The Ant1 / panx1 pathway sensitizes patients with NAFLD to lifestyle interventions

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1 **The Ant1 / panx1 pathway sensitizes patients with NAFLD to**
2 **lifestyle interventions**

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Abstract

Background: Different patients have different sensitivities to lifestyle interventions (LSI) and thus different outcomes (improved or unchanged). In the present study, we aim to find key markers that can identify patients sensitive to lifestyle interventions.

Methods: Raw data were obtained from the Liver Clinic and Obesity Clinic of the Antwerp University Hospital that divided patients into LSI responders and non-responders based on the effectiveness of the intervention at the pathologic level. The limma / voom algorithm was used for difference analysis; Over representation analysis was used for functional enrichment analysis. Functional class scoring was used for gene set enrichment analysis. WGCNA and PPI / MCODE were used to identify valuable modules and genes. Western blot and immunohistochemistry were used for experimental validation.

Results: Specific features of LSI responders were extracted based on differential expression and functional enrichment. The CIBERSORT and ESTIMATE algorithm reconfirmed that there were differences in the immune cell composition of LSI responders versus non-responders. Next, the WGCNA algorithm was used to integrate analyses of associations between immune cell phenotypes and genes. PPI and MCODE algorithms were used to screen for gene clusters with higher weights. The test set and validation set data were subjected to correlation analysis of the filtered genes to obtain the hub gene - ANT1. ANT1 was highly expressed in LSI responders, while it was lowly expressed in non-responders. Mechanistically, ANT1 responds to lifestyle intervention by activating Panx1, which is key to lesion improvement in LSI responder populations.

Conclusions: In the study, we confirm that LSI responders populations respond to exercise intervention via the ANT1 / Panx1 pathway.

Keywords: NAFLD, Lifestyle interventions sensitivities, Immune state, Transcriptome, ANT1

Background

With a worldwide prevalence of approximately 25%, NAFLD has become the most common chronic liver disease[1]. Its prognosis mainly depends on the degree of hepatic inflammation and fibrosis. The 10-year cirrhosis incidence is 4% in simple hepatic steatosis and up to 22% in NASH[2]. In NAFLD at least 25% belong to NASH. There are currently no specific clinical drugs for the treatment of NASH, and guidelines still recommend lifestyle intervention as the primary treatment, but this is often not effective due to the difficulty of patient adherence. However, study has also found limited disease remission even when patients adhere to lifestyle modification[3]. This suggests that there may be different subtypes of patients with NASH who respond differently to lifestyle interventions. Therefore, if a therapeutic strategy of lifestyle intervention is adopted for insensitive patients, this will progress to worsening because of poor disease control.

Inflammatory infiltrates within liver lobules are a hallmark of NASH and are associated with resident specialized immune cells[3, 4]. During NASH, hepatocyte death releases danger associated molecular patterns (DAMPs, such as high mobility group box 1, heat shock proteins, ATP, and extracellular vesicles filled with lipotoxic fatty acids and chemokines) to induce macrophage M1 type polarization. The collapse of mitochondrial membrane potential triggered by dysfunctional mitochondria, together with the release of ROS, mitochondrial DNA, is one of the major causes of hepatocyte death[5, 6].

Adenine nucleotide translocase 1 (ANT1) is an ADP / ATP translocase located in the inner mitochondrial membrane and is essential in maintaining mitochondrial membrane potential and

permeability[7-9]. Overexpression of ANT1 significantly enhanced mitochondrial permeability and was required for NLRP3 inflammasome activation under pathological conditions[7]. Meanwhile, ANT1 is also a key protein in the mitochondrial permeability transition pore (MPTP) - dependent apoptosis pathway[10]. Studies have shown that metabolites produced by apoptosis, released in a panx1 dependent pathway, can regulate the gene expression of phagocytes in vivo, closely related to the body inflammatory response[11].

We found that different patients differ in their sensitivity to lifestyle interventions, the specific mechanisms of which are not known. In this study, we compared differences in gene profile expression between treatment sensitive and - insensitive NASH patients using a combined analysis of functional enrichment, WGCNA, and PPI to identify a single key molecule-ANT1. In the follow-up verification at three levels (*in silico*, *in vitro*, *in vivo*), it was confirmed that in lifestyle sensitive patients, ANT1 regulates the expression of panx1 in response to exercise style and improves liver lesions. This study clarified the distinctions among different subtypes of NASH patients and elucidated the key mechanisms of lifestyle interventions, which will be helpful for patients to develop personalized treatment strategies.

Methods

Data sources and processing

Raw data and clinical information of GSE106737 and GSE83452 on patients were downloaded from the Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>). Data were RMA_normalized using oligo/bi conductor (DOI: 10.18129/B9.bioc.oligo) and corrected for batch effects using SVA/Bioconductor package[12] in the R suit (version: 3.3.3). Differentially expressed

genes (DEGs) were screened by the “limma” package[13], and a volcano map of DEGs was drawn using the “ggplot2” package[14]. All patients were consecutively recruited at the Liver Clinic and Obesity Clinic of the Antwerp University Hospital and underwent hepatologic and metabolic work-ups[3]. NASH patients were treated with a one-year lifestyle intervention (LSI). NASH patients with improvement in lobular inflammation and/or ballooning were defined as LSI responders after 1-year follow-up, whereas NASH patients without improvement in ballooning and/or lobular inflammation were defined as non-responders.

Functional enrichment and Gene set enrichment analysis

Gene Ontology (GO) enrichment analysis on DEGs were carried out with clusterProfiler package[15] to explore the characteristic function. Ontology gene sets (c5.all.v7.4.symbols) were used for gene set enrichment analysis (GSEA) through the clusterProfiler package. A false discovery rate (FDR) < 0.25 and $p < 0.05$ were considered significant enrichment.

Protein-protein Interaction Enrichment Analysis

For each given gene list, protein-protein interaction enrichment analysis (PPI) has been carried out with the following databases: STRING[16], BioGrid[17], OmniPath[18], InWeb_IM[18]. Only physical interactions in STRING (physical score > 0.132) and BioGrid are used. The resultant network contains the subset of proteins that form physical interactions with at least one other member in the list. If the network contains between 3 and 500 proteins, the Molecular Complex Detection (MCODE) algorithm[19] has been applied to identify densely connected network components. Pathway and process enrichment analysis has been applied to each MCODE

component independently, and the three best-scoring terms by p-value have been retained as the functional description of the corresponding components, shown in the tables underneath corresponding network plots within Figure 6.

Weighted gene co-expression network analysis (WGCNA)

We used the goodSamplesGenes method of R software package WGCNA[19] on gene expression matrix to remove outlier genes and samples, and constructed a scale free co expression network. At first, the Pearson's correlation matrices and average linkage method were both performed for all pair-wise Genes. Then, a weighted adjacency matrix was constructed using a power function:

$$a_{mn} = |c_{mn}|^{\beta}$$

(c_{mn} = Pearson's correlation between Gene_m and Gene_n; a_{mn} = adjacency between Gene_m and Gene_n; β was a soft-thresholding parameter that could emphasize strong correlations between Genes and penalize weak correlations.)

After choosing the power of 9, the adjacency was transformed into a topological overlap matrix (TOM), which could measure the network connectivity of a Gene defined as the sum of its adjacency with all other Genes for network Gene ration, and the corresponding dissimilarity (1-TOM) was calculated.

$$TOM_{mn} = \frac{d_{mn} + a_{mn}}{\min(e_m + e_n) + 1 - a_{mn}}$$

(d_{mn} = Sum of the adjacency coefficients of the nodes connected by Gene_m and Gene_n;
 e = Sum of the adjacency coefficients of the given gene with all other genes in the weighted network)

To classify Genes with similar expression profiles into Gene modules, average linkage

hierarchical clustering was conducted according to the TOM-based dissimilarity measure with a minimum size (Gene group) of 30 for the Genes dendrogram. To further analyze the module, we calculated the dissimilarity of module eigen Genes, chose a cut line for module dendrogram and merged some module. Average linkage hierarchical clustering was performed according to a TOM-based dissimilarity measure with a minimum size (gene set) of 30 for the Genes dendrogram to classify Genes with similar expression profiles into gene modules. Further, the dissimilarity of module eigengenes were calculated to merged certain modules (distance less than 0.25). Finally, a total of 4 co-expression modules were obtained, in which the grey module was considered as the set of genes that could not be assigned to any module

For hub gene screening[20], gene significance (GS) was obtained by calculating the expression correlation with the gene. Module membership (mm) was obtained by calculating the expression correlation of module eigenvectors with genes. Based the cut-off criteria ($|MM| > 0.76$ and $|GS| > 0.6$), 13 genes with high connectivity in the clinical significant module were identified as hub genes. The protein abundance of hub genes was retrieved from the Human Protein Atlas database (<https://www.proteinatlas.org/>).

Western Blot

Samples were lysed by RIPA lysis buffer (Beyotime Institute of Biotechnology, China) containing 1% phenylmethanesulfonyl fluoride. A total of 30 μ g protein was loaded onto 10%, 12.5% SDS-polyacrylamide gels, and transferred to PVDF membranes, which incubated overnight at 4°C with primary antibody followed by an incubation with the horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (cat. no. A0208; 1:10,000; Beyotime Institute of Biotechnology)

and goat anti-mouse IgG (cat. no. A0216; 1:10,000; Beyotime Institute of Biotechnology) at room temperature for 1 h. The protein bands were visualized by ECL Moon from Beyotime Biotechnology. The blots were scanned by using an Odyssey Two-color Infrared Laser Imaging System (Li-Cor, Lincoln, NE, USA). The primary antibodies were as follow: Anti-ANT1 (cat. no. TD6674; 1:1,000; Abmart), anti-Panx1 (cat. no. 12595-1-AP; 1:1,000; ProteinTech Group, Inc.), anti-Caspase-3 (cat. no. T55051; 1:1,000; Abmart), anti-Cleaved Caspase-3 (cat. no. Asp175; 1:1,000; Cell Signaling Technology, Inc), anti-VDAC1 (cat. no. 55259-1-AP; 1:1,000; ProteinTech Group, Inc.), and anti- β -actin (cat. no. MA1-140; 1:10,000; Thermo Fisher Scientific, Inc.).

Histopathological and Immunohistochemistry Analysis

Samples were fixed in 4% paraformaldehyde for 24 h at temperature. For the preparation of paraffin embedded tissues, tissues routinely dehydrated at room temperature using an increasing ethanol gradient followed by xylene. The tissues were then embedded in paraffin. For the preparation of frozen tissues, tissues routinely dehydrated at room temperature using an increasing sucrose followed by optimal cutting temperature compound embedding, - 80°preservation. 5 μ m liver sections were respectively stained with hematoxylin eosin dye, hematoxylin-eosin staining to observe hepatic lipid droplets. For immunohistochemical staining, sections were immersed in 0.01 M sodium citrate for antigen retrieval. Endogenous peroxidase activity was quenched by incubating with 3% H₂O₂ for 10 min. The sections were then incubated with 10% bovine serum albumin for 1 h, and then incubated with as follow: Anti-ANT1 (cat. no. TD6674; 1:50; Abmart), anti-Panx1 (cat. no. 12595-1-AP; 1:50; ProteinTech Group, Inc.), at 4°C overnight. A second antibody specific to the primary antibody was selected and allowed to react at room temperature for 60 min. DAB

(Sigma) color reaction solution was used for slice color development.

Statistical Analysis

All statistical analyses were carried out in the R suit (version: 3.3.3). One-way analysis of variance was used for comparison of three or more groups, and after one-way analysis, Dunnett-t was used for post-hoc test. All data were expressed as mean $\pm$ standard error of the mean (SEM). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Biological functions of the two isoforms in patients with NASH

Different patients have different sensitivities to lifestyle interventions and thus different outcomes. In the present study, we aim to find key markers that can identify patients sensitive to lifestyle interventions. Along this line, we divided the patients in the baseline data into two subgroups (LSI responders and non-responders) using the pathological outcome at 1-year follow-up data as a reference. The workflow is shown in figure1.

On this basis, we used R language software for differential gene analysis. A total of 628 differentially expressed genes (DEGs) were identified, including 362 up-regulated and 266 down-regulated genes, as shown in heatmaps and volcano plots (Figure 2A-B). Then, we further analyzed the potential functional implication of these DEGs. GO enrichment analysis showed that DEGs were mainly related to metabolic process and immune state (Figure 2C). The top 10 enriched GO terms mainly included lipid metabolic process, cholesterol biosynthetic process and immune response (Table 1).

Next, a gene set enrichment analysis (GSEA) was performed on the two subtypes to clarify the difference of biological functions and revalidation. Consistent with the results of GO analysis, GSEA showed that adaptive immunity related pathways were mainly enriched on LSI responders subtypes (Figure 3). The results of the enrichment analysis suggest that differences in the immune status of the two subtypes are key to responding to lifestyle intervention.

Differences in immune status among subtypes of patients with NASH

To decipher the immune heterogeneity between these two subtypes, CIBERSORT algorithm[21] and Estimate algorithm[22] were used for further analysis. The CIBERSORT algorithm calculated the constituent proportions of 22 immune cells in the two subtypes to assess the discrimination of immune responses (Figure 4A). The results showed significant differences in the content of lymphocytes, monocytes, and mast cells in both subtypes. Notably, the LSI responders group had a higher proportion of M2 type macrophages, whereas the non-responders group had a higher proportion of M1 type macrophages (Figure 4B). Consistent with this, LSI responders had significantly higher stromal and immune scores by computing Estimate algorithm compared to non-responders (Figure 4C). These results suggest that differences in immune cell composition among different subtypes may have differential sensitivity to lifestyle interventions.

Identification of key modules based on gene and immune cell expression matrices in the two subtypes

WGCNA was used to construct networks based on the expression matrix of genes and immune cells from the two subtypes to explore key genes responsible for differences in immune cell composition.

The soft threshold power ($\beta = 9$) was set to build a scale free network (Figure 5A-B). Similar expression patterns clustered into the same modules, a hierarchical clustering tree was constructed using dynamic hybrid cutting and cut height < 0.25 were merge (Figure 5C). Four co-expression modules were generated: turquoise, blue, brown, gray (Figure 5D-E). The gray module contained genes that could not be incorporated into any other module. The eigengenes of co-expression module were shown in Figure 5F. Among the four modules, the blue and turquoise modules were strongly correlated with macrophages. In detail, the turquoise module was positively correlated with macrophage M2 type, while the blue module was negatively correlated with macrophage M2 (Figure 5G-H). These correlations were confirmed through analysis of hierarchical clustering and adjacency relationships. The brown and turquoise modules were used for further analysis.

Screening of hub genes associated with immune cell phenotypes in two subtypes

On the basis of WGCNA, standard cutoff thresholds were set at 0.75 and 0.6 for the Module Membership and gene trait significance, respectively. In the blue module, 27 genes were identified (15 for m0). In the turquoise module, 65 genes were identified (38 for m0). In addition, we also performed protein-protein interactions (PPI) network on the basis of differential genes. MCODE algorithm was then applied to this network to identify neighborhoods where proteins are densely connected and a total of 12 MCODES were computed (Figure 6A). To further test the discrimination efficiency of these MCODES, we validated them using the GSE83452 dataset as a validation set[23](Figure 6B). Among these MCODES, MCODE4 and MCODE8 reached a high level (AUC = 0.948; AUC = 0.906). The gene markers obtained by the two algorithms were overlapped, and two genes (ANT1 and PSMD8) were selected as hub genes (Figure 6C). Finally, correlation analysis between hub

genes and immune cells showed that ANT1 and PSMD8 were positively correlated with macrophages M2 and negatively correlated with macrophages M0, which was consistent with the results of WGCNA (Figure 6D).

The capacity of hub genes to identify LSI responders populations

Can the hub gene serve as a key marker to distinguish sensitive populations for lifestyle intervention?

We further used the training set and validation set to explore the relationship between the hub genes and disease states, respectively.

In the training set, the two hub genes were significantly up-regulated in the LSI responders group, showing good discrimination. Meanwhile, to confirm the correlation of hub gene and treatment sensitivity, we compared the expression changes of hub gene before and after lifestyle intervention. In the LSI responders group, the hub gene significantly decreased as the lifestyle intervention proceeded. The non-responders group did not change (Figure 7A). At the same time, we verify the relationship of hub gene and LSI responders again in the validation set (Figure 7B). ANT1 was specifically highly expressed in the LSI responders group and decreased after lifestyle intervention. ANT1 was lowly expressed in the non-responders group and was unresponsive to lifestyle intervention. These results indicate that ANT1 is a key gene in response to lifestyle intervention and a key marker to distinguish individuals sensitive to lifestyle intervention.

ANT1 responds to LSI intervention by regulating Panx1

Why is high ANT1 expression sensitive to exercise? We performed experimental validation to complement and refine the bioinformatic analysis. The HPA database showed that the abundance of

ANT1 in liver parenchymal cells was higher than that in other cells (Figure 7C-D). On this basis, different gradients of FFAs (0.5mM, 0.75mM, 1mM) were used to stimulate AML12 cells to mimic an in vitro model of NAFLD while inducing ANT1 expression. ANT1 was higher with increasing FFA concentrations. Cleaved caspase3 and Panx1 were also elevated along with ANT1, showing a high degree of correlation (Figure 8). High expression of ANT1 would induce apoptosis and activate caspases[24]. Panx1 channel is activated by cleaved caspase during apoptosis[11]. Activation of Panx1 channels is associated with exercise as well as M2 type macrophages[11, 25, 26]. There may be a regulatory relationship between Panx1 and ANT1. Therefore, the loss of function experiment was designed for further analysis.

SiRNA was transfected to knock down ANT1 in an in vitro model of NAFLD, and ANT1 siRNA3 was selected out for subsequent experiments (Figure 8). Cleaved caspase3 and Panx1, which are elevated in response to FFA, decreased with ANT1 knockdown, confirming that these indexes were affected by ANT1 (Figure 8). We also verified the relationship between the two in vivo model. In three animal models (control, HFHC, HFHC & exercise), we found that the expression of ANT1 was significantly up-regulated in NAFLD model induced by high-fat and high cholesterol diet, while exercise intervention on this basis significantly down regulated the expression of ANT1 (Figure 8). At the same time, Panx1 is also positively regulated with the change of ANT1, which is consistent with the above.

In the disease state, the highly expressed ANT1 affected the apoptotic activity by regulating Panx1. Exercise attenuates the onset of apoptosis in response to lipid accumulation, which explains why populations characterized by high expression of ANT1 can be sensitized to exercise.

Discussion

Nonalcoholic fatty liver disease is currently the most common chronic disease, endangering human health. Currently, lifestyle interventions remain the main treatment. But in clinical work, there are often some patients who cannot adhere to or tolerate exercise, which is an urgent problem[3]. Along this line, we want to clarify the effective mechanism of lifestyle intervention. During the analysis of sequencing data, we found that not all NASH patients were sensitive to lifestyle intervention, even if they received one-year intervention. This makes us realize that patients with NASH may have different subtypes. In clinical work, different treatment methods should be taken for patients with different subtypes of NASH.

In this experiment, we analyzed the differences between different subtypes of NASH patients (LSI responders and Non-responders) at baseline. Considering the similar pathological characteristics of different NASH patients, we set a small threshold to screen the largest gene set. On this basis, we performed functional enrichment analysis of differentially expressed genes. The results showed that the immune response was enriched in the LSI responders group, indicating that the immune characteristics of patients at baseline determined the efficacy of lifestyle intervention. The immune cell infiltration and microenvironment analysis (ESTIMATE and CIBERSORT) algorithm was used to analyze the immune characteristics of LSI responders group and non-responders, which further confirmed that LSI responders patients had a unique immune microenvironment at the baseline level, which was sensitive to lifestyle intervention. Furthermore, we used different module division methods (WGCNA, PPI) to identify a common feature, that is, the high expression subtype of ANT1 is sensitive to lifestyle intervention. In addition, we also analyzed the correlation between ANT1 gene and immune cells. ANT1 showed a significant

correlation with macrophage M2 activation. Meanwhile, Ant1 is localized to the inner mitochondrial membrane and is responsible for transporting ATP. High expression of Ant1 would induce apoptosis and activating caspases[24]. And the results of apoptosis metabolomics showed that metabolites (ATP) and caspases would stimulate the opening of Panx1 channels[11]. Panx1 channels are also one of the key channels through which exercise exerts its utility[25, 26]. We speculated that it may be the key to the effectiveness of lifestyle intervention.

To confirm this idea, we de validated the relationship between ANT1 and LSI at three different levels. First, *in silico*, we chose two different datasets to compare ANT1 expression in the LSI responders and non-responders groups separately to verify ANT1 specificity. Then, to verify the sensitivity of Ant1 to exercise, we compared gene level changes before and after intervention. These results indicate that ANT1 is a key gene in response to lifestyle intervention and a key marker to distinguish individuals sensitive to lifestyle intervention. Second, why is high ANT1 expression sensitive to exercise? ANT1 is a key protein in the mitochondrial permeability transition pore (MPTP) - dependent apoptosis pathway[10]. Studies have shown that metabolites produced by apoptosis, released in a panx1 dependent pathway, can regulate the gene expression of phagocytes *in vivo*, closely related to the body inflammatory response[11]. Thus, ANT1 may respond to lifestyle intervention through Panx1. As described earlier, we confirmed the existence of a regulatory relationship between ANT1 and Panx1 by functional rescue experiments at the *in vitro* level. Finally, at the *in vivo* level, we explored the relationship of exercise, ANT1 and panx1. ANT1 and Panx1 could be significantly upregulated in mice fed a high-fat, high cholesterol diet, and were effectively downregulated by an exercise intervention based on this result. These results indicate that the HFHC diet model successfully mimics the characteristics of the LSI responders population and that the

corresponding expression of ANT1 and Panx1 is altered under exercise intervention, providing further evidence that the LSI responders population may respond to exercise intervention through the ANT1 / Panx1 pathway. The discovery of this mechanism is expected to provide a new direction for the personalized treatment of patients with NAFLD.

Conclusions

In conclusion, our study identified key gene (ANT1) that can identify patients sensitive to lifestyle interventions through bioinformatic analysis and experimental validation. We found that lifestyle intervention may act through the Ant1 / panx1 pathway.

Abbreviations

ANT1:Adenine nucleotide translocase 1; DEGs:Differentially expressed genes; FDR: false discovery rate; GEO:Gene Expression Omnibus; GO:Gene Ontology; GS:gene significance; GSEA :gene set enrichment analysis; HFHC:high-fat and high cholesterol diet; LSI :lifestyle interventions; MCODE:Molecular Complex Detection ; MPTP :mitochondrial permeability transition pore ; NAFLD: non-alcoholic fatty liver disease; PPI:protein-protein interaction enrichment analysis; WGCNA :Weighted gene co-expression network analysis;

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Authors' contributions

Conceptualization, Bo Sun, Linying Lai, Han Zhang, Wenzhuo Yang; Investigation, Bo Sun, Linying Lai; Formal

analysis, Bo Sun, Linying Lai; Visualization, Bo Sun, Linying Lai; Experiment, Bo Sun, Linying Lai; Writing, Bo Sun, Linying Lai; Supervision, Bo Sun, Linying Lai, Han Zhang, Wenzhuo Yang; Funding acquisition, Wenzhuo Yang. All authors read and approved the final manuscript.

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Availability of data and materials

RNA -seq datasets from this study are available through the NCBI Gene Expression Omnibus (GEO) database, with the accession number GSE106737 and GSE83452.

Declarations

Ethics approval and consent to participate

All the specimens were collected with informed consent, according to the regulations of the Liver Clinic and Obesity Clinic of the Antwerp University Hospital.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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375 **References**

- 376 1. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M: Global epidemiology of
377 nonalcoholic fatty liver disease-Meta-analytic assessment of prevalence, incidence, and
378 outcomes. *Hepatology* 2016; 64:73-84.
- 379 2. Loomba R, Friedman SL, Shulman GI: Mechanisms and disease consequences of nonalcoholic
380 fatty liver disease. *Cell* 2021; 184:2537-2564.
- 381 3. Haas JT, Vonghia L, Mogilenko DA, Verrijken A, Molendi-Coste O, Fleury S, et al: Transcriptional
382 Network Analysis Implicates Altered Hepatic Immune Function in NASH development and
383 resolution. *Nat Metab* 2019; 1:604-614.
- 384 4. Vonghia L, Michielsen P, Francque S: Immunological mechanisms in the pathophysiology of
385 non-alcoholic steatohepatitis. *Int J Mol Sci* 2013; 14:19867-19890.
- 386 5. Machado MV, Diehl AM: Pathogenesis of Nonalcoholic Steatohepatitis. *Gastroenterology* 2016;
387 150:1769-1777.
- 388 6. Foroughi M, Maghsoudi Z, Khayatzadeh S, Ghiasvand R, Askari G, Iraj B: Relationship between
389 non-alcoholic fatty liver disease and inflammation in patients with non-alcoholic fatty liver. *Adv*
390 *Biomed Res* 2016; 5:28.
- 391 7. Guo W, Liu W, Chen Z, Gu Y, Peng S, Shen L, et al: Tyrosine phosphatase SHP2 negatively
392 regulates NLRP3 inflammasome activation via ANT1-dependent mitochondrial homeostasis.
393 *Nat Commun* 2017; 8:2168.
- 394 8. Kroemer G, Galluzzi L, Brenner C: Mitochondrial membrane permeabilization in cell death.
395 *Physiol Rev* 2007; 87:99-163.
- 396 9. Clmenon B, Babot M, Trzguet V: The mitochondrial ADP/ATP carrier (SLC25 family):
397 pathological implications of its dysfunction. *Mol Aspects Med* 2013; 34:485-493.
- 398 10. Halestrap AP, McStay GP, Clarke SJ: The permeability transition pore complex: another view.
399 *Biochimie* 2002; 84:153-166.
- 400 11. Medina CB, Mehrotra P, Arandjelovic S, Perry JSA, Guo Y, Morioka S, et al: Metabolites released
401 from apoptotic cells act as tissue messengers. *Nature* 2020; 580:130-135.
- 402 12. Parker HS, Leek JT, Favorov AV, Consideine M, Xia X, Chavan S, et al: Preserving biological
403 heterogeneity with a permuted surrogate variable analysis for genomics batch correction.
404 *Bioinformatics* 2014; 30:2757-2763.
- 405 13. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al: limma powers differential expression
406 analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res* 2015; 43:e47.
- 407 14. Ginestet C: ggplot2: Elegant Graphics for Data Analysis. *Journal of the Royal Statistical Society:*
408 *Series A (Statistics in Society)* 2011; 174:245-246.
- 409 15. Yu G, Wang LG, Han Y, He QY: clusterProfiler: an R package for comparing biological themes
410 among gene clusters. *Omics* 2012; 16:284-287.
- 411 16. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al: STRING v11: protein-
412 protein association networks with increased coverage, supporting functional discovery in
413 genome-wide experimental datasets. *Nucleic Acids Res* 2019; 47:D607-d613.
- 414 17. Stark C, Breitkreutz BJ, Reguly T, Boucher L, Breitkreutz A, Tyers M: BioGRID: a general
415 repository for interaction datasets. *Nucleic Acids Res* 2006; 34:D535-539.

18. Li T, Wernersson R, Hansen RB, Horn H, Mercer J, Slodkowitz G, et al: A scored human protein-protein interaction network to catalyze genomic interpretation. *Nat Methods* 2017; 14:61-64.
19. Bader GD, Hogue CW: An automated method for finding molecular complexes in large protein interaction networks. *BMC Bioinformatics* 2003; 4:2.
20. Tang J, Kong D, Cui Q, Wang K, Zhang D, Gong Y, et al: Prognostic Genes of Breast Cancer Identified by Gene Co-expression Network Analysis. *Front Oncol* 2018; 8:374.
21. Newman AM, Liu CL, Green MR, Gentles AJ, Feng W, Xu Y, et al: Robust enumeration of cell subsets from tissue expression profiles. *Nat Methods* 2015; 12:453-457.
22. Yoshihara K, Shahmoradgoli M, Martínez E, Vegesna R, Kim H, Torres-Garcia W, et al: Inferring tumour purity and stromal and immune cell admixture from expression data. *Nat Commun* 2013; 4:2612.
23. Lefebvre P, Lalloyer F, Baugé E, Pawlak M, Gheeraert C, Dehondt H, et al: Interspecies NASH disease activity whole-genome profiling identifies a fibrogenic role of PPAR α -regulated dermatopontin. *JCI Insight* 2017; 2.
24. Jang JY, Choi Y, Jeon YK, Aung KC, Kim CW: Over-expression of adenine nucleotide translocase 1 (ANT1) induces apoptosis and tumor regression in vivo. *BMC Cancer* 2008; 8:160.
25. Suvorava T, Cortese-Krott MM: Exercise-Induced Cardioprotection via eNOS: A Putative Role of Red Blood Cell Signaling. *Curr Med Chem* 2018; 25:4457-4474.
26. Casas M, Buvinic S, Jaimovich E: ATP signaling in skeletal muscle: from fiber plasticity to regulation of metabolism. *Exerc Sport Sci Rev* 2014; 42:110-116.

Figure1. Analytical flow diagram of the experiment.

Figure2. Functional analysis of differentially expressed genes. (A) Heatmap, (B) volcano plot, (C) the top10 and top 40 of GO term between LSI responders and non-responders

Figure3. Gene set enrichment analysis of c5.all.v7.5.1.symbols between the two subtypes. (A-B)

Top10 gene set positively associated with LSI responders subtype.

Figure4. Evaluation and visualization of Immune microenvironment. (A) Compositional proportions of 22 immune cells in each sample. (B) Histogram of immune scores between the two subtypes. (C) Violin diagram of immune cells between two subtypes.

448

449 **Figure5.** WGCNA analysis of combined gene and immune phenotypes. (A-B) Scale independence
450 and average connectivity were analyzed by network topology to filter out soft thresholds. (C-E)
451 Genes were divided into distinct modules by hierarchical clustering. (F) Heatmap of the correlation
452 between module eigengenes (MES) and immune cells. (G) Scatter plots of GS score and MM for
453 macrophage in the turquoise modules. (H) Scatter plots of GS score and MM for macrophage in the
454 blue modules.

455

456 **Figure6.** Screening of hub genes associated with immune cell phenotypes in two subtypes. (A) The
457 dense regions in PPI network are clustered by molecular complex detection. (B) ROC curve of
458 MCODE. (C) Venn diagram of WGCNA and PPI. (D) Validation of co-expression network of hub
459 genes and immune cells.

460

461 **Figure7.** Expression of hub genes in LSI responders and non-responders groups. (A) Expression of
462 ANT1 in both subtypes in the training set at baseline and before and after the intervention. (B)
463 Expression of ANT1 in both subtypes in the validation set at baseline and before and after the
464 intervention. (C-D) Abundance of Ant1 in different cells from the HPA database. Liver parenchymal
465 cells are selected out by the red box.

466

467 **Figure8.** ANT1 responds to LSI intervention by regulating Panx1. (A-B) Protein expression and
468 quantification of Ant1, panx1 and c-casp3 stimulated by different concentrations of FFA. (C)
469 Screening of Ant1 siRNA silencing efficiency. (D-E) Protein expression and quantification of Panx1

470 and C-CASP3 after ANT1 knockdown. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group.

471

472 **Graphic abstract** Schematic diagram of LSI responders mode

Figures

Figure 1

Analytical flow diagram of the experiment.

Figure 2

Functional analysis of differentially expressed genes. (A) Heatmap, (B) volcano plot, (C) the top10 and top 40 of GO term between LSI responders and non-responders

Figure 3

Gene set enrichment analysis of c5.all.v7.5.1.symbols between the two subtypes. (A-B) Top10 gene set positively associated with LSI responders subtype.

Figure 4

Evaluation and visualization of Immune microenvironment. (A) Compositional proportions of 22 immune cells in each sample. (B) Histogram of immune scores between the two subtypes. (C) Violin diagram of immune cells between two subtypes.

Figure 5

WGCNA analysis of combined gene and immune phenotypes. (A-B) Scale independence and average connectivity were analyzed by network topology to filter out soft thresholds. (C-E) Genes were divided into distinct modules by hierarchical clustering. (F) Heatmap of the correlation between module eigengenes (MES) and immune cells. (G) Scatter plots of GS score and MM for macrophage in the turquoise modules. (H) Scatter plots of GS score and MM for macrophage in the blue modules.

Figure 6

Screening of hub genes associated with immune cell phenotypes in two subtypes. (A) The dense regions in PPI network are clustered by molecular complex detection. (B) ROC curve of MCODE. (C) Venn diagram of WGCNA and PPI. (D) Validation of co-expression network of hub genes and immune cells.

Figure 7

Expression of hub genes in LSI responders and non-responders groups. (A) Expression of ANT1 in both subtypes in the training set at baseline and before and after the intervention. (B) Expression of ANT1 in both subtypes in the validation set at baseline and before and after the intervention. (C-D) Abundance of Ant1 in different cells from the HPA database. Liver parenchymal cells are selected out by the red box.

Figure 8

ANT1 responds to LSI intervention by regulating Panx1. (A-B) Protein expression and quantification of Ant1, panx1 and c-casp3 stimulated by different concentrations of FFA. (C) Screening of Ant1 siRNA silencing efficiency. (D-E) Protein expression and quantification of Panx1 and C-CASP3 after ANT1 knockdown. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group.

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