Circulation Research

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Imbalance of APOB Lipoproteins and Large HDL in Type 1 Diabetes Drives Atherosclerosis

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BACKGROUND: Individuals with type 1 diabetes (T1D) generally have normal or even higher HDL (high-density lipoprotein)-cholesterol levels than people without diabetes yet are at increased risk for atherosclerotic cardiovascular disease (CVD). Human HDL is a complex mixture of particles that can vary in cholesterol content by >2-fold. To investigate if specific HDL subspecies contribute to the increased atherosclerosis associated with T1D, we created mouse models of T1D that exhibit human-like HDL subspecies. We also measured HDL subspecies and their association with incident CVD in a cohort of people with T1D.

METHODS: We generated LDL receptor–deficient (*Ldlr*$^{-/-}$) mouse models of T1D expressing human APOA1 (apolipoprotein A1). *Ldlr*$^{-/-}$*APOA1*^{Tg} mice exhibited the main human HDL subspecies. We also generated *Ldlr*$^{-/-}$*APOA1*^{Tg} T1D mice expressing CETP (cholesteryl ester transfer protein), which had lower concentrations of large HDL subspecies versus mice not expressing CETP. HDL particle concentrations and sizes and proteins involved in lipoprotein metabolism were measured by calibrated differential ion mobility analysis and targeted mass spectrometry in the mouse models of T1D and in a cohort of individuals with T1D. Endothelial transcytosis was analyzed by total internal reflection fluorescence microscopy.

RESULTS: Diabetic *Ldlr*$^{-/-}$*APOA1*^{Tg} mice were severely hyperglycemic and hyperlipidemic and had markedly elevated plasma APOB levels versus nondiabetic littermates but were protected from the proatherogenic effects of diabetes. Diabetic *Ldlr*$^{-/-}$*APOA1*^{Tg} mice expressing CETP lost the atheroprotective effect and had increased lesion necrotic core areas and APOB accumulation, despite having lower plasma APOB levels. The detrimental effects of low concentrations of larger HDL particles in diabetic mice expressing CETP were not explained by reduced cholesterol efflux. Instead, large HDL was more effective than small HDL in preventing endothelial transcytosis of LDL mediated by scavenger receptor class B type 1. Finally, in humans with T1D, increased concentrations of larger HDL particles relative to APOB100 negatively predicted incident CVD independently of HDL-cholesterol levels.

CONCLUSIONS: Our results suggest that the balance between APOB lipoproteins and the larger HDL subspecies contributes to atherosclerosis progression and incident CVD in the setting of T1D and that larger HDLs exert atheroprotective effects on endothelial cells rather than by promoting macrophage cholesterol efflux.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: atherosclerosis ■ cardiovascular diseases ■ diabetes mellitus ■ cholesterol ■ lipoproteins, HDL ■ transcytosis

Individuals with well-controlled type 1 diabetes (T1D) generally have normal or even less atherogenic lipoprotein profiles than people without diabetes, but with suboptimal blood glucose control, plasma levels of low-density lipoprotein cholesterol (LDL-C) and APOB (apolipoprotein B) can become elevated, and elevated non-high-density lipoprotein cholesterol (non-HDL-C) and LDL-C predict cardiovascular disease (CVD).[1–3]

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For Sources of Funding and Disclosures, see page 348.

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Novelty and Significance

What Is Known?

- Low levels of high-density lipoprotein-cholesterol (HDL-C) strongly associate with an increased risk of atherosclerotic cardiovascular disease, but human HDL (high-density lipoprotein) varies widely in size, concentration, and cholesterol content.
- Type 1 diabetes greatly increases the risk of cardiovascular disease despite little changes in HDL-C levels and atherogenic APOB (apolipoprotein B)-containing lipoproteins.
- Hyperlipidemic mice expressing the human form of APOA1 (apolipoprotein A1), HDL's major protein, have small and large HDLs similar in sizes to that of human HDL.

What New Information Does This Article Contribute?

- Diabetic mice expressing human APOA1 and human cholesteryl ester transfer protein exhibit lower levels of large HDLs and increased atherosclerosis despite decreased plasma levels of atherogenic APOB-containing lipoproteins.
- Large HDLs prevent endothelial transcytosis of atherogenic APOB-containing lipoproteins through the scavenger receptor class B type 1, suggesting a cardioprotective mechanism distinct from cholesterol efflux.
- Translational studies demonstrate that high levels of large HDLs relative to APOB100 strongly and negatively predicted incident cardiovascular disease independently of HDL-C in humans with type 1 diabetes.

HDL particles range widely in size and cholesterol content. To investigate the cardioprotective properties of different sizes of HDL, we generated hyperlipidemic mouse models expressing human APOA1, the major HDL protein. When diabetes was induced in the presence of cholesteryl ester transfer protein, the concentration of large HDL particles was lower and atherosclerosis increased despite a decrease in plasma atherogenic APOB-containing lipoproteins. However, one proposed cardioprotective action of HDL—cholesterol efflux capacity—was not reduced in those animals. Instead, mechanistic studies demonstrated that large HDLs blocked the transcytosis of LDL across endothelial cells, a key step in atherogenesis. Translational studies demonstrated that high levels of large HDLs relative to APOB100 negatively predicted incident cardiovascular disease independently of HDL-C levels in humans with type 1 diabetes. Collectively, these observations suggest a novel hypothesis: that large HDLs are cardioprotective by blocking the transport of LDL across the endothelium of coronary arteries in humans.

Nonstandard Abbreviations and Acronyms

AAV	adenoassociated virus
ALK1	activin A receptor-like type 1
APOA1	apolipoprotein A1
APOB	apolipoprotein B
APOB100	APOB100, full-length APOB
APOC3	apolipoprotein C3
CEC	cholesterol efflux capacity
CETP	cholesteryl ester transfer protein
CVD	cardiovascular disease
HDL	high-density lipoprotein
HDL-P	high-density lipoprotein particle concentration
IMA	ion mobility analysis
LCMV	lymphocytic choriomeningitis virus
LDL	low-density lipoprotein
L-HDL	large high-density lipoprotein particle
M-HDL	medium high-density lipoprotein particle
S-HDL	small high-density lipoprotein particle
SRB1	scavenger receptor class B type 1
T1D	type 1 diabetes
VLDL	very low-density lipoprotein

Analyses of lipoprotein subpopulations by calibrated differential ion mobility analysis (IMA) or nuclear magnetic resonance suggest that small and medium-sized LDL (low-density lipoprotein) particles are elevated, but larger HDL (high-density lipoprotein) particles are decreased in individuals with T1D and CVD.[4,5]

Because large HDL particles contain more cholesterol than smaller HDLs, this finding is consistent with studies in the general population showing a negative relationship between HDL-C and CVD.[6] However, the discoveries that genetic variations or pharmacological interventions that affect HDL-C levels do not associate with CVD[7,8] have led to the realization that the function of HDL particles, rather than their cholesterol content, may be critical in determining CVD risk.

Human HDL is a complex mixture of particles ranging from ≈7 to ≈12 nm in diameter.[9] The protein and lipid composition and various putative cardioprotective functions of HDLs are not distributed evenly across the HDL size spectrum,[10,11] and HDL subspecies may have distinct cardioprotective effects. Thus, small HDLs (S-HDLs) are more efficient than medium-sized HDL (M-HDLs) and L-HDLs in mediating cholesterol efflux from macrophages by the transporter ABCA1 (ATP binding cassette subfamily A member 1).[9,11–13] Conversely, early studies suggested that more buoyant HDL2 particles may be more effective than denser HDL3 particles at protecting the endothelium.[14] Whether different HDL subpopulations indeed have different biological functions in terms of cardioprotection, and whether differences in HDL's function contribute to the increased risk of CVD associated with T1D is unknown.

To address this question, we generated mouse models of T1D with different concentrations of HDL subspecies. We also investigated whether the relationship between APOB plasma levels and distinct HDL subspecies predicts incident CVD in participants in the CACTI Study (Coronary Artery Calcification in T1D).

Our results suggest that the balance between concentrations of APOB100 lipoprotein particles and larger HDL particles governs lesion APOB accumulation, atherosclerosis, and incident CVD in T1D and that these larger HDL subspecies exert atheroprotective effects on endothelial cells.

METHODS

Data Availability

The authors declare that all supporting data, analytic methods, and study materials are available within the article, in the Supplemental Material, or from the corresponding author upon reasonable request.

Mouse Models of T1D

All animal studies were approved by the Animal Care and Use Committee of the University of Washington (3154-01). *APOA1*^{Tg} (001927) mice were purchased from the Jackson Laboratory. The LDL receptor-deficient (*Ldlr*$^{-/-}$) T-cell–induced *GP*^{Tg} mouse model of T1D-accelerated atherosclerosis has been described previously.[15] These mice express the lymphocytic choriomeningitis virus LCMV GP (glycoprotein) transgene under the control of the insulin promoter, allowing reliable induction of diabetes due to CD8^{+} T cell–mediated β-cell destruction following LCMV injection.[15,16]

Because mice normally exhibit only one HDL particle population in the medium-size range,[17] human *APOA1*^{Tg} mice (which have more HDL particle diversity) were crossed with *Ldlr*$^{-/-}$*GP*^{Tg} mice to generate *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} mice and *Ldlr*$^{-/-}$*GP*^{Tg} littermates expressing the GP transgene. Moreover, a cohort of *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} mice was injected with a liver-targeted adeno-associated virus (AAV-DJ/8 or AAV8; 1×10^{11} genome copies/mouse) to express human CETP (cholesteryl ester transfer protein), a plasma protein normally absent in mice. CETP mediates the bidirectional net exchange of cholesteryl ester in HDL for triglycerides in APOB-containing lipoproteins.[18] All mice were females on the C57BL/6J background, 8 to 12-week of age. We studied female mice because the relative CVD risk is higher among women than in men with T1D.

We designed 2 types of studies, both including a diabetes duration of 4 weeks. The goal of the first study was to characterize the mouse models in terms of lipid and lipoprotein changes. *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} mice were injected with liver-directed TBG-AAV-DJ/8 (thyroid hormone-binding globulin-adenoassociated virus DJ/8) or TBG-AAV8 (thyroid hormone-binding globulin-adenoassociated virus DJ/8) to express either eGFP (enhanced green fluorescent protein) control or human CETP. When CETP plasma activity had reached a steady level, diabetes was induced by LCMV injection, as described.[19] Nondiabetic littermates received saline injections. The mice were fed a low-fat semipurified diet.[15] Diabetic mice received sufficient exogenous insulin to prevent extensive weight loss and ketonuria.

The second study investigated the effects of diabetes on preexisting advanced lesions of atherosclerosis. For this study, female *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} mice were fed a high-fat diet containing 1.25% cholesterol[15] for 16 weeks to allow advanced lesions to develop and were then switched to standard laboratory diet for 2 weeks, and then cohorts of *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} mice were injected with the eGFP or CETP AAV. Two weeks after injection, mice were injected with LCMV to induce diabetes, or saline for nondiabetic controls. Once diabetic, the mice were maintained for 4 weeks on the low-fat semipurified diet. *Ldlr*$^{-/-}$*GP*^{Tg} mice without the APOA1 (apolipoprotein A1) transgene served as controls. We have previously shown that diabetes increases lesion progression using a similar study design in these mice.[16]

Analysis of Atherosclerosis

Aortas were dissected and opened longitudinally (from the heart to the iliac bifurcation), and the Sudan IV-positive area was quantified, as described.[15,16] Aortic sinuses were cross-sectioned and stained using Movat pentachrome stain to visualize lesion morphology.[15,16] Immunohistochemistry is described in the Supplemental Materials (Figure S1 and Major Resources Table).

Isolation, Digestion, and Proteomics Analysis of Lipoproteins and Serum

HDL (density, 1.063–1.21 g/mL) was isolated from the plasma of *Ldlr*$^{-/-}$*APOA1*^{Tg} mice or healthy human subjects by density gradient ultracentrifugation.[20] Digestion and proteomics analyses were performed as described in the Supplemental Materials. LDL was isolated by density ultracentrifugation (density, 1.063–1.019 g/mL).

Determination of HDL and LDL Particle Concentrations and Sizes and HDL Cholesterol Efflux Capacity

HDL and LDL particle concentrations and sizes were quantified by calibrated differential IMA, as described.[5] Cholesterol efflux capacity (CEC) of serum HDL was quantified using cAMP-stimulated J774 macrophages and baby hamster kidney cells with mifepristone-inducible expression of the cholesterol exporter ABCA1.[5]

Endothelial LDL Transcytosis and Uptake

Endothelial uptake of DiI-labeled LDL was performed as previously described,[21] and endothelial transcytosis of DiI-labeled LDL was analyzed using total internal reflection fluorescence microscopy.[22] HDL subpopulations were isolated from pooled plasma of 5 healthy individuals to provide a sufficient quantity of each HDL subpopulation for these experiments (details provided in Supplemental Materials). In some experiments, expression of SRB1 (scavenger receptor class B type 1) or ALK1 (activin A receptor-like type 1) was silenced using siRNA. Efficient protein knockdown was verified by immunoblots.

Human Samples

The 181 samples from a case cohort in the CACTI study on participants with T1D have been described.[16] The subcohort consisted of 145 randomly selected subjects with T1D from the overall CACTI cohort. The clinical characteristics of the selected subcohort and the overall CACTI cohort were similar, with the exception of HbA1c and total cholesterol levels, which were both lower in the subcohort (Table). CVD cases included all 47 subjects with CVD events, defined as myocardial infarction, coronary artery bypass grafting, angioplasty, or coronary artery disease death that occurred after baseline blood collection. All subjects were free of known CAD at the time of blood collection. All human subjects provided informed consent. The studies were approved by the Colorado Combined Institutional Review Board (97-661), UW Institutional Review Board (00012123), and the Unity Health Toronto Research Ethics Board (19–314). Samples from the CLEAR study (Carotid Lesion Epidemiology and Risk) were used to isolate HDL subpopulations for lipid and proteomics analyses.[11] The CLEAR study was approved by institutional review boards at the University of Washington, Virginia Mason Medical Center, and Veterans Affairs Puget Sound.

Table. Baseline Characteristics for Selected Versus Nonselected CACTI Study (Coronary Artery Calcification in Type 1 Diabetes) Participants

Variable	Nonselected (n=402)	Selected (n=145)	*P* value
Age, y	36±9	37±9	0.3229
Diabetes duration, y	23±9	23±8	0.8512
Sex, male, n (%)	184 (46)	66 (46)	0.9580
BMI, kg/m^2	25.9±4.2	26.3±4.4	0.3891
Square root CAC volume	2.8±6.6	2.6±5.5	0.7130
HbA1c, %	8.1±1.3	7.7±1.2	0.0020
Fasting glucose, mg/dL	188±97.8	189±91.2	0.9259
Daily insulin dose per kg body weight, units	0.63±0.23	0.65±0.29	0.4235
eGFR	65±15	65±13	0.9363
Total cholesterol, mg/dL	177±34	171±34	0.0491
LDL cholesterol, mg/dL	101±30	97±28	0.0869
HDL cholesterol, mg/dL	57±17	55±16	0.1919
Triglycerides, mg/dL	93±51	95±73	0.7064
APOB, mg/dL	92±24	89±24	0.1186
Current smoker, n (%)	49 (12.7)	17 (11.8)	0.7895
On lipid treatment, n (%)	72 (17.9)	26 (17.9)	0.9956
On statins, n (%)	71 (17.7)	24 (16.6)	0.7614

Statistical analysis by univariate analysis of the characteristics of those selected vs those not selected, using *t* test for continuous variables and χ^2 test for categorical variables. APOB indicates apolipoprotein B; BMI, body mass index; CAC, coronary artery calcium; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; and LDL, low-density lipoprotein.

Statistical Analyses

Power calculations were based on a previous atherosclerosis study using a similar study design.[23] These power calculations showed that 17 mice per group would provide 80% power to detect a difference in lesion necrotic core area (α, 0.05). Statistical analyses were performed by 2-way ANOVA, aligned rank transform ANOVA,[24–26] or 1-way ANOVA followed by Tukey multiple comparison tests, or Kruskal-Wallis test followed by Dunn multiple comparison tests depending on if one or more individual groups within the data set were non-normally distributed (D'Agostino and Pearson test). Comparison of two groups used unpaired 2-tailed Mann-Whitney *U* test, χ^2 test, or *t* test, depending on if normality was established. Univariate and multivariate models for human samples were built with Cox proportional hazards ratio regression. Hazard ratios (HRs), 95% CIs, and *P* values were calculated using a case-cohort design and the R package cchs, function cch with default Prentice weighting. HRs are reported per 1 SD increment of the ratios of serum levels of APOB100 to concentrations of HDL-P. Ratios were log-transformed because the original ratios were non-normally distributed. Two-sided $P<0.05$ were considered statistically significant. Statistical analyses were performed using GraphPad Prism, Version 10 (San Diego) or the R 3.6.2 statistical computing environment (http://www.R-project.org).

See the Major Resources Table and further details on methods and statistical analyses in the Supplemental Material.

RESULTS

Characterization of T1D Mouse Models With Different HDL Particle Profiles

To investigate the effect of different HDL subspecies on atherosclerosis in T1D, we needed to generate mouse models of T1D with different HDL subpopulation profiles. To achieve this goal, we utilized mice expressing human APOA1, because these mice exhibit a human-like HDL subspecies profile,[17] and crossed them with our *Ldlr$^{-/-}$GP^{Tg}* model of T1D.[15] To generate diabetic mice with lower concentrations of L-HDL, we expressed human CETP by using a liver-targeted AAV.

We first confirmed that the CETP expression and models worked as predicted. Plasma CETP activity increased 2 weeks after AAV injection and remained stable throughout the study (Figure 1A). Diabetes did not alter plasma CETP activity. Plasma CETP activity in *Ldlr$^{-/-}$GP^{Tg}APOA1^{Tg}* mice expressing hepatic CETP was ≈2.5-fold higher than that of human plasma (Figure 1A). As *APOA1^{Tg}* mice have 2.5 to 3.0-fold higher plasma APOA1 levels than humans, the ratio of APOA1/CETP was comparable to humans.[27–29] CETP

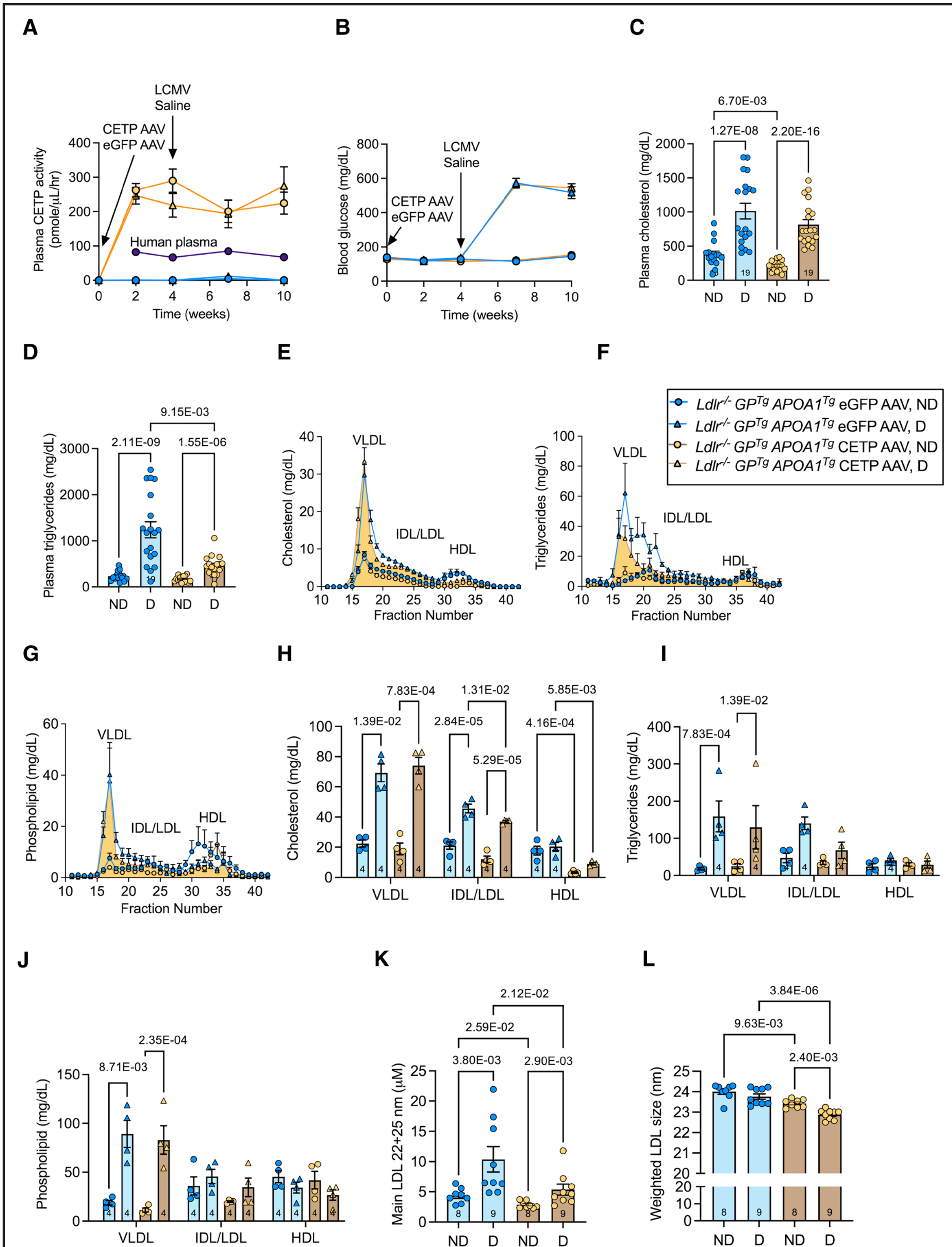

Figure 1. Characterization of the *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} CETP (cholesteryl ester transfer protein) type 1 diabetes (T1D) model. Female *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} mice were injected with liver-targeted TBG-AAV-DJ/8 (thyroid hormone-binding globulin-adenoassociated virus DJ/8) or TBG-AAV8 (thyroid hormone-binding globulin-adenoassociated virus 8) to express human CETP or eGFP (enhanced green fluorescent protein) control. Four weeks after adeno-associated virus (AAV) injection, diabetes (D) was induced by lymphocytic choriomeningitis virus (LCMV). Nondiabetic (ND) littermates received saline. At the onset of diabetes, the mice were switched to a low-fat diet and maintained for 4 weeks. **A**, Plasma CETP activity. A human plasma sample was measured in quadruplicates as an internal control. **B**, Blood glucose during the study. **C** and **D**, Plasma cholesterol and triglyceride levels at the end of the study. **E** through **G,** At the end of the study, lipoprotein profiles cholesterol (**E**), triglycerides (**F**), and phospholipid (**G**) were analyzed in a subset (n=4) of mice. Areas under the curves of diabetic mice (*Continued*)

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expression did not change blood glucose levels in nondiabetic or diabetic mice (Figure 1B) or alter body weights (Figure S2A).

Diabetic *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} mice exhibited higher plasma cholesterol and triglyceride levels, as compared with nondiabetic controls (Figure 1C and 1D). Plasma lipid levels tended to be higher in diabetic *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} mice than in previous studies on this diabetes model in the absence of human APOA1,[15,16] consistent with another study on diabetic *APOA1*^{Tg} mice,[30] perhaps explained by increased VLDL (very low-density lipoprotein) secretion due to increased hepatic HDL-delivery of cholesterol in combination with reduced clearance of triglyceride-rich lipoproteins in diabetic mice.

Plasma lipoprotein profiles revealed that diabetes increased VLDL cholesterol and triglyceride levels and to a lesser extent intermediate-density lipoprotein (IDL)/LDL cholesterol levels (Figure 1E, 1F, 1H, and 1I). Consistent with previous literature,[31] CETP-reduced HDL-C levels in nondiabetic and diabetic mice (Figure 1E and 1H). Diabetes also markedly increased VLDL phospholipids independently of CETP (Figure 1G and 1J). A right-ward shift in the HDL phospholipid peak suggested that CETP resulted in smaller HDL particles (Figure 1G).

Because CETP inhibitors can increase LDL size in humans,[32] we next analyzed LDL particle concentration and size using calibrated IMA. Diabetes increased LDL particle concentration in mice with or without CETP expression, but levels were lower in diabetic mice expressing CETP (Figure 1K). Consistent with human studies,[32] CETP expression slightly reduced LDL size in both nondiabetic and diabetic mice (from 24.0±0.13 to 23.4±0.07 nm diameter in nondiabetic mice [mean±SEM; n=8] and from 23.8±0.13 to 22.9±0.08 nm in diabetic mice [mean±SEM; n=9]; Figure 1L).

To quantify HDL subspecies, we again used calibrated IMA to obtain absolute concentrations of HDL particles. We identified three HDL-P subpopulations in *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} mice, ranging in size from small HDL (S-HDL; 7.6–8.2 nm diameter), medium HDL (M-HDL; 9.5±0.05 nm) to large HDL (L-HDL; 11.2±0.03 nm). The S-HDL subpopulation contained a population of extra-small particles with a mean diameter of ≈7.8 nm, which was not analyzed separately.

The total plasma HDL-P was reduced by half by CETP in nondiabetic mice, but not in diabetic mice (Figure 2A). This finding was explained by a redistribution of HDL subspecies in the 4 groups of mice: while CETP caused a marked reduction in M-HDL-P in nondiabetic mice and in L-HDL-P in both nondiabetic and diabetic mice, diabetes resulted in increases in S-HDL-P and increased M-HDL-P in the presence of CETP (Figure 2B through 2D). CETP increased the S-HDL-P to >60% of total HDL-P whereas the L-HDL-P was reduced to <20% of total HDL-P (Figure 2E and 2F).

We next analyzed changes in the HDL proteome by targeted liquid chromatography-tandem mass spectrometry. In general, the effects of diabetes were distinct from those of CETP, likely due in part to the differences in HDL subspecies (Figure S2B and original Supplemental Material). Hepatic expression of CETP caused detectable human CETP in HDL, as expected[33] (Figure 2G). CETP also reduced the APOA1 content in HDL (Figure 2H).

Our findings support the suitability of these mouse models to investigate the role of HDL subspecies in atherosclerosis in the setting of T1D.

Diabetic Mice With Low L-HDL-P Do Not Show Reduced CEC

The only known function of HDL that predicts incident CVD in humans is its ability to mediate cholesterol efflux from cells.[33] We, therefore, measured basal and ABCA1-specific CEC of APOB-depleted serum (serum HDL) from the mice, using cAMP-stimulated J774 macrophages, which exhibit increased ABCA1 expression, and baby hamster kidney (BHK) cells with inducible ABCA1 expression. Consistent with the total HDL particle concentrations (Figure 2A), basal and cAMP-stimulated J774 cholesterol efflux were lower in nondiabetic mice expressing CETP, as compared with mice expressing eGFP (Figure 2I). Moreover, serum HDL from diabetic mice expressing CETP showed higher basal and cAMP-specific cholesterol efflux than nondiabetic mice expressing CETP (Figure 2I and 2J) rather than lower efflux as would be expected if L-HDL contributed significantly to CEC. There were no differences in HDL CEC between diabetic mice with and without CETP in J774 cells (Figure 2I and 2J). These findings are consistent with smaller HDLs being most effective at ABCA1-mediated CEC. Similar results were obtained using ABCA1-expressing BHK cells (Figure 2K and 2L). Despite the higher levels of S-HDL and CEC, diabetes resulted in a marked accumulation of cholesteryl esters in peritoneal macrophages, with no differences between mice with and without CETP (Figure 2M).

These findings are consistent with the interpretation that diabetic mice with low concentrations of larger HDLs induced by CETP do not have impaired HDL ABCA1-specific CEC.

Figure 1 Continued. expressing CETP are shaded in yellow. **H** through **J**, Cholesterol (**H**), triglyceride (**I**), and phospholipid (**J**) levels in VLDL (very low-density lipoprotein; fraction 17), intermediate-density lipoprotein (IDL)/LDL (low-density lipoprotein; fraction 21), and HDL (high-density lipoprotein; fraction 31) peak fractions (n=4). **K** and **L**, LDL particle concentration (LDL-P; **K**) and LDL size (**L**) were measured by calibrated IMA. Number of mice per group are indicated in the panels and in the original Supplemental Material. Data are expressed as scatter plots and mean±SEM. Statistical analysis by ART ANOVA followed by Tukey multiple comparisons tests (see original Supplemental Material).

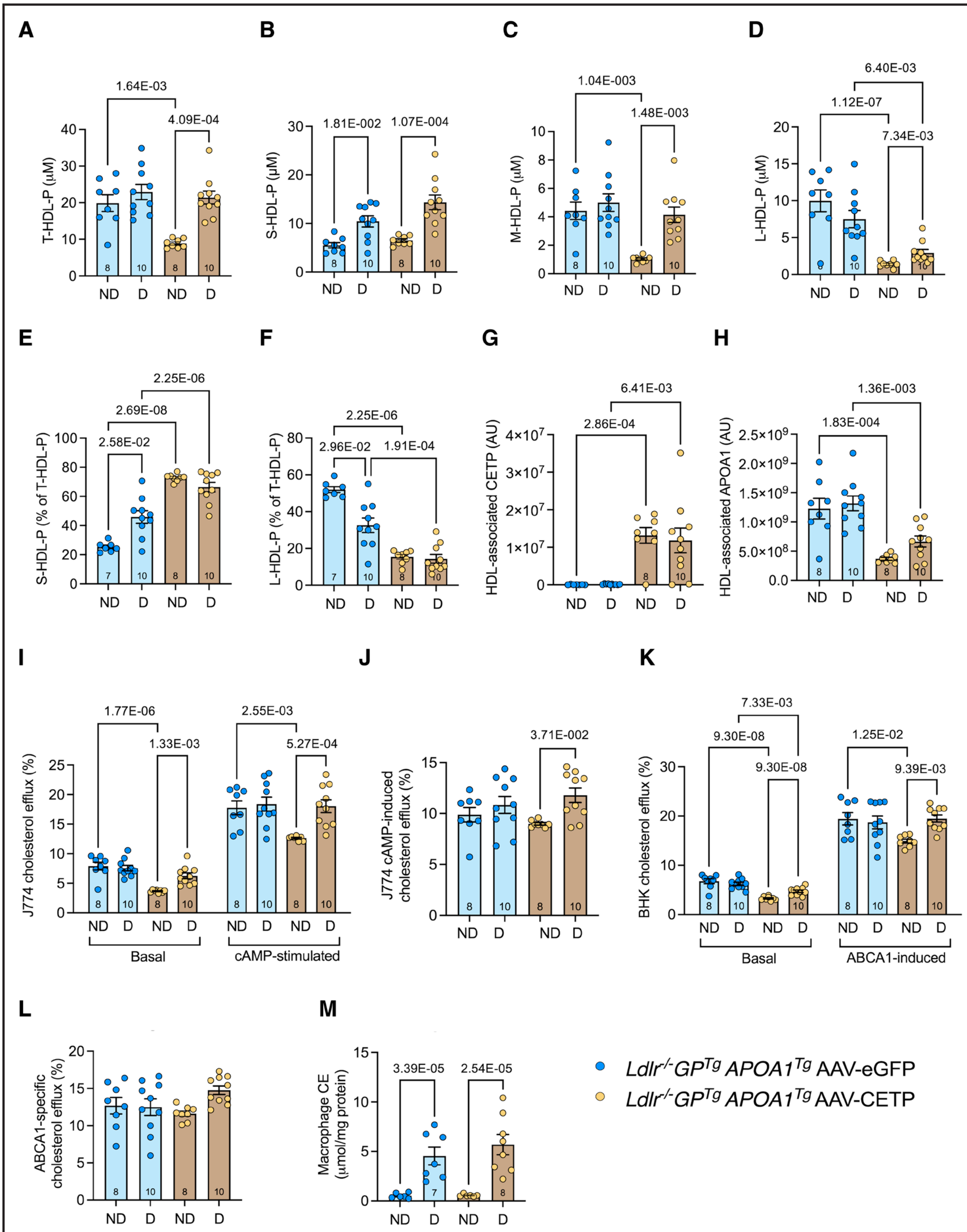

Figure 2. Low L-HDL (large high-density lipoprotein) levels in diabetic mice expressing CETP (cholesteryl ester transfer protein) do not cause reduced ABCA1 (ATP binding cassette subfamily A member 1)-mediated cholesterol efflux.

A through **E**, At the end of the study in Figure 1, concentrations of HDL (high-density lipoprotein) particles were quantified using calibrated ion mobility analysis (IMA). **A**, Total HDL-P (high-density lipoprotein particle concentration). **B**, S-HDL-P (small HDL-P). **C**, M-HDL-P (medium HDL-P). **D**, L-HDL-P (large HDL-P). **E**, S-HDL-P (%). **F**, L-HDL (%) and **G-H**, HDL-associated human CETP and APOA1 (apolipoprotein A1) analyzed by tandem mass spectrometry. **I** through **L**, Cholesterol efflux capacity (CEC) of APOB (apolipoprotein B)-depleted serum was measured in J774 macrophages and ABCA1-expressing baby hamster kidney (BHK) cells. **M**, Resident peritoneal macrophages were adhesion-purified for 1 hour and then used to measure cholesteryl ester contents. Statistical analysis by 2-way ANOVA or aligned rank transform (ART) ANOVA followed by Tukey multiple comparisons tests (see also original Supplemental Material). Data are expressed as scatter plots and mean±SEM. Number of samples per group are indicated in the panels and in the original Supplemental Material.

Low Plasma Concentrations of L-HDL Are Associated With Progression of Atherosclerosis and Lesional APOB Accumulation in Diabetic Mice

As our previous data showed that diabetes promotes the progression of preexisting atherosclerotic lesions and necrotic core expansion,[16,23] we used a similar experimental design (Figure 3A) to investigate how altering HDL subspecies affects atherosclerosis. $Ldlr^{-/-}GP^{Tg}APOA1^{Tg}$ mice were fed a high-fat diet for 16 weeks to allow advanced lesions to develop. One week after switching them to a standard laboratory diet to lower plasma lipids, the mice were injected with eGFP control AAV or CETP AAV targeted to the liver. Two weeks later, the mice were injected with LCMV to induce diabetes or with saline for nondiabetic littermate controls, at a time-point defined as baseline, and were then fed a low-fat semipurified diet starting at the onset of diabetes. Groups of nondiabetic and diabetic $Ldlr^{-/-}GP^{Tg}$ mice were additional controls.

Consistent with previous studies,[16,23] 4 weeks of diabetes did not increase the size of preexisting aortic atherosclerotic lesions as determined by en face analysis of Sudan IV-positive area or aortic sinus cross-sectional lesion area (Figure 3B through 3E). As expected, $Ldlr^{-/-}GP^{Tg}APOA1^{Tg}$ mice before induction of diabetes (baseline) were largely protected from atherosclerosis, as compared with $Ldlr^{-/-}GP^{Tg}$ mice[34,35] (Figure S3). This resulted in markedly reduced Sudan IV-positive aortic lesions and aortic sinus lesions in all $APOA1^{Tg}$ mice, regardless of the presence of diabetes or CETP for the last 4 to 6 weeks (Figure 3C and 3D).

However, the short duration of diabetes resulted in a larger necrotic core area in mice not expressing the *APOA1* transgene, as we have observed previously[23] (Figure 3F). $Ldlr^{-/-}GP^{Tg}APOA1^{Tg}$ mice were protected from the increase in necrotic core area, but $Ldlr^{-/-}GP^{Tg}APOA1^{Tg}$ mice expressing CETP showed a similar phenotype as the control $Ldlr^{-/-}GP^{Tg}$ diabetic mice (Figure 3F). A similar pattern was observed for lesion smooth muscle α-actin (Figure 3G). All groups of mice carrying the *APOA1* transgene had a low content of lesion macrophages (Figure 3H). There were no differences in lesion collagen bundles between the groups (Figure 3I; Figure S4).

We have previously shown that diabetic $Ldlr^{-/-}GP^{Tg}$ mice exhibit an increased accumulation of APOB immunoreactivity in early lesions,[16] despite similar levels of plasma APOB in nondiabetic and diabetic mice.[16,23] Deposition of proatherogenic APOB lipoproteins in the artery wall is a key factor in atherogenesis. We, therefore, asked whether arterial accumulation of APOB is enhanced in the mouse models of T1D. Immunostaining of sinus lesions revealed overall higher levels of immunoreactive APOB in lesions of diabetic mice as compared with nondiabetic mice (Figure 3J; Figure S4). Diabetic $Ldlr^{-/-}GP^{Tg}APOA1^{Tg}$ mice were protected from accumulation of APOB in the absence of CETP (Figure 3J). Like for the necrotic core size, CETP expression significantly increased lesion APOB immunoreactivity in diabetic $Ldlr^{-/-}GP^{Tg}APOA1^{Tg}$ mice (Figure 3J). A similar pattern was observed for APOE immunoreactivity (Figure 3K), which could be derived from lipoproteins or lesion cells. Importantly, no difference in APOB immunoreactivity in aortic sinus lesions in $Ldlr^{-/-}GP^{Tg}APOA1^{Tg}$ mice was observed at baseline, as compared with $Ldlr^{-/-}GP^{Tg}$ mice (Figure S5).

APOA1 immunoreactivity was abundant in all lesions. Diabetes did not affect mouse or human APOA1 immunoreactivity, suggesting no increased trapping of HDL (Figure S6A and S6B).

These observations suggest that APOB-lipoprotein particles accumulate in lesions of diabetic mice concomitant with increased necrotic core expansion. The increased APOB immunoreactivity could indicate increased accumulation of LDL or remnant lipoprotein particles in diabetic mice that have low L-HDL levels.

Lesion Progression in Diabetic Mice With Low L-HDL Is Not Explained by Plasma Lipids or Plasma Apolipoproteins

Diabetic mice exhibited higher plasma cholesterol and triglycerides than their nondiabetic controls but the cholesterol and triglyceride levels in diabetic $Ldlr^{-/-}GP^{Tg}APOA1^{Tg}$ mice expressing CETP were lower than those without CETP (Figure 4A and 4B). There were no differences in blood glucose between the 3 diabetic groups (Figure 4C).

To further investigate plasma levels of key apolipoproteins involved in atherosclerosis, we quantified mouse and human APOA1, human CETP, APOB, APOC3, and APOE by targeted LC-ESI–MS/MS (liquid chromatography electrospray ionization tandem mass spectrometry). As expected, plasma human APOA1 was detected only in $APOA1^{Tg}$ mice, and levels were lower in both nondiabetic and diabetic mice expressing CETP (Figure 4D), likely due to increased clearance of APOA1. Expression of human APOA1 was associated with a striking reduction in mouse APOA1 levels (Figure 4E), consistent with previous studies.[17,28,36] Plasma APOB was markedly increased in diabetic $Ldlr^{-/-}GP^{Tg}APOA1^{Tg}$ mice, as compared with diabetic $Ldlr^{-/-}GP^{Tg}$ mice, although they were less elevated in diabetic mice expressing CETP (Figure 4G), consistent with the lower lipid levels in those mice (Figure 4A and 4B). Plasma APOC3 and APOE levels were similarly increased in diabetic mice with and without CETP (Figure 4H and 4I). Thus, the diabetic mice with and without CETP appeared to have a similar impairment of triglyceride-rich lipoprotein clearance, likely due to insulin insufficiency.[16]

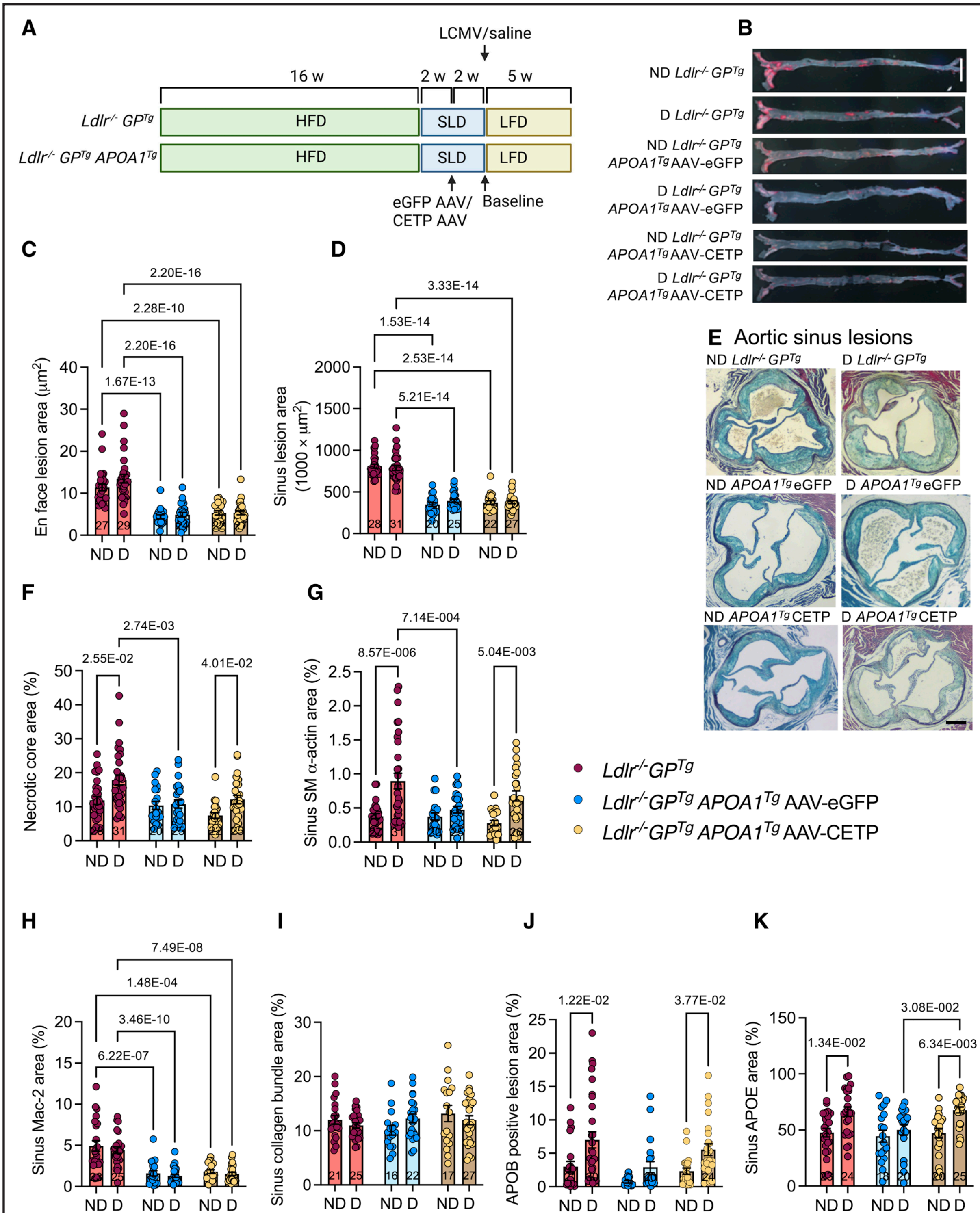

Figure 3. Diabetic mice with low levels of L-HDL (large high-density lipoprotein) exhibit increased lesion progression and accumulation of APOB (apolipoprotein B).

A, Study design. Female *Ldlr*$^{-/-}$*GP*^{Tg} and *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} mice were fed a high-fat diet (HFD) for 16 weeks to allow advanced lesions to form and then were switched to a standard laboratory diet (SLD; chow). After 1 week on SLD, cohorts of *Ldlr*$^{-/-}$*GP*^{Tg}*APOA1*^{Tg} mice were injected with liver-targeted AAV-CETP (cholesteryl ester transfer protein) or AAV-eGFP (enhanced green fluorescent protein). Two weeks after AAV injection, the mice were injected with lymphocytic choriomeningitis virus (LCMV) to induce diabetes or saline for nondiabetic littermates. At the onset of diabetes, mice were maintained on a low-fat semipurified diet (LFD) for 4 weeks. **B** and **C**, En face aortic preparations and quantification of Sudan IV-positive area. **D**, Quantification of aortic sinus lesion cross-section area. **E**, Images of aortic sinus lesion cross-sections stained with Movat pentachrome stain. **F**, Quantification of aortic sinus lesion necrotic core area (%). **G**, Quantification of % aortic sinus smooth muscle (SM) α-actin area. **H**, Quantification of percent aortic sinus lesion Mac-2 area in a subset of mice. **I**, Quantification of percent aortic sinus lesion (*Continued*)

Monocytosis contributes to impaired atherosclerosis regression in diabetic mice and in mice with hyperglycemia.[37,38] Moreover, diabetic *APOA1^{Tg}* mice have been shown to be protected from monocytosis and impaired lesion regression in another mouse model of diabetes.[39] We, therefore, quantified circulating monocytes by flow cytometry. Neither diabetes nor the presence of human APOA1 or CETP altered the numbers of circulating monocytes or Ly6C^{hi} monocytes in our models (Figure S6C and S6D), indicating that altered levels of circulating monocytes are unlikely to explain the atherosclerosis phenotypes.

Therefore, the increased lesion progression in diabetic $Ldlr^{-/-}GP^{Tg}APOA1^{Tg}$ mice expressing CETP cannot be explained by increased plasma cholesterol, triglyceride, or APOB levels, or by monocytosis. Instead, the results are consistent with the interpretation that low levels of L-HDL are associated with a worsened lesion outcome.

L-HDL Protects the Endothelium From LDL Transcytosis Via SRB1

What might explain the increased APOB accumulation in atherosclerotic lesions from mice with low L-HDL-P concentrations? We conducted two types of experiments to address this question. First, we measured human coronary artery endothelial cell uptake of DiI-labeled human LDL in the presence of human S-HDL, M-HDL, and L-HDL. All HDL subpopulations suppressed DiI-LDL uptake, but L-HDL was more effective than S-HDL (Figure 5A and 5B).

Next, we analyzed transcytosis of DiI-LDL in the presence of S-HDL, M-HDL, and L-HDL using total internal reflection fluorescence microscopy.[22] As shown by Figure 5C, all HDL subpopulations interfered with LDL endothelial transcytosis, but L-HDL was the most effective, having a statistically significant effect compared with that of both M-HDL and S-HDL. To investigate whether L-HDLs prevent LDL transcytosis via SRB1 or ALK1, which both mediate LDL transcytosis,[40–42] we silenced SRB1 and ALK1 expression and repeated the transcytosis experiments. As shown by Figure 5D, in cells in which SRB1 had been silenced, the ability of L-HDL to suppress LDL transcytosis was lost. Conversely, silencing of ALK1 did not prevent the ability of L-HDL to suppress transcytosis. Efficient knockdown of SRB1 and ALK1 was verified by immunoblots (Figure S7). These findings demonstrate that L-HDL acts through protecting the endothelium from LDL transcytosis via an SRB1-mediated mechanism.

To compare the composition of the three HDL subpopulations we isolated S-HDL, M-HDL, and L-HDL from 20 participants in the CLEAR study.[11] The larger HDL particles contained less total protein and more phospholipid, total cholesterol, free cholesterol, cholesteryl esters, and triglycerides per particle (Figure S8). Although all HDL particles contained similar proteomes, the relative abundance of several proteins differed between the particles. L-HDL particles had relatively lower APOA1 per particle and were enriched in APOCs, APOD, APOE, and CETP, among others (Table S1). The size and composition of L-HDL likely contribute to the increased ability to compete with LDL for SRB1 binding.

Higher Ratios of Larger HDL Subspecies to APOB100 Negatively Predict Incident CVD Independently of HDL-C in Subjects With T1D Participating in the CACTI Study

Finally, we investigated the concept that the balance between concentrations of larger HDL subspecies and APOB100-containing lipoproteins (LDL, IDL, and VLDL) promote incident CVD by analyzing whether this ratio predicts incident CVD in people with T1D involved in the CACTI study. We used three peptides specific to APOB100 to allow measurements of LDL and VLDL/IDL without interference from chylomicrons and chylomicron remnants (which carry the truncated APOB48 in humans). Because there is one APOB molecule per particle in all APOB-containing lipoproteins, this measure estimates the number of APOB100-containing particles. As expected, higher serum levels of APOB100 positively predicted incident CVD with a HR of 1.65 ([95% CI, 1.19–2.29]; P=0.003) in unadjusted analyses.

Likewise, the calibrated IMA HDL-P measurements determine HDL particle concentration. As shown in Figure 6A, in unadjusted analyses, higher ratios of M-HDL-P to APOB100 negatively predicted incident CVD events in subjects with T1D with a HR of 0.72 (95% CI, 0.52–0.99). The ratios of L-HDL-P to APOB100 and total HDL-P to APOB100 showed similar trends with HRs of 0.84 (95% CI, 0.61–1.14) and 0.78 (95% CI, 0.56–1.08), respectively.

When the models were adjusted for HDL-C levels, the M-HDL-P to APOB100 and L-HDL-P to APOB100 HRs became more prominent and reached statistical significance, with HRs of 0.55 (95% CI, 0.37–0.83; P=0.0040) and 0.50 (95% CI, 0.30–0.83; P=0.0078), respectively. In contrast, the ratio of S-HDL to APOB100 did not significantly predict incident CVD in the unadjusted or adjusted models. Our analyses suggested that the ratios of HDL-P to APOB100 for total HDL-P, M-HDL-P, and

Figure 3 Continued. collagen area in a subset of mice. **J**, Quantification of percent aortic sinus lesion APOB-positive area in a subset of mice. **K**, Quantification of percent aortic sinus lesion APOE (apolipoprotein B)-positive area in a subset of mice. Data are shown as mean±SEM. Statistical analysis by 2-way ANOVA or ART ANOVA followed by Tukey multiple comparisons tests (see original Supplemental Material). Scale bar: 0.5 cm (**B**) and 100 μm (**E**). Data are expressed as scatter plots and mean±SEM. Number of samples per group are indicated in the panels and in the original Supplemental Material.

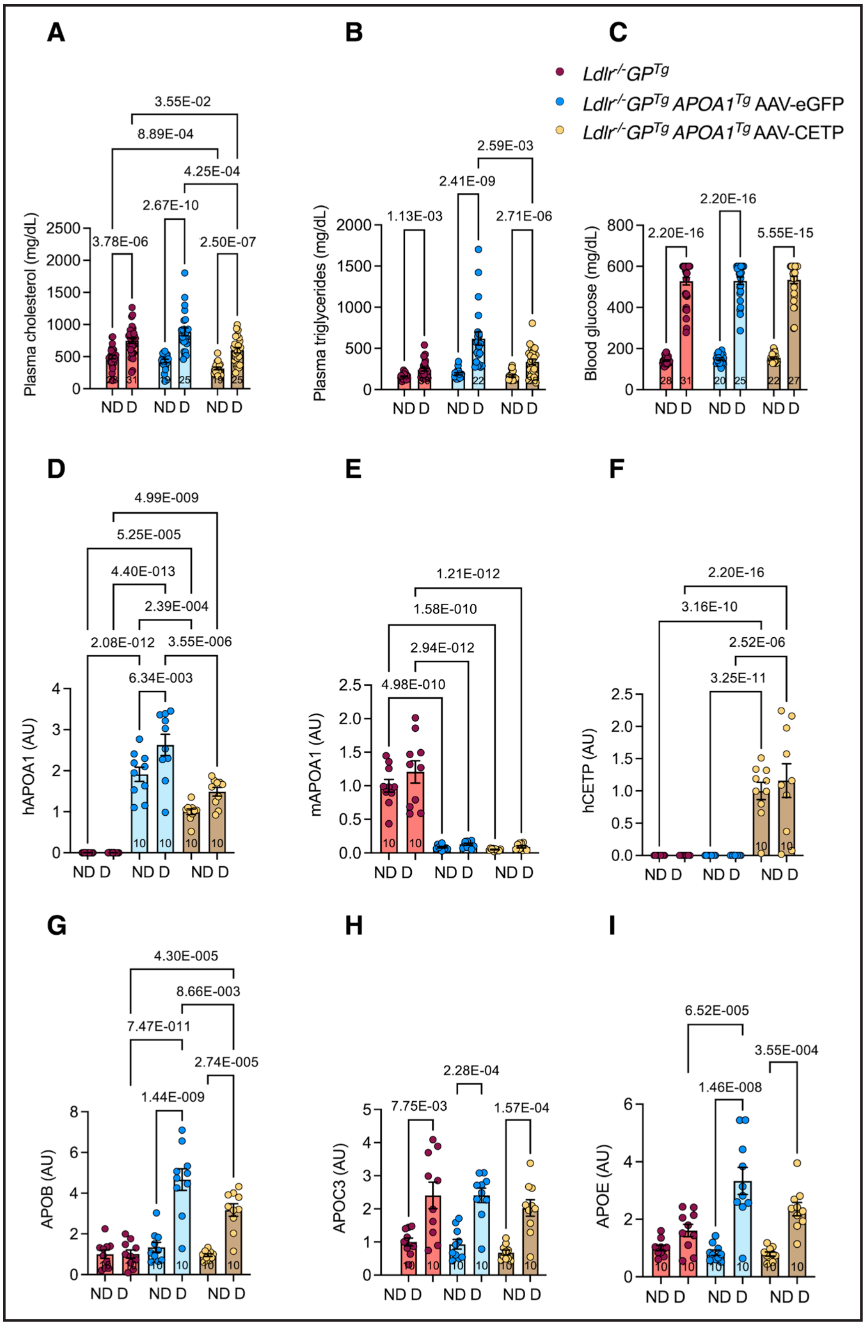

Figure 4. Diabetic mice with low L-HDL (large high-density lipoprotein) concentrations exhibit lower plasma lipids and APOB (apolipoprotein B). Plasma lipids, blood glucose, and apolipoproteins were measured in the mice in Figure 3. **A** and **B**, Plasma cholesterol and triglycerides at the end of study. **C**, Blood glucose. **D** and **I**, Plasma human APOA1 (apolipoprotein A1), mouse APOA1, human CETP (cholesteryl ester transfer protein), APOB, APOC3 (apolipoprotein C3), and APOE (apolipoprotein E) analyzed by targeted mass spectrometry. Statistical analysis by 2-way ANOVA or aligned rank transform (ART) ANOVA followed by Tukey multiple comparisons tests (see also original Supplemental Material). Data are expressed as scatter plots and mean±SEM. Number of samples per group are indicated in the panels and in the original Supplemental Material.

L-HDL-P were highly and negatively predictive at equal HDL-C levels.

When the models were further adjusted for age, sex, diabetes duration, and blood pressure together with HDL-C levels, the ratios of M-HDL-P to APOB100 and L-HDL-P to APOB100 kept significant and negative associations with incident CVD with HRs of 0.59 (95% CI, 0.38–0.92) and 0.39 (95% CI, 0.22–0.69), respectively (Figure 6B). The ratio of total HDL-P to APOB100 also negatively associated with incident CVD with a HR of 0.61 (95% CI, 0.40–0.95), while the ratio of S-HDL-P to APOB100 did not associate with incident CVD in this adjusted model (HR, 1.34 [95% CI, 0.88–2.04]).

Thus, high concentrations of larger HDL subspecies (M-HDL and L-HDL) relative to APOB100 negatively predict incident CVD in individuals with T1D in the CACTI study independently of HDL-C levels and other common clinical confounders.

DISCUSSION

The focus on HDL as a cardioprotective factor has shifted over the years from HDL-C levels to functional and structural differences in HDL and its subspecies. The finding that HDL-P size is a critical determinant of ABCA1-mediated cellular cholesterol export[9,12] led to the increased realization that different HDL subspecies

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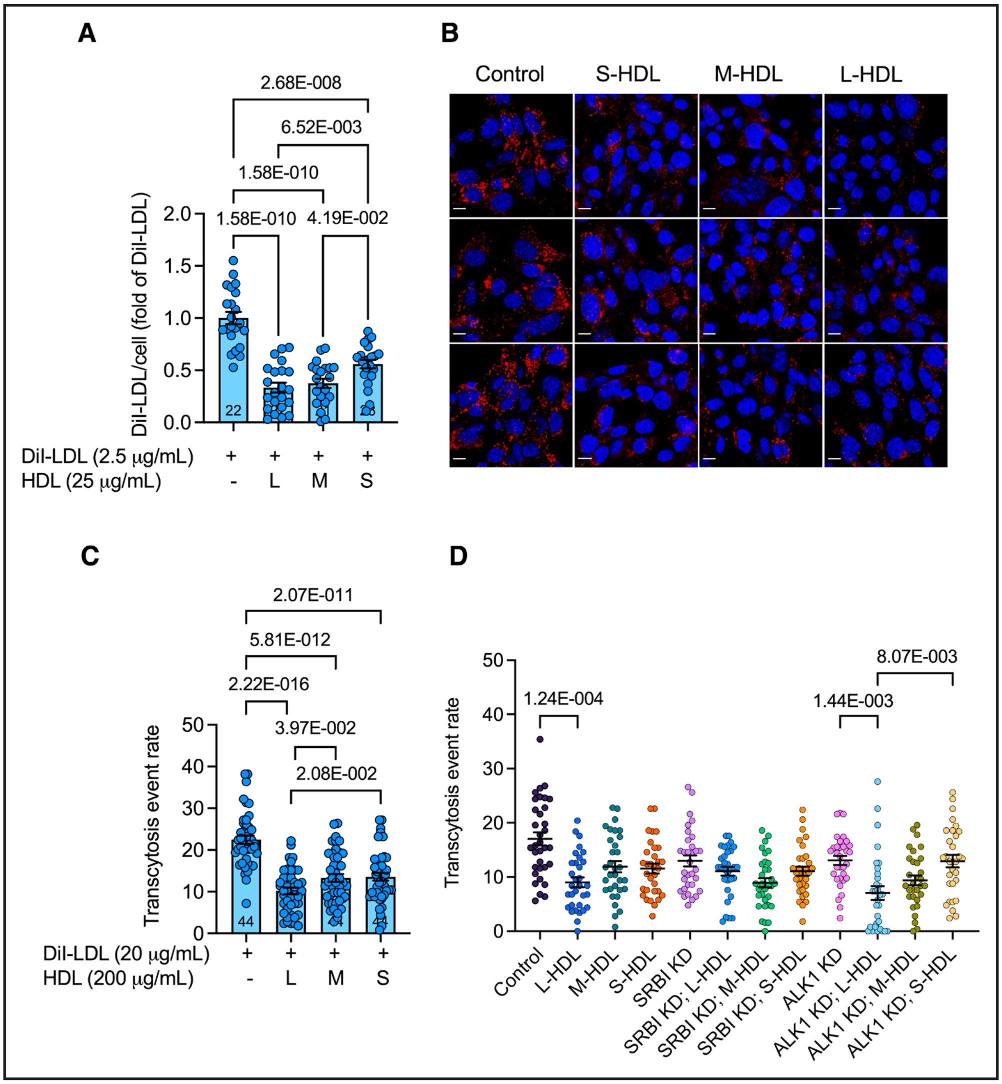

Figure 5. L-HDL (large high-density lipoprotein) is superior at protecting endothelial cells from LDL (low-density lipoprotein) transcytosis and LDL internalization.

A, Human Dil (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate)-LDL endothelial cell uptake in the presence of S-HDL (small high-density lipoprotein), M-HDL (medium high-density lipoprotein), and L-HDL (low high-density lipoprotein). **B**, Representative images of endothelial cells (nuclei stained with DAPI [4′,6-diamidino-2-phenylindole]; blue) and Dil-labeled LDL (red). The experiment was performed twice with similar results. Each data point represents one tissue culture well. Scale bar, 10 µm. **C**, Human Dil-LDL transcytosis was analyzed in the presence of human S-HDL, M-HDL, and L-HDL. The experiment was repeated 4× with similar results. Transcytosis rate represents the average number of exocytosis events per cell that occurred over 15 seconds of each video recording. **D**, LDL transcytosis experiments were repeated in endothelial cells treated with a control siRNA, SRB1 (scavenger receptor class B type 1) siRNA, or an ALK1 (activin A receptor-like type 1) siRNA. The experiment was performed 3×. Statistical analysis by 1-way ANOVA followed by Tukey multiple comparisons tests or Kruskal-Wallis followed by Dunn multiple comparisons tests (see original Supplemental Material). Data are expressed as scatter plots and mean±SEM. Number of samples per group are indicated in the panels and in the original Supplemental Material.

might have different biological or cardioprotective functions. The functional differences between different HDL subspecies are not well understood.

To investigate the distribution and biological functions of HDL subpopulations, we used calibrated differential IMA to quantify particle concentrations of the major HDL-P subspecies. There is currently no consensus on which HDL subpopulation is most beneficial in terms of predicting cardioprotection, but medium-sized HDL particles have most frequently been suggested to be protective in subjects with and without T1D.[5,43,44] A recent study showed that low levels of extra-small HDL-Ps are most strongly associated with incident CVD in men with T1D, although low levels of M-HDL and L-HDL also associated with increased CVD risk.[45] However, it is unclear if the association between HDL-P and CVD risk is causal. It is also unclear if the relative contribution of different HDL subpopulations to cardioprotection diverge in different populations.

The best-known functional differences between HDL subspecies is the superior ability of small HDLs to promote cholesterol efflux from macrophages.[9,11–13]

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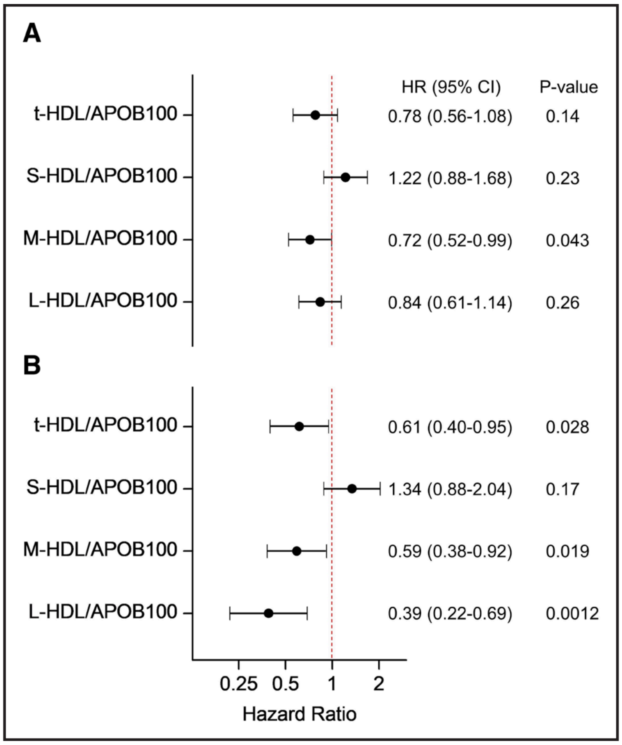

Figure 6. Increased ratios of larger HDL (high-density lipoprotein) particles to APOB (apolipoprotein B) 100 particles negatively predict incident cardiovascular disease (CVD) in humans with type 1 diabetes (T1D).
A, HDL-P/APOB100, unadjusted (human). Serum APOB100 was measured by targeted mass spectrometry and HDL subspecies were measured by calibrated ion mobility analysis (IMA) in human samples from the CACTI Study (Coronary Artery Calcification in T1D; 47 subjects with events; 181 total subjects). Data are shown as hazard ratio (HR)±95% CI. Statistical analysis was performed by weighted Cox proportional hazards linear regression. Unadjusted analysis of HR for HDL-P/logAPOB100 and incident CVD in subjects with T1D. Strata were analyzed for each CVD event, with subcohort subjects serving as controls for each stratum CVD event that occurred while the subcohort member remained at risk for a CVD event (eg, before experiencing a CVD event and before censoring). **B**, HDL-P/APOB100, unadjusted (human). The data in **A** were adjusted for age, sex, diabetes duration, blood pressure and HDL-cholesterol levels. t-HDL indicates total HDL particle concentration.

Importantly, our study identifies a different cardioprotective function of the larger HDL subspecies (M-HDL and L-HDL). We show that these larger HDL particles exert protective effects by acting on the endothelium, leading to reduced LDL uptake and LDL transcytosis. Although L-HDL was the most effective at protecting the endothelium on a per-particle basis, M-HDL had a similar effect, and L-HDL was more effective than S-HDL. In humans, M-HDL particles are the most abundant, perhaps explaining the positive association between M-HDL-P and protection from CVD.[5] Consistently, endothelial dysfunction in a human cohort was associated with lower plasma concentrations of M-HDL and L-HDL, but not S-HDL.[46] Our results are also consistent with those findings because the ratios of serum L-HDL-P/APOB100 and M-HDL-P/APOB100 negatively predicted incident CVD independently of HDL-C in CACTI participants with T1D.

The detrimental effect of CETP (low concentrations L-HDL) on lesion progression and APOB accumulation in human APOA1-transgenic diabetic mice occurred despite lower levels of plasma lipids and APOB, as compared with CETP-deficient APOA1 transgenic diabetic mice. The lower levels of APOB are most likely explained by an increased hepatic clearance of APOB-containing particles. Although LDL diameter was slightly (≈1 nm) smaller in CETP-expressing diabetic mice, potentially allowing it to more readily enter the lesion, our findings are consistent with the proposal that larger HDLs exert cardioprotective effects by acting on the endothelium, preventing transcytosis of LDL and perhaps small VLDL or VLDL remnants.

We show that the mechanism behind the protective effect of larger HDLs on endothelial cells is mediated by SRB1 and not ALK1. Larger and less dense HDL particles bind better (have a greater affinity) to SRB1 than smaller HDL particles,[47] and LDL transcytosis is known to be mediated by SRB1[40,42] and ALK1.[41] Larger HDLs might also prevent remnants derived from triglyceride-rich lipoproteins from entering the lesion.[21]

Do these results suggest that specific CETP inhibitors will be effective in people with T1D who also have elevated APOB100 levels? Several CETP inhibitors (torcetrapib, dalcetrapib, evacetrapib) have failed in large-scale randomized cardiovascular clinical outcome trials in subjects without T1D.[18] Only one CETP inhibitor, anacetrapib, resulted in the prevention of coronary heart disease events, but the protective effect was attributed to this drug's ability to reduce LDL-cholesterol rather than to an increase in HDL-C. However, recent biomarker-weighted drug target Mendelian randomization analyses suggest that the failures of CETP inhibitors were compound-rather than target-related, and that on-target CETP inhibition is likely to decrease CVD risk.[48] Moreover, genetic CETP deficiency is associated with a lower risk of cardiovascular morbidity and mortality, but with a higher risk of age-related macular degeneration,[49] although another study suggested adverse effects on CVD risk with CETP reduction, possibly explained by adverse effects in subjects with hypertension or on antihypertensive drugs.[50] Analyses of lipoprotein particle subpopulations by nuclear magnetic resonance indicated that higher CETP concentrations are associated with lower levels of L-HDL, consistent with our study, and with more small VLDL particles.[51] It is possible that highly specific next-generation CETP inhibitors, such as obicetrapib[52] will be more effective. In addition, CETP inhibitors appear to reduce incident type 2 diabetes through an unclear mechanism.[53] The relevance of these findings to T1D is also unclear.

In summary, our study suggests that the balance between APOB lipoproteins and larger HDL particles governs lesion APOB accumulation, atherosclerosis progression, and

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incident CVD in T1D, and that larger HDLs exert atheroprotective effects on endothelial cells rather than by promoting macrophage cholesterol efflux. Ultimately, our study supports the conclusion that controlling LDL and VLDL remnants, as well as distinct HDL subspecies will be important for prevention of CVD associated with T1D.

ARTICLE INFORMATION

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Disclosures

K.E. Bornfeldt serves on the Scientific Advisory Board of Esperion Therapeutics. The other authors report no conflicts.

Supplemental Material

Expanded Materials and Methods including Table S1
Figures S1–S8
Original Data Supplement

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