**ORIGINAL ARTICLE**

**Xinnaokang improves cecal microbiota and lipid metabolism to target atherosclerosis**

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**Significance and Impact of the Study:** Atherosclerosis, a common clinical disease, contributes to the primary pathological basis of ischemic cardiovascular and cerebrovascular diseases. Xinnaokang is used for atherosclerosis treatment, while the detailed mechanisms are obscure. We clarified the underlying mechanism of Xinnaokang in atherosclerosis treatment and provided a theoretical basis for applying traditional Chinese medicine compounds in treating disease.

**Keywords**  
cecal microbiology, metabolism, microbial physiology, microbial structure, molecular epidemiology.

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**Abstract**

This study aims to explore the potential mechanisms of Xinnaokang in atherosclerosis treatment. Firstly, the active components of Xinnaokang were analyzed by HPLC, which contains ginsenoside Rg1, puerarin, tanshinone, notoginsenoside R1, ammonium glycyrrhizate and glycyrrhizin. Network pharmacology analysis showed there were 145 common targets of Xinnaokang, including the chemical stress, lipid metabolite, lipopolysaccharide, molecules of bacterial origin, nuclear receptor and fluid shear stress pathways. Then, the animal experiment showed that Xinnaokang reduced the body weight and blood lipid levels of atherosclerotic mice. Vascular plaque formation was increased in atherosclerotic mice, which was markedly reversed by Xinnaokang. In addition, Xinnaokang reduced CAV-1 expression and increased ABCA1, SREBP-1 and LXR expressions in the vasculature. Xinnaokang promoted SREBP-2 and LDLR expressions in the liver but decreased IDOL and PCSK9 expressions, indicating that Xinnaokang regulated lipid transport-related protein expression. Cecal microbiota diversity was reduced in atherosclerotic mice but increased after Xinnaokang treatment. Xinnaokang treatment also improved gut microbiota communities by enriching Actinobacteria, Bifidobacteriales and Bifidobacteriaceae abundances. Metabolic profile showed that Xinnaokang significantly reduced homogentisate, phenylacetylglycine, alanine and methionine expressions in the liver of atherosclerotic mice. Xinnaokang effectively alleviated atherosclerosis, and this effect might be linked with the altered features of the liver metabolite profiles and cecal microbiota.

**Introduction**

Atherosclerosis is the formation of fibrous fatty lesions in arterial walls, which causes morbidity and mortality worldwide, including the majority of myocardial infarctions and strokes, as well as disabling peripheral artery disease (Libby et al. 2019). Atherosclerosis is characterized by lipid accumulation in the arterial walls, inflammatory response, cell death and fibrosis (Wolf and Ley 2019). Lipid metabolism disorder is the pathological basis of atherosclerosis (Neeland et al. 2019). Current studies have shown that immune cells and inflammation and elevated low-density lipoprotein (LDL) levels play critical roles in the development of atherosclerosis (Schaftenaar et al. 2016). Studies have indicated that LDL levels are positively correlated with the risk of atherosclerosis, while
high-density lipoprotein (HDL) levels are negatively related to it. The concept of reverse cholesterol transport is based on the hypothesis that HDL displays a cardioprotective function, which is a process involved in the removal of excess cholesterol that is accumulated in the peripheral tissues (e.g., macrophages in the aortae) by HDL, transporting it to the liver for excretion into the faeces via the bile (Wang et al. 2017). Thus, the targeted regulation of lipid levels and lipid metabolism in the liver could help reduce the risk of atherosclerosis.

Researchers compared gut microbiota from 218 patients and 187 healthy controls and found a significant increase in the abundance of Enterobacteriaceae and Streptococcus and a decrease in the abundance of beneficial flora such as Clostridium tetissima (Barrington and Lusis 2017; Jie et al. 2017). Levels of certain molecules with metabolic or transport functions associated with cardiovascular health were increased. The abundance of Streptococcus was positively correlated with blood pressure. Besides that, the abundance of Enterobacteriaceae was positively correlated with myocardial indicators (Barrington and Lusis 2017; Jie et al. 2017). Based on a risk prediction model of atherosclerosis in China from 47 types of gut microbiota, the AUC reached 86%, which proves the association between gut microbiota and atherosclerosis (Barrington and Lusis 2017; Jie et al. 2017). Faecal microbiota transplantation studies confirmed that the pro-inflammatory gut microbiota could aggravate inflammation and accelerate the formation of atherosclerosis in mice with a susceptible genetic background, which is related to the reduction in SCFA-producing bacteria (Brandsma et al. 2019). Microbiota can also directly influence the formation of atherosclerotic plaques, causing chronic inflammation and affecting endothelial cell function (Kamaroff 2018). These studies suggest that changes in the gut microbiota affect atherosclerosis formation.

Bile acid regulates the core pathways of lipid and glucose metabolism. It maintains intestinal homeostasis, barrier integrity and immunity. It could also control host metabolic pathways and regulates inflammatory responses, which is a crucial mediator of enteric–liver communication (Schneider et al. 2018). The bidirectional interaction between gut microbiota and bile acid mediates enteric–liver communication, which is expected to be a therapeutic target for various liver diseases (Chen et al. 2016; Ridlon et al. 2016). Liver metabolites affect gut microbiota composition and barrier integrity, and intestinal factors regulate liver bile acid synthesis and glycolipid metabolism (Tripathi et al. 2018). Recent studies have shown that butyrate can inhibit atherosclerosis and hepatic steatosis and regulate gut microbiota by upregulating the expression of ABCA1 in macrophages in ApoE−/− mice fed a high-fat diet (Du et al. 2020). These studies suggest that lipid metabolism in the enteric–hepatic axis plays an essential role in regulating lipid metabolism disorders associated with atherosclerosis.

Xinnaokang comprises pseudo-ginseng, American ginseng, Pueraria root, Fructus trichosanthis, Salvia miltiorrhiza and licorice. It promotes blood circulation, eliminates blood stasis, lowers blood lipid and blood pressure and dilates blood vessels. Clinically, it is mainly used to prevent and treat hypertension, diabetes, hyperlipidaemia, coronary arteriosclerosis, cerebral arteriosclerosis, arterial plaque and other cardiovascular and cerebrovascular diseases. In this study, ApoE−/− mice were used to construct an atherosclerosis model to analyse the changes in cecal microbiota structure, blood lipid levels and liver lipid metabolism function of Xinnaokang in the treatment of atherosclerosis. Through experiments and analysis, we clarified its underlying mechanism and provided a theoretical basis for applying traditional Chinese medicine compounds in treating disease.

Results and discussion

Identification and network pharmacological analysis of Xinnaokang

At present, an increasing number of gut microbiota metabolites (from food and the corresponding hosts) and traditional Chinese medicine compounds transformed by gut microbiota are used for disease prevention and treatment (Feng et al. 2019). HPLC analysis of the medicinal components of Xinnaokang showed that ginsenoside Rgl, puerarin and tanshinone were the most abundant, followed by notoginsenoside R1 and ammonium glycyrrhizinate, with glycyrrhizin content being the least abundant (Fig. 1a). Based on the network pharmacological analysis of the constituent targets and disease targets, 145 common targets were obtained, which could be used as the predictive targets of Xinnaokang’s action on atherosclerosis (Fig. 1b). A PPI network with common targets of Xinnaokang and atherosclerosis was constructed (Fig. 1c). GO enrichment analysis showed that a total of 2355 biological process-related pathways, 64 cell composition-related pathways and 151 molecular function-related pathways were enriched. The cellular response to chemical stress, lipopolysaccharides, molecules of bacterial origin and nutrient levels were significantly enriched in biological processes. Nuclear receptor activity in cell components and membrane rafts in molecular function were also significantly enriched (Fig. 1d). KEGG pathway enrichment was screened by correcting P-values <0.05, showing co-enrichment with 165 signalling pathways, including AGE-RAGE signalling pathway in diabetic complications, lipid and atherosclerosis and fluid shear stress and atherosclerosis were significantly enriched (Fig. 1e). The results
Xinnaokang treats atherosclerosis

Figure 1 Identification and network pharmacological analysis of Xinnaokang. (a) Identification of the specific components of Xinnaokang via HPLC. 1, puerarin; 2, glycyrrhizin; 3, notoginsenoside R1; 4, ginsenoside Rg1; 5, tanshinone; 6, ammonium glycyrrhizinate. (b) Venn diagram showed the Xinnaokang and atherosclerosis targets. (c) Potential enrichment targets of Xinnaokang were analysed using Cytoscape. (d) GO enrichment was performed to analyse the potential functional pathways of Xinnaokang. (e) KEGG analysis showed the signalling pathways related to Xinnaokang.
showed that the drug composition of Xinnaokang might be related to chemical stress, lipid metabolite, lipopolysaccharide, molecule of bacterial origin, nuclear receptor and fluid shear stress of atherosclerosis. However, the specific mechanism required further study.

Xinnaokang inhibited the formation of vascular plaques in atherosclerotic mice

Animal experiments were performed to analyse the effects of Xinnaokang on atherosclerotic mice. Compared with the CON group, mice in the model group weight increased, while liver, kidney and heart indexes decreased. However, the weight of mice decreased in the rosuvastatin (RSV) and Xinnaokang treatment groups compared with the CON group. Mice in the model group weight increased, while liver, kidney and heart indexes decreased. These results indicated that Xinnaokang treatment could restore the bodyweight of atherosclerotic mice, inhibit the formation of vascular plaques and maintain vascular function.

Xinnaokang regulated blood lipid levels and the expression of proteins related to lipid metabolism

Lipoproteins play an essential causal role in the development and progression of atherosclerosis, so maintaining optimal lipid levels is necessary to achieve optimal cardiovascular health (Ference et al. 2018). Dyslipidaemia, more specifically, high-serum LDL and low-serum HDL levels are known risk factors for cardiovascular disease (CVD) (Ference et al. 2018). The effect of Xinnaokang on blood and liver lipid metabolism in atherosclerotic mice was also analysed. Compared with the CON group, the total cholesterol (TC), triglycerides (TG) and low-density lipoprotein cholesterol (LDL-C) levels were significantly increased, while high-density lipoprotein cholesterol (HDL-C) levels were significantly decreased in the model group. However, the levels of TC, TG and LDL-C were significantly reduced, while HDL-C levels were significantly increased in the RSV, X-L and X-H groups compared with the model group (Fig. 3a). Compared with the CON group, the expression of ABCA1, SREBP-1 and LXR in the vascular tissues of mice was significantly decreased in the model group. Furthermore, compared with the model group, the expression of ABCA1, SREBP-1 and LXR in the vascular tissues of mice was significantly increased in the RSV, X-L and X-H groups (Fig. 3b,c). Dynamic changes in liver, very LDL content and hepatic steatosis are the main events affecting atherosclerosis (Fisher 2016; Muhammad et al. 2018; Nagareddy et al. 2018). Typically, lower-density lipids activate intracellular pathways to increase local and systemic inflammation, monocyte adhesion, endothelial cell dysfunction and apoptosis, and smooth muscle cell proliferation, leading to foam cell recruitment and atherosclerotic plaque formation (Helkin et al. 2016). In contrast, higher-density lipids may prevent or reduce atherosclerosis (Helkin et al. 2016).

Further analysis of the expression of lipid metabolism-related proteins in liver tissue showed that compared with the CON group, the expression of SREBP-2 and LDLR was significantly decreased. In contrast, the expression of IDOL was significantly increased in the model group. Compared with the model group, the expression of SREBP-2 and LDLR was significantly increased in the RSV group and X-H group, and the expression of IDOL was significantly decreased in the RSV, X-L and X-H groups (Fig. 3d,e). Immunohistochemistry analysis of the expression of PCSK9 in liver tissue showed that compared with the CON group, the expression of PCSK9 was significantly increased in the model group. Compared with the model group, the expression of PCSK9 in the liver tissues of mice in the RSV, X-L and X-H groups was significantly decreased (Fig. 3f,g). Studies have shown that naringin, the main flavonoid in grapefruit peel, can also downregulate PCSK9/IDOL through the gut microbiota–liver–cholesterol axis to promote reverse cholesterol transport and reduce the incidence of atherosclerosis (Wang et al. 2020). Other studies have shown that quercetin can also prevent the development of atherosclerosis in ApoE−/− mice by regulating the expression of PCSK9, LXRα and ABCA1 (Jia et al. 2019). These results suggested that Xinnaokang could regulate the level of blood lipids in atherosclerotic mice and promote lipid metabolism by regulating the expression of lipid metabolism-related proteins in the blood vessels and liver tissues.

Xinnaokang regulated the structure and function of cecal microflora in atherosclerotic mice

Traditional Chinese medicine has been used for over 2000 years to treat CVD, which is believed to be caused by the regulation of gut microbiota as a novel mechanism to alter the pathogenesis of CVD in mice and rats (Ji et al. 2020). Emerging data suggest that the gut microbiota is strongly associated with the development of CVD risk factors, such as atherosclerosis, inflammation, obesity and plasma lipid abnormalities (Duttaroy 2021). Several studies have demonstrated that the gut microbiota–liver–
cholesterol axis is a potential pathway for the treatment of atherosclerosis (Chen et al. 2016; Kasahara et al. 2017; Wang et al. 2021). Changes in the gut microbiota and metabolic profiles play essential roles in the occurrence and development of atherosclerosis (Xue et al. 2021). 16S rDNA sequencing was performed to analyse the structure and function of the cecal microflora in atherosclerotic mice. The Venn diagram showed 422 common micro-

Figure 2 Effect of Xinnaokang on body weight, vascular plaque formation and CAV-1 expression in mice with atherosclerosis. (a) Changes in body weight, liver, kidney and heart indexes of mice in different treatment groups. (b) Abdominal aortic vessel histopathology was observed via HE staining. (c) The expression of the CAV-1 gene in abdominal aortic vessels was analysed using qRT-PCR. (d) Western blot was performed to detect the expression of CAV-1 protein in abdominal aortic vessels. (e, f) The distribution of CAV-1 protein in abdominal aortic vessels was analysed via IHC. Scale bar = 100 µm. The magnification is 100 times. Compared with the CON group, *P < 0.05; compared with the MODEL group, #P < 0.05.
organisms in the K, M, Y, XD and XG groups. There were two endemic micro-organisms in the K group, one endemic micro-organism in the M group, three endemic micro-organisms in the Y group, eight endemic micro-organisms in the XD group and three endemic micro-organisms in the XG group (Fig. 4a). Principal component analysis (PCA) showed that the PC1 and PC2 indices were 32.53 and 9.09% respectively (Fig. 4b). Alpha-diversity indexes analysis showed that the alpha-diversity indexes decreased in the M group compared with the K group. The alpha-diversity indexes in the Y, XD and XG groups increased significantly after the intervention (Fig. 4c). The micro-organisms at the phylum level mainly included Bacteroidetes, Verrucomicrobia, Actinobacteria, Cyanobacteria, Patescibacteria, Firmicutes, Proteobacteria, Deferribacteres and Tenericutes (Fig. 4d). LefSe analysis showed that Prevotellaceae was significantly enriched in the K group (Fig. 4e). Caulobacteraceae and Caulobacteriales were significantly enriched in the M group (Fig. 4e). Marinilaceae, Rikenellaceae, Tannerellaceae, Deferribacteraceae, Deferribacteres, Planococcaceae, Carnobacteriaceae, Christensenellaceae, Clostridiaceae_1, Clostridiales_vadinBB60_group, Family_XIII, Lachnospiraceae, Ruminococcaceae, un_o_Clostridiales, Clostridia and un_p_Firmicutes were significantly enriched in the XD group (Fig. 4e). KEGG enrichment analysis showed that methane metabolism, sporulation, cytoskeleton proteins, starch and sucrose metabolism, porphyrin and chlorophyll metabolism were decreased in the M group. This result was reversed after the intervention in the XD group (Fig. 4f). Ingestion of Bifidobacterium动物学 SP. Lactis LKM512 (Bifal) and arginine (Arg) has been reported to prevent or reduce the risk of atherosclerosis by up-regulating blood spermidine levels (Sugiyama et al, 2018; Matsumoto 2020). These results suggested that Xinnaokang treatment could restore the diversity of cecal microflora and related functional pathways.

Xinnaokang regulated liver metabolism in mice with atherosclerosis

To further analyse the effect of Xinnaokang on liver metabolic function in atherosclerotic mice, 1H NMR was performed to investigate the changes in liver metabolic patterns. Partial least squares discrimination analysis (PLS-DA) showed that the metabolite spectra of liver tissue of mice in each group were significantly different. The metabolite spectra of the M and XG groups were significantly dispersed. This indicated that the liver metabolism of mice underwent significant changes after successful modelling (Fig. 5a). Compared with the K group, the expression of homogentisate was significantly increased. In contrast, the expression of phenylacetylglycine, alanine and methionine was significantly decreased in the liver tissue of mice in the M group (Fig. 5b,c). Compared with the M group, the expression of homogentisate, phenylacetylglycine, alanine and methionine was significantly decreased in the XG group (Fig. 5b,d). It is known that there is a close relationship between changes in amino acid (AA) metabolism and the development of atherosclerosis. AA metabolism (e.g. L-arginine, high arginine and L-tryptophan) is also considered a key regulator of vascular homeostasis (Nitz et al. 2019; Zaric et al. 2020). A dietary intake high in arginine, taurine and glycine ameliorate atherosclerosis through endothelial remodelling (Zaric et al. 2020). These results indicated that Xinnaokang treatment could improve the liver metabolic function of atherosclerotic mice. Our study revealed the role of Xinnaokang in the treatment of atherosclerosis by regulating lipid and amino acid metabolism in blood vessels and liver tissues by restoring intestinal micro-organism homeostasis, which provided a theoretical basis for the application of traditional Chinese medicine in CVD.

Materials and methods

Grouping and treatment of animals

Fifty 4-week-old male ApoE−/− mice were used in this study and weighed after adaptive feeding for 1 week. They were then randomly divided into five groups: normal group (K/CON), model group (M/MODEL), positive control group (Y/RSV), Xinnaokang low-dose group (XD/X-L) and Xinnaokang high-dose group (XG/X-H), with 10 rats in each group. The mice in the normal group were fed a normal diet, and the mice in the other groups were fed a high-fat diet with free food and water intake. The Xinnaokang low-dose and high-dose groups were given one and four times the human dose of the compound, at 0.78 and 3.12 g kg−1 respectively. The positive control group was administered an equivalent dose of
RSV after conversion. The specific administration method included the dissolution of each group of drugs in distilled water, gavaged once a day at an administration volume of 1 ml per 100 g body weight, with continuous treatment for 4 weeks. The animal use protocol listed below has been reviewed and approved by the Experimental Animal Ethics Committee of the Hunan University of Traditional Chinese Medicine (lbb201911120002).

Pharmaceutical component identification
According to the Chinese Pharmacopeia (2015 edition) requirements, the components of Xinnaokang, including puerarin, glycyrrhiza, notoginsenoside R1, ginsenoside Rg1, tanshinone and ammonium glycyrrhizinate, were identified via high-performance liquid chromatography (HPLC). The specific experimental process was as follows: 1 g Xinnaokang powder was obtained, diluted to 50 ml with 50% methanol, followed by ultrasound for 30 min and then the test solution was obtained via filtration. We analysed the solution by the HPLC (Waters 2489). Chromatographic separation was performed on a Venusil XBP C18 column (4.6 x 260 mm, 5 μm) at 30°C. Acetonitrile (A) and 0-1% phosphoric acid (B) were used as the mobile phases for analysis. The flow rate was set to 1 ml min⁻¹. The detection wavelength was 203 nm. The following gradient programme was used to apply the elution conditions: 95–81% B at 0:01–35 min, 81–71% B at 35–55 min, 71–60% B at 55–70 min, 71–60% B at 70–100 min and 60–40% B at 100–120 min. A 10 μl sample was injected into the HPLC system for analysis.

Network pharmacological analysis
Based on the TCMSP database (Ru et al. 2014) (https://tcmspw.com/tcmspw.php), compounds of pseudo-ginseng, American ginseng, Pueraria root, F. trichosanthis, S. miltiorrhiza and licorice were retrieved. Target prediction of the included compounds was also carried out using the TCMSP database. All targets were corrected using the UniProt database (Anonymous 2019) (https://www.uniprot.org/), and non-human targets were removed. Disease targets were then obtained using the GeneCards (Safran et al. 2010) database (https://www.genecards.org/), NCBI database (Anonymous 2018) (https://www.ncbi.nlm.nih.gov/) and OMIM database (Amberger et al. 2015) (http://ctdbase.org/). Human gene retrieval was conducted using ‘atherosclerosis’ as a keyword. The screened drug targets and disease targets were inputted into Venny 2.1, a Venn diagram making software and the common targets were obtained, which were used as the prediction targets of drugs on diseases. The common targets of drugs and diseases were inputted into the STRING database (Szklarczyk et al. 2019) (https://string-db.org/cgi/input.pl) to construct the PPI network. The species was set as ‘Homo sapiens’ and a reliability score >0.7 was used to obtain the PPI network. The PPI network was imported into Cytoscape 3.8.0 (Doncheva et al. 2019), and topology analysis was carried out using NetworkAnalyzer. Genes with scores more significant than the average were selected as the key targets after degree sorting. Core genes were screened using the MCODE analysis (Sun et al. 2017). Then, based on the included components, a component–disease–target network diagram was constructed for the therapeutic diseases and action targets. Cytoscape 3.8.0 was also used for topology analysis and the creation of a network diagram. Enrichment analysis of the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways was carried out on the common disease targets of the drugs. The STRING database was used to select items with a P-value <0.05. The ClusterProfiler, enrichplot and ggplot2 packages were installed and used to generate histograms and bubble plots.

Detection of blood lipid levels
The levels of TC, TG, HDL-C and LDL-C in serum were measured using a blood lipid biochemical measuring instrument (Beckman Coulter AU5800, Beckman Coulter Inc., Brea, CA, USA). All the samples were repeated three times.

Haematoxylin–eosin staining
The abdominal aortic vessels tissues of mice were fixed in 4% paraformaldehyde for 24 h and then dehydrated with a 20 and 30% sucrose solution. The abdominal aortic vessels tissues of mice were cut into thin sections, dehydrated, embedded in paraffin, sliced using a paraffin sectioning machine, adhered to treated slides and baked at 60°C for 12 h. The sections were stained with haematoxylin (Wellbio, Changsha, China) for 5–10 min, rinsed with distilled water and then with PBS (Wellbio,
(a) Scaled proportionally to R2X
Colored according to classes in M10

(b) R2x[2] = 0.156

(c) Metabolite concentration

(d) Ellipse: Hotelling’s T2 (95%)

Homogenisate Phenylacetylglycine Alanine Methionine

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Figure 5 Xinnaokang regulated liver metabolism in mice with atherosclerosis. (a) PLSDA was used to analyse the metabolic differences between the groups. (b) The bar chart showed the differentially expressed metabolites. (c) The liver metabolic atlas of atherosclerotic mice. 1, Guanidoacetate; 2, alanine; 3, glutamine; 4, myo-inositol; 5, phenylacetylglycine; 7, β-glucose; 8, homogentisate; 9, methanol; 10, methionine; 11, glycerol; 12, betaine; 13, β-glucose; 14, NAD^+; 15, ethanolamine; 16, N,N-dimethylglycine; 17, phosphocholine; 18, glycerophosphocholine; 19, P-hydroxyphenylacetate; 20, taurine; 21, α-glucose; 22, glycine; 23, choline. (d) Changes in liver metabolism of atherosclerotic mice after Xinnaokang treatment. 1, α-Glucose; 2, guanidoacetate; 3, alanine; 4, myo-inositol; 5, phenylacetylglycine; 6, β-glucose; 7, homogentisate; 8, methionine; 9, β-glucose; 10, glycerol; 11, betaine; 12, ethanolamine; 13, ethanolamine; 14, N,N-dimethylglycine; 15, phosphocholine; 16, threonine; 17, glycerophosphocholine; 18, P-hydroxyphenylacetate; 19, taurine; 20, ethanol; 21, sarcosine; 22, glycine; 23, phenylalanine; 24, histidine; 25, choline; 26, adenosine monophosphate. Compared with the K group, *P < 0.05; Compared with the M group, #P < 0.05.

Changsha, China). The slices were then stained with eosin for 3–5 min, and then washed with distilled water. The slices were then dehydrated with gradient alcohol (95–100%) for 5 min. The slices were placed in xylene for 10 min twice, sealed with neutral gum and observed under a microscope (Motic, BA210T, Xiamen, China).

Quantitative real-time PCR
After treatment, the mice’s abdominal aortic vessels and liver tissues were collected, and total RNA was extracted using TRIzol (Thermo, ThermoFisher Scientific, Waltham, MA, USA). cDNA was synthesized using a HiFiScript cDNA Synthesis Kit (Cown Biosciences, Changsha, China). The sequences for primers were list in the following: LDLR, Sense 5'-CAGCTACCAAGGGCCACCCGAAGC-3'; antisense 5'-CCCTCTGGCACCCATGTCA-3'. Caveolin-1, Sense 5'-GCCAAAGTTGATGCAGCCGAG-3'; antisense 5'-GC CGTATTCACCTTGCTTC-3'. SREBP-2, Sense 5'-GGCT GTCCGGTGTCGA-3'; antisense 5'-CTGTGACAACTGTAG CATCTCG-3'. IDOL, sense 5'-CAGGAGCAACAAGGCA TATC-3'; antisense 5'-GCTGTATATGCTGGCAACG-3'. β-actin, sense 5'-ACATCCGTAAAGACCTTATGGCC-3'; antisense 5'-TACCTCGTTGCTGATCCAC-3'. β-actin was used as an internal reference, and the relative mRNA expression levels were analysed using the 2^{-ΔΔCT} method after quantitative real-time-PCR.

Western blot
After treatment, the abdominal aortic vessels and liver tissues of mice from different treatment groups were collected, radio-immunoprecipitation analysis lysis buffer was added and the bicinchoninic acid method was performed to determine the total protein concentration. Protein samples (200 μg) were separated using 12% sodium dodecyl sulphate–polyacrylamide gel electrophoresis. The isolated proteins were transferred to a polyvinylidene fluoride membrane that has been activated with methanol, blocked with 5% skim milk and dried at room temperature for at least 1 h. The membranes were then incubated overnight with the primary antibodies at 4°C. The primary antibodies used were anti-Caveolin-1 (CAV-1) (16447-1-AP, 1:3000; Proteintech, Rosemont, IL, USA), anti-LDLR (ab52818, 0.4 μg ml^{-1}; Abcam, UK), anti-SREBP-2 (28212-1-AP, 1:1000; Proteintech), anti-IDOL (15455-1-AP, 1:500; Proteintech) and anti-β-actin (60081-1-lg, 1:5000; Proteintech). Afterward, the membranes were incubated with secondary antibodies, including anti-mouse IgG (SA00001-1, 1:5000; Proteintech) and anti-rabbit IgG (SA00001-2, 1:6000; Proteintech) at 37°C for 90 min. The membranes were then visualized through chemiluminescence (Millipore, Burlington, MA, USA) and analysed using imaging software (GE Healthcare Life Sciences, NJ, USA).

Immunohistochemistry
The abdominal aortic vessels and liver tissues were fixed in 4% paraformaldehyde for 24 h and then dehydrated using 20 and 30% sucrose solutions. The mice’s blood vessels and liver tissues were cut into thin sections, dehydrated, embedded in paraffin, sliced using a paraffin sectioning machine, adhered to treated slides and baked at 60°C for 12 h. The slides were then placed in xylene three times for 20 min each time. Next, the slides were placed in 100, 95 and 75% ethanol, with each step taking place for 5 min. The slides were immersed in 0.01 mol l^{-1} citrate buffer solution (pH 6-0) and heated to boiling in an electric furnace or microwave oven. After continuous boiling for 20 min, the slides were removed and cooled to room temperature. The slides were then washed with 0.01 mol l^{-1} PBS (pH 7.2–7.6) for 3 min thrice. Next, the slides were incubated with 1% periodic acid at room temperature for 10 min to inactivate the endogenous enzymes. The slides were then rinsed thrice with PBS for 3 min each time. Then, the slides were incubated with primary antibodies overnight at 4°C. The primary antibodies used were anti-ABCA1 (ab18180, 1:200; Abcam), anti-SREBP-1 (ab28481, 1:200; Abcam), anti-LXR (14351-1-AP, 1:200; Proteintech), anti-PCSK9 (bs-6060R, 1:200; Bioss, Peking, China) and anti-CAV-1 (NBPI-76914, 1:100; Novus, Germany). The slides were then incubated with the corresponding secondary
antibodies, anti-mouse/rabbit-IgG antibody-HRP polymer and incubated at 37°C for 30 min. Afterward, the slides were washed with PBS three times for 5 min each time. Finally, diaminobenzidine (ZSGB-Bio, Peking, China) substrate was used to visualize the sections. Haematoxylin was used for redyeing, followed by alcohol gradient dehydration, section sealing and microscopic observation. Image analysis and processing were performed using ImagePro Plus software.

**16S rDNA sequencing**

To investigate the changes in the cecal microbiota in atherosclerotic mice, microbial genomic DNA was extracted from each stool sample (200 mg) using the QIAAMP® Fast DNA Stool Mini Kit (Qiagen, Germany), as recommended by the manufacturer. An enzyme standard instrument (Multiskan™ GO) was used to detect the concentration of the extracted DNA. The Illumina library construction strategy was used to build a 16S library, quantified using Qubit 3.0. Out-machine double-ended data (raw reads) of samples were obtained using the Illumina HiSeq/MiniSeq sequencing platform. Flash software was used for quality control to get high-quality clean reads. Chimera_check was then carried out. Finally, QiIME software was used for OTU cluster analysis. The MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/) platform was used for subsequent bioinformatics analyses. Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt, https://github.com/picrust/picrust2) and KEGG (https://www.kegg.jp/) were used to predict microbial function. LDA effect size was used for differential functional pathways.

**1H NMR**

The collected liver tissue was transferred to a −4°C refrigerator to thaw, and 150 mg of liver tissue was weighed and cut into pieces in a sterile EP tube. The processed tissue was successively mixed with 300 l water and 600 l methanol. Then, they were homogenized with a homogenizer until no visible tissue masses were found. The liver tissue homogenate was mixed with a homogenizer until no visible tissue masses were found. The liver tissue homogenate was mixed with 300 l double-steamed water and 600 l methanol. Then, they were homogenized with a homogenizer until no visible tissue masses were found. The liver tissue homogenate was mixed with 300 l chloroform, followed by ultrasound treatment for 5 min and centrifugation at 400 g min⁻¹ for 15 min at 4°C (D3024R; SCILOGBX). After centrifugation, 850 l of the supernatant was transferred to another sterile EP tube. Methanol (M116118-500 ml; Aladdin, Shanghai, China) was then added to concentrate the sample, which was removed using a nitrogen blower to dry the sample fully. Before testing, 600 l D₂O solution containing 0-015% TSP was added, followed by ultrasound for 10 min and centrifugation at 400 g min⁻¹ for 15 min at 4°C (D3024R; SCILOGBX). From this, 500 l of the supernatant was transferred to a 5 mm NMR tube, sealed with a rubber cap and placed into a Bruker 600 MHz spectrometer (Bruker AV600; Bruker, Germany) for 1H NMR detection. The specific scanning parameters of the NOESY-PR-1D pulse sequence were as follows: spectrum width = 12.019 kHz, relaxation time = 320 ms, scanning times = 64, FID conversion LB = 0.3 Hz, PW = 30°C (12.7 μs) and RD = 1:0 s. After obtaining the metabolite map, noise reduction, baseline and phase correction were performed on all 1H NMR spectra using Mestrenova software (Mestrenova ver. 9.0.1), and peak alignment was performed on all spectra using the TSP peak at 0 ppm as the reference. After alignment, the water peaks (4.75−4.85 ppm) were removed. The spectra were integrated at 0–1 ppm intervals in the δ = 0.6–9.5 ppm region, and then the integral values were normalized to make up for the differences in concentration among samples. The normalized integral values were imported into SIMCA-P software and analysed via PCA of unsupervised pattern recognition and orthogonal partial least squares analysis (OPLS-DA) of supervised pattern recognition. The independent sample t-test (P < 0.05) and the S-plots of OPLS-DA model VIP ≥1 were used for differential metabolite screening. The online website of MetaboAnalyst (https://www.metaboanalyst.ca/) was used for the metabolic pathway analysis.

**Data analysis and processing**

The statistical software spss (ver. 21.0; IBM, New York, NY, USA) was used to analyse the data. Measurement data were expressed as the mean ± standard deviation. First, normality tests and homogeneity of variance were carried out to determine if the values were consistent with the normal distribution and homogeneity of variance respectively. An unpaired t-test was used between groups, while one-way ANOVA or ANOVA of repeated measurement data followed by Tukey’s post-hoc test was used to compare more groups. A P-value <0.05 indicated that differences were statistically significant.

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Author contributions
Rong Yang and Shunxiang Li designed the research. Rong Yang and Dongliang Yin performed the research. Dihe Yang and Yu Pan provided the drugs. Xinxuan Liu, Qun Zhou and Juan Li analysed the data. All authors contributed to the writing and revision of the manuscript. All authors reviewed the manuscript.

Conflict of Interest
All authors declared no conflict of interest.

Data availability statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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