

Comparison of the measurement of lipids and lipoproteins versus assay of apolipoprotein B for estimation of coronary heart disease risk: a study in familial combined hyperlipidemia

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Abstract

We compared in 506 members of families with familial combined hyperlipidaemia (FCH), two approaches to selecting subjects with an apparent increased risk for coronary heart disease: assay of apolipoprotein (apo) B only versus measurement of plasma lipids and lipoproteins. When comparing both criteria, there was an overlap of 81.2% at apo B levels ≤ 1250 mg/l and of 86.9% at apo B levels > 1250 mg/l. At apo B ≤ 1250 mg/l all subjects were normolipidemic. However, 18.8% of these subjects had sub-normal HDL-cholesterol concentrations (< 0.9 mmol/l) but were not considered to have an increased risk because of very low LDL-cholesterol levels (< 2.5 mmol/l). At apo B concentrations > 1250 mg/l we observed a group with normal plasma lipid levels (13.1%). In this group, defined as normolipidemic hyperapobetalipoproteinemia, and considered to have an increased risk for coronary heart disease, apo B determination was thus most informative. The selection of the subgroup with 'normolipidemic hyperapobetalipoproteinemia' on the basis of the conventional approach could be refined using a cut off limit for plasma triglycerides < 1.5 mmol/l. This limit distinguished optimally between an atherogenic very dense LDL pattern versus a dense and buoyant pattern. Thus, based on the results of our study, the determination of apo B appeared to be, if not superior, at least as effective as the conventional lipid and lipoprotein parameters in classifying subjects at increased risk for coronary heart disease. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

At present, the most commonly used approach to estimate coronary heart disease risk is based on the measurement of plasma cholesterol, triglycerides and high-density lipoprotein cholesterol (HDL-cho), with calculation of low-density lipoprotein cholesterol (LDL-cho) by means of the Friedewald formula [1–4]. The variable fasting conditions of the examined patients constitute the major limitation of this approach. In addition, the information that dense LDL is more atherogenic than buoyant LDL is not included [5]. There is consensus that apoprotein (apo) B of LDL is a

more accurate clinical measure of atherogenic risk than is total cholesterol or LDL-cho [6–10], but it is doubtful whether this is also true when risk estimation includes plasma lipids and HDL-cho. Sniderman and Cianflone [11] recently proposed apo B measurement as the first line risk estimator for coronary heart disease instead of the conventional lipoprotein-oriented approach.

This proposal prompted us to review our results on measurement of lipids, lipoproteins, LDL-subfractions and apo B measurement in 40 families with familial combined hyperlipidemia (FCH). FCH is the most common form of heritable lipid disorder accompanied by an increased incidence of cardiovascular disease [12]. In these families, the occurrence of dense LDL is typical [13] and normolipidemic hyperapobetalipo-

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proteinemia is more frequent [14]. Thus, this sample group provided interesting study material to compare the validity of the lipoprotein-based and apoprotein B-based approaches in risk evaluation of coronary heart disease.

2. Subjects and methods

2.1. Design

The recruitment of FCH families took place through probands exhibiting a combined hyperlipidemia with both plasma cholesterol and triglycerides above the 90th percentile, adjusted for age and gender, as derived from the Prospective Cardiovascular Munster (PRO-CAM) study [15]. These values were consistent over several measurements in which the probands had not been given any lipid-lowering drug. Families were included when a multiple type hyperlipidemia with levels of total plasma cholesterol and/or triglycerides above the 90th percentile was present. Besides a proband presenting a combined hyperlipidemia, the presence of at least one first-degree relative with hypertriglyceridemia or hypercholesterolemia was obligatory. Furthermore, at least one of the first-degree relatives should have cardiovascular disease before the age of 60 years. All probands were tested for an underlying cause of hyperlipidemia (i.e. diabetes mellitus, hypothyroidism and hepatic or renal impairment). The presence of one of these causes excluded them and their families from further analyses. None of the probands in these families was homozygous for the apo ϵ_2 allele and none of the first-degree relatives had tendon xanthoma. In addition, to refine the selection procedure, the 95th percentile for plasma cholesterol and triglycerides was used if the body mass index exceeded 30 kg/m², or alcohol consumption if more than two units per day. In total, 506 members including the probands were studied from multigenerational families. All individuals were Caucasian above the age of 12 years. Everyone filled out a questionnaire in order to provide information on medical status, medication use, alcohol intake and smoking habits. The ethical committee of the University Hospital of Nijmegen approved the study protocol. Details about the study population have been described elsewhere [13,14].

2.2. Blood sampling procedure

Patients had not taken lipid-lowering medication for at least 4 weeks. After an overnight fast blood was drawn by venipuncture into EDTA-containing vacutainer tubes. Non-local participants were visited at their homes, and blood was transported immediately to the laboratory. Plasma was isolated within 3 h for determi-

nation of the lipid, lipoprotein, apolipoprotein B levels and the LDL subfraction profile.

2.3. Analytical methods

Plasma cholesterol and triglycerides were measured on the Hitachi 747 analyzer with enzymatic, commercially available reagents (Boehringer–Mannheim, Germany). HDL-chol was determined by the PEG-6000 method [16], LDL-chol was calculated by the Friedewald formula [4]. For other purposes, not reported here, VLDL-chol and LDL-chol were determined by ultracentrifugation [17]. Both series of LDL values obtained with different methods agreed well (Pearson correlation coefficient $r = 0.97$, $n = 506$) and were used in the risk estimation study. For risk evaluation, the upper limits for total cholesterol, LDL-chol, and triglycerides were 6.5, 4.5 and 2.0 mmol/l, respectively, as normal limits for HDLchol we used ≥ 0.90 mmol/l [18]. Subjects were defined to have abnormal plasma lipids or lipoproteins when at least one of the values was abnormal.

Total plasma apo B concentrations were determined by immunonephelometry following modifications of a previously reported approach [19]. As antigen we used LDL (d 1.025–1.038 g/ml). The antiserum appeared monospecific and did not react with purified apo A-I, apo A-II, apo C-II, apo C-III, apo E or human albumin as tested by immunodiffusion. The analyses were performed on the Behring Nephelometer II (Behringwerke AG, Marburg). Before use, the antiserum was treated with 7.5% (w/v) PEG-4000 1:2 (v/v). After incubating overnight at room temperature, the mixture was centrifuged and filtered through a 0.20 μ m filter. Sample (30 μ l), diluted 20–100 times with saline, was incubated with 40 μ l of antibody solution for 20 min followed by immunonephelometry. Results of both dilutions had to be within 5% and, after acceptance, were averaged. The assay was calibrated against freshly isolated LDL. The LDL protein content was determined by the method of Lowry et al. [20] with chloroform extraction of the coloured solution before reading the absorbance. To evaluate the standardisation of the measurements, two sets of fresh-frozen plasma samples were shipped in dry ice to the Northwest Lipid Research Laboratories at the University of Washington, Seattle. One set was composed of 14 untreated plasma samples, while the other set was composed of 14 samples to which we added 600 mg/l of saccharose prior to freezing at -80°C . Multiple apo B analyses were performed on these samples at the Northwest Lipid Research Laboratories using a standardised nephelometric approach calibrated by the use of the World Health Organisation International Federation of Clinical Chemistry (WHO-IFCC) First International Reference preparation for apo B [21].

LDL subfractions were detected by single spin density gradient ultracentrifugation, according to a method described in detail elsewhere [22]. Up to five LDL subfractions could be distinguished, concentrated in the following density ranges: LDL1 (1.030–1.033 g/ml); LDL2 (1.033–1.040 g/ml); LDL3 (1.040–1.045 g/ml); LDL4 (1.045–1.049 g/ml) and LDL5 (1.049–1.054 g/ml). The ultracentrifugation tubes, containing the LDL subfractions stained with Coomassie Brilliant Blue R, were placed in a specially designed rack and photographed [23]. Accurate documentation of the different LDL subfraction patterns was obtained by scanning the obtained slides in triplicate on a LKB 2202 ultrascan laser densitometer gel scan program (Pharmacia-LKB, Upsala, Sweden). The mean peak heights (h_1 – h_5) of the LDL subfractions (LDL1–LDL5) on the three scans were used to calculate the parameter K as a continuous parameter, that best describes each individual LDL subfraction pattern [24]. The relative contribution of each LDL subfraction, expressed by its peak height ($\%h_1$ – $\%h_5$), relative to the total LDL subfraction profile (total LDL [100%] = $\%h_1 + \%h_2 + \%h_3 + \%h_4 + \%h_5$) was calculated. The relative peak heights of LDL3 and the less frequently occurring LDL4 and/or LDL5 were added to give $\%h_3' = (\%h_3 + \%h_4 + \%h_5)$, where LDL (100%) = $LDL1 \times [\%h_1] + LDL2 \times [\%h_2] + LDL3 \times [\%h_3']$. When a subfraction profile was characterised by a predominance of buoyant LDL particles ($h_1 - h_3 > 0$), parameter K was calculated by $K = (\%h_1 - \%h_3') / (\%h_2 - \%h_3' + 1)$. In the case of a predominance of dense LDL subfraction ($h_1 - h_3 < 0$), parameter K was calculated by: $K = (\%h_1 - \%h_3) / (\%h_2 - \%h_1 + 1)$. A buoyant LDL subfraction profile was defined by values of $K \geq 0$, whereas a dense LDL subfraction profile was reflected by parameter $K < 0$ [24]. In those subjects with predominantly dense LDL subfractions LDL3, LDL4 and LDL5, parameter K was < -0.25 . So, the dense LDL subfraction profile ($K < 0$) was subdivided into 2 groups: dense ($-0.25 \leq K \leq 0$) and very dense ($K < -0.25$) LDL subfraction profile.

2.4. Statistics

A one-way analysis of variance (ANOVA) was used to analyse the differences in plasma lipid and lipoprotein levels between subjects stratified by three different LDL subfraction profiles. The Student's t -test was used to analyse the differences in mean values between the distinctive groups. Pearson correlation coefficients were computed to determine the correlation between LDL values calculated by Friedewald formula and determined by ultracentrifugation. The correlation between apo B values obtained in our laboratory and these obtained at the Northwest Lipid Research Laboratories (NWLRL) in Seattle was also determined by

computing Pearson correlation coefficients. All values are presented as mean \pm S.D. Statistical analysis were performed with procedures available in SPSS/PC software (Statistical Package for the Social Sciences) Software package version 4.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Standardisation of apo B measurements

To evaluate the accuracy of the apo B measurements, we prepared two sets of fresh-frozen samples, encompassing a large range of apo B values. Saccharose was added as a cryopreservative to one set of samples while the other set did not contain any additive. Multiple apo B analyses were performed on each sample, in our laboratory and at the Northwest Lipid Research Laboratories (NWLRL) in Seattle. The NWLRL has been the leading laboratory of the IFCC International Standardisation of apo A-1 and apo B, and consequently these parameters are measured by a highly standardised nephelometric approach calibrated against the WHO-IFCC International Reference Materials with accuracy-based target values assigned as previously described in details [21,25]. An excellent agreement was found between the apo B values obtained in our laboratory and those obtained at the NWLRL with a correlation coefficient of 0.995 for the untreated samples and a correlation coefficient of 0.998 for the samples containing saccharose. However, there was a systematic bias between the apo B values obtained in our laboratory and those obtained at the NWLRL and this bias was practically identical in the two sets of data. The following equation, $y = 0.73x + 49$, where x represents the Nijmegen data, provides the magnitude of the bias and this equation was used to harmonise our data to those traceable to the WHO-IFCC International Reference Material. When corrected, the average bias and the absolute bias for apo B measurements were 0.5 and 2.7%, respectively. These biases were independent of the plasma triglyceride concentration up to a value of 8.0 mmol/l. Considering the excellent correlation between the two methods, the inaccuracy of our data, prior to correction, was most likely related to the assay calibration due to differences in the method used for LDL purification and/or in the Lowry method used to assign a target value to our primary calibrator.

3.2. The diagnostic power of apolipoprotein B versus lipid and lipoprotein profile

We compared the validity of apo B assay as a selection criterion for coronary heart disease versus conventional risk estimation on the basis of measure-

ment of lipids and lipoproteins. Subjects were grouped according to the cut off limits for total cholesterol, LDL-chol, and triglycerides (6.5, 4.5, and 2.0 mmol/l, respectively) [18], and stratified as having an increased risk of coronary heart disease when a value was outside the normal range. In total, 246 subjects had an elevated total cholesterol and/or triglycerides and/or LDL-chol level and were defined as subjects at risk. In all these subjects the apo B level was > 1250 mg/l. So, values of apo B above 1250 mg/l were considered to identify subjects at risk for coronary heart disease.

In Table 1 we show the comparison of both diagnostic criteria for selecting subjects with an apparent increased risk for coronary heart disease. Within the group of subjects with apo B concentration ≤ 1250 mg/l no subject had elevated total cholesterol or triglyceride concentrations. A subgroup of 42 subjects (18.8%) was found with HDL-chol < 0.9 mmol/l without other abnormal lipid levels, apparently not being at an increased risk because of the concomitant sub-normal total cholesterol and low LDL-chol concentrations (< 2.5 mmol/l). Thus, at an apo B concentration ≤ 1250 mg/l, there was an overlap between both diagnostic criteria in 81.2% of the subjects. With inclusion of a second risk estimator of the lipid/lipoprotein approach, there was complete identity between both selection criteria. On the other hand, the group with apo B > 1250 mg/l included 37 subjects (13.1%) with normal plasma lipid and lipoprotein parameters (Table 1). This subgroup is referred to normolipidemic hyperapobetalipoproteinemia in whom the risk estimation on the basis of apo B appears thus more accurate. At an apo B concentration > 1250 mg/l, there was an overlap between both diagnostic criteria in 86.9% of the subjects.

The phenomenon of normolipidemic hyperapobetalipoproteinemia subjects ($n = 37$) was studied further with regard to their triglyceride concentrations. All these 37 subjects had a plasma triglyceride concentration between 1.5 and 2.0 mmol/l. When the cut-off limit

Table 1
Comparison of both diagnostic criteria (apolipoprotein B values versus lipid and lipoprotein profile) for selecting subjects with an apparent increased risk for coronary heart disease.

| | Apo B ≤ 1250 mg/l | Apo B > 1250 mg/l |
|---|---------------------------|------------------------|
| Total group ($n = 506$) | 223 | 283 |
| Normal lipids | 181 | 37 |
| Elevated Chol and/or TG and/or LDL-chol* | 0 | 246 |
| Decreased HDL-chol only** | 42 | 0 |

* Defined as Chol > 6.5 mmol/l and/or TG > 2.0 mmol/l and/or LDL-chol > 4.5 mmol/l.

** Defined as HDL-chol < 0.9 mmol/l without other abnormal lipid concentrations.

for normal triglycerides was lowered to 1.5 mmol/l, the overlap between both selection criteria in the subjects with apo B > 1250 mg/l increased from 86.9 to 97.8%. Decreasing the cut-off limit for plasma triglycerides from 2 to 1.5 mmol/l could thus enhance the selection power. To underscore this, we re-analysed subjects with a plasma triglyceride concentration between 1.5 and 2 mmol/l ($n = 76$). Of the 76 subjects, only nine had a normal apo B concentration, which stresses the additional power of lowering the cut-off limit for normal triglycerides from 2.0 to 1.5 mmol/l for inclusion of the patients with normal versus hyperlipidemic hyperapobetalipoproteinemia.

3.3. Dense LDL as another risk estimator

The total group of 506 subjects was subdivided according to their LDL-subfraction profile defined by parameter K . In 132 subjects a buoyant LDL subfraction profile ($K > 0$, group 1) was found. A dense LDL subfraction profile, defined by parameter $K \geq -0.25$ and $K \leq 0$, was found in 151 subjects (group 2), whereas 223 subjects showed a very dense LDL subfraction profile ($K < -0.25$, group 3). As expected, in FCH a very dense LDL subfraction profile was frequently observed (Table 2).

Table 2 shows the concentrations of plasma lipids, lipoproteins and apolipoprotein B stratified by LDL-subfraction profile. A significant difference for all parameters was found (ANOVA, $P < 0.05$). A very dense LDL subfraction profile was associated with significantly increased levels of total cholesterol, triglycerides, LDL-chol, apo B and decreased concentration of HDL-chol and ratio LDL-chol/plasma apo B, compared to a dense and buoyant LDL subfraction profile (Table 2).

In Fig. 1 we show that a triglyceride concentration of 1.5 mmol/l is a sharp cut off limit for segregating subjects with very dense LDL versus dense and buoyant LDL, confirming the rationale to lower the threshold for normal triglyceride value from 2 to 1.5 mmol/l. In addition Fig. 2 shows that an apo B value of 1370 mg/l, near to the cut-off limit of 1250 mg/l used in the present evaluation, can optimally distinguish subjects with very dense versus dense and buoyant LDL.

4. Discussion

In this study, using subjects with familial combined hyperlipidemia and their relatives, we show that assay of apo B results in almost the same selection of subjects with an increased risk for coronary heart disease as measurement of lipids and lipoproteins. Thus, on the basis of one single deviating parameter (apolipoprotein B) we obtained 81.2% of agreement between both crite-

Table 2
Plasma lipids, lipoproteins and apolipoprotein B concentrations in subjects stratified according to their LDL-subfraction profile^a

| Parameter K^b | Buoyant LDL $K > 0$ Group 1 | Dense LDL $-0.25 \leq K \leq 0$ Group 2 | Very dense LDL $K < -0.25$ Group 3 |
|--|--------------------------------|--|---------------------------------------|
| Total group (n) | 132 | 151 | 223 |
| Age (years) | 36.5 ± 16.3 | 37.7 ± 16.6 | 46.4 ± 15.9^d |
| Plasma cholesterol | 5.23 ± 1.13 | 5.48 ± 1.35 | 6.40 ± 1.30^d |
| Plasma triglycerides ^c | 0.88 ± 0.51 | 1.42 ± 1.42^c | 2.69 ± 1.84^d |
| HDL-cholesterol ^c | 1.45 ± 0.33 | 1.24 ± 0.33^c | 0.99 ± 0.25^d |
| LDL-cholesterol ^c | 3.40 ± 1.04 | 3.68 ± 1.26^c | 4.27 ± 1.09^d |
| Plasma apo B ^c | 1050 ± 272 | 1218 ± 338^c | 1616 ± 378^d |
| LDL-cholesterol/plasma apoB ^c | 3.31 ± 0.40 | 3.06 ± 0.50^c | 2.65 ± 0.57^c |

^a Plasma concentrations of lipids and lipoproteins are given in mmol/L and of apo B in mg/L.

^b Parameter K is defined in materials and methods section.

^c Dense LDL versus buoyant LDL $P < 0.05$, Student's t -test.

^d Very dense LDL versus dense LDL $P < 0.05$, Student's t -test.

^e Significant difference for the presented variable ANOVA $P < 0.005$.

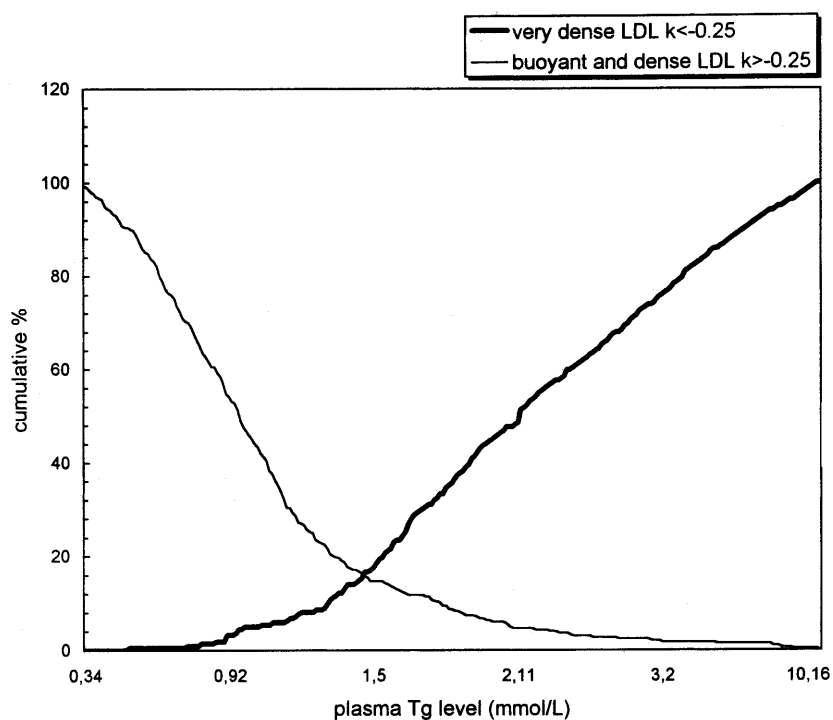


Fig. 1. Cumulative frequency distribution of plasma triglycerides in the groups with very dense versus dense and buoyant LDL. Note: the frequency distribution for the dense and buoyant group is combined and reversed for better establishing the cut-off limit that optimally distinguishes both groups. The intercept of both curves is at a triglyceride concentration of 1.5 mmol/l.

ria in the subjects with normal apo B concentrations (< 1250 mg/l) and 86.9% agreement in the subjects with increased apo B concentrations (> 1250 mg/l). With inclusion of a second parameter, we obtained 100% agreement in the first group, whereas, in the group with increased apo B concentration, an additional 10.9% of subjects with normal lipid concentrations could be included if the upper limit for triglycerides was decreased to 1.5 mmol/l. Thus, irrespective of knowing the coronary heart disease conditions assessed by invasive techniques we were able to

show that the claim of Sniderman and Cianflone [11] about the predictive value of total apo B measurement in plasma is indeed justified. The predictive value of apo B is slightly superior to that obtained with the conventional risk estimation for which a fasting condition is needed and three different parameters should be measured according to strict quality control criteria in combination with calculation of LDL-cholesterol. Evaluation of treatment by diet or drugs would be less problematic if only the change in one parameter has to be taken into account instead of positive and/or nega-

tive changes in three to four different parameters. The additional value of apo B measurement is especially relevant in subjects with the so called normolipidemic hyperapobetalipoproteinemia [6]. Patients with this phenotype are characterised by a typical LDL-subfraction pattern with a predominance of very dense LDL; the protein to cholesterol content of this LDL is increased [13,14,26]. This explains their preferential selection on the basis of apo B versus LDL-chol. A dense LDL subfraction is usually associated with an increased plasma triglyceride concentration [22–24]. It is surprising that we were able to detect a group of normolipidemic subjects with a plasma triglyceride concentration in the upper part of the normal range (between 1.5 and 2.0 mmol/l). It is likely that the subjects participating in our family study strictly adhered to the prescriptions for fasting.

Regarding the large biological variation of the fasting plasma triglyceride concentration [27], it cannot be excluded that these patients have temporary episodes of hypertriglyceridemia throughout the year. The half-life of VLDL, the main triglyceride carrier, is several hours [28,29], while that of LDL is several days [29,30]. In addition, the parameter LDL-apo B is independent of typical lipid exchange reactions, in contrast to LDL-chol. Apo B is thus a more stable metabolic estimate of the lipoprotein status similarly HbA1c which is a long term metabolic control of glucose homeostasis. Consequently, strict fasting conditions on the day preceding

the blood sampling procedure or an increased physical activity in the evening or morning before blood sampling can normalise plasma triglycerides in contrast to LDL-chol and especially to LDL-apo B. The fact that fasting conditions are mandatory for the lipid-oriented approach makes it more attractive to concentrate on apo B measurements. On the other hand, increased apo B concentrations are the result of quantitative changes in LDL rather than qualitative changes. Indeed, only 10.9% of our subjects with increased apo B concentrations belong to the last group with mainly qualitative changes in LDL. The apo B approach is thus primarily directed towards the atherogenic LDL particle. Consequently, this approach diminishes the significance of an altered triglyceride mechanism as a risk factor for coronary heart disease. On the basis of epidemiological and case control studies, hypertriglyceridemia in combination with slightly decreased HDL-chol level is considered atherogenic [15]. However, the group with normolipidemic hyperapobetalipoproteinemia had, in our study, apo B values that were lower than those in the group with hypertriglyceridemia and decreased HDL-chol levels. That decreasing the cut off limit for normal triglycerides to 1.5 mmol/l results in the inclusion of this group, could mean that normolipidemic hyperapobetalipoproteinemia is a marginal problem within two valid diagnostic approaches. Nevertheless, these patients have a very dense LDL pattern. On the basis of this it is tempting to decrease, in the classical

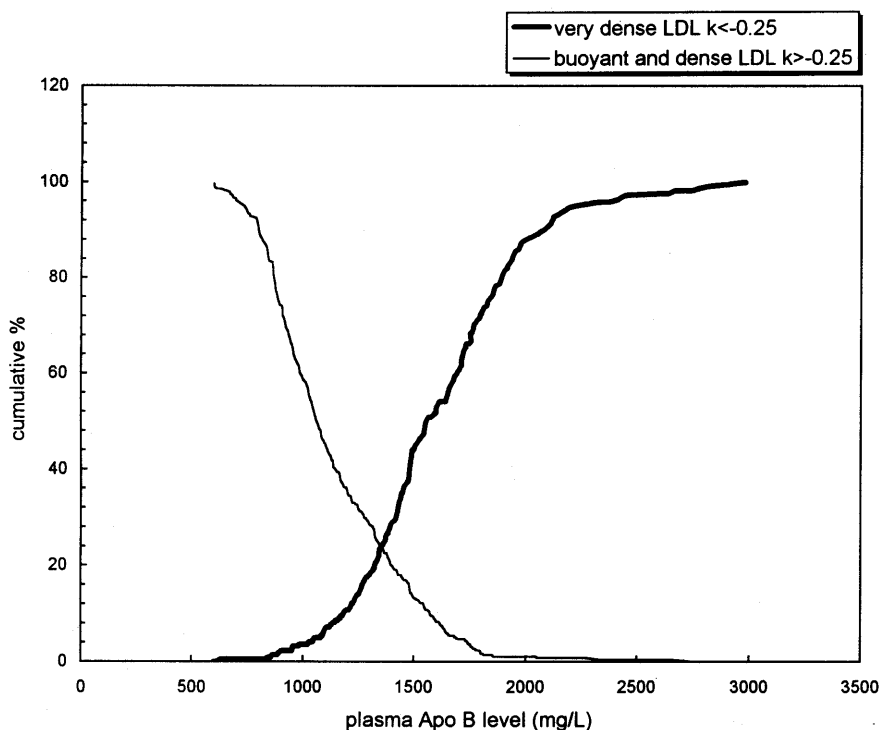


Fig. 2. Cumulative frequency distribution of plasma apo B in the groups with very dense versus dense and buoyant LDL. For explanation see Fig. 1. The intercept of both curves lays at an apoB concentration of 1370 mg/l.

lipid approach, the normal limit for plasma triglycerides to 1.5 mmol/l. A similar approach was followed by Austin et al. [27]. This cut-off value could have relevance for the metabolic control of VLDL secretion.

In our study, the apo B value appeared to be, if not superior, at least as effective as lipid and lipoprotein values in identifying subjects at increased risk for coronary artery disease. However, to reach uniformity across the studies, it is imperative that apo B is measured by standardized methods that are documented to be traceable to the WHO-IFCC International Reference Material. Recently, population-based reference values for apo B obtained by WHO-IFCC standardised methods showed an excellent agreement between the apo B cut-off values obtained in the different populations [31–33]. Another potential problem in the accuracy of apo B measurements may be related to improper storage of the samples, which may result in denaturation of apo B [34]. In this study, we show that the storage at -80°C does not affect the apo B assay and that addition of saccharose to fresh-frozen quality control samples is not needed. However, in lyophilized samples used as assay calibrator or quality control, saccharose addition may be important to prevent LDL denaturation [35].

In conclusion, we have shown that apo B values obtained on accurate assay are, if not superior, at least as effective as the conventional lipid and lipoprotein parameters in classifying subjects at increased risk for coronary artery disease.

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