Peer Review File

Article information: https://dx.doi.org/10.21037/atm-22-1010

First External Peer Review

Reviewer A

This was an interesting paper with a unique approach.

The study used gene set enrichment and immune cell infiltration analysis to identify differential expressed genes and possible immune cells discrepancies that may contribute to metabolic syndrome between the skeletal muscle of wild type and obese mice. Data set results include the identification of Mast and Dendritic cells as being elevated in obesity along with seven acute phase response genes. Findings from the analyses were followed by experiments in mice that received a normal diet or a high fat diet. The follow-up studies were able to confirm Mast cell infiltration and upregulation in a portion of the identified genes. However, Dendritic cells and 3 of genes identified in the gene analysis were not elevated in high fat diet mice.

While this is an interesting study I have a few concerns.

Minor

Comment 1: Abstract (Line 49) – Mentions eight genes, yet only seven are listed?

Reply 1: Thank you very much for pointing out this problem. We screened out seven hub genes (*Saa1, Saa2, Orm1, Hp, Shh, Igf2,* and *Cela1*), but accidentally wrote eight genes. We feel very sorry for our carelessness.

Changes in the text: We modified "eight genes" to "seven genes" (see Page 4, line 53). According to the suggestions of Reviewer C, we asked an English editor to revise the full paper, so the position of the text was changed.

Comment 2: Introduction (Line 93) – IGF2 is discussed as a positive regulator of skeletal muscle health, then listed as a possible mechanism of diabetes. Can you provide additional details to connect these thoughts?

Reply 2: Thank you for your kind and careful guidance on our manuscript. We have supplemented the introduction section according to your suggestions to improve the introduction of IGF2.

Changes in the text: We added some contents about the introduction of IGF2. "Insulin-like growth factor 2 (IGF2), a widely expressed polypeptide hormone, is mainly synthesized and secreted by the liver. IGF2 is a pro-myogenic molecule that plays a key role in the development and growth of muscles in the foetus and after birth and helps in muscle recovery after injury. Moreover, IGF2 is closely related to metabolism; studies have shown that serum IGF2 levels are higher in patients with obesity and patients with high IGF2 levels are more likely to develop diabetes. IGF2 expression in porcine skeletal muscle is positively correlated with intramuscular lipid content, and its overexpression promotes endoplasmic reticulum stress leading to β cell dysfunction,

which may be the mechanisms of IGF2 leading to metabolic disorders." (see Page 6, line 98-107)

Major

Comment 3: My primary concern is with the confocal immune cell images. They are not very convincing. They are difficult to see the number of nuclei seem very low and no additional marker(s) are used to identify the cells location in the skeletal muscle samples. Typically, some form of membrane, extracellular matrix or fiber identifying marker should be used to help verify the cells are where we would expect to see them. I think it would add value to the data if more convincing images were available.

Reply 3: Thank you for your constructive suggestions. It would be better to mark the location of the skeletal muscle cells as you mentioned, so as to indirectly determine the location of the immune cells. Tryptase is a mast cell specific marker, which is the main component of mast cell secretion granules and the most abundant protein in mast cells. As shown in the following figure, mast cells are identified by the criteria that the cytoplasm is filled with tryptase and the tryptase surrounds the nucleus. Many studies also identified mast cells in this way:

1) Qin B, Peng Y, Zhong C, et al. Mast Cells Mediate Inflammatory Injury and Aggravate Neurological Impairment in Experimental Subarachnoid Hemorrhage Through Microglial PAR-2 Pathway. Frontiers in Cellular Neuroscience. 2021;15. 10.3389/fncel.2021.710481
2) Gupta K, Idahosa C, Roy S, et al. Differential Regulation of Mas-Related G Protein-Coupled Receptor X2-Mediated Mast Cell Degranulation by Antimicrobial Host Defense Peptides and Porphyromonas gingivalis Lipopolysaccharide. Infect Immun. 2017(10):e00246-17. 10.1128/IAI.00246-17
3) Manorak W, Idahosa C, Gupta K, et al. Upregulation of Mas-related G Protein coupled receptor X2 in asthmatic lung mast cells and its activation by the novel neuropeptide hemokinin-1. Respir Res. 2018;19(1):1. 10.1186/s12931-017-0698-3
4) Oottamasathien S, Jia W, Roundy LM, et al. Physiological relevance of LL-37 induced bladder inflammation and mast cells. J Urol. 2013;190(4 Suppl):1596-602. 10.1016/j.juro.2013.01.002

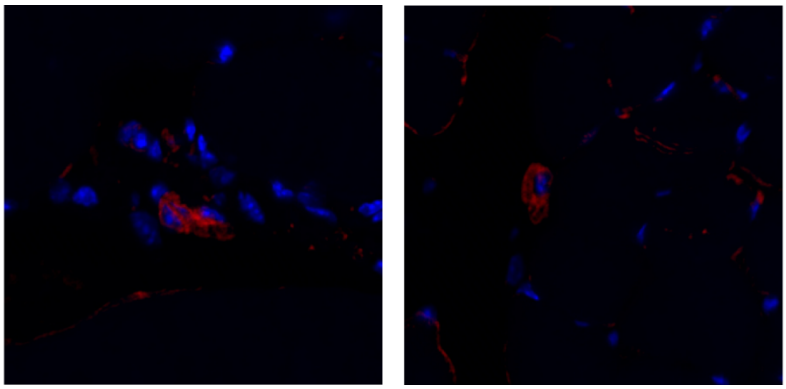
Co-localization of CD86 and CD11c is the specific marker of active dendritic cells. Similarly, the criterion for identifying active dendritic cells is that CD86 and CD11c co-localization and they surround around the nucleus. Some studies also identified active dendritic cells in this way:

1）Takenaka S, Safroneeva E, Xing Z, et al. Dendritic cells derived from murine colonic mucosa have unique functional and phenotypic characteristics. J Immunol. 2007;178(12):7984-93. 10.4049/jimmunol.178.12.7984

2）Morelli AE, Coates PT, Shufesky WJ, et al. Growth factor-induced mobilization of dendritic cells in kidney and liver of rhesus macaques: implications for transplantation. Transplantation. 2007;83(5):656-62. 10.1097/01.tp.0000255320.00061.e9

Your suggestion is very rigorous and constructive. However, because of the COVID-

19 outbreak in Shanghai of China, our lab was shut down for more than a month, biological companies in Shanghai also were shut down and we were sequestered in the dormitory, so we couldn't do this experiment. If you think we still need to do this experiment, could you please give us more time until everything in Shanghai returns to normal? Thank you very much.



Reviewer B

This manuscript titled Identification of hub genes and infiltrating immune cells in skeletal muscle under obesity was set to identify key biomarkers of skeletal muscle during obesity for prevention and treatment of MetS. This is an interesting study considering the effort to find cure for MetS. However, data presentation and text description of figures is very poor for me critically review this manuscript. For instance, almost all the figures are not clear. I cannot read the texts on the figures and the quality of all the figures are below standard.

For editor

Comment 1: I think this manuscript in its current should be not accepted. The authors could be advised to overhaul the quality of all figures and submit it again.

Reply 1: Thank you for your valuable advice. We have uploaded the full manuscript and all the original figures, which meet the requirements of the journal. The original figures should be available in the system. However, the system automatically generated the PDF version, and we found that the figures in the PDF version were blurred, which was caused by file conversion, and we don`t know how to avoid this problem. We can only attach the figures and tables for the full text below, but the following figures may not be as clear as the original figures in the system. We`re really sorry about that.

Table 1 P-value of correlation analysis between hub genes molecules

	HFD vs ND				ob/ob vs WT			
	SAA1	*SAA2*	*ORM1*	*HP*	*SAA1*	*SAA2*	*ORM1*	*HP*
SHH	0.021679	0.037605	0.009771	0.014893	0.045027	0.078461	0.055559	0.038506
MAFB	0.122827	0.186904	0.069383	0.027884	0.118143	0.202819	0.033867	0.132701
MECOM	0.578370	0.759932	0.185669	0.424563	0.406863	0.505385	0.342975	0.670521
SIN3A	0.278942	0.347226	0.091424	0.177620	0.815112	0.877007	0.972791	0.912114
IGF2	0.026663	0.036096	0.007606	0.010150	0.043434	0.087859	0.004584	0.020718
NR1H3	0.645306	0.737784	0.506240	0.410363	0.846728	0.758220	0.384628	0.640547
RORA	0.229494	0.187485	0.391920	0.201011	0.312522	0.341297	0.199811	0.434619
CELA1	0.003424	0.007910	0.057371	0.001004	0.015725	0.039290	0.000162	0.003046
SOX4	0.389868	0.432718	0.066716	0.277109	0.056704	0.096250	0.065182	0.073140

Figure 1 Summary of the workflow adopted in this study.

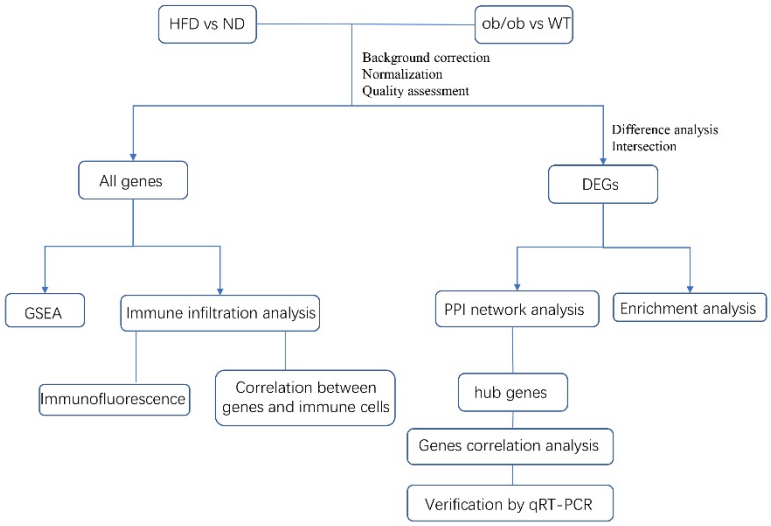


Figure 2 The results of gene set enrichment analysis. **(A)** The predicted biological processes and **(C)** pathways between HFD mice and ND mice. **(B)** The predicted biological processes and **(D)** pathways between ob/ob group and WT group.

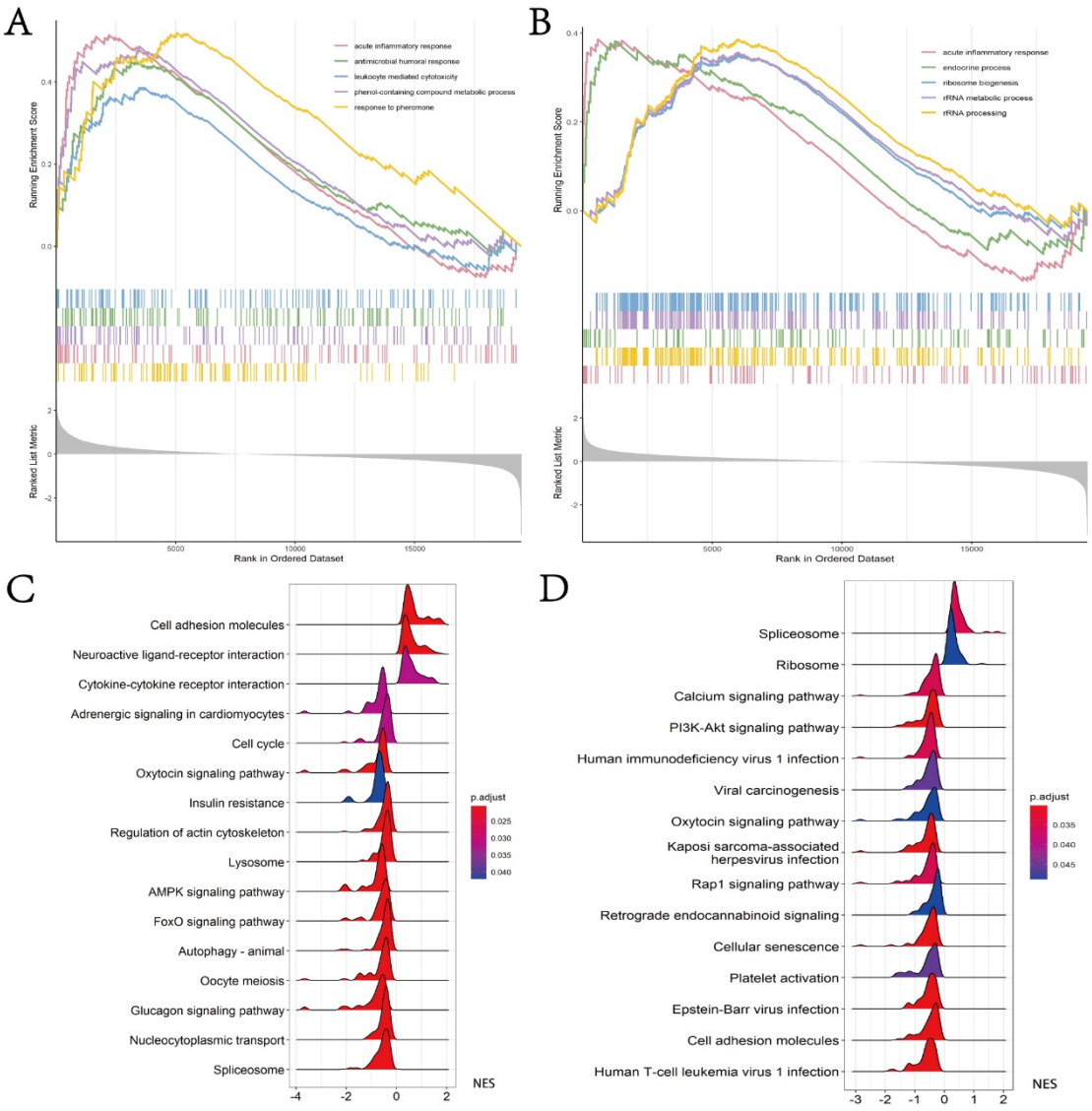


Figure 3 DEGs between groups. DEGs were shown in the volcano plot **(A)** between HFD mice and ND mice and **(B)** between ob/ob group and WT group, with red dots and blue dots respectively representing up-regulated genes and down-regulated genes. **(C)** The intersection between DEGs of all groups. **(D)** GO (BP) analysis of intersection genes of DEGs.

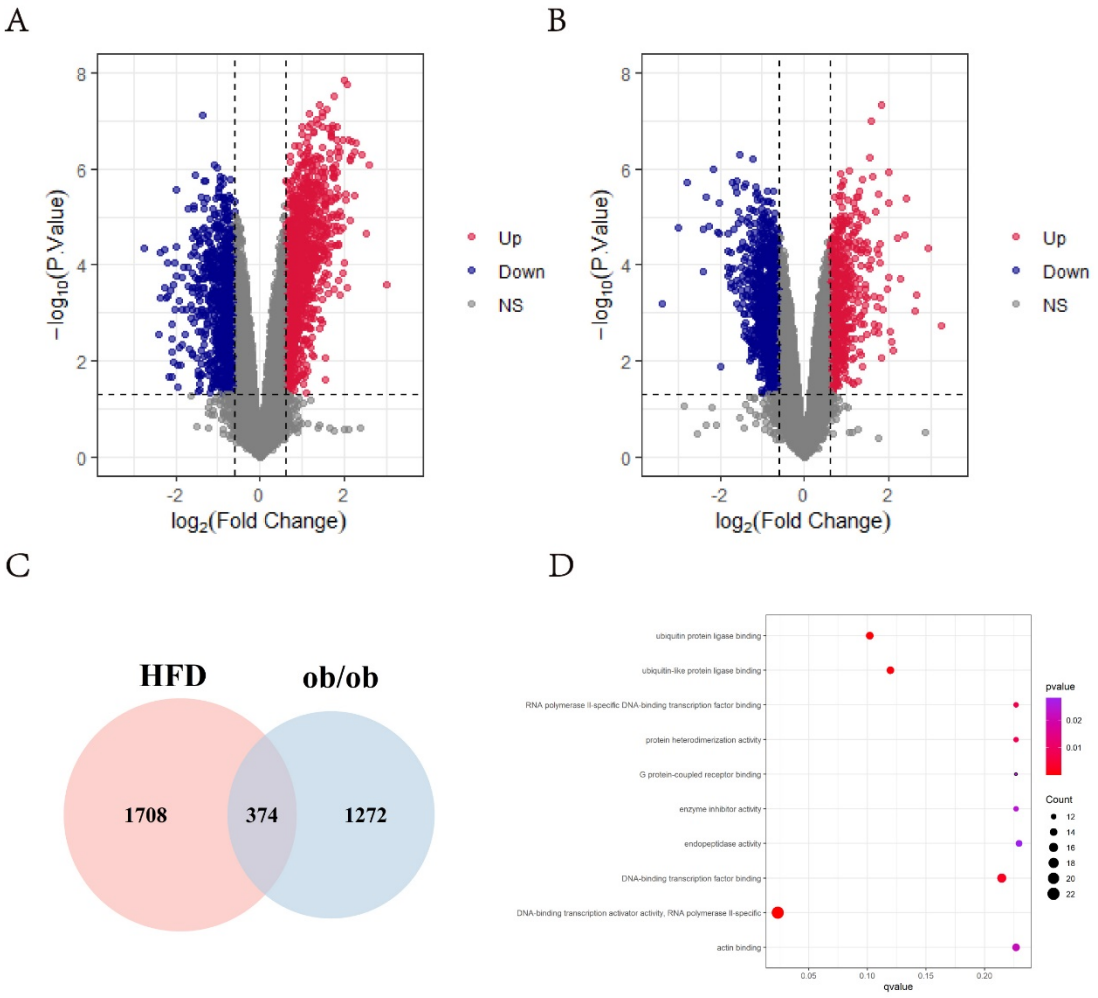


Figure 4 PPI network analysis. **(A)** PPI network of intersection genes. **(B)** Cluster 1, **(C)** cluster 2, **(D)** cluster 3 and **(E)** cluster 4 were shown.

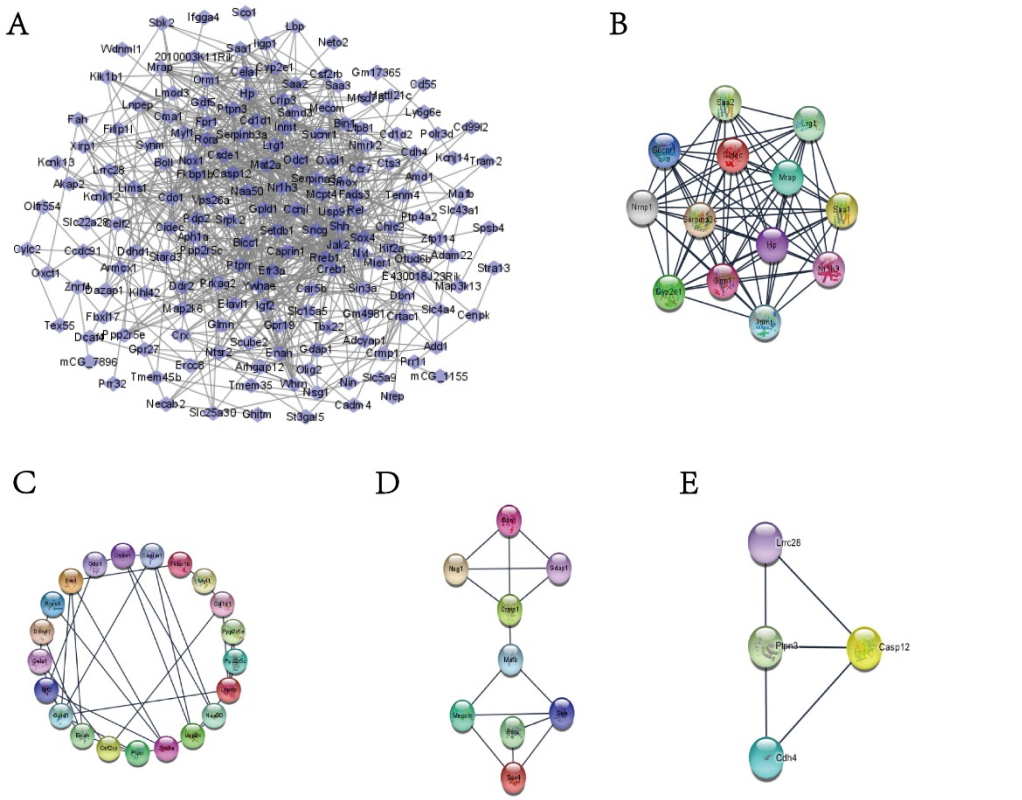


Figure 5 Immune infiltration analysis between HFD mice and ND mice and between ob/ob group and WT group.

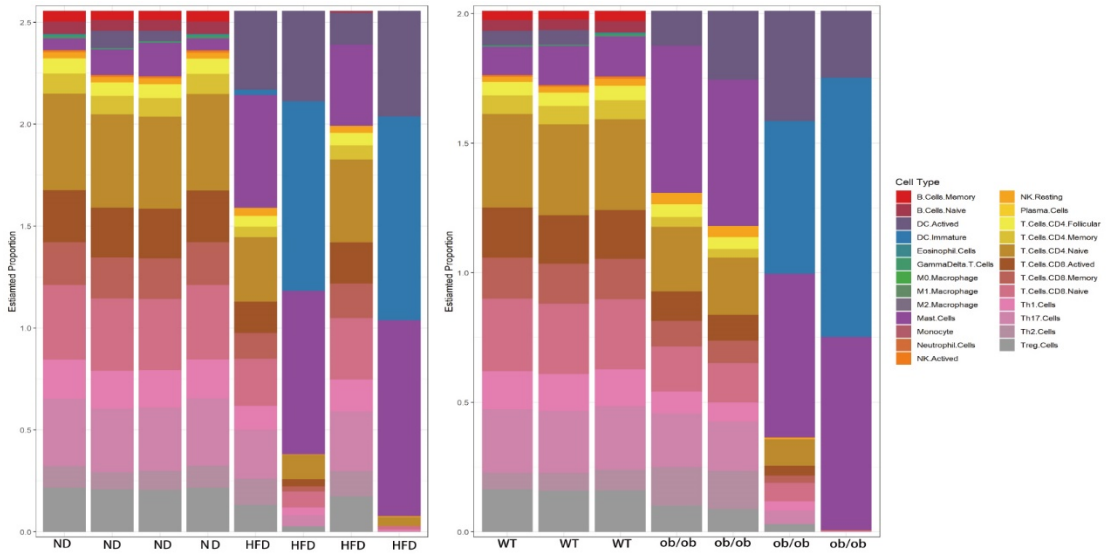


Figure 6 Immunofluorescence staining of mast cells. **(A)** The tryptase antibody labeled mast cells, and **(B)** the results showed more MCs were present in the skeletal muscle of HFD mice than of ND mice. Arrowheads indicate MCs. *, $p<0.05$;

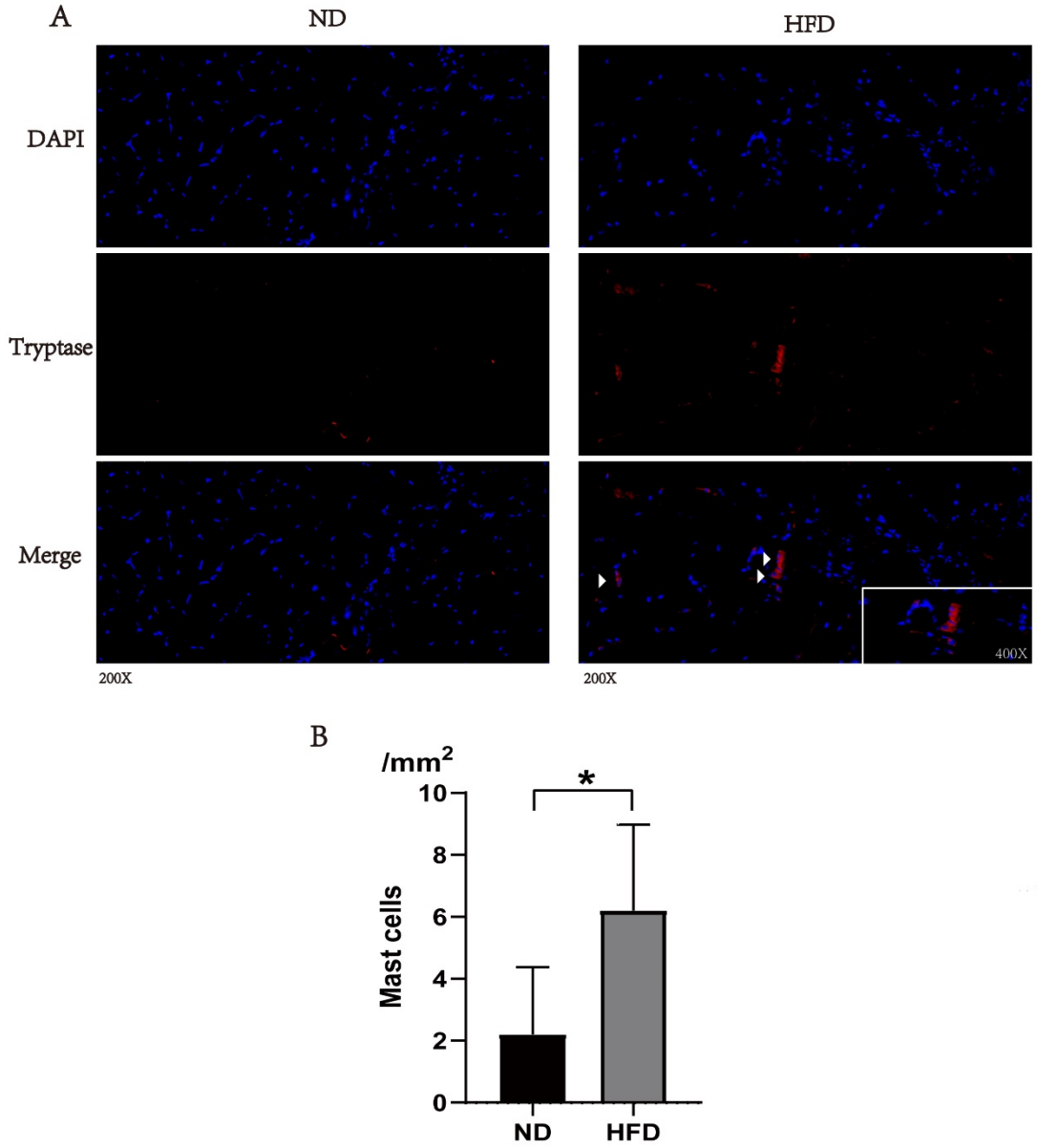


Figure 7 Correlation analysis between acute-phase-response genes and transcription-regulating genes **(A)** in HFD mice and **(B)** in ob/ob mice. **(C)** Chord plot depicts the relationship between clusters genes and biological processes. **(D)** Heatmap of hub genes expression in the skeleton muscle of all groups.

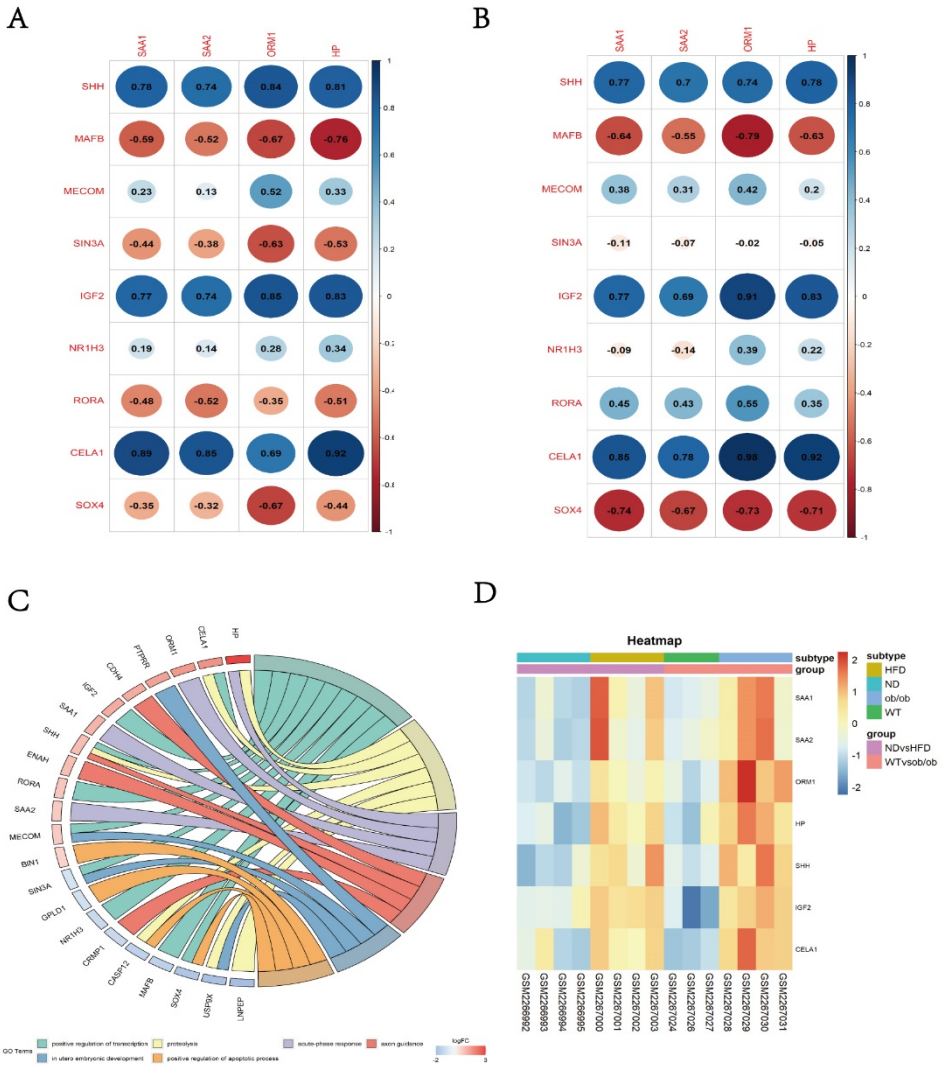


Figure 8 Correlation analysis between molecules and the proportion of infiltrating immune cells. The abscissa indicates the value of gene expression and the ordinate indicates the proportion of immune cells.

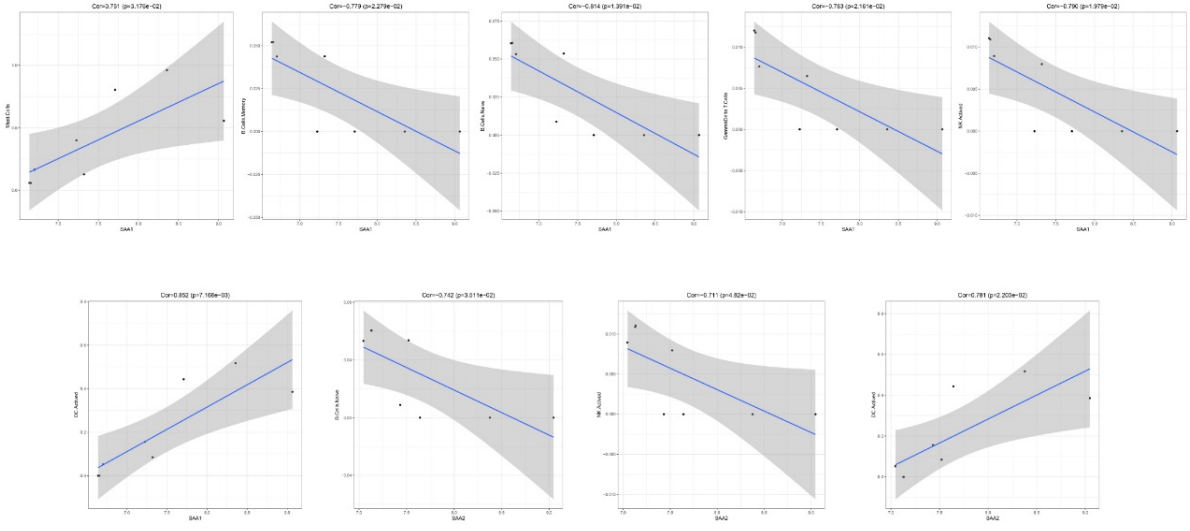
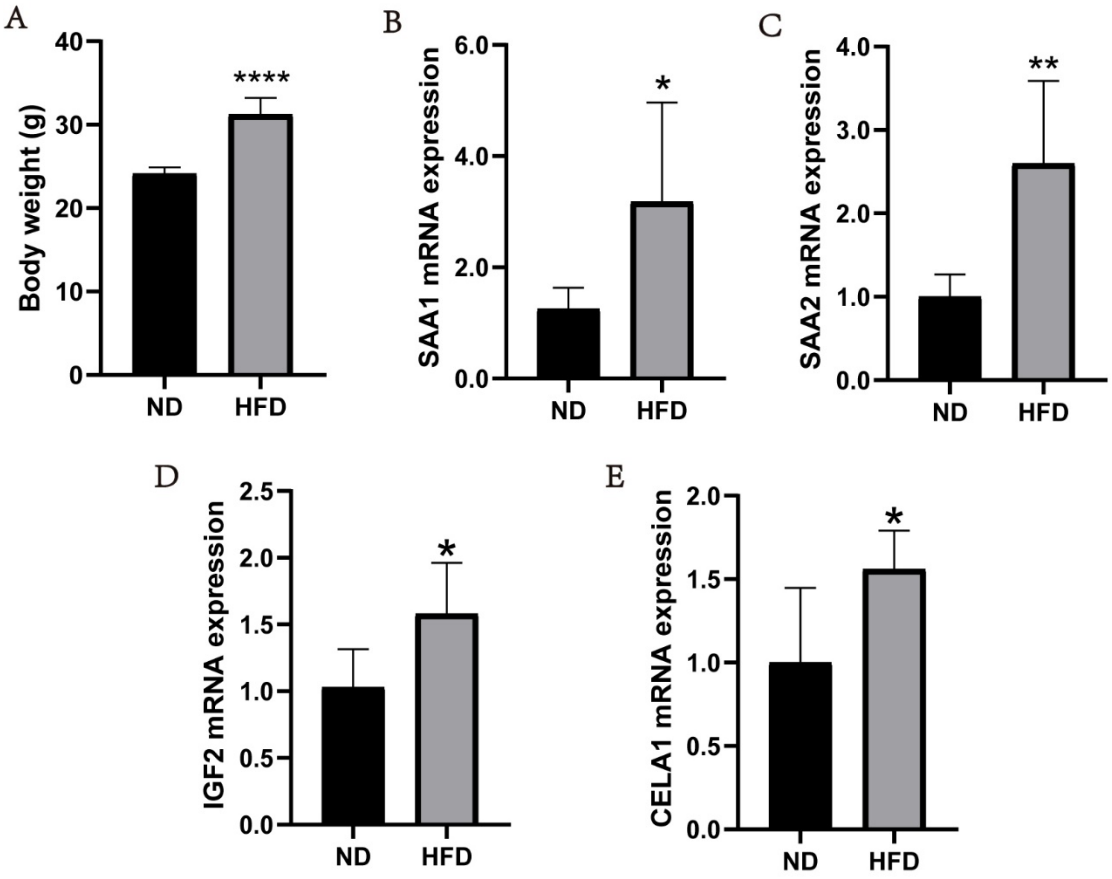


Figure 9 Determination of hub genes expression with qRT-PCR. **(A)** Comparison

between body weight of HFD mice and ND mice. qRT-PCR results showed that *SAA*1 **(B)**, *SAA2* **(C)**, *IGF2* **(D)** and *CELA1* **(E)** genes had significantly higher expression in HFD mice than in ND mice. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001.



Reviewer C

The present study demonstrated that obese skeletal muscle elevated MCs infiltration using bioinformatics analysis. This manuscript certainly deserves publication in the Annals of Translational Medicine. Altogether, the methodology appears adequate and the data are convincing.

However, I have some relatively minor suggestions.

Comment 1: Please specify the number of animals used per group in the methods.
Reply 1: Thank you very much for pointing out this problem. We have specified the number of mice in each group in the method section according to your suggestion.
Changes in the text:
1) bioinformatics analysis:
"Among the microarray datasets, four groups of male mice were selected, and each group contained four gastrocnemius samples." (see Page 8, line 121-122).
2) molecular biology experiments:
"Age- and weight-matched mice were randomly assigned to two groups: HFD (60% kcal from fat, 20% kcal from carbohydrate, 20% kcal from protein; D12492, Research Diets Inc, USA) or ND (14% kcal from fat, 62% kcal from carbohydrate, and 24% kcal from protein, NIH-31, Harlan Teklad), with five mice in each group." (see Page 12, line197-200)

Comment 2: Spelling and grammatical errors are too numerous to list individually. Please seek an English editor.
Reply 2: Thank you for your valuable suggestions. We have asked a professional English editor to polish the whole paper.

Comment 3: Line 108: The authors need to describe WT and ob/ob mice.
Reply 3: We are really grateful for the valuable advice. Ob/ob mice and WT mice were described in "Data Source", and these descriptions were quoted from the original author of the chip data.
Changes in the text: "The other two groups from the Jackson Laboratory comprised 10-week-old ob/ob male mice and their lean male controls, which were fed with ND and water ad libitum. The mice were kept in a clean room on a light/dark cycle for 12 hours. Before experiments, these mice were acclimatized to living conditions for at least 10 to 14 days. Before the gastrocnemius muscle was harvested, all mice were subjected to 4–5 h food withdrawal." (see Page 8, line 128-133)

Comment 4: Line 112: Please add the information about HFD. Also, the authors need to clearly confirm how this choice in LFD matches the HFD with the exception of the total amount of fat.
Reply 4: Thank you for your serious and constructive suggestions. We have

supplemented information about HFD. Normal diet (ND) and HFD are mainly different in content of three productive nutrients (protein, fat and carbohydrate), and we used ND that matches HFD in many studies. We also supplemented the information of ND, which is quoted from the original author of the chip data:
Yang L, Li P, Yang W, et al. Integrative Transcriptome Analyses of Metabolic Responses in Mice Define Pivotal LncRNA Metabolic Regulators. Cell Metab. 2016;24(4):627-39. 10.1016/j.cmet.2016.08.019
Changes in the text:
We have supplemented information about HFD and ND:

1) "From 6 weeks of age, groups 1 and 2 were fed a normal diet (ND) (14% kcal from fat, 62% kcal from carbohydrate, and 24% kcal from protein, NIH-31, Harlan Teklad) and high-fat diet (HFD) (60% kcal from fat, 20% kcal from carbohydrate, and 20% kcal from protein; D12492, Research Diets Inc, USA), respectively for 12 weeks." (see Page 8, line 124-128)
2) "Age- and weight-matched mice were randomly assigned to two groups: HFD (60% kcal from fat, 20% kcal from carbohydrate, 20% kcal from protein; D12492, Research Diets Inc, USA) or ND (14% kcal from fat, 62% kcal from carbohydrate, and 24% kcal from protein, NIH-31, Harlan Teklad), with five mice in each group." (see Page 12, line 197-200)

Comment 5: Line 114: Which skeletal muscle was harvested?
Reply 5: Thank you very much for pointing out this problem. We extracted the gastrocnemius samples, which we have supplemented in the manuscript.
Changes in the text:

1) "Before the gastrocnemius samples were harvested, all mice were subjected to 4–5 h food withdrawal." (see Page 8, line 132)
2) "At 15 weeks of age, the mice were weighed, and their gastrocnemius was harvested." (see Page 13, line 202-203)

Comment 6: Fig. 9: The authors should also supply the body weight data and gene expression data for WT and ob/ob groups. Also, why are the protein levels not shown?
Reply 6: Thank you for your constructive suggestions.

1) The "Animal" description in the first paragraph of the methods section may be misleading. In this study, raw chip data of skeletal muscle of HFD mice and ob/ob mice and their control mice were downloaded from GEO database, and cleaned and analyzed. To verify the results of the bioinformatics analysis, we raised obese mice on a high-fat diet, extracted their gastrocnemius, and performed qPCR and immunofluorescence experiments. GEO chip data provider only provided skeletal muscle chip data of ob/ob mice and wild-type control mice, but did not provide body weight and gene expression data of ob/ob mice and control mice.
2) The microarray data we downloaded were transcriptome data, so we only verified the key genes of skeletal muscle by qPCR, but did not verify the protein level. However, in the future, we will further study the protein levels of these key

molecules and their relationship with metabolism of skeletal muscle.

Changes in the text:

We transferred the description of "Animal" in the method section after "PPI Network Analysis", in order to first describe the methods of bioinformatics analysis, and then describe the methods of molecular biology experiments.

Second External Peer Review

Reviewer A

I believe the authors have sufficiently addressed my minor concerns by adding, removing or clarifying text. However, they were unable to address my major concern, which is to provide more convincing images of the immune cell infiltration. That said, I know the covid situation in China has been difficult if not impossible for allowing lab experiments. Thus, the authors have directed me to other studies that have used similar Histology samples. While I still believe that the images should be improved, I feel limited by the situation.

Comment 1: The Editorial Office would like to suggest authors add Limitations to the Discussion section, and explain the situation that the experiments could not further proceed.

Reply: Thank you for your constructive suggestions. We have added limitations to the discussion section.

Changes in the text: We added the content, "Moreover, due to the COVID-19 epidemic, we could not optimize the experiment to use the marker of membrane or extracellular matrix to verify the location of immune cells, but we will supplement this experiment in the future", to the discussion section (see Page 26, line 429-432)

Reviewer B

This manuscript titled Identification of hub genes and infiltrating immune cells in skeletal muscle under obesity was set to identify key biomarkers of skeletal muscle during obesity for prevention and treatment of MetS. This is an interesting study considering the effort to find cure for MetS. I am happy with improvements in the data presentation. Skeletal muscle is the major organ responsible for glucose disposal but do not receive sufficient attention compared with liver and adipose tissue in the obesity/signaling field. For most of the manuscript the authors present a broad analysis of skeletal muscle in in obese mice including immune cell infiltration, mRNA expression of some hub genes and bioinformatics. They showed that skeletal muscle SAA and IGF2 are increased in obesity and also implicate a role for CELA1 in obesity. The mechanisms underlying the effects of these hub genes and immune cells on the skeletal muscle in obesity are not described. Overall, this study will provide a foundation for future studies that will delineate the roles of skeletal muscle in obesity and potentially metabolic syndrome. I support the publication of the manuscript in ATM.