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LDL-Containing Immune Complexes in the DCCT/EDIC Cohort: Associations with Lipoprotein Subclasses

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Abstract

Immune complexes containing modified LDL (LDL-IC) and NMR-determined total LDL particle concentrations are significantly associated with intima-medial thickness (IMT). We analyzed the associations between concentrations of NMR-determined lipoprotein subclasses and LDL-IC in the DCCT/EDIC cohort. LDL-IC concentrations in women and men of the DCCT/EDIC cohort did not differ significantly and were positively associated with Total LDL particle concentrations in men and women ($r=0.34$, $r=0.32$, respectively; $p<0.01$) and with Small LDL concentration ($r=0.22$, $r=0.13$, respectively; $p<0.01$). In women, Large LDL concentrations were also associated with LDL-IC ($r=0.20$, $p<0.01$) while in men, the association was more modest ($r=0.11$, $p<0.05$). Thus, both Small and Large LDL are associated with LDL-IC formation. Based on the results from statistical mediation analyses, we concluded that plasma concentrations of LDL-IC may provide a physiological link between the statistically significant association of Total LDL particle concentration with carotid artery intima-media thickness in subjects with Type 1 diabetes. Furthermore, after adjusting for conventional risk factors, there was a decrease in LDL-IC concentration even in the presence of high Total LDL particle concentrations in those women with high concentrations of Large HDL but the association was not evident in men. This suggests that the associations between Large HDL and Total LDL particle concentrations, and their associations with LDL-IC levels, differ by gender and suggest that LDL-IC partially mediates the contribution of Total LDL particle concentration to increased carotid IMT in diabetic men.

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Keywords

Diabetes; Lipoprotein subclasses; LDL autoantibodies; Intima-media thickness; Statistical mediation analysis

INTRODUCTION

Enhanced oxidative stress and dyslipoproteinemia have been proposed as significant factors contributing to the accelerated development of macrovascular complications in diabetes (1–3). Multiple lines of evidence support the role of immune complexes containing modified LDL (LDL-IC) in inflammation and in atherosclerosis in diabetes (4). First, formation of autoantibodies is generally enhanced in Type 1 diabetes due to the multiple immunoregulatory defects associated with the disease (5). Second, diabetic patients are at a higher risk of forming LDL-IC because LDL glycation favors oxidation and advanced glycation end-product (AGE) formation (6,7). These LDL modifications promote immune complex formation detectable in both serum (4) and in atheroma (8,9). LDL-IC levels were significantly elevated in a small group of patients with Type 2 diabetes and coronary heart disease (CHD) when compared with levels in control subjects and in non-diabetic patients with CHD (10). Levels of LDL-IC were also significantly higher at entry into a prospective case-controlled sub-study of the (Type 1 diabetes) Pittsburgh Epidemiology of Diabetes Complications Study cohort in the group of patients in whom macrovascular disease developed within a 7-year follow-up period compared to the matched control group without macrovascular disease (11,12). These results were confirmed and extended in our recent investigations of the Diabetes Control and Complications Trial/Epidemiology of Diabetes and Complications (DCCT/EDIC) cohort that demonstrated that LDL-IC concentration was independently associated with the 5-year progression of carotid intima-media (IMT) thickness, a surrogate marker for CHD (13).

We have previously analyzed in detail using Nuclear Magnetic Resonance (NMR) spectroscopy the concentrations of lipoprotein subclasses in patients with Type 1 diabetes enrolled in the DCCT/EDIC cohort (14–18). We have determined that male gender and poor glycemic control are associated with a potentially more atherogenic NMR-determined lipoprotein subclass profile (15). The concentrations of Medium and Small LDL were significantly increased, while those of Large HDL were significantly decreased in men from this cohort. We also determined that the total LDL particle concentration was positively correlated with concurrent HbA1c levels. Most importantly, we determined that total LDL particle concentration quantitated using NMR spectroscopy was positively associated with internal carotid IMT in both sexes after adjusting for conventional risk factors (14).

Prior to the present study, the contribution of specific LDL subclasses to LDL-IC formation had not been investigated. Therefore, we determined the associations between the NMR-determined lipoprotein subfraction profile and the concentrations of LDL-IC in the DCCT/EDIC cohort of Type 1 diabetic subjects. We now report that Total LDL particle concentration, and the concentrations of both Small and Large LDL, were associated with increased LDL-IC formation in the DCCT/EDIC cohort. Of equal importance, increased concentrations of Large HDL were related to decreased LDL-IC formation.

METHODS

Patients and Blood Samples

Serum was obtained initially from fasting blood collected from 1063 patients with Type 1 diabetes enrolled in the Epidemiology of Diabetes Intervention and Complications (EDIC)

study who agreed to participate in a collaborative study between EDIC and the Medical University of South Carolina (MUSC). Blood was collected by venipuncture at the EDIC clinics from patients who consecutively appeared between May 1997 and May 2000 for their biennial evaluation of lipid levels and physical exam according to the DCCT/EDIC research protocol (19). Blood was clotted at room temperature for 1 h, and centrifuged to separate serum. EDTA (1 mg/ml) was added to the serum to prevent LDL oxidation and aliquots were immediately stored at -70°C until they were shipped monthly on dry ice to MUSC. The Institutional Review Board on record at every participating clinical research center approved the study, and each participant provided written informed consent. The Institutional Review Board of the Medical University of South Carolina approved the experimental protocol.

Unfortunately, not all of the 1063 EDIC participants in the MUSC ancillary study provided adequate sample volume to perform both NMR lipoprotein analyses and LDL-IC measurements. Lipoprotein subclass distribution was evaluated using NMR in 932 of the 1063 participants (88%) while LDL-IC concentration was determined in 911 (86%). A total of 845 patients had both measurements. Thus, analysis of the associations of NMR-determined lipoprotein concentrations with LDL-IC was based on the 845 participants with both data points available. Clinical characteristics reported in Table 1 for these 845 patients did not differ significantly from those in the 1,301 patients who comprise the overall DCCT/EDIC cohort (data not shown). Additional clinical characteristics of these patients have been reported in our previous publications (13–18).

Characterization of Soluble Immune Complexes (IC) Precipitated with Polyethylene Glycol (PEG)

Immune complexes were precipitated from serum as described previously (10,13). In brief, serum (3 mL) was gently mixed under constant and gentle stirring with an equal volume of a freshly prepared and sterilized 7% (v/v) polyethylene glycol (PEG) solution in borate buffered saline (BBS), pH 8.4. The samples, containing a final PEG concentration of 3.5%, were incubated overnight (4°C) and then centrifuged (3000 rpm, 20 min). The precipitates were washed once with 14 ml of chilled 3.5% PEG in BBS, centrifuged, and gently suspended at 37°C in 3 ml of PBS containing 0.01% EDTA, pH 7.4. The cholesterol content of the precipitated IC was analyzed by gas chromatography after extraction of the PEG-precipitated immune complex with a mixture of chloroform:methanol (2:1, v/v). The protein content of PEG-precipitated IC was measured by a modified Lowry assay.

NMR Analyses

The lipoprotein subclass profile was determined using NMR analysis of the fasting serum sample collected from each patient. The methodology used in this study to acquire and process the NMR data has been described in detail (20) and consists of three steps: 1) acquisition of 250-MHz proton NMR spectra of the serum specimens at 45°C , using a Bruker WM-250 spectrometer; 2) deconvolution of the lipid methyl group signal envelope appearing in these spectra at ≈ 0.8 ppm, yielding the derived signal amplitudes broadcast by 18 modelled lipoprotein subclasses; and 3) conversion of these signal amplitudes to lipoprotein subclass concentrations by using experimentally determined standards that relate the signal amplitudes of isolated subfraction standards to their chemically measured cholesterol and triglyceride concentrations. To simplify data analysis, diameters of LDL subclasses were reported as Large (21.2–23 nm) (15) and Small LDL subclasses (18–21.2 nm). HDL subclasses were grouped as Large (8.8–13 nm), Small (7.3–8.2 nm) and Medium HDL subclasses (8.2–8.8 nm) (15).

Evaluation of Carotid Artery Intima Media Thickness

Carotid IMT data were obtained within 4 years of NMR lipoprotein analysis as previously described (21). In brief, a single longitudinal lateral view of the distal 10mm of both common carotid arteries and three longitudinal views in different imaging planes of each internal carotid artery were obtained by B-mode ultrasound (21). The internal carotid artery was defined as including both the carotid bulb and 10mm of the internal carotid artery distal to the tip of the flow divider separating the external and internal carotid arteries (21). The maximum IMT of the common carotid artery was defined as the mean of the maximum IMT for near and far walls in both right and left sides. The maximum IMT of the internal carotid artery was defined similarly and the results from three scans were averaged (anterior, lateral, and posterior views on both sides). Videotapes of the studies were graded at the DCCT/EDIC Central Ultrasound Reading Unit at New England Medical Center by certified sonographers who were masked to study details.

Statistical analysis

The primary hypothesis was that selected lipoprotein subclass concentrations known to be associated with vascular disease were associated with LDL-IC concentrations, and that the LDL-IC concentrations partially mediated the association of lipoprotein subclass concentrations with carotid IMT. To address this hypothesis systematically, a combination of descriptive and regression-based techniques were used. Due to gender differences in the lipoprotein subclass profiles (15) and in the associations of lipoprotein levels with IMT (14), all analyses were stratified by sex.

Descriptive statistics were calculated for demographic variables, the variables of interest, and known confounders of LDL-IC. Spearman's rho was used to measure the correlation of demographic variables with LDL-IC. The following conventional risk factors were considered as adjustment variables in all adjusted regression models: age, the patients' assigned DCCT treatment group, concurrent HbA1c level, and waist-to-hip ratio. In the cohort, it was determined that 6.8% of the men and 4.5% of the women were taking lipid lowering medications while 9% and 7.8%, respectively, received anti-hypertension medication. Therefore, prior to formal analyses, the association of serum LDL-IC concentration with these medications was examined to determine if additional factors should be included as adjustment variables. It was found that lipoprotein subclass distribution in participants taking anti-hypertensive medications did not differ significantly from those not taking antihypertensive medications ($p=0.12$ and $p=0.42$, respectively for men and women). In contrast, serum LDL-IC concentration in patients taking lipid-lowering medications (32 men, 17 women) was significantly different from those not taking lipid-lowering medications ($p=0.004$ and $p=0.039$ in men and women, respectively); therefore, all analyses were additionally controlled for treatment with lipid-lowering medications.

To facilitate graphical illustration and interpretation, LDL-IC and NMR particle concentrations were grouped into tertiles, thereby enabling standard ANOVA/ANCOVA methods (note: regression models treating the variables as continuous measures yielded comparable findings). Analysis of covariance (ANCOVA) models were constructed to investigate the effect of Large HDL concentration on LDL-IC controlling for the Total LDL particle concentration and conventional risk factors. The Tukey-Kramer (22,23) correction factor was used to adjust p-values for multiple comparisons that resulted from *post hoc* comparisons of adjusted cell means (lsmeans).

A mediation analysis was performed to test the extent that LDL-IC cholesterol concentrations mediated the association between total NMR-determined LDL particle concentrations and carotid IMT. Prior to analysis, all variables (IMT, LDL-IC, and total

LDL particle concentration) were standardized (i.e., transformed to follow a standard normal distribution). Three measures of the dependent variable, IMT, were assessed; Internal IMT, Common IMT, and Combined IMT as measured at EDIC Year 6 (21). The extent of mediation is quantified by the difference in the unadjusted association of NMR with IMT (denoted as τ) and the adjusted effect (τ'). To test the statistical significance of the mediated effect, the bootstrap procedure developed by Lockwood and MacKinnon (24) was used. A total of 2000 bootstrap replicates were used to determine the mean indirect effect and corresponding 95% confidence interval.

SAS software (Version 9.1.3, SAS Institute, Cary, NC) was used in all statistical analyses. The bootstrap estimate of the mediated effect was calculated by a SAS MACRO (25). All significant tests were two-sided and were considered statistically significant at $p < 0.05$.

RESULTS

We investigated the Spearman correlation of LDL-IC concentration with clinical characteristics (Table 1) and determined that BMI, but not diabetes duration, was significantly associated with LDL-IC cholesterol levels in both sexes. Although the concentration of LDL-IC in women and men did not differ significantly (Table 1), LDL-IC concentrations were not significantly correlated with WHR in women but were associated with concurrent HbA1c. In contrast in men, LDL-IC concentration was significantly correlated with WHR. LDL-IC concentrations in both sexes were significantly and positively associated with Total LDL, Large LDL, and Small LDL particle concentrations.

There was a highly significant, negative association of Large HDL particle concentration with LDL-IC in both sexes. In women, the concentrations of Total as well as Small and Medium sized HDL particles were not significantly associated with LDL-IC. In men, the concentration of Small HDL particles was, unexpectedly, positively associated with LDL-IC suggesting that this HDL subclass may differ metabolically from Small HDL in diabetic women.

We investigated further the associations of Small LDL, Large LDL, and Total LDL with increasing LDL-IC concentration separated by tertile using ANCOVA procedures after adjustment for conventional risk factors (age, prior DCCT treatment group, concurrent HbA1c level, waist-to-hip ratio, and treatment with lipid lowering medications). These results are shown in Figure 1. The concentration of LDL-IC was similar in both men and women, respectively, in the Low (87 ± 27 vs. 82 ± 29), Medium (171 ± 25 vs. 169 ± 25), and High (352 ± 123 vs. 336 ± 131 $\mu\text{g/L}$) LDL-IC tertiles. When LDL concentration was examined in each tertile of LDL-IC, in men there was a statistically significant increase in Small LDL particle concentration in the high compared to both the low tertile concentration of LDL-IC ($p < 0.001$) as well as in the medium tertile ($p < 0.01$). Women also showed increases in Small LDL concentration but the differences reached statistical significance only between the low and high LDL-IC tertiles ($p < 0.05$). Large LDL concentration increased only moderately between the low and medium LDL-IC tertiles ($p < 0.05$) in men, while in women there were significant increases between the low and both the medium LDL-IC tertile ($p < 0.05$) as well as the high concentration tertile ($p < 0.01$). In both men and women, there were statistically significant increases in Total LDL concentration between the low and high ($p < 0.001$) LDL-IC tertiles. In men, there was also a significant increase in Total LDL concentration between the medium and high LDL-IC tertiles while in women, the increase was significant in the medium compared to the low LDL-IC tertiles ($p = 0.005$). Thus, LDL-IC concentration increases significantly with increases in Total LDL particle concentration. In men, the increase in LDL particle concentration results primarily from a significant increase in Small LDL concentration, while in women, Large LDL is the primary contributor.

The particle concentrations of Large HDL were negatively associated with LDL-IC concentrations while those for Total LDL were positively associated. Therefore, we investigated the impact of Large HDL particle concentration on the association between Total LDL particle and LDL-IC concentrations by modelling associations between tertile concentrations of Total LDL particles and of Large HDL particles with LDL-IC concentration while controlling for conventional risk factors. These results are graphically presented in Figure 2. Total LDL particle concentration was associated with LDL-IC concentration ($p < 0.0001$) in both men (Figure 2A) and women (Figure 2B). There was a decrease in LDL-IC concentration, even in the presence of high Total LDL concentration, in those women with high concentrations of Large HDL. In contrast, even high concentrations of Large HDL were not associated with lower LDL-IC concentrations in the group of men with high Total LDL particle concentration.

Two factors, Total LDL particle concentration and LDL-IC concentration, each have been shown to be significantly associated with a single outcome, e.g. carotid intima-media thickness (13,14). Statistical mediation is used to evaluate the contribution made by one of the factors, in this instance LDL-IC concentration, to mediate the association between the other factor (Total LDL particle concentration) and the outcome variable (IMT). Table 2 displays results of the statistical mediation analysis. To establish the mediated effect, the association of Total LDL particle concentrations with carotid IMT measurements was first considered although this analysis is generally regarded as a non-essential step for a formal mediation analysis (24). This effect, denoted as τ , was statistically significant for all three IMT measurements in men but in women, the association was statistically significant for the internal or common carotid, or for the combined thickness measurement. The unadjusted effect of Total LDL particle concentrations on LDL-IC levels, denoted as α , was then evaluated and this analysis demonstrated that Total LDL particle concentrations were significantly associated with LDL-IC levels in both men and women in agreement with the analyses reported in Table 1. Regression equations were then calculated to estimate the effect of LDL-IC concentration on IMT while adjusting for the contribution of Total LDL particle concentration (β) and to estimate the effect of Total LDL particle concentration on IMT while adjusting for the contribution of LDL-IC concentration on IMT (τ'). Statistical mediation analysis, reported as the difference between the unadjusted and adjusted regression estimates of Total LDL particle concentration with IMT ($\tau - \tau'$), support the conclusion that LDL-IC concentration partially mediated the association of Total LDL particle concentration with internal carotid IMT in both men and women ($p < 0.05$); however, the association was significant with common carotid IMT in men only ($p < 0.05$).

DISCUSSION

We have shown for the first time that the concentration of immune complexes formed against modified lipoproteins (LDL-IC) is significantly associated with the Total LDL particle concentration in both women and men with Type 1 diabetes, and persisted even after controlling for conventional risk factors. Furthermore, there was evidence that the LDL-IC levels partially mediated the previously reported association of total LDL particle concentrations with cardiovascular disease reflected by IMT (14) in the internal carotid artery in men but the mediation effect in women failed to reach statistical significance.

Native, unmodified LDL is not immunogenic in humans. Therefore, the presence of LDL-IC can only be associated with the presence of modified LDL. Interestingly, neither Small nor Large LDL subclasses were uniquely associated with LDL-IC levels. The concentrations of both Large and Small LDL subclasses were strongly associated with the LDL-IC levels in women but only that of the Small LDL subclass was significantly associated with the LDL-IC concentration in men (Table 1). However, it is important to note that in women the levels

of Large LDL are approximately 1.5 times higher than those of Small LDL. Conversely in men, the levels of Small LDL are higher than those of Large LDL. Thus, these data suggest that both (Small and Large) LDL subclass particles can be modified and promote LDL-IC formation.

Our data suggest that both larger diameter LDL particles as well as the potentially more atherogenic smaller LDL may contribute to LDL-IC formation, but future, prospective, studies are needed to support a causal link. Most importantly, the Total LDL particle concentration, even the lower Total LDL particle concentrations found in women, is associated with LDL-IC formation. This observation complements the data reported by Mora *et al.* (26) in their lipoprotein analyses of the MESA cohort. These authors reached the conclusion that both Small LDL and Large LDL are associated with increased carotid IMT and although Small LDL may be more readily oxidized, the total number of LDL particles is the final determinant of CVD risk or IMT progression. Thus, if the total LDL particle concentration is high, even if Small LDL levels are low, CVD risk is increased.

We were interested in examining the relationship between lipoprotein levels and LDL-IC because it is well known that native LDL does not alter intracellular cholesterol homeostasis or promote atherogenesis. Atherosclerosis is promoted by modified lipoproteins which may lead to the production of modified LDL antibodies and we have demonstrated that the modified forms of LDL in circulation are responsible for the formation of LDL-containing IC (27). Both modified LDL, but particularly LDL-IC, have pro-inflammatory properties and contribute to the induction and perpetuation of vascular inflammation. We previously have clearly shown that LDL-IC prepared with human oxLDL and human oxLDL antibodies are potent stimulators of macrophages and promote release of pro-inflammatory cytokines and chemokines (28,29). These pro-inflammatory cytokines induce overexpression of adhesion molecules and promote endothelial dysfunction (4). Thus, there is ample evidence proving that LDL-IC are potent triggers of inflammation. Furthermore, modified LDL-IC can stimulate scavenger receptor expression and also promote transformation of macrophages into foam cells due to their uptake by the Fcγ receptor (30). Therefore determining which LDL subclass or subclasses elicit LDL-IC formation is important due to the major role these IC play in the development of cardiovascular disease.

In addition to the strong positive association between Total LDL particle concentration and LDL-IC concentration in both men and women (Table 1), we found an equally powerful but negative association between LDL-IC concentration and the concentration of the Large HDL subclass. The Large HDL subclass is generally considered to be atheroprotective. Therefore, we investigated the potentially atheroprotective influence of high concentrations of Large HDL on the potentially atherogenic contribution of Total LDL particle concentration to LDL-IC formation in this Type 1 diabetes cohort (Figure 2). The relationship between Large HDL particle concentration and Total LDL concentration with LDL-IC concentration was strikingly different in women and men. In women, increasing concentrations of Large HDL appeared to function in an atheroprotective manner and to moderate the positive association between LDL particle concentration and LDL-IC formation. Importantly, high concentrations of Large HDL reduced LDL-IC concentration even in those women with the highest Total LDL particle concentration. In contrast in Type 1 diabetic men, Large HDL particle concentration did not modulate the positive contribution of Total LDL particle number to LDL-IC levels. Surprisingly, LDL-IC concentrations were generally higher in men with the highest concentrations of Large HDL particles regardless of the Total LDL concentration. This suggests that the Large HDL subclass in Type 1 diabetic men may be less atheroprotective than the comparable HDL subclass in diabetic women. Although the effects of LDL and HDL concentration on LDL-IC concentration differed substantially between sexes, the association of Large HDL concentration with LDL-IC concentration did

not attain statistical significance in either gender when controlling for these same factors ($p=0.21$ and $p=0.52$). This may have resulted from low statistical power due to the limited numbers of subjects with high Large HDL particle concentration occurring concomitantly with high levels of Total LDL particle concentration ($n=11$ in women and $n=14$ in men). Regardless, the results of the present study strongly support the need for further investigation of HDL function in diabetes.

We have shown previously that both Total LDL particle concentration (14) and circulating concentrations of LDL-IC (12,13) are statistically associated with carotid intima-media thickness. Based on the results from statistical mediation analyses, we now suggest for the first time that plasma concentrations of LDL-IC may provide a physiological link between the statistically significant association of Total LDL particle concentration with carotid artery intima-media thickness in subjects with Type 1 diabetes (Table 2, ($\alpha\beta = \tau - \tau'$)). Increasing LDL particle concentrations, regardless of LDL diameter, were positively associated with LDL-IC concentrations in the circulation (Table 1). Our mediation analysis now suggests that LDL-IC formation may be one potential mechanism whereby increasing LDL concentration contributes to macrovascular disease in these diabetic patients. The schematic diagram presented in Figure 3 presents possible mechanisms whereby LDL-IC mediates the statistically significant association between increasing plasma LDL particle concentration and macrovascular disease evidenced by increased IMT. In our study, increasing LDL concentration was statistically associated with internal carotid, but not common carotid, intima-media thickness in women. We have observed previously (13) that the association of LDL-IC and other relevant risk factors with intima-media thickness differs significantly between the internal and common carotid arterial beds. This may result because of increased thickness of the intima-media layer in the internal, compared to the common, carotid artery in this cohort (21) which may contribute to increased sensitivity and selectivity of the measure. In addition, because atherosclerosis is a multifactorial disease, multiple, atherogenic factors may contribute differently to disease development in the internal, compared to the common carotid artery. The extent of macrovascular disease (IMT) was significantly greater in men than women in this Type 1 diabetes cohort (21) and the associations between carotid IMT and other coronary artery risk factors differed significantly between men and women (13). This suggests that other factors in addition to LDL-IC concentration may contribute to IMT. These results clearly support the need for further investigation of macrovascular disease development in diabetes.

Abbreviations

LDL-IC	LDL-containing immune complexes
LDL	Low Density Lipoprotein
HDL	High Density Lipoprotein
NMR	nuclear magnetic resonance
DCCT/EDIC	Diabetes Control and Complications Trial/Epidemiology of Diabetes Intervention and Complications
IMT	intima-media thickness

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FIGURE 1.

Estimated means and standard errors of NMR-determined Small, Large and Total LDL particle concentrations in Type 1 diabetes patients grouped according to the LDL-IC concentration separated by tertile: Tertile 1 (<131), Tertile 2 (131 to 217), and Tertile 3 (>217) $\mu\text{g/ml}$. All mean LDL particle concentrations were adjusted for conventional risk factors (age, prior DCCT treatment group, concurrent HbA1c level, waist-to-hip ratio, and treatment with lipid lowering medications). Separate ANCOVA models were run for each of the three LDL particle groups (Small, Large, and Total). Statistically significant comparisons after the Tukey-Kramer adjustment ($\alpha=0.05$) are shown. * $P < 0.001$; ** $P < 0.01$; # $P < 0.05$

FIGURE 2.

Influence of increasing Large HDL particle concentration on the association between Total LDL particle and LDL-IC cholesterol concentrations in men and women with Type 1 diabetes. The particle concentration of Large HDL averaged $<6.2\mu\text{M}$, $6.2\text{--}9.7\mu\text{M}$, and $>9.7\mu\text{M}$ for the Low, Medium, and High tertiles, respectively. The Total LDL particle concentration averaged $<966\text{nM}$, $966\text{--}1289\text{nM}$, and $>1289\text{nM}$ in the Low, Medium, and High tertiles, respectively. Estimated means and standard errors for the particle concentrations for each combination of LDL and HDL were adjusted using the conventional risk factors detailed in Figure 1. Statistically significant comparisons after the Tukey-Kramer adjustment ($\alpha=0.05$) are discussed for each panel. All LDL and HDL groupings are listed as “Total LDL Tertile/Large HDL Tertile”, so the label ‘High/Low’ refers to the cell pertaining to a high Total LDL concentration with a low Large HDL concentration.

Panel A: In men, the concentration of LDL-IC in the High/Low group was greater than both the Low/Low and Low/High groups ($p=0.008$ and $p=0.043$, respectively). The concentration of LDL-IC in the High/Medium group was also greater than that in the Low/Low group ($p=0.024$). **Panel B:** In women, the concentration of LDL-IC in the Medium/Medium group was significantly greater than both the Low/Medium and Low/High groups ($p=0.022$ and $p=0.004$, respectively). Also, the concentration of LDL-IC in the High/Low group was greater than in both the Low/Medium and Low/High groups ($p=0.022$ and $p=0.006$, respectively). Increasing Total LDL particle concentration was significantly associated ($p<0.001$) with LDL-IC concentration in both women and men when controlling for these factors but the association of LDL-IC with Large HDL failed to reach statistical significance. No significant interaction term of Total LDL particle concentration by Large HDL particle concentration was observed; however, this term was retained in the model.

FIGURE 3.

Schematic depicting the potential mechanisms whereby increased concentrations of immune complexes formed with modified LDL may mediate the association between elevated Total LDL particle concentration in plasma and increased carotid artery intimal media thickness (IMT) used as a surrogate marker of macrovascular disease. Increased plasma Total LDL particle concentration is associated with IMT in the DCCT/EDIC cohort (14). We also have shown that LDL-IC concentration is independently associated with the 5-year progression of carotid IMT (13) in the same patients. We now demonstrate that increased Total LDL particle concentration, regardless whether increased concentrations of Small LDL or Large LDL concentration contributed, was positively associated with LDL-IC concentration in the DCCT/EDIC cohort of Type 1 diabetic patients. Furthermore, using statistical mediation analyses, we now demonstrate that the increase in LDL-IC concentration may mediate this previously observed, positive association between Total LDL particle concentration and IMT. LDL-IC may mediate this pathogenic metabolism through multiple mechanisms. We have shown that LDL-IC metabolised by phagocytic cells (30) can activate the cell (29) which results in increased release of matrix metalloproteinases and pro-inflammatory cytokines which can contribute to unstable plaques and vascular inflammation, respectively (4). LDL-IC metabolism by phagocytic cells also leads to cholesteryl ester accumulation in

the cell (1) which may be sufficient to result in foam cell formation. Collectively, these processes may both contribute to macrovascular disease in diabetes.

TABLE 1

Clinical characteristics and Spearman correlation analyses of LDL-containing immune complex cholesterol concentration in Type 1 diabetic women and men

	VARIABLE	MEAN $\pm$ SD	SPEARMAN CORRELATION COEFFICIENT
WOMEN N = 409	LDL-IC Cholesterol (μ g/L)	190 $\pm$ 125	-
	Age (y)	38 $\pm$ 7.2	0.03
	Diabetes Duration	17.8 $\pm$ 4.9	0.05
	HbA1c (%)	8.2 $\pm$ 1.4	0.11 *
	BMI (kg/m^2)	26.4 $\pm$ 4.3	0.13 *
	WHR	0.8 $\pm$ 0.1	0.03
	Total LDL (nM)	1096 $\pm$ 416	0.32 **
	Small LDL (nM)	411 $\pm$ 438	0.13 *
	Large LDL (nM)	663 $\pm$ 214	0.20 **
	Total HDL (μ M)	35.7 $\pm$ 6.3	- 0.01
	Small HDL (μ M)	21.3 $\pm$ 6.6	0.10
	Medium HDL (μ M)	4.6 $\pm$ 4.9	- 0.04
	Large HDL (μ M)	9.8 $\pm$ 3.6	- 0.18 **
MEN N = 502	LDL-IC Cholesterol (μ g/L)	209 $\pm$ 138	-
	Age (y)	39 $\pm$ 6.7	0.12 **
	Diabetes Duration	17.3 $\pm$ 4.8	0.02
	HbA1c (%)	8.3 $\pm$ 1.3	0.09
	BMI (kg/m^2)	27.1 $\pm$ 3.8	0.18 **
	WHR	0.9 $\pm$ 0.1	0.17 **
	Total LDL (nM)	1288 $\pm$ 480	0.34 **
	Small LDL (nM)	682 $\pm$ 562	0.22 **
	Large LDL (nM)	580 $\pm$ 258	0.11 *
	Total HDL (μ M)	36.0 $\pm$ 5.0	0.07
	Small HDL (μ M)	26.1 $\pm$ 5.7	0.11 *
	Medium HDL (μ M)	2.9 $\pm$ 4.0	0.03
	Large HDL (μ M)	6.9 $\pm$ 3.5	- 0.13 **

*
p < 0.05

**
p < 0.01

Normal Theory and Bootstrapped Estimates of Mediation Effect of LDL-IC on EDIC Year 1 Intima-media Thickness by Sex.

Table 2

Carotid intima-media thickness measurement location	Normal Theory Regression Estimates ^(a)			Bootstrapped Estimates of Mediation Effect ^(b)	
	Unadjusted effect of Total LDL particle concentration on IMT measurement (τ)	Unadjusted effect of Total LDL particle concentration on LDL-IC levels (α)	Effect of LDL-IC levels on IMT after adjusting for Total LDL particle concentration (β)	Effect of Total LDL particle concentration on IMT after adjusting for LDL-IC levels (τ')	Measure of the indirect mediation effect (αβ= τ· τ')
MEN (n=469)					
Internal	0.145**	0.330**	0.119*	0.103	0.037* [0.001, 0.081]
Common	0.167**	0.330**	0.134**	0.120*	0.046* [0.015, 0.086]
Combined	0.188**	0.330**	0.155**	0.134*	0.050* [0.015, 0.093]
WOMEN (n=376)					
Internal	0.082	0.376**	0.109*	0.045	0.047* [0.001, 0.105]
Common	0.033	0.376**	0.094	-0.001	0.037 [-0.005, 0.082]
Combined	0.068	0.376**	0.118*	0.025	0.048* [0.005, 0.099]

* P < 0.05

** P < 0.01

(a) Three regression models were used to test mediation for each measurement of carotid intima-media thickness. The first regression model estimated the effect of the Total LDL particle concentration (denoted as τ) on predicting carotid intima-media thickness. The second regression model estimated the main effect of Total LDL particle concentration (denoted as α) on LDL-IC. Note: all values of α were equal within sex strata since only one regression model was fit within stratum. The third regression model estimated the adjusted effect of LDL-IC (denoted as β) on predicting carotid intima-media thickness when Total LDL particle concentration was also in the model (effect denoted as τ'). All variables were standardized to N(0,1) prior to regression procedures.

(b) Bootstrap estimates of the mediated effect (αβ= τ· τ') and associated 95% confidence interval was computed. The reported measure of the indirect mediation effect is the mean of the 2000 bootstrap replicates. As such, it differs slightly from the direct difference in normal theory based estimates. The 95% confidence interval may be used to test H₀ (αβ) = 0, or that LDL-IC is not a mediator for the association of total LDL particle concentrations on carotid IMT. The data suggest that LDL-IC partially mediates the association of Total LDL particle concentrations with carotid IMT for all models except for the common carotid IMT measurement in women.