High levels of oxidized LDL in circulating immune complexes are associated with increased odds of developing abnormal albuminuria in Type 1 diabetes

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Abstract

Background. Modified low-density lipoprotein (LDL) immune complexes (IC) have proinflammatory properties and play a role in albuminuria development.

Methods. We measured oxidized LDL (oxLDL) and advanced glycation end-product (AGE)–LDL in IC isolated from sera of Type 1 diabetic subjects followed for 14–20 years and studied their association with abnormal albuminuria. Patients with albumin excretion rates (AER) <40 mg/24 h at baseline and follow-up (n = 302) were deemed resistant to developing abnormal albuminuria. Patients with AER <40 mg/24 h at baseline whose AER levels progressed to >40 mg/24 h were considered prone to abnormal albuminuria (n = 185), those who progress to AER >299 mg/24 h were considered as having macroalbuminuria (n = 57). The odds of developing abnormal albuminuria were estimated by logistic regression based on natural log-transformed levels of oxLDL and AGE–LDL in IC and stratified by baseline AER decile.

Results. OxLDL and AGE–LDL were significantly higher in IC isolated from patients progressing to abnormal albuminuria. In unadjusted conditional logistic analysis, an increase of 1 SD in oxLDL and AGE–LDL levels in IC significantly increased the odds ratio (OR) for development of macroalbuminuria, respectively, by a factor of 2.5 and 1.8 (P < 0.001, P = 0.008). The increased odds of developing macroalbuminuria remained significant when adjusted for treatment group, diabetes duration, retinopathy, baseline hemoglobin A1c and LDL (OR = 2.5 and 1.8, respectively, P < 0.01).

Conclusion. Higher levels of oxLDL and AGE–LDL in circulating IC were associated with increased odds to develop abnormal albuminuria.

Keywords: albuminuria; diabetic nephropathy; immune complexes; modified lipoproteins

Introduction

Diabetic nephropathy is the major cause of end-stage renal failure in the Western world [1]. However, there is a great degree of uncertainty concerning the pathogenic mechanisms responsible for its development. Increased levels of albumin in the urine characterize the early stages of renal disease in diabetes. Urine albumin levels exceeding 30–40 mg/day but <300 mg/day [2, 3] define microalbuminuria, while levels exceeding 300 mg/day define macroalbuminuria. Abnormal albuminuria has been shown to be associated with an increased risk for the development of nephropathy [4, 5] and cardiovascular disease [6] in diabetic patients.

The pathological mechanism(s) responsible for the development of microalbuminuria are ill defined. Several metabolic and hormonal factors have been suggested to play a significant role in the onset of glomerular disease in diabetes [7], and there is strong evidence implicating endothelial dysfunction and inflammation in the early stages of renal disease [8–12]. The deposition of glycated proteins [13, 14] and the generation of reactive oxygen species (ROS) [14] in the kidney glomeruli have been proposed as key factors intimately linked to the inflammatory process, particularly because the deposition of advanced glycation end-product (AGE)-modified proteins can be directly or indirectly implicated in the generation of ROS [15, 16]. The involvement of AGE-modified proteins in diabetic nephropathy is supported by the development of glomerular sclerosis in rats receiving AGE-modified rat albumin and the significance of the interaction of AGE-modified proteins and cells expressing the RAGE receptor is supported by experiments showing that
the administration of soluble RAGE can slow down the development of nephropathy in db/db mice [17].

AGE-modified proteins [e.g. AGE–low-density lipoprotein (LDL)] are detected in the circulation [18] and tissues, including the kidney mesangial area and capillary walls [19] and atheromatous plaques [20] and are immunogenic [21]. Antibodies to AGE–LDL have been purified and characterized and they have been shown to react predominantly with carboxymethyl-lysine (CML) epitopes [21]. The co-existence of AGE–LDL and corresponding antibodies leads to the formation of circulating immune complexes (IC), and it is highly likely that the same IC will form in extravascular spaces. Modified LDL (mLDL)-containing IC can be isolated from the peripheral blood of patients with Type 1 diabetes [21]. The mLDL included in those IC is a mixture of AGE–LDL, with CML as its major modification and oxidized LDL (oxLDL), with malondialdehyde (MDA) as its major recognizable modification [21]. The generation of MDA-LDL is a consequence of lipoxidation pathways activated as a consequence of tissue oxidative stress, which appears to take place in the kidney of patients with diabetes, where both glycoxidation and lipoxidation products are co-localized in the glomeruli [19]. The antibodies involved in IC formation with mLDL are predominantly of the IgG isotype, subclasses 1 and 3 [21, 22], with strong proinflammatory potential [23].

Earlier studies using the cholesterol content of isolated circulating IC as an indicator of the concentration of mLDL in the isolates showed that the mLDL-IC concentration was higher in patients with insulin-dependent diabetes mellitus (IDDM) and macroalbuminuria than in patients with microalbuminuria or patients with normal albuminuria [24] and that higher concentrations of LDL in IC were a predictor of nephropathy [25]. With the development of capture assays for different LDL modifications in our laboratory [26], we were in a position to add specificity to our studies since we can now assay the contents of different forms of mLDL in circulating IC and determine their contribution to the development of diabetic nephropathy.

Materials and methods

The Diabetes Control and Complications Trial (DCCT) was a randomized controlled trial of 1441 patients who were 13–39 years of age and had Type 1 diabetes for 1–15 years at study entry [2]. The participants were randomized to intensive or conventional insulin therapy and followed for an average of 6.5 years before the study was terminated early, in 1993, because of its obvious positive impact. In 1994, ~95% of the DCCT participants enrolled into the Epidemiology of Diabetes Interventions and Complications (EDIC) study. The goal of EDIC was to assess the development of macrovascular disease in Type 1 diabetes [27]. During the EDIC observational phase, all patients were under the care of their personal physicians and encouraged to practice intensive insulin therapy. Each EDIC participant underwent a standardized annual history, physical examination, resting electrocardiogram (ECG) and routine laboratory analysis that included hemoglobin A1c (HbA1c) levels [27, 28]. Lipid profiles and 4-h urine collections to measure albumin excretion rates (AER) were obtained in alternate years [27, 28].

This study was performed on a subgroup of DCCT/EDIC participants to test the hypothesis that DCCT baseline values of mLDL-IC would be associated with the development of abnormal albuminuria. Participants

![Fig. 1. Recruitment flow chart. 1441 subjects were recruited into the study at DCCT baseline. 905 of the subjects had longitudinal follow-up and blood collection for the MUSC Program Project during the EDIC phase of the study. A total of 518 of those subjects had sufficient samples (>800 mL of serum) to perform the measurement of oxLDL and AGE–LDL in isolated IC. Of those 518 subjects, 28 had elevated AER at baseline (>40 mg/24 h) and were excluded from the analysis. Additionally, three more subjects were removed due to a lack of EDIC AER data. The remaining 487 were then assessed for albuminuria status. 302 subjects had no AER values >40 mg/24 h for the duration of DCCT and EDIC and were considered resistant to the development of abnormal albuminuria. One hundred and eighty-five subjects had one or more AER measurements ≥40 mg/24 h and were considered as having abnormal albuminuria. Additionally, 57 of the 185 subjects developed macroalbuminuria during follow-up (AER ≥ 300 mg/24 h).](image-url)
included in the study had normal (<40 mg/24 h) AER at DCCT baseline and stored specimens with sufficient volume for analysis (Figure 1). Those patients who developed AER >40 mg/24 h during DCCT or up to Year 9 of the EDIC phase of the study (14–20 years of follow-up) were defined as prone to developing abnormal albuminuria. Participants with normal AER (<40 mg/24 h) throughout DCCT and up to EDIC Year 9 were defined as albuminuria resistant. A total of 487 patients, 185 (38%) of them considered prone to develop abnormal albuminuria, met sampling criteria and were included in this analysis.

Samples

Fasting serum samples obtained during DCCT/EDIC were sent to the DCCT/EDIC central laboratory for standard lipid analysis. Aliquots of these samples were archived for future research purposes. In 1999–2000, as part of Medical University of South Carolina Program Project Grant funded by the National Institutes of Health/Juvenile Diabetes Foundation, serum samples collected during DCCT were provided by the DCCT/EDIC Coordinating Center and National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) to complement the serum samples collected during EDIC. The serum samples had been stored at −70°C and refreezing effects were minimized by preparing aliquots of the serum when thawed for the first time and using a new frozen aliquot for each new test performed. The IRB at Medical University of South Carolina and all participating DCCT/EDIC centers approved the sample collection procedures. Written informed consent was obtained from all participants.

Methods

Measurement of oxLDL and AGE–LDL in isolated IC

Cirulating IC were isolated by precipitation with 3.5% polyethylene glycol, condition that results in negligible precipitation of free LDL [24, 29]. The washed and resuspended IC were dialyzed versus 0.02 M NaHCO3 buffer, pH 7.3, containing 0.5 NaCl and fractionated by affinity chromatography in protein G-Sepharose columns equilibrated with the same buffer. Given that human antibodies to mLDL are of low-to-moderate affinity [30] and that the interaction of IgG with protein G is of high affinity, the sodium bicarbonate-NaCl buffer can be dissociate IC-LDL and elute mLDL, while IgG antibodies remained bound to protein G [21, 24]. We measured the oxLDL and AGE–LDL in the mLDL-rich fraction by capture assays developed in our laboratory [26, 31]. Coefficients of variation for 50 samples measured in two separate assays were 5.2% for oxLDL and 8.3% for AGE–LDL. Cholesterol was measured in the IC isolated from 1 mL of serum as well as in the first eluate from the protein G column containing the mLDL portion of the IC. The measurements of cholesterol were performed to correct for possible variations in the recovery of mLDL from isolated IC. The final values of mLDL are expressed as the concentration of the specific mLDL per milliliters of serum.

Other methods

At the baseline DCCT examination, each participant completed a physical examination, medical history, ECG and laboratory testing including serum creatinine, lipid profile and hemoglobin A1c (HbA1c) [27, 32]. Four-hour urine collections for measurement of AER and creatinine clearance were also obtained during EDIC in alternate years [27, 32]. Baseline covariates for the current analyses were obtained from DCCT baseline history, physical examination and laboratory data (fasting lipids and renal function). The methodology used to perform all the routine measurements used as conventional risk factors in this study have been described elsewhere [27, 32, 33].

Statistical analysis

Standard descriptive statistics were used to summarize the general demographic and clinical data. A Wilcoxon Rank Sum test was used to compare continuous demographic and clinical measures between patients with abnormal albuminuria and subjects resistant to develop abnormal albuminuria. Pearson chi-square test was used to compare categorical variables between patients prone and resistant to develop abnormal albuminuria. Development of macroalbuminuria was defined by one or more AERs >300 mg/24 h during follow-up.

From the 1441 subjects randomized at the DCCT baseline, two subsets were selected post hoc to comprise the sample of the 302 resistant and the 185 prone to develop abnormal albuminuria. To minimize the effects of ‘confounding’ that may have been introduced into the study sample by the selection process on the analysis, conditional (stratified) logistic regression was used to quantify the association of mLDL-IC levels with subsequent development of micro or macroalbuminuria [34]. Baseline AER values were grouped into deciles, used as a stratification variable in the conditional logistic regression model. In conditional logistic regression, the effects of baseline AER levels have been removed (conditioned) out of the estimation process in a manner similar to a stratified Cox regression [35]. The primary parameter of interest in the conditional logistic regression model was the change in the log-odds (with 95% Wald confidence intervals) for the development of micro/macrotubulaminuria for the main effect of baseline natural-log-transformed mLDL-IC levels after controlling for DCCT-randomized treatment, retinopathy cohort at DCCT baseline, duration of diabetes at DCCT baseline as well as LDL and HbA1c also at DCCT baseline. The mLDL-IC levels were natural log-transformed to normalize their distribution.

To further measure the effect of the mLDL-IC measurements on the development of abnormal albuminuria, the strength of the association of the covariates was quantified using the change in the −2 log likelihood indices as well as the entropy R-squared (R̂ entropy) [36]. Statistical significance for the change was computed using likelihood ratio tests for nested regression models. Albuminuria measure sensitivity was conducted to compare several levels of albuminuria severity. The primary measures of interest are shown in comparison with the use of AER >100 mg/24 h during the course of the study.

All statistical analyses were performed using the SAS System version 9.2. The Type I error rate was controlled at 0.05 for all analysis, and P-values have not been adjusted for multiple comparisons.

Results

Demographic and clinical differences at DCCT baseline between subjects resistant to the development of abnormal albuminuria and the micro/macrotubulaminuria subgroups are summarized in Table 1. There were statistically significant differences in several clinical factors—duration of diabetes at study entry, AER, HbA1c, presence of mild retinopathy and levels of triglycerides and high-density lipoprotein (HDL). There was also a statistically significant difference in the length of follow-up between those deemed prone as compared to those deemed resistant. The difference, roughly of 6 months, is not considered as clinically relevant. No subjects were taking angiotensin-converting enzyme inhibitors, angiotensin II receptor blockers or HMG-CoA reductase inhibitor (statin) drugs at DCCT baseline when the serum was collected to measure mLDL in the IC.

Both oxLDL and AGE–LDL levels in circulating IC at DCCT baseline were higher in the group prone to develop abnormal albuminuria than in the resistant group (4.9 ± 0.8 versus 4.6 ± 0.9, P < 0.01 for oxLDL in IC and 1.5 ± 1.1 versus 1.3 ± 1.1, P = 0.02, for AGE–LDL in IC, respectively).

In unadjusted conditional logistic analysis, an increase of 1 SD (0.9) in the log-transformed oxLDL-IC increased the odds of subsequent development of abnormal albuminuria by a factor of 1.4 [odds ratio (OR) = 1.4 [confidence interval (CI): 1.1–1.7], P = 0.001]. This point estimate remained stable and significant with the addition of primary retinopathy at baseline, DCCT treatment group, duration of diabetes at baseline, baseline LDL and HbA1c as adjustment variables [Adjusted OR = 1.3 (CI: 1.1–1.7), P = 0.01] (Table 2). Similarly, the effects of an increase of 1 SD in the log-transformed oxLDL-IC increased the odds of further progression to macroalbuminuria by a factor of 2.4 [OR = 2.4 (CI: 1.7–3.4), P < 0.001]. The strength of the
relationship remained strong with the addition of the same covariates [OR = 2.5 (CI: 1.6–3.9), P < 0.001] (Table 2).

The unadjusted analysis of 1 SD increase (1.04) in the log-transformed AGE–LDL-IC did not significantly increase the odds for the development of abnormal albuminuria [OR = 1.2 (CI: 1.0–1.4), P = 0.097]. With the addition of the same covariates (see Table 2), the effect of AGE–LDL-IC on the development of abnormal albuminuria remained insignificant [OR = 1.2 (CI: 1.0–1.5), P = 0.136]. However, the effect of an increase of 1 SD in the log-transformed AGE–LDL in IC significantly increased the odds to develop macroalbuminuria by a factor of 1.7 [OR = 1.7 (CI: 1.2–2.4), P = 0.002]. The addition of the covariates to the model (Table 2) did not diminish the strength of the association [OR = 1.8 (CI: 1.2–2.4), P = 0.008]. The adjustment variables HbA1c and DCCT treatment group were statistically significant (P < 0.001) in both adjusted models (Table 2).

In order to determine the strength of the association of the covariates in the model with macroalbuminuria, likelihood ratio tests were performed for the full models against the model with only the five adjustment variables included as well as a model with only DCCT treatment group assignment as a predictor (Table 3). The model with only the DCCT treatment group assignment predicting nephropathy outcomes attained a −2 log likelihood of 234.7 (one parameter estimated) while that of the model with the addition of primary retinopathy at baseline, baseline diabetes duration, baseline LDL and baseline HbA1c had a significantly lower value of 179.5 (five parameters estimated; P < 0.001). When the LDL-IC measures were (individually) included in the model, a significant increase in model fit was achieved. With the inclusion of AGE–LDL in IC, the −2 log likelihood was 160.7 and with the inclusion of oxLDL in IC the −2 log likelihood was 164.2 (six parameters estimated; P < 0.001 for both). Thus, the addition of oxLDL and AGE–LDL in IC to the predictive model improves the fit over the inclusion of preliminary indicators alone. The AER stratified conditional model with only the DCCT treatment group assignment had a likelihood ratio chi-square of 24.2 (P < 0.001) by itself with an approximate $R^2$ of 0.093. When primary retinopathy at baseline, baseline diabetes duration, baseline LDL and HbA1c were added to the model, the joint chi-square was 55.2, P < 0.001 with an approximate $R^2$ of 0.307. When AGE and oxLDL in IC measures were (individually) added to the model with the five covariates, a significant increase in model fit was achieved. The chi-square test of the addition of AGE–LDL in IC was 15.3, P < 0.001, $R^2_F = 0.366$ and that for the addition of oxLDL was 18.8, P < 0.001, $R^2_F = 0.379$. Thus, the addition of the oxLDL and AGE–LDL in IC improved the fit of the model with the other covariates.

The results of the sensitivity analysis for the various levels of albuminuria are shown in Table 4. It can be seen that the magnitude of the association changes positively with the increase in albuminuria.

### Discussion

The contribution of dyslipidemia to the development of nephropathy is well established [8, 37]. The role of IC in the pathogenesis of nephropathy is also well established,
Table 2. Conditional logistic regression results for the development of micro/macroalbuminuria

<table>
<thead>
<tr>
<th>Variable</th>
<th>Abnormal albuminuria</th>
<th>Macroalbuminuria</th>
<th>OR</th>
<th>P</th>
<th>OR</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OxLDL-IC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.33 (1.06–1.66)</td>
<td>0.014</td>
<td>2.50 (1.60–3.92)</td>
<td>&lt;0.001</td>
<td></td>
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</tr>
<tr>
<td>Experimental treatment group</td>
<td>0.56 (0.37–0.84)</td>
<td>0.006</td>
<td>0.13 (0.05–0.31)</td>
<td>&lt;0.001</td>
<td></td>
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</tr>
<tr>
<td>Baseline HbA1c %</td>
<td>1.45 (1.27–1.66)</td>
<td>&lt;0.001</td>
<td>2.22 (1.70–2.91)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary retinopathy group</td>
<td>0.71 (0.39–1.28)</td>
<td>0.250</td>
<td>0.83 (0.29–2.40)</td>
<td>0.732</td>
<td></td>
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</tr>
<tr>
<td>Diabetes duration (months)</td>
<td>1.00 (0.99–1.01)</td>
<td>0.250</td>
<td>1.00 (0.99–1.02)</td>
<td>0.425</td>
<td></td>
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</tr>
<tr>
<td>LDL</td>
<td>1.00 (0.99–1.01)</td>
<td>0.497</td>
<td>1.00 (0.98–1.01)</td>
<td>0.384</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>AGE–LDL-IC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.17 (0.95–1.45)</td>
<td>0.136</td>
<td>1.75 (1.22–2.39)</td>
<td>0.008</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental treatment group</td>
<td>0.54 (0.35–0.81)</td>
<td>0.003</td>
<td>0.10 (0.04–0.26)</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline HbA1c %</td>
<td>1.47 (1.28–1.68)</td>
<td>&lt;0.001</td>
<td>2.29 (1.76–2.97)</td>
<td>&lt;0.001</td>
<td></td>
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</tr>
<tr>
<td>Primary retinopathy group</td>
<td>0.69 (0.38–1.24)</td>
<td>0.218</td>
<td>0.78 (0.27–2.24)</td>
<td>0.648</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes duration (months)</td>
<td>1.00 (0.99–1.01)</td>
<td>0.264</td>
<td>1.00 (0.99–1.02)</td>
<td>0.432</td>
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<td></td>
</tr>
<tr>
<td>LDL</td>
<td>1.00 (0.99–1.01)</td>
<td>0.812</td>
<td>1.00 (0.99–1.01)</td>
<td>0.790</td>
<td></td>
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</tbody>
</table>

<sup>a</sup> OR listed for a 1 SD change in natural logged value. Adjusted for DCCT treatment group, baseline retinopathy cohort, duration of diabetes, baseline HbA1c % and baseline LDL. Analysis was stratified on baseline AER measurement decile.

Table 3. Model fit statistics for conditional logistic regression model for the development of macroalbuminuria

<table>
<thead>
<tr>
<th>Model</th>
<th>−2 Log likelihood, parameters</th>
<th>$R_E^2$&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DCCT treatment</td>
<td>234.7, 1</td>
<td>0.093&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DCCT treatment + other covariates&lt;sup&gt;c&lt;/sup&gt;</td>
<td>179.5, 5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.159&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>AGE–LDL-IC full</td>
<td>164.2, 6&lt;sup&gt;df&lt;/sup&gt;</td>
<td>0.300&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
<tr>
<td>OxLDL-IC full</td>
<td>160.7, 6&lt;sup&gt;df&lt;/sup&gt;</td>
<td>0.315&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>$R_E^2$ is a measure of improvement in the Model fit. It is similar to the standard $R^2$ in that it measures the percentage change in the log likelihood attributable to the variables added to the model.

<sup>b</sup>As compared to intercept only model.

<sup>c</sup>Covariates include DCCT treatment group, primary retinopathy group at baseline, diabetes duration at baseline, LDL at baseline and HbA1c %.

<sup>d</sup>P < 0.001 improvement in fit with the additional variables added to the treatment group model.

<sup>e</sup>P < 0.001 as compared to DCCT treatment only model.

<sup>f</sup>P < 0.05 improvement in fit with the additional variables added to the covariate only model.

Table 4. Counts and ORs using increasing levels of albuminuria

<table>
<thead>
<tr>
<th>Albuminuria/nephropathy definitions</th>
<th>Alternative</th>
<th>AER ≥ 40 mg/24 h</th>
<th>AER ≥ 100 mg/24 h</th>
<th>AER ≥ 300 mg/24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prone (n)</td>
<td>185</td>
<td>99</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>Resistant (n)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>302</td>
<td>302</td>
<td>302</td>
<td></td>
</tr>
<tr>
<td>OxLDL-IC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.3 (1.1–1.7)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.8 (1.3–2.4)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.5 (1.6–3.9)&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>AGE–LDL-IC&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.2 (1.0–1.5)&lt;sup&gt;e&lt;/sup&gt;</td>
<td>1.4 (1.1–1.8)&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1.8 (1.2–2.6)&lt;sup&gt;ef&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Resistant subjects defined as those who had a normal AER at baseline (<40 mg/24 h) and maintained normal AER throughout the duration of their study participation.

<sup>b</sup>Adjusted for DCCT treatment group, baseline retinopathy cohort, duration of diabetes, baseline HbA1c % and baseline LDL. Analysis is stratified on baseline AER measurement decile.

<sup>c</sup>P = 0.014.

<sup>d</sup>P < 0.001.

<sup>e</sup>P = 136.

<sup>f</sup>P = 0.029

<sup>ef</sup>P = 0.008.
although most of the examples have been in glomerulopathies other than diabetic nephropathy [38, 39]. The possible involvement of IC in the pathogenesis of diabetic nephropathy had been suggested by us [40] and others [41] in studies based on non-specific assays for IC, later supported by data generated using assays in which the cholesterol content of isolated IC was measured [24, 25]. The data presented in this study clearly shows that higher baseline AGE and oxLDL levels in isolated IC were associated with increased odds to develop macroalbuminuria in the DCCT/EDIC cohort.

Several points need to be considered in our study. Approximately, equal numbers of patients from a primary and a secondary cohort were enrolled in the DCCT study. The primary cohort had no complications, and the secondary cohort had mild-to-moderate retinopathy and AER values <200 mg/24 h. Also, there was a difference in diabetes duration between the two groups (1–5 years in the primary group and 1–15 years in the secondary group). Since we could not examine the whole cohort and the number of patients who developed abnormal albuminuria up to EDIC Year 9 was relatively small, we studied all the patients with abnormal albuminuria (cases), defined as having an AER >40 mg/24 h during follow-up at least in one measurement and studied two to three patients with normal AER (controls) per case. The selection was random but due to the pre-selection criteria, the subgroup of cases included more patients enrolled as part of the DCCT secondary prevention (mild retinopathy), although none of the patients with microalbuminuria (cases) or DIABETES AER was ≥40 mg/24 h at entry into the study was included. Patients with abnormal albuminuria ~20 years later included a larger number of patients enrolled as part of the secondary retinopathy prevention group due to the longer diabetes duration in that cohort. Therefore, the frequency of retinopathy and duration of diabetes were higher at baseline in the group considered prone to develop abnormal albuminuria compared to the group resistant to the development of abnormal albuminuria. Our analysis was appropriately adjusted for all these factors.

Interestingly, a lower level of HDL cholesterol and a higher level of triglycerides as well as elevated HbA1c were observed in the group prone to develop abnormal albuminuria although at entrance into the study the levels of these parameters in the primary and secondary prevention subgroups were similar. As part of the DCCT design, lipid levels were ‘normal’ to qualify for enrollment. However, even at baseline, a slight but significant increase of triglycerides as well as a lower HDL cholesterol level was observed in the patients prone to develop albuminuria. No significant difference was, however, observed in LDL-cholesterol (LDL-C) levels. It is noteworthy that triglycerides as well as HDL-cholesterol levels, are well within ‘normal limits’ in both groups. LDL-C was, by present guidelines, slightly elevated in both groups although not significantly different. This is not the case for HbA1c; the levels of HbA1c were higher in the group prone to develop abnormal albuminuria and the difference in absolute levels between the two groups is considerably higher than that observed in lipid levels. Thus, higher baseline oxLDL and AGE-LDL levels in IC were associated with increased odds to develop abnormal albuminuria and eventually progression to macroalbuminuria. In the conventionally treated patients, the levels of oxLDL in IC were associated to an increase in odds to develop abnormal albuminuria quite similar to that observed with high levels of HbA1c. In contrast, higher baseline LDL-C was not associated with increased odds to develop abnormal albuminuria. In other words, the level of oxLDL in IC measured in conventionally treated patients is as good predictor for the development of nephropathy as HbA1c.

The fact that patients with IDDM have circulating IC containing modified AGE and oxLDL strongly suggests a new mechanism by which AGE modification and LDL oxidation can be significantly involved in the early stages of diabetic nephropathy. Circulating oxLDL- and AGE–LDL-IgG antibodies can easily diffuse to the extravascular space, thus creating the necessary conditions for the formation of proinflammatory IC in the vessel walls, both in the kidneys and in other areas of the systemic circulation. The deposition of AGE-modified proteins starts very early in the evolution of diabetes [42], and perhaps as a consequence of local oxidative stress, oxidized proteins are also generated and co-localize with AGE-modified proteins in the expanded mesangium and glomerular capillary walls of patients with diabetic nephropathy [19]. Among all the modified proteins that can emerge as a consequence of glyco-oxidation and lipid peroxidation, LDL seems to be particularly important. Studies in animal models and humans suggested that macrophages and hypercholesterolemia played a key role in the evolution of glomerulosclerosis [43]. The obvious implication would be that hyperlipidemia, common in patients with diabetes, was linked to the development of diabetic nephropathy. It seems likely that hyperlipidemia will facilitate the infiltration of LDL into the extravascular space, including the glomerular mesangium. Activated mesangial cells have been shown to oxidize LDL in vitro [44]. This observation has significant implications because in situ oxidation of LDL would create the necessary conditions for the combination with oxLDL antibodies and formation of oxLDL-IC in the glomeruli. The activation of mesangial cells by mLDL-IC is particularly significant in the context of diabetic nephropathy in IDDM because mesangial expansion seems to be the earliest morphological evidence of the transition to microalbuminuria [45]. As the lesions progressed, inflammatory cells would be recruited and activated leading to the release of proinflammatory cytokines and growth factors, followed by a self-perpetuating cycle of mesangial cell activation and proliferation of mesangial cells and expansion of the extracellular matrix resulting in glomerulosclerosis [46, 47].

Further studies are needed to clearly detail the pathogenic mechanisms by which oxLDL- and AGE–LDL-IC lead to diabetic nephropathy but the present study provides strong clinical evidence that a link may likely exist between the formation and/or deposition in the glomeruli of IC containing AGE or oxLDL and the initiation and perpetuation of renal disease in patients with Type 1 diabetes.

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