection of marker genes from LASSO and SVM-RFE models led to the selection of six marker genes (EGR1, IL-6, JUN, NR4A1, SOCS1, and ZFP36) as diagnostic genes for NAFLD for further investigation (Fig. 3D).

Logistic regression models were developed using the R package GLM based on the aforementioned six characteristic genes. The ROC curves demonstrated that these models, utilizing the six marker genes, effectively discriminated between standard and NAFLD samples ($AUC = 0.946$; Fig. 3E). Additionally, ROC curves were constructed for these six marker genes to evaluate their capability in distinguishing between samples with NAFLD and those without it. As illustrated in Figure 3F, the AUCs of all six genes exceeded 0.8, indicating their high diagnostic accuracy. These findings suggest that the logistic regression model surpasses individual marker genes in terms of accuracy and specificity when differentiating NAFLD samples from control samples.

Marker genes were intimately connected to several NAFLD-related pathways

We conducted a single-gene GSEA-KEGG pathway analysis to further investigate the potential role of marker genes in distinguishing NAFLD samples from control samples. Figure S1 displays the top six pathways enriched for each marker gene. After comprehensive research, we found that these genes are involved in ribosomes, cell cycle, immune response (T-cell receptor signaling pathway, extracellular matrix-receptor interaction, and B-cell receptor signaling pathway), amino acid synthesis and metabolism (calcium, leucine, and isoleucine degradation, tyrosine metabolism, and amino acid



Figure 2. DE-FRG functional analyses. Circles (A) and barplots (B) were used to display the GO findings. Bubble (C) and circle (D) graphs were used to display the KEGG findings. (See online version for color figure.)

biosynthesis), and various disease pathways (type 2 diabetes, hypertrophic cardiomyopathy, dilated cardiomyopathy, tumors, and renal cell carcinoma) were abundant. Additionally, we observed that these marker genes were enriched in the “P53 signaling pathway,” “MAPK signaling pathway,” “NOD-like receptor signaling pathway,” “chemokine signaling pathway,” “JAK-STAT signaling pathway,” “Toll-like receptor signaling pathway,” and “Wnt signaling pathways.”

We used GSVA to compare the activation pathways across groups with high and low expression levels based on the degree of expression of each marker gene. As shown in Figure S2, the low expression of EGR1 may be involved in the pathogenesis of NAFLD through immune response (extracellular matrix receptor interaction), “hematopoietic cell signaling,” and “cytokine interaction.” In contrast, its high expression is associated only with the “protein export” pathway. Upregulation of IL-6 activates pathways related to the cell cycle, amino acid metabolism, and DNA production, whereas its downregulation activates “hypertrophic cardiomyopathy,” “right ventricular arrhythmogenic,” and “myocardial contraction.” In addition to EGR1 and IL-6, JUN, NR4A1, and SOCS1 were associated with the NOD-like receptor signaling pathway in the pathogenesis of NAFLD. Furthermore, low expression of IL-6, JUN, NR4A1, SOCS1, and ZFP36 was all directly related to the “JAK-STAT signaling pathway.”

Immune landscape analysis

The immunological microenvironment and NAFLD are inextricably linked (Van Herck et al. 2019). Thus, using the CIBERSORT method, we investigated the variations in the immune microenvironment between NAFLD and normal samples. The results showed that the top three classes of immune cells in NAFLD tissues were M2 macrophages, $\gamma\delta$ T cells, and resting-memory CD4 T cells, which accounted for approximately 50% of the immune cell infiltration in NAFLD (Fig. 4A). We observed that the infiltration of neutrophils, plasma cells, activated dendritic cells, activated mast cells, and naive B cells was considerably reduced in NAFLD tissues compared to normal tissues. Conversely, the infiltration of M1 and M2 macrophages, resting dendritic cells, and resting mast cells was significantly increased in NAFLD tissue (Fig. 4B).

Finally, we aimed to further study the relationship between our discovered marker genes and immune cells. To this end, we investigated the correlation between the expression of the six marker genes and the presence of 22 resistant cell types, as shown in Figure 4C. Regarding the expression of IL-6, NR4A1, SOCS1, and ZFP36, activated mast cells and resting mast cells had positive and negative correlations, respectively. Additionally, we found that the expression of IL-6 was positively and negatively associated with activated

dendritic cells and M2 macrophages, respectively. Furthermore, SOCS1 was positively correlated with activated dendritic cells and monocytes but negatively correlated with $\gamma\delta$ T cells. ZFP36 was negatively correlated with resting dendritic cells but positively correlated with monocytes and activated Mast cells. Finally, we observed that NR4A1 was positively correlated with monocytes.

Prediction of marker gene-targeted drugs

We utilized the DGIdb database to identify additional potential therapeutic targets for the marker genes and utilized Cytoscape tools to visualize the associations (Fig. S3). We assessed 53 medications targeting marker genes: 2 for SOCS1, 20 for IL-6, 1 for EGR1, 20 for JUN, and 10 for NR4A1. However, no drugs targeting ZFP36 were found in the DGIdb database. Among the 20 drugs targeting IL-6, six were IL-6 inhibitors. The relationship between marker genes and medications targeting them is presented in Table S6.

A ceRNA network based on marker genes

After that, we used the Targetscan, MiRanda, MiRDB, and spongeScan databases to construct a ceRNA network based on the six marker genes. In total, 290 edges and 248 nodes were present in the network containing six marker genes, 119 miRNAs, and 123 lncRNAs (Fig. S4). Our analysis revealed that 51 lncRNAs might competitively bind hsa-miR-766-3p, hsa-miR-149-3p, hsa-miR-561-3p, hsa-miR-324a-5p, and 11 other miRNA-regulated SOCS1. Moreover, hsa-miR-766-3p and hsa-miR-149-3p were found to be regulated by 39 lncRNAs. For JUN, we identified 30 lncRNAs that regulated JUN expression through competitive binding with hsa-miR-524-5p, hsa-miR-1972, hsa-miR-542-3p, hsa-miR-758-3p, and hsa-miR-940. Among them, lncRNA LINC00917, AC079586.1, and RP11-157B13.7 can target both hsa-miR-542-3p and hsa-miR-758-3p. We also found that 20 lncRNAs can regulate JUN expression through the modulation of hsa-miR-515-5p, hsa-miR-760, hsa-miR-302a-5p, hsa-miR-149-5p, which in turn affects IL-6 expression. Among these, hsa-miR-515-5p expression was regulated by 15 lncRNAs. In the ceRNA network of NR4A1, eight and nine lncRNAs were identified to bind to hsa-miR-3425p and hsa-miR-665, respectively, to regulate the gene. In total, five lncRNAs were found to compete with hsa-miR-3787-3p for binding, leading to the regulation of EGR1 expression. Detailed information on the ceRNA network is provided in Table S7.

Expression of the marker gene in the NAFLD mouse model

To validate the reliability of the identified marker genes, NAFLD mouse models were induced by feeding C57BL/6 mice with an HFD for an additional 20 weeks. H&E stain-

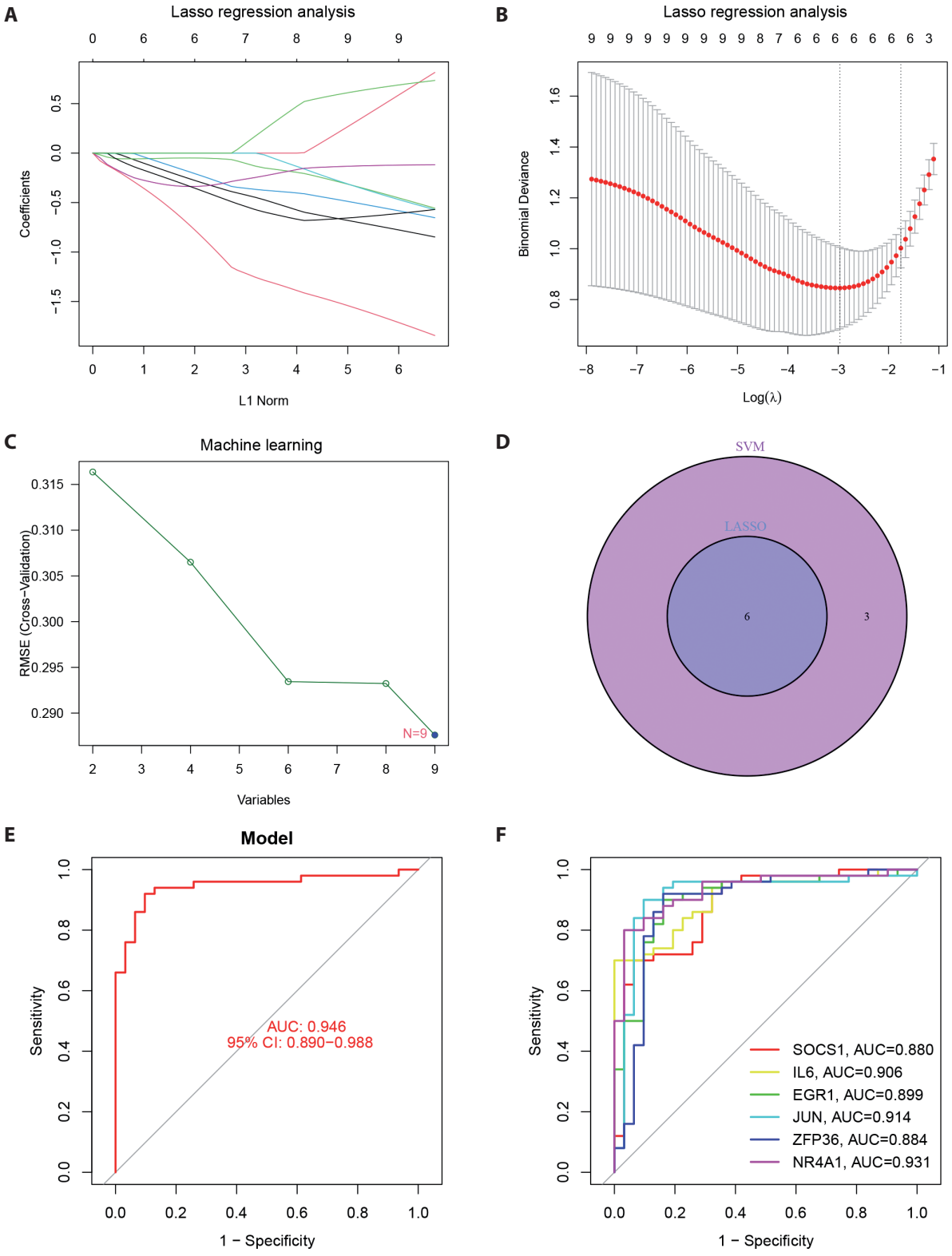


Figure 3. A. LASSO regression of the nine Ferroptosis-related DEGs. B. Partial likelihood deviance for the LASSO coefficient profiles. Six genes were selected at the value ($\lambda_{\min}$). C. SVM-RFE algorithm filtered 9 DE-FRGs and finally screened all nine genes as the best diagnostic genes. D. NAFLD marker genes were obtained from the LASSO model and the SVM-RFE model. E. Determination of logistic regression model for AUC of disease samples. F. ROC curves of 6 marker genes. (See online version for color figure.)

ing confirmed the establishment of NAFLD in the mouse models (Fig. 5A,B). Subsequently, we validated the marker genes in the NAFLD mouse model using qRT-PCR. The results indicated differential expression of the four marker genes in the livers of HFD-fed mice (Fig. 5C–F). Specifically, HFD-fed mice exhibited significantly lower hepatic expression levels of IL-6, EGR1, SOCS1, and NR4A1 compared to CON mice ($p < 0.05$).

Discussion

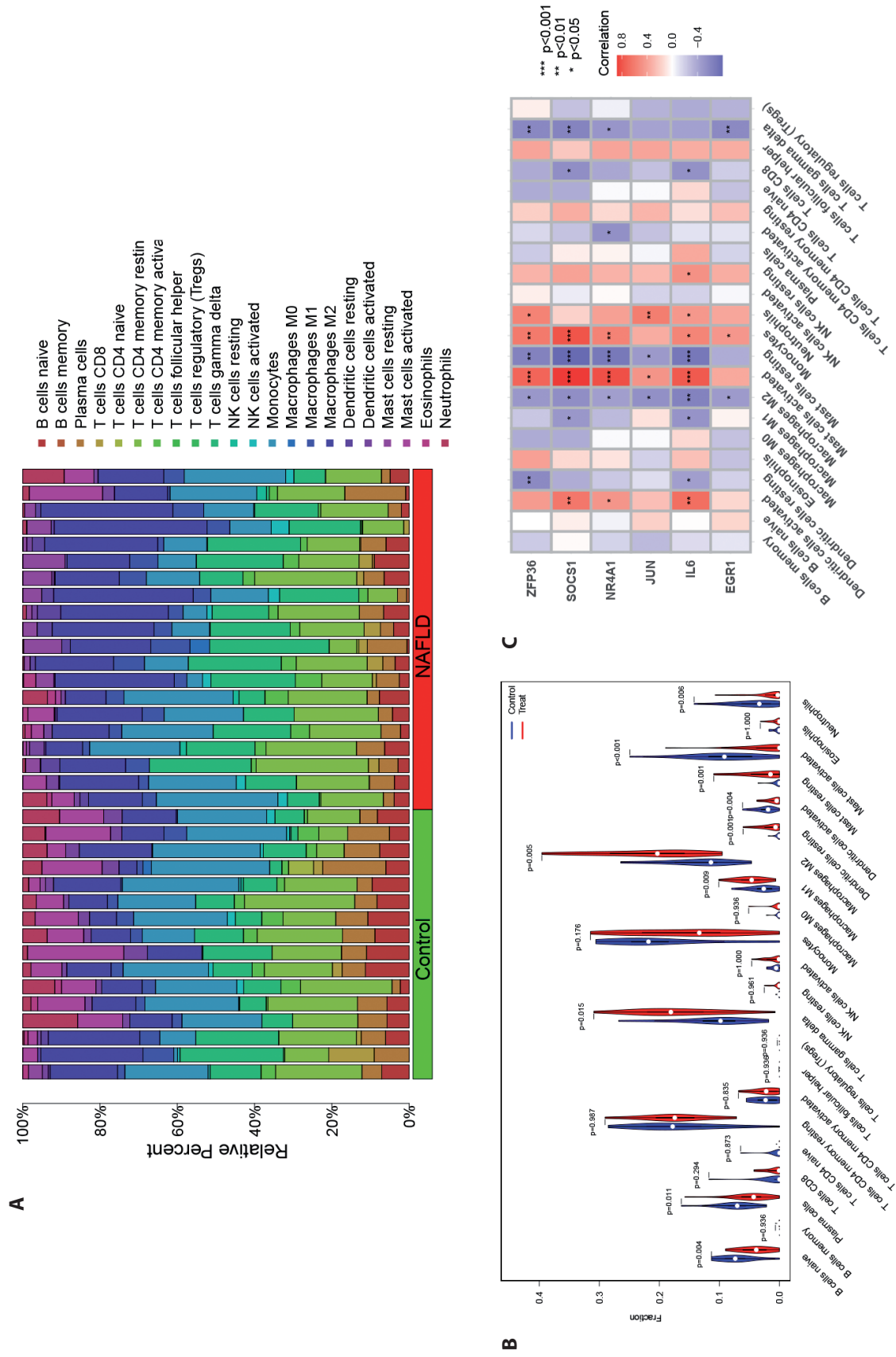
Hepatocyte death constitutes a pivotal mechanism in various liver injuries, encompassing multiple cell death pathways, including autophagy, pyroptosis, and programmed necrosis, alongside apoptosis and necrosis (Qian et al. 2021; Knorr et al. 2022). The liver is particularly susceptible to oxidative damage, with many liver disorders marked by an overabundance of iron accumulation (Wu et al. 2021). NAFLD is closely related to the mechanism of ferroptosis lipid peroxidation, and ferroptosis suppression has been shown to substantially alleviate NAFLD (Tsurusaki et al. 2019). Therefore, ferroptosis could be an essential target for treating and preventing NAFLD. In this study, we used bioinformatics approaches to analyze the genetic differences in ferroptosis between NAFLD and normal samples. We screened diagnostic markers, explored the molecular pathogenesis of ferroptosis in NAFLD, and validated these genes in an established NAFLD mouse model. Our findings offer valuable clinical insights for preventing and treating NAFLD.

In our study, we assessed six DEGs implicated in ferroptosis in NAFLD: EGR1, IL-6, JUN, SOCS1, ZFP36, and PTGS2. The AUC values of the ROC curves for these genes exceeded 0.8, indicating their reliable accuracy and specificity in distinguishing NAFLD cases from healthy samples. Due to the challenge of obtaining human liver samples, we induced NAFLD in C57BL/6 mice by feeding them a HFD to further validate the expression of these genes. This model closely mimics the development of human NAFLD and is commonly used in metabolic studies (Eng and Estall 2021). Our findings demonstrate a notable decrease in the expression levels of EGR1, IL-6, NR4A1, and SOCS1 in NAFLD mice compared to the control group, validating the forecasts generated by our analysis.

EGR1 is a transcription factor primarily involved in tissue damage, immune response, and fibrosis (Ma et al. 2023). By targeting and suppressing the expression of miR-15a-5p, the knockdown of EGR1 can increase the protein expression of GPX4 (Fan et al. 2021), which is the primary endogenous inhibitor of ferroptosis (Bersuker et al. 2019). This implies that low levels of EGR1 can contribute to the disease by preventing ferroptosis. Our findings indicate a downregulation of EGR1 expression in NAFLD mice,

implying a potential organismal defense mechanism. It is conceivable that an EGR1/GPX4 axis, by modulating ferroptosis, contributes to the regulation of NAFLD development. SOCS1, a member of the cytokine signaling repressor protein family, functions as a negative feedback regulator, inhibiting cytokine signaling pathways within cells (Dai et al. 2023). It specifically inhibits the JAK/STAT signaling pathway by targeting unphosphorylated JAK and blocking JAK phosphorylation through the kinase inhibitory region of SOCS1 (Liau et al. 2018). Furthermore, GSVA analysis of SOCS1 confirmed the upregulation of the JAK/STAT signaling pathway in the low-expression group. SOCS1 plays a critical role in regulating ferroptosis in cancer cells, thereby indirectly enhancing cellular susceptibility to lipid oxidation and ferroptosis (Yan et al. 2023). Remarkably, our model demonstrated downregulation of SOCS1 expression, suggesting its role in inhibiting ferroptosis and its protective function in NAFLD. Additionally, in mice subjected to a high-fat diet, the knockdown of NR4A1 exacerbates insulin resistance and liver steatosis, whereas NR4A1 overexpression mitigates hepatic triglyceride accumulation (Sun et al. 2021). NR4A1 regulates tumor ferroptosis (Ye et al. 2021) and is consequently involved in both ferroptosis and NAFLD. The GSVA analysis of the ferroptosis-related gene NR4A1 showed upregulation of the “JAK-STAT signaling pathway” and “NOD-like signaling pathway” in the low-expression group. This observation suggests that NR4A1 plays a role in NAFLD development by suppressing these pathways. Additionally, IL-6 induces chondrocyte ferroptosis by triggering cellular oxidative stress and perturbing iron homeostasis (Bin et al. 2021). This is because IL-6 has isozyme-specific effects on GPX expression and can reduce its transcript concentration (Bin et al. 2021). Increased IL-6 expression in the presence of hepatic steatosis is associated with the severity of NAFLD as well as insulin resistance in the liver and adipose tissue (Cobbina and Akhlaghi 2017). Nevertheless, our analysis uncovered a reduction in IL-6 expression in NAFLD. This observation indicates the complexity of IL-6 involvement in NAFLD, with the decrease in IL-6 levels potentially attributed to excessive loss induced by ferroptosis in the liver.

The liver harbors numerous innate and adaptive immune cells pivotal for preserving immune homeostasis (Kubes and Jenne 2018), and immune dysregulation is implicated in NAFLD development. Consequently, we investigated immune infiltration in the livers of both NAFLD patients and healthy individuals employing the CIBERSORT algorithm. Our results revealed significantly elevated levels of M1 and M2 macrophages, as well as resting dendritic cells and mast cells, in the livers of NAFLD patients compared to those of healthy controls. Notably, M2 macrophages and $\gamma\delta$ T cells predominated in liver tissue, underscoring their importance in the immunological milieu of NAFLD.



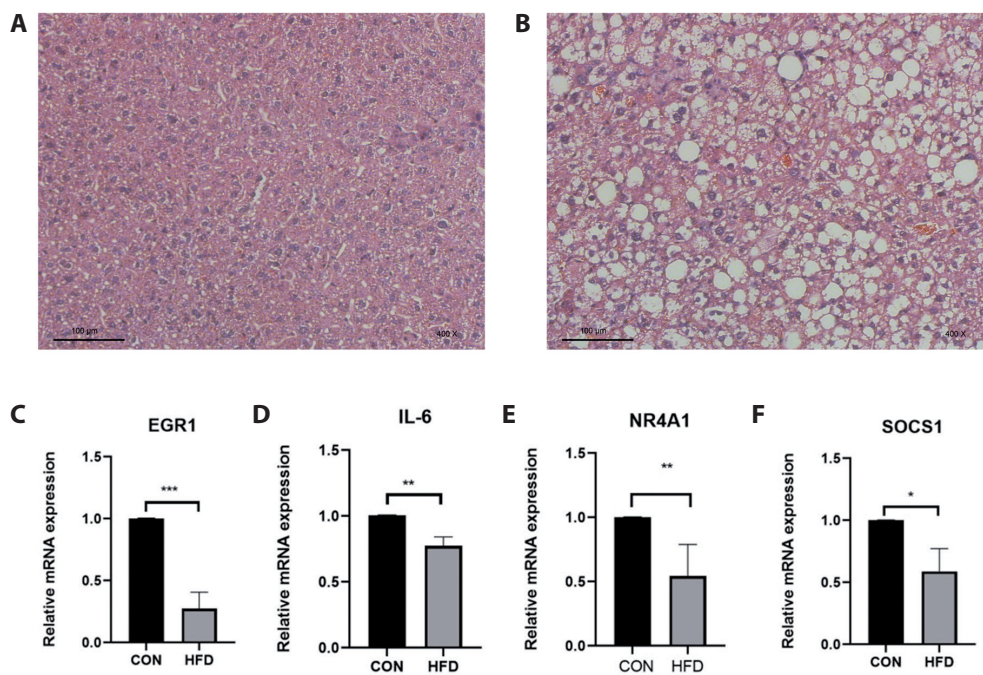


Figure 5. Marker genes expression in high-fat diet (HFD)-fed mice. **A,B.** Hematoxylin and eosin (H&E) staining of liver slices (magnification $\times 400$, scale bar = 100 μm). EGR1 (**C**), IL-6 (**D**), NR4A1 (**E**), and SOCS1 (**F**) mRNA levels were considerably lower in the HFD group than in the CON group. Values are shown as the mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Simple steatosis triggers macrophage infiltration in the liver, with the macrophage count escalating alongside the severity of steatosis and liver inflammation (Barreby et al. 2022). Macrophage activation and polarization profoundly influence NAFLD progression (Kazankov et al. 2019). During the reparative phase of NASH, hepatic macrophages polarize towards an inflammatory and pro-fibrotic M2 phenotype, thereby fostering fibrosis (Kazankov et al. 2019). This observation suggests that while dysfunctional immune cells may exacerbate liver damage, an early inflammatory response could facilitate the recovery and regeneration of liver tissue (Ahmed et al. 2021). Nevertheless, our study revealed a notable abundance of hepatic M1 macrophages in NAFLD patients compared to normal individuals. This phenomenon may stem from the plasticity and adaptability of macrophages, allowing them to partially reverse their phenotype (Sica et al. 2014). $\gamma\delta$ T cells represent a distinct subset of T lymphocytes crucial for host defense, immune surveillance, and immune system homeostasis. IL-17A, a pivotal pro-inflammatory cytokine in the liver, is predominantly secreted by $\gamma\delta$ T cells (Mills 2023). The escalating presence of $\gamma\delta$ T cells in the liver during NAFLD progression and exacerbation of steatohepatitis modulates CD4 T cells and augments IL-17 expression (Xi et al. 2019). This finding aligns with our study results, which unveiled a negative correlation between the expression levels of NAFLD ferroptosis-related genes and $\gamma\delta$ T cells. KEGG enrichment analysis revealed the predominant enrichment of these genes in the IL-17 signaling pathway, which facilitates NAFLD progression by recruiting neutrophils and stimulating ROS produc-

tion. Knockdown of the pivotal gene IL-17RA within this pathway impedes NAFLD progression (Harley et al. 2014). Consequently, $\gamma\delta$ cells, responsible for IL-17A production, emerge as the principal regulatory cells in NAFLD progression, suggesting that diminishing the hepatic enrichment of $\gamma\delta$ cells could offer an effective strategy to halt NAFLD advancement.

The expression levels of various ferroptosis-related genes exhibited consistent correlations with distinct immune cell populations. In our investigation, IL-6, SOCS1, and NR4A1 displayed a robust positive correlation with activated mast cells but a pronounced negative correlation with resting mast cells, while EGR1 showed a significant negative association with $\gamma\delta$ T cells. Additionally, the expression of these genes was downregulated in NAFLD patients. These findings suggest that the altered immune microenvironment in NAFLD patients may be associated with the expression of these genes. We hypothesize that the regulation of immune cell aggregation by the ferroptosis-related genes EGR1, IL-6, SOCS1, and NR4A1 occurs through immunological or inflammatory pathways, thereby influencing the progression of NAFLD. Nevertheless, these conclusions rely primarily on the analysis of existing data, and additional clinical trials or animal studies are warranted to corroborate and substantiate our findings.

Our analysis of drugs targeting the marker and ceRNA networks revealed six out of the 20 drugs as IL-6 antagonists: siltuximab, clazakizumab, sirukumab, elsilimomab, pf-04236921, and olokizumab. Although these drugs have been utilized in conditions like rheumatoid arthritis, Crohn's

disease, and myeloma, their efficacy in NAFLD treatment remains unreported. Additionally, genipin, which targets SREBP-1c and reduces hyperlipidemia and hepatic lipid accumulation in mice, exhibits beneficial effects, such as enhancing pancreatic β -cell function in energy metabolism for the treatment of metabolic disorders (Wang et al. 2022). Non-coding RNAs, such as miR-34a-3p, miR-23a-3p, miR-200b, and miR-200c, play essential roles in lipid metabolism in NAFLD (Li et al. 2021; Xu et al. 2021; Goncalves et al. 2023). Nevertheless, the effectiveness of these targeted drugs and non-coding RNAs in treating NAFLD remains uncertain, necessitating additional research to elucidate their exact mechanisms. Consequently, these gene-targeting drugs and non-coding RNAs selected for study merit further exploration as prospective NAFLD treatments. However, this study has several limitations. Firstly, the reliance on mouse models instead of human samples may hinder the validation of disparities in NAFLD marker gene expression. Secondly, the sample size is relatively small; underscoring the need for larger sample sizes in future animal studies and clinical cohort investigations.

Conclusion

In conclusion, this study employed a comprehensive bioinformatics approach to investigate ferroptosis-related genes and their implications in NAFLD. The expression levels of EGRI, IL-6, SOCS1, and NR4A1 were found to be significantly diminished in an established NAFLD model. This observation implies their involvement in ferroptosis in NAFLD and potentially in modulating the immunological milieu within the livers of NAFLD patients. While further experiments are necessary to validate the molecular interactions between these ferroptosis-related genes and NAFLD, our findings are crucial for advancing our understanding of NAFLD pathophysiology and treatment. Consequently, we will continue to explore these genes in future research endeavors to enhance our comprehension of NAFLD management.

Conflict of interest. The authors report no conflicts of interest in this work.

Author contributions. XXY, KY designed study, XXY, KY and ZHF developed methodology, KY, ZHG and ZCD analyzed data, XXY, TTH and CXL wrote the manuscript and ZHF and CL revised the full text.

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