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# The mediation effect of HDL-C: Non-HDL-C on the association between inflammatory score and recurrent coronary events

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ABSTRACT

*Background:* Inflammation and lipids are both involved in the pathogenesis of coronary heart disease (CHD). However, the mediation effect of lipoproteins on the association between inflammation and recurrent coronary events in CHD patients remains unclear.
*Methods:* This was a retrospective study including CHD patients hospitalized in the Department of Cardiovascular Medicine in Sun Yat-sen Memorial Hospital between January 2011 and December 2012 with the endpoint of recurrent coronary events. The study calculated inflammatory score based on six serum inflammatory markers, including complement C3, complement C4, hypersensitive CRP, fibrinogen, D-dimer, and white blood cell count. Logistic regression analysis, subgroup analysis and mediation analysis were performed to assess the associations between inflammatory score and recurrent coronary events in different subpopulations and the identification of mediators. Inflammatory cytokine expression, cholesterol efflux capacity, and hepatic cholesterol influx were performed in additional CHD patients and healthy controls.
*Results:* There were 191 CHD patients included in the analysis with a median inflammatory score of −0.78 (−2.17, 1.35) and 63 cases of recurrent coronary events. Subgroup logistic regression analysis demonstrated that inflammatory score was positively associated with recurrent coronary events only in the diabetic subgroup [OR: 1.241 (1.004, 1.534), $P < 0.046$]. HDL-cholesterol (HDL-C): non-HDL-C performed 46.74 % of negative mediation effect on this association. CHD patients had lower cholesterol efflux capacity than healthy controls, which was mediated by HDL: non-HDL ratio of 0.4. No difference was found in hepatic cholesterol influx between the two groups.
*Conclusion:* Inflammatory score was associated with recurrent coronary events mediated by HDL-C: non-HDL-C ratio in diabetic CHD patients, indicating that lipoproteins might aggravate the

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inflammatory effect on atherosclerosis under hyperglycemia. Our findings suggested that anti-inflammatory and lipid-lowering therapies might be beneficial for this population.

## 1. Introduction

Atherosclerotic cardiovascular disease is a dominant cause of high mortality and heavy health burden [1,2]. Inflammation is an important underlying mechanism of atherosclerosis, which participates in the process of focal lesions of medium and large arteries [3,4]. High levels of complement, D-dimer, and white blood cells (WBC) were demonstrated to be associated with proatherogenic lipid profiles [5–7]. Anti-inflammation therapy in addition to lipid-lowering drugs and other traditional anti-atherosclerotic strategies has emerged as effective and safe according to previous evidence from clinical trials [8]. Interleukin (IL)-1β antibody and colchicine succeed in reducing the recurrence of coronary events and systematic inflammatory burden in patients with a previous MI history, indicating that chronic inflammation increases the risk of recurrent coronary events and should be suppressed [9,10]. Efforts have been made to develop an inflammatory score for quantifying systemic inflammatory status, and the $z$ score summation of various serum-based inflammatory markers is one of the most common methods. The advantage of using the $z$ score was that adjusting it by the mean of each inflammatory marker narrowed the range and lowered the standard deviation. Although different inflammatory parameters, including complement C3, C-reactive protein (CRP), white blood cell count (WBC), and fibrinogen, were included in various inflammatory score algorithms, the inflammatory score was demonstrated to be associated with ideal cardiovascular health and the occurrence of carotid restenosis [11–13]. The inflammatory score may be a novel surrogate inflammatory marker, and its association with recurrent coronary events remains to be investigated.

Low high-density lipoprotein (HDL)-cholesterol (HDL-C) and high non-HDL-C are two traditional cardiovascular risk factors. HDL carries cholesterol from macrophages back to the liver for cholesterol clearance as a “good” cholesterol [14], while low-density lipoprotein (LDL) and other remnant lipoprotein particles are retained in the intima of injured arteries as a “bad” cholesterol to form unstable plaques with necrotic cholesterol cores in patients with atherosclerotic diseases [8]. Moreover, HDL particles also exert anti-inflammatory effects by increasing alpha-1-antitrypsin binding [15], while oxidized non-HDL particles interact with immune cells and endothelial cells to activate the inflammatory process [16,17]. Previous mediation analysis showed that hyper-sensitive CRP (hsCRP) partly mediated the correlation between dyslipidemia and the risk of CHD [18]. However, the mediation effect of the combination of HDL and non-HDL particles on the mechanism of inflammation promoting atherosclerosis is still unclear.

Inflammation is associated with atherosclerosis with impaired lipid profiles, and proatherogenic lipid profiles are also correlated with inflammation, indicating that impaired lipid profiles mediate atherosclerosis. Mediation analysis helps to unravel the direct and indirect effects of inflammation on recurrent coronary events in the presence of proatherogenic lipid profiles [18]. In this study, we investigated the association between the inflammatory score and recurrent coronary events in patients with previous coronary heart disease (CHD) and the correlation between lipids and the inflammatory score. Besides, the study also examined the mediation effect of the HDL-C: non-HDL-C ratio on this association. Furthermore, in vitro translational experiments were employed to explore the inflammatory change mediated by different ratios between HDL and non-HDL on inflammatory cytokine expression, cholesterol efflux from macrophages, and hepatic cholesterol influx.

## 2. Materials and methods

### 2.1. Study population and sample preparation

The study was a retrospective study approved by the Ethics Committee of Sun Yat-sen Memorial Hospital (No. SYSEC-KY-KS-2021-265) on October 17th, 2021, and the requirement for informed consent was waived with no private health information included in the research data. The study followed the ethical principles of the World Medical Association, also known as the Declaration of Helsinki, for conducting research with human participants. The inclusion criteria were patients diagnosed with CHD hospitalized in the Department of Cardiovascular Medicine in Sun Yat-sen Memorial Hospital between January 2011 and December 2012, and the primary sample size was 236. CHD was defined as $\geq$50 % stenosis in any of the four major coronary arteries as detected by coronary angiography [19]. Forty-five patients who lacked data on complement C3, complement C4, hsCRP, fibrinogen, D-dimer, or WBC were therefore excluded from the analysis. The final analytical sample consisted of 191 patients.

Three CHD patients and three healthy controls were recruited for blood sample collection, after they provided informed consent. Four milliliters of fasting blood samples per person were collected in an ethylene diamine tetraacetonitrile acid (EDTA) anticoagulant tube. After centrifugation at 2100$\times g$ for 15 min, the plasma was collected from the supernatant and stored at −80 °C. HDL-C and LDL-C were measured by an enzymatic method with blood lipid profile test paper (MYCURA, Infopia Co., Korea). The average values of HDL-C and LDL-C were 1.6 mmol/L and 1.8 mmol/L in healthy controls and 0.8 mmol/L and 1.6 mmol/L in CHD patients, respectively.

### 2.2. The primary endpoint

The primary endpoint was a recurrent coronary event with a follow-up period from 2013 to 2021. A recurrent coronary event was defined as the composite of recurrent chest distress or chest pain, non-fatal MI, and coronary artery revascularization.

### 2.3. Inflammatory score

The inflammatory score was calculated as the sum of the *z* scores of inflammatory parameters according to previous studies [11–13], and it included complement C3, complement C4, hsCRP, fibrinogen, D-dimer, and WBC. The *z* score was calculated as the ratio of the difference between the observed value and the mean value and the standard deviation of certain variables among complement C3, complement C4, hsCRP, fibrinogen, D-dimer, and WBC.

### 2.4. Blood lipid parameters

Baseline and last total cholesterol (TC), triglycerides (TG), HDL-C, low-density lipoprotein-cholesterol (LDL-C), apolipoprotein A1 (APOA1) and apolipoprotein B (APOB) were recorded. Non-HDL-C was calculated as TC minus HDL-C. HDL2-C and HDL3-C were measured by the heparin-$Mn^{2+}$(Wako, Osaka, Japan) precipitation method [20]. Total HDL-C was measured in the heparin-$Mn^{2+}$ supernatant, and HDL2-C was further precipitated by dextran sulfate (DS) (Sigma, St. Louis, MO, USA) from the heparin-$Mn^{2+}$ supernatant. HDL3-C was measured in the heparin-$Mn^{2+}$-DS supernatant, and HDL2-C was calculated as TC minus HDL3-C. The ratio of HDL-C/HDL2-C/HDL3-C: LDL-C/non-HDL-C and APOA1: APOB was also calculated.

### 2.5. Clinical variables

In addition to inflammatory and lipid parameters, age, gender, the presence of hypertension and diabetes, fasting blood glucose (FBG), creatinine kinase (CK), creatinine kinase-MB (CK-MB), lactate dehydrogenase (LDH), and N-terminal pro-B type natriuretic peptide (NT-proBNP) were also collected at admission. Hypertension was defined as an average of ≥2 careful readings of blood pressure of 140/90 mmHg or higher obtained on ≥2 occasions, previously diagnosed hypertension, or the use of oral antihypertensive drugs [21]. Diabetes was defined as the presence of typical diabetic symptoms together with a random blood glucose level ≥11.1 mmol/L, a fasting blood glucose level ≥7.0 mmol/L, a 2-h blood glucose level after an oral glucose tolerance test ≥11.1 mmol/L, previously diagnosed diabetes, or receiving diabetic treatment [22].

### 2.6. Ultracentrifugation for HDL

HDL and non-HDL subfractions were isolated by the first step of a two-step discontinuous density-gradient ultracentrifugation method [23]. The density of each plasma sample with a volume of 4 mL was adjusted to a density of 1.24 g/mL with KBr (0.3816 g/mL) and was added to a 13.2 mL centrifuge tube (Beckman); then, it was slowly overlaid with KBr/phosphate buffer solution (0.0834 g/mL, d = 1.063 g/mL). The samples were centrifuged at 280,000×*g* for 4 h at 15 °C in a Beckman Optima™ XE-100 equipped with an SW 41 Ti swinging-bucket rotor (Beckman Instruments, Fullerton, CA, USA). Non-HDL subfractions (<1.063 g/mL) located at the top of the tube were collected, and HDL subfractions (1.063<d < 1.21 g/mL) located in the middle of the tube were then collected.

### 2.7. Luminex liquid suspension chip detection for interleukin-6 (IL-6), macrophage inflammatory protein-1β (MIP-1β), tumor necrosis factor-α (TNF-α), and monocyte chemoattractant protein-1 (MCP-1)

Luminex liquid suspension chip detection was performed by Wayen Biotechnologies (Shanghai, China). The MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel (containing IL-6, MIP-1β, TNF-α, and MCP-1) was used in accordance with the manufacturer's instructions. In brief, 5 mg/dL (0.13 mmol/L) HDL and different concentrations of non-HDL (HDL: non-HDL = 0.3, 0.4, 0.5, and 0.6) were added to 500 μl Dulbecco's modified Eagle's medium (DMEM) with 10 % fetal bovine serum (FBS) and 1 % penicillin–streptomycin (PenStrep), and 5 mg/dL and 17 mg/dL LDL were used as controls. The medium of J774 macrophages treated with different ratios of HDL and non-HDL was incubated in 96-well plates embedded with microbeads for 1 h and then incubated with detection antibody for 30 min. Subsequently, streptavidin-phycoerythrin was added to each well for 10 min, and values were read using the BioPlex MAGPIX System (Bio-Rad).

The results of Luminex liquid suspension chip detection were the average value of two repeated measurements for each sample.

### 2.8. Radiolabeled assays

**a. Efflux assays.** The efflux assay was performed by the detection of tritium-labeled cholesterol ([$^3$H]-chol, PerkinElmer, Boston, MA) from J774 macrophages. J774 macrophages were cultured for at least two passages prior to seeding at 100,000 cells per well in 24-well plates in DMEM, 10 % FBS, and 1 % PenStrep on Day 1.

On Day 2, the culture media was removed, and macrophages were washed twice with PBS. Serum-free DMEM with 5 % bovine serum albumin (BSA) and 1 % PenStrep was incubated with J774 macrophages for 4 h. The DMEM-based media comprising [$^3$H]-chol (2 μCi/mL), 5 % BSA, and 2 μg/ml Sandoz, an ACAT inhibitor to prevent esterification of [$^3$H]-chol, was formulated as labeling media. Then, 500 μL of [$^3$H]-chol labeling media was added (1 μCi per well) and incubated for 24 h.

On Day 3, [$^3$H]-chol labeling media was removed, and then 500 μL of the synchronization media (DMEM, 1 % PenStrep, 2 μg/mL Sandoz, and 5 vol % BSA) was incubated for 4 h. A total of 300 μL of the upregulation media (DMEM, 1 % PenStrep, 300 μM cAMP, 2 μg/mL Sandoz, 1 % BSA) with 26.7 % HDL and different concentrations of non-HDL (HDL: non-HDL = 0.3, 0.4, 0.5, and 0.6) were added to 300 μl of serum-free DMEM with 0.5 % BSA, and 1 % PenStrep was added to upregulate the canonical cholesterol efflux

receptor ABCA1 after removal of the synchronization media for 4 h. Both 5 mg/dL and 17 mg/dL LDL were used as controls. Efflux media was then removed and added to 500 μL Optiphase Hisafe 3 scintillation fluid (PerkinElmer, Boston, MA) for scintillation counting. A separate triplicate cohort of [$^3$H]-chol-loaded macrophages was washed, added to 300 μl of 1 % Triton X-100, air dried, and incubated at 37 °C to extract intracellular [$^3$H]-chol for scintillation counting with 500 μL Optiphase Hisafe 3 scintillation fluid. The radio-luminescence intensity was detected by a MicroBeta$^2$ Microplate Counter (PerkinElmer, Boston, MA), and the cholesterol efflux capacity was calculated as extracellular luminescence/(extracellular + intracellular luminescence) × 100 %.

**b. Tandem efflux-influx assays.** For the efflux-influx experiments, efflux from J774 cells was conducted as described above. HepG2 hepatocytes were plated at 100,000 cells/well in DMEM, 10 % FBS, and 1 % PenStrep in 24-well plates on Day 1. On Day 3, immediately after 4 h of incubation with serum-free DMEM with 0.5 % BSA and 1 % PenStrep, HepG2 hepatocytes were washed twice in PBS, and then efflux media from J774 macrophages was removed and added directly to HepG2 cells. Influx was allowed to proceed for 4 h prior to harvesting. Influx media supernatant was then collected and processed in the same manner as the efflux media described above. [$^3$H]-Chol-loaded HepG2 hepatocytes were washed, added to 300 μl of 1 % Triton X-100, air dried, and incubated at 37 °C to extract intracellular [$^3$H]-chol for scintillation counting with 500 μL Optiphase Hisafe 3 scintillation fluid, which was detected by a MicroBeta$^2$ Microplate Counter (PerkinElmer, Boston, MA). The cholesterol influx to HepG2 hepatocytes was calculated as intracellular luminescence/(extracellular + intracellular luminescence) × 100 %.

The results of radiolabeled assays were the average value of two repeated measurements for each sample.

### *2.9. Confocal microscopy imaging for hepatic cholesterol intake of NBD-cholesterol to HepG2 hepatocytes*

HepG2 cells were cultured in DMEM containing 10 % FBS and 1 % PenStrep. One day prior to treatment, cells were plated at 100,000 cells/well in confocal glass dishes. On the day of treatment, the cells were washed three times with PBS, and fresh medium containing 5 μg/mL NBD-cholesterol was then added to each well with or without a delivery agent according to the treatment regimen. Uptake was allowed to proceed for 30 min with 26.7 vol % HDL and a 2.5-fold molar concentration of non-HDL (HDL: non-HDL = 0.4) in 1 ml DMEM with 10 % FBS and 1 % PenStrep when the cells were fixed and prepared for confocal microscopy. Cells were fixed in 4 % PFA for 10 min at rt. The cells were then washed three times in PBS, stained with DAPI (300 nM in PBS) for 5 min, and washed two more times in PBS. Coverslips were then mounted on glass slides and allowed to seal at rt for at least 24 h prior to imaging. Confocal microscopy was performed using a Zeiss LSM800 with Airyscan (Carl Zeiss AG, Oberkochen, Germany), and image processing was conducted with Zeiss imaging software for microscopy (Carl Zeiss AG, Oberkochen, Germany) [24].

### *2.10. Statistical analysis*

Statistical analyses were performed by using SPSS software (version 25, IBM Corporation, Armonk, NY, USA) and the R tool (version 4.0.5, R Foundation for Statistical Computing, Vienna, Austria).

The comparison of baseline covariates between inflammatory score ≥ -0.8 and <-0.8 was performed in the total population and stratified by gender and the prevalence of diabetes. Continuous variables were expressed as the means with standard deviations if they conformed to a normal distribution or medians with interquartile ranges otherwise. Categorical variables were expressed as the number with the percentage. The intergroup differences for continuous variables were compared using an independent sample *t*-test (conforming to a normal distribution) or the Wilcoxon-Mann–Whitney test (not conforming to a normal distribution), while categorical

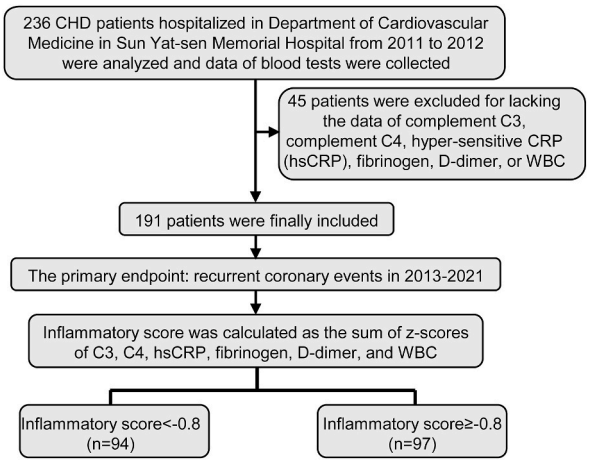


**Fig. 1.** Flow chart of the inclusion and grouping of 191 CHD patients. Flow chart of patient inclusion of the study on the association between the inflammatory score and recurrent coronary events in CHD patients. The cut-off point of the inflammatory score was defined to be its median, −0.8, to avoid imbalanced sample sizes in the two groups. Abbreviation: CHD: coronary heart disease; hsCRP: hypersensitive C reactive protein; WBC: white blood cell count.

variables were compared by the $\chi^2$ test.

A linear correlation model was performed to identify the association between the inflammatory score and lipid parameters. Univariable logistic regression analyses were used to estimate the association of the inflammatory score and recurrent coronary events. Subgroup analysis of the logistic regression model was performed in subgroups of age ≥ or <70 years, female gender or not, complicated with diabetes or not, complicated with hypertension or not, HDL-C ≥ or <1.17 mmol/L (median), HDL2-C ≥ or < 0.42 mmol/L (median), HDL3-C ≥ or < 0.37 mmol/L (median), LDL-C ≥ or < 2.63 mmol/L (median), and non-HDL-C ≥ or < 3.07 mmol/L (median). The odds ratios (ORs) and their 95 % confidence intervals (95 % CIs) were expressed as forest plots with a null line at OR = 1. The interaction effect of subgroup variables and the inflammatory score were calculated to identify potential bias. Mediation analyses were performed to identify the mediation effect of lipid parameters on the association between inflammatory score and recurrent coronary events. Average causal mediation effects (ACME) and average direct effects (ADE) represented the mediation effect of lipid parameters on the association between inflammatory score and recurrent coronary events and the direct effect of inflammatory score on recurrent coronary events, respectively. The results with *P* values < 0.05 were regarded as statistically significant. The power calculation was described in the Supplementary Material section.

**Table 1**
The baseline characteristics of 191 CHD patients.

| | Total population (n = 191) | Inflammatory score < -0.8 (n = 94) | Inflammatory score ≥ -0.8 (n = 97) | *P* |
|---|---|---|---|---|
| Age (year) | 68 (60, 75) | 70 (63, 76) | 66 (56, 75) | 0.030* |
| Female gender (%) | 72 (37.7) | 38 (40.4) | 34 (35.1) | 0.444 |
| Diabetes (%) | 50 (26.2) | 22 (23.4) | 28 (28.9) | 0.391 |
| Hypertension (%) | 129 (67.5) | 64 (68.1) | 65 (67.0) | 0.874 |
| TC (mmol/L) | 4.26 (3.49, 5.24) | 4.00 (3.35, 4.75) | 4.79 (3.71, 5.58) | 0.001* |
| TG (mmol/L) | 1.29 (0.95, 1.92) | 1.16 (0.85, 1.57) | 1.46 (1.05, 2.40) | 0.001* |
| HDL-C (mmol/L) | 1.17 (1.04, 1.36) | 1.19 (1.07, 1.46) | 1.14 (1.00, 1.31) | 0.092 |
| HDL2-C (mmol/L) | 0.42 (0.31, 0.53) | 0.44 (0.30, 0.56) | 0.42 (0.31, 0.50) | 0.206 |
| HDL3-C (mmol/L) | 0.37 (0.28, 0.48) | 0.40 (0.31, 0.50) | 0.37 (0.25, 0.46) | 0.044* |
| HDL2-C:HDL3-C | 1.14 (0.756, 1.74) | 1.13 (0.73, 1.61) | 1.17 (0.80, 1.86) | 0.406 |
| LDL-C (mmol/L) | 2.63 (2.05, 3.40) | 2.45 (1.93, 2.93) | 2.91 (2.20, 3.59) | <0.001* |
| Non-HDL-C (mmol/L) | 3.07 (2.34, 4.09) | 2.84 (2.15, 3.40) | 3.46 (2.52, 4.41) | <0.001* |
| APOA1 (mmol/L) | 1.07 (0.98, 1.17) | 1.08 (1.01, 1.19) | 1.03 (0.94, 1.17) | 0.025 |
| APOB (mmol/L) | 0.74 (0.59, 0.98) | 0.66 (0.54, 0.85) | 0.85 (0.66, 1.06) | <0.001* |
| HDL-C:non-HDL-C | 0.38 (0.29, 0.51) | 0.45 (0.35, 0.58) | 0.32 (0.25, 0.44) | <0.001* |
| HDL-C:LDL-C | 0.45 (0.35, 0.58) | 0.51 (0.41, 0.63) | 0.39 (0.31, 0.50) | <0.001* |
| HDL2-C:non-HDL-C | 0.13 (0.09, 0.20) | 0.15 (0.11, 0.23) | 0.12 (0.08, 0.17) | <0.001* |
| HDL2-C:LDL-C | 0.15 (0.11, 0.23) | 0.18 (0.13, 0.25) | 0.14 (0.10, 0.20) | 0.001 |
| HDL3-C:non-HDL-C | 0.12 (0.08, 0.18) | 0.15 (0.10, 0.20) | 0.10 (0.07, 0.14) | <0.001* |
| HDL3-C:LDL-C | 0.14 (0.10, 0.20) | 0.16 (0.12, 0.22) | 0.12 (0.08, 0.17) | <0.001* |
| APOA1:APOB | 1.41 (1.07, 1.89) | 1.68 (1.31, 2.08) | 1.21 (0.98, 1.54) | <0.001* |
| C3 (g/L) | 774 (642, 1027) | 685 (519, 795) | 966 (760, 1295) | <0.001* |
| z-score of C3 | −0.27 (−0.58, 0.31) | −0.48 (−0.86, −0.22) | 0.17 (−0.31, 0.93) | <0.001* |
| C4 (g/L) | 220 (162, 293) | 178 (134, 222) | 286 (220, 377) | <0.001* |
| z-score of C4 | −0.23 (−0.50, 0.12) | −0.43 (−0.64, −0.22) | 0.09 (−0.23, 0.52) | <0.001* |
| hsCRP (mg/L) | 4.33 (1.37, 17.16) | 1.81 (0.87, 3.65) | 10.01 (4.62, 31.58) | <0.001* |
| z-score of hsCRP | −0.31 (−0.38, −0.03) | −0.37 (−0.39, −0.33) | −0.19 (−0.31, 0.30) | <0.001* |
| Fibrinogen (g/L) | 3.06 (2.65, 3.62) | 2.68 (2.41, 3.01) | 3.61 (3.08, 4.31) | <0.001* |
| z-score of fibrogen | −0.18 (−0.65, 0.47) | −0.62 (−0.93, −0.24) | 0.45 (−0.16, 1.26) | <0.001* |
| D-dimer (mg/L FEU) | 0.41 (0.22, 0.84) | 0.32 (0.19, 0.57) | 0.62 (0.29, 1.54) | <0.001* |
| z-score of D-dimer | −0.15 (−0.15, −0.12) | −0.15 (−0.16, −0.13) | −0.13 (−0.15, −0.07) | <0.001* |
| WBC ( $\times 10^9$/L) | 7.20 (6.03, 8.89) | 6.50 (5.49, 7.50) | 8.19 (7.01, 10.46) | <0.001* |
| z-score of WBC | −0.25 (−0.66, 0.35) | −0.49 (−0.85, −0.14) | 0.10 (−0.31, 0.90) | <0.001* |
| Inflammatory score | −0.78 (−2.17, 1.35) | −2.18 (−3.10, −1.60) | 1.34 (0.12, 3.47) | <0.001* |
| FBG (mmol/L) | 4.8 (4.4, 5.7) | 4.7 (4.3, 5.1) | 5.0 (4.5, 6.1) | 0.015* |
| CK (U/L) | 89 (66, 140) | 83 (63, 122) | 94 (67, 202) | 0.076 |
| CK-MB (U/L) | 13 (10, 16) | 12 (10, 16) | 13 (10, 20) | 0.115 |
| LDH (U/L) | 202 (178, 251) | 186 (169, 211) | 232 (195, 325) | <0.001* |
| NT-proBNP (pg/ml) | 315.3 (76.5, 1646.0) | 254.6 (57.5, 986.4) | 587.8 (103.1, 2903.0) | 0.004* |
| Recurrent coronary events (%) | 63 (33.0) | 28 (29.8) | 35 (36.1) | 0.355 |

*$P < 0.05$.

Abbreviation: ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; CAD: coronary artery disease; CK: creatine kinase; CK-MB: creatine kinase-isoenzymes; HDL-C: high-density lipoprotein-cholesterol; HDL2-C: high-density lipoprotein 2-cholesterol; HDL3-C: high-density lipoprotein 3-cholesterol; hsCRP: hypertensive C reactive protein; LDH: lactate dehydrogenase; LDL-C: low-density lipoprotein-cholesterol; non-HDL-C: non-high-density lipoprotein-cholesterol; NT-proBNP: N-terminal pro-B-type natriuretic peptide; TC: total cholesterol; TG: triglyceride; WBC: white blood cell count.

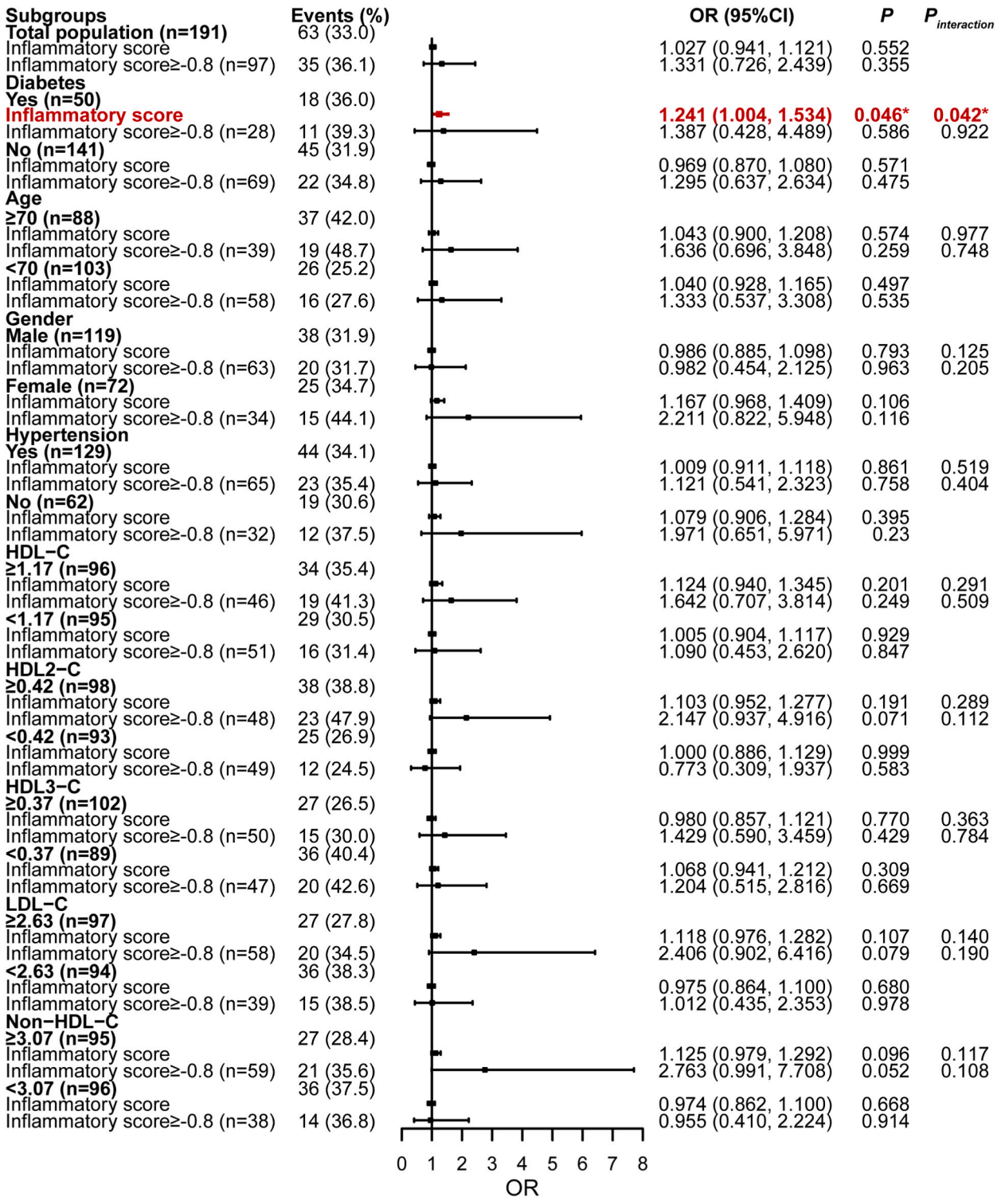


**Fig. 2.** Univariable logistic regression model and subgroup analysis of the association between inflammatory score and recurrent coronary events. *$P < 0.05$ Abbreviation: CI: confidence interval; HDL-C: high-density lipoprotein-cholesterol; HDL2-C: high-density lipoprotein 2-cholesterol; HDL3-C: high-density lipoprotein 3-cholesterol; LDL-C: low-density lipoprotein-cholesterol; non-HDL-C: non-high-density lipoprotein-cholesterol; OR: odds ratio.

## 3. Results

### 3.1. Comparisons of baseline characteristics between patients with inflammatory score ≥ −0.8 and < −0.8

A total of 191 patients were included in the analysis (Fig. 1). The median age of the total population was 68 (60, 75) years, and the median inflammatory score was −0.78 (−2.17, 1.35). The median HDL-C, HDL2-C, HDL3-C, LDL-C, and non-HDL-C levels were 1.17 (1.04, 1.38) mmol/L, 0.42 (0.31, 0.53) mmol/L, 0.37 (0.28, 0.48) mmol/L, 2.63 (2.05, 3.40) mmol/L, and 3.07 (2.34, 4.09) mmol/L, respectively. Based on the median inflammatory score, patients were divided into two groups: inflammatory score ≥ −0.8 (n = 97) and < −0.8 (n = 94). TC, TG, LDL-C, non-HDL-C, APOB, FBG, LDH, NT-proBNP, complement C3, complement C4, hsCRP, fibrinogen, D-dimer, WBC, and their *z* score were significantly higher in patients with inflammatory score ≥ −0.8. The total occurrence of recurrent coronary events was 63 cases (33.0 %), with no difference between patients with inflammatory score ≥ −0.8 and < −0.8. Age, HDL3-C, HDL-C: non-HDL-C, HDL-C: LDL-C, HDL2-C: non-HDL-C, HDL2-C: LDL-C, HDL3-C: non-HDL-C, HDL3-C: LDL-C, and APOA1: APOB were significantly lower in patients with inflammatory score ≥ −0.8 than in those with inflammatory score < −0.8 ($P < 0.05$) (Table 1).

In the analysis grouped by gender, the average age among female patients was higher than that among male patients. Hypertension was more prevalent in female patients with inflammatory score ≥ −0.8. HDL-C levels, especially HDL2-C, were increased in female patients, while LDL-C and non-HDL-C were also increased in female patients with inflammatory score <0.8. When the analysis was grouped by diabetes, diabetic patients were older than non-diabetic patients with lower HDL-C and HDL2-C levels. There was no significant difference in inflammatory markers, HDL-C: non-HDL-C ratio, or the morbidity of recurrent coronary events in the analyses grouped by gender or diabetes, but fibrinogen was significantly higher in diabetic patients with inflammatory score < -0.8.

### 3.2. The association between the inflammatory score and lipid parameters

The inflammatory score was inversely associated with HDL-C, HDL2-C, HDL3-C, HDL-C: LDL-C, HDL-C: non-HDL-C, HDL2-C: LDL-C, HDL2-C: non-HDL-C, HDL3-C: LDL-C, HDL3-C: non-HDL-C, APOA1, and APOA1: APOB (Figs. S1A, S1B, S1C, S1G-L, S1O, and S1Q). There were positive associations between the inflammatory score and LDL-C, non-HDL-C, TC, TG, and APOB (Figs. S1E and S1F, S1M, S1N, and S1P). However, there was no significant associations between the inflammatory score and HDL2-C: HDL3-C and FBG (Figs. S1D and S1R).

### 3.3. The association between the inflammatory score and recurrent coronary events and subgroup analyses

In logistic regression models, the inflammatory score was not associated with recurrent coronary events in the total population, regardless of the categorical and continuous variables of the inflammatory score. Among the subgroups of age, gender, diabetes, hypertension, HDL-C, HDL2-C, HDL3-C, LDL-C, and non-HDL-C, the inflammatory score was positively associated with recurrent coronary events only in the diabetic subgroup [OR: 1.241 (1.004, 1.534), $P = 0.046$]. The interaction between diabetes and the inflammatory score was significant ($P = 0.042$) (Fig. 2).

**Table 2**
The mediation effect of lipids on the association between inflammatory score and recurrent coronary events in diabetic CHD patients.

| | ACME (95%CI) | ADE (95%CI) | Total effect (95%CI) | Proportion of mediation (95%CI) |
|---|---|---|---|---|
| TC | 0.003 (−0.011, 0.020) | 0.049 (0.006, 0.100)* | 0.052 (0.013, 0.090)* | 0.037 (−0.200, 0.640) |
| TG | −0.002 (−0.013, 0.010) | 0.053 (0.013, 0.090)* | 0.051 (0.012, 0.090)* | −0.030 (−0.338, 0.130) |
| HDL-C | −0.008 (−0.026, 0.000) | 0.061 (0.023, 0.090)* | 0.053 (0.018, 0.090)* | −0.165 (−0.823, 0.050) |
| HDL2-C | −0.001 (−0.009, 0.010) | 0.052 (0.014, 0.090)* | 0.052 (0.013, 0.080)* | −0.003 (−0.229, 0.13) |
| HDL3-C | 0.002 (−0.004, 0.010) | 0.048 (0.008, 0.080)* | 0.051 (0.010, 0.090)* | 0.042 (−0.105, 0.360) |
| LDL-C | −0.001 (−0.017, 0.010) | 0.052 (0.009, 0.100)* | 0.051 (0.013, 0.090)* | 0.004 (−0.367, 0.330) |
| Non-HDL-C | −0.001 (−0.019, 0.020) | 0.052 (0.008, 0.100)* | 0.052 (0.013, 0.090)* | 0.002 (−0.399, 0.390) |
| HDL2-C:HDL3-C | 0.006 (−0.004, 0.020) | 0.046 (0.008, 0.090)* | 0.052 (0.014, 0.090)* | 0.094 (−0.118, 0.580) |
| HDL-C:non-HDL-C | −0.025 (−0.051, −0.010)* | 0.078 (0.036, 0.110)* | 0.053 (0.018, 0.090)* | −0.467 (−1.687, −0.010)* |
| HDL2-C:non-HDL-C | −0.009 (−0.027, 0.000) | 0.061 (0.021, 0.090)* | 0.052 (0.014, 0.090)* | −0.142 (−0.823, 0.000) |
| HDL3-C:non-HDL-C | −0.000 (−0.014, 0.010) | 0.051 (0.008, 0.080)* | 0.051 (0.011, 0.080)* | 0.004 (−0.269, 0.450) |
| HDL-C:LDL-C | −0.027 (−0.053, −0.010)* | 0.080 (0.039, 0.110)* | 0.053 (0.019, 0.090)* | −0.478 (−1.859, −0.120)* |
| HDL2-C:LDL-C | −0.008 (−0.025, 0.000) | 0.060 (0.020, 0.090)* | 0.052 (0.014, 0.090)* | −0.116 (−0.729, 0.010) |
| HDL3-C:LDL-C | 0.001 (−0.012, 0.010) | 0.050 (0.007, 0.080)* | 0.051 (0.011, 0.080)* | 0.021 (−0.232, 0.480) |
| ApoA1:ApoB | −0.015 (−0.032, 0.000) | 0.067 (0.027, 0.100)* | 0.052 (0.016, 0.090)* | −0.293 (−1.093, 0.020) |

*$P < 0.05$.

Abbreviation: ACME: average causal mediation effect; ADE: average direct effect; ApoA1: apolipoprotein A1; ApoB: apolipoprotein B; CAD: coronary artery disease; CI: confidence interval; HDL-C: high-density lipoprotein-cholesterol; HDL2-C: high-density lipoprotein 2-cholesterol; HDL3-C: high-density lipoprotein 3-cholesterol; LDL-C: low-density lipoprotein-cholesterol; non-HDL-C: non-high-density lipoprotein-cholesterol; TC: total cholesterol; TG: triglyceride.

### 3.4. The mediation effect of the HDL-C: non-HDL-C ratio on the association between the inflammatory score and recurrent coronary events in diabetic patients with CHD

Mediation effect analysis was performed to identify whether blood lipids mediate the association between inflammatory score and recurrent coronary events in diabetic patients with CHD. Among all lipid parameters, only the HDL-C: non-HDL-C ratio and HDL-C: LDL-C ratio had a significant mediation effect on the association between the inflammatory score and recurrent coronary events in diabetic patients with CHD (Table 2). The HDL-C: non-HDL-C ratio accounted for 46.74 % of the negative mediation effect, with ACME = −0.025 and ADE = 0.078, while the HDL-C: LDL-C ratio accounted for 47.76 % of the negative mediation effect, with ACME = −0.027 and ADE = 0.080 (Fig. 3).

### 3.5. In vitro experiments for the change in inflammatory cytokines, cholesterol efflux capacity, and influx capacity mediated by HDL and non-HDL

The concentrations of IL-6, MIP-1β, TNF-α, and MCP-1, cholesterol efflux from J774 macrophages and cholesterol influx to HepG2 hepatocytes were compared among HDL, HDL: non-HDL ratios of 0.3, 0.4, 0.5, and 0.6, as well as non-HDL in both CHD patients and healthy controls. Interestingly, J774 macrophages treated with an HDL: non-HDL ratio of 0.4 in CHD patients had a significantly lower cholesterol efflux capacity than those in healthy controls. Furthermore, the combination of HDL and non-HDL mediated higher cholesterol efflux compared to single treatment of HDL or non-HDL or PBS (Fig. 4A). Moreover, MIP-1 concentrations were significantly higher in CHD patients among all ratios of HDL and non-HDL treatment. MCP-1 concentrations were significantly higher in CHD patients in HDL and non-HDL single treatment, as well as HDL: non-HDL = 0.5 and 0.6 groups. However, no significant differences in IL-6, TNF-α, and cholesterol influx to HepG2 hepatocytes were revealed between CHD patients and healthy controls among all subgroups of different HDL: non-HDL ratios (Fig. 4B and S2). Furthermore, a significantly higher intensity of NBD-cholesterol was detected in HepG2 hepatic cells treated with CHD LDL, while a low intensity of NBD-cholesterol was detected in HepG2 heptaic cells treated with heathy LDL, HDL and an HDL: non-HDL ratio of 0.4 from both CHD patients and healthy controls (Fig. S3).

## 4. Discussion

In this study, we investigated the association between the inflammatory score and recurrent coronary events and found a positive association between the inflammatory score and recurrent coronary events in diabetic subpopulations from CHD patients, which was negatively mediated by the HDL-C: non-HDL-C ratio. Furthermore, inflammatory cytokine expression, cholesterol efflux capacity, and hepatic cholesterol influx mediated by HDL and non-HDL particles were also compared between CHD patients and healthy controls, and a significant reduction in cholesterol efflux mediated by HDL: non-HDL with a ratio of 0.4 in CHD patients was revealed compared to healthy controls. These results support the cardio-protective role of HDL and the cooperative effect of non-HDL particles on HDL-mediated cholesterol efflux in different health states.

Inflammatory scores comprehensively reflect the systematic inflammatory burden in the human body by the quantitative integration of various circulatory inflammatory markers, which was regarded as an independent variable in this study and was positively associated with ideal cardiovascular health and the occurrence of carotid restenosis [11–13]. The inflammatory score was also demonstrated to be positively associated with recurrent coronary events in diabetic patients with CHD. Chronic inflammatory burden is one of the determinant processes linking the development and progression of dysglycemia and atherosclerotic diseases [25]. Glucose-lowering therapies, including sodium-glucose cotransporter type 2 inhibitors and metformin, have not only glucose regulatory effects but also anti-inflammatory functions [26,27]. Dyslipidemia also plays a central role in the atherosclerotic process, with cholesterol accumulation in the intima of injured arteries. HDL-mediated cholesterol efflux from macrophages and non-HDL-induced cholesterol retention in macrophages are acknowledged to manipulate the lipid and inflammatory balance in atherosclerotic plaques [8]. The ratio of non-HDL-C and HDL-C was demonstrated to better predict a lower risk of CHD in diabetes compared to low LDL-C

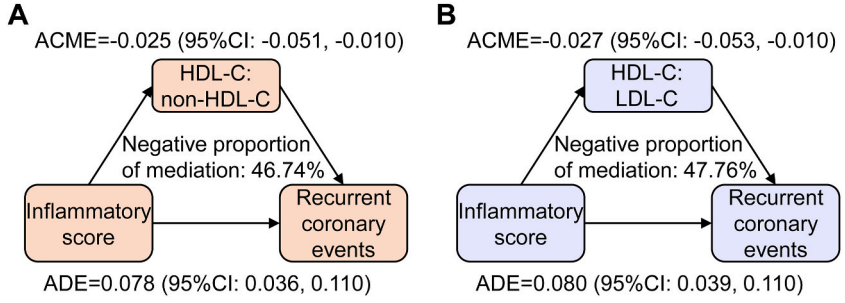


**Fig. 3.** Mediation effect of HDL-C/non-HDL-C (A) and HDL-C/LDL-C (B) on the association between inflammatory score and recurrent coronary events. Abbreviation: ACME: average causal mediation effect; ADE: average direct effect; CHD: coronary heart disease; CI: confidence interval; HDL-C: high-density lipoprotein-cholesterol; LDL-C: low-density lipoprotein-cholesterol; non-HDL-C: non-high-density lipoprotein-cholesterol; TC: total cholesterol; TG: triglyceride.

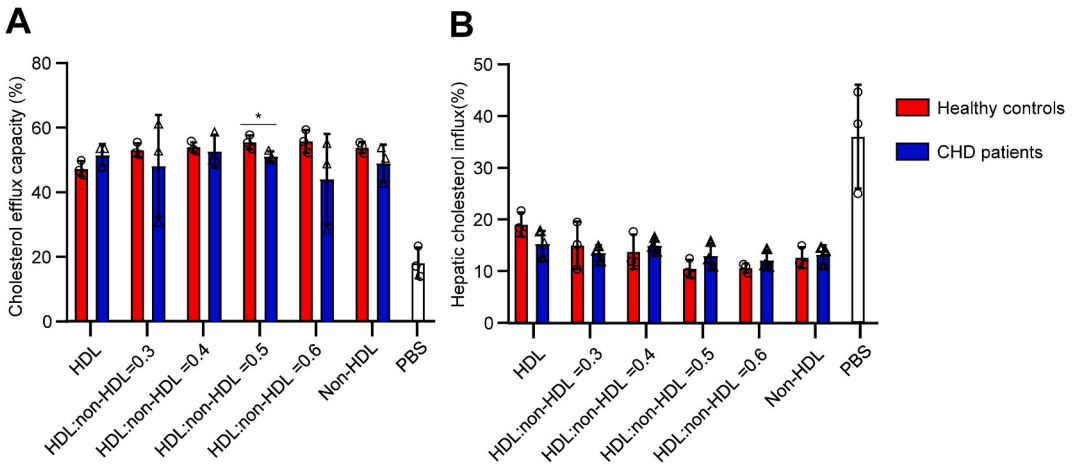


**Fig. 4.** Comparison of cholesterol efflux from J774A.1 macrophages and hepatic cholesterol influx to HepG2 hepatic cells among different HDL:non-HDL ratios. Abbreviation: CHD: coronary heart disease; HDL: high-density lipoprotein; non-HDL: non-high-density lipoprotein; PBS: phosphate buffer solution.

levels in a large sample-based prospective study [28]. Previous mediation analysis showed that the inflammatory marker hsCRP partly mediated the correlation between dyslipidemia and the risk of CHD with a direct effect of 0.621 and an indirect effect of 0.056 [18], indicating the interplay of inflammation and lipids in the process of atherosclerosis. Our findings of the negative mediation effect of the HDL: non-HDL ratio on the association between inflammatory score and recurrent coronary events in diabetic patients with CHD also provide clinical evidence for the potential regulatory effect of lipids on chronic inflammation, which increases the atherosclerotic risk in diabetes.

The strength of this study is that we involved some translational in vitro experiments that shed light on the clinical findings of the potential mediation effect of HDL: non-HDL on the inflammatory effect on atherosclerosis. Excessive cholesterol is removed from macrophages by the mediation of HDL through membrane cholesterol transporters, especially adenosine triphosphate-binding cassette transporter A1 (ABCA1). However, whether non-HDL particles affect HDL-mediated cholesterol efflux from macrophages remains unclear. Adorni et al. found that LDL apheresis significantly reduced HDL levels and serum-mediated cholesterol efflux capacity in patients with familial hypercholesterolemia (FH), but it also reduced macrophage cholesterol loading capacity [29]. The increase in LDL clearance is an effective therapeutic measure for FH patients, but the role of LDL in HDL functions in the healthy population is still poorly understood. The combination of HDL and non-HDL particles increased cholesterol efflux from macrophages with no significant increase in cholesterol influx, which indicated that there might be an assistant effect of non-HDL particles for HDL particles in a healthy population. Interestingly, the cholesterol efflux capacity was impaired in CHD patients with a HDL: non-HDL ratio of 0.4, the average among the included population, compared to healthy controls, which demonstrated that non-HDL might assist with the cholesterol transport functions of HDL.

Inflammatory markers, including MIP-1 and MCP-1, were increased in macrophages treated with HDL and non-HDL in CHD patients compared to healthy controls. HDL from CHD patients also renders higher intake of NBD-cholesterol in HepG2 hepatic cells. Although non-HDL was incubated with HDL, the proinflammatory effect of non-HDL was significant in CHD patients, which was consistent with previous transcriptome analysis of the non-HDL-induced proinflammatory response in macrophages [30]. Non-HDL in CHD has a proinflammatory effect and may impair HDL anti-atherogenic capacity. However, non-HDL particles may assist HDL functions in healthy populations. A recent experimental study found that oxidized LDL could not facilitate platelet aggregation in vitro [31], indicating that impairment by oxidized LDL might require an atherogenic environment. This study also found that the combination of HDL and non-HDL was not associated with impaired cholesterol efflux capacity. The atherosclerosis-associated functions of non-HDL in a healthy population can be further investigated. There were also limitations of this study. First, the sample size for this retrospective study of the association between inflammatory score and recurrent coronary events was small, which requires further prospective studies with larger sample sizes to validate the association. Moreover, the mediation effect of the HDL: non-HDL ratio on the association between inflammation and cardiovascular risk in the general population is also important to be investigated. The explorative subgroup analyses in this study were not predefined, and the subgroups were chosen based on traditional risk factors in the Framingham risk score [32]. This mediation analysis pilot to design a study properly powered to search for mediating effects of HDL: non-HDL to inflammation and atherosclerotic process in patients with dysglycemia.

## 5. Conclusions

In this study, we found that both the HDL-C: non-HDL-C ratio and HDL-C: LDL-C ratio had a negative mediation effect on the positive association between the inflammatory score and recurrent coronary events. The cholesterol efflux capacity affected by the ratio of HDL and non-HDL should be considered and validated in further experiments in lipid research.

## Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

## CRediT authorship contribution statement

**Jie Zhang:** Writing – original draft, Investigation, Formal analysis, Data curation. **Hongwei Li:** Writing – original draft, Visualization, Methodology, Investigation. **Runlu Sun:** Investigation, Data curation. **Zhengyu Cao:** Visualization, Validation. **Jingjing Huang:** Validation, Methodology. **Yuan Jiang:** Methodology. **Mingxing Mo:** Visualization. **Lingyu Luo:** Data curation. **Qi Guo:** Methodology. **Qian Chen:** Methodology. **Yuling Zhang:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

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