INSIGHTS IN LIPIDS IN CARDIOVASCULAR DISEASE: 2021

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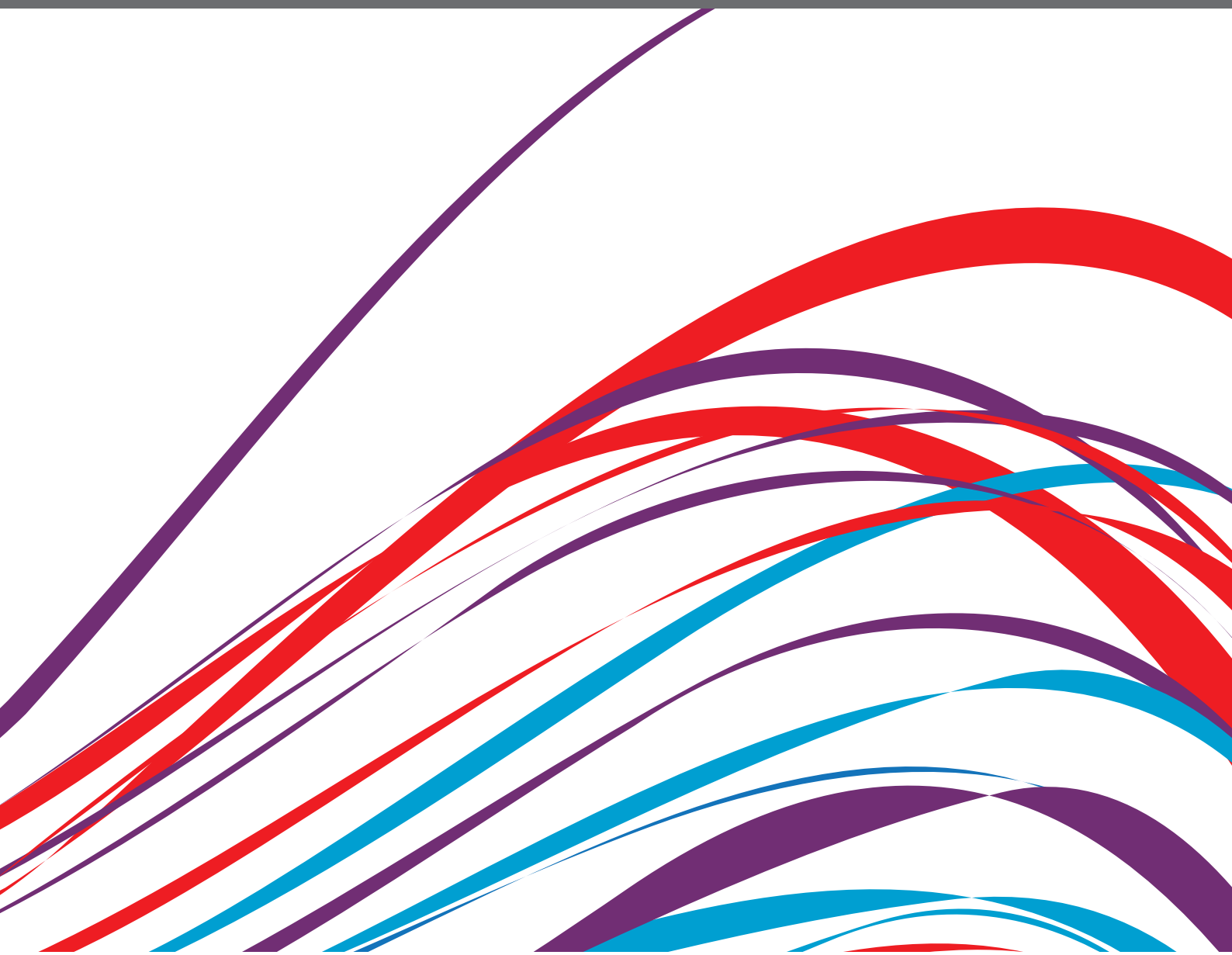

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INSIGHTS IN LIPIDS IN CARDIOVASCULAR DISEASE: 2021

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Editorial: Insights in lipids in cardiovascular disease: 2021

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LDL, VLDL, HDL, triglycerides, lipidomics

Editorial on the Research Topic
Insights in Lipids in Cardiovascular Disease: 2021

The connection between lipids and cardiovascular disease emerged almost a 100 years ago when it was recognized that familial hypercholesterolemia, a genetic disorder that results in high levels of low-density lipoproteins (LDL) leads to a high risk of atherosclerosis. Since then large-scale clinical and epidemiological studies have revealed a direct association between LDL and cardiovascular disease (CVD), whereas high-density lipoproteins (HDL) were found to be negatively correlated. The vast majority of studies that have investigated the effects of LDL and HDL have primarily focused on their cholesterol content. In this Research Topic, we included several articles that focus on LDL and HDL heterogeneities, as well as on triglyceride rich lipoproteins, from perspectives other than their cholesterol content and their less well-studied effects.

Not all LDLs are created equal

It is well recognized that there is significant heterogeneity in the composition and proatherogenic properties of LDL particles with small, dense LDL (sdLDL) being more proatherogenic than larger particles. A review article by Jin et al. on "*Small, Dense Low-Density Lipoprotein-Cholesterol and Atherosclerosis: Relationship and Therapeutic Strategies*" provides an in-depth analysis of the properties, mechanisms of action, and methods of detection of sdLDL, and describes therapeutic interventions. One of the main conclusions is that the small size and higher density of sdLDL particles enhance their ability to penetrate the vascular wall affecting lipid metabolism and promoting inflammation. The molecular mechanisms specific to sdLDL-induced proatherogenic effects are not well understood and further studies are needed to address these questions.

HDL: Quality, not quantity

Another staple of lipoprotein research that emerged over the past decade is the complexity of HDL. Despite epidemiological evidence for inverse association of HDL cholesterol content (HDL-C) with CVD, genetic studies and therapeutic interventions elevating HDL-C do not support the causal role of HDL-C in CVD.

A meta-analysis of Lee et al. "*Cholesterol Efflux Capacity and Its Association With Adverse Cardiovascular Events: A Systematic Review and Meta-Analysis*" provides a new perspective highlighting the capacity of HDL to mediate cholesterol efflux rather than HDL-C concentration. Analyzing 20 trials, the authors show that an increase in cholesterol efflux capacity (HDL-CEC) is associated with improved outcomes even when adjusted for the levels of HDL-C. This analysis shows that HDL-CEC rather than HDL-C concentration should be taken into the account both for clinical applications and for basic research.

A review by Diab et al. "*HDL Composition, Heart Failure, and Its Comorbidities*" comprehensively summarizes the state of the knowledge of the relationship between HDL composition and function and heart failure, both from the perspective of known HF comorbidities (inflammation, diabetes, obesity, and renal disease) on HDL and from the perspective of altered HDL functions: including anti-inflammatory, anti-oxidative and anti-fibrotic. In particular, the role of apoA-I/SRBI, apoM, and sphingosine-1-phosphate is emphasized in relation to endothelial and vascular protection. Notably, the discussion explores the positive results of some HDL targeted therapeutics that showed promising results in relation to HF.

These two articles highlight that HDL should not be completely written off after the failures of the CETP inhibitor trials and emphasize the promising areas where HDL could play an important protective role.

Beyond LDL

It is also increasingly recognized that multiple lipids, beyond the major lipoprotein species, play a major role in CVD and more studies are needed to elucidate these effects. In this Research Topic, we present several studies that explore the lipidomics of cardiovascular disease.

Triglycerides (TG), esters of glycerol and free fatty acids, are a major component of larger lipoproteins [triglyceride rich lipoproteins (TRL)], especially very low-density lipoproteins (VLDL), and are associated with increased CVD, even in people with well controlled LDL-C. However, the mechanisms of TRL-induced detrimental effects are not well understood. The study by Lin et al. on "*Dietary-Induced Elevations of Triglyceride-Rich Lipoproteins Promote Atherosclerosis in the Low-Density Lipoprotein Receptor Knockout Syrian Golden Hamster*" develops a new animal model to study the effects of TRL and discriminate between the effects of TRL and LDL. The authors show that blocking intestinal uptake of cholesterol results in a decrease in the TRL levels but not LDL and that this is sufficient to reduce atherosclerosis. This model is a powerful new tool to elucidate the mechanisms by which triglyceride rich lipoproteins contribute to CVD.

One of the apolipoproteins that emerged as a regulator of TRLs and a potential therapeutic target is apolipoprotein C-III (APOC3) through its effects on LPL activity and liver lipoprotein uptake. Olivieri et al. "*High Plasma Concentration of Apolipoprotein C-III Confers an Increased Risk of Cerebral Ischemic Events on Cardiovascular Patients Anticoagulated With Warfarin*" explores yet another role of the APOC3 as pro-coagulant in people on warfarin and its association with increased risk of cerebral ischemic events. The study shows that people with high APOC3 had a 3x higher risk of stroke or transient ischemic attack than those with low APOC3. Although the specific molecular mechanism whether due to the TRL elevating effect of APOC3 or APOC3s pro-coagulation activity remains to be elucidated, the study highlights yet another way by which APOC3 contributes to cardiovascular disease.

An underappreciated subfraction of TRLs, is intermediate density lipoprotein (IDL), a class of lipoprotein that encompasses at least some portion of remnant lipoproteins (RLP), lipoproteins formed by lipolysis of VLDL and chylomicrons. While RLPs are not easy to measure, the measurement of IDL may serve as their surrogate marker. In their study "*Clinical Significance of Intermediate-Density Lipoprotein Cholesterol Determination as a Predictor for Coronary Heart Disease Risk in Middle-Aged Men*" Yoshida et al., quantify IDL-C using anion-exchange chromatography to demonstrate a significant association of IDL-C with an increased 10-year risk of CVD as estimated by Framingham risk score (FRS). Notably, in a step-wise multivariate logistic analysis, the IDL-C was a stronger predictor of the higher FRS than VLDL-C or other traditional risk factors.

Collectively, these studies demonstrate that targeting LDL-C as the only lipoprotein therapeutic target is not sufficient and that both smaller (HDL) and larger (TRLs and especially RLP and/or IDL) lipoproteins play important roles in residual cardiovascular risk after controlling LDL-C.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Plasma Lipidomics Identifies Unique Lipid Signatures and Potential Biomarkers for Patients With Aortic Dissection

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Aortic dissection (AD) is a catastrophic cardiovascular emergency with a poor prognosis, and little preceding symptoms. Abnormal lipid metabolism is closely related to the pathogenesis of AD. However, comprehensive lipid alterations related to AD pathogenesis remain unclear. Moreover, there is an urgent need for new or better biomarkers for improved risk assessment and surveillance of AD. Therefore, an untargeted lipidomic approach based on ultra-high-performance liquid chromatograph-mass spectrometry was employed to unveil plasma lipidomic alterations and potential biomarkers for AD patients in this study. We found that 278 of 439 identified lipid species were significantly altered in AD patients ($n = 35$) compared to normal controls ($n = 32$). Notably, most lipid species, including fatty acids, acylcarnitines, cholesteryl ester, ceramides, hexosylceramides, sphingomyelins, lysophosphatidylcholines, lysophosphatidylethanolamines, phosphatidylcholines, phosphatidylinositols, diacylglycerols, and triacylglycerols with total acyl chain carbon number $\geq$54 and/or total double bond number $\geq$4 were decreased, whereas phosphatidylethanolamines and triacylglycerols with total double bond number $<$4 accumulated in AD patients. Besides, the length and unsaturation of acyl chains in triacylglycerols and unsaturation of 1-acyl chain in phosphatidylethanolamines were decreased in AD patients. Moreover, lysophosphatidylcholines were the lipids with the largest alterations, at the center of correlation networks of lipid alterations, and had excellent performances in identifying AD patients. The area under the curve of 1.0 and accuracy rate of 100% could be easily obtained by lysophosphatidylcholine (20:0/0:0) or its combination with lysophosphatidylcholine (17:0/0:0) or lysophosphatidylcholine (20:1/0:0). This study provides novel and comprehensive plasma lipidomic signatures of

AD patients, identifies lysophosphatidylcholines as excellent potential biomarkers, and would be beneficial to the pathogenetic study, risk assessment and timely diagnosis and treatment of AD.

Keywords: aortic dissection, plasma, lipidomics, phospholipid, triglyceride, sphingolipid, fatty acid, biomarker

INTRODUCTION

Aortic dissection (AD), defined as the progressive separation of aortic wall layers, is a catastrophic cardiovascular emergency with an acute onset, a poor prognosis, and little preceding symptoms (1, 2). The incidence of thoracic AD is estimated to be 2.9–4.3 cases per 100,000 individuals per year, but deemed to be underestimated due to the large undiagnosed population (1). Notably, in patients with acute ascending AD who do not have surgery, ≥50% die within 48 h, and up to 90% die within 3 months (3). Moreover, even with surgical repair, the 24-h mortality rate for acute type A AD can reach 10%, 13% at 7 d, and about 20% at 30d (4). Therefore, research on AD pathogenesis and its diagnosis and treatment is of great importance and urgency.

Accumulating data indicate that abnormal lipid metabolism is closely related to the pathogenesis of AD (5–9). Lipid and atherosclerotic profiles revealed that total cholesterol, low density lipoprotein cholesterol, and apo A were significantly lower in patients with AD compared to patients with abdominal aortic aneurysms, even though less lipid lowering drugs were administrated (5). Targeted serum metabolomics discovered that the trimethylamine N-oxide level was significantly higher, whereas those of choline, betaine, and carnitine were significantly lower in patient with AD compared to normal controls, and that trimethylamine N-oxide had significant positive correlations with parameters on AD severity, including interleukin-6, D-dimer, C-reactive protein, and maximum aortic diameter on admission (6). Besides, the trimethylamine N-oxide level was significantly increased in AD patients with plaque rupture compared to AD patients without plaque rupture, and not affected by the incidence of hypertension (6). In addition, knockout of vascular smooth muscle cell-specific E-prostanoid receptor 4 gene induced AD in angiotensin II-infused mice with severe degradation of aortic elastic fiber, smooth muscle cell dedifferentiation, increased vascular NADPH oxidase 1 activity, reactive oxygen species generation, macrophage infiltration, matrix metalloproteinase-2/9 levels, and monocyte chemoattractant protein-1 expression, and higher blood pressure (7). *In vitro* investigations further showed that vascular smooth muscle cell-specific E-prostanoid receptor 4 gene deficiency significantly enhanced angiotensin II-triggered mesenteric arterial vasoconstriction, probably via the activation of intracellular calcium release in vascular smooth muscle cells (7). Moreover, phospholipases and unsaturated fatty acids (FAs) were also demonstrated to have vital roles in the pathogenesis of AD (8).

Owing to the diagnostic challenges, such as rapidly propagating pathology, non-specific signs, analogy with other acute conditions, non-discrete symptomatology, and lack of management infrastructure, untimely diagnosis and/or misdiagnosis of AD remain common, which could markedly deteriorate patient outcomes (10). Currently, only D-dimer was used as the clinically relevant biomarker in the condition of suspected AD, with a specificity of 47% and a sensitivity of 97% (11). As with many other diseases, the search for new or better markers is needed to improve the risk assessment and surveillance of AD, since the diagnostic specificity of available markers is insufficient (12). Lipids, accounting for nearly or more than 50% of the metabolome in many biological samples, are drawing more and more interests as potential biomarkers for the prediction of cardiovascular events (12). On the other hand, comprehensive lipid alterations related to the pathogenesis of AD remain unclear. Accordingly, an untargeted lipidomic approach based on ultra-high-performance liquid chromatograph-mass spectrometry was employed to discover the unique lipid signatures of patients with AD and relevant potential biomarkers in this study.

MATERIALS AND METHODS

Subjects

After obtaining informed consent from all subjects and approval from the Ethics Committee of The First Affiliated Hospital of Nanchang University, the plasma samples were collected from 32 normal controls (healthy individuals) and 35 patients with AD. All patients with AD were suffering of an episode of chest and/or back pain lasting 5 min or longer within 3 d. Furthermore, we confirmed the diagnosis of AD according to computed tomography angiography, and further excluded the patients with Marfan syndrome, other connective tissue disorders or hemodialysis. The blood sample was collected immediately after the diagnosis of AD at the Department of Cardiovascular Surgery. Meanwhile, the normal controls were collected from the medical examination center, and excluded from those with atherosclerotic diseases, aneurysms, or valvular diseases. All the subjects fasted for at least 8 h prior to the blood sample collection. The blood sample was collected in the tube with EDTA, and immediately centrifuged at 1,500 g for 15 min at 4°C to prepare the plasma sample, which was stored at −80°C for subsequent preparation.

Demographic characteristics of the subjects were summarized in **Supplementary Table 1**. There were no significant differences in gender ratio (m/f, 18/14 vs. 23/12), age (52.0 ± 3.5 vs. 55.6 ± 11.1 y), and the incidence of chronic obstructive pulmonary disease (0 vs. 5.7%) between normal controls and patients with AD. Notably, the incidence of hypertension in patients with AD (82.9%) was significantly higher than that in normal controls (12.5%).

Materials

HPLC-grade acetonitrile, methanol, and isopropanol were purchased from Merck (Germany). Distilled water was obtained from Watsons (Hong Kong). Ammonium acetate and methyl tert-butyl ether were gained from Sigma-Aldrich (USA).

Sample Preparation

Following thawing on ice, the plasma sample was mixed thoroughly, and then 50 μL of the plasma sample was pipetted to a 2-mL centrifuge tube. Three hundred microliter of pre-cooled methanol, containing 0.32 μg/mL of sphingomyelin (SM, d18:1/12:0) and 0.25 μg/mL of FA 18:0-d3 as the internal standard in the positive and negative mode, respectively, was subsequently added to the sample, followed by 1-min vortex oscillation. Then, the sample was added with 1 mL of methyl tert-butyl ether, vortexed for 1 min, and gently vibrated for another 1 h. After that, the sample was added with 300 μL of water, and vortexed for 1 min. After equilibration at 4°C for 10 min, the sample was centrifuged at 14,000 g for 15 min. Two 400-μL aliquots of the upper lipid extract were separately pipetted into the new centrifuge tube, vacuum-dried, and then stored at −80°C. Finally, the dried lipid extract was dissolved with 120 μL of acetonitrile/isopropanol/water solution (v/v/v = 65:30:5) for the instrumental analysis in the negative ion mode. Meanwhile, 30 μL of the above dissolved sample was further diluted by 60 μL of the above-mentioned acetonitrile/isopropanol/water solution, and then used for the instrumental analysis in the positive ion mode.

To evaluate the analytical performance of the lipidomic approach, quality control samples were prepared by mixing equal parts of all plasma samples, and processed with the same parameters as the analytical sample during all processes involved in the sample preparation, instrumental analysis, and data processing.

Instrumental Analysis

The plasma lipid profiling was acquired by a Q Exactive Plus high-resolution mass spectrometer (Thermo Scientific, USA) equipped with an Ultimate 3000 UHPLC system (Thermo Scientific, USA). Parameters on the chromatographic separation in the positive ion mode were the same as those in the negative ion mode. Five microliter of the dissolved sample was injected for the lipid separation by a BEH C_8 column (100 × 2.1 mm, 1.7 μm, Waters Co., USA). The column temperature was 55°C. The lipids were eluted by the binary mobile phase A (acetonitrile/water solution, v/v = 6:4, containing 10 mM ammonium acetate) and B (isopropanol/acetonitrile solution, v/v = 9:1, containing 10 mM ammonium acetate). The flow rate was 0.26 mL/min. The elution gradient was conducted as follows: initial 32% B maintained for 1.5 min, linearly increased to 85% B from 1.5 to 15.5 min, to 97% B from 15.5 to 15.6 min, maintained at 97% B from 15.6 to 18.0 min, and then decreased to 32% B from 18.0 to 18.1 min, finally maintained at 32% B from 18.1 to 20.0 min. The temperature of the sample manager was 10°C.

The lipids eluted from the column were ionized by electrospray ionization in the mass spectrometer, and the mass signals were detected in full scan MS and -data dependent MS/MS ($ddMS^2$) mode, with the resolution of 70,000 and 17,500, respectively. The spray voltage (kV) was +3.8 and −3.0 in the positive and negative ion mode, respectively. Other parameters were identical in the positive and negative ion mode, and set as follows: capillary temperature (°C), 320; aux gas heater temperature (°C), 350; sheath gas flow rate (arb), 35; aux gas flow rate (arb), 8; S-lens RF level, 50; mass scanning range (m/z), 100–1,500; TopN (N, the number of the fragmentation ions with the highest abundance), 10; stepped normalized collision energy (NCE), 25, 35, and 45%.

Data Pre-processing

A peak table containing the retention time, m/z, peak area, and lipid identification results was obtained by peak matching and structural identification using the MS-DIAL software (13). Briefly, the raw MS data were converted to the common file format of Reifycs Inc. (.abf) using the Reifycs ABF converter. After that, MS-DIAL software was used for feature detection, spectra deconvolution, lipid identification, and peak alignment among samples. MS/MS spectra-based lipid identification was performed in MS-DIAL software by searching the acquired MS/MS spectra against the internal *in silico* MS/MS spectra database. It includes MS1 and MS/MS information of common lipid species. Tolerances for MS1 and MS/MS searches were set to 0.01 and 0.05 Da, respectively. Data collected in the positive ion mode were normalized to the internal standard SM (d18:1/12:0), and then multiplied by 8×10^8. Meanwhile, data collected in the negative ion mode were normalized to the internal standard FA 18:0-d_3, and then then multiplied by 1×10^{10}. After that, data from the positive and negative ion mode were combined and defined as relative abundances of lipids, which were then used for subsequent statistical analysis.

Statistical Analysis

Chi-square test was done using PASW Statistics 18 (SPSS Inc., USA). Following unit variance scaling, principal component analysis, partial least square discriminant analysis, classical univariate receiver operating characteristic curve analysis, multivariate exploratory receiver operating characteristic curve analysis, and support vector machine algorithm for the feature ranking and sample classification were performed by MetaboAnalyst 5.0 (14). Two-tailed Mann-Whitney U test was carried out via MultiExperiment Viewer 4.9.0 (15). After unit variance scaling, the data were used for heat map plot using MultiExperiment Viewer 4.9.0. Correlation networks of the lipid signatures were constructed using Cytoscape 2.8.2 (16). Spearman correlation analysis was performed by MATLAB (MathWorks Inc., USA). The level of significance was 0.05, and further corrected by the false discovery rate employing Benjamini-Hochberg correction via MultiExperiment Viewer 4.9.0.

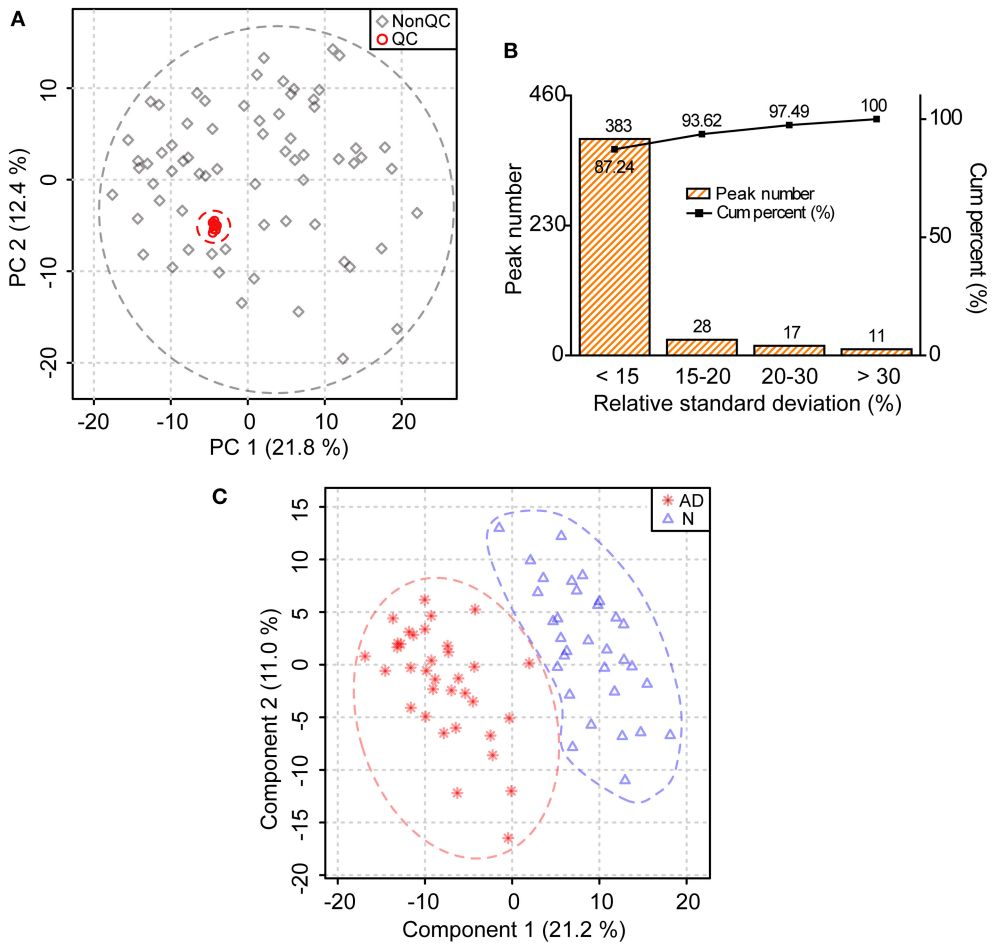


FIGURE 1 | Substantial changes in the lipid profile of patients with AD. **(A)** Distribution of quality control (QC) samples in the score plot of principal component analysis. The explained variances are provided in brackets. **(B)** Distribution of relative standard deviations of ions in QC samples. **(C)** Changes in the lipid profile of patients with AD in the score plot of partial least square discriminant analysis. The explained variances are provided in brackets. $n = 32$ and 35 in the normal (N) and AD group, respectively.

RESULTS

Substantial Changes in the Lipid Profile of Patients With AD

The score plot of principal component analysis showed that 10 quality control samples were located closely to each other (**Figure 1A**). Besides, it was clear from the relative standard deviation distribution that among the 439 lipids identified, there were 383, 411, and 428 lipids, separately accounting for 87.24, 93.62, and 97.49%, with relative standard deviations <15, 20, and 30%, respectively (**Figure 1B**). Above data demonstrated that the lipidomic approach was highly repeatable, stable, and reliable in this study (17, 18). Furthermore, we found that the lipid profile of patients with AD differed greatly from that of normal controls in the score plot of partial least square discriminant analysis, indicating substantial changes in lipid metabolism in patients with AD (**Figure 1C**).

Characteristic Lipid Metabolism in Patients With AD

In total, 278 of 439 identified lipid species were found to be significantly altered in patients with AD compared to normal controls, including 42 FAs, 13 acylcarnitines (ACs), 1 cholesteryl ester (CE), 7 ceramides (Cers), 5 hexosylceramides (HexCers), 40 SMs, 24 lysophosphatidylcholines (LPCs), 6 lysophosphatidylet hanolamines (LPEs), 59 phosphatidylcholines (PCs), 9 phos phatidylethanolamines (PEs), 11 phosphatidylinositols (PIs), 17 diacylglycerols (DGs), and 44 triacylglycerols (TGs) (**Figure 2A**; **Supplementary Table 2**). Heatmap plot showed that levels of most lipids were significantly decreased, including FAs, ACs, CE, Cers, HexCers, SMs, LPCs, LPEs, PCs, PIs, DGs, and TGs with the total number of carbons in the acyl chains ≥54, while levels of most PEs were significantly increased in patients with AD compared to those in normal controls (**Figure 2B**; **Supplementary Table 2**). Consistently, total levels of FAs, ACs,

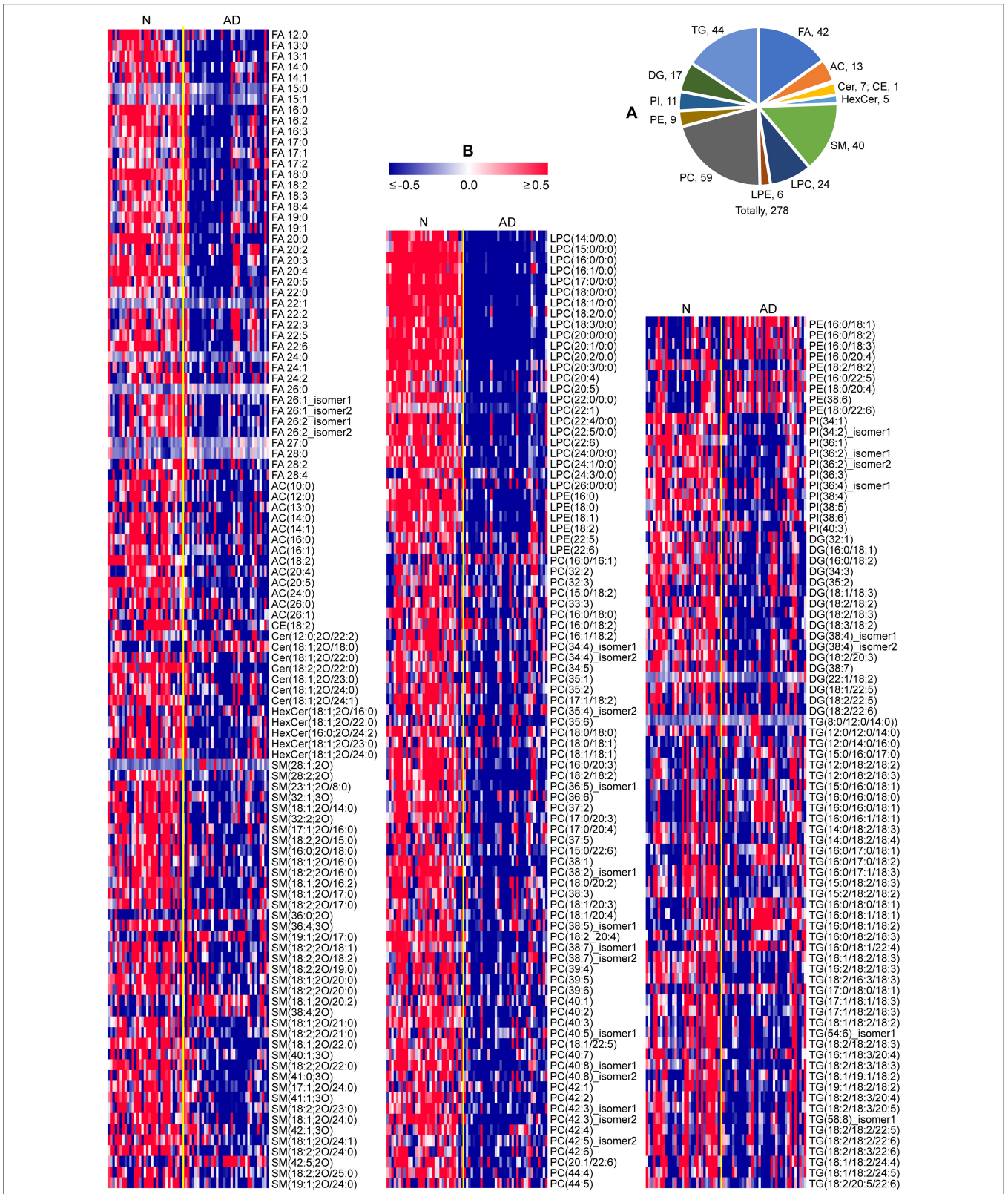


FIGURE 2 | Characteristic lipid metabolism in patients with AD. **(A)** Pie chart indicating the number of lipids significantly altered in patients with AD ($P < 0.05$, two-tailed Mann-Whitney *U* test). **(B)** Heat map plot of lipid alterations in patients with AD. Red/blue color: high/low abundance. $n = 32$ and 35 in the normal (N) and AD group, respectively. FA, fatty acid; AC, acyl carnitine; CE, cholesteryl ester; Cer, ceramide; HexCer, hexosylceramide; SM, sphingomyelin; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; DG, diglyceride; TG, triglyceride.

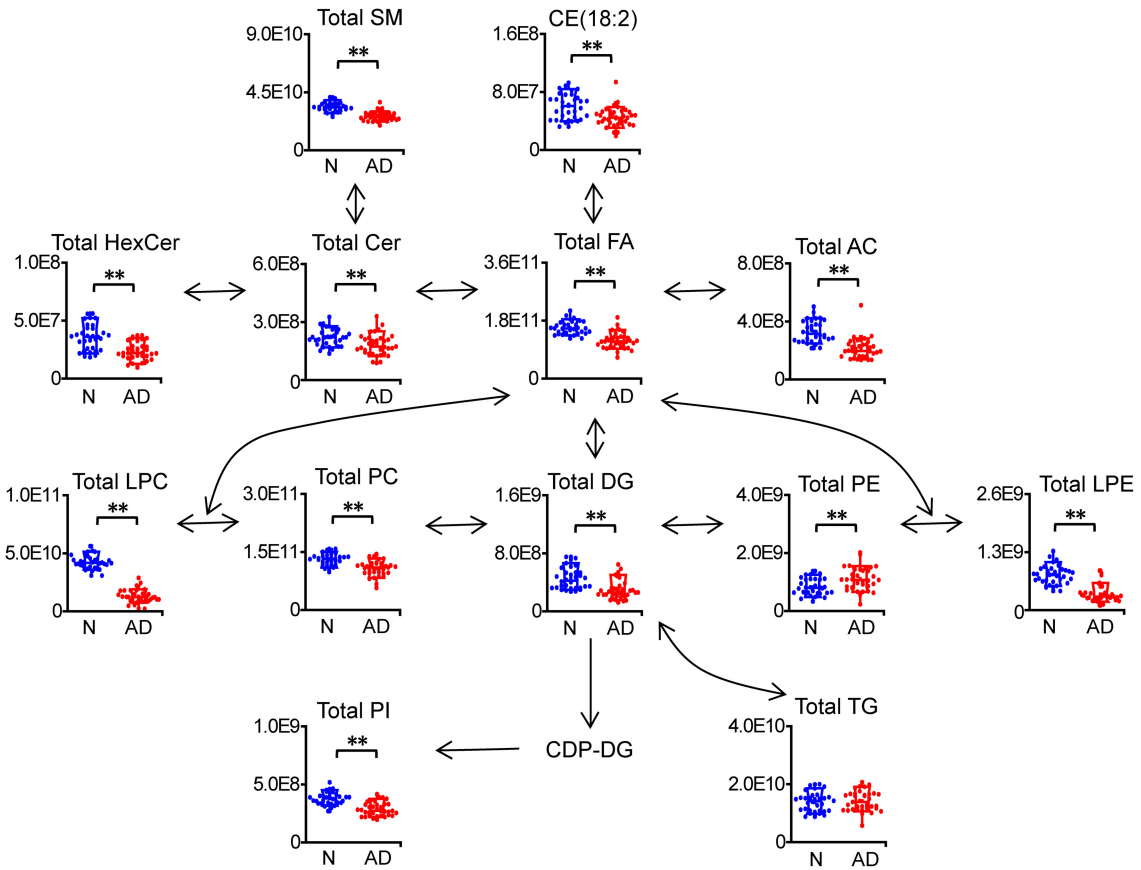


FIGURE 3 | Characteristic changes in total lipids of each category in patients with AD. $n = 32$ and 35 in the normal (N) and AD group, respectively. $^{**}P < 0.01$, two-tailed Mann-Whitney *U* test. The relative abundances of lipids were used for the box plot. Arrows indicate the conversion between lipids. FA, fatty acid; AC, acyl carnitine; CE, cholesteryl ester; Cer, ceramide; HexCer, hexosylceramide; SM, sphingomyelin; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; DG, diglyceride; TG, triglyceride.

CE, Cers, HexCers, SMs, LPCs, LPEs, PCs, PIs, and DGs were significantly decreased, while the total level of PEs were significantly increased in patients with AD compared to those in normal controls (**Figure 3**; **Supplementary Table 3**). However, significant changes in the total level of TGs were not observed in patients with AD compared to normal controls, which could be due to the phenomena that changes in TGs with the total carbon number of acyl chains <54 were different from those in TGs with the total carbon number of acyl chains ≥54 in patients with AD (**Figure 3**; **Supplementary Table 3**). Above data demonstrated decreases in most lipid species and accumulation of PEs in the plasma of patients with AD compared to normal controls, which suggested that most lipid species might accumulate, while PEs might decrease in the dissection of patients with AD compared to normal controls.

Characteristic Changes in the Acyl Chains of TGs in Patients With AD

Since changes in TGs with the total carbon number of acyl chains <54 were different from those in TGs with the total carbon number of acyl chains ≥54, we further investigated changes in the composition and percentage of the acyl chains in TGs. It was clear from the total carbon distribution in the acyl chains of TGs that most TGs with the total carbon number of acyl chains ≥54 were significantly decreased in patients with AD, which was consistent with the results observed in **Figures 2**, **4A**; **Supplementary Table 2**. In addition, it could be clearly observed from the double bond distribution in the acyl chains of TGs that most TGs with the total number of double bonds in the acyl chains ≥4 were significantly decreased, while most TGs with the total number of double bonds in the acyl chains <4 were significantly increased in patients with AD (**Figure 4B**). Moreover, from the changes in the percentage of the acyl chains in TGs, we could observe that polyunsaturated FA chains (the number of double bonds ≥2, i.e., 15:2, 18:2, 20:4, 22:5, 24:4) and FA chains with carbon number >18 (i.e., 19:1, 20:4, 20:5, 22:5, 22:6) were significantly decreased, while 16:0, 16:1, 17:0, 18:0, and 18:1 were significantly increased in patients with AD (**Figure 4C**; **Supplementary Table 4**). These data demonstrated decreases in the length, polyunsaturation, and total number of carbons in the acyl chains of TGs in the plasma of patients with AD.

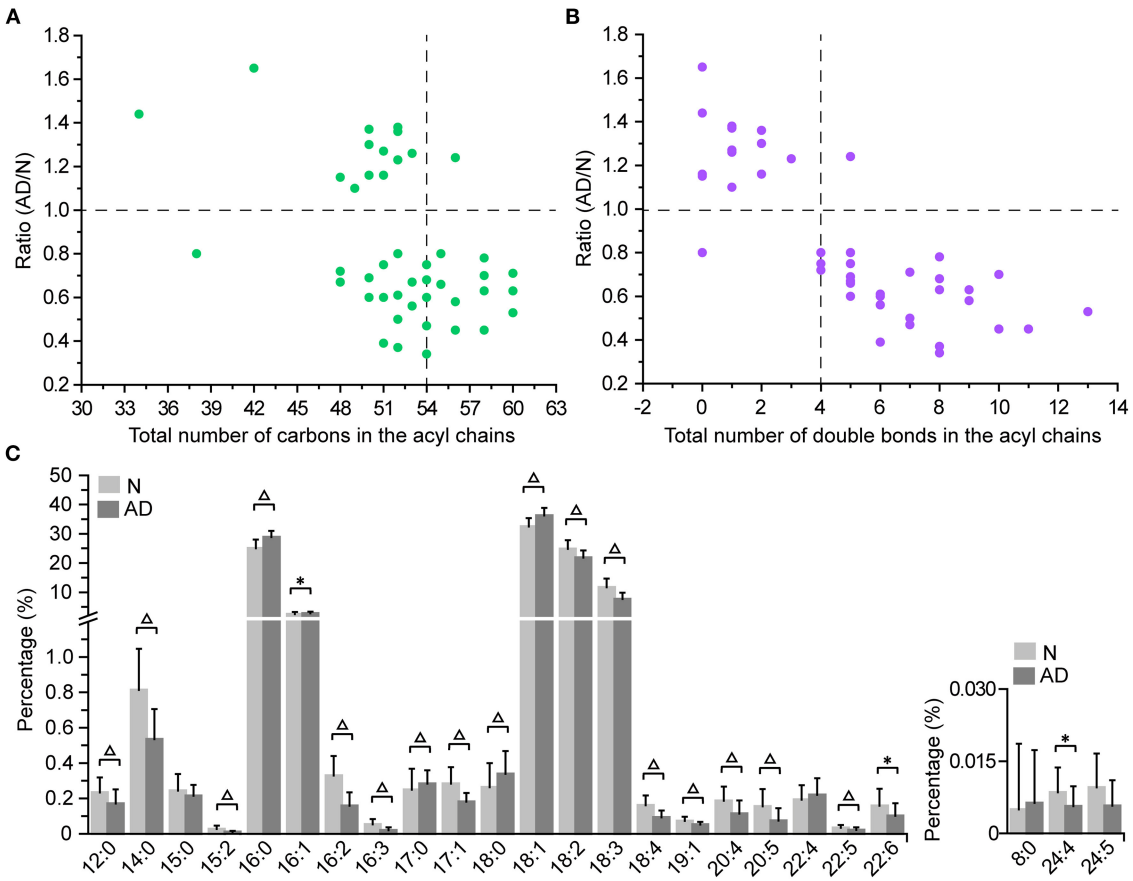


FIGURE 4 | Characteristic changes in the acyl chains of TGs in patients with AD. Changes in the total number of carbons **(A)** and double bonds **(B)** in the acyl chains of TGs. **(C)** Changes in the percentage of the acyl chains of TGs. The column denotes the mean plus standard deviation. n = 32 and 35 in the normal (N) and AD group, respectively. *$P < 0.05$; Δ$P < 0.01$, two-tailed Mann-Whitney *U* test.

Characteristic PE Accumulation and Its Acyl Chain Alterations in Patients With AD

We found that most PE accumulated, including PE (16:0/18:1), PE (16:0/18:2), PE (16:0/18:3), PE (16:0/20:4), PE (16:0/22:5), PE (18:0/20:4), PE (38:6), and PE (18:0/22:6), while PE (18:2/18:2) was decreased in patients with AD (**Figure 5A**; **Supplementary Table 2**). Consistently, the total PE level was also increased in patients with AD (**Figure 3**; **Supplementary Table 3**). Subsequently, characteristic changes in the acyl chains of PEs in patients with AD were further examined (**Figure 5B**; **Supplementary Table 5**). It was showed that saturated acyl chains were only located at the sn-1 position of the glycerol moiety, and that unsaturated acyl chains were mainly distributed at the sn-2 position of the glycerol moiety. Additionally, the percentage of 16:0 at the sn-1 position was significantly increased, while that of 18:2 at the sn-1 position was significantly decreased, which indicated an increase in the saturation at the sn-1 position of PEs in patient with AD. Moreover, the percentages of 18:1 and 22:6 at the sn-2 position were significantly increased, while that of 20:4 at the sn-2 position was significantly decreased, indicating significant acyl chain remodeling at 2-acyl chains of PEs in patients with AD.

Correlation Networks of Lipid Alterations in Patients With AD

To determine the latent relationships between lipid alterations in patients with AD, correlation networks were further constructed with the standard that the absolute values of Spearman correlation coefficients were >0.75 (**Figure 6**). Totally, 1,086 edges and 240 lipids (including 40 FAs, 11ACs, 6 Cers, 4 HexCers, 35 SMs, 24 LPCs, 5 LPEs, 48 PCs, 9 PEs, 5 PIs, 14 DGs, and 39 TGs) were retained in the correlation networks. Besides, the correlation coefficients between lipids in the correlation networks were all positive. Moreover, it was clear that LPCs and PCs were located at the center of the correlation networks, suggesting potential pivotal roles in the molecular pathogenesis of AD. These data demonstrated the high correlations among lipid alterations and potential involvement of lipids in the molecular pathogenesis of AD, especially LPCs and PCs.

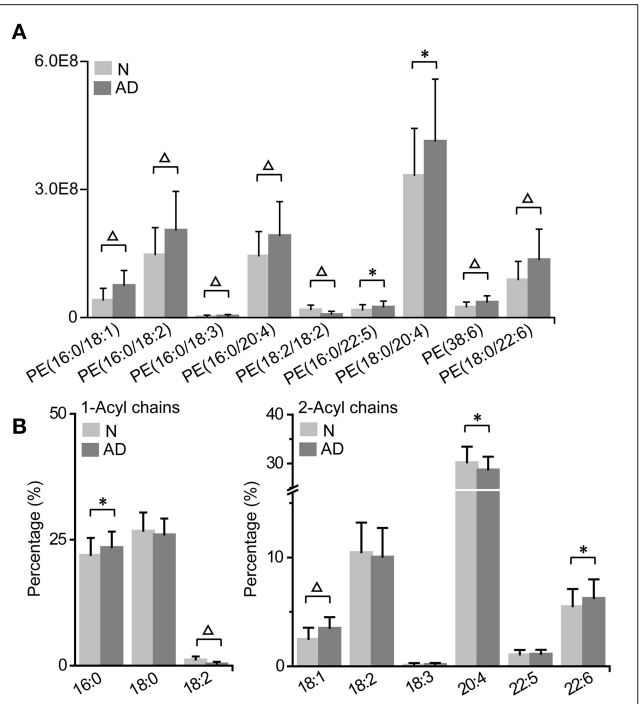


FIGURE 5 | Characteristic PE accumulation **(A)** and its acyl chain alterations **(B)** in patients with AD. The column denotes the mean plus standard deviation. $n = 32$ and 35 in the normal (N) and AD group, respectively. $^*P < 0.05$; $\Delta P < 0.01$, two-tailed Mann-Whitney U test. **(A)** The relative abundances of lipids were used for the histogram.

Potential Biomarkers for the Identification of Patients With AD

The volcano plot showed that LPCs were the lipids with the largest alterations according to the *P*-value, since the top 10 lipids with the lowest *P*-values were all LPCs, including LPC (20:0/0:0), LPC (17:0/0:0), LPC (18:0/0:0), LPC (18:1/0:0), LPC (16:0/0:0), LPC (20:1/0:0), LPC (20:2/0:0), LPC (15:0/0:0), LPC (22:0/0:0), and LPC (18:3/0:0) (**Figure 7A**; **Supplementary Table 2**). Subsequently, the above 10 LPCs were used separately as the potential biomarker for the classical univariate receiver operating characteristic curve analyses and identification of patients with AD based on the support vector machine algorithm (**Figure 7B**). The results showed that when LPC (20:0/0:0), LPC (17:0/0:0), LPC (18:0/0:0), LPC (18:1/0:0), LPC (16:0/0:0), LPC (20:1/0:0), LPC (20:2/0:0), LPC (15:0/0:0), and LPC (22:0/0:0) were used as the potential biomarker, respectively, the area under the curve could be above 0.99, and the accuracy rate could be above 95%, demonstrating that above 9 LPCs had excellent diagnostic performances in identifying patients with AD from normal controls. It was clear from the box plot that the above 10 LPCs were all significantly decreased in patients with AD (**Figure 7C**). Although 100% of both patients with AD and normal controls could be correctly identified by LPC (20:0/0:0) alone, the predicted class probability of one patient with AD (0.48) was close to the classification threshold (0.5) (**Figure 7D**). To improve the classification performance, the multivariate exploratory receiver operating characteristic curve analysis based on the support vector machine algorithm was used for further feature selection from the above 10 LPCs and relevant sample classification. It was revealed that when selecting 2–10 feature variables to identify patients with AD, LPC (20:0/0:0), LPC (17:0/0:0), and LPC (20:1/0:0) were screened as the feature variables with the highest frequency among the 10 LPCs, and the value of each area under the curve could reach 1.0. Therefore, LPC (17:0/0:0) and LPC (20:1/0:0) were separately combined with LPC (20:0/0:0) as the biomarker combination to distinguish patients with AD from normal controls. It was showed that patients with AD could be more clearly distinguished from normal controls by the above combinatorial biomarkers compared to the single biomarker LPC (20:0/0:0), especially the combination of LPC (20:0/0:0) and LPC (17:0/0:0). These data demonstrated that LPCs owned excellent diagnostic performances in discriminating patients with AD from normal controls.

DISCUSSION

FAs are located at the metabolic center of lipids, which can interconvert with other lipids with acyl chains, provide energy through oxidative degradation, and act as signaling molecules to mediate many pathophysiological processes. Significant decreases in most plasma FAs (including saturated, monounsaturated, and polyunsaturated FAs) and ACs in patients with AD in this study suggested potential disorders of lipolysis, FA synthesis, transport and/or oxidation in patients suffering from AD. It was found that the plasma level of superoxide dismutase was decreased, while that of malondialdehyde was increased in patients with AD compared to normal controls (19). Consistently, proteomics and western blotting investigations showed that the malondialdehyde level was increased, while levels of total, Cu/Zn^{-}, and extracellular superoxide dismutase were decreased in the aortic tissue of patients with AD compared to normal controls (20). It was revealed that melatonin treatment increased levels of superoxide dismutase, sirtuin 1, and nuclear factor erythroid 2-related factor 2, decreased the malondialdehyde level, and prevented the deterioration of AD in β-aminopropionitrile fumarate-treated mice, evinced by decreases in the incidence, aneurysmal dilation and vascular stiffness, improvement of aortic morphology, and the inhibition of matrix metalloproteinase expression, elastin degradation, macrophage infiltration, oxidative stress damages and vascular smooth muscle cell loss, and that suppressing sirtuin 1 signaling decreased the protective effects of melatonin on AD (21). Above data indicated increases in oxidative stress and polyunsaturated FA peroxidation, and a decrease in the antioxidant capacity in patients with AD compared to normal controls, and that decreases in polyunsaturated FAs might be related to the increased oxidative degradation (22). Notably, the metabolism of polyunsaturated FAs in oxidative states would produce some lipid signaling mediators, such as prostaglandins, thus affecting the pathogenesis of AD via mediating molecular processes related to inflammation

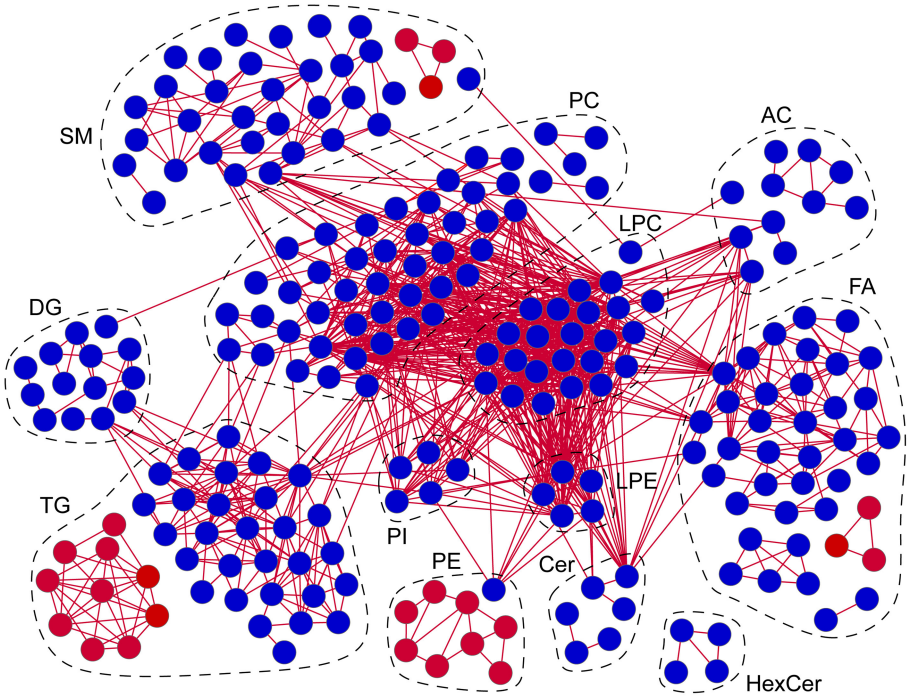
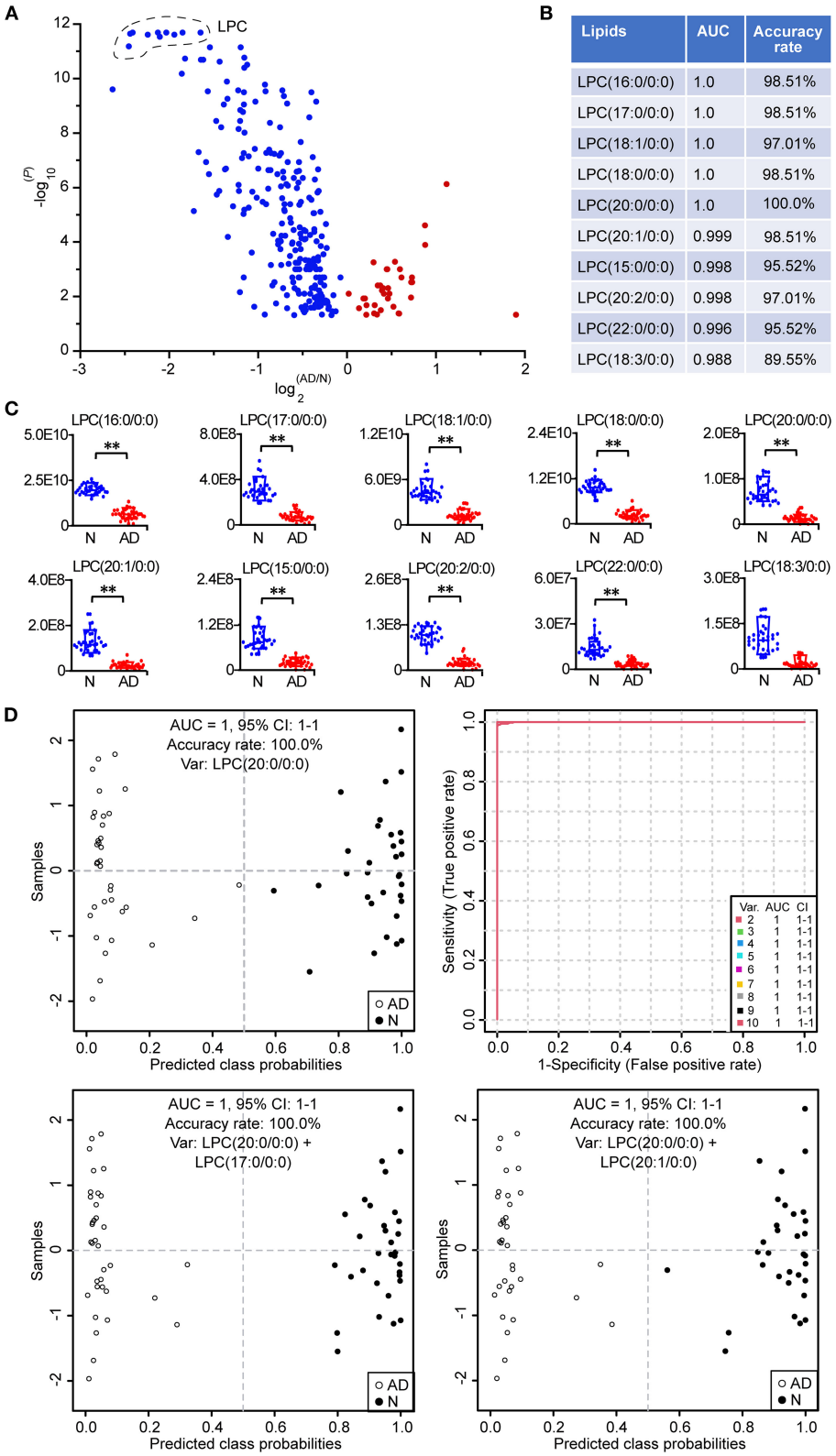


FIGURE 6 | Correlation networks of lipid alterations in patients with AD. Red/blue circles: lipids significantly increased/decreased in patients with AD. Red lines: positive correlations. Absolute values of Spearman correlation coefficients were set to be higher than 0.75. $n = 67$. FA, fatty acid; AC, acyl carnitine; Cer, ceramide; HexCer, hexosylceramide; SM, sphingomyelin; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; DG, diglyceride; TG, triglyceride.

and oxidative stress (7, 23). Moreover, 13 odd-chain FAs, averagely accounting for 1.18% of total FAs in all the subjects, were found to be significantly decreased in AD patients in this study. Changes in odd-chain FAs and some lipids containing odd-chain FAs, such as LPC (17:0), CE (17:0), and monoacylglycerol (15:0), were discovered to be significantly associated with the incidence of ischemic heart disease and type 2 diabetes (24, 25).

We observed decreases in the percentages of 18:2 sn-1 and 20:4 sn-2 in PEs and polyunsaturated FA chains in TGs (such as 18:2, 20:4, 22:5, and 24:4), and increases in the percentages of 18:1 and 22:6 in PEs patients with AD in this study, which indicated the involvement of the unsaturated acyl chains of PEs and TGs in the pathogenesis of AD. Phospholipases and lysophospholipid acyltransferases mediate the deacylation and acylation, respectively, thus modulating the composition and percentage of acyl chains in phospholipids and related alterations in biological functions (26, 27). It was revealed that secreted phospholipase A2 group V from endothelial cells in the angiotensin II-treated aorta of mice could mobilize linoleate and oleate, which could ameliorate endoplasmic reticulum stress, and enhance the expression of lysyl oxidase, thus stabilizing the extracellular matrix, and that dietary linoleate or oleate supplementation abolished the susceptibility to AD in secreted phospholipase A2 group V-deficient mice (8). On the other hand, lysophosphatidylcholine acyltransferase 3 could enhance contents of phospholipids containing polyunsaturated FAs, and mediate arachidonic acid remodeling among different lipid species and eicosanoid release in macrophages, thus affecting the pathogenesis of cardiovascular diseases (27–29). Moreover, serum lipidome found that 3 DGs (including 18:0/18:2, 18:1/18:2, and 18:2/18:2) and 7 TGs (including 16:0/18:1/18:2, 16:0/18:2/18:2, 18:0/18:2/18:2, 18:1/18:1/18:2, 18:1/18:2/18:2, 18:2/18:2/18:2, and 18:2/18:2/20:4), all containing 18:2, were significantly correlated with the incidence of abdominal aortic aneurysm in human, and that the combination of above DGs and TGs with traditional risk factors significantly improved the diagnosis of abdominal aortic aneurysm compared to traditional risk factors alone (30). Besides, the increases in the percentages of short and saturated fatty acyls in TGs were correlated with elevated cardiovascular diseases (31).

Notably, we found that LPCs were the lipids with the largest alterations in the plasma lipidome of patients with AD, and located at the center of metabolic correlation networks, and that LPCs were demonstrated to be excellent potential biomarkers for identifying patients with AD in this study. LPCs can be acylated and deacylated by lysophospholipid acyltransferases and phospholipases to generate PCs and release FAs, respectively, thus affecting the pathogenesis of AD. Additionally, LPCs can acts as the ligands to activate peroxisome



Lipids	AUC	Accuracy rate
LPC(16:0/0:0)	1.0	98.51%
LPC(17:0/0:0)	1.0	98.51%
LPC(18:1/0:0)	1.0	97.01%
LPC(18:0/0:0)	1.0	98.51%
LPC(20:0/0:0)	1.0	100.0%
LPC(20:1/0:0)	0.999	98.51%
LPC(15:0/0:0)	0.998	95.52%
LPC(20:2/0:0)	0.998	97.01%
LPC(22:0/0:0)	0.996	95.52%
LPC(18:3/0:0)	0.988	89.55%

FIGURE 7 | Potential biomarkers for the identification of patients with AD. **(A)** Volcano plot of lipid alterations. Only the differential lipids ($P < 0.05$, two-tailed Mann-Whitney *U* test) are shown in the plot. **(B)** Classical univariate ROC (receiver operating characteristic) curve analyses of the top 10 lipids with the lowest *P*-value. **(C)** Box plot of the top 10 lipids with the lowest *P*-value. The relative abundances of lipids were used. $^{**}P < 0.01$, two-tailed Mann-Whitney U test. **(D)** Discovery of important potential biomarkers by multivariate

(Continued)

FIGURE 7 | ROC curve analyses and the performance. The predicted class probability of each sample was obtained from the 100 cross-validations using the support vector machine algorithm. The cutoff value of probabilities for the sample classification was 0.5. Relevant potential biomarkers used for the sample classification were provided in the graph. LPC, lysophosphatidylcholine. $n = 32$ and 35 in the normal (N) and AD group, respectively. AUC, area under the curve; CI, confidence interval.

proliferator-activated receptors, which are commonly used as the targets for treating metabolic diseases, such as diabetes and cardiovascular diseases (32, 33). The decreases in LPCs in patients with AD in this study were consistent with their mediatory roles in the expression of peroxisome proliferator-activated receptors (31). Besides, it was discovered that levels of LPCs (including 16:0, 18:0, and 18:1) and lipoprotein-associated phospholipase A_2 expression were increased in human atherosclerotic plaques, and that high levels of LPCs (16:0, 18:0, and 18:1) were correlated with increases in tumor necrosis factor-α, interleukin-1β, interleukin 6, macrophage inflammatory protein-1β, monocyte chemoattractant protein-1, macrophages, and lipids, and decreases in smooth muscle cells in human atherosclerotic plaques (34). Moreover, serum metabolomics discovered that a total of 8 LPCs, including LPC (16:0), LPC (16:1), LPC (18:0), LPC (18:1), LPC (18:2), LPC (18:3), LPC (20:3), and LPC (22:6), were significantly decreased in patients with AD (Stanford type A or B) compared to normal controls, and that levels of above LPCs did not vary with gender or blood pressure in patients with AD (Stanford type A or B) (9). However, the diagnostic performance of LPCs as potential markers for the identification of AD patients, such as the sensitivity, specificity, area under the curve, and accuracy rates, were not conducted in the study (9). Large-scale blood metabolomic profiling identified negatively strong associations of LPC (18:1) and LPC (18:2) with the risk of cardiovascular diseases in an age-dependent manner, stronger negative associations in older individuals (35). Decreases in LPCs in this study indicated potential disorders of phospholipid/lysophospholipid lipolysis, lysophospholipid acylation, and/or relevant lipid transport in AD patients. However, the specific role of LPCs in the pathogenesis of AD still needs further functional experiments to discover and verify.

Significant decreases in most plasma sphingolipids, including SMs, Cers, and HexCers, in patients with AD were also observed in this study, which indicated potential disorders of sphingolipid synthesis, degradation, and/or transport in the pathogenesis of AD (36). SMs, as the most abundant sphingolipid in plasma and lipoproteins, accumulated in both lipoproteins and atherosclerotic plaques (36, 37). Sphingomyelinase in atherosclerotic plaques could induce aggregation of low density lipoproteins enriched in Cers via hydrolysis of SMs, thus resulting in the initiation and progression of atherosclerosis (37). Moreover, both SMs and Cers are emerging as promising predictive and/or prognostic biomarkers for the diagnosis of many metabolic diseases, such as cardiovascular diseases (36).

In summary, we provided a comprehensive and novel presentation on plasma lipidomic signatures of patients with AD, which were characterized by decreases in most FAs, ACs, CE, Cers, HexCers, SMs, LPCs, LPEs, PCs, PIs, DGs, and TGs (with the total carbon number of acyl chains ≥54 and/or the total number of double bonds in the acyl chains ≥4), accumulation of PEs and triacylglycerols with the total number of double bonds in the acyl chains <4, and decreases in the length and unsaturation of acyl chains in TGs and unsaturation of 1-acyl chain in PEs. Additionally, pivotal pathophysiological processes and potential therapeutic targets related to above lipidomic signatures of AD were described and discussed. Moreover, LPCs were demonstrated to be useful as potential biomarkers for identifying AD, an accuracy of >95% could easily achieved with a single LPC. The results indicate that verifying the applicability of LPCs as suitable markers for the identification of AD using large-scale samples from multi-centers has a good prospect, which would be of great benefit to improving the prediction, diagnosis, and/or prognosis of AD.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Nanchang University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

HH, GY, S-qL, S-lC, B-cY, and J-cL conceived and designed this study. H-xZ, BY, Q-cW, LW, QW, X-lZ, W-jW, Y-pC, and J-fH collected the written informed consent and plasma samples. GY carried out the lipidomic data analysis, interpreted the data, wrote, and revised the manuscript. S-lC conducted the sample preparation, instrumental analysis, and data pre-processing for the lipidomic approach. HH, S-qL, B-cY, and J-cL checked the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.757022/full#supplementary-material

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Dietary-Induced Elevations of Triglyceride-Rich Lipoproteins Promote Atherosclerosis in the Low-Density Lipoprotein Receptor Knockout Syrian Golden Hamster

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Elevated triglycerides are associated with an increased risk of cardiovascular disease (CVD). Therefore, it is very important to understand the metabolism of triglyceride-rich lipoproteins (TRLs) and their atherogenic role in animal models. Using low-density lipoprotein receptor knockout ($LDLR^{-/-}$) Syrian golden hamsters, this study showed that unlike $LDLR^{-/-}$ mice, when $LDLR^{-/-}$ hamsters were fed a high cholesterol high-fat diet (HFD), they had very high plasma levels of triglycerides and cholesterol. We found that $LDLR^{-/-}$ hamsters exhibited increased serum TRLs and the ApoB100 and 48 in these particles after being fed with HFD. Treatment with ezetimibe for 2 weeks decreased these large particles but not the LDL. In addition, ezetimibe simultaneously reduced ApoB48 and ApoE in plasma and TRLs. The expression of LRP1 did not change in the liver. These findings suggested that the significantly reduced large particles were mainly chylomicron remnants, and further, the remnants were mainly cleared by the LDL receptor in hamsters. After 40 days on an HFD, $LDLR^{-/-}$ hamsters had accelerated aortic atherosclerosis, accompanied by severe fatty liver, and ezetimibe treatment reduced the consequences of hyperlipidemia. Compared with the serum from $LDLR^{-/-}$ hamsters, that from ezetimibe-treated $LDLR^{-/-}$ hamsters decreased the expression of vascular adhesion factors in vascular endothelial cells and lipid uptake by macrophages. Our results suggested that in the $LDLR^{-/-}$ hamster model, intestinally-derived lipoprotein remnants are highly atherogenic and the inflammatory response of the endothelium and foam cells from macrophages triggered atherosclerosis. The LDL receptor might be very important for chylomicrons remnant clearance in the Syrian golden hamster, and this may not be compensated by another pathway. We suggest that the $LDLR^{-/-}$ hamster is a good model for the study of TRLs-related diseases as it mimics more complex hyperlipidemia.

Keywords: triglyceride-rich lipoproteins, ezetimibe, atherosclerosis, low-density lipoprotein receptor, Syrian golden hamster

INTRODUCTION

Cardiovascular diseases are a leading cause of death and are associated with metabolic syndromes including hyperlipidemia, non-alcohol fatty liver, diabetes, hypertension, and obesity (1, 2). Atherosclerosis is the underlying pathology of many cardiovascular diseases (CVD), while hyperlipidemia is the most common contributing factor. So far, animal models of atherosclerosis have demonstrated that plaque formation begins with the infiltration and oxidation of low-density lipoproteins (LDL) in the arterial wall (3). However, the role of triglyceride-rich lipoproteins (TRL) is poorly understood, and hence, the role of TRLs in atherosclerosis needs to be further investigated (4). While it has previously been demonstrated that severe hypertriglyceridemia promoted atherosclerosis in lipoprotein lipase knockout ($LPL^{-/-}$) mice, disease progression was very slow, taking more than 1 year to detect atherosclerosis (5). It is possible that very large lipoprotein particles in LPL-deficient animal models have difficulty entering the arterial wall. Further, different types of hypertriglyceridemia detected in clinical settings suggest that atherogenic mechanisms are more complex than previously thought. Therefore, a more appropriate animal model is required to investigate the role of TRLs in atherosclerosis.

The Syrian golden hamster is widely used to study lipid metabolism. This is because hamsters and humans have comparable lipid metabolisms, including that of cholesteryl ester transfer protein (CETP) activity and the "LDL-based" lipoprotein profile in the blood (6–8). Previous studies have shown that, unlike in mice and rats, blood lipid levels in wild type (WT) hamsters were regulated by lipids and fructose in the diet, where blood triglyceride levels increased significantly (9, 10). Interestingly, it has been shown that the characteristics of postprandial hyperlipidemia were more evident in LDL receptor knockout ($LDLR^{-/-}$) hamsters (11–14). Further, lipid-lowering drugs had differing effects on hamsters to mice and rats. Inhibition of cholesterol synthesis by statins was very toxic in the hamster, especially when fed a cholesterol-rich diet (11, 15, 16). Moreover, inhibition of cholesterol absorption by ezetimibe completely reversed the increased plasma lipid levels in hamsters fed atherogenic diets, and this effect was not altered by a compensatory increase in cholesterol synthesis (9, 17, 18).

Using the $LDLR^{-/-}$ hamster, this study investigated lipid metabolism and atherosclerosis in mixed hypercholesterolemia and severe hypertriglyceridemia. As previous reports have shown that hamster plasma lipid levels were affected by cholesterol from the intestine, this study aimed to assess the lipid profile of $LDLR^{-/-}$ hamsters when fed high cholesterol high fat (simply termed high-fat diet HFD for ease) diets either with or without ezetimibe treatment. Parallel comparison of $LDLR^{-/-}$ hamster and mouse responses to ezetimibe have not been reported, as well as the comparison of $LDLR^{-/-}$ hamster responses between chow diet (CD) and HFD. Because of the cholesterol absorption inhibitory effect of ezetimibe, this study sought to determine the characteristics of the hyperlipidemia $LDLR^{-/-}$ hamster animal model and evaluate the effects of intestinally-derived cholesterol on a range of lipoproteins and atherogenesis. Through this, it was hoped that the analysis of lipid metabolism and its influence on the disease would determine the appropriateness of using the $LDLR^{-/-}$ hamster for such studies in the future.

Abbreviations: α-SMA, α-smooth muscle actin; ABCG5, ATP-binding cassette transporter G5; ACC1, Acetyl-CoA carboxylase 1; ACSL5, Long-chain lipid coenzyme A synthase 5; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; ApoC3, apolipoprotein C3; ApoE, apolipoprotein E; BSA, bovine serum albumin; ECM, Endothelial Cell Medium; CD, chow diet; CD36, cluster of differentiation 36; CD86, cluster of differentiation 86; CETP, cholesteryl ester transfer protein; CM, chylomicrons; CVD, cardiovascular diseases; DGAT1, Diamide glycerin-based transferase 1; DGAT2, Diamide glycerin-based transferase 2; DTT, dithiothreitol; EZE, ezetimibe; FATP4, fatty acid transport protein4; FFA, free fatty acid; FPLC, fast protein liquid chromatography; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPIHBP1, glycosylphosphatidylinositol-anchored high-density lipoprotein binding protein 1; FAS, Fatty acid synthase; H&E, hematoxylin-eosin staining; HDL, high-density lipoprotein; HFD, high cholesterol high-fat diet; HMG-CoA, 3-hydroxy-3-methyl-glutaryl coenzyme A; HUVEC, human umbilical vein endothelial cells; ICAM-1, intercellular cell adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; LCAT, lecithin cholesterol acyltransferase; LDL, low-density lipoprotein; LDLR, low-density lipoprotein receptor; LPL, lipoprotein lipase; LRP-1, Lipoprotein receptor-related protein 1; mRNA, messenger ribonucleic acid; MTP, microsomal triglycerides transfer protein; NPC1L1, Niemann-Pick C1-Like 1; OCT, optimal cutting temperature; Ox-LDL, oxidative low-density lipoprotein; PBS, phosphate buffer saline; PCR, polymerase chain reaction; RIPA, radioimmunoprecipitation assay; RNA, ribonucleic acid; RPM, revolutions per minute; RT, reverse transcription; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SR-A, scavenger receptor A; SRB1, scavenger receptor B1; SREBP1c, sterol regulatory element-binding protein 1c; TNF, tumor necrosis factor; TRLs, triglyceride-rich lipoproteins; VCAM-1, vascular cell adhesion molecule-1; VLDL, very-low-density lipoprotein; VLDLR, very-low-density lipoprotein receptor; WT, wild type.

MATERIALS AND METHODS

Animals

Syrian golden hamsters and C57BL/6 mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). $LDLR^{-/-}$ hamsters were generated by CRISPR/Cas9 genetic editing system in our lab and bred at the SPF animal facility of Hebei Ex&Invivo Biotechnology Co. (Hebei, China) as described previously (9). $LDLR^{-/-}$ mice were provided by the genetically modified animal model platform of the Key Laboratory of Molecular Cardiovascular Sciences of the National Science and Technology Ministry (Beijing, China). All animals were fed with a normal CD (20% protein and 4% fat; purchased from Beijing Ke'ao Company, Beijing, China) or a high-fat diet (HFD) [0.5% cholesterol, 15% lard (w/w) based on CD] and water *ad libitum*. All animals were kept in a temperature-controlled environment on a light-dark cycle of 12 light/12 dark. The experimental procedures were handled according to the guidelines of the laboratory animal care (NIH publication no. 85Y23, revised 1996) and approved by the Animal Care and Use Committee of the Peking University Health Science Center (LA2015-012).

Considering the life span of the two kinds of rodents, we compared the mid-life animals in the experiments. As we have known, mice are generally considered to live for 1 year. According to the report (19) and our observation, the overall average life span of hamsters is 2 years. Therefore, female

Syrian golden hamsters at 12 months of age, and 6-month-old female C57BL/6 mice were used in this study in order to induce atherosclerotic lesions more quickly. Thirty $LDLR^{-/-}$ hamsters were divided into four groups: CD, $n = 5$; CD and ezetimibe (CD + EZE), $n = 5$; HFD, $n = 10$; HFD and ezetimibe (HFD + EZE), $n = 10$. Then, 20 WT hamsters were used as controls and were divided into four groups (see above) where for each group, $n = 5$. The WT or $LDLR^{-/-}$ mice were divided into HFD and HFD+EZE groups ($n = 10$) as controls for the investigations involving the lipid-lowering treatment (ezetimibe) in hamsters. Ezetimibe was administrated by gavage dissolved in saline, and saline alone was the control. The dose of ezetimibe for hamsters was 2 mg/kg/day and for mice was 3 mg/kg/day, which is the equivalent dose for hamsters and mice based on body surface area. Hamster plasma was collected 1- and 2-weeks post-treatment for analysis of plasma lipid levels and lipoprotein profiles. After 40 days, hamsters were anesthetized with sodium pentobarbital and euthanized for tissue harvest.

Plasma Lipid Analysis

Experimental animals were fed an HFD for 2 weeks, then animals fasted overnight and anticoagulated blood was collected and centrifuged (4,000 rpm, 4°C, 10 min) to separate plasma. The total cholesterol and triglyceride content was measured using commercially available assays from Biosino Biotechnology and Science, Inc. (Beijing, China). Fractions of plasma lipoproteins were separated and collected using an ÄKTA fast protein liquid chromatography (FPLC) system (Amersham Biosciences, USA). Pooled plasma (100 μl) from 5 aliquots per group were eluted with buffer at a constant flow rate of 1 ml/min. For each fraction, 500 μl eluate was collected for triglyceride and cholesterol concentration measurements. A unique feature of the HFD-fed $LDLR^{-/-}$ hamster was the presence of high plasma concentrations of chylous, discernable upon visual inspection, which had not been previously observed in other HFD animal models in this study. The plasma lipoproteins from the HFD-fed $LDLR^{-/-}$ hamsters were too large to be processed with a column because the column (superpose 6 HR10/30, GE) would be damaged. Pooled plasma was then centrifuged (15,000 rpm, 30 min) and the lower phase was used for loading to remove very large particles, which accounted for ∼68% of triglyceride and 45% of the cholesterol of the estimated lipid content in the pre-treatment samples. The concentration of triglycerides and cholesterols in total plasma and the lower phase was determined, respectively, to calculate the number of large particles removed.

Cell Culture

Human umbilical vein endothelial cells (HUVEC, 8000, ScienCell, USA) were maintained in an endothelial cell medium (1001, ScienCell, USA) at 37°C, 5% CO_2 environment. Cells were subcloned into 6-well plates and equilibrated with 0.2% BSA-ECM for 6 h. Then, 5% (w/w) serum, which was separated from CD treated, HFD treated, and HFD with ezetimibe treated hamsters, was added into the cells for a further 24 h. Culture mediums were discarded and cells were washed 3 times with PBS. Protein was extracted by RIPA Lysis Buffer (R0020, Solarbio, Beijing, China) to prepare samples for subsequent western blot analysis.

SDS-Page and Western Blot Analysis

Triglyceride-rich lipoproteins were separated by ultracentrifugation at 42,000 rpm for 18 h from 5 ml plasma of WT or $LDLR^{-/-}$ hamsters with Optima XPN-100 Ultracentrifuge (Beckman Coulter, USA). Then, TRL samples with equal triglyceride concentrations were delipidated with an organic solvent and used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The gel was dyed with Coomassie Brilliant Blue to show apolipoprotein bands. For western blot analysis, 1 μl plasma (animal experiments) or 20 μg protein (cell experiments) were prepared with an SDS and dithiothreitol (DTT) buffer by heating at 95°C for 10 min. The samples were loaded to 10 or 6% SDS-PAGE gels and underwent electrophoresis at 110 V. Proteins were transferred onto nitrocellulose membranes for 60–180 min at 220 mA. Membranes were then blocked with 5% BSA, and hybridized with the following antibodies: anti-ApoB, anti-ApoE, or anti-ApoAI (ab20737, ab20874, ab20453 rabbit polyclonal IgG respectively, Abcam, U.K) for plasma analysis, or anti-ICAM-1 or anti-VCAM-1 (CST 4915s or 13662 respectively, Cell Signaling Technology) for cell lysate analysis. Because the same volumes of plasma were used for electrophoresis, no internal reference was required as the sample loading control. The anti-GAPDH (ab8245, Abcam, U.K) antibody was used for the internal reference and the loading control for cell lysate analysis. Target proteins were visualized upon incubation with horseradish peroxidase-conjugated secondary antibodies followed by enhanced chemiluminescence detection (Molecular Imager Gel Doc XR System, Bio-Rad, Hercules, CA, USA).

Pathology Analysis

Tissues were fixed in 4% paraformaldehyde for 24 h and equilibrated in 20% sucrose for 24 h. For oil red O (Sigma-Aldrich, St. Louis, MO, USA) staining, heart and liver were embedded in optical coherence tomography (OCT), frozen at an approximate temperature of −20°C, and sectioned at 7 μm with a freezing microtome (Leica, Switzerland). The quantitative analysis of atherosclerosis was represented as the percentage of *en face* lesion area ratio to the whole area of the full-length aorta, and the total area of the aortic root lesion by image J software. For H&E, sirius red, and immunohistochemical staining, the tissue samples from the aortic arch for all hamster samples after *en face* analysis were embedded in paraffin and sectioned at 3 μm with a Leica microtome, following a standard protocol referring to our pathology platform. Immunohistochemical staining was performed with anti-VCAM-1 and anti-α-SMA antibodies (BA3840 and A03744 rabbit polyclonal IgG respectively, Boster, USA).

Tissue Lipid Analysis

Lipid extraction was referred to as a modified method by Bligh and Dyer (20). Briefly, 100 mg of tissue was homogenized in 1 ml cold PBS. Then, lipids were extracted in 5 ml glass tubes to

TABLE 1 | The primers used in real-time polymerase chain reaction (PCR).

Gene	Forward	Reverse
ABCG5	CCATTCTGACTTACGGAGAGTTG	CAGGGGTAACCACAGTTATTGAA
ApoB	GTGTACGGCTTCAACCCTGA	TCAGGAATGGCCAGCTTGAG
CETP	TCCATAAGCTGCTCCTGCAC	GCCCTTGTGATGGGACTCAA
GAPDH	GACTCATGACCACAGTCCATGC	AGAGGCAGGGATGATGTTCTG
HMGCoA Synthetase	TCCTCCGTGGATTGCTCCTT	TCGGTCACTGTCTCCACCTT
HMGCoA Reductase	TGATGGGAGCTTGCTGTGAG	ACCAAGACCTATTGCCCTGC
MTP	AGAGGAAAACCTGGACTCCTATG	AGCATTTTGGACATCAGATCACT
NPC1L1	ATGGCCACTCACTGTCTTGG	CGTCGTGGAAAGCCTTCTCT
SRB1	TGCCCGTCATCTACCAGTTG	TTTGGGACCCTACAGCTTGG
SREBP1c	GCGGACGCAGTCTGGG	ATGAGCTGGAGCATGTCTTCAAA
VLDLR	GCCTACCAGCACCACAGATT	GGTCACATTGATCCTTTGACACT

avoid polymer contamination by vortex with the same volume of chloroform/methanol (v:v = 2:1) for 90 s and then centrifuged at 2,000 rpm for 20 min. The chloroform layer at the lower phase was transferred using a glass syringe and the rest was repeated as above. The collected chloroform layer was dried under nitrogen. Lipids were dissolved with 3% TritonX-100 (T8200, Solarbio, Beijing, China) for analysis. The cholesterol and triglyceride content were measured with the kits described above.

Quantitative Real-Time PCR Assay

Total RNAs were extracted with Trizol reagent (12183555, Invitrogen, USA) and 50 μg RNA was RT by a commercial RT kit (18091200, Invitrogen, USA). Real-time polymerase chain reaction (PCR) was performed using the AriaMx Real-Time PCR System with Top Green PCR Master Mix (AQ131-01, TransGen Biotech, USA). The primers used in real-time PCR are listed in **Table 1** and GAPDH was used as the internal reference.

Quantification and Statistical Analysis

All data were expressed as the mean ± SEM, and statistical tests are specified in figure legends. GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA) was used for all statistical analyses. For a comparison between two groups, the Mann-Whitney test was used. For comparisons between three or more groups, one-way ANOVA was used. $P < 0.05$ were considered statistically significant.

RESULT

Dietary Cholesterol Increased Triglyceride-Rich Lipoproteins in LDLR$^{-/-}$ Hamsters

As we have previously reported, hamster plasma triglyceride levels were sensitive to dietary cholesterol (9–11, 14, 21). In this study, we found that LDLR$^{-/-}$ hamsters had significantly higher total cholesterol and triglyceride levels in the plasma when fed with CD, compared with LDLR$^{-/-}$ mice (**Figures 1A,B**). Interestingly, an HFD diet dramatically increased plasma lipids levels in LDLR$^{-/-}$ hamsters, especially triglyceride levels. In addition, in WT hamsters, an HFD also significantly increased the level of plasma cholesterol and triglycerides (**Supplementary Figure 1A**). Thus, whether the LDL receptor was present or knocked out, HFD-feeding in hamsters resulted in these marked changes. Therefore, we concluded that compared with other rodents, the hamster is special for studying lipid metabolism and related diseases with their obvious phenotypes.

After ezetimibe treatment for 2 weeks, there was a 30% decrease in total plasma cholesterol levels in CD-fed LDLR$^{-/-}$ hamsters. Ezetimibe had no significant effect on plasma lipids levels in CD-fed WT hamsters or LDLR$^{-/-}$ mice (**Supplementary Figure 1A** and **Figures 1A,B**). Ezetimibe completely prevented HFD-induced elevation of both cholesterol and triglycerides in hamsters and mice. Without ezetimibe treatment, plasma cholesterol and triglyceride concentrations were more than 3 times greater in hamsters up to more than 2,000 mg/dl. But in mice, HFD can only induce elevation of cholesterol, from about 200–500 mg/dl. But there was no significant increase of triglyceride concentration after HFD-induced in mice.

We also analyzed plasma lipoprotein profiles by FPLC to investigate which lipoproteins changed in response to diet and ezetimibe. The results showed that in LDLR$^{-/-}$ hamsters, HFD-induced cholesterol changes occurred predominantly in the TRL fraction. The effect of ezetimibe was also restricted to the cholesterol in the TRL fraction (**Figures 1C,D**). In contrast, in WT hamsters, changes in plasma cholesterol in response to an HFD were observed in both TRL and LDL particles (**Figures 1E,F**). Increased triglycerides in TRLs indicated an increase in the number of these particles (**Figures 1D,F**).

This data suggested that in LDLR$^{-/-}$ hamsters, nutritional, not endogenous, cholesterol contributed to the increase in blood lipids. In LDLR$^{-/-}$ hamsters, it is the TRL fraction that was regulated by intestinally-derived cholesterol, but in WT hamsters, both TRL and LDL fractions were regulated. It was also evident that intestinally-absorbed cholesterol may be cleared by the LDL receptor in hamsters. Together, these current and previous (8, 21) results showed that in rodents, Syrian golden hamsters were more sensitive to changes in dietary lipids and more susceptible to hypertriglyceridemia. The LDL receptor might play an important role in this process. The LDL receptor was mainly involved in the clearance of triglycerides in plasma, and not in VLDL secretion (**Supplementary Figure 2**).

Ezetimibe Prevented HFD-Induced Increase of Plasma ApoB48 and ApoE

In order to better understand the different plasma lipoprotein profiles, in particular the apolipoproteins, in LDLR$^{-/-}$ and WT hamsters, TRLs were analyzed using SDS-PAGE and 1 μl plasma was analyzed using western blot, respectively. Consistent with our findings on lipid concentrations and lipoprotein fractions, ApoB and ApoE in TRLs from LDLR$^{-/-}$ hamsters increased compared with WT hamsters (**Figure 2A**). In LDLR$^{-/-}$ hamsters, HFD led to a marked increase in both

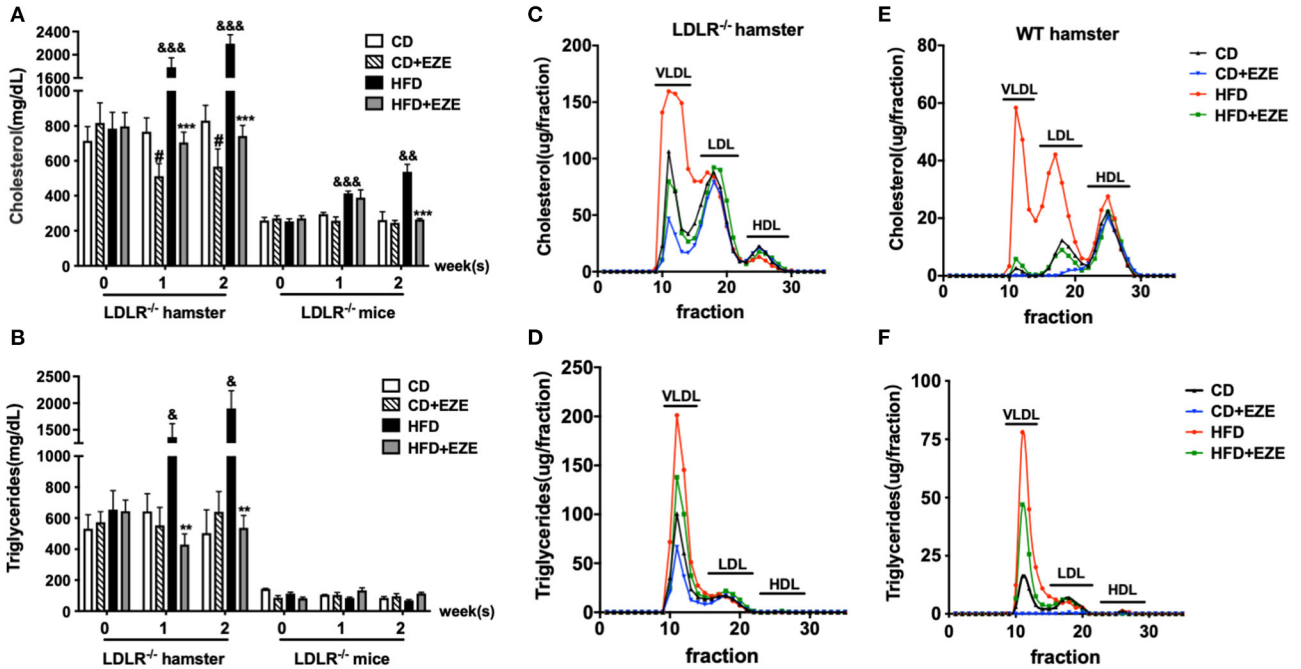

FIGURE 1 | The responses to high-fat diet (HFD) and ezetimibe (EZE) in hamsters and mice. Total cholesterol **(A)** and triglyceride **(B)** levels were determined from plasma collected after 2 weeks of HFD and EZE treatment from LDLR$^{-/-}$ hamsters ($n = 10$) and mice ($n = 5$). Data are shown as mean ± SEM. $^{\#}p < 0.05$ CD+EZE vs. CD; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ CD vs. HFD; $^{**}p < 0.01$, $^{***}p < 0.001$ HFD + EZE vs. HFD, where a two-way ANOVA with Tukey's multiple comparisons test was performed. Lipid profiles of LDLR$^{-/-}$ **(C,D)** and WT **(E,F)** hamsters. The plasma lipoprotein profile of hamsters 2 weeks after different treatments (CD, CD ± EZE, HFD, HFD ± EZE) was analyzed by FPLC. Each sample was of pooled plasma of five hamsters in each group, and 100 μL of the sample was loaded into the column. The cholesterol (upper) and triglycerides (bottom) of every collected fraction were determined and each data point was connected to make a curve on the graph. Each peak represents chylomicrons/VLDL, LDL, HDL fractions, respectively, from left to right.

ApoB48 and ApoE in plasma, with no significant changes in ApoB100 and ApoAI, and ezetimibe largely prevented the increase of ApoB48 and ApoE (**Figures 2B,C**). However, in WT hamsters, ezetimibe treatment decreased ApoB100 levels both in CD and HFD, in addition, a decrease of ApoE after HFD. As known in the Syrian golden hamster, ApoB100 and ApoB48 are derived from liver and intestinal synthesis, respectively. These results further supported the notion that the dietary cholesterol increased TRLs in LDLR$^{-/-}$ hamsters originated from an intestinal source. The changes in hamster lipoproteins very much resemble postprandial hyperlipidemia in humans. On the other hand, it suggested that the clearance capacity of chylomicrons and their remnants might be poor in the hamster, especially when the LDL receptor is deficient. Consequently, the LDL receptor likely plays an important role in the metabolism of these lipoproteins.

In addition, we examined ApoB100 and 48 contents in separated TRLs and FPLC TRLs' fractions of LDLR$^{-/-}$ hamsters (**Supplementary Figures 4A,B**). From the results, the ApoB100 and 48 both increased in TRLs in the HFD group, and the ratio of ApoB100:48 was consistent in TRLs separated by ultracentrifuge and FPLC (about 1:1-1.3). However, ezetimibe can reverse this increase in TRLs fractions. Analysis by SDS-PAGE, ApoA5, and ApoC3 increased in the HFD group, while the ApoC2 did not change (**Supplementary Figure 4C**). These results also suggested that increased TRLs in HFD-fed hamsters were intestinally derived.

TRLs Promoted Early-Stage Atherosclerosis Due to High LDL Levels

Most of the previous atherosclerosis studies were based on mouse and rabbit models which do not have severe hypertriglyceridemia (21–23). In this study, LDLR$^{-/-}$ hamsters had severe hypertriglyceridemia and diet-affected lipoproteins allowed us to investigate the atherogenic properties of TRL particles. As the results indicated thus far, ezetimibe affected large lipoprotein particles in LDLR$^{-/-}$ hamsters. Following this, we compared atherosclerosis with or without ezetimibe treatment to evaluate the role of TRLs. Our previous study showed that spontaneous atherosclerosis occurred in LDLR$^{-/-}$ hamsters that were older than 15 months. Here, we were able to accelerate atherogenesis and successfully induce early atherosclerosis in 12-month-old LDLR$^{-/-}$ hamsters, after 40 days of HFD. The levels of total cholesterol and triglycerides exceeded 3,000 mg/dl in HFD-fed LDLR$^{-/-}$ hamsters (**Supplementary Figure 1C**).

The analysis of the pathology of the aortic root and the full length of the aorta *en-face* showed early-stage atherosclerotic lesions in LDLR$^{-/-}$ hamsters (**Figures 3A,B**) and of note, this was not found in WT hamsters (Data not shown). Following

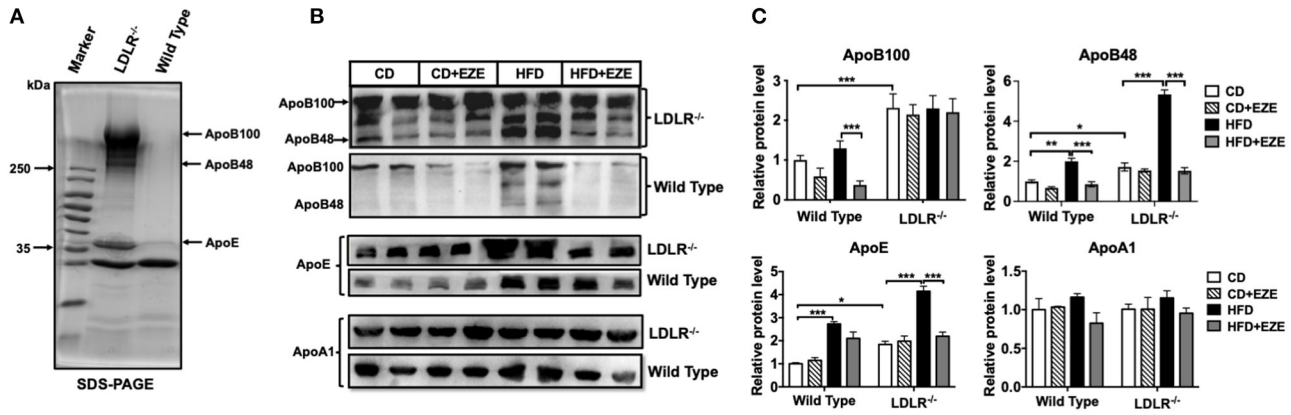

FIGURE 2 | Apolipoproteins of TRLs were analyzed by SDS-PAGE **(A)** and western blotting **(B)** for ApoB, ApoE, and ApoAI in plasma of LDLR$^{-/-}$ and WT hamsters with or without HFD and ezetimibe treatment after 2 weeks. **(C)** Is the quantitative bar chart analysis for **(B)**, the relative protein level was ratio to the CD group of WT hamsters. Separated TRLs from the plasma of fasted hamsters fed a CD were used for SDS-PAGE analysis. For each sample, 1 μL of plasma was used for analysis. Data are shown as mean ± SEM. Three-way ANOVA with Tukey's multiple comparison test, *, **, *** represent $p < 0.05$, 0.01, and 0.001, respectively.

ezetimibe administration, the lesion area decreased significantly by 75% at the aorta root and 50% at the full length of the aorta.

Sectioning of the aortic arch and staining with H&E (**Figure 3C**) found that fibrous cap structures have appeared in the plaques of HFD-induced hamsters, most of them were stable without necrotic cores, and also found the existence of unstable plaques in individual animals. In ezetimibe treated hamsters, the plaques are much reduced. Of note, ezetimibe can slow down the formation of atherosclerosis. However, when lesions from LDLR-/- hamsters with or without ezetimibe treatment were compared, no significant difference in inflammation or smooth muscle cell migration by immunohistochemistry staining was found (**Supplementary Figure 4**). In addition, when HUVECs were treated with serum from HFD-fed LDLR$^{-/-}$ hamsters after ezetimibe was treated, ICAM-1 and VCAM-1 expression significantly reduced (**Figures 3D,E**).

Therefore, we believe that high concentrations of TRLs increased the susceptibility of atherosclerosis in the LDLR$^{-/-}$ hamster which has high LDL levels. This may begin with an inflammatory response by endothelial cells in response to TRLs, and lipid deposition becomes severe with remnants and LDL uptake by macrophages.

Severe Accumulation of Lipids in the Liver Was Significantly Reduced by Dietary Cholesterol Inhibition of Ezetimibe

After 40 days of HFD, a severe accumulation of lipids in the livers of both LDLR$^{-/-}$ and WT hamsters was found by H&E and oil red O staining, and upon lipid extraction. Ezetimibe treatment significantly reduced lipid deposition in the liver of both the LDLR$^{-/-}$ (**Figures 4A,B**) and WT hamster (**Supplementary Figure 4**) induced by HFD-fed. Hepatic triglycerides were reduced by about 31% in the HFD-fed group treated with ezetimibe (**Figure 4B**). Hepatic cholesterol was reduced by about 30% in the CD-fed group and 45% in the HFD-fed group when treated with ezetimibe, respectively (**Figure 4C**). Unlike the plasma, lipid concentrations in the liver were not entirely reversed by ezetimibe, probably due to the upregulated synthesis of triglycerides but no significant increase of VLDL secretion (**Supplementary Figure 2**). Interestingly, H&E staining, along with anti-CD68 and anti-TNF-α immunohistochemistry staining, all showed an abundance of inflammatory cells in the liver of HFD-fed LDLR$^{-/-}$ hamsters (**Figures 4A,D**). Sirius red staining showed that an HFD promoted fibrosis in the liver of LDLR$^{-/-}$ hamsters. Ezetimibe attenuated both the inflammatory response and fibrosis (**Figures 4D,E**). In WT hamsters, the same results were found in the liver (**Supplementary Figure 5E**).

We quantified the expression levels of several major genes that are known to be involved in cholesterol (both intestinal and hepatic) and triglyceride metabolism and uptake in LDLR$^{-/-}$ hamsters (**Figure 5** and **Supplementary Figure 6**). Ezetimibe treatment significantly upregulated the expression of HMGCoA synthetase and reductase in both the liver and intestine, suggesting an increase in endogenous cholesterol synthesis. The expression of ABCG5 was upregulated in the liver and intestine in HFD-fed LDLR$^{-/-}$ hamsters and ezetimibe treatment downregulated expression in the liver but not in the jejunum. SRB1 was downregulated by an HFD but was reversed in the liver upon ezetimibe treatment. NPC1L1 expression did not change in all groups. SREBP1c and VLDL receptor were both upregulated by HFD-fed LDLR$^{-/-}$ hamsters and was attenuated by ezetimibe in the liver suggesting a response to dietary cholesterol. On the other hand, the genes of triglyceride synthesis and secretion were all no change during the CD but they were upregulated after HFD-fed, such as FAS and ACC1 in the liver and DGAT1, MTP, and ApoB in the intestine. Fas and ACC1, which are key enzymes that regulate fatty acid synthesis, were upregulated in the liver, suggesting that the synthesis of fatty acids in the liver was increased. DGAT1 and DGAT2, which are the key

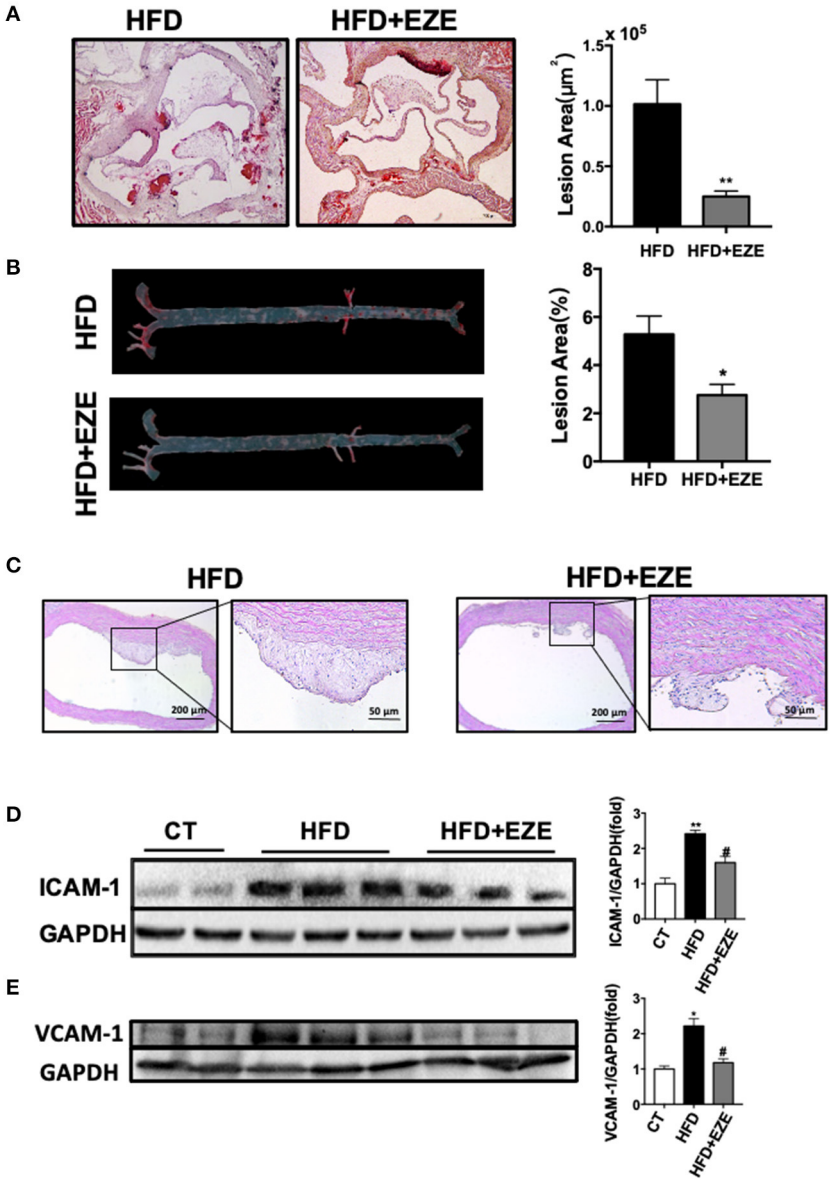

FIGURE 3 | Atherosclerosis is attenuated in LDLR$^{-/-}$ hamsters treated with ezetimibe after 40 days of HFD. Representative pictures of histochemistry sections of the aortic root **(A)** and *en face* analysis of the whole length of the aorta **(B)** by oil red O staining ($n = 10$). Scale bars, 500 μm. Total absolute oil red O positive area in all sections of the aortic root **(B)** and relative oil red O-positive area of the aorta *en face* ($n = 10$). Data are shown as mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ compared with the HFD group, where the one-way ANOVA with Tukey's multiple comparisons test was performed. **(C)** Representative images of H&E staining of sections of the aortic arch. Scale bar = 200 or 50 μm, respectively. The expression of ICAM-1 **(D)** and VCAM-1 **(E)** in cultured HUVECs was analyzed by western blotting, the right panel was the quantitative bar chart analysis of the left panel. The serum from normal CD-fed WT hamsters was used as the control, the expression of ICAM-1 and VCAM-1 incubated with serum from HFD diet-fed hamsters was upregulated and downregulated with the serum from HFD-fed hamsters with ezetimibe administration. Data are shown as mean ± SEM. Two-tailed unpaired *t*-test, # HFD+EZE vs. HFD, $^{\#}p < 0.05$, and the one-way ANOVA with Tukey's multiple comparisons test was performed.

enzyme in the last step of synthesizing triglycerides. From the result, we found the expression of DGAT1 was increased in the jejunum, which suggested that the TG synthesis was increased in the jejunum. MTP is a key protein for the transfer of triglycerides into the blood, the increased expression of MTP indicated more triglycerides into the blood. FATP4 and ACSL5 have participated in triglycerides synthesis in the jejunum. CD36 is responsible for fatty acid intake. However, the expression of FATP4, ACSL5, and CD36 did not change after HFD-fed. The results suggested that accelerated synthesis of triglyceride but no change of secretion in the liver resulted in the fatty liver, and accelerated secretion in the intestine resulted in the plasma triglyceride increase.

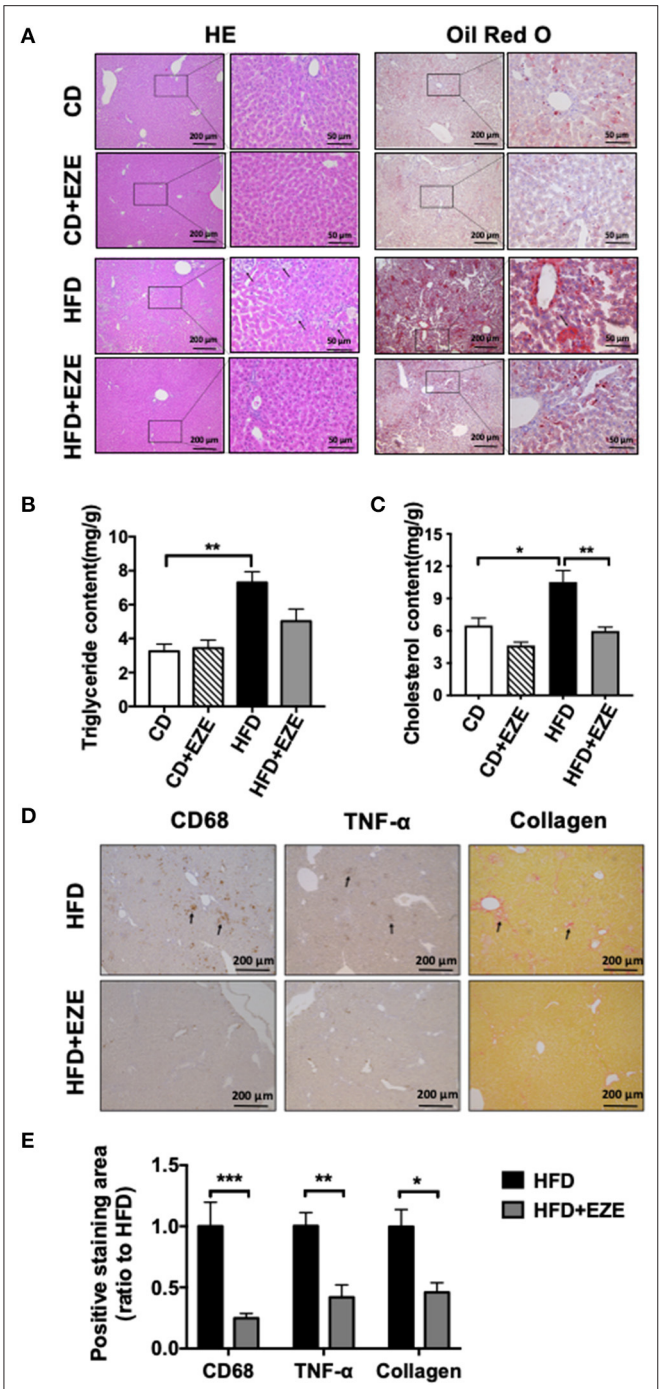

FIGURE 4 | Hepatic morphological analysis and lipid extraction. LDLR$^{-/-}$ hamsters were fed CD or HFD for 40 days and administrated with ezetimibe (+EZE) or solvent. **(A)** Representative images of liver cross-sections with H&E and oil red O staining. Scale bar = 200 or 50 μm, respectively. **(B)** Hepatic triglyceride content ($n = 5$), and **(C)** hepatic cholesterol content ($n = 5$). **(D)** Representative immunohistochemistry stained images of CD68 and TNF-α, and sirius red-stained images and **(E)** the relative quantization. Scale bar = 200 μm. Data are shown as mean ± SEM. Two-way ANOVA with Tukey's multiple comparison test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

DISCUSSION

This study described characteristics of the plasma lipid profile of a hyperlipidemia rodent model, the Syrian golden hamster, which was sensitive to, and regulated by, dietary lipids (11–14). Most significantly, in this model triglyceride levels were markedly increased by intestinally-derived cholesterol. The plasma lipid profile of the LDLR$^{-/-}$ hamster after HFD-feeding was similar to that of postprandial hyperlipidemia in humans. Therefore, the LDLR$^{-/-}$ hamster is a useful tool to study postprandial hyperlipidemia and the regulation of TRLs metabolism and associated diseases.

Our results showed that dietary lipids predominantly increased large particles of plasma lipoproteins in the Syrian golden hamster, especially in the LDLR$^{-/-}$ hamster model. Unlike the ApoE knockout mouse (17), in these hamsters, the plasma lipid concentrations were elevated particularly high. These large lipoproteins contributed to increased plasma cholesterol and triglyceride levels by more than 80% in LDLR$^{-/-}$ hamsters. The susceptibility of TRLs being elevated by an HFD is responsible for the particular increase of triglyceride in hamsters when compared with mice. But as with most rodents, the cholesterol level was also very high in TRLs.

The Syrian golden hamster has plasma CETP activity, and remarkably, this is a feature similar to human lipid metabolism. CETP has been reported to promote triglyceride synthesis and secretion by cells and impair triglyceride clearance in plasma (24–26). CETP activity significantly increased during the postprandial state, almost in parallel with an increase of plasma triglycerides (27, 28). A CETP inhibitor can increase triglycerides in TRLs, ApoE, and ApoC2 to promote the clearance of TRLs (29). Our data showed that ApoC3 and ApoA4 increased in the TRLs separated from HFD-fed LDLR$^{-/-}$ hamsters. Therefore, it is possible that HFD-fed LDLR$^{-/-}$ hamsters have increased TRLs and remnants, much like that in humans, due to CETP transferring HDL to VLDL and LDL, and changed apolipoproteins also may affect the characteristic of TRLs, thus delaying the clearance of triglycerides. We found that by knocking out the LDL receptor, there was a dramatic delay in the clearance of plasma triglycerides (**Supplementary Figure 2**). VLDL secretion did not change enough to affect blood lipid levels by diet, genotype, or ezetimibe (**Supplementary Figure 2**). The LDL receptor was also the receptor for lipoprotein remnants. Our data showed that expression of LRP1, another remnants receptor, did not change significantly in the liver (**Supplementary Figure 6A**). Extremely high levels of triglycerides in the LDLR$^{-/-}$ hamster may largely be due to the delayed clearance of TRLs by LDL receptors. Therefore, compared with other rodents, hamsters have their distinguishing features in TRLs metabolism and further study needs to clarify the application of these characteristics into disease models.

Similar to humans, the editing of ApoB in hamsters happens only in the intestine (7), and upon apolipoprotein analysis, increases in ApoB48 and ApoE in plasma of HFD-fed LDLR$^{-/-}$

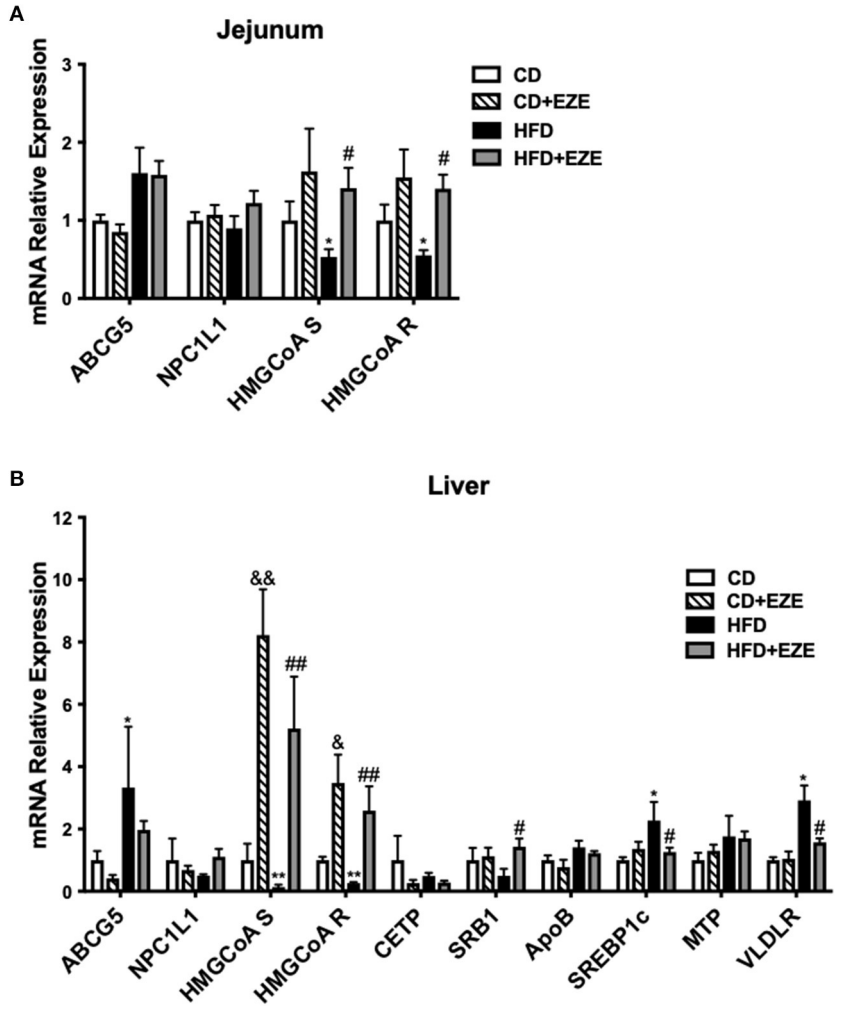

FIGURE 5 | The expression of genes involved in lipid metabolism was determined by real-time PCR. LDLR$^{-/-}$ hamsters were fed CD or HFD for 40 days with ezetimibe (+EZE) or solvent treatment. Total mRNA was extracted from the jejunum **(A)** or liver **(B)** for real-time PCR. Data are shown as expression relative to CD by mean ± SEM, $n = 5$. Two way ANOVA with Tukey's multiple comparison test, * HFD vs. CD, $^{*}p < 0.05$, $^{**}p < 0.001$; & CD + EZE vs. CD, $^{\&}p < 0.05$, $^{\&\&}p < 0.001$; # HFD+EZE vs. HFD, $^{\#}p < 0.05$, $^{\#\#}p < 0.001$.

hamster indicated an accumulation of chylomicron remnants. Through analysis of TRLs of CD-fed hamsters, it was also found that the levels of ApoB100/48 and ApoE in LDLR$^{-/-}$ hamsters were significantly higher than in WT hamsters. Hence, our data also supported that chylomicron remnants accumulated when LDL receptor-deficient.

The study of Heek et al. (18) pointed out that ezetimibe alone may lead to a reduction in plasma cholesterol and triglycerides in humans with combined hyperlipidemia, such as obese insulin-resistant and/or patients with type 2 diabetes. In order to investigate the effect of dietary cholesterol, we used an NPC1L1 inhibitor, ezetimibe, to inhibit the absorption of cholesterol in HFD-fed animal models. Treatment with ezetimibe in LDLR$^{-/-}$ hamsters resulted in a significant decrease in plasma triglycerides and TRLs. This showed that dietary cholesterol led to an accumulation of TRLs in hamsters, and this was mediated by LDL receptor deficiency. The study of Xia et al. (30) reported that ezetimibe may enhance RCT and expression of PPARγ to lower the lipids levels in high cholesterol high-fat diets. Additionally, we showed the effects on lipids levels in CD-fed LDLR$^{-/-}$ hamsters. These results suggested that dietary cholesterol might play an important role in triglyceride metabolism in the Syrian golden hamster, a unique feature not seen in other rodents. Therefore, it will be helpful for understanding triglyceride metabolism and related diseases.

Unexpectedly, using FPLC analysis, it was found that HFD-fed LDLR$^{-/-}$ hamsters had increased TRLs in the plasma only and ezetimibe only decreased these lipoprotein particles. LDL levels did not change in our experiments. Therefore, this feature can lend to the exploration of the effects of large lipoprotein particles and triglycerides. Analysis of FPLC data found that cholesterol accumulated in these large lipoprotein particles. We believe that these TRLs may be much more atherogenic. In this study, early-stage lesions, after 40-days of HFD, had reduced when ezetimibe

inhibited TRLs but not LDL from the beginning. This is evidence of an important role of TRLs in the formation of initial lesions. Of note, these TRLs were mainly chylomicron remnants and relatively cholesterol-rich.

The study of Zilversmit proposed that increased chylomicrons/VLDL or their remnants in the postprandial plasma were major atherogenic lipoproteins (31). In the WT hamster, both TRLs and LDL were significantly increased after an HFD, and both contributed to the development of atherosclerosis. Thus, we are unable to delineate the roles of TRLs and LDL in this model. Based on genetically modified mouse models, such as LPL$^{-/-}$, GPIHBP1$^{-/-}$, and ApoC3 transgenic mice, triglycerides were demonstrated to promote atherosclerosis (5, 32–34). However, in these models, ApoB editing occurs in both the liver and intestine (35), along with the fact that they are insensitive to dietary lipids, which means that Apo48 levels in these models do not represent chylomicrons and their remnants, and one cannot distinguish postprandial lipoproteins. In LDLR$^{-/-}$ hamsters, we offered clear evidence to support the association between TRLs and the formation of atherosclerotic lesions, as there was a significant increase of ApoB48 lipoproteins. These characteristics of the LDLR$^{-/-}$ hamster provide a new experimental model to evaluate TRLs in the development of diseases in the context of atherosclerosis susceptibility.

Triglyceride-rich lipoproteins and their remnants activated inflammation and stress pathways, which in turn impaired endothelial function, leading to the infiltration of monocytes (35). Relatively small remnants in TRLs could also infiltrate into the artery wall like LDL (36). Subintimal lipoproteins may be modified and taken up *via* receptors on macrophages, such as SR-A, LRP1, the LDL receptor, the VLDL receptor, and so on, promoting the formation of foam cells and atherosclerotic lesions (37). These studies help us to understand hypertriglyceridemia as an independent risk factor for CVD. We also found that plasma with high TRL levels and remnants stimulated the upregulation of ICAM-1 and VCAM-1 when incubated with cultured HUVECs (**Figures 3F,G**). They activated the mitogen-activated protein kinase (MAPK) signaling molecules (32). Together, this suggested that atherosclerosis might begin with TRLs activating endothelial cells and was then promoted by increased monocyte adhesion and lipoprotein infiltration. Interestingly, we had found that macrophages from LDLR$^{-/-}$ hamsters had a greater accumulation of lipids compared with WT hamsters (data not shown). Further, incubation of WT peritoneal macrophages with plasma from HFD-fed LDLR$^{-/-}$ hamsters, which is rich in TRLs, resulted in an accumulation of lipids within these cells (data not shown). Whereas, TRLs incubated with LDLR$^{-/-}$ hamster-derived macrophages also resulted in an accumulation of lipids within these macrophages. The hamsters can serve as an ideal model to investigate the effects of TRLs on diseases due to their TRLs levels can be manipulated (through HFD or ezetimibe). In other animal models, the effects of non-LDL lipoproteins on the development of atherosclerosis were not definitively excluded or properly investigated.

The liver plays an important role in lipid metabolism and fatty liver is usually associated with CVD. HFD-fed LDLR$^{-/-}$ hamsters showed a significant accumulation of lipids in the liver. Interestingly, when ezetimibe inhibited the absorption of cholesterol, lipid deposition in the liver was also inhibited. This finding suggested that in hamsters, exogenous cholesterol plays an important role in both plasma and liver lipid metabolism and may be associated with atherosclerosis. The analysis of pathological sections also showed infiltration of inflammatory cells in liver tissue, suggesting that TRLs may induce inflammation in the liver, consistent with a previous report of liver inflammation in LDLR$^{-/-}$ mice fed a cholesterol-rich diet (38).

Gene expression of cholesterol synthesis genes in the liver and intestine, such as HMG-CoA synthetize and reductase, were downregulated in HFD-fed LDLR$^{-/-}$ hamsters and significantly upregulated after ezetimibe treatment. This reflects the effects of ezetimibe on cholesterol absorption and inhibition. Further, the finding was that ABCG5, a key gene for cholesterol efflux, was regulated by HFD and ezetimibe, which was also expected. The upregulation of Srebp1c and VLDLR after HFD-feeding may be associated with elevated TRLs. However, an understanding as to why SRB1 is downregulated is still unclear. LDLR$^{-/-}$ mice did not show these changes (**Supplementary Figure 7**) related to triglyceride metabolism. The expressions of genes involved in triglyceride synthesis and secretion were not found to change in LDLR$^{-/-}$ hamster in CD, suggesting that LDL receptor deficiency mainly caused the abnormal clearance of TRLs for elevated triglyceride. But these expressions upregulated in the liver or intestine after HFD, such as FAS, ACC1, MTP, ApoB, and DGAT1, indicated that the intestine derived lipoproteins and liver triglyceride accumulation in LDLR$^{-/-}$ hamster after HFD fed.

In conclusion, the changes in lipoproteins in LDLR$^{-/-}$ hamsters after HFD-feeding and ezetimibe treatment suggested that TRLs initiate and promote the factors involved in atherosclerosis. In addition, the LDL receptor may play an important role in triglyceride metabolism as the TRLs receptor. However, the patterns of lipid metabolism in hamsters need further investigation to understand its characteristics for better application. Although there are many aspects of the lipid metabolism of hamster more like humans, hamster also has many features, unlike humans, for example, extremely high lipids in plasma after HFD fed, a too easy rise of triglyceride, and maybe the difference of cholesterol ratio in lipoproteins. We hope that with the continuous application of hamsters in experiments, we can be more and more clear about which scientific problems are suitable to be solved with hamsters.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care and Use Committee of the Peking University Health Science Center (LA2015-012).

AUTHOR CONTRIBUTIONS

XL performed most of the experiments. YW and XL wrote the original draft. PM, CY, JW, KH, and GC participated in the experiments and provided some data. WH, JF, and XX gave methodological conduct including molecular biology and pathology. YW and GL conceived the study and supervised the experiments. GL gave guidance in conceptualization and analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.738060/full#supplementary-material

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Clinical Significance of Intermediate-Density Lipoprotein Cholesterol Determination as a Predictor for Coronary Heart Disease Risk in Middle-Aged Men

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Background: Not only low-density lipoprotein (LDL) cholesterol but also non-high-density lipoprotein cholesterol (non-HDL-C), very low-density lipoprotein (VLDL) cholesterol (VLDL-C), and intermediate-density lipoprotein (IDL) cholesterol (IDL-C) are reported to be significant risk markers for coronary heart disease (CHD). We reported the relevance of IDL-C to Framingham risk score (F-score), but the present study addressed the relevance of IDL-C to Suita score (S-score), a risk score for coronary heart disease (CHD) developed for the Japanese individuals in addition to F-score.

Methods: The cholesterol levels of lipoproteins, including triglyceride (TG)-rich lipoproteins (IDL and VLDL), were measured by an anion exchange high-performance liquid chromatography (AEX-HPLC). This study enrolled 476 men, aged mean 51 years and free of CHD and stroke.

Results: Non-HDL-C, IDL-C, and VLDL-C significantly correlated with F-score and S-score. In the multiple stepwise regression analysis, IDL-C as well as body mass index (BMI) significantly correlated with both F-score and S-score in both the total subjects and the subjects without drug therapy. The multivariate logistic analysis with the model composed of BMI and IDL-C as the predictor variables demonstrated that 1 SD increase in IDL-C was an independent predictor for 10-year CHD risk >10% of F-score (*OR* 1.534, 95% CI 1.266–1.859, $p < 0001$) and that of S-score (*OR* 1.372, 95% CI 1.130–1.667, $p = 0.0014$) in the total subjects. Even in the subjects without the drug therapy, the increased IDL-C, as well as BMI, were significant predictors for 10-year CHD risk >10% of S-score as well as F-score.

Conclusion: These results suggest the significant relevance of the increased IDL-C for CHD risk scores in middle-aged men free of CHD and stroke. Further investigations are needed in women and elderly subjects.

Keywords: Framingham risk score, IDL-cholesterol, Non-HDL cholesterol, VLDL-cholesterol, Suita score

INTRODUCTION

A high level of serum low-density lipoprotein (LDL) cholesterol (LDL-C) is established as a primary risk factor for atherosclerotic cardiovascular disease (ASCVD), including coronary heart disease (CHD) (1–3). However, a residual ASCVD risk remains after LDL-C reduction under the target level by LDL-lowering therapy (1–6). In addition to LDL-C, total cholesterol (TC) minus high-density lipoprotein (HDL) cholesterol (HDL-C), namely non-HDL-C, is of importance as a risk marker for ASCVD (6–10). Non-HDL is composed of apolipoprotein B (ApoB)-containing lipoproteins, including triglyceride (TG)-rich lipoproteins [very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and remnant lipoprotein]. Recently, a high level of non-HDL-C attracts attention because of its important significance and clinical usefulness in relation to the determination of ASCVD risk (6).

Not only LDL-C but also VLDL cholesterol (VLDL-C) and IDL cholesterol (IDL-C) are reported to be significant risk markers for CHD (7, 11, 12). The results from subjects aged ≥30 years and free of CHD at baseline in the Framingham Heart Study suggest that non-HDL-C and VLDL-C are stronger predictors of CHD risk than LDL-C regardless of the serum TG levels, indicating that VLDL-C may play a critical role in the development of CHD (7). The Copenhagen General Population Study reported that VLDL-C explained one-half myocardial infarction risk relevant to the cholesterol levels of ApoB-containing lipoproteins and indicated that IDL-C was a stronger predictor for myocardial infarction risk (11). In addition, VLDL-C explained a large fraction of excess myocardial infarction risk in obese individuals (12). We reported that IDL-C may contribute as a useful marker to CHD risk determination in the Japanese men free of CHD and stroke, indicating the significant association of increased IDL-C levels with high levels of Framingham risk score (F-score) (13).

As mentioned above, VLDL-C and IDL-C may be the significant markers for ASCVD risk. However, each method for the determination of VLDL-C and IDL-C is different among the previous studies. The measurement methods in the Framingham Heart Study and the Copenhagen General Population Study were ultracentrifugation (7) and an NMR spectroscopy platform (11, 12), respectively. On the other hand, our study used an anion exchange-high performance liquid chromatography (AEX-HPLC) method, convenient and inexpensive as compared with ultracentrifugation and NMR (13, 14). VLDL-C and IDL-C measured by the AEX-HPLC method are sufficiently correlated with those measured by an ultracentrifugation method (14–16). Meanwhile, the Suita score (S-score) has been established for predicting a 10-year probability of developing CHD, which is based on the findings of a large cohort study in Japan (17). The F-score overestimated the 10-year risk of CHD for the Japanese population as compared with the S-score (17).

Consequently, we investigated the relevance of cholesterol levels of TG-rich lipoproteins (VLDL-C and IDL-C), measured by the AEX-HPLC method, to CHD risk estimated by S-score and F-score in men free of CHD and stroke.

METHODS

The present cross-sectional study enrolled 476 middle-aged men who underwent annual medical checkup examination in Tobu Medical Center (Shizuoka, Japan), and who did not suffer from CHD, stroke, and any cancer according to the medical questionnaire. At entry, written informed consent was obtained from all the participants. The study protocol was approved by the institutional review board of Tobu Medical Center (approval no. 2010–01). In our previous study (13), 487 men were enrolled, but in the present study, 476 men were enrolled because of the assessment of SS scores targeted at individuals aged 35 years and over.

The dataset of our previous study (13) was used for the present study. The measurement methods for main laboratory data are given below. The cholesterol levels of five lipoprotein classes were measured by using AEX-HPLC as described previously (13–16). Briefly, the HPLC system was composed of non-porous polymer-based gel with diethylaminoethyl ligands as separation media and sodium perchlorate buffers as elution reagents. TC was calibrated using the Lipopropak calibrator (LT-S01A, TC 271.8 mg/dL) (Tosoh, Tokyo, Japan), the value of which was assigned according to the reference materials JCCRM223-36 (TC level 137.1, 171.4, and 207.3 mg/dL; ReCCs). The analysis conditions of AEX-HPLC were optimized with VLDL [density (d) < 1.006 g/ml, IDL (1.006 < d < 1.01 g/ml), LDL (1.019 < d < 1.06 g/ml), and HDL (d > 1.063 g/ml)], and the samples for the calibration procedure were separated by ultracentrifugation. Each lipoprotein cholesterol concentration measured by AEX-HPLC was correlated with those measured by the ultracentrifugation method, and the accuracy of these cholesterol levels was reported (14, 16). In the five lipoprotein classes determined by the AEX-HPLC method, the data of "other fraction," include lipoprotein(a) in addition to chylomicron (14, 18).

Cholestest-CHO, Cholestest-HDL, Cholestest-LDL, Cholestest-TG (Sekisui Medical, Tokyo, Japan), GA08 (A and T Corp, Yokohama, Japan), and HLC-723G8 (Tosoh Corporation, Tokyo, Japan) were used to measure TC, HDL-C, LDL-C, TG, blood glucose, and hemoglobin (Hb) A1c, respectively. Non-HDL-C was calculated by subtracting HDL-cholesterol from TC. In addition, the estimated glomerular filtration rate (eGFR) was calculated using the following formula: 194 × creatinine −1.094 × age (years) −0.287 (19).

We determined F-score [National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATPIII) version] levels of 476 subjects, incorporating data of age, sex, TC or LDL-C, and HDL-C concentrations, blood pressures, anti-hypertensive drug medication, smoking and diabetic status into the calculation (20, 21). The S-score levels also were calculated similarly, using data on age, sex, TC or LDL-C, HDL-C, SBP, DBP, smoking, diabetes, and eGFR (17, 22). A distinct difference between the two scores is that e-GFR is incorporated into S-score but not into F-score.

The data were presented as mean ± SD. Student's *t*-test or Mann–Whitney *U*-test was used to compare the variables between Group 1 (<6 points of F-score) and Group 2 (≥6 points of F-score) or between Group 3 (<41 points of S-score) and

TABLE 1 | Clinical characteristics, biochemical data, Framingham risk score and Suita score of the study subjects.

	Total (n = 476)	Group 1 FRS < 6 points (n = 294)	Group 2 FRS > 6 points (n = 182)	P-value Group 1 vs. Group 2	Group 3 SS < 41 points (n = 202)	Group 4 SS > 41 points (n = 274)	P-value Group 3 vs. Group 4
Framingham risk score; FRS (total point)	4.5 ± 2.9	2.7 ± 2.1	7.5 ± 1.5	< 0.0001	2.1 ± 2.1	6.4 ± 2.0	<0.0001
Suita score; SS (total point)	42.1 ± 9.8	37 ± 7	51 ± 6	< 0.0001	33 ± 5	49 ± 6	<0.0001
Basic data							
Age (years)	51 ± 8	49 ± 7	56 ± 7	<0.0001	46 ± 6	55 ± 6	<0.0001
Body mass index (kg/cm^2)	24.2 ± 3.2	23.7 ± 3.0	24.9 ± 3.4	<0.0001	23.5 ± 2.9	24.7 ± 3.3	<0.0001
Syematic blood pressure (mmHg)	122 ± 15	118 ± 13	129 ± 16	<0.0001	115 ± 13	128 ± 14	<0.0001
Diastonic blood pressure (mmHg)	77 ± 10	75 ± 10	81 ± 10	<0.0001	73 ± 10	80 ± 9	<0.0001
Smoker, *n* (%)	194 (41)	90 (31)	104 (57)	<0.0001	68 (34)	126 (46)	<0.01
Fasting blood glucose (mmol/L)	5.84 ± 0.99	5.64 ± 0.71	6.18 ± 1.25	<0.0001	5.55 ± 0.64	6.06 ± 1.13	<0.0001
Glycated hemoglobin A1c (%)	5.9 ± 0.6	5.8 ± 0.5	6.1 ± 0.8	<0.0001	5.7 ± 0.5	6.0 ± 0.7	<0.0001
Estimated GFR (mL/min/1.73 m^2)	79.7 ± 13.9	80.9 ± 13.7	77.7 ±14.1	< 0.05	82.3 ± 12.9	77.8 ± 14.4	<0.0005
Lipid data							
Total cholesterol (mmol/L)	5.29 ± 0.84	5.15 ± 0.82	5.52 ± 0.83	<0.0001	5.10 ± 0.80	5.44 ± 0.84	<0.0001
HDL cholesterol (mmol/L)	1.47 ± 0.35	1.55 ± 0.36	1.34 ± 0.29	<0.0001	1.53 ± 0.33	1.43 ± 0.36	<0.005
LDL cholesterol (mmol/L)	3.31 ± 0.80	3.13 ± 0.76	3.60 ± 0.78	<0.0001	3.11 ± 0.74	3.46 ± 0.81	<0.0001
Non-HDL cholesterol (mmol/L)	3.82 ± 0.89	3.60 ± 0.84	4.18 ± 0.83	<0.0001	3.60 ± 0.84	4.01 ± 0.87	<0.0001
Triglyceride (mmol/L)	1.56 ± 1.01	1.37 ± 0.85	1.85 ± 1.17	<0.0001	1.35 ± 0.83	1.71 ± 1.10	<0.0001
Lipoprotein data by anion-exchange liquid chromatography							
HDL cholesterol (mmol/L)	1.41 ± 0.37	1.49 ± 0.38	1.28 ± 0.31	<0.0001	1.47 ± 0.35	1.37 ± 0.38	<0.005
LDL cholesterol (mmol/L)	3.55 ± 0.87	3.37 ± 0.81	3.83 ± 0.88	<0.0001	3.36 ± 0.81	3.69 ± 0.88	<0.0001
IDL cholesterol (mmol/L)	0.203 ± 0.081	0.190 ± 0.081	0.224 ± 0.078	<0.0001	0.189 ± 0.081	0.213 ± 0.080	<0.005
VLDL cholesterol (mmol/L)	0.49 ± 0.39	0.42 ± 0.33	0.59 ± 0.46	<0.0001	0.42 ± 0.32	0.53 ± 0.43	<0.005
Other cholesterol (mmol/L)	0.104 ± 0.068	0.108 ± 0.077	0.099 ± 0.051	NS	0.112 ± 0.083	0.099 ± 0.055	NS
Therapy for diseases							
Hypertension, *n* (%)	83 (17)	43 (15)	40 (22)	<0.05	18 (8.9)	65 (24)	<0.0001
Dyslipidemia, *n* (%)	55 (12)	27 (9.2)	28 (15)	<0.05	19 (9.4)	36 (13)	NS
Diabeties mellitus, *n* (%)	32 (6.7)	15 (5.1)	17 (9.3)	NS	9 (4.5)	23 (8.4)	NS

Data are expressed as means ± SD. NS means "not significant".

Group 4 (≥41 points of S-score). Namely, Groups 1 and 3 are regarded as being at a low-risk stage, and Groups 2 and 4 are regarded as being at a high-risk stage (10-year CHD risk more than 10%). Assuming an α level of 0.05, 80% power, and 0.3 effect size, the required number of patients for each group to observe a difference in IDL-C was determined ≥154 in Group 1 and ≥230 in Group 2. The correlations were estimated by Spearman's rank test. A multiple stepwise regression analysis was performed to assess the independent relationship of the variables, body mass index (BMI), and cholesterol levels of IDL, VLDL, and the other fraction [chylomicron and lipoprotein(a)] (14, 18). The TC or LDL-C and HDL-C concentrations were incorporated into the calculation of F-score and S-score. Therefore, TC, LDL-C, and non-HDL-C were not applied to the explanatory factors of multivariate analysis. TG was considered as one of the explanatory factors for F-score and S-score, but TG also was not applied to the explanatory factors of multivariate analysis because of natural collinearity between TG and TG-rich lipoprotein cholesterol (VLDL-C and IDL-C).

In addition, the univariate and multivariate logistic regression analyses were performed to analyze the relationship between the nominal variables (high-risk stage of 10-year CHD risk at F-score points with ≥6 or S-score points with ≥41) and continuous variables (1 SD increase in BMI and cholesterol levels of TG-rich lipoproteins), with the results expressed as odds ratios (*OR*) and 95% *CI*s, and the predictive values of BMI and TG-rich lipoprotein cholesterol for the high-risk stage were investigated. The P values < 0.05 were considered significant. The statistical analyses were performed using STATFLEX software (version 7.0, Artech, Osaka, Japan).

RESULTS

The clinical characteristics, biochemical data, F-score, and S-score are shown in **Table 1**. The predicted 10-year CHD risk values (7–8%) calculated by the F-score (4.5 points) were higher than those (about 2%) estimated by the S-score (42.1 points) as reported previously (17). In **Table 1**, Groups 1 and 2 show

TABLE 2 | Simple correlations of body mass index and serum lipids to Framingham risk score and Suita score.

	Framingham risk score			Suita score		
	Rank correlation coefficient	*t*-value	*P*-value	Rank correlation coefficient	*t*-value	*P*-value
Total subjects ($n = 476$)						
Basic data						
Body mass index (kg/m^2)	0.218	4.867	<0.0001	0.194	4.295	<0.0001
Lipid data						
Total cholesterol (mmol/L)	0.297	6.778	<0.0001	0.234	5.239	<0.0001
Non-HDL cholesterol (mmol/L)	0.417	9.978	<0.0001	0.295	6.727	<0.0001
Triglyceride (mmol/L)	0.338	7.821	<0.0001	0.255	5.747	<0.0001
Lipoprotein data by anion-exchange liquid chromatography						
IDL cholesterol (mmol/L)	0.313	7.181	<0.0001	0.240	5.391	<0.0001
VLDL cholesterol (mmol/L)	0.288	6.557	<0.0001	0.190	4.220	<0.0001
Other cholesterol (mmol/L)	0.008	0.170	NS	−0.035	−0.760	NS
Subjects without drug therapy ($n = 341$)						
Basic data						
Body mass index (kg/m^2)	0.211	3.978	<0.0001	0.183	3.429	<0.001
Lipid data						
Total cholesterol (mmol/L)	0.344	6.744	<0.0001	0.313	6.071	<0.0001
Non-HDL cholesterol (mmol/L)	0.457	9.467	<0.0001	0.362	7.140	<0.0001
Triglyceride (mmol/L)	0.366	7.247	<0.0001	0.272	5.212	<0.0001
Lipoprotein data by anion-exchange liquid chromatography						
IDL cholesterol (mmol/L)	0.313	6.069	<0.0001	0.241	4.577	<0.0001
VLDL cholesterol (mmol/L)	0.313	6.070	<0.0001	0.211	3.966	<0.0001
Other cholesterol (mmol/L)	−0.008	−0.147	NS	−0.054	−0.990	NS

NS means "not significant". Other cholesterol means cholesterol of chylomicron and lipoprotein(a).

data of subjects with F-score < 6 points and F-score ≥ 6 points, respectively. The percentages of subjects with hypertension and dyslipidemia were higher in Group 2 than in Group 1. Groups 3 and 4 show data of subjects with S-score <41 points and S-score ≥41 points, respectively. The patients with hypertension but not with dyslipidemia were more common in Group 4 than in Group 3. However, the prevalence of patients with diabetes was comparable both between Groups 1 and 2 and between Groups 3 and 4.

Both the F-score and S-score were calculated by age, sex-difference, TC or LDL-C, HDL-C, blood pressures, and status of smoking and glycemic control, and S-score also incorporated eGFR into the calculation. In the lipid data, LDL-C and HDL-C were excluded from the investigation because they were used in the calculation of risk scores. TC, TG, and non-HDL-C significantly correlated with the levels of F-score and S-score in the 476 men (**Table 2**). In TG-rich lipoproteins of non-HDL, IDL-C and VLDL-C significantly correlated with F-score and S-score. However, the individuals treated with drug therapy for hypertension, dyslipidemia, and diabetes were included in the 476 men (**Table 1**). Then, a part of the study subjects ($n = 341$) without the drug treatment was further investigated. Any drug users for dyslipidemia, diabetes, and hypertension were excluded from the sub-study subjects. The medication information was acquired from the annual medical checkup records. **Table 2** shows the similar correlations of lipid levels to F-score and S-score in the 341 subjects as in the total subjects ($n = 476$). Furthermore, BMI significantly correlated with F-score and S-score both in the subjects without the drug therapy and in the total subjects (**Table 2**).

Subsequently, a multiple stepwise regression analysis was performed to test the independent relationships of IDL-C and VLDL-C with F-score and S-score (**Table 3**). At first, BMI independently correlated with F-score and S-score levels both in the total subjects and in the subjects without the drug therapy. In the total subjects, IDL-C independently correlated with both the F-score and S-score levels, but the independent correlation of VLDL-C was found only in F-score. In the subjects without drug therapy, only IDL-C independently correlated with the F-score and S-score levels.

These results as above show that the high levels of IDL-C would be a potent marker for the F-score and S-score high-risk stage (10-year CHD risk >10%) as well as BMI. Then, the predictive values of BMI and IDL-C for 10-year CHD risk >10% were investigated by the univariate logistic regression analysis (**Figure 1**). The 1 SD increase in BMI was significantly associated with the F-score 10-year CHD risk >10% (*OR* 1.430 in total subjects and *OR* 1.431 in the subjects without drug therapy). In addition, the 1 SD increase in BMI was significantly associated with the S-score 10-year CHD risk >10% (*OR* 1.420 in total

TABLE 3 | Multiple stepwise regression of body mass index and cholesterol levels of triglyceride-rich lipoproteins to Framingham risk score and Suita score.

	Framingham risk score			Suita score		
	Partial correlation coefficient	*t*-value	*P*-value	Partial correlation coefficient	*t*-value	*P*-value
Total subjects ($n = 476$)						
Basic data						
Body mass index (kg/m^2)	0.173	3.930	0.0001	0.154	3.410	0.0007
Lipoprotein data by anion-exchange liquid chromatography						
IDL cholesterol (mmol/L)	0.217	4.407	<0.0001	0.187	3.698	0.0002
VLDL cholesterol (mmol/L)	0.111	2.204	0.0280	0.044	0.851	0.3950
Other cholesterol (mmol/L)	−0.074	1.715	0.0870	−0.071	1.603	0.1096
Subjects without drug therapy ($n = 341$)						
Basic data						
Body mass index (kg/m^2)	0.160	2.959	0.0033	0.157	2.817	0.0051
Lipoprotein data by anion-exchange liquid chromatography						
IDL cholesterol (mmol/L)	0.192	3.228	0.0014	0.170	2.783	0.0057
VLDL cholesterol (mmol/L)	0.118	1.885	0.0604	0.021	0.331	0.7411
Other cholesterol (mmol/L)	−0.085	1.664	0.0972	−0.094	1.782	0.0757

Other cholesterol means cholesterol of chylomicron and lipoprotein(a).

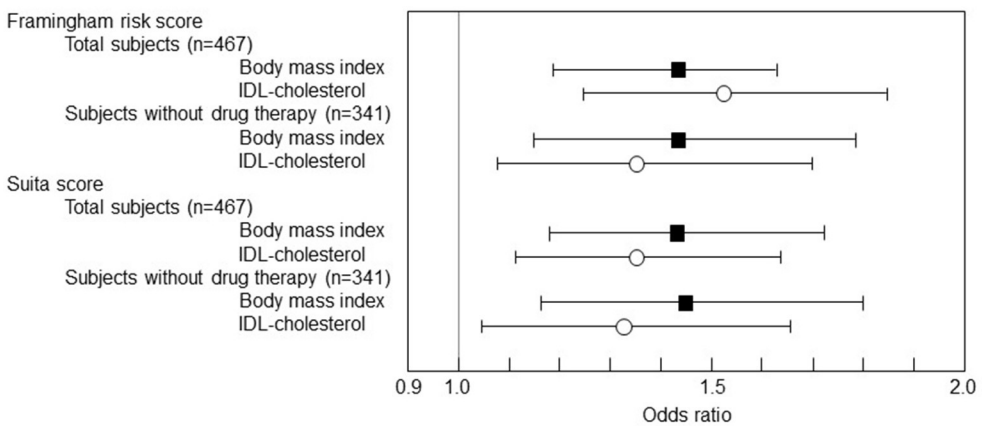

FIGURE 1 | A univariate logistic regression analysis of body mass index (BMI) and cholesterol levels of intermediate-density lipoprotein (IDL) to Framingham risk score (F-score) and Suita score (S-score). The logistic regression results were shown as odds ratios (*OR*s) and 95% *CI*s. The 1 SD increase in BMI was significantly associated with F-score 10-year CHD risk> 10% (*OR* 1.430, 95% *CI* 1.187–1.722, $p = 0.002$ in total subjects and *OR* 1.431, 95% *CI* 1.146–1.787, $p < 0.0001$ in the subjects without drug therapy). The 1 SD increase in BMI was also associated with S-score 10-year CHD risk >10% (*OR* 1.420, 95% *CI* 1.175–1.717, $p = 0.0003$ in total subjects and *OR* 1.445, 95% *CI* 1.158–1.804, $p = 0.0011$ in the subjects without drug therapy). Besides, the 1 SD increase in IDL-C was associated with F-score 10-year CHD risk >10% (*OR* 1.520, 95% *CI* 1.248–1.850, $p < 0.0001$ in total subjects and *OR* 1.350, 95% *CI* 1.070–1.703, $p = 0.0114$ in the subjects without drug therapy) and also associated with the S-score 10-year CHD risk> 10% (*OR* 1.348, 95% *CI* 1.107–1.642, $p = 0.003$ in total subjects and *OR* 1.319, 95% *CI* 1.047–1.662, $p = 0.019$ in the subjects without drug therapy).

subjects and *OR* 1.445 in the subjects without drug therapy). Besides, the 1 SD increase in IDL-C was significantly associated with the F-score 10-year CHD risk >10% (*OR* 1.520 in total subjects and *OR* 1.350 in the subjects without drug therapy) and with S-score 10-year CHD risk >10% (*OR* 1.348 in total subjects and *OR* 1.319 in the subjects without drug therapy).

Subsequently, a multivariate logistic regression analysis was performed with the 1 SD increase in BMI and IDL-C as the predictor variables in the multivariate analysis model (**Figure 2**). IDL-C and BMI were independent predictive markers for the F-score and S-score high-risk stage of 10-year CHD risk >10% both in the total subjects and in the subjects without the drug therapy.

DISCUSSION

The present study demonstrates that the increased levels of IDL-C among the TG-rich lipoproteins of non-HDL significantly correlated with the levels of F-score and S-score independently

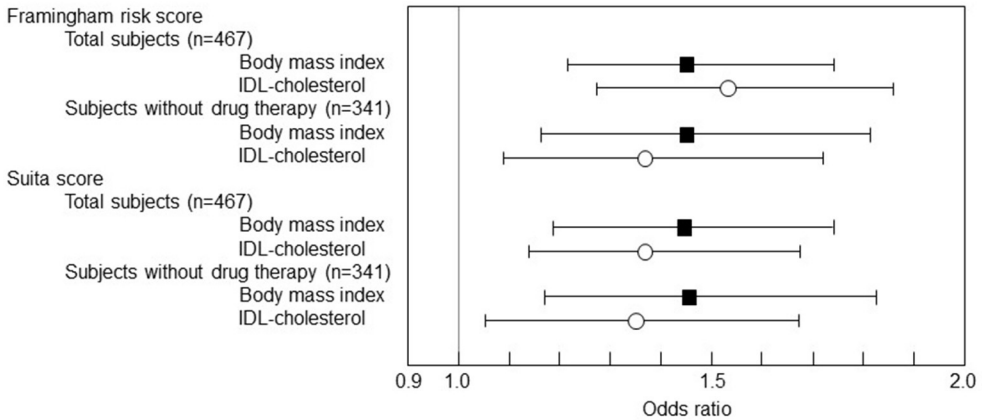

FIGURE 2 | The multivariate logistic regression analysis of BMI and cholesterol levels of IDL to F-score and S-score. The logistic regression results were shown as *ORs* and 95% *CIs*. The 1 SD increase in BMI was significantly associated with F-score 10-year CHD risk > 10% (*OR* 1.448, 95% *CI* 1.206–1.738, $p = 0.0001$ in total subjects and *OR* 1.450, 95% *CI* 1.163-1.808, $p < 0.0001$ in the subjects without drug therapy). The 1 SD increase in BMI was also associated with S-score 10-year CHD risk > 10% (*OR* 1.441, 95% *CI* 1.194–1.739, $p = 0.0001$ in total subjects and *OR* 1.462, 95% *CI* 1.173–1.823, $p = 0.0007$ in the subjects without drug therapy). Besides, the 1 SD increase in IDL-C was associated with F-score 10-year CHD risk > 10% (*OR* 1.534, 95% *CI* 1.266–1.859, $p < 0.0001$ in total subjects and *OR* 1.368, 95% *CI* 1.091–1.716, $p = 0.0066$ in the subjects without drug therapy) and also associated with S-score 10-year CHD risk > 10% (*OR* 1.372, 95% *CI* 1.130–1.667, $p = 0.0014$ in total subjects and *OR* 1.337, 95% *CI* 1.067–1.675, $p = 0.0116$ in the subjects without drug therapy).

of BMI, and also showed that the increased IDL-C would be a predictor for F-score and S-score 10-year CHD risk more than 10% in men free of CHD and stroke.

The previous papers from Framingham Heart Study and Copenhagen General Population Study demonstrate that the elevated levels of VLDL-C provide a certain contribution to ASCVD risk among the cholesterol levels of non-HDL, namely ApoB-containing lipoproteins (7, 11, 12). In our cross-sectional study with 476 individuals without CHD and stroke, however, we found the significant relevance of IDL-C rather than VLDL-C to CHD risk scores (FRS and SS). In the Copenhagen General Population Study, the multivariate-adjusted hazard ratios (*HR*s) for myocardial infarction for a 1-mmol/L (39 mg/dl) higher cholesterol content were 5.38 (95% *CI*: 3.73–7.75) for IDL, 2.07 (95% *CI*: 1.81–2.36) for VLDL, 1.86 (95% *CI*: 1.62–2.14) for LDL, and 1.49 (95% *CI*: 1.39–1.60) for non-HDL, presumably indicating the remarkable relevance of increased IDL-C to myocardial infarction risk (11). This attributable risk of IDL-C to myocardial infarction is presumably similar in effect to the IDL-C association with F-score and S-score in the present study. The similar messages from these previous cohort studies (Framingham Heart Study and Copenhagen General Population Study) show the significant contribution of elevated VLDL-C levels to CHD risk, but the present study suggested that the increased IDL-C rather than VLDL-C among the TG-rich lipoproteins significantly correlated with the levels of F-score and S-score. This discrepancy might be attributable in part to the differences in the methods for the determination of VLDL and IDL between the two studies and the present study. The Monitored Atherosclerosis Regression Study (MARS, $n = 180$), using data of IDL and VLDL measured ultracentrifugally, demonstrated that IDL but not VLDL or LDL was associated with the progression of carotid artery intima-media thickness, suggesting evidence for the atherogenicity of IDL independent of the levels of LDL and VLDL (23). However, which is a better predictor of CHD risk between VLDL-C and IDL-C remains inconclusive and it needs further large-scaled investigations.

Nishimura et al. reported that F-score might overestimate the CHD incidence in the Japanese general population, while S-score could improve the estimation power for CHD risk in the Japanese individuals (17). However, another study reported that the discrimination of S-score for estimating CHD was slightly better compared with F-score in whole individuals, but that the performance was comparable when the study subjects were divided into men and women (24). The reason for more accurately predicting CHD events by S-score than F-score in the Japanese individuals might be due to the incorporation of CKD factor into S-score calculation (17, 24). The incidence of myocardial infarction in the Japanese patients with hemodialysis with no history of ASCVD was independently associated with high non-HDL-C and low HDL-C, indicating that the elevated non-HDL-C predicts ASCVD events in the patients with hemodialysis (25–27). Especially, the increased IDL-C and decreased HDL-cholesterol levels in the patients with hemodialysis persisted even at very-low levels of serum lipids (27–30). In the patients with diabetes, VLDL-C was elevated but did not differ among the stages of diabetic nephropathy, whereas IDL-C was increasingly higher as the disease stage was advanced (27, 28). The previous studies with the AEX-HPLC method also showed increased levels of IDL-C and VLDL-C in the patients undergoing hemodialysis or continuous ambulatory peritoneal dialysis (CAPD) as compared with the healthy subjects (29, 30). In addition, elevated IDL-C levels in the patients with CAPD were found regardless

of CAPD duration (30). The increased IDL-C would be a significant biomarker for CHD risk in individuals with kidney dysfunction.

Then, we previously reported that the cholesterol levels of IDL in TG-rich lipoproteins were significantly correlated with the F-score independently of BMI, regardless of the medications for dyslipidemia, diabetes, and hypertension although the multivariate logistic regression analysis was not performed (13). In the present study, IDL-C was significantly correlated not only with the F-score but also with the S-score, incorporating CKD in the CHD risk score calculation. Tatami et al. reported that the increased IDL-C was associated with the severity of coronary artery disease, estimated by the coronary lesion scores determined by coronary angiographic data, indicating the contribution of IDL to the development of CAD (31). Consequently, an increase in IDL-C among the cholesterol levels of non-HDL lipoproteins may be considered a more significant biomarker for ASCVD.

LIMITATIONS

The present study has several limitations that need to be mentioned. One of the limitations is that this research was a cross-sectional study, which provides no evidence of a causal relationship between the IDL-C and ASCVD. Second, the interpretation of study results is limited to Japanese middle-aged men, and the extrapolation to other populations, such as women and elderly subjects should be validated by further studies. Third, because the methods to measure IDL-C and VLDL-C were different between the present study and the previous two studies, the direct comparison about the clinical significance of IDL-C and VLDL-C as an ASCVD risk biomarker could not be discussed. Fourth, IDL is considered a transient intermediate in the delipidation cascade from VLDL to LDL but also is known to be increased in the patients with high risk for CHD, including diabetes and kidney dysfunction. The normalization of ApoB-containing lipoprotein cholesterol levels by ApoB concentrations could adjust the individual status of LDL receptor activity and TG-rich lipoprotein metabolism, but unfortunately, the present study did not measure the ApoB concentrations.

CONCLUSION

In conclusion, these results for the first time suggest the significant relevance of increased IDL-C for CHD risk score estimated by S-score as well as F-score in middle-aged men free of CHD and stroke. Admittedly, non-HDL-cholesterol is simple and inexpensive as a potential marker of ASCVD risk but is just the aggregated cholesterol amount of ApoB-containing lipoproteins. Therefore, when non-HDL-cholesterol is high, IDL-cholesterol is considered a CHD risk biomarker to be measured in middle-aged men.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board of Tobu Medical Center. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

HY was mainly responsible for writing this paper. YH and SM mainly handled the statistical analyses. KI mainly took charge of collecting the samples and data. All authors confirmed they have contributed to the intellectual content of this paper, have discussed the data of this study, and have read the manuscript.

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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SYSTEMATIC REVIEW
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Cholesterol Efflux Capacity and Its Association With Adverse Cardiovascular Events: A Systematic Review and Meta-Analysis

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Background: Serum high-density lipoprotein cholesterol (HDL-C) levels are inversely associated with cardiovascular disease events. Yet, emerging evidence suggests that it is the functional properties of HDL, in particular, reverse cholesterol transport, which is a key protective mechanism mediating cholesterol removal from macrophage cells and reducing plaque lipid content. Cholesterol efflux capacity (CEC) measures the capacity of HDL to perform this function. A systematic review and meta-analysis were conducted to explore the association of CEC and adverse cardiovascular events.

Methods: A comprehensive literature review of Embase, PubMed, and Web of Science Core Collection from inception to September 2019 was performed for all studies that examined the association between CEC and cardiovascular outcomes. The primary outcome was adverse cardiovascular events, which were inclusive of atherosclerotic cardiovascular disease (ASCVD) or mortality.

Results: A total of 20 trials were included. Compared with low CEC levels, high CEC levels were associated with a 37% lower risk of adverse cardiovascular events (crude RR = 0.63; 95% CI, 0.52–0.76; $P < 0.00001$). Every SD increase of CEC was associated with a 20% lower risk of adverse cardiovascular events (HR = 0.80; 95% CI, 0.66–0.97; P = 0.02). The association remained significant after adjusting for cardiovascular risk factors, medications, and HDL-C levels (HR = 0.76; 95% CI, 0.63–0.91; $P = 0.004$). A significant CEC-endpoint relationship was observed ($P = 0.024$) such that for every 0.1 unit increase in CEC, there was a 5% reduced risk for adverse cardiovascular events (RR = 0.95; 95% CI, 0.91–0.99).

Conclusions: Higher CEC is associated with lower adverse cardiovascular outcomes. These findings warrant further research on whether CEC is merely a biomarker or a mechanism that could be targeted as a pharmacologic intervention for improving clinical outcomes.

PROSPERO Registration Number: CRD42020146681; https://www.crd.york.ac.uk/prospero/.

Keywords: acute coronary syndrome, atherosclerosis, acute myocardial infarction, cholesterol, cholesterol efflux capacity (CEC)

INTRODUCTION

An inverse relationship between high-density lipoprotein cholesterol (HDL-C) concentration and atherosclerotic cardiovascular disease (ASCVD) has been established through numerous observational studies and clinical trials (1, 2). However, the mechanisms underlying this association are not completely understood. Pharmacological studies have challenged the hypothesis that increasing levels of HDL-C would decrease ASCVD risk (3, 4). Mendelian randomization studies have demonstrated that genetic variants associated with high HDL-C levels were not associated with low ASCVD risk (5–7). Rather than crude HDL-C concentrations, emerging evidence has suggested that a quantitative measure of HDL functionality may be a better predictor of ASCVD risk.

A key mechanism by which HDL mitigates the development of atherosclerosis is through reverse cholesterol transport, which promotes cholesterol efflux from macrophages within atherosclerotic plaques. HDL functions to transport excess cholesterol to the liver, thereby reducing the formation of foam cells, which is a key component of atherosclerosis development. Cholesterol efflux capacity (CEC) measures the ability of HDL to promote cholesterol efflux from macrophages, the first step in reverse cholesterol transport. Greater CEC or improved HDL function, rather than higher HDL-C concentrations, is hypothesized to be a mechanism of ASCVD risk reduction.

Recent literature has shed light on the association between increased CEC and decreased ASCVD risk in the outpatient setting. Most notably, the Dallas Heart Study reported an inverse relationship between CEC and incident cardiovascular events, and the EPIC-Norfolk study found consistent results using a nested case-control design (8, 9). The present study aimed to review and synthesize the current evidence regarding the association between CEC and adverse cardiovascular events.

METHODS

Search Strategies and Selection Criteria

Systematic literature searches were performed in Embase, PubMed, and Web of Science Core Collection. The searches included a set of keywords, wildcards, truncation and medical subject headings, including cholesterol efflux capacity, atherosclerotic cardiovascular disease, atherosclerosis, coronary artery disease (CAD), acute coronary syndrome, myocardial infarction, stroke, cerebrovascular event, mortality, and death. The search terms were organized in thematic building blocks that could be combined as required. Human studies, published as original research articles, letters, or abstracts, that reported measurement of cholesterol efflux capacity at baseline as well as adverse cardiovascular events, including ASCVD or mortality were included. All searches were limited to English language and the time from inception to September 2019. Duplicates were removed before screening references. Detailed queries are provided in **Supplementary Table S1**.

Data Extraction

Data extracted from each study included baseline characteristics, methods for CEC measurement, CEC levels, and frequencies or risk estimates for adverse cardiovascular events. Database search, article screening, and study selection were performed independently by two investigators using a standardized approach. Disagreement in extracted data was adjudicated by a third investigator. A flow diagram depicting the process of literature search and screening is provided in **Supplementary Figure S1**.

Quality Assessment

Two independent investigators assessed the quality of case-control studies and cohort studies in accordance with the Newcastle–Ottawa Scale. Disagreement in the quality assessment was resolved by discussion and consensus. The quality assessment criteria and forms are provided in **Supplementary Tables S2–S5**.

Study Endpoints and CEC Measurements

The primary endpoint is adverse cardiovascular events, defined as the composite of ASCVD or all-cause mortality. ASCVD was inclusive of acute coronary syndrome, stroke, arterial revascularization, atherosclerotic plaque (including coronary carotid and femoral atherosclerotic detected by angiography or ultrasonography), and cardiovascular death. Death from all causes and death from cardiovascular causes were also evaluated.

Global CEC was captured from each study for assessment of association with adverse cardiovascular events. Normalized CEC levels (expressed as arbitrary units [AU]) in reference to the CEC of serum controls were used in assessing the strength of the CEC-endpoint relationships. To document the methodological variability of quantifying CEC, information regarding the type of cholesterol donor cell (mouse macrophage cell line [J774] or human macrophage cell line [THP-1 macrophages] and cholesterol tracer were extracted from each study.

Statistical Analysis

Several approaches were deployed to investigate the relationship between CEC and endpoints (including adverse cardiovascular events, ASCVD, death from all causes, and death from

Abbreviations: ASCVD, atherosclerotic cardiovascular disease; AU, arbitrary unit; CAD, coronary artery disease; CEC, cholesterol efflux capacity; CI, confidence interval; CKD, chronic kidney disease; df, degree of freedom; eGFR, estimated glomerular filtration rate; HR, hazard ratio; HDL-C, high-density lipoprotein cholesterol; HR, hazard ratio; IV, inverse variance; OR, odds ratio; RR, relative risk; SD, standard deviation; SE, standard error.

cardiovascular causes). First, the relative risk (RR) of high CEC group vs. low CEC group was examined. High CEC group was defined as the group above the median CEC or the top quartile or tertile (i.e., better CEC), whereas the low CEC group was the group below the median CEC or the bottom quartile or tertile (i.e., worse CEC). Second, the risk of outcomes associated with each standard deviation (SD) increment of CEC was assessed. Third, the strength of CEC-endpoint relationships were explored using the *dosresmeta* package in R. In brief, the relationship between the log-transformed CEC and endpoint for each study was estimated by fitting a linear regression model based on the number of cases and controls as well as cohort size from at least three quantitative exposure categories. The generalized least squares method was applied to estimate the covariances and the vector of the regression coefficients. The CEC concentration assigned to each level of functionality category was approximated from the mean or median as reported by the studies. Pooled RR with Wald-type confidence interval (CI) associated with every 0.1 unit increase in CEC was calculated. Subsequently, to test the potential non-linear association, a restricted cubic spline model was constructed, with three knots located at 10th, 50th, and 90th percentiles of the aggregated exposure distribution. Non-linearity was assessed under the null hypothesis that the coefficient of the second spline (i.e., between 10th and 50th percentiles) was equal to zero. The two regression coefficients and the variance-covariance matrix within each study were then combined in a random-effects meta-analysis. Last, a separate analysis was performed among the case-control studies to compare the mean CEC level between cases (individuals with adverse cardiovascular events, with ASCVD, or died) and controls (individuals without adverse cardiovascular events, without ASCVD, or survived).

Measures of effect included relative risk (RR), odds ratio (OR), and hazard ratio (HR), with or without adjustment as reported by the studies. The DerSimonian-Laird random-effects model was fitted to derive the combined overall estimate of the treatment effects. Heterogeneity among the studies was evaluated using Cochran's Q test (with the threshold of $P > 0.10$) and Higgins's I^2 statistic (with the values of 0.25, 0.50, and 0.75 indicating a low, moderate, and high degree of heterogeneity, respectively).

Contour-enhanced funnel plots (with a significance level of 1, 5, and 10%) and Egger's test were employed to detect small-study effects for the endpoints with a study number of ten or more. The trim-and-fill method was used to adjust for publication bias. Subgroup analysis was performed to examine the robustness of the association among three subsets: (1) individuals without cardiovascular risk factors or chronic kidney disease (CKD); (2) individuals with cardiovascular risk factors (e.g., with underlying or a history of CAD, dyslipidemia, family history of myocardial infarction); and (3) individuals with CKD (e.g., estimated glomerular filtration rate [eGFR] <90 ml/min/1.73 m^2, patients on dialysis, or renal transplant recipients). All analysis was performed using R software (Version 3.5.2; The R Foundation for Statistical Computing), Review Manager (Version 5.3; The Nordic Cochrane Center, The Cochrane Collaboration), Stata (StataCorp. 2019. Stata Statistical Software: Release 16. College Station, TX: StataCorp LLC), and SAS (Version 9.4; SAS Institute Inc.).

RESULTS

A total of 25,132 subjects from 20 studies were included in the meta-analysis and summarized in **Table 1** (8–27). Ten studies included individuals with cardiovascular risk factors, four studies included patients with CKD, and six studies included general populations without cardiovascular risk factors or CKD. The mean age ranged from 42 to 69 years. The proportion of males ranged from 26.4 to 100%. The follow-up duration varied from 1 to 16 years. Three and 17 studies used THP-1 (human) and J774 (mouse) as the macrophage cell type donating labeled cholesterol in the CEC assay, respectively. Five studies measured the efflux of a fluorescent sterol (BODIPY-cholesterol), whereas 15 used radioisotope labeling ([^{3}H]-cholesterol) in the CEC assays. The quality of the studies was generally high, with scores ranging from 5 to 9, as evaluated with the Newcastle-Ottawa Scale (**Supplementary Tables S2–S5**).

Association With Adverse Cardiovascular Events

Increased CEC was significantly associated with reduced adverse cardiovascular events. Compared with the lowest CEC, the highest levels of CEC were associated with a 37% lower risk of adverse cardiovascular events (RR = 0.63; 95% CI, 0.52 to 0.76; $P < 0.00001$; **Figure 1**). Every SD increase of CEC was associated with a 20% lower risk of adverse cardiovascular events (HR = 0.80; 95% CI, 0.66–0.97; $P = 0.02$; **Figure 2**). The association remained significant even after adjusting for cardiovascular risk factors (e.g., with underlying or a history of CAD, dyslipidemia, family history of myocardial infarction), medications, and HDL-C levels (HR = 0.76; 95% CI, 0.63–0.91; $P = 0.004$; **Figure 3**). The I^2 values ranged from 82 to 89%, indicating a high degree of heterogeneity. There were significant small-study effects as determined by the funnel plots (**Supplementary Figures S16**, **S17**) and Egger's test (**Supplementary Table S6**). After controlling for publication bias, high CEC remained associated with an improved cardiovascular outcome (RR = 0.79; 95% CI, 0.65–0.97; **Supplementary Table S6**), and the risk of adverse cardiovascular events was 19% lower with every SD increment of CEC (HR = 0.81; 95% CI, 0.66–0.98; **Supplementary Table S6**).

In the restricted cubic spline model, the relationship between CEC levels and adverse cardiovascular events are depicted in **Figure 4**. The risk of adverse cardiovascular events did not vary with CEC concentrations in a log-linear fashion (non-linearity $P = 0.075$). A significant CEC-adverse cardiovascular relative risk relationship was observed ($P = 0.024$) such that for every 0.1 unit increase in CEC, there was a 5% reduced risk for adverse cardiovascular events (RR = 0.95; 95% CI, 0.91–0.99).

Subgroup analyses on the association with adverse cardiovascular events are summarized in **Supplementary Figures S19**, **S20**. Compared with the low CEC group, the high CEC group had a lower risk among the individuals without cardiovascular risk factors or CKD (RR = 0.54; 95% CI, 0.41–0.71; $P < 0.0001$) and individuals

TABLE 1 | Summary of included studies.

Author (Year)	Study Design	Population	N	Mean Age	Male	Follow-Up	Endpoints [number of events/cases]	Donor Cell	Labeling	NOS
Ebtehaj et al. (10)	Case-control; prospective	General population	705	59.0	71.2%	12 years	ASCVD (cardiovascular death and hospitalization for MI/coronary revascularization) [351]	J774	[^{3}H]-Cholesterol	8
Cahill et al. (11)	Case-control; prospective	General population	1,397	63.0	100%	16 years	ASCVD (nonfatal MI and fatal CHD) [701]	J774	[^{3}H]-Cholesterol	8
Guerin et al. (12)	Cohort; prospective	Patients with acute MI	1,609	63.0	75.7%	1.9 years	Death (all-cause mortality) [239]	THP-1	[^{3}H]-Cholesterol	7
Chindhy et al. (13)	Cohort; prospective	CKD vs non-CKD patients	2,805	NR	NR	11.3 years	ASCVD (nonfatal MI, stroke, cardiovascular death) [131]; CVD [187]	J774	BODIPY-Cholesterol	8
Tejera-Segura et al. (14)	Case-control; cross-sectional	RA patients	401	57.2	26.4%	N/A	ASCVD (presence of atherosclerotic plaque in carotid artery) [66]	J774	BODIPY-Cholesterol	6
Khera et al. (15)	Case-control; prospective	Individuals with LDL-C <130 mg/dL and hsCRP ≥2.0 mg/L	1,050	69	71.6%	1.9 years	ASCVD (MI, hospitalization for unstable angina, arterial revascularization, stroke, or cardiovascular death) [314]	J774	[^{3}H]-Cholesterol	8
Bauer et al. (16)	Cohort; prospective	CKD patients	526	65	59%	4.6 years	ASCVD (MI, arterial revascularization, stroke, lower extremity amputation, or cardiovascular death) [114]	J774	[^{3}H]-Cholesterol	7
Gall et al. (17)	Case-control; cross-sectional	Patients with dyslipidemia	1,202	56.4	51.3%	N/A	ASCVD (presence of atherosclerotic plaque in carotid [7] or femoral artery [72])	J774	[^{3}H]-Cholesterol	8
Kopecky et al. (18)	Cohort; prospective	Patients with T2DM on hemodialysis	1,147	66.3	54.8%	4.1 years	ASCVD (cardiovascular death, nonfatal MI, and stroke) [423]; Death [561]	THP-1	[^{3}H]-Cholesterol	7
Javaheri et al. (19)	Cohort; prospective	Cardiac transplant recipients with CAV	35	44.4	85.7%	1 year	Death (all-cause mortality) [15]	J774	[^{3}H]-Cholesterol	7
Mody et al. (20)	Cohort; prospective	General population and a subgroup with FHx of MI	1,972	44.9	44%	9.4 years	ASCVD (nonfatal MI, nonfatal stroke, coronary revascularization, or cardiovascular death) [97]	J774	BODIPY-Cholesterol	9
Liu et al. (21)	Cohort; prospective	Patients with CAD	1,737	63.5	65.2%	3.8 years	Death (all-cause mortality) [166]; CVD (cardiovascular death) [122]	J774	BODIPY-Cholesterol	9
Zhang et al. (22)	Cohort; prospective	Patients with SAP or ACS	429	66.2	74.8%	3 years	ACS [214]; ASCVD (nonfatal MI, nonfatal stroke, or cardiovascular death) [34]; CVD [22]	J774	[^{3}H]-Cholesterol	8
Ogura et al. (23)	Cohort; cross-sectional	Patients with HeFH	227	57	44.5%	N/A	ASCVD (MI, stroke, angina pectoris with significant stenosis >75% on coronary angiogram, and coronary revascularization) [76]	J774	[^{3}H]-Cholesterol	5
Annema et al. (24)	Cohort; prospective	Renal transplant recipients	495	51.6	54.3%	7.0 years	Death [102]; CVD [54]	THP-1	[^{3}H]-Cholesterol	8
Ishikawa et al. (25)	Case-control; cross-sectional	Patients with suspected CAD	254	65.7	78.0%	N/A	ASCVD (native coronary atherosclerosis with >50% stenosis) [182]	J774	[^{3}H]-Cholesterol	8

(Continued)

TABLE 1 | Continued

Author (Year)	Study Design	Population	N	Mean Age	Male	Follow-Up	Endpoints [number of events/cases]	Donor Cell	Labeling	NOS
Saleheen et al. (9)	Case-control; prospective	General population	3,494	65.5	64.5%	12 to 16 years	ASCVD (unstable angina, stable angina, and fatal/nonfatal MI) [1745]	J774	[^{3}H]-Cholesterol	7
Rohatgi et al. (8)	Cohort; prospective	General population	2,924	42	43%	9.4 years	ASCVD (nonfatal MI, stroke, cardiovascular death, and coronary revascularization) [132]; CVD (cardiovascular death) [42]	J774	BODIPY-Cholesterol	9
Li et al. (26)	Case-control; cross-sectional	General population (angiography & outpatient cohort)	1,727	60.6	54.1%	N/A	ASCVD (MI/CAD/coronary revascularization) [1017]	J774	[^{3}H]-Cholesterol	7
Khera et al. (27)	Cohort; cross-sectional	Patients with CAD and controls	996	57.5	58.5%	N/A	ASCVD (angiographically confirmed coronary artery disease with >50% stenosis in a major coronary vessel) [442]	J774	[^{3}H]-Cholesterol	7

ACS, acute coronary syndrome; ASCVD, atherosclerotic cardiovascular disease; CAD, coronary artery disease; CAV, cardiac allograft vasculopathy; CHD, coronary heart disease; CKD, chronic kidney disease; CVD, cardiovascular death; FHx, family history; HeFH, heterozygous familial hypercholesterolemia; hsCRP, high-sensitivity C-reactive protein; LDL-C; low-density lipoprotein cholesterol; MI, myocardial infarction; N/A, not applicable; NOS, Newcastle–Ottawa Scale; NR, not reported; RA, rheumatoid arthritis; SAP, stable angina pectoris; T2DM, type 2 diabetes mellitus.

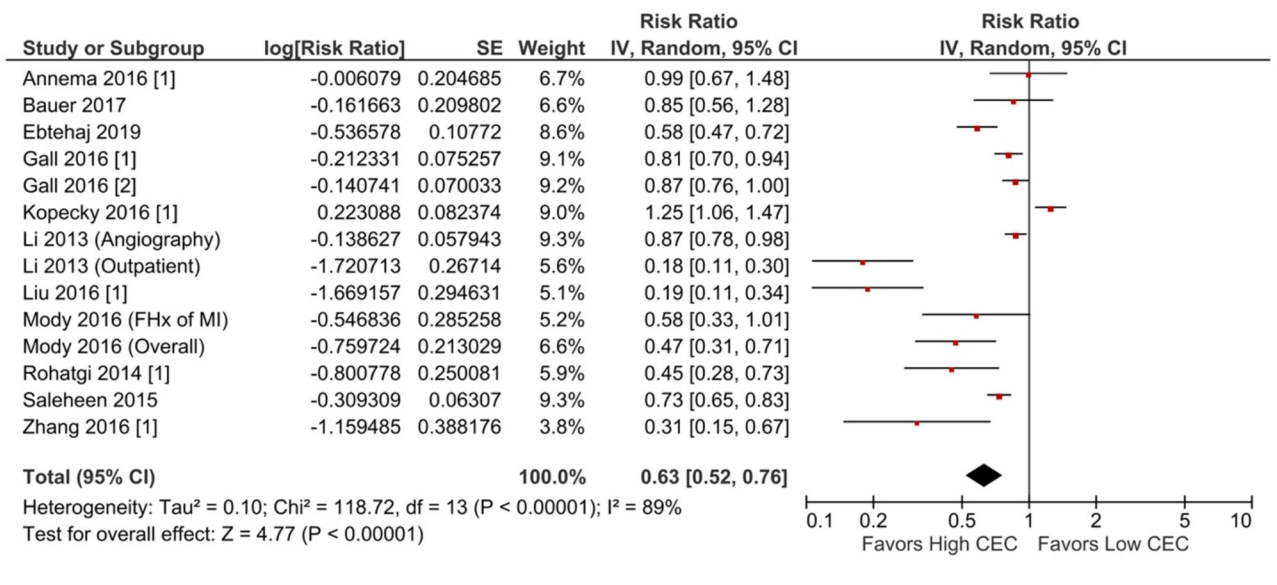

FIGURE 1 | Adverse cardiovascular event: High CEC vs. low CEC (RR) (14 studies). CEC, cholesterol efflux capacity; CI, confidence interval; df, degree of freedom; FHx, family history; IV, inverse variance; MI, myocardial infarction; RR, risk ratio; SE, standard error.

with cardiovascular risk factors (RR = 0.54; 95% CI, 0.38–0.78; $P = 0.001$). Of note, the inverse relationship was not observed in CKD patients (RR = 1.08; 95% CI, 0.86–1.38; $P = 0.50$). The association with adverse cardiovascular events was heterogeneous across subgroups ($P = 0.0002$, $I^2 = 88.6\%$). Similarly, for each SD increment of CEC, a significantly decreased risk was observed in individuals without cardiovascular risk factors or CKD (HR = 0.69; 95% CI, 0.59 −0.82; $P < 0.00001$) and individuals with cardiovascular risk factors (HR = 0.49; 95% CI, 0.29–0.82; $P = 0.006$), but not in patients with CKD (HR = 1.05; 95% CI, 0.87–1.27; $P = 0.62$). There was a significant difference across subgroups ($P = 0.0007$, $I^2 = 86.3\%$).

Association With Atherosclerotic Cardiovascular Disease

Higher CEC (i.e., better CEC) was significantly associated with lower ASCVD risk. Compared with the lowest CEC (i.e., worse CEC), the highest levels of CEC were associated

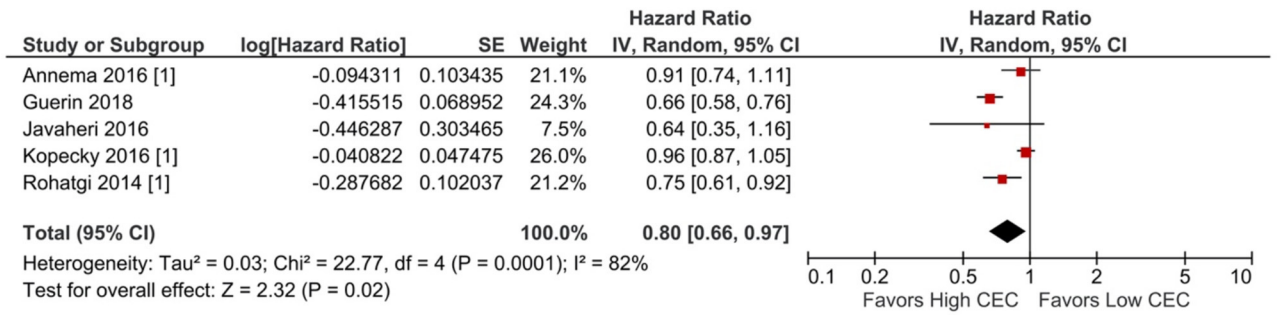

FIGURE 2 | Adverse cardiovascular event: Per SD increment of CEC (HR) (5 studies). CEC, cholesterol efflux capacity; CI, confidence interval; df, degree of freedom; HR, hazard ratio; IV, inverse variance; SD, standard deviation; SE, standard error.

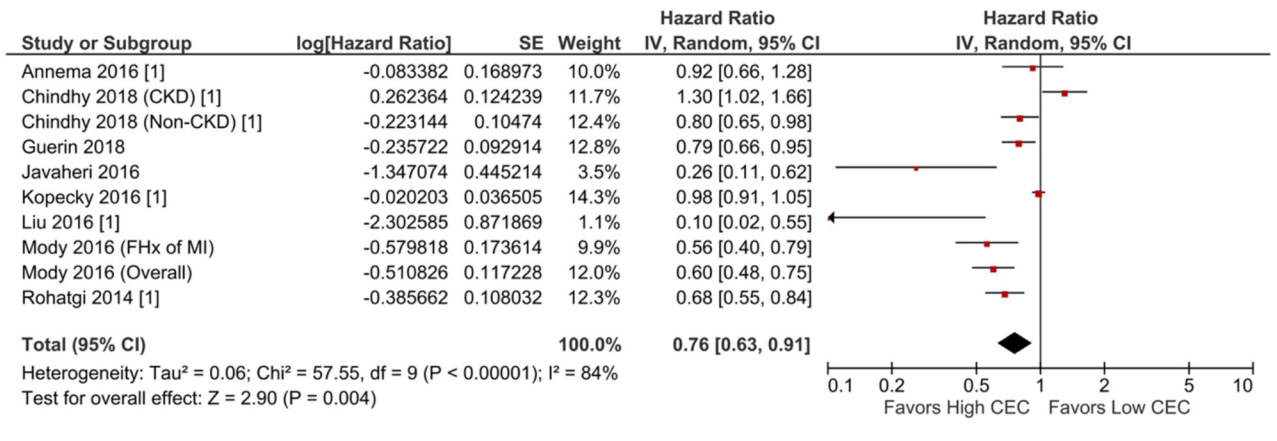

FIGURE 3 | Adverse cardiovascular event: Per SD increment of CEC (adjusted HR) (10 studies). CEC, cholesterol efflux capacity; CI, confidence interval; CKD, chronic kidney disease; df, degree of freedom; FHx, family history; HR, hazard ratio; IV, inverse variance; MI, myocardial infarction; SE, standard error.

with a 34% lower risk of ASCVD (RR = 0.66; 95% CI, 0.55–0.80; $P < 0.0001$; **Supplementary Figure S2**). After adjustment for cardiovascular risk factors, medications, and HDL-C levels, high CEC remained associated with a 21% lower risk compared with low CEC (RR = 0.79; 95% CI, 0.65–0.97; $P = 0.02$; **Supplementary Figure S3**). With respect to predicting ASCVD risk with each SD increase of CEC, there was no significant association either without adjustment (HR = 0.86; 95% CI, 0.68–1.10; $P = 0.23$; **Supplementary Figure S4**) or with adjustment (HR = 0.80; 95% CI, 0.64–1.00; $P = 0.05$; **Supplementary Figure S5**). With respect to differentiating ASCVD cases from controls, each SD increase of CEC was associated with a 20% lower odds of ASCVD (OR = 0.80; 95% CI, 0.66–0.97; $P = 0.02$; **Supplementary Figure S6**) and 19% lower odds after adjustment (OR = 0.81; 95% CI, 0.73–0.90; $P = 0.0002$; **Supplementary Figure S7**). The I^2 values ranged from 79 to 91%, indicating a high degree of heterogeneity. There were significant small-study effects as determined by the funnel plots (**Supplementary Figure S18**) and Egger's test (**Supplementary Table S6**). After controlling for publication bias, high CEC remained associated with an improved cardiovascular outcome (RR = 0.78; 95% CI, 0.64–0.94; **Supplementary Table S6**).

Subgroup analysis on the association with ASCVD was summarized in **Supplementary Figure S21**. Compared with the low CEC group, the high CEC group had a lower risk among individuals without cardiovascular risk factors or CKD (RR = 0.54; 95% CI, 0.41–0.71; $P < 0.0001$) and individuals with cardiovascular risk factors (RR = 0.75; 95% CI, 0.60–0.93; $P = 0.009$). Of note, the inverse relationship was not observed in patients with CKD (RR = 1.08; 95% CI, 0.75–1.56; $P = 0.67$). There was a significant heterogeneity across subgroups ($P = 0.01$, $I^2 = 77.8\%$).

Association With Death From All-Causes and Death From Cardiovascular Causes

The high CEC group did not have a significantly different risk of all-cause mortality compared with the low CEC group (RR = 0.61; 95% CI, 0.27–1.41; $P = 0.25$; **Supplementary Figure S8**). The risk of mortality did not vary significantly with per SD

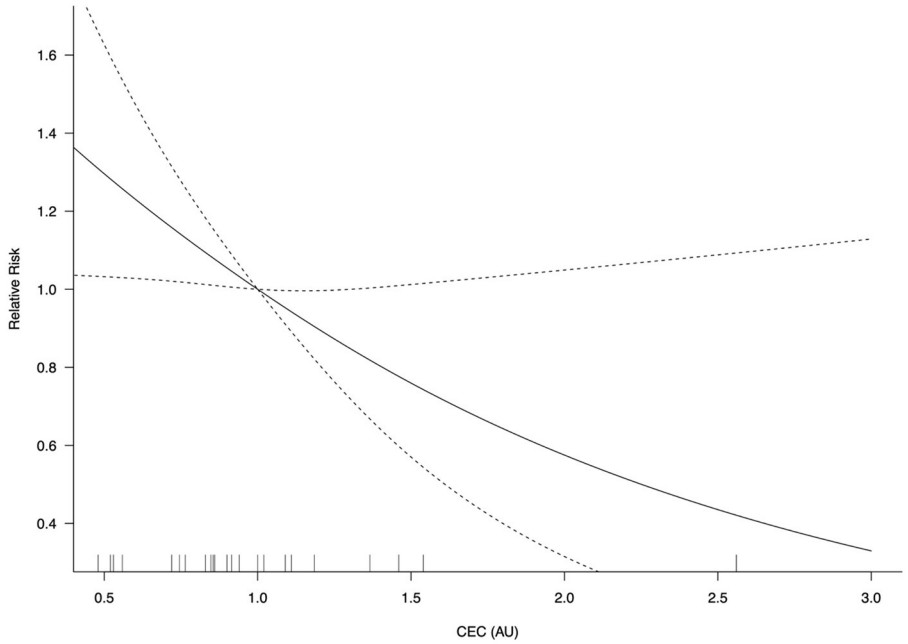

FIGURE 4 | CEC-adverse cardiovascular event relative risk relationship ($P = 0.024$). AU, arbitrary unit; CEC, cholesterol efflux capacity; CI, confidence interval. Log-transformed CEC was used.

increment of CEC either without adjustment (HR = 0.81; 95% CI, 0.64–1.02; $P = 0.08$; **Supplementary Figure S9**) or with adjustment (HR = 0.77; 95% CI, 0.58 −1.02; $P = 0.07$; **Supplementary Figure S10**). The I^2 values ranged from 81 to 94%, indicating a high degree of heterogeneity.

Similarly, a significant association between CEC and cardiovascular mortality was not observed. The high CEC group had a comparable risk of all-cause mortality with the low CEC group (RR = 0.48; 95% CI, 0.14–1.62; $P = 0.24$; **Supplementary Figure S11**). The risk of cardiovascular mortality did not vary per SD increment of CEC after adjustment (HR = 1.08; 95% CI, 0.72–1.62; $P = 0.71$; **Supplementary Figure S12**). The I^2 values ranged from 71 to 89%, indicating a high degree of heterogeneity.

Difference in CEC Between Cases and Controls

In this separate analysis of case-control studies, mean CEC levels between cases (individuals with adverse cardiovascular events, with ASCVD, or died) and controls (individuals without adverse cardiovascular events, without ASCVD, or survived) were compared. Compared with controls, a lower level of CEC was observed in cases who developed adverse cardiovascular events (mean difference, −0.08; 95% CI, −0.12 to −0.04; $P < 0.00001$; **Supplementary Figure S13**), cases who developed ASCVD (mean difference, −0.09; 95% CI, −0.16 to −0.02; $P = 0.007$; **Supplementary Figure S14**), and cases who died (mean difference, −0.07; 95% CI, −0.11 to −0.04; $P < 0.0001$; **Supplementary Figure S15**). The I^2 values ranged from 83 to 92%, indicating a high degree of heterogeneity.

DISCUSSION

In this meta-analysis, higher CEC levels were associated with favorable cardiovascular outcomes (**Figure 5**). Compared with the lowest CEC group, the highest CEC group had a 37 and 34% reduced risk of adverse cardiovascular events and ASCVD, respectively. Every SD increase in CEC [equivalent to 0.27 unit in the study by Ebtehaj et al. (10)] was associated with a 20% lower risk of adverse cardiovascular events. When fitting a restricted cubic spline model, there was an inverse concentration-dependent relationship, with a 5% lower risk of adverse cardiovascular events for every 0.1 unit increase of CEC.

The conventional "HDL hypothesis" posits that interventions that increase the plasma level of HDL-C reduce the risk of coronary heart disease. However, HDL-C-raising therapies, such as fibrates, niacin, and cholesteryl ester transfer protein inhibitors have not consistently demonstrated cardiovascular benefits (28). Furthermore, Mendelian randomization studies did not demonstrate a causal relationship between genetically-altered plasma HDL-C levels and cardiovascular risk (29–33). Rather than focusing on HDL-C levels, emerging evidence has highlighted the functional aspects of HDL in improving cardiovascular outcomes, known as the "HDL flux hypothesis"(34) In contrast to the HDL hypothesis, the HDL flux hypothesis postulates that interventions promoting CEC and reverse cholesterol transport may stabilize atherosclerosis

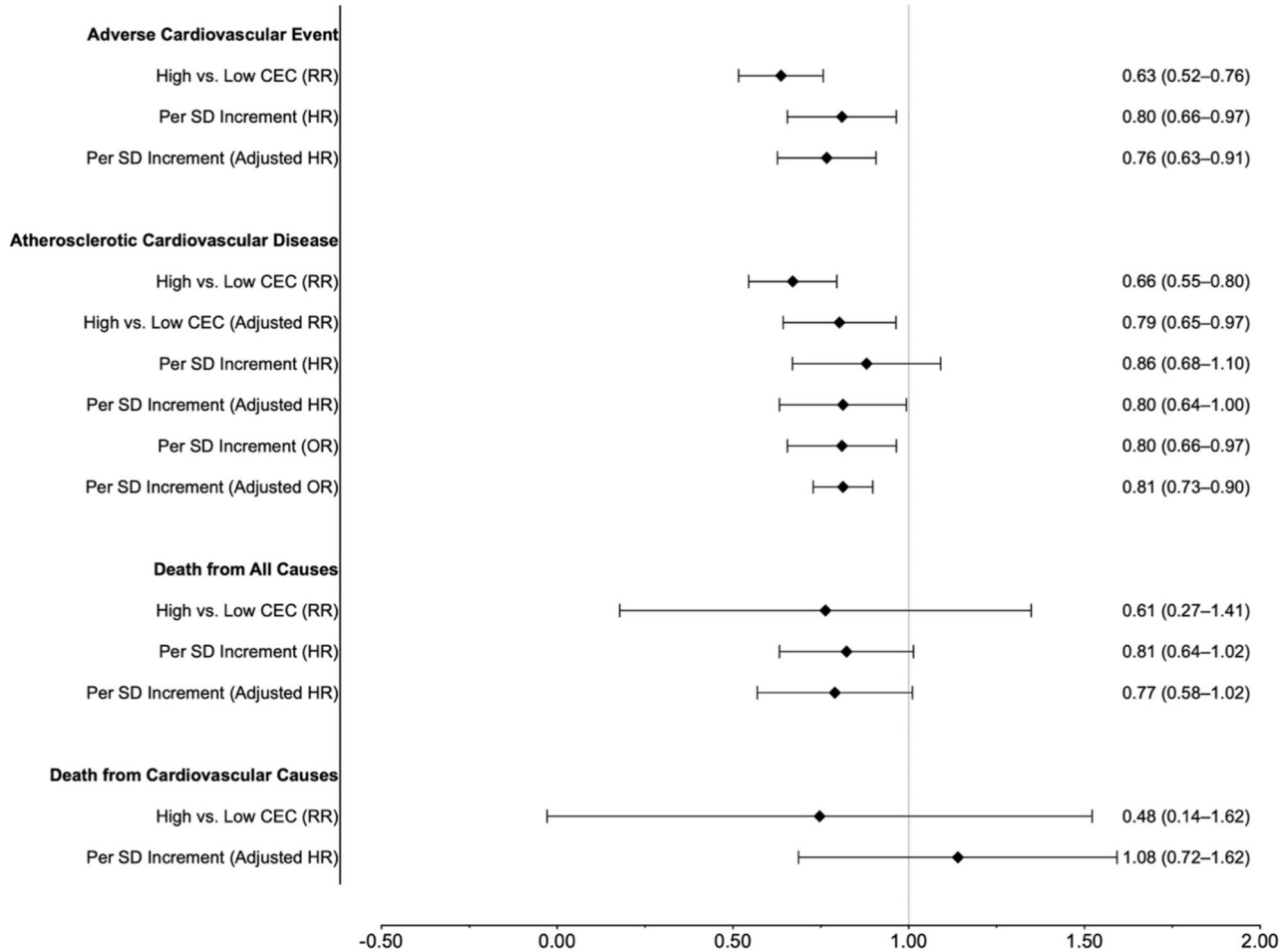

FIGURE 5 | Summary of results.

and reduce the risk of coronary heart disease, regardless of whether it affects plasma HDL cholesterol levels (35). Similar to our findings, a previous meta-analysis of 14 studies showed that there was a relationship between CEC and cardiovascular risk (36). Additionally, the highest CEC group was associated with 44% reduced risk of cardiovascular events compared with the lowest CEC group, and per SD increase in CEC was associated 13% reduced risk.

Although the association of CEC with all-cause mortality was not statistically significant (**Supplementary Figures S8–S10**), the current analysis demonstrates that there may be a trend toward lower mortality with higher CEC. Among the three studies available for all-cause mortality sub-analysis (**Supplementary Figure S8**), the inverse association was evidence in one study where the adjusted HR of the highest CEC quartile compared to the lowest quartile was 0.24 (95% CI, 0.13–0.44; $P < 0.001$) (21). Moreover, deceased patients had significantly lower mean levels of CEC, as opposed to the survived patients (<0.0001; **Supplementary Figure S15**). Results from the current analysis of 20 studies were generally consistent with the work by Qiu et al. The present analysis further demonstrated that there was a significant difference in the mean CEC between cases (those who had adverse cardiovascular events or ASCVD) and controls. In addition to all-cause mortality, the present analysis shows that the association with cardiovascular mortality was not significant. Notably, the performance of CEC as a prognostic indicator of cardiovascular risk among patients with CKD was shown to be limited compared to its performance among patients with normal renal function. Of the three studies included in this analysis, the definitions of the CKD varied vastly, and included renal transplant recipients, (24) patients with eGFR 15 to 89 ml/min/1.73m^2 (2, 16), and patients on hemodialysis (18). This suggests a significant heterogeneity in patients with CKD that were included in this analysis. Accordingly, further primary research among patients with CKD is required to explore whether CEC or other functional properties of HDL particles can assist with cardiovascular risk prediction.

The gold standard for measuring CEC in humans has not yet been established. It is possible that the choice of CEC assay may influence its association with adverse cardiovascular outcomes.

For instance, the Cohort on Diabetes and Atherosclerosis Maastricht (CODAM) study found no association between CEC and subclinical or clinical atherosclerosis among participants with normal glucose metabolism, prediabetes, or diabetes using human THP-1 cells as the cholesterol donor (37). In contrast, a significant correlation was observed when remeasured using murine J774 cells among a subset of samples, suggesting the impact of cholesterol donor on CEC measurement (37). In this study, the majority (85%) of the included studies used J774 as the cholesterol donor. In the stratified analysis by the type of cholesterol donor (J774 vs. THP-1), high CEC was associated with a lower risk of adverse cardiovascular event among the 12 studies that used J774 as the donor (**Supplementary Figure S22**). In contrast, among the two studies that used THP-1 as the donor, high CEC was related to a greater risk. Regarding the association of per SD increment of CEC with adverse cardiovascular event (**Supplementary Figures S23**, **S24**), there was no significant difference between the J774 subgroup and THP-1 subgroup. More studies using THP-1 as the cholesterol donor are needed to examine the relationship between CEC and adverse cardiovascular event. In the stratified analysis by the cholesterol tracer ([^{3}H]-Cholesterol vs. BODIPY-Cholesterol), high CEC was associated with a lower risk of adverse cardiovascular event among the 10 studies that used [^{3}H]-Cholesterol as well as in the four studies that used BODIPY-Cholesterol (**Supplementary Figure S25**), with a greater magnitude of association observed in the BODIPY-Cholesterol subgroup (RR = 0.40 [95% CI, 0.26–0.61]) than in the [^{3}H]-Cholesterol subgroup (RR = 0.74 [95% CI, 0.61–0.89]). Regarding the association of per SD increment of CEC with adverse cardiovascular event (**Supplementary Figures S26**, **S27**), there was no significant difference between the [^{3}H]-Cholesterol subgroup and BODIPY-Cholesterol subgroup. Last, the difference in laboratory protocols (e.g., using whole serum vs. apolipoprotein B-depleted serum) across studies may have contributed to the heterogeneity of results.

Several interventions have been shown to improve the HDL function. For instance, in the STAMPEDE sub-study, bariatric surgeries, including Roux-en-Y gastric bypass and sleeve gastrectomy), were found to improve HDL functionality as evaluated by the CEC assay at five years. (38) In addition, eicosapentaenoic acid (EPA) supplementation has been associated with a dose-dependent increase of CEC from macrophages mediated by ATP-binding cassette transporter A1 (ABCA1) (39), which may help explain the anti-atherogenic properties and cardiovascular benefits of EPA in high-risk patients from recent trials (40). Furthermore, CETP inhibitors have been shown to significantly improve CEC along with HDL level (41–43). In further support of the HDL flux hypothesis, a novel infusible ApoA-I agent named CSL112 has been associated with an immediate and pronounced increase in CEC in patients with stable atherosclerotic disease and in healthy individuals (44). To test the safety and tolerability of CSL112, the AEGIS-I trial (ApoA-I Event Reducing in Ischemic Syndromes I) was a multicenter, randomized double blind placebo controlled trial that demonstrated four weekly infusions of CSL112 were feasible, well tolerated, and not associated with significant changes in hepatic or renal function among patients with an acute myocardial infarction (45). Importantly, the AEGIS-I trial demonstrated that compared with placebo, CSL112 was associated with improved CEC (45). To determine if improving cholesterol efflux is associated with improved cardiovascular outcomes, the AEGIS-II trial (ApoA-I Event Reducing in Ischemic Syndromes II) is underway and will evaluate the efficacy and safety of CSL112 in reducing the risk of major adverse cardiovascular events in patients with acute myocardial infarction.

Limitations

This meta-analysis has several limitations that should be considered. First, the follow-up duration and case definitions for adverse cardiovascular events and ASCVD vary across the studies. Therefore, this analysis was unable to ascertain the association of CEC with specific components of the composite endpoints. Second, covariates included in the multivariable models (such as cardiovascular risk factors, medications, and the lipid panel) were not consistent and may impact the accuracy of the adjusted risk estimates. Third, only three endpoint comparisons had ten or fewer studies available. For this analysis, tests of small-study effect and subgroup analyses were performed. Few studies were available for examining the association with all-cause death and cardiovascular death; however, the lack of association with these endpoints may reflect a lack of statistical power. More data are warranted to validate the association of CEC with specific cardiovascular outcomes while accounting for individual risk profile and CEC method. Last, the cutoff value for defining high versus low CEC varied across the included studies. As this was a study-level meta-analysis based on aggregated data, a uniform cutoff value of CEC could not be applied to the analysis. Future patient-level meta-analysis is required to validate the findings.

CONCLUSION

The meta-analysis demonstrates an inverse relationship between CEC levels, a quantitative measure of HDL functionality, and the risk of adverse cardiovascular events or atherosclerotic cardiovascular disease. Future studies should examine whether CEC can serve as a therapeutic target for improving cardiovascular outcomes.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

JL and GC: concept and design, acquisition, analysis, or interpretation of data, and drafting of the manuscript. CF, SK, AK, SK, DD, AS, BK, RY, DB, and CG: critical revision of the

manuscript for important intellectual content. CG: supervision. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.774418/full#supplementary-material

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Optimal Low-Density Lipoprotein Cholesterol Levels in Adults Without Diabetes Mellitus: A Nationwide Population-Based Study Including More Than 4 Million Individuals From South Korea

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Background: Although the strong association between low-density lipoprotein cholesterol (LDL-C) and cardiovascular disease (CVD) is well-known, the threshold LDL-C level at which the risk of CVD begins to increase in individuals without diabetes mellitus (DM) remains unknown. We aimed to evaluate the association between incident CVD and serum LDL-C levels with or without statin use in individuals without DM.

Methods: We identified 4,182,117 individuals without previous CVD who underwent a health screening examination in 2009 and 2011 from the Korean National Health Insurance Cohort database. The primary endpoint was a composite of cardiovascular deaths, myocardial infarction (MI) cases, and ischemic stroke cases.

Results: During the median follow-up of 6 years, there were 51,961 CVD events that included 17,392 MI cases, 33,779 ischemic stroke cases, and 2,039 cardiovascular deaths. The LDL-C levels that were associated with an increased risk of CVD were ≥100 mg/dL in non-statin users and ≥130 mg/dL in statin users. However, even in individuals with lower LDL-C levels, all those with fasting plasma glucose (FPG) levels ≥110 mg/dL had a significantly higher risk of CVD.

Conclusions: We demonstrated that LDL-C levels ≥100 mg/dL were correlated with an increased risk of CVD in individuals without DM and a history of CVD. We found that a glucose, cholesterol interaction increased CVD risk, and modestly elevated FPG levels (110–125 mg/dL) were associated with a higher CVD risk even in individuals with well-controlled LDL-C levels.

Keywords: low-density lipoprotein cholesterol, impaired fasting glucose, cardiovascular disease, mortality, glucose level

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INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of mortality globally and accounted for 31.4% of all deaths in 2012. In developed countries, age-adjusted cardiovascular mortality rates are declining; however, CVD remains the leading cause of mortality due to the rapid aging of the population (1). Low-density lipoprotein cholesterol (LDL-C) is considered to be a major causative factor in the development of atherosclerotic CVD (ASCVD). Numerous studies have robustly demonstrated that reductions in plasma LDL-C concentrations by lipid-lowering agents are strongly associated with the reduced risk of incident CVD (2, 3). Most current guidelines include the LDL-C level as a primary indicator for initiating and adjusting lipid-lowering interventions. However, these guidelines were set based on data from randomized controlled trials that investigated specific LDL-C targets for adjustments in the statin dose or from a small group of highly selected studies. Few longitudinal large-scale epidemiological studies have investigated the optimal LDL-C range for the lowest risk of CVD, especially in individuals without a prior history of CVD and diabetes mellitus (DM).

Hyperglycemia and a high LDL-C level have been considered major risk factors for CVD (4). Patients with DM have a 2–4 times higher risk of CVD and cardiovascular mortality than the general population. Individuals with DM have a higher risk of the presence of small dense LDL particles than those without DM, even at identical plasma LDL-C levels. Therefore, the current guidelines regarding lipid management recommend the use of statins according to the LDL-C levels to prevent CVD in most patients with DM. However, considering the direct effect of high plasma glucose levels on CVD independent of LDL-C levels, optimal LDL-C goals for primary prevention may differ according to the glucose levels, even in individuals without DM. Nevertheless, trials that investigated the LDL-C target for the primary prevention of CVD have been performed among both patients with DM and individuals without DM (5), as well as among different ethnic groups (6). Also, these previous studies did not consider the possible synergistic effects between higher plasma glucose levels and higher LDL-C levels.

In this study, we aimed to identify the optimal LDL-C levels associated with the lowest risk of CVD incidence in individuals without DM. We also evaluated whether fasting plasma glucose (FPG) levels magnify the risk of CVD associated with LDL-C levels in individuals without DM. Moreover, we classified the study population into statin users and non-users to investigate the optimal LDL-C level for the primary prevention of CVD in each glycemic status classification. For this analysis, we used large-scale nationwide cohort data from the Korean National Health Insurance System (NHIS) database, which represents the entire Korean population.

METHODS

Study Population

In our cohort study, we used data from the NHIS, which is a government program that was implemented in 2002 and includes data on ∼98% of the Korean population. Participants are entitled to a general health screening program every 2 years. Standardized self-reported questionnaires on medical history, lifestyle habits, anthropometric and blood pressure measurements, and regular laboratory tests using blood and urine samples are all part of the screening (7). Our research-specific database included data from 2009 to 2011 for participants aged 19–69 years who underwent at least two general health screening programs during this period. To exclude participants who experienced myocardial infarction (MI) or ischemic stroke, those who had the following International Classification of Diseases, 10th Revision (ICD-10) codes (as the main diagnosis or sub-diagnosis at baseline) were not included: I21, I22, I23, I63, or I64. We excluded those who were aged <40 years in 2009 and did not participate in a general health screening program in 2009. Thus, 4,709,862 participants were eligible for this analysis (8). Participants who had missing variables regarding cholesterol and fasting glucose levels and were already diagnosed with DM (FPG level of ≥126 mg/dL or at least one claim for the prescription of hypoglycemic drugs per year, including insulin, under the ICD-10 codes E11–14) at baseline were excluded (9). We also excluded those who had a serum glucose level of 70 mg/dL and died before 2014 or those who died due to unknown causes. Finally, 4,182,117 participants were included at baseline (**Supplementary File 1**). This study was approved by the Institutional Review Board of Yonsei University Wonju College of Medicine, Republic of Korea (no. CR318356). As the study was conducted using anonymous and de-identified data, informed consent from the participants was not obtained.

Measurements and Definitions

The NHIS data included sex, age, body mass index (BMI), height, weight, waist circumference, blood pressure, and lifestyle-related behaviors, such as the frequency of alcohol consumption per week, smoking status, and regular exercise. Regular exercise was defined as performing more than 30 min of moderate physical activity at least five times per week or more than 20 min of strenuous physical activity at least three times per week. The income level was dichotomized at the lowest 25%. Blood samples for measuring serum glucose, creatinine, and lipid levels were drawn after an overnight fast. Blood samples for measuring total cholesterol, high-density lipoprotein cholesterol (HDL-C), triglyceride (TG), and FPG levels were obtained at the health examinations after the participants fasted for at least 8 h. LDL-C levels were calculated using the Friedewald formula: LDL-C = total cholesterol – HDL-C – (TG/5). Hypertension was defined as a systolic/diastolic blood pressure of ≥140/90 mm Hg or at least one claim for antihypertensive medication prescriptions per year under the ICD-10 codes I10–I15. We defined a statin user as a person who had been prescribed statins in 2009–2011.

Study Outcomes and Follow-Up

Participants who received two or more health screenings between 2009 and 2011 and were evaluated for primary outcomes during the follow-up period from 2014 to 2017 were included in the current study. During the follow-up period, the primary endpoint was cardiovascular events, which were described as a composite of incident cardiovascular deaths, MI cases, and ischemic stroke cases. To minimize the influence of possible "reverse

TABLE 1 | Baseline characteristics of participants according to LDL-C concentrations.

Characteristics	Overall	LDL-C (mg/dL)						*P for trend
		<70	70–99	100–129	130–159	160–189	≥ 190	
N	4,182,117	158,167	954,421	1,710,682	1,031,991	274,485	52,371	
Age (years)	51.1 (7.9)	50.6 (8.2)	50.2 (8)	50.9 (7.9)	51.9 (7.7)	52.6 (7.6)	53.0 (7.5)	<0.05
BMI (kg/m^2)	23.9 (2.8)	23.5 (3)	23.4 (2.9)	23.8 (2.8)	24.2 (2.8)	24.4 (2.7)	24.6 (2.8)	<0.05
Sex (male)	2,037,288 (48.7)	53,001 (33.5)	437,295 (45.8)	831,486 (48.6)	529,958 (51.4)	121,073 (44.1)	20,235 (38.6)	<0.05
Systolic BP (mmHg)	122.6 (12.5)	124.2 (13.2)	121.9 (12.7)	122.3 (12.4)	123.2 (12.3)	123.7 (12.3)	124.2 (12.7)	<0.05
Diastolic BP (mmHg)	76.7 (8.3)	77.8 (8.8)	76.3 (8.5)	76.6 (8.3)	77 (8.2)	77.3 (8.2)	77.5 (8.4)	<0.05
eGFR (ml/min/1.73 m^2)	85.4 (19.5)	87 (19.7)	86.7 (19.6)	85.5 (19.5)	84.3 (19.3)	83.6 (18.9)	83.3 (18.4)	<0.05
Fasting plasma glucose (mg/dl)	94.3 (9.7)	95.2 (10.6)	93.7 (9.8)	94.1 (9.6)	94.8 (9.6)	95.6 (9.7)	96.1 (9.9)	<0.05
Total cholesterol (mg/dl)	199.8 (31.7)	152.4 (25.6)	170.1 (18)	195.3 (16.3)	223.2 (15.6)	252.8 (15.7)	290.0 (26.0)	<0.05
HDL-C (mg/dl)	55.8 (17.7)	55.6 (19.8)	56.3 (17.8)	55.8 (17.5)	55.3 (17.5)	55.3 (17.9)	55.7 (20.1)	<0.05
Triglycerides (mg/dl)	132.8 [77.9]	194.7 [156.7]	132.1 [87.8]	126.8 [68.6]	131.7 [62.6]	138.5 (62.0)	148.5 (75.3)	<0.05
Smoking status (%)								<0.05
Never smoker	2,614,379 (62.5)	77,620 (49.1)	577,272 (60.5)	1,076,249 (62.9)	664,294 (64.4)	182,791 (66.6)	36,153 (69.0)	
Former smoker	676,404 (16.2)	27,934 (17.7)	156,145 (16.4)	283,181 (16.6)	164,072 (15.9)	38,842 (14.2)	6,230 (11.9)	
Current smoker	891,125 (21.3)	52,604 (33.3)	220,956 (23.2)	351,155 (20.5)	203,582 (19.7)	52,840 (19.3)	9,988 (19.1)	
Alcohol consumption								<0.05
≤ 2 days/week	3,609,708 (86.3)	114,502 (72.4)	794,956 (83.3)	1,485,657 (86.9)	918,232 (89)	248,541 (90.6)	47,820 (91.3)	
3–4 days/week	405,491 (9.7)	27,970 (17.7)	110,844 (11.6)	161,468 (9.4)	83,063 (8.1)	18,874 (6.9)	3,272 (6.3)	
≥ 5 days/week	166,669 (4.0)	15,684 (9.9)	48,568 (5.1)	63,444 (3.7)	30,641 (3)	7,056 (2.6)	1,276 (2.4)	
Income (lower 25%)	870,665 (21.1)	32,854 (21.0)	201,380 (21.3)	356,338 (21.1)	211,823 (20.8)	56,917 (21.0)	11,353 (21.9)	<0.05
Regular exercise (%)	1,434,239 (34.3)	53,243 (33.7)	325,431 (34.1)	591,656 (34.6)	354,645 (34.4)	92,432 (33.7)	16,832 (32.1)	<0.05
Hypertension (%)	1,474,782 (35.3)	67,978 (43)	337,057 (35.3)	594,109 (34.7)	362,237 (35.1)	94,897 (34.6)	18,504 (35.3)	<0.05
Medication for statin (%)	699,882 (16.7)	32,525 (20.6)	116,728 (12.2)	196,774 (11.5)	210,196 (20.4)	81,596 (29.7)	15,599 (29.8)	<0.05

Data are mean (standard deviation), median [interquartile range], or percentage.

BMI, body mass index; BP, blood pressure; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein cholesterol.

**The P for trend value represent overall differences across groups, as determined by one-way analysis of variance test for continuous variables and Pearson's chi-squared test for categorical variables.*

causation," we excluded participants with cardiovascular events that occurred within 3 years after baseline measurements. The ICD-10 codes were used for the diagnoses. MI was determined by recording the ICD-10 codes I21 or I22 at least twice during hospitalization for at least 4 days. Ischemic stroke was diagnosed based on the ICD-10 codes I63 or I64 that were registered during a 4-day hospital stay with claims for brain magnetic resonance imaging or brain computerized tomography (10). The Korea National Statistical Office provided nationwide death certificate data for the follow-up analyses of cardiovascular deaths. The research was deemed complete if the participants' cardiovascular events occurred or the end of the follow-up period, whichever occurred first.

Statistical Analysis

For each group, the continuous variables are presented as means and standard deviations, and the categorical variables are presented as frequencies and percentages. The participants were classified into six groups according to the following plasma LDL-C concentrations at baseline: <70, 70–99, 100–129, 130–159, 160–189, and ≥190 mg/dL. The hazard ratios (HRs) and 95% confidence intervals (CIs) for incident CVD according to the categories of LDL-C were obtained using multivariable Cox proportional hazard models using the 70–99 mg/dL category as the reference after adjusting for age, sex, BMI, smoking status, alcohol consumption, regular exercise, income, and hypertension. We also investigated the risk of CVD according to LDL-C categories within the FPG strata. We analyzed the data using SAS version 9.4 (SAS Institute, Inc., Cary, NC) and R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Baseline Characteristics of the Participants

The NHIS data of a total of 4,182,117 participants from 2009 to 2011 were analyzed. The mean age of the participants was 51.1 years and 2,037,288 (48.7%) participants were male. Overall, 16.7% of the participants used statins. **Table 1** summarizes the baseline characteristics of the cohort groups according to the baseline LDL-C concentrations (<70, 70–99, 100–129, 130–159, 160–189, and ≥190 mg/dL). The proportion of male participants gradually increased from the lowest to the highest LDL-C categories. Participants in the highest LDL-C category (LDL-C levels ≥190 mg/dL) tended to be older, have a higher BMI, and

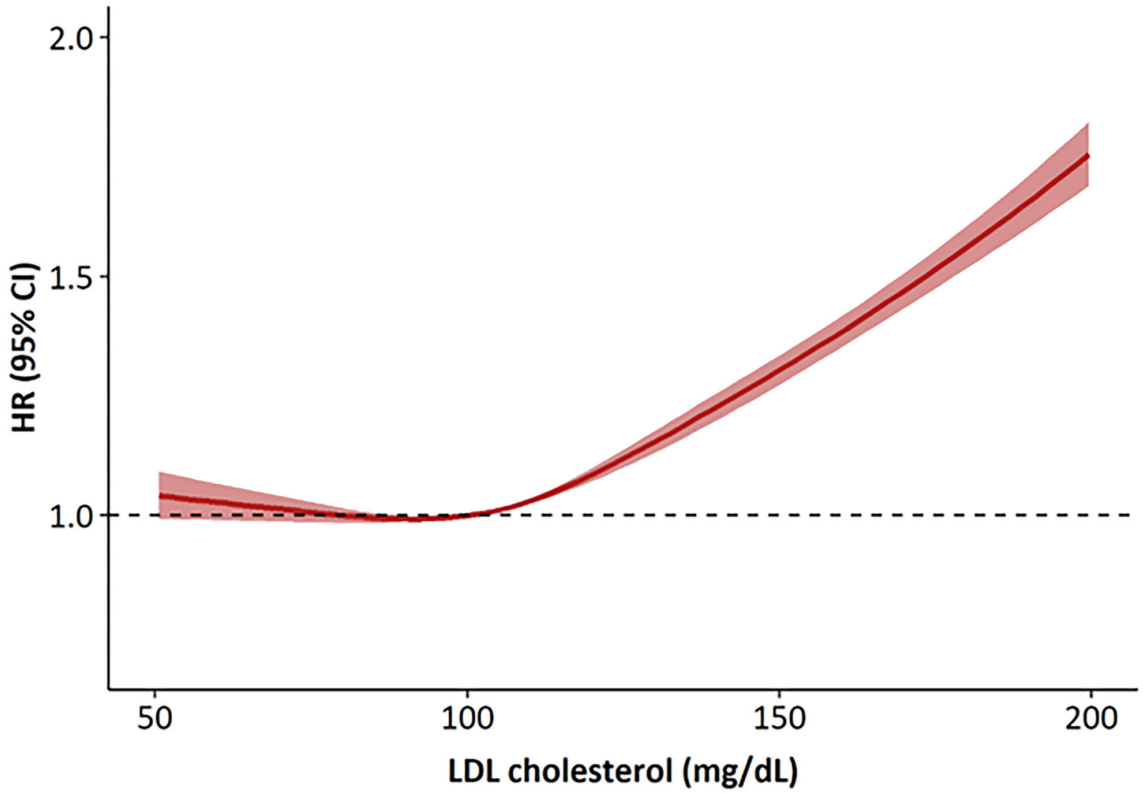

FIGURE 1 | Hazard ratios for cardiovascular disease according to LDL cholesterol levels at baseline. The hazard ratios were calculated by Cox models after adjusting for age, sex, body mass index, smoking status, alcohol consumption, regular exercise, income and the presence of hypertension. HR, hazard ratios.

were more likely to take statins than those in the other LDL-C categories. Patients with a low LDL-C level were more likely to be female, current smokers, and heavy drinkers. The HDL-C levels and blood pressure measurements were similar across the six LDL-C categories.

Risk of CVD According to the LDL-C Categories

During the median follow-up of about 6 years, there were 51,961 incident CVD events that included 17,392 cases of MI, 33,779 cases of ischemic stroke, and 2,039 cardiovascular deaths. There was a linear relationship between the LDL-C levels and CVD risk in the study population (**Figure 1**). The number of events, incidence, and HRs for CVD increased significantly in the higher LDL-C categories (**Table 2**). Using an LDL-C level of 70–99 mg/dL as the reference group, an LDL-C level of ≥100 mg/dL was associated with a significantly higher risk of CVD. In the multivariable analysis, the adjusted HRs (95% CIs) for CVD in the <70, 100–129, 130–159, 160–189, and ≥190 mg/dL LDL-C categories were 1.02 (0.97–1.07), 1.09 (1.07–1.12), 1.30 (1.26–1.33), 1.51 (1.46–1.56), and 2.01 (1.89–2.14), respectively, compared with the 70–99 mg/dL LDL-C category. An LDL-C level of ≥100 mg/dL was associated with a significantly greater risk of CVD in non-statin users. The risk of CVD increased linearly from an LDL-C level ≥130 mg/dL in statin users compared to non-statin users. When this association was stratified by the type of cardiovascular event, LDL-C levels ≥100 mg/dL in non-statin users and ≥130 mg/dL in statin users were significantly associated with a higher risk of MI (Additional File 2). LDL-C levels ≥100 mg/dL in non-statin users and ≥190 mg/dL in statin users were correlated with a significantly higher risk of ischemic stroke. LDL-C levels ≥130 mg/dL in non-statin users and ≥190 mg/dL in statin users were associated with a significantly higher risk of cardiovascular deaths.

Risk of CVD According to the LDL-C Categories Within the FPG Strata

We also analyzed the incidence values and HRs of CVD according to the LDL-C categories and stratified based on the FPG levels (70–90, 90–99, 100–109, and 110–125 mg/dL) (**Table 3** and **Figure 2**). In all the FPG categories, we observed that the risk of CVD gradually increased as the LDL-C levels increased. Using the FPG category 70–99 mg/dL and LDL-C category 70–99 mg/dL as references, the CVD risk in the higher LDL-C group increased with worsening glycemic status. Moreover, even in participants with lower LDL-C levels, all those with FPG levels ≥110 mg/dL had a significantly higher risk of CVD than participants in the reference group (**Table 3**). However, the higher risk of CVD in those with high LDL-C

TABLE 2 | Risk of primary outcome according to the baseline LDL-C levels excluding subjects who died within 2 years of follow-up.

Statin use	LDL-C (mg/dL)	Person-years	Number of events	Incident rate (10,000 person years)	Age-adjusted HR (95% CI)	Multivariable adjusted HR (95% CI) #
Overall	<70 mg/dL	1,278,498	2,079	16.26	1.1 (1.05–1.15)	1.02 (0.97–1.07)
	70–99 mg/dL	7,753,135	10,189	13.14	1.00 (Reference)	1.00 (Reference)
	100–129 mg/dL	13,934,274	19,674	14.12	1.07 (1.05–1.10)	1.09 (1.07–1.12)
	130–159 mg/dL	8,420,637	14,410	17.11	1.28 (1.25–1.31)	1.30 (1.26–1.33)
	160–189 mg/dL	2,241,436	4,467	19.93	1.50 (1.45–1.56)	1.51 (1.46–1.56)
	≥ 190 mg/dL	427,148	1,142	26.74	2.07 (1.95–2.20)	2.01 (1.89–2.14)
Statin non-user	<70 mg/dL	1,011,432	1,381	13.65	1.07 (1.01–1.14)	1.01 (0.95–1.07)
	70–99 mg/dL	6,788,255	7,876	11.60	1.00 (Reference)	1.00 (Reference)
	100–129 mg/dL	12,303,761	16,121	13.10	1.10 (1.08–1.13)	1.11 (1.08–1.15)
	130–159 mg/dL	6,679,456	10,924	16.35	1.36 (1.32–1.40)	1.37 (1.33–1.41)
	160–189 mg/dL	1,310,427	2,613	19.94	1.69 (1.62–1.77)	1.69 (1.61–1.76)
	≥190 mg/dL	170,941	505	29.54	2.58 (2.36–2.83)	2.47 (2.26–2.70)
Statin user	<70 mg/dL	267,066	698	26.14	1.03 (0.94–1.12)	0.99 (0.91–1.08)
	70–99 mg/dL	964,879	2,313	23.97	1.00 (Reference)	1.00 (Reference)
	100–129 mg/dL	1,630,513	3,553	21.79	0.99 (0.94–1.04)	1.02 (0.97–1.07)
	130–159 mg/dL	1,741,181	3,486	20.02	0.99 (0.94–1.04)	1.06 (1.00–1.11)
	160–189 mg/dL	931,009	1,854	19.91	1.04 (0.98–1.10)	1.16 (1.09–1.24)
	≥190 mg/dL	256,206	637	24.86	1.36 (1.25–1.49)	1.51 (1.38–1.66)

 # adjusted for age, sex, body mass index, smoking status, alcohol consumption, regular exercise, income and hypertension.
LDL-C, low lipoprotein cholesterol; HR, hazard ratios.

and high FPG levels was more attenuated in statin users than in non-statin users (**Figure 2**).

DISCUSSION

We evaluated the risk of incident CVD according to the LDL-C and FPG levels in participants without DM. We observed significant positive associations between the increased risk of CVD and high LDL-C levels in participants without DM, and the risk of CVD increased in participants without DM who had LDL-C levels ≥100 mg/dL. We demonstrated that elevations in the FPG levels, even in the same LDL-C category, were associated with an increased risk of CVD. The risk of CVD increased more significantly in participants with FPG levels ≥110 mg/dL than in those in the other FPG categories. These findings suggested that elevations in the FPG and LDL-C levels independently contributed to the increased risk of CVD in participants without DM. To the best of our knowledge, this was the first nationwide study to investigate the optimal ranges of LDL-C that were associated with the lowest risk of CVD in East Asian adults without DM.

Previous epidemiological investigations have consistently demonstrated a strong positive, continuous, independent, and graded relationship between LDL-C levels and the incidence of CVD (11, 12). Furthermore, recent meta-analyses of Mendelian randomization studies involving over 300,000 participants and 80,000 CVD cases provided convincing evidence regarding the causal correlation between LDL-C levels and the risk of ASCVD. Moreover, they showed that the causal impact of LDL-C levels on ASCVD might essentially be independent of the mechanism by which LDL-C levels are "lowered" (13). Therefore, most international guidelines suggest strategies for managing LDL-C levels and setting LDL-C targets for the primary prevention of CVD. These guidelines consider DM to be a major risk factor for CVD and recommend the use of statins, regardless of the LDL-C levels, for the prevention of CVD in patients with DM (14–16). However, the implementation of guidelines among members of the general population, especially among those without DM, has been a challenge for a long time. The American College of Cardiology/American Heart Association (ACC/AHA) guidelines removed specific target LDL-C levels since 2013 and emphasized a strategy of fixed-dose statin therapy based on cardiovascular risk in individuals without DM (14). However, calculating the ASCVD risk is challenging due to the lack of time and complexities in clinical calculations. Moreover, this ASCVD risk calculator was designed based on data mainly from populations other than those from East Asia. Considering the established strong graded relationship between LDL-C levels and incident CVD, determining the optimal LDL-C range for the primary prevention of CVD in individuals without DM is needed in the Asian population.

The present study found that among participants without DM and a history of CVD, there was an increased risk of CVD in those with LDL-C levels ≥100 mg/dL This cut-off value was in line with that recommended by several international guidelines. The 2019 European Society of Cardiology/European Atherosclerotic Society guidelines suggest an LDL-C goal of <116 mg/dL for the primary prevention of CVD in individuals with a low risk of CVD (16). However, guidelines for dyslipidemia management in Korea suggest an LDL-C goal of <130 mg/dL in individuals without

TABLE 3 | Risk of primary outcome according to the baseline LDL-C levels excluding subjects who died within 2 years of follow-up stratified by fasting glucose level at baseline.

Glucose (mg/dL)	LDL-C (mg/dL)	Person-years	Number of events	Incident rate (10,000 person years)	Age-adjusted HR (95% CI)	Multivariable adjusted HR (95% CI)#
70–90 mg/dL	<70 mg/dL	421783.9	568	13.47	1.20 (1.10–1.31)	1.05 (0.96–1.15)
	70–99 mg/dL	2891630.2	3,185	11.01	1.00 (Reference)	1.00 (Reference)
	100–129 mg/dL	4877874.2	5,948	12.19	1.03 (0.99–1.08)	1.06 (1.02–1.11)
	130–159 mg/dL	2,664,123	4,067	15.27	1.19 (1.14–1.25)	1.23 (1.18–1.29)
	160–189 mg/dL	647856.6	1,165	17.98	1.33 (1.25–1.43)	1.40 (1.31–1.50)
	≥ 190 mg/dL	117293.0	310	26.43	1.94 (1.73–2.18)	2.03 (1.81–2.29)
90–99 mg/dL	<70 mg/dL	469637.3	716	15.25	1.23 (1.14–1.34)	0.99 (0.91–1.07)
	70–99 mg/dL	3000266.7	3,759	12.53	1.04 (1.00–1.09)	0.96 (0.92–1.01)
	100–129 mg/dL	5609484.6	7,685	13.70	1.09 (1.05–1.14)	1.06 (1.02–1.10)
	130–159 mg/dL	3461224.7	5,689	16.44	1.24 (1.19–1.29)	1.23 (1.18–1.29)
	160–189 mg/dL	921301.7	1,806	19.60	1.41 (1.34–1.50)	1.45 (1.37–1.54)
	≥ 190 mg/dL	172577.0	457	26.48	1.85 (1.68–2.04)	1.92 (1.74–2.12)
100–109 mg/dL	<70 mg/dL	257539.9	484	18.79	1.42 (1.29–1.56)	1.05 (0.95–1.15)
	70–99 mg/dL	1328820.6	2,189	16.47	1.26 (1.20–1.33)	1.05 (1.00–1.11)
	100–129 mg/dL	2503745.5	4,074	16.27	1.23 (1.17–1.29)	1.09 (1.04–1.14)
	130–159 mg/dL	1656633.4	3,134	18.92	1.38 (1.31–1.45)	1.27 (1.21–1.34)
	160–189 mg/dL	478705.6	1,004	20.97	1.48 (1.38–1.59)	1.43 (1.33–1.53)
	≥ 190 mg/dL	95308.2	244	25.60	1.77 (1.56–2.02)	1.69 (1.48–1.93)
110–125 mg/dL	<70 mg/dL	129537.5	311	24.01	1.73 (1.54–1.94)	1.17 (1.04–1.32)
	70–99 mg/dL	532416.8	1,056	19.83	1.43 (1.33–1.53)	1.09 (1.02–1.17)
	100–129 mg/dL	943169.8	1,967	20.86	1.49 (1.41–1.58)	1.22 (1.15–1.29)
	130–159 mg/dL	638655.6	1,520	23.80	1.68 (1.58–1.79)	1.44 (1.36–1.54)
	160–189 mg/dL	193572.2	492	25.42	1.75 (1.59–1.93)	1.58 (1.44–1.74)
	≥ 190 mg/dL	41969.3	131	31.21	2.10 (1.77–2.51)	1.95 (1.63–2.32)

 # adjusted for age, sex, body mass index, smoking status, alcohol consumption, regular exercise, income and the presence of hypertension.
LDL-C, low lipoprotein cholesterol; HR, hazard ratios.

DM and a moderate CVD risk, and <160 mg/dL in individuals without DM and a low CVD risk (15). The suggested target LDL-C levels according to the Korean guidelines in individuals without DM were significantly higher than those in our study (LDL-C levels <100 mg/dL). However, the Korean dyslipidemia guidelines were made based on data from other countries. Further prospective randomized controlled studies are warranted to determine the optimal LDL-C levels for the initiation of pharmacological intervention for the primary prevention of CVD in Korean adults without DM.

In the current study, we observed a graded positive trend for CVD risk starting from an LDL-C level of 100 mg/dL, which increased for the higher LDL-C categories among participants who were non-statin users. The risk of CVD was higher whose LDL-C levels were ≥130 mg/dL in statin users than in those in the LDL-C reference group. From these results, we suggest that the uptitration of statins may be considered if LDL-C levels are ≥130 mg/dL during statin treatment, with consideration of the CVD risk in individuals without DM. Furthermore, the risk of CVD was relatively more attenuated in statin users than in non-statin users, even in the same LDL-C categories. The attenuated CVD risk in statin users was prominent in those in the higher LDL-C and FPG categories. It demonstrated the benefit of using statin for the primary prevention of CVD, regardless of the LDL-C levels in individuals without DM. This finding was consistent with those of previous primary prevention trials that demonstrated the benefits of statin therapy (17). These studies showed that statins can reduce CVD risk through pleiotropic effects, including the inhibition of inflammation (6, 18).

We found that modestly elevated FPG levels (110–125 mg/dL), even it is not suitable level for diagnosing diabetes, were independently associated with a higher risk of CVD compared to the reference groups (FPG level of 70–99 mg/dL and LDL-C level of 70–99 mg/dL). A higher risk of CVD in participants with modestly elevated FPG levels was still observed even when their LDL-C levels were low (70 mg/dL). This suggested that well-controlled LDL-C levels might not be protective against a higher CVD risk in individuals with modestly elevated FPG levels. Moreover, we observed that the combination of higher FPG and LDL-C levels synergistically elevated the risk of CVD in participants without DM. This finding was consistent with the biological synergistic interaction between glucose and cholesterol levels reported in previous studies (19, 20). There are some possible biological mechanisms that support the interaction between glucose and LDL-C. It is widely believed that the oxidation of LDL-C plays an important role in atherogenesis,

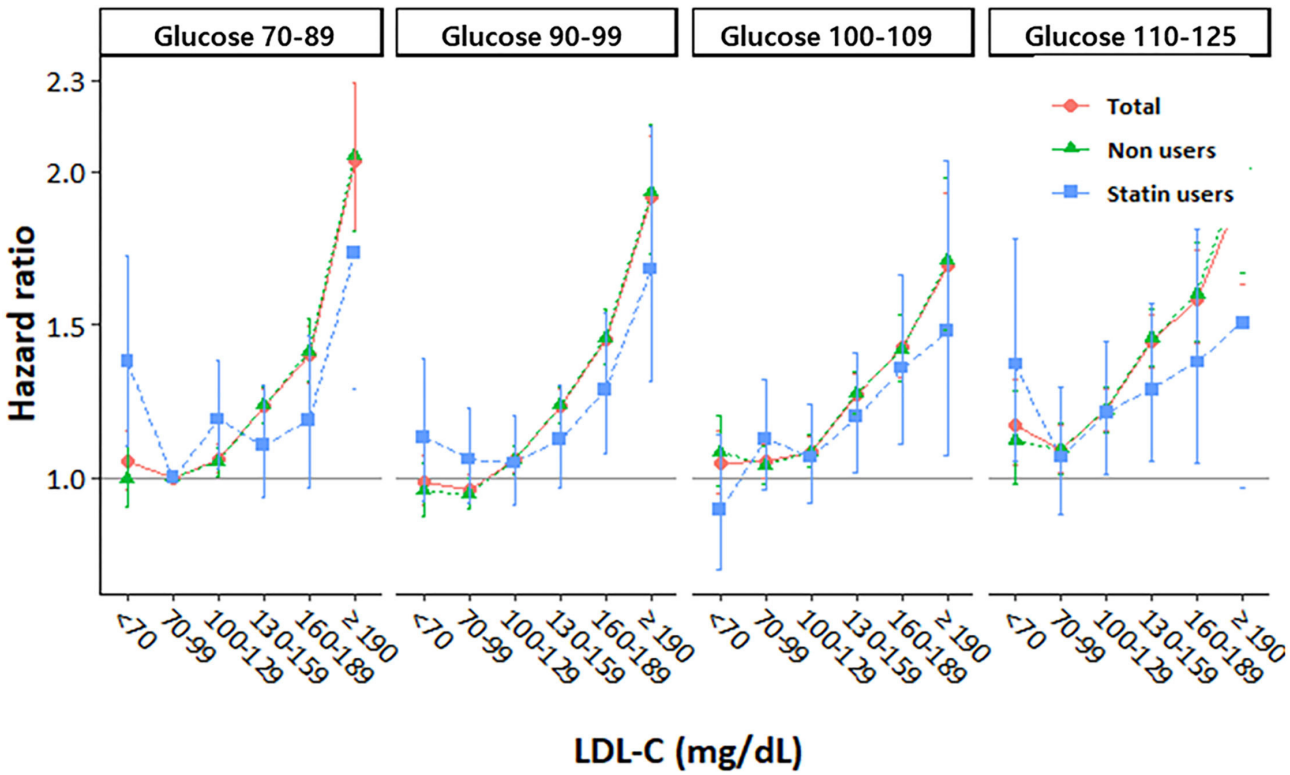

FIGURE 2 | Hazard ratios for cardiovascular disease according to LDL cholesterol levels stratified by fasting glucose level at baseline.

and excess circulating glucose levels might facilitate cholesterol peroxidation (21). It was found that DM might be related to oxidative stress, which is linked to atherogenesis (22–24). This biological interaction indicates that optimal LDL-C goals might differ according to glucose levels, which might be clinically significant. Our findings warrant a clinical trial to determine whether using glucose levels to advise about cholesterol control would improve outcomes. Our findings indicated that the target LDL-C goal for the primary prevention of CVD should be lower, and more aggressive statin use may be considered in individuals with FPG levels of 110–125 mg/dL, similar to those with DM.

Our study had several limitations. First, as the NHIS database relies on the issuance of a diagnostic code for CVD by physicians, there might have been a risk of misdiagnosis, which might have contributed to the underestimation or overestimation of the prevalence of CVD. Second, day-to-day variabilities might have influenced the findings due to laboratory errors or biological variations, as we used the results of a single LDL-C and FPG test in the analyses. Additionally, as we could not directly measure LDL-C levels and used the Friedewald formula instead, it might have led to the underestimation of the LDL-C levels. Third, there was lack of data on antidyslipidemic medication use among our participants during the follow-up period. Over time, cholesterol levels can increase, which could have led to statin use even among non-users, thereby mitigating the observed risk of CVD. We did not perform time-varying Cox regression considering these factors and this is limitation of our study. Fourth, we did not obtain data on changes in medications or interventions during the follow-up period. Furthermore, we did not account for many confounders in our study, such as genetic factors, medication use, and socioeconomic status, which might have influenced our results. Fifth, we could not calculate the 10 year risk of ASCVD due to lack of data and consider individual cardiovascular risks in our analysis. Finally, as the present study only included the Korean population, our findings could not be generalized to other ethnicities. However, the major strengths of the current study were its large sample size, with approximately 4,000,000 relatively healthy general populations, and use of longitudinal data. Thus, our results reflect "real-world" evidence on the association of LDL-C levels with CVD risk in individuals without DM on a national scale. Finally, we used only fasting glucose level as a surrogate marker for glycemic status because HbA1c measurement was not included the national health screening program in our country during the study period.

CONCLUSIONS

In conclusion, we demonstrated that LDL-C levels ≥100 mg/dL increased the risk of CVD in individuals without DM. Furthermore, we found that a glucose–cholesterol interaction magnified the CVD risk even in those without DM, and the CVD risk was more attenuated in statin users than in non-statin users.

We observed a graded positive trend in CVD risk starting from an LDL-C level of 100 mg/dL, and this risk increased in the higher LDL-C categories. Thus, more active initiation of statin treatment for the primary prevention of CVD in Korean adults without DM can be considered when their LDL-C levels are ≥100 mg/dL. Moreover, given that participants with modestly elevated FPG levels (especially FPG levels ≥110 mg/dL) had a high risk of CVD after adjusting for confounders, even those with lower LDL-C levels, earlier initiation of statin treatment for the primary prevention of CVD may be considered for these participants. Further large-scale, long-term, follow-up randomized control studies are warranted to clearly determine the optimal LDL-C target for the primary prevention of CVD in individuals without DM.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the datasets generated and analyzed during the current study are not publicly available due to rule of Korea National health insurance system. Requests to access the datasets should be directed to kimjang713@gmail.com.

ETHICS STATEMENT

This study was approved by Institutional Review Board of Yonsei University Wonju College of Medicine, Republic of Korea (no. CR318356). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JH and SP conceived the study concept and design. T-HG and DK acquired data and performed statistical analyses. JH and SP wrote the first draft and conducted the literature search. JH, S-HL, and J-YK analyzed and interpreted data. J-YK is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data, and the accuracy of the data analysis. All authors contributed to critical revision of the manuscript, read, and approved the final submitted version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.812416/full#supplementary-material

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High Plasma Concentration of Apolipoprotein C-III Confers an Increased Risk of Cerebral Ischemic Events on Cardiovascular Patients Anticoagulated With Warfarin

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Introduction: Apolipoprotein C-III (Apo CIII) is a crucial regulator of triglyceride-rich lipoproteins (TRLs) and influences the risk of cardiovascular diseases. High levels of Apo CIII have been also associated with cerebrovascular events and earlier works showed procoagulant effects of Apo CIII. The main aim was to assess whether the plasma concentration of Apo CIII could confer an increased risk of cerebral ischemic events in anticoagulated patients at high-risk of cardioembolism.

Methods: We systematically checked medical records and quantified cerebral ischemic events in a selected cohort of 118 subjects [median age 68 with interquartile range (IQR) 59–75 years, 66.9% males, 52.5% with coronary artery disease (CAD)], taking anticoagulant therapy with warfarin because of atrial fibrillation (AF) and/or mechanical prosthetic heart valves. All the subjects, enrolled between May 1999 and December 2006, were prospectively followed until death or July 31, 2018. Assessments of complete plasma lipid and apolipoprotein profiles, including Apo A-I, B, CIII, and E, were available for all subjects at enrollment.

Results: After a median follow-up of 109 months (IQR, 58–187), 24 subjects (20.3%) had cerebral ischemic events: stroke ($n = 15$) and TIA ($n = 9$). Subjects with plasma concentration of Apo CIII above the median value (10.3 mg/dL) had an about three-fold increased risk of stroke/TIA than those with lower levels of Apo C-III [hazard ratio 3.08 (95%CI, 1.22–7.77)]. This result was confirmed in multiple Cox regression models adjusted for gender, age, CAD, AF, diabetes, hypertension, plasma lipids, and CHA_2DS_2-VASc score. By stratifying the sample on the basis of Apo CIII level and CHA_2DS_2-VASc score, an additive effect was observed with the highest risk in subjects with both high Apo C-III concentration and CHA_2DS_2-VASc score.

Conclusion: High Apo CIII plasma levels may be associated with an increased risk of ischemic stroke/TIA in high-risk cardiovascular patients anticoagulated with warfarin.

Keywords: apolipoprotein C-III, cerebral ischemic events, anticoagulant therapy, warfarin, atrial fibrillation

INTRODUCTION

Stroke is not only a major cause of death, but it is burdened by high morbidity with up to 50% of survivors being chronically disabled, thus having enormous health and social-economic consequences (1). Ischemic strokes account for the great majority of all types of strokes. The analysis of secular trends in ischemic stroke subtypes demonstrates that the proportion of cardioembolic stroke increased in recent years more extensively as compared with large artery stroke and small vessel stroke (2), probably as a consequence of a more intensive management of atherogenic risk factors. Notably, cardioembolic clots from heart valves or chambers are not only one of the most frequent but also a potentially preventable mechanism (3–5). Atrial fibrillation (AF) and mechanical prosthetic heart valves, as major determinants of embolic strokes, require appropriate anticoagulant prophylaxis, that is currently mandatory in the management of the patients affected by these conditions, the former if associated with an increased risk profile defined by clinical prediction rule like CHA_2DS_2-VASc score (3–5).

Despite being treated with anticoagulants, a substantial number of these patients still develop cardioembolic complications and are exposed to the risk of ischemic stroke and/or transient ischemic attack (TIA) (6, 7). Notably, although several factors (e.g., older age, female sex, previous stroke/TIA, previous aspirin use, high prediction rule scores, and renal impairment) have been statistically associated with such risk (8), little is known regarding the precise mechanisms involved in such residual risk and able to breach the anticoagulant protection. In this context, we recently published a prospective study in a cardiovascular cohort of 950 subjects with or without coronary artery disease (CAD) showing that high apolipoprotein CIII (Apo CIII) plasma concentration may be associated with an increased risk of ischemic stroke or transient ischemic attack (TIA) (9). The effect of Apo CIII on cerebrovascular risk appeared stronger in subjects without signs of relevant atherosclerotic disease but with valvular heart disease (9), including patients with mechanical prosthetic heart valves and/or atrial fibrillation, i.e., well recognized conditions at risk of cardioembolic stroke. Such observation prompted us to consider that Apo CIII may influence the risk of cerebrovascular events beyond atherosclerosis-related pathways. Notably, in previous works our group also showed that high Apo CIII plasma concentration is associated with a prothrombotic diathesis, characterized by enhanced thrombin generation (10), increased factor II coagulant activity (11), and higher activated factor VII-antithrombin complex (FVIIa-AT) (12).

TABLE 1 | Clinical and laboratory characteristics of the study sample at time of enrollment considered as a whole and subdivided on the basis of the occurrence of non-fatal ischemic stroke or TIA events during the follow-up.

	All the subjects (n = 118)	**No Stroke/TIA (n =94)**	**Stroke/TIA (n = 24)**
Age (years)	68 (59–75)	68 (59–75)	68 (60–76)
Male gender (n, %)	79, 66.95	65, 69.15	14, 58.33
BMI (kg/m²)	26.45 (23.23–29.88)	26.63 (23.14–29.74)	25.89 (23.37–29.95)
Coronary artery disease (n, %)	62, 52.54	50, 53.19	12, 50.00
Previous stroke/TIA (n, %)	8, 6.78	6, 6.38	2, 8.33
Congestive heart failure (n, %)	10, 8.47	10, 10.63	0, 0.00
Atrial fibrillation (n, %)	62, 52.54	50, 53.19	12, 50.00
Diabetes (n, %)	25, 21.18	20, 21.28	5, 20.83
Hypertension (n, %)	66, 55.93	51,54.26	15, 62.50
Smoke habit (n,%)	50, 49.15	48, 51.06	10, 41.67
CHA_2DS_2-VASc score (n, %)			
0	18, 15.25	16, 17.02	2, 8.33
1	26, 22.03	21, 22.34	5, 20.83
≥2	74, 62.71	57, 60.64	17, 70.83
Laboratory parameters			
Creatinine (μmol/L)	90.00 (77.70–105.03)	91.95 (79.28–105.10)	82.15 (75.72–94.21)
Total Cholesterol (mmol/L)	4.88 (4.02–5.89)	4.75 (3.98–5.72)	5.08 (4.37–5.97)
LDL Cholesterol (mmol/L)	3.15 (2.64–4.00)	3.17 (2.59–4.09)	3.12 (2.66–4.01)
HDL Cholesterol (mmol/L)	1.24 (1.04–1.46)	1.26 (1.05–1.49)	1.15 (1.01–1.38)
Triglyceride (mmol/L)	1.50 (1.10–1.98)	1.41 (1.09–1.98)	1.60 (1.13–2.03)
Apo AI (g/L)	1.32 (1.14–1.50)	1.33 (1.13–1.50)	1.27 (1.19–1.54)
Apo B (g/L)	0.95 (0.78–1.18)	0.94 (0.77–1.14)	1.09 (0.87–1.24)
Apo CIII (mg/dL)	10.30 (9.00–12.34)	9.89 (8.86–12.12)	11.51 (10.24–14.52)
Apo E (g/L)	0.036 (0.029–0.046)	0.037 (0.031–0.047)	0.034 (0.026–0.044)

Distributions of continuous variables were expressed as median value with interquartile range (IQR). Categorical variables were expressed as proportions.

On the basis of these premises, we hypothesized that high Apo CIII levels leading to a prothrombotic diathesis could be associated with an increased risk of ischemic stroke/TIA in the specific clinical setting of patients taking anticoagulant therapies. Therefore, in order to investigate the factors potentially contributing to cerebrovascular risk despite anticoagulation, we selected from the previous study cohort (9) the subgroup of patients who were treated with warfarin for either atrial fibrillation (AF) or mechanical prosthetic heart valves (MPHV), which are well-recognized clinical conditions at high-risk of cardioembolic events. In particular, we reassessed whether the plasma concentration of Apo CIII could still contribute to the mechanisms leading to cerebral ischemic events in cardiovascular patients taking anticoagulant therapy with warfarin.

MATERIALS AND METHODS

Study Population

A detailed description of the original study population has already been reported in a previous work (9). This observational study was performed within the framework of the Verona Heart Study (VHS), a regional survey that assessed cardiovascular risk factors in subjects with angiographic documentation of the state of their coronary vessels (9–12). All the subjects in the VHS are required to have no history of any acute illness in the month preceding the enrollment. CAD patients with acute coronary syndromes were excluded from this study. Subjects with severe renal failure (estimated glomerular filtration rate <30 mL/min) and those with severe hepatic impairment (clinically defined diagnosis of liver cirrhosis) were also excluded from this study. Briefly, in the original analysis (9) a total of 950 subjects enrolled between May 1999 and December 2006 with angiographic documentation of their coronary vessels, who lived in the District of Verona and for whom both prospective data on cerebrovascular events and laboratory data on baseline plasma lipids were available, were included in the original analysis. Among those patients we selected all the 118 subjects who were taking anticoagulant therapy with warfarin, because of AF and/or MPHV ($n = 62$ and $n = 56$, respectively), i.e., clinical conditions at high-risk of cardioembolic events. All subjects had a complete routinary laboratory evaluation, including Apo A-I, Apo B, Apo CIII and Apo E plasma concentrations (for analytical methods, see ref. (9)). CHA_2DS_2-VASc score was calculated according to the ACC/AHA/HRS 2019 guidelines (13).

Assessment of Outcome

The subjects were followed until death or July 31, 2018. Follow-up data were available for all the subjects in the sample of the present analysis. Study subjects' mortality status was determined by searching in the National Population Register. Survival times were calculated starting from the date of enrolment. The electronic medical records of all the Hospitals in the District of Verona, Northeast Italy, including data of Emergency Units admissions were obtained for all subjects; ambulatory or telephone survey was performed in case of clinical doubt. In order to be included in the statistical computation, non-fatal ischemic stroke/TIA events had to be confirmed and validated by two independent neurologists after careful evaluation of all the available information (9). Ischemic stroke was defined as a focal neurological deficit with acute onset and presence of a corresponding lesion on diffusion weighted magnetic resonance imaging [DWI] or, if no MRI was acquired, signs of early ischemic injury on CT (14). TIA was defined as an acute onset focal neurological deficit of presumed ischemic origin without a corresponding lesion on DWI or, if no MRI was acquired, lasting <24 h (15). According to the Trial Org 10172 in Acute Stroke Treatment (TOAST) classification, etiologic ischemic stroke/TIA subtypes were the following: (1) large-artery atherosclerosis, (2) cardioembolism, (3) small-vessel occlusion, (4) stroke of other determined etiology, and (5) stroke of undetermined etiology (15). Diagnoses are based on clinical features and on data collected by tests such as brain imaging (CT/MRI), cardiac imaging (echocardiography, etc.), duplex imaging of extracranial arteries, arteriography, and laboratory assessments for a prothrombotic state.

TABLE 2 | Clinical and laboratory characteristics of the study sample at time of enrollment subdivided according to Apo CIII plasma concentration with the median value (10.3 mg/dL) as threshold level.

	Apo CIII < 10.3 mg/dL ($n = 59$)	Apo CIII ≥ 10.3 mg/dL ($n = 59$)
Age (years)	67 (60–76)	69 (59–74)
Male gender (*n*, %)	47, 79.66	32, 54.24
BMI (kg/m²)	27.16 (23.23–28.98)	25.48 (23.19–30.15)
Coronary artery disease (*n*, %)	29, 49.15	33, 55.93
Previous stroke/TIA (*n*, %)	4, 6.78	4, 6.78
Congestive heart failure (*n*, %)	6, 10.17	4, 6.78
Atrial fibrillation (*n*, %)	29, 49.15	33, 55.93
Diabetes (*n*, %)	8, 13.5	17, 28.81
Hypertension (*n*, %)	27, 45.76	39, 66.10
Smoke habit (*n*, %)	26, 44.07	32, 54.24
CHA_2DS_2-VASc (*n*, %)		
0	15, 25.42	3, 5.08
1	11,18.64	15, 25.42
≥2	33, 55.93	41, 69.49
Laboratory parameters		
Creatinine (μmol/L)	92.40 (79.73–105.10)	86.30 (76.44–104.25)
Total Cholesterol (mmol/L)	4.47 (3.80–5.14)	5.40 (4.43–6.33)
LDL Cholesterol (mmol/L)	2.96 (2.34–3.62)	3.59 (2.76–4.35)
HDL Cholesterol (mmol/L)	1.20 (1.04–1.34)	1.26 (1.04–1.59)
Triglyceride (mmol/L)	1.13 (0.95–1.49)	1.89 (1.46–2.37)
Apo AI (g/L)	1.20 (1.02–1.42)	1.42 (1.23–1.58)
Apo B (g/L)	0.86 (0.71–1.04)	1.09 (0.94–1.32)
Apo CIII (mg/dL)	9.02 (7.92–9.68)	12.30 (11.41–15.76)
Apo E (g/L)	0.034 (0.029–0.040)	0.039 (0.032–0.049)
Follow-up events		
Ischemic Stroke/TIA (*n*, %)	6, 10.17	18, 30.51

Distributions of continuous variables were expressed as median value with interquartile range (IQR). Categorical variables were expressed as proportions.

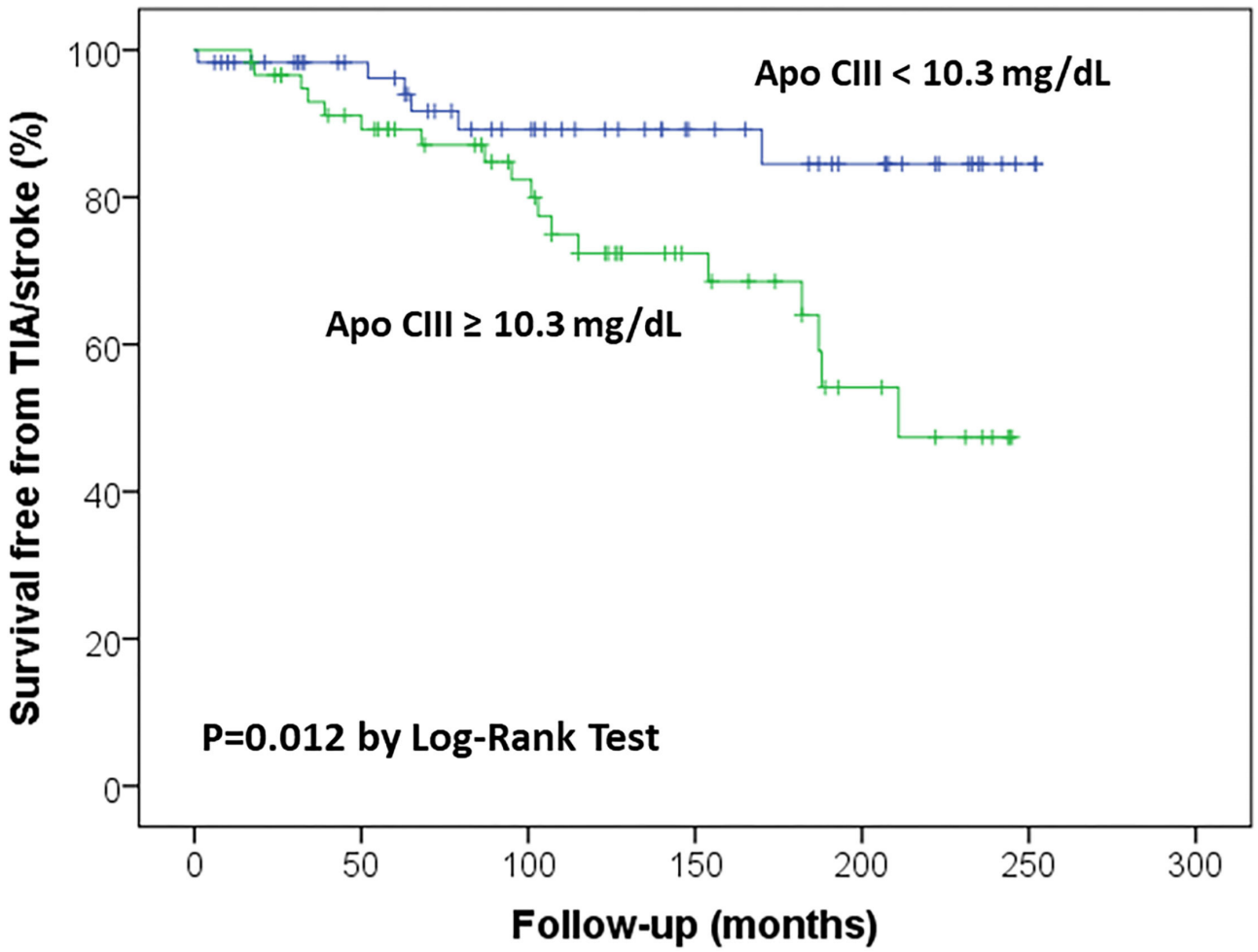

FIGURE 1 | Kaplan-Meier survival curves for ischemic stroke/transient ischemic attack (TIA) in the study sample according to Apolipoprotein C-III (Apo CIII) plasma concentration, with the median level (10.3 mg/dL) as threshold value.

Statistical Analysis

Statistical analyses were performed using the SPSS 23.0 (SPSS Inc., Chicago, IL, USA) and STATA 13.0 (StataCorp, College Station, TX, USA) statistical packages. Distributions of continuous variables were expressed as median value with interquartile range (IQR). Categorical variables were expressed as proportions. Quantitative data distributions were assessed using Mann-Whitney test and the comparisons were presented also as median difference with 95% confidence interval (CI). Qualitative data were analyzed by χ^2 test or χ^2 for linear trend analysis when indicated. Ischemic stroke/TIA event rates during the follow-up period were assessed by using the Kaplan–Meier method with Log-rank statistic and Cox regression. Kaplan–Meier curves were used for survival plots, which stratified the study population according to Apo CIII plasma concentration. Taking into account the well-recognized role of CHA_2DS_2-VASc score in predicting the risk of cardioembolic events [e.g., it is a codified clinical prediction rule for estimating the risk of stroke in subjects with AF (13)], we stratified the whole study sample by Kaplan-Meier curves for ischemic stroke/TIA events according to both Apo CIII levels and CHA_2DS_2-VASc score. Multivariate Cox proportional models for ischemic stroke/TIA events were performed considering the Apo CIII median value (10.3 mg/dL) as threshold and including in the different models potential confounding factors, like sex, age, CAD diagnosis, atrial fibrillation, diabetes, hypertension, all plasma lipid parameters, and CHA_2DS_2-VASc score. Considering the high number of variables in this analysis, a final model with backward stepwise selection of variables was performed with $P > 0.10$ as the critical value for excluding variables in the model. Subjects with missing data were excluded from multivariate analysis in Cox regression models. Hazard ratios (HRs) and 95% CIs are reported with two-tailed probability values. A value of $P < 0.05$ was considered statistically significant.

RESULT

After a median follow-up of 109 months (IQR, 58–187), 24 subjects (20.3%) had non-fatal ischemic stroke ($n = 15$) or TIA ($n = 9$) events despite the reported anticoagulant

treatment. In 23 out of 24 subjects the cerebral ischemic events were classified as cardioembolic. Clinical and laboratory characteristics of the study population, as a whole and divided on the basis of ischemic stroke/TIA events during the follow-up, are summarized in **Table 1** and **Supplementary Table S1**. Subjects with cerebral ischemic events had generally, although without statistical significance, higher plasma concentrations of both Apo B and Apo CIII (**Table 1** and **Supplementary Table S1**). On the basis of the previous results in the original VHS cohort, suggesting Apo CIII levels as a predictor of cerebral ischemic events (9), we compared subjects with Apo CIII plasma concentrations below and above the median level (10.30 mg/dL), whose clinical and biochemical features are reported in **Table 2** and **Supplementary Table S2**. Subjects with high Apo CIII levels had an increased rate of ischemic stroke/TIA during the follow-up [30.51% (18/59) vs. 10.17% (6/59), as compared with subjects with low Apo CIII levels, $P = 0.006$]. As expected, subjects with high Apo CIII levels had an unfavorable plasma lipid profile, with increased concentrations of both total and LDL cholesterol, triglycerides, Apo B and Apo E (**Table 2** and **Supplementary Table S2**). Subjects with high Apo CIII levels had also an increased prevalence of diabetes and hypertension. When clinical history elements were assessed by CHA_2DS_2-VASc score, patients with higher Apo CIII had more frequently an increased score ≥ 2 (**Table 2**).

Kaplan-Meier survival curves confirmed that subjects with high Apo CIII levels ($\geq$10.30 mg/dL) had an increased rate of ischemic stroke/TIA (**Figure 1**), with an about three-fold increased risk by Cox regression analysis as compared with those with low Apo CIII levels (**Table 3**). High Apo CIII levels remained associated with ischemic stroke/TIA by including progressively potential confounding factors in the regression models, like age, sex, CAD, AF, diabetes, hypertension, plasma lipids and apolipoproteins, and CHA_2DS_2-VASc score (**Table 3**), and even including all these parameters into a Cox regression model with backward stepwise selection of variables (HR 5.22 with 95%CI 1.43–19.01).

Considering the observed relation between Apo CIII levels and CHA_2DS_2-VASc score, which was associated with ischemic stroke/TIA in the study sample (**Figure 2**), a possible synergic additive effect was hypothesized. Kaplan-Meier curves were used to further dissect their association with cerebral ischemic events. We stratified the study sample in four subgroups according to the different combination of these two variables: low/high Apo CIII (threshold level at median value 10.3 mg/dL) and low/high CHA_2DS_2-VASc score (threshold level 2 or more points, which connote moderate to high-risk according to guidelines (13)). By this analysis, subjects with both high Apo CIII and CHA_2DS_2-VASc score had the highest risk of ischemic stroke/TIA as compared with those with both low Apo CIII and CHA_2DS_2-VASc score who had the lowest risk of cerebral ischemic events, while subjects with either low Apo CIII/high score or high Apo CIII/low score showed an intermediate risk (**Figure 3**).

TABLE 3 | Association between high plasma concentration of Apo CIII ($\geq$10.3 mg/dL), and ischemic stroke/TIA events by different Cox Regression models (subjects with low plasma concentration of Apo CIII, <10.3 mg/dL, are considered as reference group).

	Coefficient B	SE	Hazard ratio
Unadjusted	1.12	0.47	3.08 (1.22–7.77)
Model 1	1.09	0.49	2.97 (1.14–7.74)
Model 2	1.05	0.49	2.86 (1.10–7.53)
Model 3	0.99	0.49	2.70 (1.03–7.13)
Model 4	1.32	0.57	3.76 (1.24–11.42)
Model 5	1.32	0.58	3.73 (1.20–11.61)

Model 1: sex- and age-adjusted.
Model 2: adjusted for sex, age, CAD diagnosis, and atrial fibrillation.
Model 3: adjusted for sex, age, CAD diagnosis, atrial fibrillation, diabetes, and hypertension.
Model 4: adjusted for sex, age, CAD diagnosis, atrial fibrillation, diabetes, hypertension, and all plasma lipid parameters (i.e., total, LDL, and HDL-cholesterol, triglycerides, Apo AI, Apo B, and Apo E).
Model 5: adjusted for sex, age, CAD diagnosis, atrial fibrillation, diabetes, hypertension, all plasma lipid parameters (i.e., total, LDL, and HDL-cholesterol, triglycerides, Apo AI, Apo B, and Apo E), and CHA_2DS_2-VASc score.

DISCUSSION

As a necessary preliminary consideration, we must recognize that the current results are the by-product of a larger analysis showing that Apo CIII levels may confer an increased risk of ischemic cerebrovascular events in cardiovascular patients (8). This study was not originally designed to investigate factors influencing the residual risk of ischemic stroke in high-risk patients treated with anticoagulants. The sample size is small with consequent reduction of study power. All these issues may reduce the potential significance of our findings. Nonetheless, taking into account the proofs indicating a procoagulant effect of Apo CIII (10–12), we hypothesized that its high plasma concentration may be particularly harmful in clinical conditions requiring anticoagulation. Therefore, according to the available data, we selected a subgroup of 118 anticoagulated patients and attempted to assess whether Apo CIII could play a prognostic role in this specific clinical setting.

The findings showed that was just the case. With the limitations due to the above-mentioned premise, the present results are the first to provide evidence suggesting such a role for Apo CIII, with its high levels being associated with an about three-fold increased risk of ischemic cerebrovascular events during an about 10-year follow-up. Notably, three-quarters of subjects (18/24) with ischemic stroke/TIA during the follow-up had basal Apo CIII plasma concentrations above the median value (**Table 2**). Apo CIII is a crucial regulator of lipoprotein metabolism and a specific marker of TG-rich lipoproteins (TRLs). High plasma levels of Apo CIII are well-recognized to be associated with atherosclerotic diseases and inhibiting Apo CIII is now considered an attractive way of reducing not only triglyceride levels but also residual cardiovascular risk (16). Our results are consistent with this position which attributes

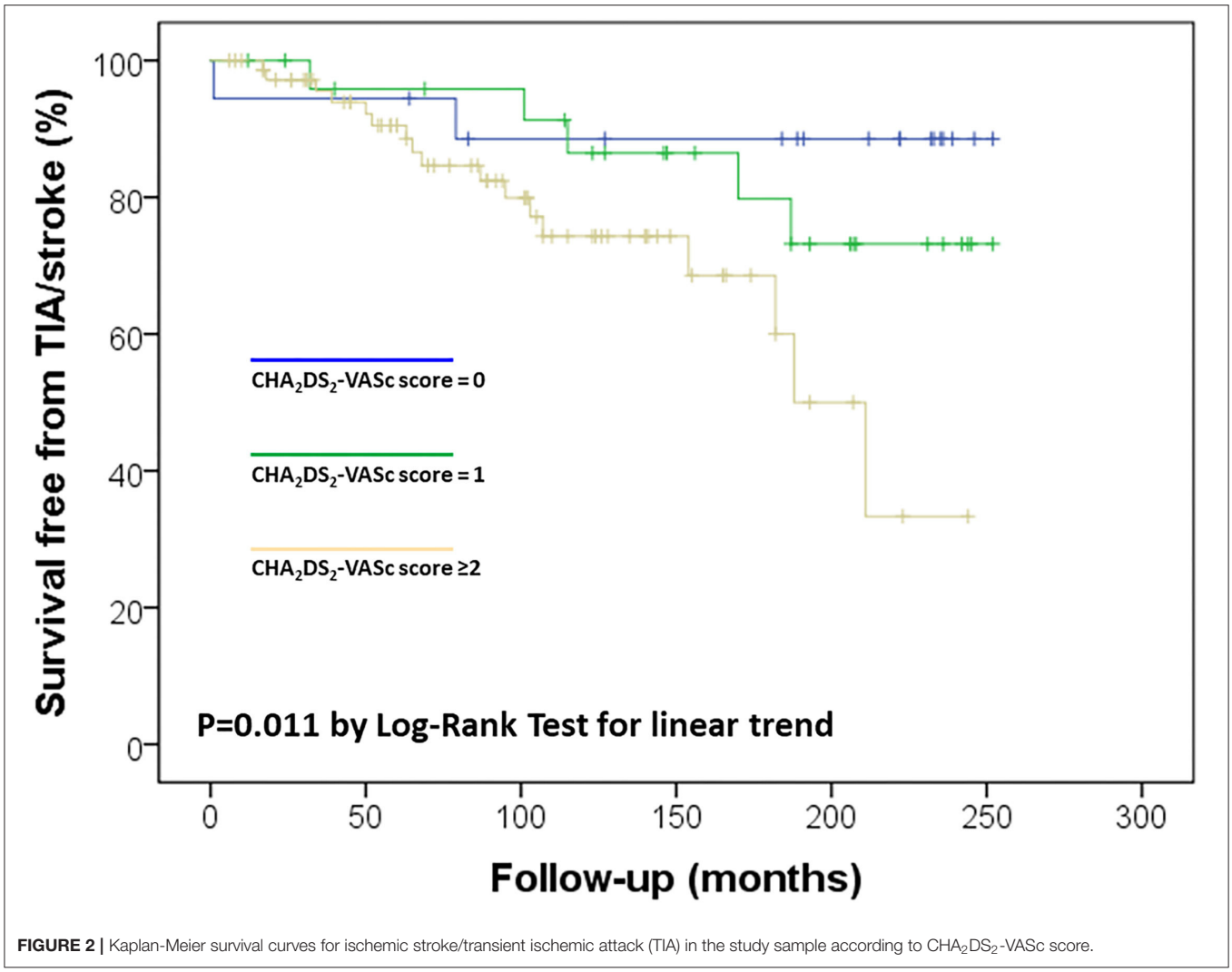

FIGURE 2 | Kaplan-Meier survival curves for ischemic stroke/transient ischemic attack (TIA) in the study sample according to CHA_2DS_2-VASc score.

a prominent role to Apo CIII in the setting of prevention of cardiovascular disease. Our data show that in the VHS population a substantial risk of cerebral ischemic events in the context of secondary prevention is associated with Apo CIII (and, thus, with the metabolism of TRLs), even despite anticoagulant protection. Previous reports in patients treated with vitamin K antagonists or direct oral anticoagulants (DOACs) indicated hyperlipidemia as a possible risk factor for recurrent cerebral ischemic events (6–8, 17). However, the type of hyperlipidemia was not clearly specified (6, 17) and generally emphasis was more devoted to total and LDL cholesterol rather than TG or TRLs (18). Accordingly, LDL-cholesterol-lowering treatment by statins, ezetimibe, and/or PCSK9 inhibitors is preferentially indicated for secondary prevention of stroke so far (19). However, statins are also potent Apo C III-lowering drugs (20) and their cardiovascular protective role may express by affecting both LDL and TRLs metabolism. Therefore, our present and previous results (9), suggesting a role of Apo CIII in cerebral ischemic disease, may be considered as consistent with the recommendations in current therapeutic guidelines which indicate high-dose statin (e.g., atorvastatin 80 mg), if needed also associated with ezetimibe and/or PCSK9 inhibitors, to reduce the risk of stroke recurrence (18). It should be noted that it is generally difficult to dissect the individual role of lipoproteins that share common lipid and protein-based components. In similar way, the lipid profile of our patients with high Apo CIII plasma concentration corresponded to so called "atherogenic dyslipidemia" (21, 22) that is mainly characterized by elevated TRLs levels, but also by increased Apo B, total and LDL cholesterol levels (**Table 2**). Nonetheless, in our study cohort among the assessed plasma lipids and apolipoproteins only Apo CIII was associated with cerebral ischemic events after adjustment for all plasma lipids, as well as for other potential confounding factors like diabetes and hypertension (**Table 3**).

Earlier studies identified older age, female sex, renal impairment, previous stroke/TIA, previous aspirin use, and higher CHA_2DS_2-VASc score as risk factors for recurrence of cerebral ischemic events (6–8, 17). In our study sample some of these parameters were not available among the collected data for analysis (e.g., previous aspirin use) and the small sample size did not allow to investigate adequately these associations.

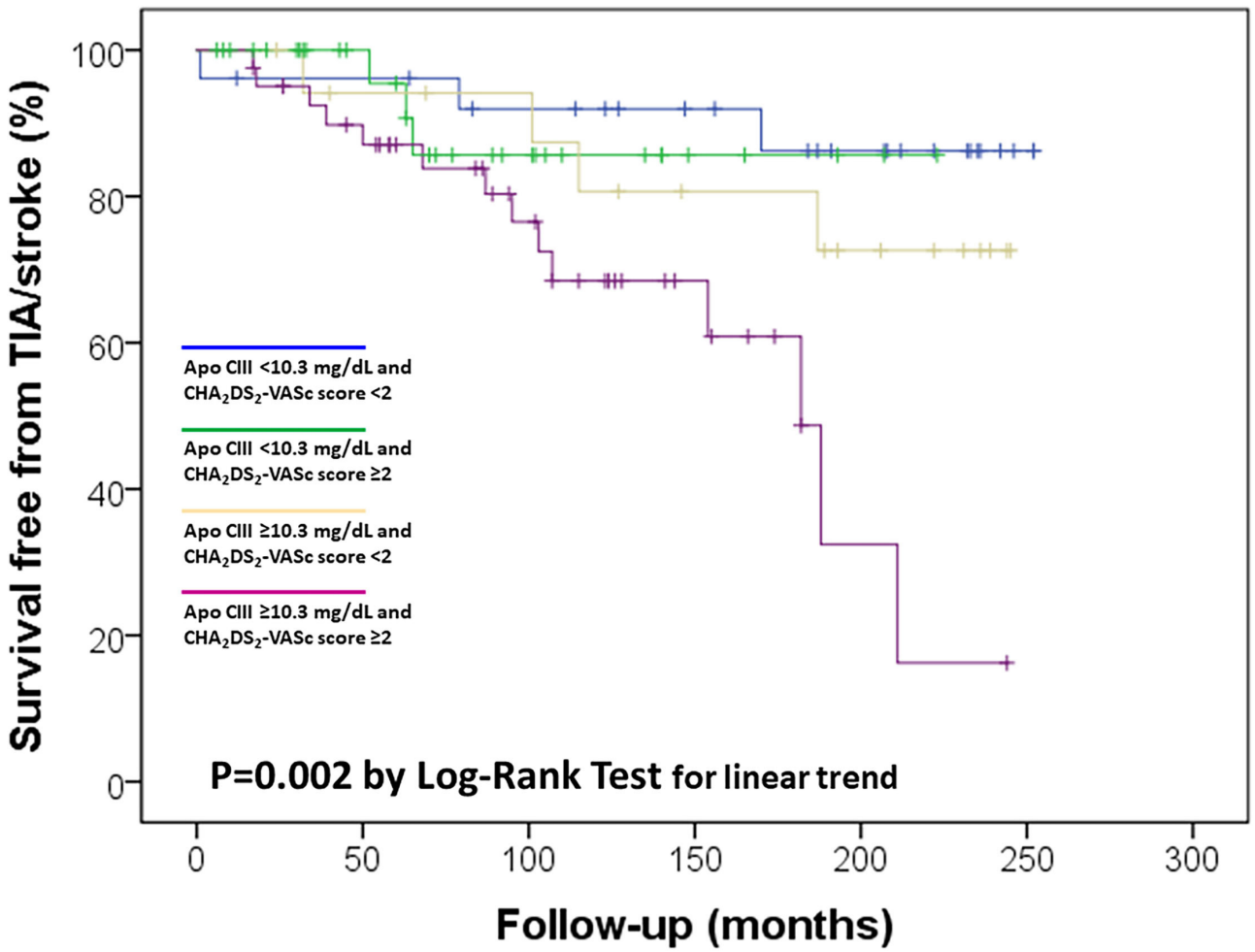

FIGURE 3 | Kaplan-Meier survival curves for ischemic stroke/transient ischemic attack (TIA) in the study sample according to Apolipoprotein C-III (Apo CIII) plasma concentration, with the median level (10.3 mg/dL) as threshold value, and CHA_2DS_2-VASc score, with ≥2 as threshold value.

CHA_2DS_2-VASc score—as expression of a combination of several clinical features—was confirmed as an important risk factor, notably with an evident synergic role with Apo CIII levels (**Figure 3**). In other words, subjects with both atherogenic dyslipidemia marked by high Apo CIII concentrations and a clinical history coherent with an elevated risk marked by high CHA_2DS_2-VASc score resulted at the highest risk of ischemic stroke/TIA in spite of anticoagulant treatment, thus being potentially the "best target" for more aggressive therapeutic approaches aiming at the reduction of cerebral ischemic events. The current results also raise concerns about the prothrombotic potential triggered by high concentrations of Apo CIII, apparently potent enough to hinder the protection afforded by anticoagulation. Coagulation pathway needs lipids and high levels of cholesterol and triglyceride have been related with increased coagulation activity (23). Some previous results suggested specifically a procoagulant activity of Apo CIII in the setting of both arterial atherosclerotic vascular disease and venous thromboembolism. In a prospective study of subjects with angiographically proven CAD, we first demonstrated that thrombin generation was amplified in patients with elevated Apo CIII concentrations (10). Similarly, high levels of Apo CIII (but not other plasma lipids or apolipoproteins) were associated with a progressive increase in factor II coagulant activity (11). Apo CIII was also strongly associated with the activated FVII–antithrombin (FVIIa-AT) complex, which is an indirect marker of intravascular exposure of tissue factor (TF), thus providing suggestion for an Apo CIII-related activation of the extrinsic coagulation pathway (12). In a recent study involving 127 patients with venous thromboembolism and 299 controls, concentrations of Apo CI, CII, CIII and E were associated with several coagulation factors, including vitamin K-dependent factors, as well as factor XI, factor VIII, and von Willebrand factor levels (24). Finally, within the framework of VHS, in a cohort of 1,020 of patients with cardiovascular disease, subjects with high Apo CIII concentrations had an about three-fold increased risk to experience venous thromboembolic events within a long-term period of 12 years as compared with those with low Apo CIII levels (25). All these lines of evidence are consistent with an active interplay between TG-rich/Apo

CIII-rich lipoproteins and the mechanisms of coagulation and, thereby, may represent a plausible biological ground for the results of the current analysis. Nevertheless, the present findings do not allow us to get any causal inference to why anticoagulated patients with higher levels of Apo CIII are less protected from the risk of ischemic stroke over time. Our results only show a mere statistical association between two objective parameters, i.e., apolipoprotein plasma concentration and number of cerebral ischemic events. This work has some significant limitations which should be acknowledged beyond the previously mentioned hindrances due to a sub-analysis not primarily designed for such purpose and with a small sample size. Notably, our results pertain substantially to dicumarolic anticoagulants. All the subjects of this analysis were treated with warfarin at time of enrollment between May 1999 and December 2006, but we cannot exclude for patients with AF without mechanical prosthetic heart valves the possibility of a shift to DOACs in the last period of follow-up, i.e., since April 2013 (when dabigatran became the first approved DOAC for stroke prevention in AF in Italy). Most importantly, we have not been able to control compliance to anticoagulant drugs over time. However, it is also difficult to hypothesize an asymmetric distribution of drug compliance according to Apo CIII concentration. Taking into account all these limitations, the present results provide, for the first time, an unexpected basis for suspecting a role of Apo CIII in the residual risk of stroke in patients with AF and/or mechanical prosthetic heart valves treated with anticoagulants. There is now a great need of research investigating the mechanisms of stroke recurrence and improving secondary prevention (26). If our data would be corroborated by further studies, patients with promptly identified increased Apo CIII levels could speculatively take advantage of a more intensive treatment strategy, including higher anticoagulant dosages and/or specific lipid (Apo CIII)-lowering therapies. Anyway, future investigations will be needed to fully confirm these observations and the related clinical implications, as well as to clarify the underlying molecular mechanisms.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because data cannot be shared publicly due to the privacy of individuals that participated in the study. The data will be shared on reasonable request to the corresponding author. Requests to access the datasets should be directed to oliviero.olivieri@univr.it and nicola.martinelli@univr.it.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Azienda Ospedaliera Universitaria Integrata of Verona, Italy. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

OO, GT, and NM contributed to conception and design of the study. FS, GT, and MC organized the database. NM, FP, and SF performed the statistical analysis. AC, NO, MC, and AB participated to data acquisition and analysis. NM and OO drafted the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2021.781383/full#supplementary-material

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BRIEF RESEARCH REPORT
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Lipoprotein(a) and Pulmonary Embolism Severity-A Retrospective Data Analysis

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Aim: We aimed to investigate a correlation between PE severity and Lp(a) levels.

Methods: We performed a retrospective data analysis from our medical records of PE patients admitted to the University Hospital Graz, Austria. Patients with an Lp(a) reading within a 1-year interval before and after PE diagnosis were included. In accordance with the 2019 ESC guidelines for the diagnosis and management of acute PE, severity assessment was carried out classifying patients into four groups: low risk (LR), intermediate low risk (IML), intermediate high risk (IMH) and high risk (HR). The study period of interest was between January 1, 2002 and August 1, 2020.

Results: We analyzed 811 patients with PE, of whom 323 (40%) had low-risk PE, 343 (42%) had intermediate-low-risk PE, 64 (8%) had intermediate-high-risk PE, and 81 (10%) had high-risk PE, respectively. We did not observe an association between PE severity and Lp(a) concentrations. In detail, median Lp(a) concentrations were 17 mg/dL [25–75th percentile: 10-37] in low-risk PE patients, 16 mg/dL [10–37] in intermediate-low-risk PE patients, 15mg/dL [10–48] in intermediate-high-risk PE patients, and 13mg/dL [10–27] in high-risk PE patients, respectively (Kruskal-Wallis $p = 0.658$, p for linear trend = 0.358).

Conclusion: The current findings suggest no correlation between PE severity and Lp(a) levels.

Keywords: Lipoprotein(a), pulmonary embolism, severity, venous thromboembolism, Lp(a)

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HIGHLIGHTS

- We aimed to investigate a correlation between PE severity and Lp(a) levels.
- Potential pathomechanisms of Lp(a) include similarities of Lp(a) to plasminogen, resulting in a decrease of plasmin synthesis and inhibition of fibrinolysis, which is mainly observed under laboratory conditions. It, however, remains elusive whether this inhibitory effect is strong enough to play a significant role in the development of venous thrombotic events (VTE) such as pulmonary embolism (PE).
- The current findings suggest no correlation between PE severity and Lp(a) levels.

INTRODUCTION

Lipoprotein(a) (Lp(a)) is a genetically determined low-density lipoprotein (LDL) particle. In the absence of acute inflammation, the Lp(a) level is stable through an individual's lifetime, regardless of lifestyle (1). Elevated Lp(a) levels are strongly associated with the development of atherosclerotic cardiovascular diseases (ASCVD) such as stroke, peripheral artery disease or coronary heart disease (2, 3). A Lp(a) level over 50 mg/dL is generally considered as an additional factor that indicates a high risk of ASCVD, whereas the highest risk is strongly restricted to those with very high Lp(a)-concentrations (3). Therefore, the European Society of Cardiology (ESC) recommends measuring Lp(a) levels in selected patients at high risk of ASCVD (4). Potential pathogenic mechanisms of Lp(a) include their propensity to oxidize after entry into the vessel wall, creating highly immunogenic and proinflammatory phospholipids, the presence of lysine binding sites that allow accumulation in the arterial wall, and similarities of Lp(a) to plasminogen, resulting in a decrease of plasmin synthesis and inhibition of fibrinolysis (5, 6). It, however, remains elusive whether this inhibitory effect is strong enough to play a significant role in the development of venous thrombotic events (VTE) such as pulmonary embolism (PE) (7).

PE is a leading cause of death worldwide, especially when massive PE is present (8, 9). In the current ESC guidelines for the management of PE (10), PE-related severity is stratified based on clinical presentation and factors contributing to haemodynamic collapse, reflecting acute right ventricular (RV) dysfunction (11). According to these guidelines we aimed to investigate a potential correlation between PE severity and Lp(a) levels in a single-center cohort by retrospective data analysis.

METHODS

Study Design and Patient Population

We performed a retrospective chart review study from our medical records of PE patients with an available Lp(a) value admitted to the University Hospital Graz, Austria. At our center, admission of patients with newly-diagnosed PE to an inpatient ward is local standard-of-care. Although Lp(a) is thought to be relatively stable within patients over time, latency between PE diagnosis and Lp(a) determination was a maximum of 1 year, i.e., only patients with an Lp(a) reading within a 1-year interval before and after PE diagnosis were included. In accordance with the 2019 ESC guidelines (10) for the diagnosis and management of acute PE, severity assessment was carried out classifying patients into four groups: low risk (LR), intermediate low risk (IML), intermediate high risk (IMH) and high risk (HR). These guidelines report a PE risk stratification based on immediate and early mortality risk. The presence of haemodynamic instability is the main determinant to classify patients as having a high risk PE. In these guidelines other patients are divided into intermediate-high risk (no hemodynamic instability, but clinical criteria of severity, positive Pulmonary Embolism Severity Index (PESI) simplified positive Pulmonary Embolism Severity Index (sPESI), and both signs of right ventricular (RV) dilation and positive troponin), intermediate-low risk (no haemodynamic instability, presence of clinical criteria of severity, positive PESI or sPESI and either RV dilation or positive troponin), or low risk (no hemodynamic instability and a negative PESI or sPESI) (10, 11). According to these guidelines, for patients with no hemodynamic instability, signs of RV dilation and positive troponin were included in the risk stratification as well as Pulmonary Embolism Severity Index (PESI) score was assigned based on the variables of age, sex, previous PE, cancer, comorbidities, O_2-saturation, systolic blood pressure and heart rate. RV dysfunction was assessed by computed tomography (CT) by specialized radiologists, and in selected cases by point-of-care echocardiography. CT criteria for RV dysfunction included a ratio of right to left ventricular diameter (RV/LV) $>$ 1, bulging of the interventricular septum and reflux of contrast media into the inferior vena cava and hepatic veins. Echocardiographic assessments of RV dysfunction were performed on a case-by-case basis by treating physicians at our acute care treatment facilities. Laboratory data (estimated glomerular filtration rate (eGFR), Troponin T, (NT-pro) Brain Natriuretic Peptide) were extracted as close as possible to Lp(a) assessment date. In contrast, comorbidities (cancer, COPD, asthma, heart failure, kidney disease) were extracted within a time frame of seven days prior and after PE diagnosis date. The study period of interest was between January 1, 2002 and August 1, 2020. The study protocol was approved by the Ethics Committee (EK 32-646 ex 19/20) of the Medical University of Graz.

Statistical Analysis

All statistical analyses were performed with Stata (Windows Version 17.0, Stata Corp., Houston, TX, USA). Continuous variables were summarized as medians [25–75th percentile], and count data as absolute frequencies (%). Correlations between two continuous variables were evaluated with Spearman's rank-based correlation coefficient. The primary aim was the association between PE severity as indicated by the ESC PE risk stratification (4-level ordinal variable defined above) and the Lp(a) levels both as a continuous variable and as a binary variable dichotomized at a pre-defined cut-off at 50 mg/dL. For these analyses, we employed Kruskal-Wallis tests, simple and multiple linear regression models (multiple linear regression adjusted for age and sex), F-tests for linear trend, box plots, χ^2-tests, and Fisher's exact tests, as appropriate. In a pre-specified sensitivity analysis, we examined whether extremely high levels of Lp(a), defined by three Lp(a) cut-offs $>$80 mg/dL, $>$120 mg/dL, and $>$160 mg/dL, were associated with high-risk PE.

RESULTS

Cohort Description

We analyzed 811 patients with PE, of whom 323 (40%) had low-risk PE, 343 (42%) had intermediate-low-risk PE, 64 (8%) had intermediate-high-risk PE, and 81 (10%) had high-risk PE, respectively (**Table 1**). Median Lp(a) concentration was 15 mg/dL [25–75th percentile: 10-35, range: 0.6 – 254]. Median time between Lp(a) measurement and index PE was 0 days [25–75th

TABLE 1 | Baseline characteristics of the study population ($n = 811$).

Variables	Overall ($n = 811$)	Lp(a) ≤ 50 mg/dL ($n = 681$)	Lp(a) > 50 mg/dL ($n = 130$)	p-value
Age at PE diagnosis (years)	69 [54–80]	69 [53–80]	69 [56–79]	0.792
Female sex	595 (51%)	418 (49%)	177 (55%)	0.106
BMI (kg/m²)*	27 [24–30]	27 [24–30]	26 [24–30]	0.730
eGFR (ml/min/1.73 m²)*	69 [52–85]	68 [52–85]	72 [50–86]	0.563
Cancer at PE diagnosis**	73 (9%)	59 (9%)	14 (11%)	0.442
Asthma at PE diagnosis**	13 (2%)	11 (2%)	2 (2%)	0.999
COPD at PE diagnosis**	68 (8%)	54 (8%)	14 (11%)	0.284
Heart failure at PE diagnosis**	61 (8%)	46 (7%)	15 (12%)	0.058
Kidney disease at PE diagnosis**	114 (14%)	94 (14%)	20 (16%)	0.635
Troponin T (pg/mL)*	10 [10–12]	10 [10–11]	10 [10–19]	0.417
Brain natriuretic peptide (pg/mL)*	600 [125–2518]	586 [117–2518]	662 [172–2484]	0.486
PE risk stratification	/	/	/	0.430
Low-risk	323 (40%)	268 (39%)	55 (42%)	/
Intermediate-Low-risk	343 (42%)	293 (43%)	50 (38%)	/
Intermediate-High-risk	64 (8%)	50 (7%)	14 (11%)	/
High-risk	81 (10%)	70 (10%)	11 (8%)	/

*Distribution overall and by Lipoprotein(a) status. We used a Lp(a) cut-off at 50 mg/dL. Reported data are medians [25–75th percentile] for continuous variables, and absolute frequencies (column %) for count data. P-values are from rank-sum tests and χ^2-tests, as appropriate. Lp(a), Lipoprotein(a); PE, Pulmonary embolism. *closest reading to Lp(a) assessment date. **reported within 7 days prior and after PE diagnosis. Troponin T and BNP were not included in this model, as cumulative missingness in these two variables would have led to the final regression model being fitted in only n = 370 patients.*

percentile:−6–1 days, range:−359–361 days]. Higher Lp(a) did not correlate with age (Spearman's ρ = 0.04, $p = 0.271$), and was comparable between males and females (median Lp(a). 15 vs. 16, $p = 0.181$). Neither BMI, nor eGFR, nor Troponin T, nor BNP, nor comorbidities at PE diagnosis, including cancer, asthma, COPD, heart failure, and kidney disease, were associated with Lp(a) levels.

Lp(a) Concentration by PE Severity

We did not observe an association between PE severity and Lp(a) concentrations. In detail, median Lp(a) concentrations were 17mg/dL [25–75th percentile: 10-37] in low-risk PE patients, 16mg/dL [10–33] in intermediate-low-risk PE patients, 15 mg/dL [10–48] in intermediate-high-risk PE patients, and 13mg/dL [10–27] in high-risk PE patients, respectively (Kruskal-Wallis $p = 0.658$, p for linear trend = 0.358, **Figure 1**). This result prevailed also after multivariable adjustment for age, sex, BMI, eGFR, Troponin T, BNP, and comorbidities including cancer, asthma, COPD, and heart failure (Adjusted p for association between Lp(a) and PE severity = 0.212, **Table 2**). In this multivariable model, heart failure emerged as the only statistically significant predictor of Lp(a) levels.

Sensitivity Analysis–Very High Levels of Lp(a)

In this sensitivity analysis, we examined whether extremely high levels of Lp(a), defined by three Lp(a) cut-offs >80 mg/dL, >120 mg/dL, and >160 mg/dL, are associated with high-risk PE, which was not the case (**Table 3**).

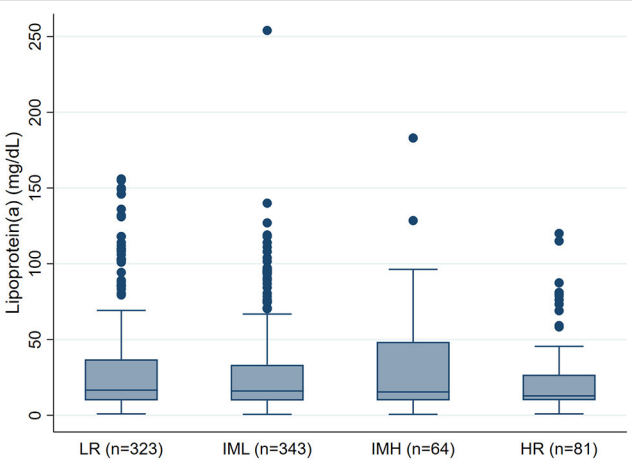

FIGURE 1 | Boxplots of Lipoprotein(a) levels according to PE severity ($n = 811$). PE, Pulmonary embolism; LR, Low-risk PE; IML, Intermediate-Low-risk PE; IMH, Intermediate-High-risk PE; HR, High-risk PE.

DISCUSSION

While several studies have shown that elevated Lp(a) levels are a causal risk factor for the development of ASCVD, the role of Lp(a) as a risk factor for VTE remains controversial (5, 7). There is evidence that Lp(a) inhibits fibrinolysis due to the similarity between apolipoprotein(a) and plasminogen (6). These potential mechanisms, however, have explicitly been described in *in-vitro* studies (5, 12). Thus, it remains unknown whether this inhibitory

effect plays a relevant role in the global fibrinolytic activity of the circulating blood that depends on many coagulation factors.

Due to impaired fibrinolysis, elevated Lp(a) levels may increase plasma clot density in patients with VTE (6). In this regard, we expected a correlation between PE severity and elevated Lp(a) levels. However, we did not observe any association between PE severity and Lp(a) concentrations. As the highest risk is strongly restricted to those with very high Lp(a)-concentrations, we also performed a sensitivity analysis, where we examined whether extremely high levels of Lp(a), are associated with high-risk PE, which was not the case. Thus, our results suggest that the fibrinolytic effect of Lp(a) may not significantly affect PE severity.

Several studies using different Lp(a) cut-off values aimed to find associations between elevated Lp(a) and VTE [13−15]. Vormittag et al. (13) for example did not find a significant association between Lp (a) plasma levels and the risk of VTE. In contrast other studies such as that from von Depka et al. (14) and that from Marcucci et al. (15) found strong associations between Lp (a) plasma levels and the risk of VTE. The cause of this contrariety is unknown. A recent study tried to clarify the conflicting results and tested whether an inhibitory effect of Lp(a) could only be visible in clot lysis assays with a relatively high tissue plasminogen activator concentration but did not find any correlation between Lp(a) concentration and lysis time (7).

Boffa et al. (12) demonstrated that a potent reduction of Lp(a) in human subjects with high Lp(a) does not affect ex *vivo* clot lysis or biomarkers of coagulation and fibrinolysis. A recent meta-analysis confirmed the questionable role of Lp(a) as a risk factor for VTE (16). Recent data revealed that only Lp(a) concentrations above the 95th percentile may be associated with an increased risk for venous thromboembolism (17). In our study, however, extremely high levels of Lp(a) were also not associated with high-risk PE.

These findings are in line with several other studies showing no association of elevated Lp(a) with deep venous thrombosis (18, 19). One potential reason why Lp(a) primarily promotes ASCVD rather than VTE could be the difference in the etiology of the diseases. VTE represents a different form of thrombosis than ischemic stroke, myocardial infarction or critical limb ischemia where atherosclerosis is concomitantly present. The association of Lp(a) with ASCVD may be attributable through its proatherogenic and proinflammatory components, such as oxidized phospholipids as primary mechanisms (5, 6). In contrast, atherosclerosis does not occur in veins. Pathogenesis of VTE can be explained by using the Virchow's Triad: stasis of blood, hypercoagulability, and endothelial vessel wall injury; which come in to effect after surgery, trauma, immobility or in cancer patients (20). Furthermore, compared with arterial thrombosis, venous thrombosis has a more fibrin-rich and platelet-poor consistency (19).

Last but not least it is noteworthy, that there is rising evidence that statins may be beneficial in preventing VTE (21). Interestingly, a recent meta-analysis revealed that statins can even significantly increase plasma Lp(a) levels (22). This is of some clinical significance, as it underscores the relevance of our findings, that elevated Lp(a) is not linked to VTE. Although the mechanism of action for statins in prevention of VTE is not entirely understood, it could offer new treatment targets.

Our study has several limitations since it is a retrospective data analysis and was based on a single center. First it is quite

TABLE 2 | A multiple linear regression model of Lipoprotein(a).

Variable	β coefficient	95%CI	*p*-value
Age at PE diagnosis (per 5 years increase)	0.99	−0.03–2.01	0.057
Female sex	2.78	−2.63–8.19	0.314
PE risk stratification	/	/	0.212
Low-risk	Ref.	Ref.	Ref.
Intermediate-Low-risk	−6.53	−13.04-(-0.02)	0.049
Intermediate-High-risk	−4.87	−15.67–5.94	0.377
High-risk	−7.03	−16.66–2.61	0.153
BMI (per 5 kg/m^2 increase)	0.39	−0.97–1.76	0.572
eGFR (per 5 ml/min/1.73 m^2 increase)	0.36	−0.26–0.98	0.260
Cancer at PE diagnosis	1.57	−8.05–11.19	0.749
Asthma and/or COPD at PE diagnosis	4.82	−4.28–13.92	0.298
Heart failure at PE diagnosis	13.18	1.96–24.39	0.021
Constant	10.31	−10.31–30.93	0.327

The β coefficient represents the change in Lp(a) per one unit change in the respective variable. 95%CI, 95% confidence interval; p, Wald test p-value; PE, Pulmonary embolism; Ref., Reference category.

TABLE 3 | Exploratory analysis of extremely high Lp(a) levels and high-risk PE according to three ascending cut-offs.

Cut-off	Group	No high-risk PE ($n = 730$)	High-risk PE ($n = 81$)	*p*-value
80 mg/dl	Lp(a) ≤ 80 mg/dL ($n = 748$)	672 (92%)	76 (94%)	0.572
	Lp(a) > 80 mg/dL ($n = 63$)	58 (8%)	5 (6%)	
120 mg/dL	Lp(a) ≤ 120 mg/dL ($n = 797$)	716 (98%)	81 (100%)	0.383
	Lp(a) > 120 mg/dL ($n = 14$)	14 (2%)	0 (0%)	
160 mg/dL	Lp(a) ≤ 160 mg/dL ($n = 809$)	728 (99%)	81 (100%)	0.999
	Lp(a) > 160 mg/dL ($n = 2$)	2 (1%)	0 (0%)	

PE, Pulmonary embolism; p, p-value from χ^2-tests or Fisher's exact test; as appropriate, Lp(a), Lipoprotein(a).

possible, that in a larger population with more participants with extremely high Lp(a) concentrations, more high risk PE's may have been observed. In addition, although Lp(a) is thought to be relatively stable within patients over time, in our study latency between PE diagnosis and Lp(a) determination was a maximum of 1 year. Thus, it cannot be excluded that Lp(a) measurements directly at the time point of PE diagnosis may have revealed slightly different estimates of the association between Lp(a) and PE severity. Next, as Lp(a) might affect fibrinolytic activity (5, 6), associations of Lp(a) levels and morphological thrombus burden may have been an important research question. However, assessment of thrombus burden in CT scans is challenging. A potential technique would be measurement of thrombus volume by means of thrombus segmentation in CT, which would require very extensive analyses of CT data that was out of the scope and resources of our study. Additionally, clot volume from CT segmentation may not represent overall clot burden, as measurements can be influenced by artifacts, peripheral clots may be underestimated in CT and additional extrapulmonary thrombus material is not represented, which will distort associations. We therefore did not include clot size data in our analysis.

In conclusion we did not observe an association between Lp(a) levels and PE severity. In light of our observations the antifibrinolytic effect of Lp(a) seems to play no significant role in the fibrinolytic activity of the circulating blood in real life, in line with findings of several other studies. Nonetheless our results should encourage other researchers to address potential procoagulant properties of Lp(a) in further studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of the Medical University of Graz. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

All authors have contributed significantly to the paper, they understand and endorse it. They have read and approved the version being submitted for publication. The article is original work of the authors.

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Small, Dense Low-Density Lipoprotein-Cholesterol and Atherosclerosis: Relationship and Therapeutic Strategies

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Low-density lipoprotein cholesterol (LDL-C) plays an important role in the formation, incidence, and development of atherosclerosis (AS). Low-density lipoproteins can be divided into two categories: large and light LDL-C and small, dense low-density lipoprotein cholesterol (sdLDL-C). In recent years, an increasing number of studies have shown that sdLDL-C has a strong ability to cause AS because of its unique characteristics, such as having small-sized particles and low density. Therefore, this has become the focus of further research. However, the specific mechanisms regarding the involvement of sdLDL-C in AS have not been fully explained. This paper reviews the possible mechanisms of sdLDL-C in AS by reviewing relevant literature in recent years. It was found that sdLDL-C can increase the atherogenic effect by regulating the activity of gene networks, monocytes, and enzymes. This article also reviews the research progress on the effects of sdLDL-C on endothelial function, lipid metabolism, and inflammation; it also discusses its intervention effect. Diet, exercise, and other non-drug interventions can improve sdLDL-C levels. Further, drug interventions such as statins, fibrates, ezetimibe, and niacin have also been found to improve sdLDL-C levels.

Keywords: small dense low-density lipoprotein-cholesterol, atherosclerosis, lipid metabolism, inflammation, endothelial injury, review

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INTRODUCTION

Atherosclerosis (AS) is the formation of fibrofatty lesions within the arterial wall, and it causes widespread morbidity and mortality worldwide together with heart muscle infarction, stroke, and disabling peripheral artery illness (1). AS could be a major condition that seriously harms human health and is understood as the major cause of mortality not only in developed countries, but globally (2). At present, inflammatory reactions (3), lipid metabolism disorders (4), and oxidative stressare the most important and widely recognized pathogenic causes of AS (5). In the field of lipid metabolism, a number of irrefutable pieces of evidence have proven the pathogenic role of low-density lipoprotein cholesterol (LDL-C) in AS, so we have extremely effective tools to reduce LDL-C levels, thus reducing the occurrence of cardiovascular events (2). Some studies have shown the correlation between different low-density lipoprotein (LDL) subgroups and the occurrence of AS, in which small, dense low-density lipoprotein cholesterol (sdLDL-C) is closely related to and has a stronger effect on AS (6). This study aimed to explore the relationship between sdLDL-C levels and AS.

DETECTION METHOD AND SOURCES OF SDLDL-C

LDL is a lipoprotein with a density between 1.006 and 1.063 g/mL. It is composed of many heterogeneous particles. They can be separated using various experimental methods. Krauss et al. used ultracentrifugation to classify LDL into four types according to density: large and light LDL- I (1.025–1.034 g/mL), intermediate density LDL- II (1.035–1.044 g/mL), low-density LDL- III (1.045–1.060 g/mL), and very low-density LDL-IV (7). Another method widely used to identify low-density lipoproteins is gradient gel electrophoresis (GGE), which separates low-density lipoprotein particles by electrophoretic mobility. In studies using GGE, LDL particles have been separated into four major subfractions, LDL I (large LDL, peak diameter 26.0–28.5 nm), LDL II (intermediate LDL, 25.5–26.4 nm), LDL III A and B (small LDL, 24.2–25.5 nm), and LDL IV A and B (very small LDL, 22.0–24.1 nm) (8). The Lipoprint LDL subcomponent rapid analysis system (Hitachi 7180) is the only diagnostic equipment certified by the United States Food and Drug Administration for the separation and detection of LDL subcomponents. Based on the charge and particle size of LDL-C, they can be divided into seven subcomponents within a short time via polyacrylamide gel electrophoresis, in which components 3–7 were defined as sdLDL-C (**Figure 1**) (9). In addition, foreign enzyme-linked immunosorbent assay (ELISA, Millipore, St. Louis, MO) kits can quickly detect the concentrations of sdLDL-C but do not rule out the possibility of cross-reactions, so it remains to be verified whether they can be used in clinical settings (10). The homogeneous method (11) can be used to measure sdLDL-C levels by removing lipoproteins other than sdLDL-C using a surfactant and sphingomyelinase, and it is a better technique compared to the traditional detection method of LDL subfraction. Its correlation, accuracy, and stability are high, which provided a cornerstone for the popularization and application of clinical sdLDL-C detection. Other analytical methods to detect sdLDL-C include gel filtration column chromatography, high performance liquid chromatography (HPLC), ion mobility analysis, and dynamic light scattering. In clinical practice, it is important to accurately analyze LDL subclasses thorough analytical methods.

For the detection of sdLDL-C, Mauree et al. developed a new equation to calculate the content of sdLDL-C {the formula of sd-LDL-C is as follows: ElbLDL-C = 1.43 × LDL-C −[0.14 × (ln (TG) × LDL-C)] − 8.99; EsdLDL-C = LDL-C−ElbLDL-C}. Equations for sdLDL-C was generated with least-squares regression analysis using the direct Denka sdLDL-C assay as reference (n = 20,171). This equation can be widely used for all patients with standard lipid groups without incurring additional laboratory testing costs. The limitation of the study is that the study population is not universal. The equation developed is based on fasting subjects. Therefore, whether this equation can be used in experiments or clinical applications remains to be verified (12).

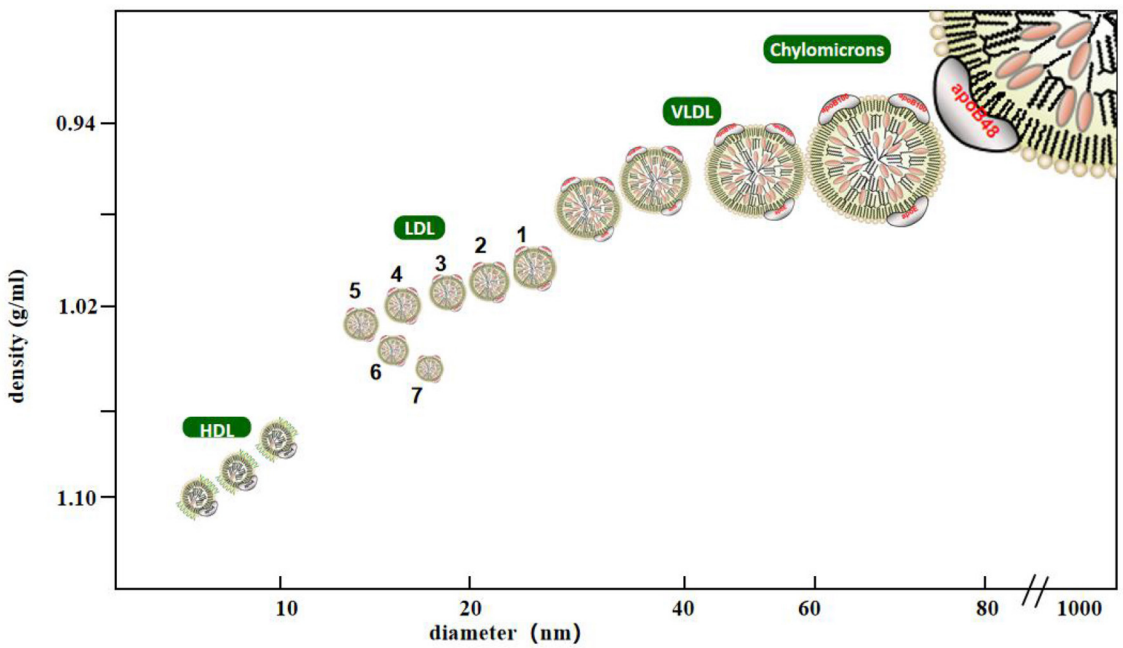

FIGURE 1 | Classification of lipoprotein cholesterol. Lipoprotein with density <0.95 g/ml, diameter of 80–500 nm is chylomicrons, density of 0.95–1.006 g/ml, diameter of 25–80 nm is very low density lipoprotein, density of 1.063–1.21 g/ml, diameter of 8–15 nm is high density lipoprotein, density of 1.006–1.063 g/ml, diameter of 18–28 nm is low density lipoprotein, Among them, low-density lipoprotein is divided into seven subtypes. The third to seventh subtypes are small and dense low-density lipoprotein with density >1,004 g/ml and diameter <25.5 nm.

About the detection method of sdLDL-C (8), ultracentrifugation involves multiple steps and requires specialized equipment and expertise to separate LDL subsets, which is more error-prone, time-consuming, laborious and costly. The resolution of gradient gel electrophoresis is very high, but it requires a lot of manpower, material and financial resources, and the sample flux is low. The polyacrylamide gel tube electrophoresis method is simple to operate and shows a satisfactory coefficient of variation between samples, but the disadvantage is that the equipment is expensive. The homogeneous method can be fully automated and can be used in the high-throughput integration platform, which is helpful for the large-scale testing of sdLDL-C.

At present, it is generally believed that there are two ways to produce sdLDL-C (7, 13). First, when the triglyceride (TG) content in the liver is high, the liver directly secretes VLDL1 (large particles and high TG content) and VLDL2 (low TG content), when the level of TG synthesized by hepatocytes decreases, the liver secretes VLDL1 (small particles and high TG content) and intermediate density lipoprotein2 (low TG content). TG-deficient lipoproteins are the precursors of larger LDL (LDL I and LDL II), while TG-rich lipoproteins are converted into sdLDL (LDL III and LDL IV) after being defatted by lipoprteinlipase (LPL) and hepatic lipase (HL). Second, there is a very active and dynamic lipid exchange between various lipoproteins in the plasma, which is mainly catalyzed by cholesterol lipid transport proteins. The total cholesterol (TC) of LDL is transferred to VLDL and the TG of VLDL is transferred to LDL, but the total amount and synthesis of LDL remains unchanged. When the TG levels in LDL increase to a certain extent, LDL will be hydrolyzed by liver lipase to remove TG, the LDL particles become smaller, then the TC content decreases, thus promoting the formation of sdLDL-C. When the plasma concentration of TG exceeds 1.5 mmol/L, lipid exchange is accelerated; higher TG levels accelerate the lipid interaction between VLDL and LDL, producing more sdLDL-C. It has been reported in the literature that the change in gene locus is also significantly related to sdLDL-C levels. In 2009, Musunuru et al. (14) proved that four genes, *CEPT*, *LIPC*, *APOA1/A5*, and *LPL* are related to the particle size and distribution of LDL or HDL. Hoogeveen (15) showed that 127 single nucleotide polymorphisms (SNPs) are considerably related to sdLDL-C. These SNPs are distributed in eight different sites on chromosomes 1, 2, 7, 8, 11, and 19, and are distributed in 14 different genes. The genetic variation of these genes is related to lipid metabolism and inflammatory pathways. This study also found that the genetic variation of the new locus *PCSK7* was also associated with sdLDL-C levels.

RELATIONSHIP BETWEEN SDLDL-C AND AS

AS mainly affects the intima of the large- and medium-sized arteries, characterized by lipid deposition, focal fibrosis, and the formation of atherosclerotic plaques, resulting in thickening, hardening, and lumen stenosis of the vessel wall, ultimately leading to ischemic changes in the corresponding organs. In recent years, some studies have confirmed that the ability of LDL-C to induce AS varies with different densities, and there is a stronger relationship between sdLDL-C and the stability of AS plaques (16, 17). Ikezaki (18) followed 2,030 men and women [median age 59 years old, no cardiovascular disease (CVD) and not taking cholesterol-lowering drugs] for five years and performed univariate, multivariate regression and least squares analysis to examine the relationship between direct sdLDL-C and other lipoproteins with the progression of carotid intimal medial thickness (cIMT). The plasma levels of direct sdLDL-C and other lipoproteins were measured using a homogeneous detection kit obtained from Denka-Seiken. The results showed that compared with LDL-C, sdLDL-C had a stronger correlation with the progress of cIMT. However, the scale of this line-up study is small and all subjects are Japanese. A larger population of different races is needed to verify this study.

Duran et al. conducted a prospective case cohort study to study the relationship between sdLDL-C and cardiovascular events. The sdLDL-C concentration in this study was directly measured using a two-stage automatic homogenization test also developed by Denka Seiken Co (Niigata, Japan). A total of 27,552 participants provided sufficient blood samples when they entered the group after taking into account the missing key exposure data, the final sample included 480 women with total CVD and 496 women whose age and smoking frequency matched. The study found that the concentration of sdLDL-C in women with myocardial infarction (MI) was much higher than that in the control group, suggested that there was a significant correlation between plasma sdLDL-C concentration and MI (17).

Balling et al. measured sdLDL cholesterol using Denka Seiken's assay in 38,322 individuals participating in the Copenhagen General Population Study from 2013 to 2017. The death and immigration information comes from the Danish civil registration system. Individuals are followed up from baseline to December 2018 for MI and atherosclerotic cardiovascular disease (ASCVD). events, death, immigration, or the end of follow-up, whichever occurs first. Covariates were measured at baseline, including smoking, lipid-lowering therapy, blood pressure, body mass index, diabetes, blood sample analysis, total cholesterol, high-density lipoprotein cholesterol, triglycerides and apolipoprotein B. The Cox regression restricted cubic spline model with multivariate adjustment was also used to examine the association between sdLDL cholesterol and the risk of MI. The median follow-up time was 3.1 years. In the multivariate adjusted spline curve, as the concentration of sdLDL cholesterol increased, the risk of myocardial infarction was observed to increase (6). A large amount of experimental data (9, 15, 19–25) showed that sdLDL-C is closely related to the occurrence of cardiovascular events, so it is necessary to monitor clinically and reduce the concentration of sdLDL-C to reduce the occurrence of cardiovascular events (**Table 1**).

TABLE 1 | The relationship between sdLDL-C and AS.

References	Research type	Detection method of sdLDL-C	Number of participants	Age	Follow-up time/years	Conclusions
Tsai et al. (9)	Retrospective analysis	Homogenous assay	4,387 atherosclerotic participants	Not mentioned	8.5	sdLDL-C was associated with CVD
Hoogeveen et al. (15)	Retrospective analysis	Homogenous assay	9,882 atherosclerotic participants	45–64	11	sdLDL-C was associated with CVD
Higashioka et al. (19)	Prospective study	Homogeneous assay	3,080 without prior CVD	>40	8.3	SdLDL-C was associated with CVD
Zhou et al. (20)	Single-centre retrospective observational study	Lipoprint LDL system	368 AIS and 165 non-AIS patients	>40	None	SdLDL-C was risk factors for increased IMT
Siddiqu et al. (22)	Ancillary study	Qualitative assay kits	130 liver transplant recipients	>47	4	sdLDL-C independently predicted CVD
Goel et al. (23)	Observational, single centre, cross sectional case control study	Enzymatic analysis	150 CAD patients and 40 healthy adults	Not mentioned	None	CAD have higher sdLDL levels compared to individuals without CAD
Williams et al. (24)	Double-blind randomized controlled clinical trial	Gradient gel electrophoresis	160 patients selected for clinical coronary disease	Men <70, Women <65	2	SdLDL-C was related to changes in coronary artery stenosis and cardiovascular events in patients with CAD and low HDL-C
Arai et al. (25)	Prospective study	Homogenous assay	2030 without cardiovascular disease	Not mentioned	11.7	sd-LDL-C was significantly associated with CVD

SdLDL-C, small, dense low-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL, high-density lipoprotein; CAD, coronary artery disease; CVD, cerebrovascular disease; AIS, acute ischemic stroke.

MECHANISM OF AS INDUCED BY SDLDL-C

The term "induration of the arteries" refers to a condition where lipids and alternative substances deposit in and on the artery walls (referred to as "plaques") that limit traditional blood flow (26). AS and the pathology of related ischemic organs are the primary causes of mortality worldwide (27). Currently, there are many theories regarding the mechanisms of AS. It is generally believed that AS is a chronic inflammatory process involving multiple cell types and cytokines (3, 28). AS begins with the deposition of LDL-C, endothelial dysfunction, the accumulation of foam cells under the endothelium, and the formation of fat streaks through the activation of pro-inflammatory f actors (1).

sdLDL-C Promotes AS by Regulating Lipid Metabolism

The metabolism of cholesterol esters (CEs) is regulated by the macrophage gene network. Studies have shown that genes such as *ATF3* (activating transcription factor 3) and *EGR2* affect AS by regulating lipid metabolism (29–32). *ATF3* is a member of the mammalian activation transcription factor/cAMP response element binding (CREB) family (33, 34). SdLDL-C can reduce the ability of ATF3 to induce type B scavenger receptor (SR-BI) by down-regulating the expression of ATF3, and promote hepatic cholesterol 12α hydroxylase (CYP8B1) by interacting with p53 and hepatocyte nuclear factor 4α, thus reducing the uptake of high-density lipoprotein, promoting visceral fat and cholesterol absorption, and inhibiting the reverse cholesterol transport of phagocytes (32). *EGR2* is another gene related to cholesterol metabolism, which is involved in the synthesis of free cholesterol (FC) and lipid droplets (LD) (35).

There were some differences in the content of components in different subgroups of LDL-C. SdLD-C showed a significant decrease in free cholesterol (FC), cholesterol ester (CE) and phospholipid (PL) than large and light LDL-C. The study of biofilm and lipid bilayer shows that the incorporation of cholesterol affects the permeability of metabolites. With the increase of FC content, the accessibility of oxidants to lipid core decreased, which may be a reasonable explanation for the protective effect of LDL particles on oxidation susceptibility. The increase of FC content in LDL particles may directly regulate the susceptibility to oxidative stress and help to prevent LDL particles from undergoing subsequent oxidative modification (36). Studies have shown that the introduction of sdLDL-C into traditional M2 macrophages can inhibit the expression of EGR2, resulting in the increase of CE production and FC efflux (29), therefore, the sensitivity of sdLDL-C to lipid peroxidation increased with the decrease of FC content in each particle. Ohmura evaluated the sensitivity to lipid peroxidation modification of low density lipoprotein by the formation of conjugated diene induced by copper, which also confirmed that sdLDL-C was more sensitive to lipid peroxidation modification (36). On the other hand, due to the efflux of FC, when macrophages accumulate a large amount of FC, it is a powerful apoptosis inducer, which will lead to the release of intracellular contents and thrombosis (37). In addition, studies have shown that the plasma retention time of apolipoproteinB-100 (apoB-100) on the surface of sdLDL-C is significantly longer than that of apoB-100 on the surface of IbLDL, which increased

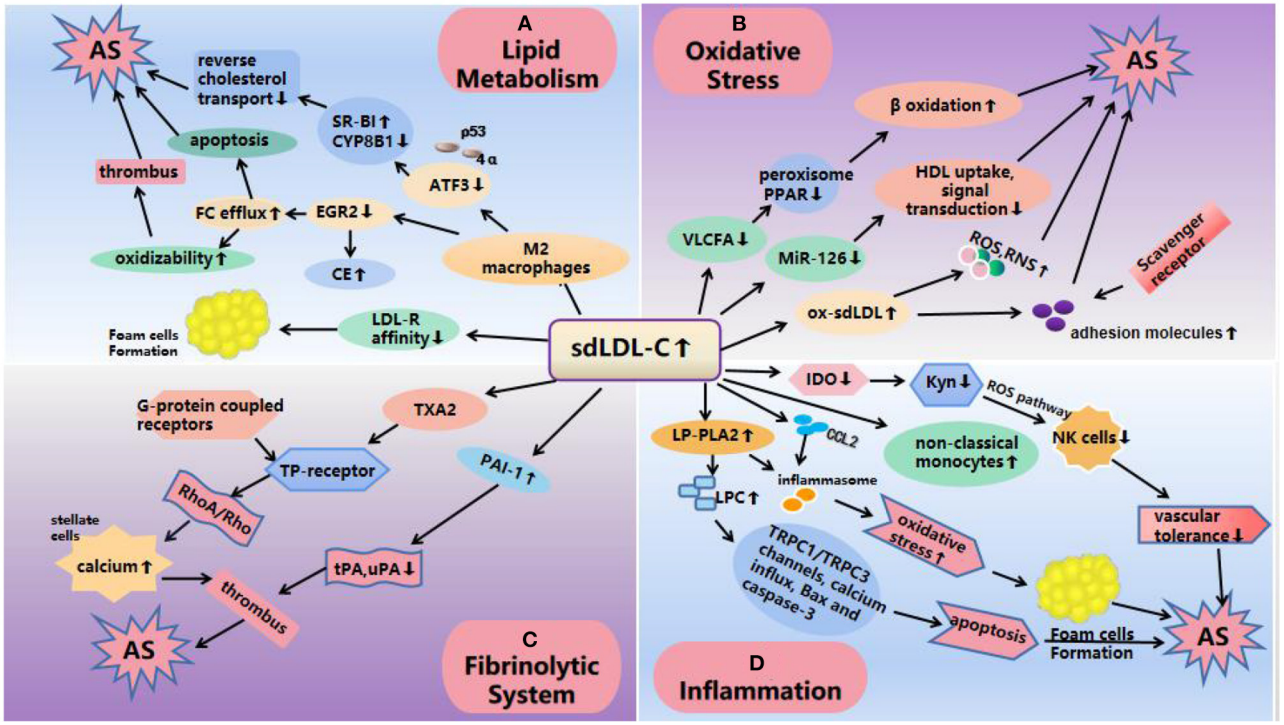

FIGURE 2 | Mechanisms of atherosclerosis induced by sdLDL-C. **(A)** Lipid metabolism: SdLDL-C reduces the expression of ATF3 and EGR2, ATF3 decreases the ability of SR-BI by interacting with p53 and 4α and promotes CYP8B1 to inhibit cholesterol reverse transport; EGR2 leads to an increase in CE production and FC outflow, thus increasing the oxidation sensitivity of sdLDL-C. On the other hand, the low affinity of apoB-100 on the surface of LDL-C receptors and sdLDL-C makes it difficult for the receptors to recognize sdLDL-C and is more easily absorbed by phagocytes to form foam cells and promote the occurrence and development of AS. **(B)** Oxidative stress: The increase of sdLDL-C level reduces the production of VLCFA and miR-126, which affects lipid metabolism and fatty acid β oxidation; miR-126 affects HDL uptake, and enhances signal transduction resulting in AS. In addition, ox-sdLDL can also increase the expression of adhesion molecules and induce excessive production of ROS and RNS, resulting in the enhancement of oxidative stress to cause AS. **(C)** Fibrinolytic system: SdLDL-C increases the levels of PAI-1 and TXA2. PAI-1 inhibits the function of u-PA and t-PA, which easily leads to thrombosis. TXA2 activates TP receptor and activates RhoA/Rho21 kinase pathway through its G protein coupled receptor, and increases calcium levels in hepatic stellate cells, resulting in vasoconstriction, platelet aggregation, thrombosis and AS. **(D)** Inflammation: SdLDL-C levels reduces IDO, causing a decrease in vascular tolerance by affecting the Kyn pathway; LP-PLA2 increased that activated TRPC1/TRPC3 channels, calcium influx, Bax and caspase-3 pathways to cause apoptosis; increased expression of inflammatory cytokines and the formation of foam cell, suggesting an inflammatory response. SdLDL-C, small, dense low-density lipoprotein cholesterol; AS, atherosclerosis; CE, cholesterol ester; FC, free cholesterol; VLCFA, very-long-chain fatty acid; ATF3, activating transcription factor 3; LDs, lipid droplets; IDO, indoleamine 2,3-dioxygenase; LP-PLA2, lipoprotein-associated phospholipase A2; LPCs, lysophosphatidylcholine; ROS, reactive oxygen species; RNS, reactive nitrogen species; PAI-1, plasminogen activator inhibitor 1; TXA2, thromboxane A2; t-PA, tissue type plasminogen activator; u-PA, urokinase type plasminogen activator.

the possibility of oxidation (38). LDL is cleared after binding to LDL-C receptors, but apoB-100 on the surface of sdLDL-C molecules and LDL-C receptors has a low affinity, which makes it difficult for the receptors to recognize sdLDL-C, and is more likely to be absorbed by phagocytes, which develop into foam cells and promote the occurrence and development of AS (39).

Current research shows that the increase of sdLDL-C level will reduce the generation of very-long-chain fatty acid (VLCFA), the ability to regulate peroxisome function and interact with peroxisome proliferator activated receptor (PPAR) was weakened (40), thus affecting lipid metabolism, fatty acid β oxidation, plasminogen (PL) biosynthesis and so on (41). This may also be one of the reasons why sdLDL-C has a stronger ability to cause AS. In addition, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to detect serum miR-126 and 122 levels in 78 patients with CAD and 60 patients without CAD in one study. There, it was suggested that miR-126 may play a role in the cholesterol metabolism of sdLDL-C. However, the mechanism by which the decrease in circulating miR-126 levels in patients with CAD is proportional to the increase in sdLDL-C is not fully understood (42). In 2012, miRNAs were reported to be important regulators of HDL metabolism and reverse cholesterol transport, including direct targeting of cellular cholesterol efflux, HDL biogenesis, liver high-density lipoprotein uptake, and the synthesis of bile acid and secretion-related genes (43). It has also been reported that miR-126 attenuates oxidized LDL (ox-LDL)-induced endothelial cell injury by inhibiting signal transduction to delay AS (44, 45). Therefore, a larger sample cohort is needed to further explain the role of miR-126 in sdLDL-C cholesterol metabolism and to understand the development of the disease (42) (**Figure 2**).

sdLDL-C Promotes AS by Inducing Inflammation

Monocytes play an important role in the early formation and maturation of plaques. They are drawn to the arteries by chemokines, such as CCL2, which are secreted by activated epithelial tissue cells (46–49) and take up lipids among the subendothelial tissue to differentiate into foam cells (50). In addition, they can also engulf precipitated cholesterol crystals (51) and oxidized lipid species (52–54) that activate the inflammasome, resulting to cell death in a highly inflammatory form called prolapse, as well as the induction of innate immune responses (51). Human monocytes are mainly divided into three types: classical ($CD14^+CD16^-$), non-classical ($CD14^-CD16^+$), and intermediate ($CD14^+CD16^+$) (55). Supported by proof from murine studies (56, 57), as well as current human observations (58), classical monocytes are believed to have the ability to differentiate into monocyte-derived macrophages and monocyte-derived dendritic cells (59) and play an indispensable role in the formation and regression of tissue inflammation. Related studies have shown that the production of sdLDL-C is related to an increase the number of non-classical monocytes and a decrease in the number of classic monocytes (60). The specific mechanism has not been detailed, but we speculate that the effect of sdLDL-C on AS may be related to the inflammatory response of monocytes. In 2017, monocytes from healthy people (stenosis degree <5%) and patients (stenosis degree >70%; single-vessel disease, two-vessel disease, three-vessel disease) were separated using a Rosette Sepkit, and macrophage colony stimulating factor (M-CSF) was used to induce them to differentiate into M2 macrophages. qRT-PCR and ELISA were used to detect MRC1 gene expression and histamine levels, respectively. After sdLDL-C treatment, the expression level of MRC1 in normal human M2 macrophages was significantly increased ($P = 0.05$), while the expression level of MRC1 gene was decreased in patients with single-vessel disease ($P = 0.05$), two-vessel disease ($P = 0.01$), and three-vessel disease ($P = 0.9$). The histamine levels secreted by M2 macrophages (after treatment for 7 day) in the case group were higher than that in the healthy control group (>3-fold, $P = 0.02$). The results illustrated that sdLDL-C granules decreased the expression of MRC1 in differentiated M2 macrophages from patients with CHD. In addition, they have a strong ability to secrete histamine (61).

Hassanpour et al. detected the effect of sdLDL-C on the changes in IDO in differentiated macrophages using RT-qPCR, colorimetric, and ELISA methods. Their results show that sdLDL-C reduces the expression and activity of IDO in macrophages (62), IDO is the first and rate-limiting enzyme in the tryptophan (Trp)-degraded kynurenine (Kyn) pathway, and its downstream metabolite is collectively called kynurenines (63). The expression of IDO was inhibited, the metabolic pathway of Trp was blocked, the formation of Kyn was decreased, the ability of cell death mediated by kyn through reactive oxygen species (ROS) pathway in natural killer (NK) cells decreased (64), vascular tolerance decreased, and promoted the occurrence and development of inflammation and AS (65, 66). In addition, ATF3 (67, 68) and EGR2 (69, 70) play crucial roles in signal transduction in the process of anti-apoptosis, anti-migration, and anti-inflammation. sdLDL-C also promotes inflammation and accelerates AS by inhibiting the expression of AFT3 and EGR2 (32).

Lipoprotein-associated phospholipase A2 (Lp-PLA2) hydrolyzes phospholipids and releases pro-inflammatory products; therefore, it is considered to be a new biomarker of vascular risk (71–74). Among the phenotypes of LDL, Lp-PLA2 preferentially binds to small, dense LDL particles (72) to produce lysophosphatidylcholine (LPC). LPC can promote the expression of inflammatory factors (75), damage arterial relaxation, increase oxidative stress, induce endothelial activation and atherosclerosis (76). SdLDL-C granules contain more Lp-PLA2. Studies have confirmed that LPC can induce apoptosis of human coronary artery smooth muscle cells by activating TRPC1/TRPC3 channels, calcium influx, Bax and caspase-3, and lead to atherosclerosis and coronary artery disease (77), which may also be a mechanism of AS induced by sdLDL-C. In addition, higher concentrations of LPC can destroy the integrity of mitochondria and enhance the release of cytochrome C in hepatocytes (78). We speculate that sdLDL-C may lead to AS through mitochondrial damage, which may be a potential mechanism (**Figure 2**).

sdLDL-C Promotes AS by Enhancing Endothelial Injury

Vascular epithelial tissue cell pathology plays an important role in the initiation and development of AS (79, 80). Endothelial cell injury increases intimal permeability and leukocyte adhesion, promoting thrombus formation and rapid malady progression (81). Both ox-LDL and cholesterol cause functional damage to the arterial intima, change the surface characteristics of endothelial cells and leukocytes (monocytes and lymphocytes), and increase the expression of adhesion molecules. The number of monocytes adhering to endothelial cells increases and gets transferred from endothelial cells to subintimal macrophages, which are then transformed into foam cells via scavenger receptor phagocytosis of ox-LDL, forming the earliest lipid streaks of atherosclerotic lesions (82–88).

Ox-LDL induces an excessive production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) *in vivo*, which induces antioxidant defense; however, the degree of oxidation exceeds the scavenging ability of oxides, resulting in tissue damage (89–92). SdLDL-C particles have the characteristics of a small size, low density, large surface area, long retention time *in vivo*, and induces great damage to endothelial cells, which leads to their increased permeability, chemotaxis of monocytes in blood vessels to form macrophages, and phagocytosis of oxidized LDL-C to form foam cells, thus further supporting the formation of AS (93). In addition, there are a few polar molecules on the surface of sdLDL-C particles, and their affinity with proteoglycans on arterial intima is enhanced, so they can easily adhere to the vascular wall and enter vascular endothelial cells, resulting in vascular endothelial damage and promoting the occurrence and development of AS (38, 94). This was verified by an experiment on the modification of LDL by methylglyoxal (MG). The modification of LDL by MG resulted in a significant

decrease in the particle size of LDL similar to that of sdLDL. Vortex-stimulation showed that sdLDL-C showed higher PG aggregation rate and degree than unmodified LDL (95).

SdLDL-C not only injures the vascular endothelium but also activates the fibrinolytic system and produces plasminogen activator inhibitor 1 (PAI-1) (96–99) and vasoconstrictor thromboxane A2 (TXA2) (100, 101), thus promoting AS (93). A previous study found that plasma PAI-1 levels were positively correlated with the concentration of sdLDL-C (102). PAI-1 inhibits the function of t-PA and u-PA by binding to tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) in a ratio of 1:1, The increase of PAI-1 expression *in vivo* will inhibit the normal fibrinolytic system, which is easy to lead to thrombosis (103). TXA2 activates TP receptors, activates RhoA/Rho kinase pathway through its G-protein coupled receptors, and increases calcium levels in hepatic stellate cells (HSC), resulting in vasoconstriction, platelet aggregation, thrombosis and atherosclerosis (104). Therefore, compared with LDL-C, sdLDL-C has a stronger effect on AS, and the damage it induces to the blood vessel wall lasts longer (105) (**Figure 2**).

ANTI-AS INTERVENTIONS INVOLVING SDLDL-C

Non-medicinal Interventions

SdLDL-C levels are closely associated with meal compositions and dietary habits. Almonds have been shown to reduce LDL-C levels; however, there is limited data regarding their effects on dyslipidemia characterized by accrued levels of VLDL and sdLDL-C particles that are related to abdominal fat and high carbohydrate intake (106). A current meta-analysis of randomized controlled clinical trials found that consumption of almonds can reduce plasma TC concentration by 0.15 mmol/L, TG concentration by 0.07 mmol/L, and LDL-C concentration by 0.12 mmol/L (107). Studies have shown that almonds and almonds with dark chocolate and cocoa ingested for four weeks have a good effect on the levels of lipids, lipoproteins, and apolipoproteins, and the combined consumption of dark chocolate, cocoa, and almonds significantly reduces levels of sdLDL-C, apoB, and the ratio of apoB/apoAI, which in turn are expected to reduce the risk of CHD (108). Avocados are a nutritious source of monounsaturated fatty acids (MUFAs), which are rich in antioxidants. Avocados have an extra effect of reducing LDL-C levels, especially sdLDL-C particles, which are prone to oxidation in the body and are associated with an increased risk of CVD (109). In another randomized, crossover, controlled feeding study of patients with elevated LDL-C levels, compared with a daily intake of pistachios (32–63 g), a twice-daily intake of pistachios (63–126 g) significantly decreased sdLDL-C levels within four weeks. This experiment adopted a double-blind crossover design in which 30 postmenopausal women with moderate hypercholesterolemia were randomly assigned to two 35-day diets supplemented with corn oil or partially hydrogenated soybean oil to diets providing energy intake for weight maintenance. The results illustrated that the decrease in sdLDL-C concentration was positively correlated with a decrease in TG (110). In a randomized, double-blind, crossover study, subjects ate 10 grams of flaxseed oil or corn oil at dinner once a day, containing 5.49 and 0.09 g α-linolenic acid, respectively. Blood samples were collected at 0.4 and 12 weeks for the analysis of serum lipids, lipid-related proteins, serum fatty acids and serum sdLDL-C. Flaxseed oil supplementation significantly decreased the concentration of sdLDL at 4 and 12 weeks (111).

A study by Mendoza et al. strengthened the relationship between weight loss and reduced sdLDL-C levels. After weight loss, the concentration of apoC-III decreased, and the average BMI decreased from 27 to 25 kg m^2, which was related to an increase in the peak particle diameter of LDL and a decrease in serum concentration of sdLDL-type (112). In addition to dietary intervention, exercise can effectively reduce the risk of cardiovascular disease (113). One hundred participants from the RESOLE trial (ages 50–70) were followed up for a year, starting with a three-week accommodation program that combined high exercise (15–20 h per week), diet restriction (500 kcal/day), and education. Forty age-matched healthy controls were recruited as a baseline reference. Lipoprint® electrophoresis was used to evaluate the distribution of lipoprotein subfractions in these subjects, allowing separation, and the results showed that sdLDL-C concentration decreased significantly after a 3-week residence plan (114). Another study conducted a six-month intervention on 30 hyperlipidemic subjects (12 males, 18 females; mean age, 64 years), focusing on moderate increases in physical activity. Clinical data before and after the intervention were observed. In addition to determining the average particle size of LDL and diacron reactive oxygen metabolites (d-ROMs) via gel electrophoresis, the risk factors for AS were also determined. The average LDL particle size after intervention was significantly larger than that before intervention (26.9 ± 0.3 vs. 27.1 ± 0.4 nm, mean ± SD, $P < 0.01$), whereas the level of sdLDL-C decreased significantly (115) (**Table 2**).

Medicinal Interventions

Statins

It is well-known that statins can effectively regulate blood lipid levels and delay the process of atherosclerosis. A study recruited 12 white men with metabolic syndrome. All subjects were treated with pitavastatin (4 mg/day) and their blood lipid levels were measured after 180 days. The results found that pitavastatin not only lowered LDL-C (−38%), sdLDL-C (sd-LDL4) and (sd-LDL5) are also effectively reduced (−27 and−33%, respectively). However, the sample size of this experiment is too small, and the sample size needs to be expanded to prove this point of view (116). A prospective, randomized, open-label, multicenter, parallel grouping comparative trial was conducted in Japan from October 2011 to November 2012. Eligible subjects (people at high cardiovascular risk over the age of 20) were treated with high-dose statins and conventional statins, respectively. The high-dose treatment group took 5 mg of rosuvastatin a day for the first four weeks, and then 10 mg a day for 8 consecutive weeks, and the low-dose statin group took 2.5 mg of rosuvastatin a day for 12 consecutive weeks. Lipid measurements were taken before, 4 and 12 weeks. The results show that both groups can reduce oxidized

TABLE 2 | Studies of non-medicinal intervention of sdLDL-C against AS.

Intervention	Method	Subjects	Targets	Effect	References
Diet	Almond	Atherogenic dyslipidemia	sdLDL-C, TC↓	Help in the maintenance of healthy blood lipid levels	(101)
	Almonds or dark chocolate	Overweight and obese individuals	sdLDL-C, LDL-C, TC↓	Improves lipid profiles	(103)
	Avocados	Overweight and obese individuals	oxLDL, sdLDL-C↓ plasma lutein↑	Reduce ox-LDL concentration and prevent AS	(104)
	Pistachios	Healthy adults	sdLDL-C, TG↓, HDL↑	Reduce cardiovascular risk	(105)
	Flaxseed oil	Healthy men	TC, LDL-C, ApoB SdLDL-C↓	Reduce sdLDL-C concentrations	(106)
Exercise	Weight loss or high carbohydrate	Overweight men	ApoB, ApoC, TG, sdLDL-C↓	Reduce sdLDL-C generation	(107)
	Physical exercise	Participants (50–70 years)	LDL-C, sdLDL-C↓, HDL↑	Improve carotid-intima-media thickness	(109)
	Moderate physical activity	30 hyperlipidemic subjects	d-ROM, sdLDL-C↓	Improve blood lipids	(110)

SdLDL-C, small, dense low-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL, high-density lipoprotein; oxLDL, oxidized low-density lipoprotein; TG, triglyceride; TC, total cholesterol; ApoB, apolipoprotein B; ApoC, apolipoprotein C; d-ROM, diacron reactive oxygen metabolites.

low-density lipoprotein cholesterol and sdLDL-C, and the effect of the high-dose group is more obvious (117). Although the high-dose group of statin therapy is effective in improving blood lipid levels, some cardiovascular events continue to occur. Therefore, high-dose statin therapy is recommended for the initial treatment of patients with high risk of atherosclerotic vascular disease (118). Another *in vitro* experiment evaluated the effect of combination therapy of Eicosapentaenoic Acid (EPA) and atorvastatin on endothelial cell function under oxidative stress conditions by measuring the release of NO and peroxynitrite (ONOO–) from human umbilical vein endothelial cells (HUVECS) to examine the comparative and time-dependent effects of these agents on endothelial dysfunction. Data shows that the combined treatment of EPA and atorvastatin can effectively reduce the level of sdLDL-C, thereby improving endothelial dysfunction, which may be because EPA contains substances that inhibit the oxidation of ApoB particles, which has a stronger antioxidant effect (119). However, some studies have shown that compared with patients who received atorvastatin for <6 days, patients who received atorvastatin for more than 90 days had significantly lower total cholesterol and LDL-C levels, but slightly lower sdLDL-C levels increased, but not significant ($p = 0.06$) (120).

Fibrates

Fibrates are agonists of peroxisome proliferator-activated receptor-α (PPAR-α), which regulate lipoprotein metabolism through transcription factors. Fibrates have shown effects in reducing fasting and postprandial TG and TG-rich lipoprotein residual particles (121). A meta-analysis of 13 studies illustrated that fibrates can reduce triglyceride levels, increase HDL-C levels, reduce the proportion of sdLDL-C, fibrates could be effective in secondary prevention considering a compound objective of non-fatal stroke, non-fatal myocardial infarction, and death of cardiovascular origin, and have fewer side effects; the most widely used drug of this class is fenofibrate (122). Other studies have also proved this point (123, 124). A retrospective study included 72 patients with type 2 diabetes. All patients received pemafibrate 0.2 mg (0.1 mg twice daily) for 24 weeks. During the entire study period, all patients did not change their exercise or diet regimens. The results show that Pemafibrate significantly reduces the levels of TG and sd-LDL-C, improves the composition of LDL and may reduce the risk of cardiovascular disease (125). It also has a better benefit-risk balance than conventional fibrates and can be applied to patients who find it difficult to use existing fibrates, such as those taking statins or those who have renal insufficiency (126).

Ezetimibe

Ezetimibe is a novel drug used for the treatment of dyslipidemia, which resists cholesterol absorption by inhibiting Niemann pick C1 like protein (NPC1L1) (127). Ezetimibe alone or in combination with statins can reduce the level of sdLDL-C (128). From October 2014 to November 2015, the author recruited patients with type 2 diabetes who had normal LDL-C levels and received statin therapy at the outpatient clinic of their institution. A total of 50 patients (31 men and 19 women) were enrolled in this study, and all subjects were randomly assigned to receive statins (statin group) or fenofibrate (160 mg/day) and ezetimibe treatment (10 mg/day), the results showed that the combination of fenofibrate and ezetimibe can effectively control the levels of sdLDL-C and TG, increase the level of HDL-C, and improve the vascular function of patients with type 2 diabetes. The effect of this combination is even better than treatment with statins alone (129). Therefore, to reduce the level of blood lipids using ezetimibe, it may be more beneficial if it is combined with other drugs. Current studies have illustrated that fenofibrate combined with ezetimibe can improve sdLDL-C levels and vascular function compared with statins (129).

Niacin

Niacin inhibits AS by activating the anti-inflammatory G protein-coupled receptor Gpr109a, also known as hydroxycarboxylic acid

receptor 2 (HCA2), expressed on immune cells, inactivating the immune response and adventitious inflammatory cell infiltration (130). Niacin treatment was shown to decrease total cholesterol, triglyceride (20–50% decrease), and LDL-C levels. Additionally, niacin decreased sdLDL-C levels, leading to a shift to massive buoyant LDL particles, delaying the progression of AS (7). However, the negative effects of nicotinic acid were in accordance with the results of the HPS2THRIVE and AIM-HIGH trials, which suggests that its clinical application requires further study (131, 132).

Omega-3 Fatty Acids

Omega-3 fatty acids are essential fatty acids found in certain fish and vegetables. These are necessary for growth and development. Numerous studies have reported that omega-3 fatty acids scale back plasma triglycerides and increase HDL levels. They have been reported to inhibit blood platelet aggregation, improve endothelial function, decrease oxidative stress, and act as a potent medication agent (133). Changes in blood lipid and lipoprotein profiles were also observed after omega-3 fatty acid treatment for 8 weeks. The results of one study also showed that sdLDL-C levels decreased significantly after intake of omega-3 fatty acids (134). The omega-3 fatty acid EPA has substiantial antioxidant activity and can protect the membrane structure, which may promote scavenging of free radicals in sdLDL-C and membrane bilayers (135).

Other Western Medicine

Proprotein convertase subtilisin/kexin type 9 (PCSK9) belongs to the proprotein convertase family of enzymes that degrades LDL-R, which directly mediates the degradation of LDL-R in lysosome, which in turn increases plasma LDL level (136, 137). PCSK9 is positively correlated with sdLDL-C levels (138). At present, PCSK9 inhibitors (PCSK-9i) have been clinically used to reduce cholesterol levels and cardiovascular events in patients (139). In addition, resin and orlistat can also reduce sdLDL-C levels (140). Baricitinib treatment can also increase LDL levels and reduce sdLDL-C particles. One of the mechanisms by which baricitinib and related interventions increase the particle size of LDL-C may be the increased activities of phospholipase A2, liver lipase, lipoprotein lipase, and endothelial lipase. It has been reported that these enzymes are increased in a state of chronic inflammation (141).

CONCLUSION AND PERSPECTIVES

Lipid metabolism disorder is an important factor leading to AS. A large amount of evidence shows the pathogenic role of increased LDL-C in AS, and SdLDL-C, as a subgroup of LDL-C, has been proved to be a specific index for the detection of AS. Compared with traditional lipid monitoring, sdLDL-C monitoring has better sensitivity and specificity, and has better clinical value in predicting AS (7, 9). At present, there are many methods for the detection of sdLDL-C, but most of them have some limitations which cannot be widely used in the laboratory and clinic because they require expensive equipment, are time consuming, labor-intensive, and other reasons. The Lipoprint LDL system is currently the mainstream method for detection of sdLDL-C because it utilizes linear polyacrylamide gel electrophoresis to separate low-density lipoprotein according to particle size and charge, which has advantages of high efficiency, high speed, and low materials consumption (6).

Because of its small size and higher density compared to larger LDL-C particles, sdLDL-C has a greater ability to penetrate the artery wall, in addition to having a longer half-life and greater susceptibility to oxidative modification. Cardiovascular diseases caused by abnormal sdLDL-C are reflected in many clinical cases. SdLDL-C plays a variety of roles in the process of AS, such as affecting lipid metabolism, promoting the release of inflammatory factors leading to inflammatory reaction, releasing excessive ROS and RNS to produce oxidative stress, activating fibrinolytic system to produce thrombus. At present, there are non-drug interventions, such as regulating diet (low carbohydrates, soybeans, corn oil, etc.). Proper exercise can effectively improve the level of sdLDL-C in patients. Medical interventions, such as statins, fibrates, ezetimibe, niacin and omega-3 fatty acids, can reduce sdLDL-C levels in the body. The main mechanism is to improve the level of blood lipids and vascular endothelial function. In recent years, Pemabet and PCSK9 inhibitors have become the focus of current research as new interventions to prevent AS.

The specific mechanism of atherosclerosis caused by sdLDL-C has not been fully explained, and it is still being explored. Further exploration of the specific mechanism and intervention measures of sdLDL-C may provide a new direction for clinical prevention, evaluation and treatment of AS. Some studies have shown that the concentration of sdLDL-C is related to the changes of gene loci, and we speculate that gene detection may provide a new reference for the study of sdLDL-C. We know that intestinal microflora is significantly associated with lipid metabolism. A randomized controlled trial shows that changing intestinal microflora in patients with hyperlipidemia can effectively reduce sdLDL-C levels in patients with hyperlipidemia (142). However, the related research on the effect of intestinal flora on sdLDL-C is insufficient, whether it can reduce the level of sdLDL-C by improving intestinal flora, so as to reduce AS, is worthy of further study. In conclusion, these studies on the role of sdLDL-C in AS may provide information regarding new targets for the prevention and treatment of AS.

AUTHOR CONTRIBUTIONS

XJ and MW designed the article and wrote the manuscript. SY and JL searched the literature and aided in the design of the illustrations. All authors contributed to the article and approved the submitted version.

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HDL Composition, Heart Failure, and Its Comorbidities

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Although research on high-density lipoprotein (HDL) has historically focused on atherosclerotic coronary disease, there exists untapped potential of HDL biology for the treatment of heart failure. Anti-oxidant, anti-inflammatory, and endothelial protective properties of HDL could impact heart failure pathogenesis. HDL-associated proteins such as apolipoprotein A-I and M may have significant therapeutic effects on the myocardium, in part by modulating signal transduction pathways and sphingosine-1-phosphate biology. Furthermore, because heart failure is a complex syndrome characterized by multiple comorbidities, there are complex interactions between heart failure, its comorbidities, and lipoprotein homeostatic mechanisms. In this review, we will discuss the effects of heart failure and associated comorbidities on HDL, explore potential cardioprotective properties of HDL, and review novel HDL therapeutic targets in heart failure.

Keywords: high-density lipoprotein (HDL), apolipoprotein A-I, apolipoprotein M, sphingosine-1-phosphate, heart failure, cardiomyopathy

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INTRODUCTION

Cardiovascular disease (CVD) is a leading cause of mortality worldwide (1). Heart failure (HF) is a common result of cardiometabolic disease and a major contributor to CVD mortality (2). The prevalence of HF in the developed world is rising and is estimated to be at 2%, while the incidence approaches 5–10 per 1,000 persons per year (3). HF is a clinical syndrome, typically presenting with symptoms of dyspnea, fluid retention, and decreased exercise tolerance. It usually follows structural or functional disorders of the endocardium, myocardium, or pericardium and is divided into three categories: HF with reduced ejection fraction (HFrEF), HF with preserved ejection fraction (HFpEF), and HF with mid-range ejection fraction (4, 5).

Multiple rationale suggest a mechanistic link between lipoproteins and HF. Interestingly, in HF patients, plasma cholesterol concentrations are inversely associated with mortality (6, 7). This observation, termed the "cholesterol paradox," could be related to malnutrition, cachexia (8, 9), and inflammation (10–14) observed in HF patients, as well as direct effects of lipoproteins on the myocardium. Moreover, recent Mendelian randomization studies support a causal effect of low-density lipoprotein cholesterol (LDL-C) and triglycerides on LV mass and myocardial remodeling (15). Analogously, a clinical trial showed that reconstituted high-density lipoprotein (HDL) infusion shortens cardiac repolarization, demonstrating the capability of HDL to alter cardiac electrophysiological properties (16). Both studies exemplify a direct role of lipoproteins on the myocardium. Furthermore, lipoproteins can function as a fuel source, an important consideration in HF patients, where the energy-starved myocardium primarily consumes ketone bodies and fatty acids (17).

Based on two large randomized trials, a case could even be made for statin use in HF patients, thus LDL-C lowering *via* statins is unlikely to exacerbate HF outcomes (18, 19). We hypothesized that decreased HDL or HDL-associated apolipoproteins could be a driver of adverse HF outcomes (18, 19). High-density lipoprotein cholesterol (HDL-C) is inversely associated with CVD risk, as large epidemiological studies, such as the Framingham Heart Study have shown (20). Nonetheless, multiple randomized trials have failed to show a decrease in CVD risk or major adverse cardiac events when increasing HDL-C levels as a therapeutic target (21, 22). One interpretation of these findings is that, rather than the steady-state cholesterol mass, HDL or its associated apolipoproteins could exert beneficial effects in the setting of HF (or even CVD or other cardiac inflammatory disorders). For instance, our group has shown that reduced pre-transplant HDL cholesterol efflux capacity is associated with the progression of cardiac allograft vasculopathy, a major cause of mortality for cardiac transplant recipients (23). This example served as a proof-of-paradigm that HDL functions may be relevant outside of traditional atherosclerosis. The cardioprotective role of HDL may be related to its anti-oxidant and anti-inflammatory properties, endothelial protection, as well as its reverse cholesterol transport capacity (24).

Many pre-clinical studies performed mainly in rodents focus on the effect of HDL in cardiac pathophysiology and have shown positive effects on the myocardium. For instance, HDL can reduce infarct size in the setting of cardiac ischemia/reperfusion injury, attenuate apoptosis, preserve mitochondrial function, and protect the myocardium against oxidative stress (25–31).

Although a broad range of anti-atherogenic properties have been attributed to HDL, many are independent of its cholesterol content and reverse cholesterol transport. The heterogeneous properties of HDL particles are relatively complex, due to the wide variety of proteomic and lipidomic cargo of the particles. These characteristics lead to specific cardioprotective functions, such as increased endothelial nitric oxide (NO) production, reduced inflammation in endothelial cells and macrophages, stimulation of insulin-independent glucose uptake in the myocardium, among others. For example, the anti-oxidative capacity of HDL is mainly attributed to its ability to protect LDL from oxidation by free radicals. Of note, antioxidant components of HDL, such as the HDL-associated enzyme Paraoxonase 1 (PON1), metabolize lipid hydroperoxides and prevent their accumulation in LDL particles, decreasing LDL endocytosis by macrophages and formation of foam cells, thus averting the formation of atherosclerotic plaque (32–37).

Recent advances in proteomic characterization have led to the identification of novel HDL subclasses that will, in all likelihood, eventually supersede the historical size and density-based characterization system (38, 39). For historical reference, larger HDL2 particles are inversely associated with CVD risk, while smaller, denser HDL3 subclass exerts anti-atherogenic, anti-oxidant, and anti-inflammatory functions (40, 41), and these subclasses are also associated with mortality in acute HF patients. Total and small HDL particles (diameter < 8.8 nm, mostly HDL3), measured by nuclear magnetic resonance spectroscopy, were inversely associated with 3-month mortality in patients with acute HF, while both large HDL and HDL-C demonstrated no significant association (42). Similarly, in HFrEF and HFpEF patients, total and small HDL were inversely associated with adverse outcomes (43).

To the best of our knowledge, the rigorous analysis of HDL proteomics has yet to be performed in advanced HF cohorts. Nonetheless, multiple preclinical and human epidemiological studies support the concept of pleiotropic effects of HDL-associated apolipoproteins (44, 45), which may play a significant role in the pathogenesis of HF. These observations led us to hypothesize that specific apolipoproteins and enzymes associated with HDL particles may potentially explain the cholesterol paradox and the underlying cardioprotective effects of HDL, which could be relevant therapeutic targets in HF. In this review, we will discuss the effects of HF and associated comorbidities on HDL, explore potential cardioprotective properties of HDL, and review novel HDL therapeutic targets in HF.

EFFECTS OF HEART FAILURE AND ASSOCIATED COMORBIDITIES ON HDL

Advanced HF is a multisystem syndrome often identified in patients with multiple cardiometabolic comorbidities; hence, both HF and its associated comorbidities can have complex effects on lipoprotein biology. Hepatic, renal, and gastrointestinal malperfusion secondary to reduced cardiac index and increased filling pressures all contribute to a vicious cycle of decreased nutritional intake, increased inflammation, metabolic stress, perturbations that can have important effects on lipoprotein homeostasis (**Figure 1**).

Effects of Chronic Inflammation on HDL

HF is characterized by a chronic inflammatory state. While the increase in pro-inflammatory cytokines in HF has been well-documented, there is still debate regarding the extent to which increased cytokines are directly responsible for deleterious results or are simply a reflection of the ongoing pathophysiological processes (46–49). Nevertheless, chronic inflammatory states, such as that observed in HF, can affect plasma HDL levels, composition, and overall function. For instance, plasma HDL contains lower cholesterol ester levels, higher free cholesterol, triglycerides, and fatty acids under inflammatory states (50). Moreover, inflammation strips HDL from key proteins that are important for its normal function (e.g., lecithin-cholesterol acyltransferase (LCAT), cholesteryl ester transfer protein, and transferrin), as well as certain important apolipoproteins (e.g., apolipoproteins A-I and M) (51–55). Apolipoprotein A-I (ApoA-I) is the primary mediator of cholesterol efflux, the key rate-limiting step of reverse cholesterol transport, and the main protein component of HDL particles (56–59). In the same context, apolipoprotein M (ApoM), a cardioprotective apolipoprotein (45), is a negative acute response protein, levels of which decrease in response to inflammation and infection (52, 60, 61). The decrease in HDL levels and alteration of its structural composition in inflammatory states impair the reverse cholesterol transport process and HDL's anti-inflammatory

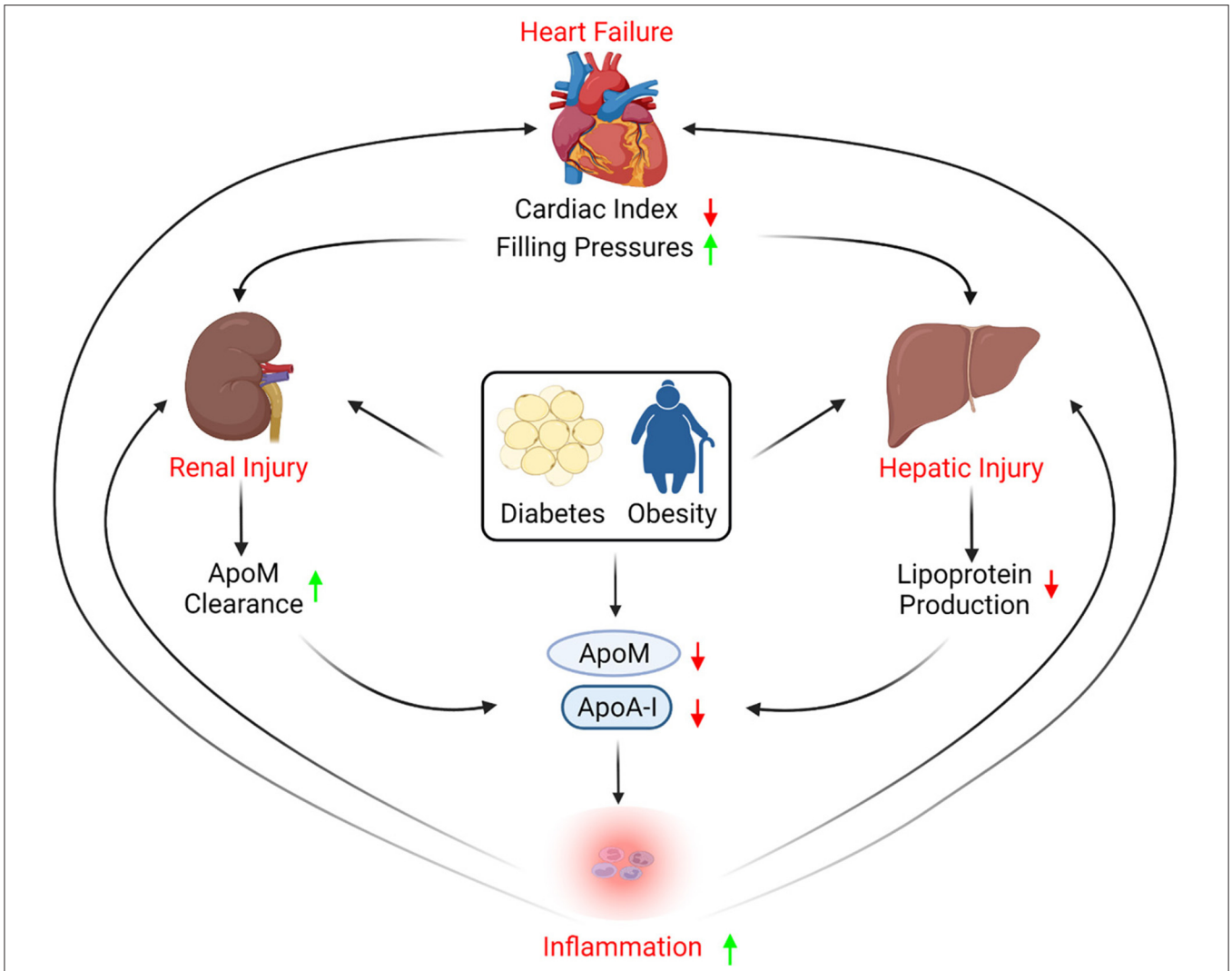

FIGURE 1 | Apolipoproteins role in heart failure progression. Heart failure causes reduced cardiac index and increased filling pressures, which subsequently leads to hepatic injury that can affect apolipoprotein production. In addition, heart failure-induced kidney injury may increase renal excretion of apolipoprotein M (ApoM). Co-morbidities such as diabetes and obesity are also known to reduce circulating apolipoproteins, contributing to inflammation, thus exacerbating kidney and hepatic injury, and provoking further cardiac dysfunction.

and anti-oxidant properties. In the long run, this can lead to the development of atherosclerosis and increased risk of CVD and HF.

How inflammation affects HDL particle number and composition is not very well-understood. In mice, endotoxin directly impairs active cholesterol efflux by ATP-binding cassettes A1 and G1 (ABCA1 and ABCG1) transporters, as well as scavenger receptor class B type I (SR-B1) mediated passive diffusion (62–64). Meanwhile, inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukins 1 and 6 (IL-1β, IL-6), upregulate the expression of endothelial lipase (EL), which exhibits an inverse association with HDL levels (65, 66). Badellino et al. showed that experimental administration of low-dose endotoxin in humans decreases HDL phospholipid, corresponding with EL peak concentration (67). Tietge et al. (68) reported that mice that overexpress secretory phospholipase A2 have changes in HDL composition, and under inflammatory conditions, exhibit increased HDL catabolism. Interestingly, in a murine model of pressure overload-induced HF, EL knockout exacerbated cardiac dysfunction compared to wild-type controls, consistent with the hypothesis that EL provides an alternative pathway for free fatty acid uptake as a source of energy and protects the failing myocardium (69). Thus, it is plausible that chronic inflammation may be upregulating the expression of EL, which leads to HDL catabolism to release fatty acids to the energy-starved myocardium at the expense of other cardioprotective components of the HDL particle.

Effects of Renal Dysfunction on HDL

Normal renal function is crucial for proper HDL function (70). Renal dysfunction induces pathologic alterations in lipoprotein metabolism in general, and HDL in particular (71). HF can

induce renal dysfunction, which is a strong independent predictor of poor cardiovascular outcomes (72, 73). Cardiorenal syndrome is a term that describes the mutual interaction between the heart and kidneys, considering that injury to one of the organs usually causes dysfunction of the other (74).

Renal dysfunction and the associated chronic inflammatory state present in cardiorenal syndrome correlate with increased oxidative stress across multiple systems (75). Oxidized HDL (ox-HDL) is a modified HDL observed during conditions of increased oxidative stress and reduced anti-oxidant capacity present in cardiorenal syndrome (76, 77). Various HDL and ApoA-1 post-translational modifications can result in ox-HDL formation (76, 78, 79), which has been linked to an increased risk of cardiovascular events (80). Myeloperoxidase (MPO) can modify ApoA-I leading to ox-HDL that is less avid in its ability to bind SR-BI receptors and dysfunctional for normal cholesterol efflux activity (81, 82). Conversely, hypochlorite-generated ox-HDL exhibits increased affinity toward SR-BI, albeit with less cholesterol efflux capacity than normal HDL (83). We propose that various post-translational modifications (for example, MPO adducts) might alter specific ox-HDL characteristics (for example, higher vs. lower affinity toward SR-BI); nonetheless, renal dysfunction can contribute toward "dysfunctional" HDL particles. Moreover, ox-HDL exhibits diminished endothelial nitric oxide synthase (eNOS) mediated endothelial protective function as well as anti-apoptotic activity, which leads to impaired endothelial repair and increased pro-inflammatory activation (84–86).

In a proteomic analysis of HDL in uremic patients, isolated HDL particles lost their anti-inflammatory properties and induced the production of inflammatory cytokines (87). HDL isolated from these patients contained high levels of serum amyloid A (SAA), a protein known to promote inflammatory cytokine production and impair the anti-inflammatory capacity of HDL (87). Furthermore, HDL from uremic patients may contribute to the systemic inflammatory state in chronic kidney disease patients by decreasing apoptosis of polymorphonuclear leukocytes (88).

Effects of Diabetes on HDL

Diabetes is a pathophysiological process that can significantly impact the biogenesis of HDL, and cause alterations in myocardial metabolism, impairing metabolic flexibility and leading to diabetic cardiomyopathy (89). Oxidative stress, intramyocardial inflammation, cardiac fibrosis, and cardiac apoptosis all contribute to diabetic cardiomyopathy (90), which can in theory be mitigated by the anti-inflammatory and anti-oxidative functions of HDL.

In diabetes, hyperglycemia-induced advanced glycation end products, oxidative stress, and inflammation can negatively affect normal HDL function and composition, potentially contributing to an increased risk of HF (91). Glycated HDL loses atheroprotective properties and cholesterol-accepting capacity, leading to the acceleration of atherosclerosis (92). HDL isolated from diabetic patients is also rendered ineffective concerning endothelial protective function (93). Many of the pleiotropic effects of HDL are attributed to ApoM-bound sphingosine-1-phosphate (S1P), which is diminished in diabetic patients mainly due to glycation of ApoM that results in the impaired binding capacity to S1P (94).

Effect of Obesity on HDL

Obesity has been established as a major risk factor for hypertension, CVD, and left ventricular hypertrophy, all risk factors for the development of HF (95). Obesity is associated with reduced HDL-C. In a large cross-sectional study, HDL-C is inversely associated with body mass index (BMI) (96). Obesity can also affect HDL subclasses and metabolism likely reflecting an underlying change in key HDL proteins and lipids (97, 98). Plasma ApoA-I exhibits a linear inverse correlation with BMI (99), while ApoM is also reduced in obese individuals (100) and is inversely associated with non-alcoholic fatty liver disease (NAFLD) (101), another comorbidity associated with obesity and an emerging risk factor for HF, in particular HFpEF (102).

Proteomic studies of HDL in patients with obesity and other comorbidities have also been informative. In NAFLD patients, quantitative changes occur in the HDL proteome, relative to morbidly obese patients without steatosis (103). One challenging aspect of studying comorbidities related to obesity is selecting the best control or reference population. Comparing obese patients with comorbidities of obesity vs. more metabolically healthy obese patients will likely minimize differences between groups. Another challenge is that many unmeasured confounders might be associated with obesity. Further prospective studies are required to unravel the complex interactions between obesity and its comorbidities, including HF, and how these interactions might be mediated by lipoproteins. In particular, the need for prospective studies is highlighted by the focus of older literature on HDL subclasses, and new studies suggesting that meals of various fat compositions can acutely affect the HDL proteome (104). Altogether, obesity itself, or its comorbidities, may alter HDL proteomic and lipidomic contents, impairing potential cardioprotective functions. This area is both complex and rapidly evolving.

Effect of Atrial Fibrillation on HDL

Metabolic disease, obesity, and HF can also result in arrhythmias. The most common arrhythmia observed in patients is atrial fibrillation (AF). Low baseline HDL-C is associated with an increased risk of AF (105–108). AF is associated with reduced HDL quality as AF was associated with reduced HDL cholesterol efflux capacity, HDL-particle number, ApoA-I levels, and reduced LCAT activity; interestingly, all these indices improved following the restoration of sinus rhythm (109). Further validation of these findings would be encouraging, especially because the mechanistic link between AF and HDL remains unclear, particularly in the acute setting. One possible theory is the role HDL plays in myocardial membrane stabilization (110). In the more chronic setting, other HDL attributes including anti-inflammatory, anti-oxidant, and anti-atherogenic properties could interact with AF development and severity.

Effect of Aging on HDL

Aging is a well-established risk factor for the development and progression of HF, resulting from the deterioration of both cardiac structure and function, as well as the high risk of co-morbidities. Elderly patients have increased HDL oxidation, which can impair the normal protective capacity against LDL oxidation, and lead to the acceleration of atherosclerosis and CVD, both risk factors for HF (111).

Holzer et al. (112) compared HDL isolated from healthy young and elderly patients and found that aging alters HDL composition and function. HDL from elderly subjects had higher SAA and sphingomyelin, while levels of total cholesterol were reduced (112). Furthermore, HDL isolated from older patients demonstrated reduced cholesterol efflux capacity, principally through the ABCA1 pathway (113). In the same context, aged murine models have exhibited reduced ApoM secretion from the liver, with consequent impairment of S1P signaling, which reduces resistance to injury-induced vascular leak and precipitates organ fibrosis (114).

Oxidative stress is one of the main pathophysiological processes associated with aging (115) and is involved in the development of HF (116). PON1 is one of the most prominent antioxidant components of HDL (112, 117, 118). In elderly patients, it has been shown that PON1 activity and ApoE levels, both having important antioxidant properties, are diminished (112, 119). Overall, these studies suggest that aging may alter HDL structure and properties, resulting in reduced antioxidant capacity and cholesterol efflux, which can contribute to higher susceptibility to CVD and advance processes associated with HF mortality.

Inflammation, renal dysfunction, diabetes, obesity, atrial fibrillation, and aging can either occur antecedent to HF or comorbid with it. These HF comorbidities, and others, can have a tremendous impact on lipid metabolism and HDL biology, which in turn may impact disease progression. In the next section, we discuss how changes in HDL may alter the development of HF or HF outcomes.

SALUTARY EFFECTS OF HDL ON PREVENTION AND OUTCOMES IN HEART FAILURE

Atheroprotective Functions of HDL

Atherosclerotic CVD can lead to ischemic cardiomyopathy, which is a major clinical cause of HF (120). LDL-C is a critical, causal factor in the pathogenesis of CVD (121). In animal models, HDL has been shown to have a protective role against the development of atherosclerosis and CVD (122). HDL exerts its protective effect on vascular endothelium mainly through stimulation of eNOS increasing NO production (123, 124). HDL is critical for the reverse cholesterol transport process, which removes excess cholesterol from the atherosclerotic plaques, reducing the risk and progression of CVD (125, 126). Additionally, HDL has anti-apoptotic, anti-inflammatory, and antithrombotic protective properties on the vascular endothelium (34, 124, 127).

Cardioprotective Functions of HDL

Both *in vitro* and *in vivo* models have repeatedly demonstrated multiple cardioprotective properties of HDL particles on many levels. HDL has shown a direct protective effect on cardiomyocytes and endothelial cells, independent of its effect on the coronary vasculature or atherosclerosis (16, 26, 128–131). It has been proposed that HDL mediates direct action on cardiomyocytes through its different array of apolipoproteins (e.g., ApoA-I and ApoM), which interact with different receptors expressed on cardiomyocytes regulating various intracellular signaling pathways. Furthermore, HDL could also indirectly protect cardiomyocytes through its systemic and local anti-inflammatory and anti-oxidative effects (132, 133).

In murine models, HDL inhibited mechanical stress-induced myocardial cell hypertrophy and autophagy through downregulation of the angiotensin II type 1 receptor (129). Angiotensin II receptors are upregulated on cardiomyocytes exposed to mechanical stress, and blockade of the renin-angiotensin pathway is a sine qua non of HF therapy. Downregulation of these receptors by HDL would be an important mechanism by which this particle may improve HF outcomes. Further, multiple *in vitro* studies show that HDL also protected against doxorubicin-induced cell injury on cultured cardiomyocytes (26, 134, 135), mostly through reducing doxorubicin-induced apoptosis. These studies are clinically important given that anthracyclines, such as doxorubicin, remain an important cause of cardiotoxicity and clinical HF.

HDL has also been associated with the preservation of endothelial barrier integrity. HDL increases NO production in endothelial cells, which enhances endothelial vasodilation and preserves endothelial integrity mainly through an SR-BI-dependent mechanism (136–140). It also modulates the contractile state of subjacent myocytes *via* paracrine mechanisms (141). Additionally, HDL contributes to endothelial repair by increasing the number and function of endothelial progenitor cells at sites of endothelial injury (128). Moreover, HDL-carried glycosphingolipids have demonstrated an anti-apoptotic capacity against stress-induced endothelial death (142). Van Linthout et al. (130) reported that HDL protects against myocardial dysfunction and hyperglycemia-induced cardiomyocyte apoptosis in diabetic murine models mainly *via* the phosphoinositide 3-kinase / protein kinase B (PI3K/Akt) pathway. Altogether, these studies suggest multiple mechanisms by which HDL may directly protect cardiomyocytes in the failing myocardium.

HDL Anti-inflammatory Properties

It has previously been established that systemic inflammatory mediators (e.g., C reactive protein (CRP), TNF-α) can contribute to the development of HF, and inflammation can induce cardiomyocyte apoptosis and endothelial dysfunction (143). Multiple studies have demonstrated anti-inflammatory properties of HDL. For instance, HDL inhibits endothelial activation and decreases the expression of adhesion molecules (e.g., VCAM-1 and ICAM-1), which prevents the recruitment of leukocytes in response to myocardial cell injury, and can attenuate the insult due to reduced chemokine secretion and impede further recruitment of inflammatory cells. HDL

also blocks T-cell binding and activation of monocytes, which results in diminished production of pro-inflammatory cytokines (144–146).

Moreover, recent studies suggest that the NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome plays a role in the development of atherosclerosis, and it has also been tied to post-ischemic remodeling, and HF (147–149). HDL can suppress the activation of the NLRP3 inflammasome, likely by simultaneous downregulation of IL-1β, and reduced activation of caspase 1 (150). Other anti-inflammatory properties of HDL are promoting the expression of anti-inflammatory cytokines, such as transforming growth factor-β2 (TGF-β2) in endothelial cells (151, 152) and neutralizing pro-inflammatory activity of both, IL-6 and CRP (153). In summary, these and other anti-inflammatory properties of HDL merit further exploration and offer a variety of targets for developing pharmacologic therapies.

HDL Anti-oxidative Properties

One of the hallmarks of HF pathophysiology is stress-induced myocardial cell death with subsequent proliferation, fibrosis, and remodeling (154, 155). HDL has demonstrated many antioxidant properties that may combat these processes. Treating cultured cardiomyocytes with HDL protects against stress-induced cell death (156). This effect has been suggested to be mediated by the anti-oxidative enzyme PON1 (157–160). Another antioxidant enzyme present on HDL is the platelet-activating factor acetylhydrolase that induces the hydrolysis of fatty acids and phospholipids peroxides (161, 162). Furthermore, HDL blocks eNOS uncoupling in myocardial cells, reducing the formation of reactive oxygen species (163–166). In addition, HDL-associated lipoproteins (ApoA-I, ApoA-II, ApoA-IV, ApoE, etc.) neutralize the remaining phospholipid hydroperoxides transferred to HDL (167). Finally, HDL can also indirectly reduce oxidative stress secondary to its anti-inflammatory properties discussed previously (168).

HDL Anti-fibrotic Properties

HDL can protect against myocardial fibrosis through inhibition of the pro-fibrotic transforming growth factor-β1 (TGF-β1), which induces collagen production and deposition in the myocardium of murine models (169, 170). In a study on aortic endothelial cells *in vitro*, HDL reduced TGF-β1-induced endothelial-mesenchymal transition and attenuated fibrosis of the vascular wall in response to various insults (171). Alternatively, HDL may exhibit anti-fibrotic properties by binding and potentially sequestering S1P (through ApoM). Although this mechanism has not been directly demonstrated in the myocardium, this type of biology has been demonstrated in the retina, where ApoM can act as a negative regulator of S1P (172).

Cardioprotective Role of ApoA-I/SR-BI Axis

ApoA-I, the most abundant protein constituent of HDL, is involved in the systemic anti-inflammatory and anti-oxidative cardioprotective properties of HDL (173). ApoA-I is the main ligand of SR-BI and thus is very important for the cardioprotective functions of HDL previously described. Low ApoA-I levels are associated with left ventricular dysfunction and adverse outcomes in patients with non-ischemic HF (173, 174). Gombos et al. have shown that ApoA-I is inversely associated with NT-proBNP and mortality in HF (175). Similarly, Florvall et al. (176) have suggested that serum ApoA-I can predict CVD and mortality in elderly men.

ApoA-I's cardioprotective properties may be related to its anti-inflammatory and antioxidative properties. ApoA-I attenuates inflammation and is inversely correlated with CRP and fibrinogen levels (173). In addition, it blocks neutrophil activation and expression of the surface adhesion proteins that regulate leukocyte migration (177, 178). Bursill et al. (144) showed that when mice were injected with ApoA-I, the expression of chemokine receptors involved in leukocyte migration was significantly reduced. ApoA-I can also enhance the proliferation of endothelial progenitor cells and stimulate angiogenesis through the cell surface F1-ATP synthase, a high-affinity receptor of ApoA-I (179). Moreover, ApoA-I accelerates endothelial regeneration and prevents transplant vasculopathy in murine models (132, 180).

ApoA-I binds to SR-BI, which is mainly expressed in the liver. SR-BI mediates selective uptake of cholesterol, as well as HDL lipid hydroperoxides, and plays a major role in modulating HDL composition and therefore its function (**Figure 2A**). Muthuramu et al. described a cardioprotective role of SR-BI (181). They performed a study using SR-BI knockout mice that received either adeno-associated virus 8 (AAV8) expressing SR-BI (via a hepatocyte-specific promoter) or a control AAV8. Notably, when SR-BI knockout mice are exposed to pressure overload, they develop worse pathological ventricular hypertrophy, interstitial and perivascular fibrosis, and myocardial apoptosis than control mice. Interestingly, in mice that received AAV8-SR-BI, the plasma lipoprotein profile normalizes, attenuates cardiac dysfunction, and mortality is lower compared to mice that received Null injection. In addition, mice that underwent SR-BI gene transfer had lower oxidative stress than those that did not (181). Similarly, Durham et al. demonstrated that pretreatment with HDL protects against myocardial cell necrosis *via* the PI3K/Akt pathway (131). This finding was not observed in SR-BI knockout cells, suggesting that SR-BI is the upstream mediator of the PI3K/Akt signaling in cardiomyocytes and that HDL could be mediating this effect through interaction with SR-BI *via* ApoA-I (131). These studies suggest that ApoA-I, *via* SR-BI, may be an important mediator of the cardio-hepatic axis.

Cardioprotective Role of the ApoM/S1P/S1PR Axis

ApoM is an apolipoprotein that binds S1P *via* its hydrophobic binding pocket, is secreted mostly by hepatocytes, and to a lesser extent by renal proximal tubular cells (182, 183). Although ApoM is only found in 5% of HDL particles, it exerts many of the beneficial effects of HDL through S1P signaling (**Figure 2B**). ApoM acts as a chaperone for S1P carrying about 70% of plasma S1P in the circulation, as well as increasing its efflux from erythrocytes to HDL (184). ApoM that is secreted from the

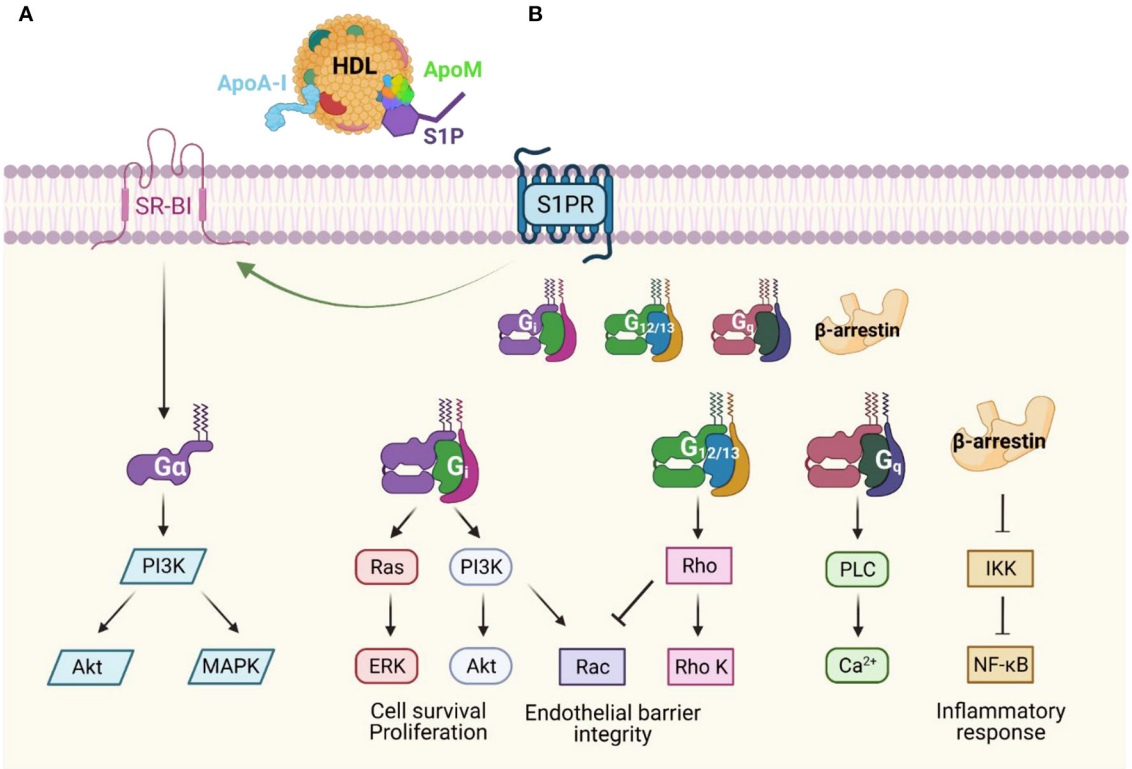

FIGURE 2 | Apolipoprotein-dependent signal transduction pathways **(A)** Apolipoprotein A-I (ApoA-I) is the major component of high-density lipoproteins (HDL), and it binds scavenger receptor class B type I (SR-BI), which mediates selective uptake of cholesterol. SR-BI may also stabilize HDL particles allowing access to S1P receptors or activate other signaling cascades. **(B)** HDL and apolipoprotein M (ApoM)-dependent activation of S1P receptors (S1PR) leads to downstream G-protein coupled receptor signaling in both endothelial cells and cardiomyocytes. This signaling promotes diverse physiological responses including maintenance of endothelial barrier integrity, promotion of cell survival, and anti-inflammatory effects. Modified from (240–242).

proximal tubular cells in the kidney also prevents renal excretion of S1P (185).

Recent studies indicate a potential protective role of ApoM on atherosclerosis and CVD (186–188). ApoM has a protective effect on vascular endothelium as ApoM transgenic LDL receptor knock out mice developed smaller atherosclerotic lesions than control mice (189, 190). Atheroprotective functions of ApoM on vascular endothelium are likely mediated by S1P through S1P receptor 1 (S1PR1) signaling (191). ApoM also has a significant anti-inflammatory effect *in vivo* and *in vitro* mainly mediated by the S1P/S1PR axis (192).

Multiple mechanisms have been invoked for how ApoM may improve myocardial health or delay disease progression. Recent studies in murine models have demonstrated that ApoM/S1P enhances endothelial barrier function and improves cardiac outcomes through different signaling pathways. For example, in LPS-treated mice, ApoM attenuated LPS-induced organ injury as well as cardiomyocyte cell death *via* PI3K/Akt downstream of S1PR1/3 (193). Moreover, *in vitro* studies of human umbilical vein endothelial cells showed that ApoM/S1P markedly reduced pro-inflammatory cytokines, including TNF-α, inhibiting the inflammatory response, and reduced endothelial injury in a PI3K/Akt and S1PR2 dependent manner (194). Furthermore, ApoM knockout mice demonstrate impaired endothelial barrier integrity compared to wild-type mice (195). Reconstitution of plasma ApoM/S1P or treatment with an S1PR1 agonist rapidly reversed the vascular leak and restores endothelial integrity (195). S1P, acting through the receptors 1-3 (S1PR1-3), plays a crucial role in the regulation of the endothelial cell cytoskeleton, and is necessary for its proper function as well as new vessel formation. Multiple studies have demonstrated S1P to be a significant mediator of angiogenesis due to its potent chemoattractant properties for endothelial cells (196–198). S1P was found to have a higher capacity for stimulation of endothelial cell migration than known molecules such as vascular endothelial growth factor or basic fibroblast growth factor (199). Furthermore, S1P acting mainly *via* S1PR1 and S1PR3 have been repeatedly demonstrated to be crucial for endothelial migration (200, 201), endothelial integrity (202–204), and normal barrier function (205, 206). S1P effects on endothelial cells are mainly mediated by pathways involving Rho GTPases (207–209) as well as the PI3K/Akt pathway (210).

In addition to its protective role on the endothelium, S1P has shown multiple cardioprotective properties. Zhang et al. (211) showed that S1P signaling through S1PR1 in murine models activated the downstream PI3K/Akt pathway

and attenuated myocardial cell injury in response to severe hypoxic stress. Similarly, Means et al. found that stimulation of S1PR2 and S1PR3 receptor activates PI3K/Akt pathway and protects against ischemia-reperfusion injury (212). Theilmeier et al. have also demonstrated that HDL and S1P, both acting through S1PR3, and NO-dependent mechanisms, protect against ischemia reperfusion-induced myocardial injury in *ex vivo* and *in vivo* mouse models (25). They also found that S1P reduced neutrophil recruitment to the site of injury and decreased cardiomyocyte apoptosis (25). Furthermore, S1PR2 showed some cardioprotective properties as well by activating signal transducer and activator of transcription 3 (STAT3) through ERK1/2 and Src-dependent mechanisms (26). STAT3 is important for myocardial adaptation to stress and has been shown to preserve cardiac function through its anti-apoptotic and anti-fibrotic effects (213–218). While these data support multiple mechanisms for S1P-mediated anti-apoptotic effects on cardiomyocytes through multiple S1P receptors, both in our experience and others, it is challenging to detect any significant S1PR2 mRNA expression in the myocardium in mice (219).

These studies support the concept that ApoM, *via* S1P, can reduce vascular leak, inflammation, and promote cell survival, all of which are likely critical targets for multiple organs in the syndrome of HF. Recently, our group measured circulating ApoM across 3 major HF cohorts comprising nearly 2,500 patients. In our study, reduced ApoM levels were significantly associated with the risk of all-cause mortality (45). These associations were independent of HDL-C and ApoA-I, natriuretic peptide levels, etiology of HF, and a commonly used HF risk score, and were observed in both HFrEF and HFpEF. Although we demonstrated a strong correlation between ApoM and S1P on HDL particles, mediation analysis suggested that ApoM could also have effects independent of S1P. Pathway analysis demonstrated that ApoM showed that the acute phase response was not only the most significant pathway associated with ApoM but also that ApoM was inversely associated with inflammation, as predicted by murine studies. In a follow-up study, we screened the plasma proteome to identify proteins that mediated the effect of diabetes on HFpEF outcomes. The only protein that fulfilled the criteria of this a priori analysis was ApoM, which was shown to mediate an astounding 70% of the effect of diabetes on HFpEF outcomes (220).

NOVEL HDL THERAPEUTICS IN CARDIOVASCULAR DISEASES

Multiple studies have shown a promising role for HDL-targeted therapies in HFrEF (221), HFpEF (221, 222), and diabetic cardiomyopathy murine models (223). In these animal models, HDL reversed pathologic features of myocardial hypertrophy, fibrosis, and stimulating reverse remodeling in pre-established HF. Multiple synthetic compounds have been designed to mimic the bioactive molecules of HDL and replicate their cardioprotective functions.

ApoA-I Milano is an ApoA-I mutant first described in Northern Italy in 1980 (224, 225). Heterozygous carriers of the mutation were thought to exhibit increased life expectancy and believed to develop atherosclerosis at lower rates compared to the normal population (226, 227). MDCO-216 is a recombinant HDL formulation of ApoA-I Milano in combination with phospholipids, which has been used to study ApoA-I Milano's potential therapeutic effects (183, 228–230).

Mishra et al. (222) reported that MDCO-216 attenuated cardiac hypertrophy, increased capillary density, and decreased interstitial fibrosis in murine models. In a subsequent study, Mishra et al. showed similar results of MDCO-216 in murine models of hypertension-associated cardiac hypertrophy (170). Aboumsallem et al. have demonstrated that MDCO-216 improves systolic and diastolic dysfunction, reduces myocardial fibrosis, and enhances myocardial vascularity in mice with HF (221). Further, Aboumsallem et al. showed that mice with diabetic cardiomyopathy that were treated with MDCO-216 presented regression of myocardial dysfunction and pathological cardiac remodeling (223). Altogether, these studies suggest MDCO-216 might be useful for HFrEF, HFpEF, or diabetic cardiomyopathy.

ApoA-I gene therapy strategies have also been employed in HF rodent models. Gordts et al. (231) evaluated if selective gene transfer may protect against the development of HF. In LDL receptor-deficient subjects to experimental MI, viral-mediated gene transfer of ApoA-I resulted in reduced infarct expansion and inhibition of left ventricular dilatation compared with controls. Similarly, Amin et al. studied the effect of selective AAV8-human ApoA-I (AAV8-ApoA-I) gene transfer on cardiac remodeling, induced by transverse aortic constriction in LDL deficient mice (232). They reported that AAV8-ApoA-I transduced mice had significantly attenuated septal wall thickness, cardiomyocyte cross-sectional area, and interstitial cardiac fibrosis compared to control mice, indicating reduced remodeling, and preserved systolic function reserve. Diastolic function was also significantly improved in mice transduced with the ApoA-I AAV8 (232).

ApoA-I mimetic peptides have also shown promise in preventing or attenuating myocardial dysfunction in murine models of MI and sepsis. Hamid et al. (233) have demonstrated that the ApoA-I mimetic peptide L-4F prevents prolonged and excessive inflammation after MI and improves post-MI LV remodeling. L-4F suppressed proliferation of myocardial pro-inflammatory monocytes and macrophages in murine models of reperfused MI (233). They suggested that L-4F could be used as a therapeutic adjunct in humans with MI to limit inflammation and alleviate the progression to HF (233). Another ApoA-I mimetic peptide D-4F has been also shown to improve vascular function, decrease myocardial inflammation, and restore angiogenic systemic sclerosis in murine models (234). Moreover, the ability of ApoA-I mimetic peptides to reduce sepsis-induced myocardial injury was studied by Moreira et al. (235). They demonstrated that the novel ApoA-I mimetic peptide D-4F reduced inflammation, attenuated vascular permeability, preserved myocardial function, and baroreceptor sensitivity in murine models of sepsis.

The AEGIS-I trial (Apo-AI Event Reducing in Ischemic Syndromes I), a multi-center, randomized, double-blind, placebo-controlled 2b trial assessed the safety of CSL112, an infusible plasma-derived ApoA-I, in patients with myocardial

infarction (236). CSL112 was generally safe and well-tolerated (236). Currently, the AEGIS-II trial is underway to evaluate whether CSL112 can reduce the risk of major adverse cardiovascular events (237). To our knowledge, there are no active trials of CSL112 in human HF, although the hypothesis that CSL112 may benefit patients with acute HF should be pursued in randomized-controlled clinical trials (238).

Beyond therapeutics targeting only ApoA-I, Swendeman et al. (239) developed a recombinant ApoM fused to the constant domain of immunoglobins (ApoM-Fc) to prevent its rapid degradation. When this novel protein was tested in multiple systems, ApoM-Fc selectively activated S1PR1, leading to enhanced endothelial barrier integrity and downstream eNOS-dependent secretion of NO and subsequent vasodilation, which could be used therapeutically to control hypertension. In addition, they demonstrated improved outcomes in murine models of myocardial ischemia/reperfusion and stroke, by promoting endothelial function and reducing further tissue inflammation.

Altogether, apolipoprotein A-I and ApoM based therapeutics have demonstrated potential in preclinical models of cardiac dysfunction. Unfortunately, to our knowledge, the human translation of these therapeutics has not been tested in randomized controlled clinical trials in HF. In addition, understanding regarding the synergistic potential of these apolipoproteins (A-I and M), as well as other functions of HDL, remains poorly understood.

CONCLUSIONS

HDL apolipoproteins remain a promising therapeutic target in patients with HF. The advances in proteomic and lipidomic technologies have permitted the discovery of HDL components, and assessment of their impact on HF pathophysiology, predominantly in preclinical models. ApoA-I and ApoM are especially promising as they have shown multiple cardioprotective properties in murine models. Further studies are needed to elucidate the functional properties of HDL proteomic and lipidomic components and to explore possible therapeutic targets in patients with HF.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Targeting Lipid—Ion Channel Interactions in Cardiovascular Disease

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General lipid-lowering strategies exhibit clinical benefit, however, adverse effects and low adherence of relevant pharmacotherapies warrants the investigation into distinct avenues for preventing dyslipidemia-induced cardiovascular disease. Ion channels play an important role in the maintenance of vascular tone, the impairment of which is a critical precursor to disease progression. Recent evidence suggests that the dysregulation of ion channel function in dyslipidemia is one of many contributors to the advancement of cardiovascular disease thus bringing to light a novel yet putative therapeutic avenue for preventing the progression of disease mechanisms. Increasing evidence suggests that lipid regulation of ion channels often occurs through direct binding of the lipid with the ion channel thereby creating a potential therapeutic target wherein preventing specific lipid-ion channel interactions, perhaps in combination with established lipid lowering therapies, may restore ion channel function and the proper control of vascular tone. Here we first detail specific examples of lipid-ion channel interactions that promote vascular dysfunction and highlight the benefits of preventing such interactions. We next discuss the putative therapeutic avenues, such as peptides, monoclonal antibodies, and aspects of nanomedicine that may be utilized to prevent pathological lipid-ion channel interactions. Finally, we discuss the experimental challenges with identifying lipid-ion channel interactions as well as the likely pitfalls with developing the aforementioned putative strategies.

Keywords: lipids, ion channels, novel therapeutics, dyslipidemia, cardiovascular disease

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INTRODUCTION

Ion channels are major regulators of cardiovascular homeostasis. The proper function of vascular cells critically depends on the modulation of ion channels which are involved in the maintenance of vascular tone (1). Cellular lipids are essential to the regulation of ion channel activity, often by direct interaction with ion channels through distinct binding (2, 3). The physiological regulation of ion channels by lipids is necessary to fine-tune ion channel activity, thereby contributing to adjustments in membrane potential (3). In the cardiovascular system, this makes lipids critical regulators of vascular tone, however, it is well-established that elevated blood lipids are associated with an increase in blood pressure, atherosclerosis, and incidence of stroke (4, 5). As such, the dysregulation of lipid levels in the cardiovascular system, as is prominent in cardiovascular diseases of the Western world, represents a major health burden. While the identified cellular

mechanisms and pathways affected by lipotoxicity are many (4), the subsequent dysregulation of ion channels repeatedly emerges as a key factor in the perturbation of cardiovascular homeostasis (6–8). Therefore, targeting specific lipid-ion channel interactions has therapeutic potential that, in combination with existing lipid-lowering strategies, may help to curb the progression of severe cardiovascular disease by restoring the appropriate level of channel function and, therefore, cardiovascular homeostasis. Here we will look at several notable examples of lipid-ion channel interactions and discuss which aspects of these interactions are well understood. We will also consider the limitations of current therapeutics and how our current knowledge of the mechanisms of lipid-ion channel interactions could be exploited to develop novel treatments for dyslipidemia. Finally, we discuss experimental difficulties specific to studying lipid-ion channel interactions that may pose a challenge to future research.

Benefits of Preventing Lipid-Ion Channel Interactions

Generally, a variety of pharmaceutical methods are utilized in dyslipidemic settings in order to lower serum lipid levels, which can prevent advancement of cardiovascular disease. In particular, common drugs include statins, which typically lower levels of LDL-cholesterol, and fibrates, which lower triglyceride levels (9). Others include ezetimibe, which inhibits cholesterol absorption (10), and niacin, which increases HDL-cholesterol and decreases LDL-cholesterol (11). However, non-specific lipid-lowering medications have a variety of clinical drawbacks. Statins in particular are limited to nightly administration due to the nocturnal synthesis of cholesterol, and have negative side effects such as myopathy, hepatotoxicity, and medication resistance (12). Bile-acid binding resins increase hepatic cholesterol uptake thereby decreasing cholesterol levels in the blood (13) but are associated with gastrointestinal side effects due low absorption in the intestines, leading to fecal excretion and high dosage frequency/concentration (14). Therefore, development of treatments targeting the specific mechanisms leading to vascular dysfunction is warranted. For a brief summary of existing therapeutics, see **Table 1**.

While the regulation of ion channel function by lipids is well established (3), a few have been shown to demonstrate particular relevance to vascular dysfunction (7, 8). However, the associations between lipids and ion channels are complex and may involve the direct binding of lipids to ion channels and/or non-specific changes to the plasma membrane (2, 15). These two mechanisms are not mutually exclusive and may both contribute to modulation of ion channel function. Furthermore, the role of additional proteins (e.g., ancillary ion channel subunits) in the plasma membrane may alter the functional effect of lipids (16). However, the apparent specificity of many lipid-ion channel interactions may permit the development of therapeutics that specifically bind to and prevent these specific interactions, thereby preventing the binding of inhibitory lipids to channel proteins. Ideally, this therapeutic would not influence ion channel function but rather allow ion channel activity to proceed in the presence of what would otherwise be an inhibitory concentration of lipids.

One such lipid-ion channel interaction of interest is that of cholesterol with Kir2, an inwardly-rectifying potassium channel expressed in numerous tissues (17). This channel appears to be involved in mechanosensing in endothelial cells and plays an important role in the regulation of vascular tone by promoting nitric oxide synthesis (7, 18). Recent studies showed that an increase in cellular cholesterol has an inhibitory effect on Kir2 channels by decreasing Kir2 channel function rather than expression. Interestingly, a reduction of flow-induced vasodilation in a high-cholesterol environment was completely reversed by both the depletion of cholesterol and the overexpression of Kir2 in mouse primary mesenteric artery endothelial cells (7), suggesting that both preventing cholesterol binding and promoting Kir2 function may be effective approaches to preventing and treating endothelial dysfunction in the presence of hypercholesterolemia. Several studies compared the effects of cholesterol with its isomers and concluded that cholesterol likely interacts directly with the channel protein to cause inhibition rather than altering the physical properties of the lipid bilayer and affecting the channel indirectly (19, 20). This process may involve phosphatidylinositol 4,5-bisphosphate (PIP2) (21, 22), the presence of which stabilizes the open state of the channel (23). It was suggested that cholesterol weakens the interaction of PIP2 with Kir2 channels, resulting in instability of the open state (22). As cholesterol binds to a distinct site from that of PIP2, it was proposed that cholesterol-Kir2 interactions caused a structural change in Kir2 that opposes that of PIP2 binding, resulting in the inability of PIP2 to stabilize the open state (**Figure 1**, top panel) (21). Therefore, targeting cholesterol-Kir2 interactions to prevent cholesterol from interacting with Kir2 channels would require generating a small molecule or drug that allows or promotes PIP2-Kir2 channel interactions (**Figure 1**, bottom panel).

The interaction of large-conductance, calcium-activated potassium (BK) channels with cholesterol has also received considerable attention. BK channels are major regulators of smooth muscle contraction in multiple tissue types in the colon (30), the urinary bladder (31) and notably the arteries, where activation of the channel results in vasodilation (32). In arterial smooth muscle cells, the pore of the channel is composed of alpha pore-forming subunits and beta auxiliary subunits which increase sensitivity to Ca^{2+}. A recent study showed that cholesterol has a direct inhibitory effect on the BK channel when only the alpha subunits are present. In contrast, elevated cholesterol activates BK channels when beta subunits are also expressed. The authors concluded that the cholesterol-mediated activation appears to be induced by increased trafficking of the beta subunit to the plasma membrane which results in increased sensitivity of BK to Ca^{2+} (16). The alpha subunit contains several conserved cholesterol recognition amino acid consensus (CRAC) motifs that may constitute the binding sites by which cholesterol inhibits BK channel activity in the absence of beta subunits, while the beta subunits also contain two CRAC motifs (32). Although the role that CRAC motifs play in cholesterol binding and effects on channel function in general is debated

TABLE 1 | Traditional vs. putative methods of targeting lipid-ion channel interactions.

		Advantages	Disadvantages	References
Traditional Methods	Statins	- Clinically effective at reducing risk of cardiovascular disease through serum LDL reduction	- Limited to night-time administration - Medication resistance - Side effects - Non-specific	(9, 12)
	Bile-Acid Binding Resins	- Clinically effective at reducing risk of cardiovascular disease through serum cholesterol reduction - Minimal and limited systemic side effects	- Gastrointestinal side effects	(13, 14)
Putative Methods	Peptides	- Highly specific	- Short half life - Immunogenic - Low oral bioavailability - High risk for side effects	(24, 25)
	Monoclonal Antibodies	- Highly specific - Consistent pharmacokinetics - Limited effect on nervous system - Long half-life	- Expensive - Difficult to produce - May not effectively bind to ion channels due to structural impediments	(24, 26)
	Nanobodies	- Smaller size allows for greater access to hard-to-reach epitopes - Intracellular generation - Can target cytosolic and extracellular epitopes	- High renal clearance leads to low retention - Potential for nephrotoxicity	(24, 27)
	Nanomaterials	- Wide range of functionality - Long half-life	- Possible unwanted accumulation - Potentially hazardous - Possibly limited to use in inflammatory conditions	(28, 29)

(19, 32, 33), the increase in cholesterol-mediated beta subunit trafficking is likely driven by a separate cellular pathway that is triggered by elevated cholesterol. This putative mechanism, which potentially serves as a protective negative feedback mechanism in the presence of high cholesterol, illustrates the potential benefit of novel therapeutics that target specific lipid-channel subunit binding. For example, in contrast to general lipid lowering therapies, a treatment specifically preventing cholesterol interaction with the BK alpha subunit at the binding site could be an effective method for preventing undesired consequences of dyslipidemia while permitting cholesterol-mediated beta subunit trafficking, thus promoting BK activation and vasodilation.

While ion channels are not currently a traditional target in dyslipidemia, the dysfunction of ion channels is implicated in the development of severe cardiovascular disease in dyslipidemic settings (7, 8). The well-established effects of lipids directly inhibiting specific vascular ion channels warrants investigation into preventing these specific lipid-ion channel interactions to prevent the progression of disease. Therefore, novel approaches in targeting lipid-ion channel interactions represent potential ways to restore proper ion channel function in dyslipidemic populations. For a detailed review of the interaction of cholesterol with Kir2 and BK channels as well additional ion channels not discussed here, including the nicotinic acetylcholine receptor (nAChR) channel and the transient receptor potential (TRP) channels, we refer the reader to (19, 34–36).

Putative Methods to Target Lipid-Ion Channel Interactions

Traditional pharmaceutical methods that target lowering blood lipid levels are clinically effective in treating cardiovascular disease (37). However, the drawbacks to lipid lowering therapies discussed above limit the overall efficacy and adherence to these drugs (38). While ion channels have not been widely studied as targets in dyslipidemia, an increasing body of evidence suggests that preventing lipid-ion channel interactions that promote inhibition of the channel may serve to restore ion channel-mediated control of vascular tone, limiting or preventing the progression of disease. In the following sections we discuss possible avenues that may be suitable for targeting specific lipid-ion channel interactions and discuss potential drawbacks to these presently putative methods.

Peptides

The ability for researchers to modify peptides to harness desirable effects presents a putative approach in preventing lipid-ion channel interactions and experimental evidence supports this avenue. A recent study showed that blocking lipid-TRPV1 (transient receptor potential vanilloid 1) channel interactions with a peptide prevented diabetes-induced endothelial dysfunction in mice (39). In this instance, lipid-mediated channel activation promoted pathophysiology and a peptide specifically targeting the lipid-channel interaction at the TRP

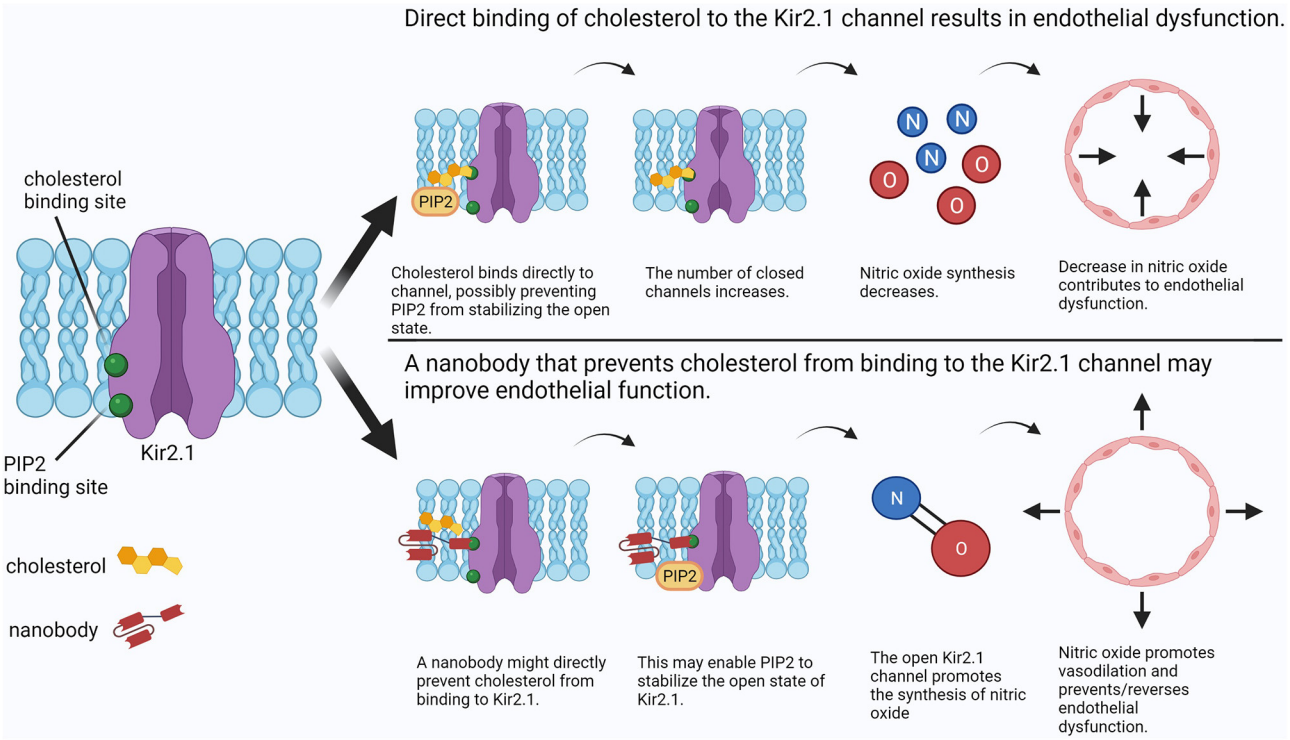

FIGURE 1 | Comparison between cholesterol-induced Kir2 channel inhibition and a putative nanobody therapeutic preventing cholesterol-Kir2 interaction. In the presence of elevated cholesterol, cholesterol may bind directly to Kir2, preventing PIP2 from stabilizing the open state and resulting in channel inhibition. Consequently, nitric oxide synthesis is decreased resulting in endothelial dysfunction. A possible treatment may use an antibody to directly prevent cholesterol from binding to Kir2 while still allowing channel regulation by PIP2. Physiological synthesis of nitric oxide is restored, preventing or reversing endothelial dysfunction. Created with BioRender.com.

box site prevented endothelial dysfunction, indicating that the presence of other conditions (e.g., diabetes) may influence which lipid-ion channel interactions should be specifically targeted in the presence of dyslipidemia. For instance, in the absence of diabetes, endothelial TRPV1 activation may promote nitric oxide production and contribute to vasodilation (40). Both cholesterol and oleic acid inhibit the channel through direct interactions, though membrane effects have not been ruled out (41). The tarantula double knot toxin (DkTx) targets TRPV1, activating the channel at low doses (39). Cholesterol has been shown to bind in the S5 helix of TRPV1, and DkTx to the S6 and S5 helices (42, 43), suggesting that DkTx could be used to simultaneously promote channel activation while preventing cholesterol-induced suppression of TRPV1 channels by blocking cholesterol binding at the S5 site. Venom peptides, well known for having specific effects on a variety of ion channels at low concentrations, have been utilized for a variety of medical advances such as the production of the angiotensin-converting enzyme inhibitor that currently treats hypertension (38). Produced by venomous animals, these peptides are potent and specific, allowing for many potential functions, including the activation/inhibition of ion channels (24). However, venom peptides have a short half-life which would entail a higher dosing frequency, complicating the noxious effects on tissues. Moreover, these peptides have the potential to be very immunogenic, leading to potential adverse reactions. Lastly, venom peptides do not have much oral bioavailability, which can cause complications in drug administration (25). Modifications made to venom peptides such that selective binding to ion channels can occur without destroying tissue is one example of how peptides should be modified to optimize desired benefits while mitigating harmful effects. Future research aimed at reducing toxicity and immunogenicity, increasing oral bioavailability, and targeting specific ion channel moieties through manipulating peptide length/sequence may allow for effective clinical usage of venom peptides to target lipid-ion channel interactions.

Monoclonal Antibodies

Monoclonal antibodies (mAbs) have been widely exploited to treat a variety of conditions, from cancer to COVID-19 (44, 45), and their use in manipulation of ion channel function has grown considerably in recent years (26). mAbs also have the potential to be utilized in targeting lipid-ion channel interactions, such as through specific binding of epitope(s) on the ion channel (26). Moreover, mAbs generated to directly target elevated lipids could potentially be used to bind a critical concentration of a specific lipid that would prevent interaction with the ion

channel yet allow a physiological concentration of lipids to exist. Many small molecules/drugs are also disadvantaged in that they may have a short half-life. In contrast, mAbs have a relatively extended half-life ranging from days to weeks, which could lead to lower dosing frequencies (24). Additionally, mAbs are highly specific, which decreases the likelihood of off-target binding, and have little variability in patient pharmacokinetics which allows for consistent dosing administrations (26). However, mAbs are expensive and difficult to generate. Furthermore, small target epitopes may lack the proper immunogenicity for mAbs, or if the target has bulky structures present, these structures may physically impede the binding of mAbs (26). Additionally, producing mAbs that do not impede channel function may be impractical. Generating mAbs that recognize a lipid antigen may be the most useful way in using mAbs to prevent specific lipid-ion channel interactions. Thus, these challenges presented by mAbs may be best addressed by nanobodies.

Nanomedicine

Nanobodies

Nanomedicine represents a groundbreaking frontier for the future of healthcare. From enhanced drug delivery to early cancer detection, the possibilities are vast (46). In this regard, nanobodies (Nbs) have immense therapeutic potential in targeting lipid-ion channel interactions in individuals afflicted by hyperlipidemia. Nbs are small (15 kDa; 2–3 nm in diameter) immunoglobulins with similar functions to mAbs (47). As described above, a common issue with mAbs is their large size, which can reduce access to epitopes. Nbs, on the other hand, are able to access hard-to-reach protein sequences along with being able to passively diffuse through membranes, allowing for access to membrane, cytosolic, and extracellular epitopes (24). An increased range of sequences can be targeted by Nbs, which allows for greater possibilities of impacting lipid-ion channel interactions. For example, and as shown in **Figure 1** (bottom panel), Nbs could be generated to bind to a cholesterol-binding region on an ion channel, acting as a competitive inhibitor of cholesterol, while allowing the ion channel to function. In contrast to mAbs, Nbs only require one domain for keeping target specificity. This allows Nbs to be functional in more environments such as the cytoplasm and membrane, where fatty acids have been shown to inhibit specific ion channels. For instance, it has been shown that cytoplasmic accumulation of long chain coenzyme A, a fatty acid metabolism intermediate, leads to the inhibition of Kir channels by direct binding (48–50). In this case, Nbs could hypothetically target the cytoplasmic binding site of these intermediates. However, more research is needed to determine if targeting such epitopes of ion channels with Nbs will show high efficacy, or if inducing genetic expression of Nbs that target ion channels is a possible avenue (24). Additionally, Nbs have a fast renal clearance which can ultimately lead to kidney toxicity (27). Nevertheless, Nbs provide a wide array of opportunities to target lipid-ion channel interactions that could have a potential role in ameliorating dyslipidemia-induced cardiovascular disease.

Nanomaterials

Along with nanobodies, the use of nanomaterials (Nms) represents another approach in the evolving field of nanomedicine. Nms are classified as materials or chemical substances that exist on a nanoscale of 1–100 nanometers (51). Nms have a wide array of functions and many unique structures along with an extended half-life to allow for reduced dosing regimens. Nms have been used to block ion channels by using hydrophobic interactions and molecular complementarity, causing ion flow to be reduced or ceased (28). Manipulating Nms to prevent lipid-ion channel interactions without impacting channel function would be a necessary alteration. Thus, Nms would need to be engineered to target binding sites of ion channels, acting as competitive inhibitors. While Nms have already been shown to bind and interact with ion channels and are therefore a potential approach in preventing lipid-ion channel interactions, Nms can also present a variety of clinical challenges. Dosing can become an issue, as it remains to be determined how to prevent Nm accumulation at non-targeted sites. Additionally, potential risks of Nms have been studied, such as the potential for induction of pathological states like pneumoconiosis and neurological disorders (28, 29). Future research aimed at abrogating the health risks associated with Nms is necessary prior to considering the use of Nms as a therapeutic use in targeting lipid-ion channel interactions in dyslipidemic populations.

For a brief overview of possible treatment strategies, see **Table 1**.

DISCUSSION

Identifying Lipid-Ion Channel Interactions, Experimental Challenges, and Theoretical Approaches

The generation of novel therapeutics such as those that may benefit ion channel function in dyslipidemia first requires a diligent and thorough investigation of the (patho)physiological consequences of the lipid-ion channel interaction in question. The nature of lipid-ion channel interactions poses unique challenges that can create roadblocks for the development of cardiovascular treatments. Furthermore, is it important to note that preventing lipid-ion channel interactions may produce unwanted effects that interact with other areas of lipid function and regulation that are important for normal biologic processes, especially with a non-specific therapeutic. For example, Kir2 channels prevent spontaneous electrical activity in excitable cells, such as cardiomyocytes (52). Inhibition of cholesterol binding in Kir2 channels could pose an issue if specific tissues cannot be targeted. In this case, cardiac Kir2 channels could be impacted and lead to arrhythmias (35). Tissue- or cell-specific targeting is just one of a variety of potential challenges that these putative methods will need to address.

While computational modeling has contributed much to the identification of potential lipid-ion channel binding sites (2, 23, 53), fundamental limitations exist in the reduction of the cellular environment to simple membranes and poor sampling

(53). Additionally, the binding of a lipid to an ion channel protein does not necessarily cause a predictable functional result. Though advances in dynamic simulations, wherein protein conformation changes in the presence of lipid contact can be observed, are rapidly progressing (54), most of the work to date was conducted on static protein structures, which does not represent the dynamic conformation changes of ion channels *in vivo*. Despite these pitfalls, computational modeling serves as an excellent, albeit time-consuming, foundational approach to identifying large numbers of lipid-ion channel interaction sites.

Following the identification of potential lipid-ion channel interaction sites by computational modeling, these sites are further explored *in vitro,* most commonly by a mutagenesis screen. In the case of investigating a specific ion channel, multiple mutations to a variety of putative lipid binding sites can be simultaneously and rapidly screened using an automated patch clamp system to ensure that a given mutation (i) does not drastically influence channel function and (ii) is functional in the presence of the otherwise inhibitory lipid of interest. However, the automated patch systems used for screening large numbers of cells at once are expensive and often researchers are left to systematically test each mutation individually in patch clamp experiments. This, along with generating the mutant plasmid constructs prior to electrophysiological testing, represents another time-consuming approach that often results in the identification of only a few mutations that confer the desired effects.

Introducing mutations with the desired effects into an animal model is the gold standard to identify if rendering an ion channel insensitive to a specific lipid promotes a physiological rescue effect on vascular function in the presence of dyslipidemia. The arrival of CRISPR technologies has drastically reduced the time and cost to generate transgenic mice, however, to investigate tissue-specific effects use of the Cre-loxP method is, at this time, required and is much more costly and time consuming. While it may seem time/cost efficient to directly screen for peptides and/or nanomaterials that may prevent lipid binding in lieu of mutagenesis studies, ensuring that preventing the specific lipid-ion channel interaction by first confirming that a physiological consequence arises from rendering the channel insensitive to lipid-induced inhibition is a necessary step in identifying novel lipid-ion channel interactions that have pathological consequence.

Generating therapeutics that prevent specific lipid-ion channel interactions with minimal adverse events presents numerous challenges, many of which are unique to the molecule of choice, with each therapeutic avenue considered requiring stringent validation for efficacy and safety. Several iterations of a theoretical therapeutic (e.g., manipulated sequences of a venom peptide predicted to bind the desired ion channel lipid binding site) may proceed in a similar fashion beginning with computational modeling to show that the molecule binds to the desired ion channel sequence and prevents lipid binding/interaction with the channel. *In vitro* screening of successful versions of the therapeutic identified in the modeling may follow to identify which versions of the therapeutic molecule restores ion channel function in the presence of the inhibitory lipids. Work in animal models will then need to determine (i) whether the therapeutic promotes a restoration of ion channel and vascular function *ex vivo/in vivo*, (ii) dosing required for maximum physiological benefit without adverse events *in vivo*, (iii) routes of administration, and (iv) clearance/toxicity to off target tissues. Ultimately, clinical trials will determine the validity of such a theoretical approach in preventing lipid-ion channel interactions to promote vascular health, perhaps in combination with lipid lowering therapies, and take the field into directions whereby differences in distinct patient populations (e.g., stratified by race, ethnicity, sex, age, and metabolic/obesity profile) can be targeted and treated. Finally, the identification of ion channel mutations in patient populations that may affect lipid binding and channel function should also be considered as the field progresses.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

EH, AB, and IF conceptualized and wrote the manuscript with editing by EH, AB, TN, and IF. EH and AB generated the figure and table which were edited by TN and IF. All references were checked for accuracy by TN and IF. All authors contributed to the article and approved the submitted version.

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