Apolipoproteins C-III and E in apoB- and non-apoB-containing lipoproteins in two populations at contrasting risk for myocardial infarction: the ECTIM study

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Abstract Apolipoprotein (apo) C-III and apoE are components of two major classes of plasma lipoproteins, i.e., apoB- and non-apoB-containing lipoproteins. To analyze the relationship between the distribution of apoC-III and apoE among lipoproteins and coronary heart disease, we compared the distribution of these two apolipoproteins in survivors of myocardial infarction (MI) and control subjects, within and between populations at contrasting risk for MI. ApoC-III and apoE concentrations were determined in plasma devoid of apoB-containing lipoproteins by immunoprecipitation using a specific anti-apoB antiserum. These assays referred to apoC-III-Lp non-B and apoE-Lp non-B, respectively. By examining the difference with total plasma apoC-III and apoE levels, we calculated apoC-III and apoE in apoB-containing lipoproteins (apoCIII-LpB and apoE-LpB, respectively). These determinations were performed in control subjects and in survivors of MI, all males aged 25 to 64 years. They were recruited in Northern Ireland and France, countries characterized by a large difference in the incidence of coronary heart disease. In univariate analysis, apoCIII-LpB appeared significantly higher and the apoC-III ratio (apoC-III-Lp non-B/apoC-III-LpB) significantly lower in MI survivors than in control subjects in both countries. ApoE-LpB was higher in MI survivors than in control subjects in Northern Ireland but not in France. The two French and Irish control populations differed for apoC-III-Lp non-B, apoC-III ratio, and apoE ratio, which were higher in France than in Northern Ireland, and for apoC-III-LpB, apoE, and apoE-LpB, which were lower. Multivariate analysis showed that no parameter involving apoC-III and apoE was more discriminatory than HDL-cholesterol, cholesterol, and triglycerides or apoA-I, apoB, and triglycerides between controls and MI subjects. In contrast, the apoC-III ratio was a better discriminatory parameter between the two control populations than the listed parameters. The differences between the two control populations are of particular interest because they are not biased by the presence of disease or the large difference of the incidence in coronary heart disease between the two countries. It is suggested that the distribution of apoC-III among lipoproteins may play a role in the different susceptibility of the two populations to the atherogenic process.—**Luc, G., C. Fievet, D. Arveiler, A. E. Evans, J-M. Bard, F. Cambien, J-C. Fruchart, and P. Ducimetiere.** Apolipoproteins C-III and E in apoB- and non-apoB-containing lipoproteins in two populations at contrasting risk for myocardial infarction: the ECTIM study. *J. Lipid Res.* 1996. **37:** 508–517.

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Apolipoprotein (apo) C-III and apoE are components of most classes of plasma lipoproteins (1). When triglyceride-rich lipoproteins, such as chylomicrons and very low density lipoproteins (VLDL), enter the circulation, they acquire apoCs and apoEs from circulating high density lipoproteins (HDL) (1–3). These exchangeable apolipoproteins are usually present in multiple copies on each triglyceride-rich particle (4). During further lipolytic degradation of chylomicrons and VLDL, apoC-II-activated lipoprotein lipase catalyzes triglyceride hydrolysis (5), and apoE facilitates hepatic removal of the resulting remnant particles (6–8). As chylomicron and VLDL lipolysis proceeds, most apoCs and a substantial part of apoEs are transferred back to HDL (9–11). Hepatic triglyceride lipase is also impli-

Abbreviations: apo, apolipoprotein; Lp, lipoproteins; ECTIM, Etude Cas-Temoins sur l'Infarctus du Myocarde; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; BMI, body mass index; MI, myocardial infarction; CHD, coronary heart disease; ELIA, enzyme-linked immunoassay.

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cated in the shift of apoE occurring from the triglyceride-rich lipoproteins to HDL (12). ApoE is thought to play a role in the clearance of lipoproteins including chylomicron and VLDL remnants, intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and HDL by receptor pathways (13, 14). Several studies suggest that the function of apoC-III is to modulate the metabolism of triglyceride-rich particles. ApoC-III inhibits apoC-II-activated lipoprotein lipase (15, 16), leading to an increase in the circulation time of the triglyceride-rich particles (17). Furthermore, in vitro and in vivo animal studies have shown that apoC-III modulates the apoE-mediated clearance of lipoproteins and that the concentration of apoC-III relative to apoE is a key determinant of interaction with cells (6). ApoC-III predominantly inhibits the apoE-dependent interaction of lipoproteins with the LDL-receptor (18) and the low density lipoprotein receptor-related protein (LRP) (19) in cell cultures. Thus, in the rat, apoC-III opposes the hepatic uptake of triglyceride-rich lipoproteins (7, 19, 20). Finally, hypertriglyceridemia observed in human apoC-III transgenic mice was the result of the increase in the apoC-III/apoE ratio in lipoprotein remnants (21).

All these mechanisms, i.e., influx of triglyceride-rich lipoproteins and HDL into the circulation, lipase activities, and lipoprotein receptor activities, appear to be important in the determination of apoC-III and apoE levels and in the distribution of these apolipoproteins among plasma lipoproteins. Results from several laboratories indicate that apoC-III in VLDL is associated with smaller VLDL subclasses (4) that are believed to be particularly atherogenic (22). Furthermore, apoE-rich lipoproteins such as β-VLDL and hypertriglyceridemic VLDL have been considered to be atherogeneous from in vitro studies because macrophages actively endocytose lipoproteins containing several molecules of apoE, and transform them into foam cells (23). Moreover, clinical data suggest that apoE-rich apoB-containing lipoproteins are associated with atherosclerosis. This is most evident in patients with type III hyperlipoproteinemia in whom β-VLDL rich in apoE are abundant (24) and in patients with myocardial infarction (25).

The World Health Organization MONICA Project is currently studying trends in CHD incidence and mortality in various regions (26). To investigate the large differences in CHD incidence between the French MONICA centers of Lille and Strasbourg, and the Northern Ireland center of Belfast, a coordinated population-based case-control study on MI was set up in each region (the Etude Cas Témoins sur l'Infarctus du Myocarde [ECTIM] study). To analyze the relationship between the distribution of apoC-III and apoE between lipoproteins and the coronary heart disease (CHD), we set up and validated a procedure to determine apoC-III and apoE in apoB-containing particles and in non-apoB-containing particles. ApoC-III and apoE were measured in total plasma and in plasma devoid of apoB-containing lipoproteins using an electroimmunoassay in each subject included in the ECTIM study (27).

MATERIALS AND METHODS

Study populations and sampling of cases and controls

Full details of the populations studied have been published elsewhere (27, 28). Briefly, the populations studied are those covered by three centers of MONICA registers participating in the ECTIM Study: Belfast and its surroundings in Northern Ireland, Lille and its surroundings in the North of France, and Strasbourg and its surroundings in the North-East of France. The ECTIM Study design has already been detailed (27). The main characteristics are briefly described as follows. Only 25- to 64-year-old men whose families had been resident in the region for at least two generations and whose four grandparents had been born in Europe were eligible for inclusion in the study. In Belfast, the grandparents had to have been born in the historical entity of Ulster. The control subjects were obtained from electoral rolls in France and from the lists of General Practitioners held by the Central Services Agency in Belfast. Among the eligible control subjects, 40% in Belfast, 47% in Lille, and 54% in Strasbourg refused to participate, did not respond, or could not be contacted. The identification of survivors of MI were obtained from the MONICA registers. MI was defined as diagnostic category 1 according to MONICA criteria (29) corresponding to at least two of the following: chest pain of at least 20 min duration, increase in cardiac enzymes, and typical electrocardiogram changes during the acute attack. Epidemiological data and blood samples from survivors of MI were obtained between 3 and 9 months after the event. The age distribution of the random control samples in French and Irish centers was chosen to match that of MI as determined by the MONICA register in each area. Informed consent was obtained from the subjects and their family doctors. In Northern Ireland, subjects were invited to attend a clinic, whereas French participants were visited at home for clinical investigation by a specially trained doctor or nurse. All subjects completed a set of questionnaires on medical history, drug intake, smoking status, and alcohol consumption. The questionnaires and the manual of operations were identical as far as translation allowed. Body weight was measured to the nearest 200 g for subjects without shoes using scales that were regularly checked

and calibrated. Height was determined to the nearest centimeter with a measuring tape on subjects without shoes standing with their back to a wall.

The total number of controls without CHD, not taking hypolipidemic drugs, and with triglyceride levels below 800 mg/dl was 489, of which 175 were recruited in Belfast and 314 in France. For the second part of the study concerning the comparison of MI patients to controls, 179 survivors of MI resident in Northern Ireland and 181 in France were included.

Methods

Venous blood was collected into EDTA-containing tubes after an overnight fast. The blood was kept at 4°C and separated within 3 h by centrifugation. After addition of preservatives (final concentrations: EDTA, 0.27 mmol/l; ε-amino caproic acid, 0.9 mmol/l; chloramphenicol, 0.6 mmol/l; and glutathione, 0.3 mmol/l), one aliquot of the plasma was stored at 4°C for no longer than 6 days and sent at 4°C to the laboratory in Lille.

The following measurements were carried out. Cholesterol and triglycerides were determined by automated enzymatic procedures (Boehringer, Mannheim, Germany) adapted to a Hitachi 705 analyzer. Cholesterol was measured in VLDL separated by ultracentrifugation (30) and in the HDL-containing supernatant after phosphotungstate/magnesium chloride precipitation of apoB-containing lipoproteins (Boehringer, Mannheim, Germany). LDL-cholesterol was calculated by subtracting VLDL- and HDL-cholesterol from total plasma cholesterol. ApoA-I and apoB were quantified by using commercial reagent immunonephelometry (Behringwerke, Marburg, Germany).

Total plasma apoC-III and apoE levels and apoC-III and apoE in plasma devoid of apoB-containing particles (apoC-III-Lp non-B, apoE-Lp non-B) were measured by an electroimmunoassay using Hydragel LpC-III and LpE kits, respectively, supplied by Sebia (Issy-les-Moulineaux, France). By difference, we calculated apoC-III and apoE levels in apoB-containing particles (apoC-III-LpB, apoE-LpB). Each kit contained ten 16-well agarose gel foils (of which 14 were utilizable) containing polyclonal antiserum to apoC-III or apoE, buffer concentrate, standard plasma (calibrated for total apoC-III and apoE), anti-apoB immunoglobulins, and acid violet stain and destaining in concentrated solutions. The antiserum to apoC-III and apoE was prepared in rabbits as described earlier (31). Both were monospecific for their respective apolipoprotein and recognized it in isolated VLDL and HDL lipoprotein fractions with the same immunological reactivity. All the main apoE isoforms were detected by the anti-apoE on immunoblotting (32).

The precipitation of all classes of apoB-containing particles was obtained by an immunological procedure using polyclonal apoB immunoglobulins from goats. These antibodies are monospecific and recognize apoB epitopes on VLDL and LDL with the same apparent affinity (33). The detailed procedure was performed according to the manufacturer's instructions. One volume of whole plasma was mixed with one volume of a ready-to-use solution of apoB immunoglobulins. After a 5-min wait at room temperature and centrifugation for 10 min at 3000 rpm, the supernatant was recovered for apoC-III and apoE determinations. This supernatant will be referred to hereafter as the treated sample and was measured as it was, without further dilution. For total plasma apoC-III and apoE determinations, each whole plasma was pre-diluted 15- and 7-fold in isotonic saline, respectively.

Four calibration points were prepared in isotonic saline by diluting a lyophilized pool of human plasma which served as a secondary standard, calibrated for apoC-III and apoE contents against purified apolipoproteins as described (31, 34). The target values of this standard were 2.6 and 4.0 mg/dl for apoC-III and apoE, respectively. The standard curve ranged from 1.3 and 7.8 and 2.0 to 12.0 mg/dl for both apolipoproteins, respectively. A quality-control sample (lyophilized plasma-calibrated to apoC-III and apoE) was analyzed as a whole plasma in each series to ensure the reproducibility of the assay. Five microliters of each standard calibration point, diluted whole and control plasma, and non-diluted treated samples were applied into each well of a gel foil and then run for 4 h at a constant voltage of 50 V. After washing in isotonic saline, gels were dried in a Sebia IS80 hot air supply and stained with a ready-to-use acid violet solution. The heights of the rockets were measured. The calibration curves were drawn with the concentrations of apoC-III or apoE of the different dilutions of the standard on the abcissa and the heights of the rockets on the ordinates. Samples and control values of apoC-III and apoE were read from each respective standard curve. The difference between the apoC-III or apoE concentrations obtained for whole (total apoC-III, total apoE) and treated (apoC-III-Lp non-B, apoE-Lp non-B) plasma corresponded to apoC-III-LpB and apoE-LpB values.

Statistical analysis

In the present analysis, the data from the two French centers were pooled. The results were analyzed using the SAS statistical software (SAS Institute Inc., Cary, NC). For univariate analysis, the means of MI and controls in each country and between controls of both countries were compared by an analysis of variance after adjustment for covariates (age, body mass index (BMI), alcohol consumption, and cigarette smoking). Pearson

correlation coefficients were computed in the whole group of controls. For some biological measurements with a highly skewed distribution (i.e., triglycerides, VLDL-cholesterol, apoC-III, apoE, apoC-III-Lp non-B, apoE-Lp non-B, apoC-III-LpB and apoE-LpB), a log transformation was first carried out. Multiple logistic regression models (LOGIST procedure) were used to identify parameters that discriminated men with MI from control subjects in each country and controls from the two countries after adjustment on the same covariates as above. Results are given for two separate models. In the first, HDL-cholesterol, total cholesterol, and triglycerides levels were also included as covariates, whereas in the second, apoA-I, apoB, and triglycerides levels were considered.

RESULTS

Analytical performances of the LpC-III and LpE assays

To ensure the complete immunoprecipitation of apoB-containing lipoproteins, we tested the supernatants corresponding to the non-precipitated fractions from plasma samples of normolipidemic, hypercholesterolemic, hypertriglyceridemic, and mixed hyperlipidemic subjects (3 of each type) for the absence of remaining apoB using an enzyme-linked immunoassay (ELIA) as previously described (35) (detection limit = 2.5 ng). Also, the absence of soluble apoB/anti-apoB complexes in the supernatants was tested by ensuring no apoB was detected after dissociation of the supernatants in 8M urea (data not shown). No apoA-I-containing lipoprotein was precipitated by immunoprecipitation with anti-apoB as apoA-I levels in total plasma and in the LpC-III supernate were identical (data not shown).

The precision of the assays was estimated according to the analysis of the variance experiment described in NC-CLS EPS-T (36). One pooled plasma that contained normal concentrations of parameters to be tested was divided into aliquots, stabilized with preservatives as described in the Methods section, and stored at 4°C. Ten aliquots were then measured as total and treated plasma each day for 4 consecutive days. Precision data for the methods are summarized in **Table 1.** The within-day variance was obtained for each day. The between-day variance was estimated by determining the variance of the means obtained for each day, which was then adjusted for the within-day variance component. For each parameter, the coefficients of variation were less than 10%.

The accuracy of the assays was evaluated by mixing various quantities of VLDL and HDL to the infranatant (d 1.006 g/ml) of three corresponding hypertriglyceridemic plasma samples. Each fraction was quantified for apoC-III and apoE before and after mixing. The mean percentages of added apolipoproteins accounted for were 103.0 and 100.2%, for apoC-III and apoE, respectively.

TABLE 1. Precision of LpC-III and LpE immunoassays

	Mean	SDw	SDb	SDtotal	CV
	mg/dl				%
Total apoC-III	2.40	0.20	0.12	0.23	9.7
ApoC-III-Lp non-B	2.13	0.16	0.03	0.16	7.7
Total apoE	5.19	0.39	0.27	0.47	9.1
ApoE-Lp non-B	3.40	0.21	0.11	0.24	7.0

SDw, within-day standard deviation; SDb, between-day standard deviation; SDtotal, $\sqrt{(SDw^2 + SDb^2)}$; CV, coefficient of variation = (SDtotal/mean) × 100.

Correlations in general population

The correlations between apoC-III, apoE, apoC-III and apoE in Lp non-apoB- and in LpB-containing particles, and the plasma lipids, lipoproteins, and apolipoproteins were calculated in control subjects and are shown in **Table 2.** The plasma apoC-III and apoC-III-LpB were strongly and positively correlated with triglyceride, VLDL-cholesterol, and apoB levels. The triglyceride, VLDL-cholesterol, and apoB levels were positively correlated with apoC-III-Lp non-B, but the coefficient of correlation was lower ($r < 0.45$) than with apoC-III-LpB ($r > 0.45$). HDL-cholesterol and apoA-I were positively correlated with apoC-III-Lp non-B and inversely with apoC-III-LpB. The ratio apoC-III-Lp non-B/apoC-III-LpB (apoC-III ratio) was negatively correlated with triglycerides, VLDL-cholesterol, and apoB, but also strongly and positively correlated with HDL-cholesterol and apoA-I. The apoE and its fractions, apoE-LpB and apoE-Lp non-B, showed similar correlations as did apoC-III and its subfractions with lipid parameters. Indeed, apoE, apoE-LpB, and apoE-Lp non-B were positively correlated with triglycerides, VLDL-cholesterol, and apoB. However, if apoE-LpB was negatively and apoE-Lp non-B positively correlated with HDL-cholesterol, as were the corresponding apoC-III fractions with HDL-cholesterol, the strength of the relation of these apoE fractions was clearly lower than with apoC-III fractions. The apoE ratio was significantly correlated among the classical plasma lipids and apolipoproteins only with HDL-cholesterol and apoA-I. This ratio was moderately correlated with the apoC-III ratio ($r = 0.28$), but strongly positively correlated with apoE-Lp non-B ($r = 0.58$) and negatively with apoE-LpB ($r = 0.61$).

Plasma total apoC-III was strongly correlated with apoC-III-Lp non-B and apoC-III-LpB, $r = 0.88$ and $r = 0.83$ ($P < 0.001$), respectively. A strong negative correlation was also observed between the apoC-III ratio and apoC-III-LpB ($r = -0.68$, $P < 0.001$) simultaneously with a

TABLE 2. Correlations between apoC-III, apoE and apoC-III- and apoE-containing particles and lipids and lipoproteins parameters

	ApoC-III	ApoC-III-Lp non-B	ApoC-III-LpB	ApoC-III Ratio	ApoE	ApoE-Lp non-B	ApoE-LpB	ApoE Ratio
Cholesterol	**0.41**	0.34	**0.37**	**-0.19**	**0.27**	**0.23**	**0.18**	0.06
Triglycerides	**0.63**	0.42	**0.68**	**-0.38**	**0.53**	**0.39**	**0.48**	-0.09
VLDL-cholesterol	**0.50**	0.30	**0.58**	**-0.37**	**0.44**	**0.34**	**0.38**	-0.05
LDL-cholesterol	**0.21**	0.10	**0.27**	**-0.25**	0.08	0.03	0.12	-0.03
HDL-cholesterol	0.03	**0.32**	-0.31	**0.49**	0.04	**0.15**	**-0.22**	**0.26**
ApoA-I	**0.18**	0.45	**-0.18**	**0.46**	0.10	**0.19**	-0.14	**0.22**
ApoB	**0.47**	0.25	**0.57**	**-0.43**	**0.31**	**0.19**	**0.36**	-0.11
ApoC-III	**1**	0.88	**0.83**	**-0.28**	**0.67**	**0.55**	**0.48**	0.07
ApoC-III-Lp non-B	**0.88**	1	**0.47**	0.14	**0.58**	**0.56**	**0.24**	**0.23**
ApoC-III-LpB	**0.83**	0.47	**1**	**-0.68**	**0.57**	**0.37**	**0.61**	-0.13
ApoC-III ratio	**-0.28**	0.14	**-0.68**	**1**	-0.14	0.02	**-0.38**	**0.28**
ApoE	**0.67**	0.58	**0.57**	-0.14	**1**	**0.92**	**0.54**	**0.26**
ApoE-Lp non-B	**0.55**	0.56	**0.37**	0.02	**0.92**	**1**	**0.16**	**0.58**
ApoE-LpB	**0.48**	0.24	**0.61**	**-0.38**	**0.54**	**0.16**	**1**	**-0.61**
ApoE ratio	0.07	**0.23**	-0.13	**0.28**	**0.26**	**0.58**	**-0.61**	**1**

Bold face characters indicate $P < 0.001$. ApoC-III ratio = apoC-III-Lp non-B/apoC-III-LpB. ApoE ratio = apoE-Lp non-B/apoE-LpB.

weak and positive correlation ($r = 0.14$, ns) between the apoC-III ratio and the apoC-III-Lp non-B levels. If plasma apoE was positively correlated with apoE-Lp non-B and apoE-LpB to a significant extent ($r = 0.92$ and $r = 0.54$, respectively), these two last parameters were weakly correlated each other with ($r = 0.16$). Finally, the apoC-III and apoE levels in Lp non-B on the one hand and in LpB in the other hand were strongly correlated, the coefficients of correlation being 0.56 and 0.61, respectively.

Univariate analysis

Case-control comparison. The comparison of lipid parameters between cases and controls was carried out separately in each population, French (pooled data of Lille and Strasbourg) and Northern Irish, after adjustment for age, BMI, alcohol intake, and cigarette smoking (**Table 3**). Total cholesterol and LDL-cholesterol were not significantly different between cases and controls in the two populations. On the other hand, triglycerides, VLDL-cholesterol, and apoB were higher in cases than in controls both in France and in Northern Ireland, while the significant levels were higher in Ireland than in France. In France as in Northern Ireland, HDL-cholesterol and apoA-I were dramatically lower in cases than in controls ($P < 0.0001$). The comparison of total apoC-III and apoE and their levels in Lp non-B and LpB showed different results in both countries. Indeed, among the six measured parameters concerning apoC-III and apoE, only apoC-III-LpB was significantly ($P < 0.01$) different between cases and controls in France, apoC-III-LpB being higher in cases than in controls. No

TABLE 3. Mean (SD) levels of lipids, apolipoproteins, and apoC-III and apoE in apoB- and non-apoB-containing particles in survivors of myocardial infarction (MI) and control subjects (C) in France and in Northern Ireland

	France			Northern Ireland			Between Controls
	MI	C	*P*	MI	C	P	P
	bmg/dl			*bmg/dl*			
Cholesterol	226.5(38.0)	231.3(41.1)	ns	240.1(39.4)	235.1(42.2)	ns	ns
Triglycerides	174.9(82.4)	159.8(83.7)	0.01	199.4(87.8)	160.0(89.9)	0.0001	ns
VLDL-cholesterol	29.8(19.6)	26.2(16.9)	0.01	34.6(14.8)	27.8(16.1)	0.0001	0.03
LDL-cholesterol	154.8(33.4)	151.6(38.0)	ns	163.2(35.9)	155.9(39.0)	ns	ns
HDL-cholesterol	42.5(10.4)	53.7(16.1)	0.0001	41.8(13.0)	51.4(14.8)	0.0001	0.05
ApoA-I	126.6(19.7)	151.1(27.4)	0.0001	123.4(20.3)	145.6(26.2)	0.0001	0.02
ApoB	134(29.3)	127.9(30.9)	0.05	145.8(30.0)	131.7(32.3)	0.0001	0.04
ApoC-III	2.93(1.12)	2.92(1.12)	ns	3.32(1.35)	2.95(1.29)	0.005	ns
ApoC-III-Lp non-B	1.89(0.72)	1.99(0.71)	ns	1.82(0.66)	1.82(0.78)	ns	0.02
ApoC-III-LpB	1.03(0.54)	0.93(0.59)	0.01	1.49(0.81)	1.13(0.69)	0.02	0.0001
ApoE	4.53(1.58)	4.62(1.77)	ns	5.43(1.86)	5.16(2.47)	ns	0.0008
ApoE-Lp non-B	3.09(1.37)	3.25(1.51)	ns	3.31(1.45)	3.50(2.08)	ns	ns
ApoE-LpB	1.46(0.75)	1.38(0.77)	ns	2.13(1.11)	1.69(0.91)	0.0001	0.0001
ApoC-III ratio	2.27(1.54)	2.99(3.53)	0.004	1.43(0.68)	2.17(3.56)	0.02	0.0001
ApoE ratio	2.68(2.16)	2.88(3.07)	ns	1.95(1.40)	2.44(1.91)	0.002	0.01

ApoC-III ratio = apoC-III-Lp non-B/apoC-III-LpB. ApoE ratio = apoE-Lp non-B/apoE-LpB. *P* values represent significant levels after adjustment for age, body mass index, alcohol intake, and cigarette smoking; ns, not significant ($P > 0.05$).

significant differences were observed for total apoE levels and apoE distribution among lipoproteins between French cases and controls. In Northern Ireland, total apoC-III, apoC-III-LpB and apoE-LpB were significantly different, i.e., higher in cases than in controls. The other parameters, specifically apoC-III-Lp non-B, apoE, and apoE-Lp non-B were comparable between the two groups of Northern Ireland. The apoC-III ratio was significantly different between cases and controls, being higher in controls than in cases in both countries, but the apoE ratio was significantly higher in controls than in MI only in Northern Ireland.

Comparison of control subjects between France and Northern Ireland. The comparison of the data for controls from the two countries are shown in Table 3 (last column) and indicate different profiles in these populations. The differences between controls in Northern Ireland and in France are of particular interest because they are not biased by the presence of disease and probably reflect the actual difference between the Northern Irish and French populations. Cholesterol, triglyceride, and LDL-cholesterol levels of French and Northern Irish controls were similar. But VLDL-cholesterol and apoB were slightly, but significantly, higher in Northern Ireland than in France. On the other hand, HDL-cholesterol ($P < 0.05$) and apoA-I ($P < 0.02$) were lower in Irish than in French control subjects. Although total apoC-III levels were similar in France and in Northern Ireland, distributions of the apoC-III among lipoproteins in France and in Northern Ireland were different. Thus, apoC-III-Lp non-B was higher in France than in Northern Ireland (1.99 vs. 1.82 mg/dl: $P < 0.02$) while the opposite was true for apoC-III-LpB, 0.93 and 1.13 mg/dl in France and in Northern Ireland, respectively ($P < 0.0001$). As a consequence of the opposite and symmetric variations of apoC-III-Lp non-B and apoC-III-LpB, the apoC-III ratio was different in the two countries, 2.99 in France and 2.17 in Belfast ($P < 0.0001$). Total apoE was higher in Northern Ireland than in France ($P < 0.0008$). This difference was due to a higher apoE-LpB in Northern Ireland (1.69 mg/dl) than in France (1.38 mg/dl) ($P < 0.0001$) while apoE-Lp non-B levels were not significantly different, 3.50 and 3.25 mg/dl, respectively. Reflecting these variations, the apoE ratio was significantly higher in France than in Northern Ireland.

Multivariate analysis

To assess the independent contributions of the lipid variables to discrimination between cases and controls within each country, a series of logistic regression analyses were carried out using age, body mass index, cigarette smoking, and alcohol intake as covariates. The parameters relative to apoE did not discriminate either cases from controls in each country or French from Northern Irish controls (data not shown). Thus, the models took into account only parameters related to apoC-III, particularly the apoC-III ratio.

TABLE 4. Multivariate comparison of cases and controls by stepwise logistic regression analysis

	Northern Ireland		France	
Variable	β	*P*	β	*P*
ApoC-III ratio	-0.05	ns	+0.03	ns
HDL-cholesterol	-4.78	$<10^{-6}$	-7.42	$<10^{-6}$
Cholesterol	+0.33	ns	+0.09	ns
Triglycerides	+0.24	ns	-0.22	ns
ApoC-III ratio	+0.20	ns	+0.24	ns
ApoA-I	-4.27	$<10^{-6}$	-4.77	$<10^{-6}$
ApoB	+1.01	0.05	+0.72	ns
Triglycerides	+0.22	ns	-0.66	ns

β, standardized partial regression coefficient. A plus sign indicates that cases have a higher adjusted mean value than controls; a minus sign indicates that cases have a lower adjusted mean value than controls. *P*, level of significance; ns, not significant. ApoC-III ratio and triglycerides are log-transformed. ApoC-III ratio = apoC-III-Lp non-B/apoC-III-LpB.

As shown in **Table 4,** the apoC-III ratio did not discriminate between cases and controls, both in France and in Northern Ireland when HDL-cholesterol, total cholesterol, and triglycerides were included in the model as covariates (upper part). HDL-cholesterol differed significantly and independently in both countries, being lower in MI than in controls. In the model including apoA-I, apoB, and triglycerides as covariates (Table 4, lower half), similar results were obtained with apoA-I levels which were significantly lower in cases than in controls from both countries. Higher apoB levels were reported in Irish cases than in respective controls ($P = 0.05$), while no significant difference was found for this trait in France.

A comparison of the two control populations was carried out using the same multivariate analysis as above (**Table 5**). The apoC-III ratio differed strongly and independently between the two control groups, whichever model was used. Thus, the adjusted apoC-III ratio was lower in Northern Ireland than in France in the two models ($P < 10^{-6}$), one including HDL-cholesterol, plasma cholesterol, and triglycerides, the other apoA-I, apoB, and triglycerides, these latter parameters no longer being different between the two populations after inclusion of the apoC-III ratio in the model.

DISCUSSION

This report describes the development and the first application of an immunoassay analysis for the determination of human apoC-III and apoE levels in non-apoB- and apoB-containing particles. Several immunoassays

TABLE 5. Multivariate comparison of controls in Northern Ireland and France by stepwise logistic regression analysis

Variable	β	*P*
ApoC-III ratio	-1.86	10^{-6}
HDL-C	+1.20	ns
Cholesterol	+0.02	ns
Triglycerides	-0.29	ns
ApoC-III ratio	-1.87	10^{-6}
ApoA-I	+0.53	ns
ApoB	-0.23	ns
Triglycerides	-0.29	ns

β, standardized partial regression coefficient. A plus sign indicates that Irish controls have a higher adjusted mean value than French controls; a minus sign indicates that Irish controls have a lower adjusted mean value than French controls. *P*, level of significance; ns, not significant. ApoC-III ratio and triglycerides are log-transformed. ApoC-III ratio = apoC-III-Lp non-B/apoC-III-LpB.

have been described for apoC-III (31, 37) and apoE (34, 38), but the distribution of apoC-III and apoE in lipoproteins was only evaluated through nonspecific procedures using heparin–Mn^{2+} precipitation of lipoproteins (39), a mixture of polyethyleneglycol, dextran, and $MgCl_2$ (40), or concanavalin A (41). Although these methods are widely used, they are not as selective as the immunoprecipitation by anti-apoB antibodies. Thus, while apoC-III and apoE levels in the supernates after precipitation by heparin–Mn^{2+} and after immunoprecipitation by antibodies anti-B were significantly correlated, the levels of apoC-III and apoE in the supernates were lower after heparin–Mn^{2+} precipitation than after immunoprecipitation (42). Results of the present study show that in combination with an immunoprecipitation step, the electroimmunoassay is a specific and sensitive method for the quantitative determination of apoC-III and apoE in total plasma and in lipoprotein fractions. Furthermore, the antigen–antibody reaction seems not to be affected by factors such as particle size, and the lipid and protein composition of the lipoproteins, and thus antibodies against apoC-III or/and apoE recognize identically their respective apolipoproteins in apoB- and in non-apoB-containing lipoproteins. Enzyme-linked immunosorbent assay using monoclonal antibodies has been also studied (43) but this method needs antibodies that recognize apoB in VLDL and in LDL with the same affinity.

Using a procedure combining gel chromatography and rocket electrophoresis, Alaupovic (39) showed that apoA-I and apoB were distributed monomodally while the distribution of apoC-III and apoE was bimodal, apoC-III and apoE being clearly present in fractions of both VLDL and HDL size-range. The high correlations between apoC-III-LpB or apoE-LpB and triglycerides or VLDL-cholesterol, and between apoC-III-Lp non-B and apoA-I and HDL-cholesterol confirm this finding. The relatively weak correlations between apoE-Lp non-B and HDL-cholesterol or apoA-I could be explained by the presence of apoE in a lipoprotein fraction whose size was larger than apoA-I-containing lipoproteins (44).

Mortality and the incidence of CHD in Northern Ireland are more than three times as great as those in France (26). Differences in conventional risk factors (total cholesterol, blood pressure, and cigarette smoking) do not explain this striking contrast in incidence (45). The ECTIM study therefore offers the opportunity to determine the contribution of new factors to this risk profile even if potential biases previously detailed (27) might have been introduced in this study. Although controls were selected from general practitioner lists in Northern Ireland and from electoral rolls in France, in all probability the two selection procedures cover more than 98% of the population likely to be included. The participation rate of contacted subjects was slightly lower in France (50%) than in Ireland (60%). However, the very close means of cholesterol levels measured in the first MONICA survey in which the response rates were higher, 57% and 70% in France and Belfast, respectively, than those observed in the ECTIM study, lead us to believe that the controls included in the ECTIM study are representative of the population. Thus, the comparison of two control populations involving subjects living in two countries with a large difference of CHD incidence offered the opportunity to analyze risk markers from a new perspective.

The univariate analysis of lipid risk factors comparing MI and controls in France and in Northern Ireland showed that the MI subjects had higher apoC-III-LpB levels and apoC-III ratios in both countries. In Northern Ireland but not in France, apoE-LpB was also higher in MI than in control subjects. However, the differences in these levels did not remain significant in multivariate analysis. The comparison between control subjects of both countries showed that the Irish controls had different lipid profiles from the French controls. A previous study showed that Northern Irish controls had low levels of LpA-I, as well as high levels of apoB levels in apoE-containing particles (LpE:B) and Lp(a):B (27). Additionally, data from the present study suggest that the apoC-III ratio could be another possible risk marker for CHD in Northern Ireland because it was not the same in controls of the two countries that clearly face different levels of risk of CHD.

Several clinical studies showed that the increase of apoC-III in apoB-containing particles and/or the decrease of apoC-III in HDL could be a marker for atherogenesis or for the progression of atherosclerotic lesions. First, in two coronary angiographic trials, the CLAS (Cholesterol Lowering Atherosclerosis Study) and the MARS (Monitored Atherosclerosis Regression Study), the apoC-III levels in LpB or in Lp non-B appeared to

be the predominant risk factor of the progression of coronary lesions in some groups of subjects. Thus, in the CLAS, apoC-III in the heparin supernate lipoproteins, which approximates apoC-III in HDL, was the sole significant factor of progression in the drug-treated group with a decrease in a relative risk of progression when apoC-III in heparin supernate lipoproteins increased (46). In the MARS, Hodis et al. (47) indicated the great risk of progression when apoC-III in the heparin precipitate increased for lesions <50% diameter stenosis in subjects treated agressively for LDL-cholesterol lowering. Using the same methodology, Koren et al. (48) showed correlations between apoC-III in the heparin precipitate and the apoC-III ratio and the coronary score determined by angiography carried out in nondiabetic CHD patients. These correlations were the highest calculated between the CHD score and a number of lipoprotein measurements.

It could be hypothesized that genetic or environmental factors modified the distribution of apoC-III and apoE among lipoproteins. A genetic factor could be the influence of the apoE phenotype on the triglyceride-rich lipoproteins. Indeed, we previously described a slight difference in the frequence of apoE genotypes in Northern Ireland and in France (49) but this difference did not explain the different levels of apoE-LpB and apoC-III-LpB or apoC-III-Lp non-B in the two countries because these levels were not influenced by the apoE phenotype (G. Luc, P. Ducimetière, J-M. Bard, D. Arveiler, A. E. Evans, F. Cambien, J-C. Fruchart, and C. Fievet, unpublished observations). The role of lipoprotein lipase in the distribution of apoC-III and apoE among lipoproteins has been demonstrated by in vitro and in vivo studies (2, 9, 50–52). In our study, the lipoprotein lipase activity was not been measured, but Alaupovic (39) noted a strong relation between the apoC-III ratio and lipoprotein lipase activity. Although the apoC-III ratio (apoC-III in heparin supernate/apoC-III in heparin precipitate) used by Alaupovic (39) was calculated from apoC-III levels in lipoprotein fractions obtained by a different procedure than ours (see above), this ratio could be assimilated with the apoC-III ratio used in our study. Thus, the high correlations between apoC-III ratio and apoC-III-LpB and apoE-LpB (Table 2) suggested that the apoC-III-LpB and apoE-LpB levels partly depend on this enzyme activity.

In conclusion, many factors may differ between the Northern Irish and French populations. A high-risk profile observed in Northern Ireland, characterized by high levels of LpE:B and Lp[a]:B and a low LpA-I level, has been suggested by a previous study (27). The present study adds to this profile an other potential risk marker, the apoC-III ratio. Each of these risk markers can be biologically related to the development of CHD, which increases the plausibility of a causal association. However, this causality needs a prospective study to determine the predictive value of these lipoprotein parameters.

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