Relation of Arteriographically Defined Coronary Artery Disease to Serum Lipoprotein Particles Mapped With Monoclonal Antibodies

Catherine Fiévet, PhD; Marie-Christine Nuttens, MD; Pierre Ducimetière, PhD; Jean-Charles Fruchart, PhD; Michel Bertrand, MD; and Jean-Louis Salomez, MD

Background. This study was designed to investigate the relation of a molecular analysis of apolipoprotein B (apoB)—containing atherogenic lipoprotein particles to coronary artery disease (CAD) in middle-aged men.

Methods and Results. Two groups of men were studied. The first consisted of 97 patients with angiographically documented CAD (greater than 50% stenosis of at least one coronary artery). The second group consisted of 145 subjects without symptomatic CAD, who served as controls. In both groups, measurements were obtained for total cholesterol level, triglyceride level, cholesterol contents in apoB- and nonapoB-containing particles (LpB, LpnonB), total apoB and apolipoprotein AI (apoAI levels), lipoprotein particles recognized by monoclonal antibodies anti-apoB (LpBL3, LpBL5, LpBL7) and anti-apoAI (LpAI-2GII). Taking into account age, body mass index, hypertension, diabetes, smoking habits, and drug consumption, the analysis showed that the mean levels of cholesterol were identical in both groups but differed when cholesterol content in LpB and LpnonB subfractions were assessed, thus reflecting an increase in the low density fraction and a decrease in the high density fraction, respectively. This was confirmed by an increase in total apoB and a decrease in total apoAI. Measurements of LpBL3, LpBL5, LpBL7, and LpAI-2GII particles also discriminated between the two groups. After adjustment for cholesterol content in LpnonB particles, a difference in total apoB was no longer significant between groups, whereas LpBL3, LpBL5, and LpBL7 levels remained significantly higher in CAD patients.

Conclusions. The measurement of separate concentrations of apoB in different particles may permit a more-accurate assessment of CAD risk than measurements of total apoB levels. (Circulation 1991;84:153–159)

Coronary artery disease (CAD) is a major cause of morbidity, and procedures are currently being developed for identifying individuals who may have an increased risk of developing the disease.1,2 Many data exist about the importance of plasma lipids, lipoproteins, and apolipoproteins3 and especially the direct relations between low density lipoprotein (LDL) and CAD risk and the inverse relation between high density lipoprotein (HDL) and CAD risk are well described.4,5 However, all analyses are confounded by the evidence that lipoproteins, as classically defined on the basis of their physical properties of density and charge, are not homogeneous molecular species but represent a heterogeneous mixture of particles that differ by their apolipoprotein composition or expression of apolipoprotein epitopes.6,7 Methods of quantitative analysis of plasma lipoproteins (LDL and HDL) have used immunologic assays for their major respective apolipoproteins B and AI (apoB, apoAI), but one of the difficulties in applying such techniques is that they all depend on polyclonal antibodies, which may not be reproducible from one laboratory to another and may recognize different epitopes on the same apolipoprotein molecule.8,9 Monoclonal antibodies can recognize a specific epitope pattern and, consequently, separate lipoprotein particles that may be similar by conventional criteria.10 To evaluate the relation of CAD to this immunoheterogeneity, we quantified lipoprotein particles recognized by three anti-apoB and one anti-apoAI monoclonal anti-
bodies in patients with angiographically defined CAD and in healthy volunteers. We investigated the accuracy of assessment of angiographically defined CAD compared with classic identical tests with polyclonal antibodies and to lipids in serum or lipoprotein fractions.

Methods

Patients

The patients were men aged 30–69 years admitted to the Lille Heart Hospital for coronary angiography and suspected ischemic heart disease. They were not included if they 1) had a history of coronary artery surgery or coronary angioplasty, valvular heart disease, concomitant renal or hepatic insufficiency, or thyroid dysfunction, 2) have been affected by a myocardial infarction in the previous month, or 3) took lipid-lowering agents or heparin. The angiographic studies were performed during an 11-month period. Coronary angiography was performed by the Judkins technique,11 and multiple projections of the right and left coronary arteries were recorded. The angiograms were reviewed by two separate observers without prior knowledge of either the patient’s lipid and apolipoprotein levels. These observers classified the lesions according the following score: 0, normal vessels; 1, small irregularities; 2, stenosis less than 50%; 3, stenosis 50–75%; 4, 75–99%; 5, total occlusion. Significant vessel disease was defined as an angiogram showing one 50% or more reduction of luminal diameter in at least one of the main arteries (left main stem, left anterior descending, circumflex, or right coronary artery) or one of their major secondary branches.

Clinical data were obtained from the complete medical records acquired during the admission to the coronary care unit. Patients who had never smoked were defined as nonsmokers. Those who had stopped smoking for 3 months or more were defined as former smokers. The others were defined as present smokers. Patients were weighed while in ordinary indoor clothing with jacket and shoes removed. The body mass index was calculated according to Quetelet (weight/height2, expressed in kg and m2, respectively). History of hypertension was defined as present either if history of hypertension had been noted on the medical record, if any antihypertensive treatment had been instituted before coronary angiography, or if blood pressure was more than 160 mm Hg for the systolic or 95 mm Hg for the diastolic. Manifest diabetes mellitus was defined as present if any antidiabetic treatment had been prescribed before coronary angiography. All treatments received before the investigation were recorded.

Control Subjects

The control subjects were men aged 30–69 years who had come voluntarily to the Lille Pasteur Institute (Preventive Medicine Center) for a health control examination. Previous medical history and clinical risk factors were obtained in the same way as among the patient group. Control subjects were not included if they 1) had a history of atypical chest pain, angina pectoris, electrocardiographic signs indicative of CAD, concomitant renal or hepatic insufficiency, or thyroid dysfunction, or 2) took lipid-lowering agents or nitrates. For ethical reasons, coronary angiography was not performed in the control subjects.

Lipoprotein and Apolipoprotein Measurements

One blood sample was drawn after a strict 12-hour overnight fast, just before coronary angiography for patients or clinical examination for controls. All biochemical studies were made on fresh serum stored at 4°C for no longer than 48 hours.

Total cholesterol and triglyceride levels were determined by enzymatic methods adapted to centrifugal analysis.12,13 ApoB-containing lipoprotein particles (LPB) were selectively precipitated with concanavalin A.14,15 The supernatant was recovered by pipetting and then was assessed for cholesterol levels. This determination corresponds to cholesterol content in nonapoB-containing particles (LpnonB-C). Cholesterol level in LPB particles (LpB-C) was obtained from the difference between total determination from the sera.

Total apoB and apoAl were quantified with a noncompetitive enzyme-linked immunoassay (ELIA) calibrated against an international calibrated material (International Union of Immunological Societies [IUIS] Matrix apoAI and apoB Reference Material [Pool 1883]) and polyclonal antibodies.16,17 Lipoprotein particles recognized by three well-characterized anti-apoB (BL3, BL5, and BL7)18,19 or by one anti-apoAl (2GII) monoclonal antibodies20 were also measured in a noncompetitive immunoenzymometric assay as previously detailed.18 For calibrating, we have used a pooled sera from 4,000 subjects to which an arbitrary expression of epitopes BL3, BL5, BL7, and 2GII was assigned as 100%. The corresponding particles, LpBL3, LpBL5, LpBL7, and LpAI-2GII, were so expressed as a percentage.

The interassay coefficients of variation for total lipids, lipoprotein subfractions, lipids, and apolipoproteins ranged from 3.8% to 10.8%. They were 5.7%, 7.1%, 8.9%, and 9.1% for LpBL3, LpBL5, LpBL7, and LpAI-2GII, respectively.

Statistical Analysis

We first determined differences between CAD patients and controls in the prevalence and mean values of risk factors, including age, body mass index, smoking habits, family history of CAD, history of hypertension, diabetes, and use of β-blockers and diuretic drugs. Distribution of categorical data were compared with the χ2 or Fisher’s exact test.

Statistical significance for differences in continuous variables between the groups was tested by the Student’s t test. Age-adjusted means of lipoprotein and apolipoprotein were calculated and tested by the GLM procedure (SAS Statistical Software System, Cary, N.C.).21 For all statistical analyses, we used...
Table 1. Clinical Data in Patients With Coronary Artery Disease and Control Subjects

<table>
<thead>
<tr>
<th></th>
<th>CAD patients (n=97)</th>
<th>Control subjects (n=145)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>54.7±7.8</td>
<td>48.3±9.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Weight/height index</td>
<td>26.1±2.8</td>
<td>25.5±2.9</td>
<td></td>
</tr>
<tr>
<td>Smoking habits (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nonsmoker</td>
<td>17</td>
<td>38</td>
<td>NS</td>
</tr>
<tr>
<td>Former smoker</td>
<td>35</td>
<td>53</td>
<td>NS</td>
</tr>
<tr>
<td>Present smoker</td>
<td>45</td>
<td>54</td>
<td>NS</td>
</tr>
<tr>
<td>CAD in family (n, %)</td>
<td>26 26.8</td>
<td>28 19.3</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension (n, %)</td>
<td>44 45.4</td>
<td>15 10.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diabetes (n, %)</td>
<td>10 10.3</td>
<td>3 2.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>β-Blockers (n, %)</td>
<td>44 45.4</td>
<td>3 2.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Diuretic drugs (n, %)</td>
<td>8 8.3</td>
<td>0 0</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are mean±SD where appropriate.
CAD, coronary artery disease; NS, not significant.

logarithmic concentrations for triglycerides because of their skewed distribution. For each biochemical parameter, separate multiple logistic regression was performed to compare both groups, enabling the control of set clinical variables that may act as possible confounders. The normolipemic subjects (patients and controls) (cholesterol ≤6.5 mmol/l according to the European recommendations) were then selected. These two restricted groups were compared by separate multiple logistic regression for each biochemical parameter after adjustment upon the same set of covariates.

Results

Two hundred forty-two male subjects were studied, 97 in the CAD group and 145 in the control group. The prevalences of one-, two-, and three-vessel disease among the men with CAD were 40%, 28%, and 32%, respectively. Forty-three percent (n=42) of the patients had never suffered a myocardial infarction. Twenty-one percent (n=20) and 36% (n=35) were investigated more than 3 months and between 1 and 3 months after an acute event, respectively. Clinical data in the patient and control groups are listed in Table 1. Smoking habits, body mass index, and positive family history of CAD among both groups did not differ at the time of the metabolic evaluation. Patients with CAD were significantly older than controls. Both groups differed significantly with regard to prevalence of hypertension, diabetes, and use of β-blockers or diuretic drugs. No statistically significant difference was detected among the groups with respect to the date of blood sampling.

Table 2 gives the mean±SD values of the lipoproteins, apolipoproteins, and lipoprotein particles levels after adjustment for age. There were no significant differences between the groups with respect to total cholesterol and triglyceride levels. However, men with CAD had a significantly higher cholesterol level in LpB-C and a significantly lower cholesterol level in LpnonB-C compared with controls. Total apoB, LpBL3, LpBL5, and LpBL7 levels were significantly higher in the CAD patients than in controls. On the other hand, mean levels of total apoAI and LpAI-2GII were significantly lower in the CAD patients than in controls.

For lipoprotein, apolipoprotein, and lipoprotein particles, the CAD and control groups were compared in a separate multiple logistic regression that took into account age, body mass index, hypertension, diabetes, smoking habits, and drug consumption (Table 3). No significant difference was observed between the groups for total cholesterol and triglyceride levels. The cholesterol content in lipoprotein subfractions LpB-C and LpnonB-C was slightly decreased (p<0.05) and highly decreased (p<0.001), respectively, in the CAD patients compared with controls. Total apoB levels and apoB levels expressed in the LpBL3, LpBL5, and LpBL7 particles were significantly lower in the CAD patients than in controls.

Table 3. Comparison of Lipoprotein, Apolipoprotein, and Lipoprotein Particle Levels in Patients With Coronary Artery Disease and Control Subjects by Multiple Logistic Analysis

<table>
<thead>
<tr>
<th>Lipoprotein Particle</th>
<th>Coefficient</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>-0.008</td>
<td>NS</td>
</tr>
<tr>
<td>LpB-C</td>
<td>+0.409</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LpnonB-C</td>
<td>-1.120</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG</td>
<td>-0.039</td>
<td>NS</td>
</tr>
<tr>
<td>apo B</td>
<td>+0.506</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LpBL3</td>
<td>+0.760</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LpBL5</td>
<td>+1.030</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>LpBL7</td>
<td>+0.686</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>apo AI</td>
<td>-0.573</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LpAI-2GII</td>
<td>-0.456</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

n=97 patients with coronary artery disease; n=145 controls.
Separate regression models were fitted for each lipid parameter. Standardized regression coefficient adjusted for age, body mass index, hypertension, diabetes, smoking habits, and drug consumption.
C, cholesterol; LP, lipoprotein; TG, triglyceride; apo, apolipoprotein; NS, not significant.
significant differences in patients with CAD than in controls (p<0.01 and p<0.001, respectively). Mean levels of total apoAI and LpAI-2GII were significantly lower (p<0.01 and p<0.05, respectively) in the CAD patients than in controls. Except for LpAI2GII, which was no longer significantly different for both groups, no changes in these results occurred when we excluded from the CAD patients those who had suffered a myocardial infarction 3 months before the study.

When each parameter was successively adjusted according to LpnonB-C level, a significant difference between CAD and control groups was only observed for LpBL3 (p<0.01), LpBL5 (p<0.001), and LpBL7 (p<0.01), whereas LpnonB-C remained significantly different (p<0.001) in each analysis (data not shown).

Both groups were then restricted on the basis of normal total cholesterol level (≤6.50 mmol/l). A statistical comparison of the different biochemical parameters was then reported as before by the same type of multivariate analysis (Table 4). It disclosed a significant difference for the high density markers (LpnonB-C, total apoAI, and LpAI-2GII), but none of the low density markers differed between the two groups except for lipoprotein particles with enhanced epitopes specific to the monoclonal antibody BL5.

### Discussion

Although the use of arteriography to define CAD is considered the optimal method for determining associations of risk factors with the extent of atherosclerosis process, the strengths of associations found have varied greatly between studies because of numerous variations. In particular because valid arteriographic definitions of disease have not been determined precisely, a wide range of definitions of both the presence and absence of disease with control groups continues to be used. Consequently, it is not yet clear what defines the appropriate control group to be used in such epidemiological studies. In the present one, the control group consisted of a male, free-living group for which the mean age was 48.3±9.1 years and probably included persons with moderate, subclinical atherosclerosis. However, according to Fried and Pearson, the inclusion of such persons in a control group without disease tends to diminish the apparent relation of etiologic factors with disease, lending credence, therefore, to our results that there are no spurious associations.

The serum total cholesterol level appears not to be a predictor of the extent of disease (Tables 2 and 3). This was an unexpected finding although already noted by Lehtonen et al. When all patients taking lipid-lowering drugs were excluded, the lack of any difference may be related to dietary changes of CAD patients that would have occurred before the study or to an exceptionally high mean level of cholesterol in the controls when compared with other data from different countries, including France. Although the control group has not been built as a representative sample of the Lille area, similar levels of total cholesterol were observed in a sample of comparable age in the male population survey of the Urban Community of Lille Monica Project (6.37±1.23 mmol/l, unpublished results from our laboratory). Results of the same order of magnitude were also reported in French-speaking populations in Switzerland. No significant difference was detected among the groups for triglyceride levels, even when body weight, manifest diabetics, smoking habits, and drug consumption were considered. In the literature, the relation of whole plasma triglyceride levels to CAD risk is much debated. Initial reports of elevated levels among myocardial infarction survivors in case-control studies were subsequently confirmed in many investigations of similar design. This association has also been observed in some studies that compared lipid levels with coronary angiographic findings, but not in others, either by univariate analysis or after adjustment for other lipid levels and risk factors.

Because LDL and HDL appear to exert opposite effects on the development of CAD, it has become clinically important to develop relatively simple procedures for their separation and quantification. Their concentrations are most frequently determined and expressed in terms of their cholesterol contents (LDL and HDL). In most epidemiological studies, the determination of cholesterol subfractions is made according to the Lipid Research Clinics Program. This procedure is based on the successive use of ultracentrifugation and polyanionic precipitation (heparin-Mn2+), but several other methods exist. Some evidence has been presented that, in particular, because valid arteriographic definitions of disease have not been determined precisely, a wide range of definitions of both the presence and absence of disease with control groups continues to be used. Consequently, it is not yet clear what defines the appropriate control group...
irrespective of their hydrated sizes.\textsuperscript{5,42–44} In the present work, we used concanavalin A to separate subfractions that contain apoB (LpB) from those without apoB (LpnonB).\textsuperscript{14} These particles define qualitatively more varied molecular entities than the lipoproteins classically defined by ultracentrifugation, such as LDL or HDL. The “LpB” particle term encompasses simple particles only that contain apoB and that are essentially found in the LDL.2 fraction (1.019–1.063 g/ml) but also encompasses complex particles that contain apoB, apoCIII, and apoE that are essentially found in very low density lipoproteins (density, 1.006–1.019 g/ml) and the LDL1 (density, 1.006–1.019 g/ml) fraction.\textsuperscript{6} The same remark may be made for LpnonB particles only that contain apoAI or are associated with apoAI, apoE, and apoC(s). Because apoB is the major, if not the only, apolipoprotein of these lipoproteins, the slight increase in LpB-C in the CAD group may be in agreement with most studies in which LDL levels were assessed.\textsuperscript{4} LpnonB-C is a powerful and interesting tool that when used in analysis remains highly significantly different when taking into account the confounding factors or when only the normolipemic patients are considered (Tables 3 and 4).

There is considerable agreement that, on the average, apoB levels are higher and apoAI levels are lower in patients with CAD than in controls.\textsuperscript{3} In some studies, these levels even appeared to be better discriminators than did lipid and lipoprotein levels.\textsuperscript{45} Similar indications are suggested in Table 3 for apoB compared with LpB-C levels but not for apoAI compared with LpnonB-C levels. No definite answer, however, may be given to this question at the present time.

It is now well established that lipoproteins in plasma represent several sets of discrete particles, the structures of which are constantly changing. The apolipoproteins are specific markers for classifying lipoprotein species, and subtle modulations of apolipoprotein disposition on the surfaces of particles cause a great immunologic heterogeneity, the potential clinical implications of which must be elucidated. The development and use of monoclonal antibodies make such an approach possible. Patton et al\textsuperscript{46} reported for the first time one anti-apoB monoclonal antibody, Lp-22, which seemed to bind specific sub-species in the plasma and suggested that patients with CAD may have a significant increase in this component. Relatively few patients were retained for the study, no control group was selected, and no clinical or biological data for the patients was described. Very recently, we have published the use of such anti-apoB monoclonal antibodies in an immunoenzymometric assay and showed their usefulness in demonstrating an immunochemical heterogeneity among apoB-containing particles in patients with CAD, type IIa and type III dyslipoproteinemia, chronic renal failure, or diabetes.\textsuperscript{18,47–49} In the present study, LpBL3, LpBL5, and LpBL7 levels were significantly higher in CAD patients than in controls.

Only the mean difference of LpnonB-C levels between the groups reached the same level of statistical significance (Table 3, \(p<0.001\)). In fact, when each parameter was adjusted according to LpnonB-C level, only LpBL3, LpBL5, and LpBL7 levels remained statistically different among the two groups. The knowledge of the separate expression of apoB in the three different particles LpBL3, LpBL5, and LpBL7, according to their epitope accessibility, seems to permit a more-accurate assessment of CAD involvement than on the basis of the total apoB level. Apparently, the anti-apoAI monoclonal antibody 2GII did not show the same benefit according to total apoAI level. It presents, however, the possibility of overcoming the problems of standardization and antibody specificity that have hindered widespread acceptance of the immunoassay methods used for the determining apoAI levels in plasma.\textsuperscript{50–52}

Last, when only “normocholesterolemic” subjects are included in the analysis, differences between the CAD and control groups in low density parameters (LpB-CH, apoB, LpBL3, and LpBL7) were not so pronounced as in the total group, but accessibility of LpBL5 epitopes remained significantly different.

Modulation of apoB epitope expression has been largely discussed in the literature. Evidence exists that it is dependent not only on the conformation of the lipoprotein particles, including the lipidic and proteic environment, but also on the localization of the epitope along the apoB molecule.\textsuperscript{53–55} The epitopes specific to the antibodies BL3, BL5, and BL7 have been mapped in relation to elements of the sequence of the antigen.\textsuperscript{56,57} BL3 and BL7 antibodies recognize sequential determinants located in the thrombolytic T2 and T3 fragments, respectively, between amino acids 4082 and 4525 and between 2146 and 2375, respectively; LpBL5 failed to react with any of these fragments, suggesting it may recognize conformationally expressed epitopes. The primary map of anti-ApoAI monoclonal antibody 2GII has also been identified and located between amino acids 24 and 60, in the cyanogen bromide fragment CNBr1 (data not published). The localization of the BL3 and BL7 epitopes is reliable with a buried or a conformationally unfavorable domain.\textsuperscript{58} The recent demonstration of this on human LDL isolated from normolipemic donors, and the significantly enhanced expression of apolipoprotein epitopes that we found in this study, raises two questions: First, do these antibodies really recognize specific epitopes on atherogenic particles; second, and alternatively, is recognition due in part to a greater accessibility related to a conformational change of the apoB-containing particles? Similarly, other studies\textsuperscript{54,59} suggested that the COOH-terminal region of apoB (where the BL3 epitope is confined) is particularly susceptible to structural perturbation related to lipid composition or oxidative processes that may initiate an important atherogenic pathway. It is also possible that small LDL particles commonly found in CAD patients\textsuperscript{45} induce greater reactivity than do normal-
sized ones. We are now investigating the relationship between the expression of apoB epitopes and the physicochemical composition of such lipoproteins in patients.

The present evidence indicates that apoB particle determination might be a more efficient screening test for CAD high-risk subjects than is total apoB determination. However, one must be objective and not consider this result alone but in conjunction with other previously defined biochemical determinations. Additional long-term prospective studies would be ideally required for such an evaluation to be used on a sound epidemiological basis.

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