**Conclusions**—LDL with apoCIII strongly predicts coronary events in diabetic patients independently of other lipids and hence other lipid risk factors. In the Prospective Diabetes Study (UKPDS), high TG was significantly associated with coronary events in people with impaired glucose tolerance and diabetes even after adjustment for HDL-C and other risk factors. However, in the much larger United Kingdom Prospective Diabetes Study (UKPDS), high TG was significant only in univariate but not in multivariate analysis that included HDL-C. Lack of independence of triglyceride as a basis of apoCIII content would yield metabolically diverse lipoproteins compared with other lipid risk factors.

The metabolism of triglyceride-rich lipoproteins, chylomicrons, and some types of VLDL and LDL is abnormal in NIDDM. The production of triglyceride and VLDL by the liver is elevated, and the activity of lipoprotein lipase, which metabolizes triglyceride in VLDL, is decreased. It is possible that the plasma triglyceride concentration does not capture the full adverse effect on CHD of abnormal metabolism of triglyceride-rich lipoproteins. The same concern may apply to other lipid measurements that include triglyceride-rich lipoproteins, such as total apolipoprotein B (apoB) or non-HDL cholesterol.

ApoCIII is a small protein on the surface of apoB lipoproteins strongly affecting their metabolism. Alaupovic and colleagues recognized that VLDL and LDL (apoB lipoproteins) contain subpopulations that have apoCIII and hypothesized that classification of apoB lipoproteins on the basis of apoCIII content would yield metabolically diverse types with distinct relationships to atherosclerosis. They found that apoCIII was increased in apoB lipoproteins in patients with diabetes. ApoCIII is an inhibitor of the activity of lipoprotein lipase, which metabolizes triglyceride in VLDL and facilitates their clearance from plasma. ApoCIII also obstructs the clearance from plasma of VLDL and LDL by interfering with their interaction with hepatic lipoprotein.
receptors. The plasma concentration of apoCIII in VLDL and LDL is associated with CHD, and in patients with NIDDM. Thus, we hypothesized that VLDL or LDL that contains apoCIII (apoCIII+) is a predictor of subsequent CHD events in NIDDM.

We additionally hypothesized that apoE would lessen the adverse effects of apoCIII on VLDL or LDL on CHD, because apoE functions as a ligand for cell-surface receptors that take up VLDL and LDL from plasma. To determine directly whether the presence of apoE affects CHD, it is necessary to separate and study VLDL and LDL that have just apoCIII and those that have both apoCIII and apoE. In this study, we investigated the association of VLDL and LDL containing apoCIII, both apoCIII and apoE, or neither with recurrent coronary events in 242 patients with diabetes.

Methods

Subjects and Blood Samples

We conducted a prospective, nested case-control study in the Cholesterol and Recurrent Events (CARE) trial, a trial of pravastatin, an HMG-CoA reductase inhibitor, in 4159 patients who had experienced myocardial infarction (MI), fasting cholesterol level <240 mg/dL, LDL cholesterol 115 to 174 mg/dL, triglyceride <350 mg/dL, and glucose <220 mg/dL were inclusion criteria. Institutional review boards of the centers approved the study, all patients gave informed consent, and the procedures followed were in accordance with institutional guidelines.

There were 586 diabetic patients in the CARE trial. A total of 193 of them had at least one of the following recurrent coronary events during 5 years of follow-up: MI, coronary artery bypass grafting (CABG), or percutaneous transluminal coronary angioplasty (PTCA). Of these, 122 could be matched to diabetic patients who did not have a recurrent coronary event or stroke during follow-up using the matching criteria, age within 15 years, and sex. The baseline characteristics of the 122 diabetic patients with matches were very similar to those of the 71 without matches, eg, age 61 versus 62 years, men 83% versus 77%, body mass index 29 versus 30, smokers 16% versus 10%, and hypertensives 52% versus 52%. Baseline mean triglycerides, LDL-C, and HDL-C were the same in matched and unmatched cases. Of the 122 matched diabetic patients, I had insufficient plasma for analysis. Thus the final study group included 121 diabetic patients with recurrent events and their matched controls.

Laboratory Methods

Blood was collected in tubes containing EDTA after the patients had fasted for longer than 8 hours. Blood samples were sent by overnight delivery to the core laboratory in St Louis, Missouri, where the baseline laboratory measurements were performed on fresh plasma. Plasma aliquots were placed in 1-mL vials and stored at −80°C. One vial containing 1 mL of frozen plasma was shipped to Dr Sacks’s laboratory at the Harvard School of Public Health for analysis of lipoprotein types. Analyses were conducted by Dr Lee on 3 case-control paired samples at a time. None of the investigators at the laboratory was aware of the group identification.

Immunoaffinity chromatography was conducted with affinity-purified anti-apoCII and anti-apoE on Sepahcryn S1000 resin, as previously described and validated. Plasma (1 mL) was thawed and incubated overnight with anti-apoCII immunoaffinity resin. The unbound lipoproteins that did not contain apoCII (E−) were collected and incubated overnight on an anti-apoCII column to isolate those with (E−apoCIII+) and without apoCIII (E−apoCIII). The bound lipoproteins that contained apoE (E+) were eluted with 3 mol/L NaSCN and desalted by gel filtration. Because >95% of E+ particles also had apoCIII, they were not separated additionally and were denoted as E+CIII+ in the present study. The E+CIII− particles, especially in the LDL density range, were not detectable over 90% of diabetic patients. In the nomenclature of Alaupovic, these particle types would be labeled Lp-B-C (for E−CIII+), Lp-B-E (for E+CIII+), and Lp-B (for E−CIII−). The particle type identified by Alaupovic that has apoD and apoA-II in addition to apoE, apoCIII, and apoCII would be included in the E+CIII+ particles in our study.

VLDL (d<1.006) and LDL (1.006<d<1.050) were isolated from 3 immunofractions of plasma (E+CIII+, E+CIII−, and E−CIII−) by very-fast ultracentrifugation following the methods of Pietzsch et al and Leonhardt et al. A Beckman Optima TLX ultracentrifuge, a Beckman TL A 120.2 fixed-angle rotor, and thick-wall polycarbonate tubes (No. 343778, Beckman) were used. To prepare VLDL, we transferred 600 μL of the concentrated immunofractions (E+CIII+, E+CIII−, and E−CIII−) to ultracentrifuge tubes. The samples were overlaid with 400 μL of d=1.006 kg/L and submitted to ultracentrifugation (1 hour 17 minutes, 15°C, 625 000g). The VLDL in the top 400 μL of each tube was carefully harvested by aspiration. To prepare LDL, we mixed 81.5 μL of 40% saturated KBr solution with 18.5 μL of double-distilled water and added the 100 μL of KBr solution to the lower fraction to adjust the density to 1.050. The final volume was made up to 1 mL with d=1.050. The solute density was adjusted and verified by an optical densitometer (Bausch & Lomb). Centrifugation was performed (2 hours 30 minutes, 15°C, 625 000g), and 400 μL of the supernatant, containing LDL, was collected. The run time and speeds were standardized by comparison with the classic Lindgren method using a SW41 swinging bucket rotor (Beckman). As a result, the following 6 lipoprotein types were isolated from the plasma of each patient: VLDL E+CIII+, VLDL E−CIII+, VLDL E−CIII−, LDL E+CIII+, LDL E+CIII−, and LDL E−CIII−. In each of these lipoprotein types, apoB was measured by ELISA and cholesterol and triglyceride by enzymatic methods (Cobas Mira Plus, Roche Diagnostics).

We used d<1.050 rather than d<1.063 for LDL to isolate relatively pure LDL, minimizing contamination with lipoprotein (a) and large HDL particles. We found that recovery of LDL was virtually complete. Using non-HDL cholesterol as determined by the standard precipitation method (total cholesterol − HDL cholesterol) as the reference value, recovery of cholesterol in the sum of VLDL, LDL, and HDL was 99% and recovery of triglyceride was 98%. Finally, the bottom fraction after the LDL spin contained <5% of total apoB, indicating that we collected almost all LDL particles in the density <1.050.

Unpublished data from a smaller group of diabetic patients from the CARE study showed that the distribution of E−CIII+ in diabetic patients is 65% in LDL, 9% in HDL, and 26% in VLDL. For E+CIII+ particles, the distribution is 61% in LDL, 7% in HDL, and 32% in VLDL. For E+CIII− particles, the distribution is 94% in LDL, 3% in HDL, and 3% in VLDL. Thus, 88% to 97% of the fraction-labeled LDL in the present study is LDL.

Statistical analyses were performed at the Harvard School of Public Health using the Statistical Analysis Systems software, version 8.0 (SAS Institute, Inc). The differences between cases and controls were analyzed using a paired t test. The associations of lipoprotein types with recurrent coronary events were computed using logistic regression analysis. The distribution of measurements of lipoprotein types of the controls was divided into quartiles, and the cases and controls in each quartile were counted. The relative risks (RRs) and 95% confidence intervals (CIs) of the 2nd, 3rd, and 4th quartiles were then computed using the 1st quartile as a reference group. Univariate regression models of RR on VLDL and LDL types were performed without adjustment for risk factors and other covariates. Each VLDL and LDL type was evaluated in multivariate analysis with covariates of age and sex (the matching factors), treatment group (placebo or pravastatin), waist circumference, exercise, history of angina or CABG, fasting glucose, use of oral hypoglycemic medication, and plasma triglyceride, LDL cholesterol, and HDL cholesterol to determine whether the VLDL and LDL types predicted events independently of the standard lipid risk factors, other risk factors, and interventions. Plasma total apoB or non-HDL cholesterol was substituted for LDL cholesterol in additional models. The
influence of adjusting for the standard lipid risk factors was determined by leaving them out of the model. LDL cholesterol was the standard clinical measurement.29 P<0.05 (2-sided) was regarded as significant.

### Results

**Characteristics of Cases and Controls**

The concentrations of standard lipid risk factors, triglyceride, total cholesterol, LDL cholesterol, HDL cholesterol, non-HDL cholesterol, and glucose were similar between groups (Table 1). Baseline characteristics of this sample of the diabetic patients were similar to those of the entire group of 586 diabetic patients.30 Among the controls, 50% received pravastatin treatment compared with 42% of the cases, a nonsignificant difference (P=0.22).

**VLDL and LDL Types in Cases and Controls**

The apoB concentrations of VLDL E−CIII−, LDL E−CIII−, LDL E−CIII+, and LDL E+CIII+ were significantly higher in cases than controls (Table 2). In each instance, the cholesterol and triglyceride concentrations of these lipoprotein types were also higher in cases, although
Relative Risk Predicted by VLDL and LDL Types

VLDL CIII+ was the strongest predictor of recurrent coronary events among the VLDL and LDL particle types, RR 6.6 (95% CI, 2.6 to 14), P<0.0001 (Table 3). A high LDL CIII+ concentration was associated with increased risk in patients with plasma total apoB below or above the median concentration, 98 mg/dL, RR 5.9 (P=0.047) and RR 8.3 (P=0.004), respectively. LDL CIII+ particles comprised 10% of the total LDL and were highly enriched in cholesterol and triglycerides compared with LDL CIII− particles (Table 2), and their concentration was mildly although significantly correlated with plasma triglycerides, r=0.24 (P<0.001). The RR in multivariate analysis for the subdivided LDL CIII+, that is LDL E-CIII and LDL E+CIII+, were 3.0 (95% CI, 1.2 to 7.4) and 2.1 (95% CI, 1.0 to 4.6), respectively.

The major LDL particle type, LDL CIII−, comprising 90% of LDL particles, was also associated with increased risk of coronary events in multivariate analysis (RR 2.2; 95% CI, 0.9 to 5.0; P=0.07), although less strongly than LDL CIII+ (Table 3). The RR was 1.96 (P=0.09) in a multivariate model that included risk factors but not lipids and 1.7 (P=0.15) in univariate analysis. LDL CIII− had no correlation with triglycerides, r=−0.03.

The VLDL types had a complex relationship to coronary events. VLDL CIII− was associated with increased RR (2.3; 95% CI, 1.0 to 5.3; P=0.046) in multivariate analysis (Table 3). Substituting plasma total apoB or non-HDL cholesterol for LDL cholesterol in the model had little effect on risk, RR 1.9 (P=0.12) and 2.3 (P=0.047), respectively, nor did

<table>
<thead>
<tr>
<th>Table 3. Relative Risks for VLDL and LDL Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartiles</td>
</tr>
<tr>
<td>VLDL CIII+</td>
</tr>
<tr>
<td>Unadjusted</td>
</tr>
<tr>
<td>Adjusted</td>
</tr>
<tr>
<td>P</td>
</tr>
<tr>
<td>VLDL CIII−</td>
</tr>
<tr>
<td>Unadjusted</td>
</tr>
<tr>
<td>Adjusted</td>
</tr>
<tr>
<td>P</td>
</tr>
<tr>
<td>LDL CIII+</td>
</tr>
<tr>
<td>Unadjusted</td>
</tr>
<tr>
<td>Adjusted</td>
</tr>
<tr>
<td>P</td>
</tr>
<tr>
<td>LDL CIII−</td>
</tr>
<tr>
<td>Unadjusted</td>
</tr>
<tr>
<td>Adjusted</td>
</tr>
<tr>
<td>P</td>
</tr>
</tbody>
</table>

Mean values are apolipoprotein B concentrations (mg/dL).
Unadjusted indicates no covariates included; Adjusted, Baseline triglyceride, LDL cholesterol, HDL cholesterol, age, sex, exercise, waist circumference, CABG, angina, glucose, oral hypoglycemic use, and treatment group (placebo or pravastatin) were included.
LDL includes the IDL fraction. In the nomenclature of Alaupovic, CIII+ is Lp-B:C and CIII− is Lp-B.
leaving out the lipid risk factors (RR 1.96, P=0.08). In contrast, VLDL CIII + was associated with decreased RR, although not statistically significantly (RR 0.5; 95% CI, 0.2 to 1.1; P=0.07) in multivariate analysis (Table 3). Again, leaving out the lipid risk factors had little effect on RR (0.5, P=0.06). VLDL CIII + was much enriched with triglyceride and cholesterol compared with VLDL without apoCIII (Table 2). The RRs in multivariate analysis for the subdivided VLDL CIII +, that is VLDL E−CIII + and VLDL E+CIII +, were 0.7 (95% CI, 0.3 to 1.7) and 0.9 (95% CI, 0.4 to 1.9), respectively.

In an additional multivariate model, LDL CIII +, LDL CIII −, VLDL CIII +, and VLDL CIII − were included together to examine the relative predictive strength of these 4 particle types. The RR for LDL CIII + remained strong and highly significant (9.3, P<0.0001) (Figure). The RRs for the other lipoprotein types were similar to those in previous models (compare the Figure and Table 3), except that the reduced RR for high concentrations of VLDL CIII + became highly significant (RR 0.10, P<0.0001). Finally, the RRs were studied in the individual treatment groups, and no difference was found in the effect of the lipoprotein particle types on risk between pravastatin and placebo groups.

Discussion

The principal finding is that the plasma concentration of LDL particles that have apoCIII strongly predicted recurrent coronary events in diabetic patients who had had a myocardial infarction. This relationship between LDL CIII + and coronary events was independent of the standard lipid risk factors, plasma total apoB, non-HDL cholesterol, other baseline characteristics of the patients, and pravastatin treatment, thus demonstrating considerable robustness. The LDL CIII + particles seem to be quite potent, because a rather small increase in concentration of only 6 mg/dL, comparing the medians of the 1st versus 4th quartiles, corresponded to a 6-fold increased risk of a coronary event.

The LDL CIII + particles comprise 10% of the plasma concentration of all LDL particles and are differentiated from the predominant LDL particle type that does not have apoCIII by their large size,24 high cholesterol and triglyceride content,23,24 and positive correlation with plasma triglyceride. This result is in line with the recent finding that large LDL particles are predictors of recurrent coronary events.31 Their precursor is likely to be VLDL CIII +, because these VLDL and LDL types have similarly high cholesterol content and differ only in reduced triglyceride content in LDL CIII +. These findings suggest that the LDL CIII + particles result from partial lipolysis of triglyceride-rich VLDL particles and may be the remnant lipoproteins that have long been proposed to be atherogenic.13,32,33 The LDL CIII + in the present study includes LDL particles, and apoCIII may also contribute to the relationship between IDL and progression of coronary and carotid atherosclerosis.34,35

In contrast to the highly increased risk associated with LDL CIII +, VLDL CIII + was associated with moderately reduced RR of approximately 50%, although not reaching statistical significance. VLDL CIII + are huge particles24 that we speculate are unable to effectively pass through arterial endothelial cells into the intima to form plaque, and they become atherogenic only after shrinkage by triglyceride lipolysis. In contrast to VLDL CIII +, VLDL apoCIII − have much lower triglyceride and cholesterol contents and are smaller in size.24 VLDL CIII − was associated with moderately increased risk, approximately 2-fold, similar to LDL CIII −.

The presence of apoE on LDL CIII + and VLDL CIII + particles had little apparent relationship to coronary events. LDL E−CIII + and LDL E+CIII + were both highly significantly and similarly increased in cases. This is consistent with our previous report in the CARE population that apoE concentration in VLDL and LDL was not a predictor of coronary events in multivariate analysis that included the apoCIII concentration in VLDL and LDL.17 ApoE should enhance VLDL clearance from plasma by the liver21 and thus be beneficial. We recently reported in healthy persons that VLDL particles that had apoE were actually catabolized more slowly than those that did not have apoE, and we attributed this apparently paradoxical result to a much higher content of inhibitory apoCIII in VLDL apoE + than VLDL apoE −.36 Thus, apoCIII may play the dominant adverse role in VLDL and LDL metabolism and relationship with CHD.

These results refine those of previous reports that linked apoCIII in VLDL and LDL together with CHD.17–20 Measurements of the combined VLDL and LDL types include a diverse group of lipoprotein types that show qualitative and quantitative differences as regards CHD. Thus, enhanced specificity in measurement may improve the quantification of risk and targeting of therapy. In conclusion, the present study points toward apoCIII containing LDL particles as a potential target for diet and pharmacological therapy to reduce the high rate of coronary events in diabetic patients. This conclusion could be extended to nondiabetic persons, because they also have LDL apoCIII +.23–25 Thus, this lipoprotein type as a CHD risk factor needs to investigated in the general population.

Acknowledgments

This study was supported by an investigator-initiated grant from Bristol Myers Squibb, Inc., through the investigator-controlled...
ancillary studies fund of the Cholesterol and Recurrent Events trial. The authors are thankful to Dr Eugene Braunwald, Chair of the Steering Committee of CARE, for his encouragement and vision in establishing the ancillary studies program in the trial.

References


LDL Containing Apolipoprotein CIII Is an Independent Risk Factor for Coronary Events in Diabetic Patients
Sung-Joon Lee, Hannia Campos, Lemuel A. Moye and Frank M. Sacks

Arterioscler Thromb Vasc Biol. 2003;23:853-858; originally published online March 13, 2003; doi: 10.1161/01.ATV.0000066131.01313.EB
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/23/5/853

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/