THE ELUSIVE LP(a) LIPOPROTEIN:

A CARDIOVASCULAR RISK FACTOR

AFTER ALL?

Christa Maria Cobbaert

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THE ELUSIVE LP(a) LIPOPROTEIN: A CARDIOVASCULAR RISK FACTOR AFTER ALL?

Serum Lp(a) levels in healthy and diseased populations - an epidemiological and clinical approach

HET MYSTERIEUZE LP(a) LIPOPROTEINE: UITEINDELIJK TOCH EEN CARDIOVASCULAIRE RISICOFACTOR?

Serum Lp(a) spiegels in gezonde en zieke populaties - een epidemiologische en klinische benadering

PROEFSCHRIFT

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Chapter 1

GENERAL INTRODUCTION

BACKGROUND

Atherosclerosis is the leading cause of death and a major contributor to morbidity in the Western industrialized world. In the Netherlands about 40.000 patients die each year because of atherosclerosis, and approximately 145.000 patients are hospitalized because of its sequelae (1). Consequently, atherosclerosis introduces high costs for the community. Within the group of atherosclerotic diseases coronary atherosclerosis is most prominent (1).

Studies of coronary heart disease (CHD) have led medical investigators suggest an association with CHD for at least 246 factors (2). For the vast majority cause and effect relationships have not been demonstrated. Yet, consideration of these factors provided clues to understand CHD etiology and to gain insight into possible preventive measures. The most important risk factors recognized today are smoking, hypertension, age, male gender and dyslipidemia.

For many years, increased levels of total and hence Low Density Lipoprotein (LDL)-cholesterol have been recognized as a major risk factor (3). The dominant role of LDL in the pathogenesis and perpetuation of atherosclerotic vascular disease has emerged from a plethora of data produced in several epidemiological studies showing a graded relation between total or LDL cholesterol levels and CHD (4, 5). Subsequently, multiple clinical trials provided extensive evidence that therapeutic lowering of LDL cholesterol levels in patients with hypercholesterolemia who were initially free of CHD or who had preexisting CHD, could favorably alter the course of atherosclerosis (6-8).

By further elucidating the mechanisms responsible for atherosclerosis, researchers evolved from the "cholesterol hypothesis", to the "lipid hypothesis" and finally to the "oxidation hypothesis", moving the focus from quantity (i.e. lipid or lipoprotein levels) to quality (i.e. type and atherogenicity) of lipoprotein particles (9). Notwithstanding the increasing knowledge about precipitating and promoting risk factors for coronary heart disease, national preventive campaigns and treatment strategies at the population and the individual level, mainly aimed at lowering total or LDL-cholesterol levels (10). Yet, a focus on LDL alone is myopic because most patients with angiographically documented coronary stenosis

manifest dyslipidemias other than LDL excess. Commonly observed lipid abnormalities, besides hypercholesterolemia, include hypoalphalipoproteinemia -either in isolation or with hypertriglyceridemia- and Lp(a) lipoprotein excess. In a cohort of men younger than 60 years Genest and coworkers reported that 87.5% of the patients expressed a major lipid abnormality, the latter being defined as age- and gender-adjusted LDL-c, total triglyceride or Lp(a) levels exceeding the 90th percentiles of healthy controls, or High Density Lipoprotein (HDL)-cholesterol levels below the 10th percentile. Elevations in LDL-c were observed in 22.3% of the patients, deficiencies in HDL-c levels in 35.8% and Lp(a) excess in 15.8% (figure 1) (11). Similar findings were reported by Kwiterovich and colleagues (figure 2) (12). Recently, it was again emphasized that other disorders linked to atherosclerosis such as apoprotein E isoform differences, hyperapobetalipoproteinemia, homocysteinemia, atherogenic lipoprotein phenotype (i.e. LDL subclass B pattern) and Lp(a) excess, that are not even detected by routine lipid testing, are present in ≈30 to 50% of male Caucasians with coronary artery disease (13, 14).

This thesis deals with one of these new, so-called cardiovascular risk factors, i.e. lipoprotein(a). The work was set-up to gain better insight into the clinical relevance of this elusive lipoprotein particle by documenting serum Lp(a) across healthy and diseased populations at different risk levels of CHD, and by delineating in Caucasian males the milieus in which Lp(a) mostly exerts its postulated atherothrombogenicity.

LIPOPROTEIN(a): STRUCTURE, MECHANISMS OF VASCULAR PATHOPHYSIOLOGY AND CLINICAL SIGNIFICANCE

Lipoprotein(a) (Lp(a)) is a lipoprotein particle that is similar to LDL in terms of lipid and protein compositions, but it also contains a highly glycosylated protein, designated apolipoprotein(a) (apo(a)), which is linked through a disulfide bridge to apolipoprotein B_{100}. It was identified in 1963 as an antigenic trait in human plasma by the Norwegian genetic scientist Kare Berg (16). At that time it was considered to be a variant of LDL. By the mid-1980s it was recognized as an independent, genetically determined, risk factor for premature atherosclerotic

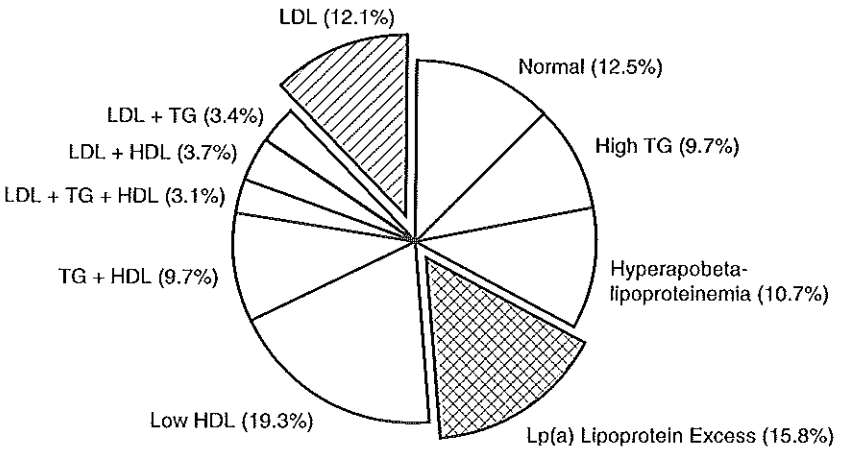

Figure 1. Prevalence of dyslipidemias in men aged 60 years with angiographic evidence for coronary atherosclerosis. Lipid and lipoprotein levels were defined by comparison with age- and sex-specific 90th percentile data for low-density lipoprotein (LDL) cholesterol, total triglycerides (TG) and Lp(a) lipoprotein, and 10th percentile data for high-density lipoprotein (HDL) cholesterol. Values are expressed as percentages. Adapted from: Genest J Jr et al.. J Am Coll Cardiol 1992; 19: 792-802.

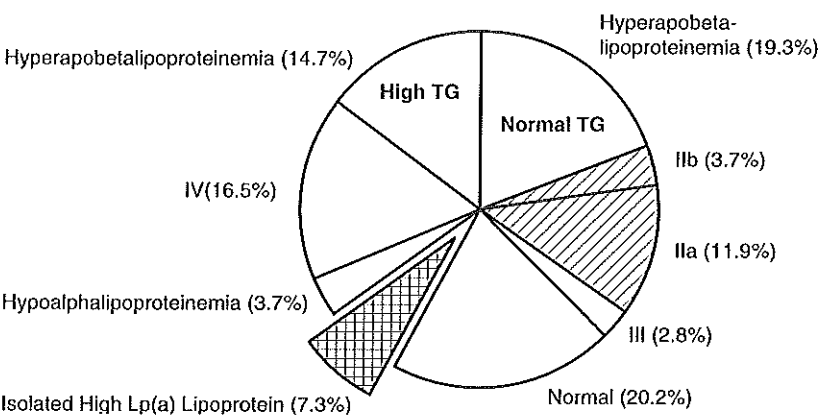

Figure 2. Prevalence of dyslipidemias in men younger than 50 years and women younger than 60 years. Dyslipidemias are categorized by lipoprotein phenotype (IIa, IIb, III and IV). Lipid, lipoprotein and apolipoprotein cutoff values were established by comparison with age- and sex-specific 90th percentile data for low-density lipoprotein (LDL) cholesterol, total triglycerides (TG), and Lp(a) lipoproteins, and 10th percentile data for high-density lipoprotein (HDL) cholesterol. Adapted from: Kwiterovich PO Jr, Coresh J and Bachorik PS. Am J Cardiol 1993; 71: 631-9.

vascular disease (17, 18). In 1987 it was established that apo(a) in Lp(a) has a striking structural similarity to plasminogen, possessing several copies of kringle 4-like repeats, a single kringle 5-like unit, and an inactive serine protease domain (figure 3) (19). The difference in the number of kringle 4-like repeats accounts for the size polymorphism of apo(a), an important contributor to the density heterogeneity of Lp(a) along with the lipid core content and composition (20). Besides contributing to the Lp(a) density heterogeneity, the genetically determined apo(a) size polymorphism is also a determinant of the serum levels of Lp(a). To date, 34 different Lp(a) genotypes have been identified that differ solely in the number of kringle 4 domains that are coded for in the apo(a) gene (21).

After the discovery of the molecular mimicricy with plasminogen, Lp(a) exerted an attraction upon researchers and clinicians like a "femme fatale" because it represented a potential link between coagulation, lipoproteins and the development of atherosclerosis. Considerable progress has been made in the last decade toward understanding the mechanism of its atherogenicity. Pathological and laboratory evidence supporting a role for Lp(a) in the atherosclerotic process stems from observations that it accumulates in atherosclerotic plaques, stimulates smooth muscle cell proliferation, binds apo B-containing lipoproteins, avidly binds to arterial proteoglycans and fibronectin, and promotes cholesterol accumulation in cells (22-24; figure 4). It may also promote thrombosis because it has structural similarities with plasminogen, binds fibrin, competes with plasminogen for binding sites on cells and exhibits antifibrinolytic actions *in vitro*. Besides, deposition of Lp(a) on fibrin surfaces is enhanced by the atherogenic amino acid homocysteine, suggesting a link with hyperhomocysteinemia-associated vascular disease (24; figure 5).

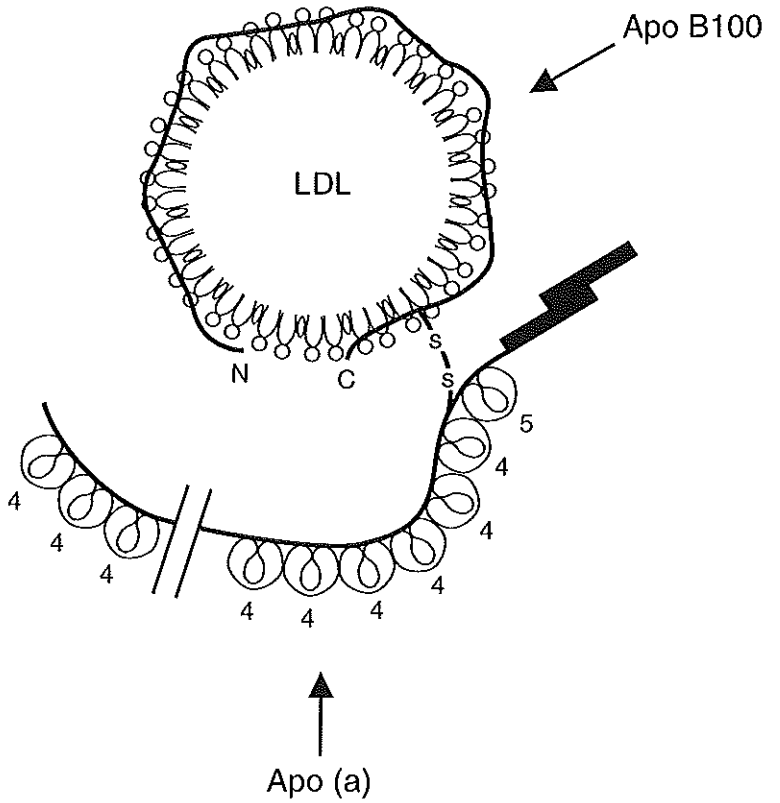

Figure 3. Schematic diagram of the structure of human Lp(a). Human Lp(a) consists of an LDL-like particle in which apoB_{100}, the characteristic protein moiety of LDL, is disulfide linked to the glycoprotein apo(a). Apo(a) contains multiple triple-loop, triple-disulfide-bonded motifs similar to the kringle 4 structure of the fibrinolytic zymogen plasminogen. Apo(a) also contains a single plasminogen-like kringle 5 structure, and an inactive protease domain.
Adapted from: Scanu AM. JAMA 1992; 267: 3326-29.

In vivo evidence for the antifibrinolytic properties of Lp(a) was shown in a study of myocardial infarct patients who had not received thrombolytic agents, in which it was found that the rate of spontaneous thrombolysis correlated inversely with Lp(a) levels (25). In myocardial infarct patients receiving thrombolytics, we and others documented that recanalization of the infarct related artery was unrelated to Lp(a) levels (26, 27). These combined findings suggest that Lp(a) levels modulate thrombolysis, having an impact in case of spontaneous

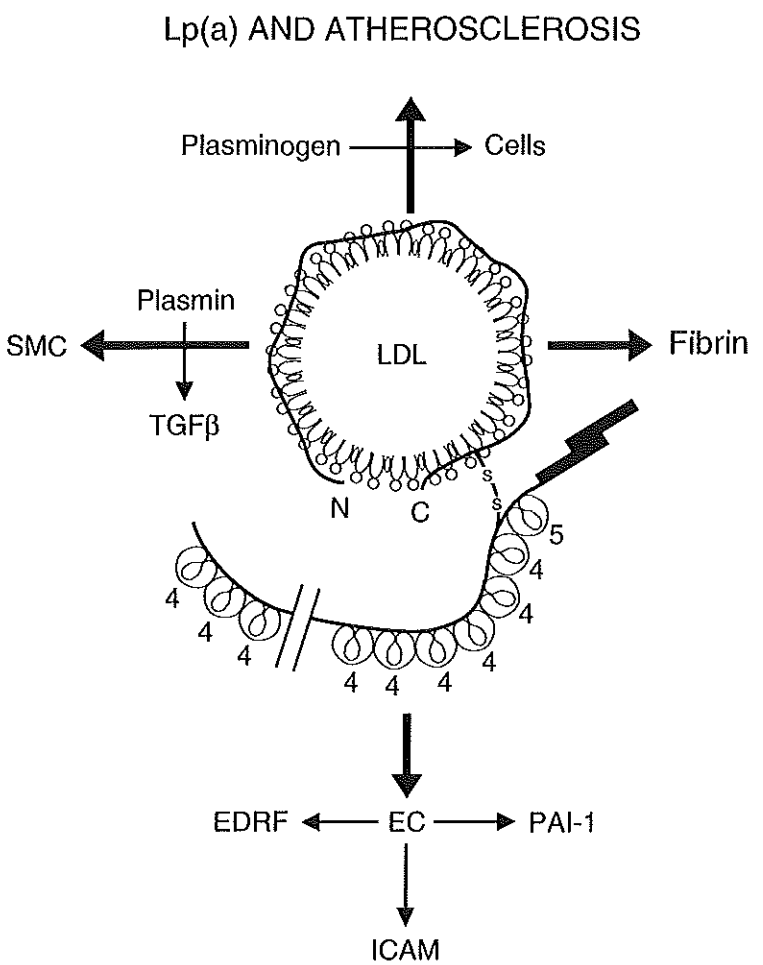

Figure 4. Working model of Lp(a) and vascular pathology. Lp(a) competes with plasminogen for binding to cell surface receptors and fibrin, preferred sites of plasminogen activation. Inhibition of plasmin generation by Lp(a) results in fibrin accumulation within atherosclerotic lesions and failure to activate transforming growth factor ß (TGFß), a modulator of smooth muscle cell (SMC) proliferation. In addition, Lp(a) may alter the endothelial cell (EC) phenotype by inducing synthesis of plasminogen-activator inhibitor-type 1 (PAI-1), expression of intercellular adhesion molecule-1 (ICAM), and release of endothelium-derived relaxing factor (EDRF) (nitric oxide).
Adapted from: Hajjar KA and Nachman RL. Annu Rev Med 1996; 47: 423-42.

thrombolysis, but being overwhelmed when large doses of thrombolytic agents are used (28).

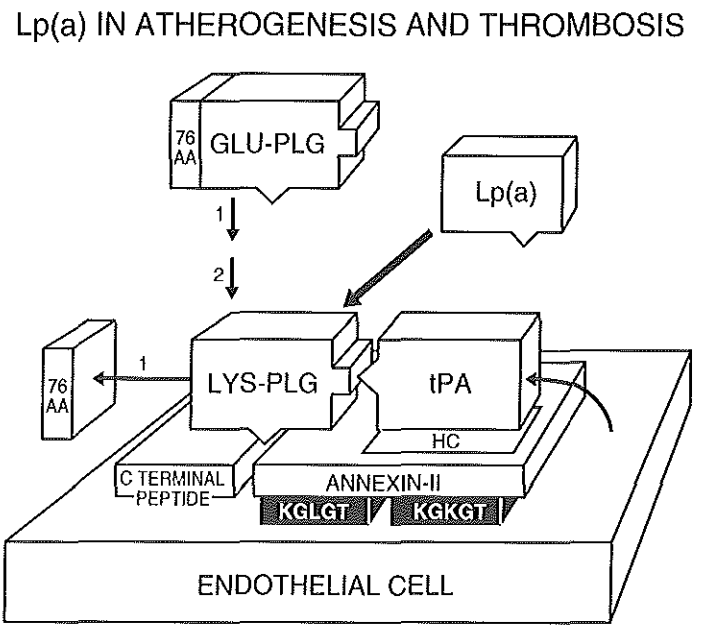

Figure 5. Hypothetical model of annexin-II mediated assembly of plasminogen and tissue plasminogen activator. Annexin II interacts with the endothelial cell surface via calcium-dependent phospholipid binding motifs (KGLGT and KGKGT). Upon binding to annexin II, circulating amino-terminal glutamic acid plasminogen (Glu-PLG) is converted to its truncated, noncirculating form, amino-terminal lysine plasminogen (Lys-PLG), through the proteolytic release of a 76-amino acid preactivation peptide (76 AA). Lys-PLG binds with high affinity to a carboxy-terminal lysine on annexin II, generated upon modification of the parent receptor by proteolytic cleavage at K307-R308. tPA, synthesized and secreted by the endothelial cell, binds to annexin II at a separate domain. Assembly of plasminogen and tPA in complex with annexin II would foster efficient generation of plasmin. Lp(a), in sufficient concentration, would compete with plasminogen for binding to annexin II, thereby decreasing production of the active protease. Homocysteine (HC) disables the tPA binding domain of annexin II, leaving the plasminogen binding domain intact.
Adapted from: Hajjar KA. Thromb Haemostasis 1995; 74: 294-301.

MEASUREMENT OF LIPOPROTEIN(a)

There are a number of potential difficulties in accurately measuring Lp(a) (29). After all, apolipoprotein(a) in Lp(a) is one of the most polymorphic proteins

in blood, possessing 10 different types of kringle 4, type 1 and types 3 to 10 being present singly. The number of copies of kringle 4 type 2 has been demonstrated to vary between 3 and 42. Most individuals have two different isoforms of Lp(a), which are genetically determined and can differ in size and structure.

With respect to the Lp(a) test kits currently in use two potential pitfalls should be considered. First, cross-reactivity with apo B or plasminogen is a potential difficulty. Therefore reliable immunoassays for Lp(a) should use antibodies immunopurified against apo B_{100} and plasminogen. Second, polyclonal and monoclonal antibodies to apo(a) will be reactive primarily to kringle 4 type 2 repeats, since these are the most common epitopes and the ones most likely to be antigenic in the host animal. The immunoreactivity of antibodies directed to kringle 4 type 2 repeats will vary depending on the size of apo(a). Because of the high degree of polymorphism and because Lp(a) levels are expressed as total Lp(a) mass or total apo(a) protein, it is impossible to match the isoform of the assay calibrator with that of the unknown samples. Consequently, Lp(a) levels in samples with apo(a) isoforms smaller than those in the calibrator are underestimated, while those with larger isoforms are overestimated (30).

A common misconception is that the use of ELISA's with apo B detection will result in measurement of Lp(a) levels independent of apo(a) isoform size. As long as the value of the assay calibrator is assigned in terms of total lipoprotein or protein mass of Lp(a), the values in the unknown samples will vary as a function of the size of apo(a) in the samples with respect to the size of apo(a) in the calibrator. Only when the assay calibrator is assigned in terms of moles of Lp(a) proteins or in terms of mass of apo B in Lp(a), the values generated by the apo B detection ELISA will be independent of the size polymorphism.

Taking into consideration that all the assays commercially available are calibrated in terms of total lipoprotein or total protein mass of Lp(a), there are no Lp(a) test kits that are unaffected by the apo(a) isoform size. The isoform dependency of Lp(a) quantification methods was reported to lead to some misclassification (30), but was shown to be of limited clinical relevance (31). In the Lp(a) test kits selected to carry out the work described in this thesis cross-reactivity was not an issue (this thesis; see 'Methods'), whereas isoform dependency of Lp(a) results might be a confounder.

OUTLINE OF THIS THESIS

High levels of Lp(a) in serum have been associated with presence and severity of atherosclerotic cardiovascular disease, at least in Caucasians. However, a role for Lp(a) in atherosclerotic cardiovascular disease, mainly based on epidemiologic data (22, 23), had not been observed in all reported clinical studies at the start of this thesis. I.e., three large prospective studies casted doubt on the role of Lp(a) in coronary heart disease because no association was found between the occurrence of cardiac events and the patients' Lp(a) levels (32-34). This promptly led to an editorial entitled "Has lipoprotein 'Little' (a) shrunk (35)?" The studies described in this thesis aimed at documenting Lp(a) levels across and within healthy and diseased populations, at delineating the conditions in which Lp(a) mostly exerts its postulated atherothrombogenic effects, and at clarifying the discrepant findings. To that end, an epidemiological and clinical approach was used.

A. Epidemiological approach:

In chapters 4, 5 and 6 serum lipoprotein(a) levels were investigated in samples of unrelated, apparently healthy Caucasian, Asian and African populations at different levels of CHD risk, this in relation to conventional serum lipid levels and other risk factors. In addition, it was examined whether sexual maturation (chapter 5) and apo E polymorphism (chapter 6) affected serum Lp(a) levels.

B. Clinical approach:

In chapters 7, 8 and 9 serum lipoprotein(a) levels were investigated in unrelated Causasian males with documented coronary artery disease. Chapters 7 and 8 deal with Lp(a) levels and changes in patients undergoing elective coronary artery bypass grafting (CABG) in a pravastatin-placebo controlled study. In chapter 9 the prognostic value of serum Lp(a) in the average Dutch male with symptomatic coronary artery disease, as included in the Regression Growth Evaluation Statin Study (REGRESS) (36), was examined. Special attention was given to define the conditions in which elevated Lp(a) levels are especially daunting and predict angiographically documented progression of the disease.

The clinical and epidemiological approaches mentioned, necessitated that Lp(a) was analyzed out of stored frozen sera. Chapter 2 deals with the effect of long-term storage and storage temperature on serum Lp(a) levels for the two Lp(a) test kits used. Besides, in order to define the required analytical imprecision of Lp(a) test kits, and critical differences between serial samplings, a comprehensive biological variation study was undertaken (chapter 3).

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Chapter 2

EFFECT OF LONG-TERM SAMPLE STORAGE ON THE ASSAY OF LIPOPROTEIN(a) BY IMMUNORADIOMETRIC AND ENZYME-LINKED IMMUNOSORBENT ASSAY KITS

ABSTRACT

Lipoprotein(a) (Lp(a) was measured by both an immunoradiometric (IRMA) kit (Mercodia, Upssala, Sweden) and an enzyme-linked immmunosorbent assay (ELISA) (Biopool Ltd, Umeå, Sweden) in split-serum samples that had been stored at -20°C and -70°C for two and a half and six years, respectively. In case of six year old samples Biopool Lp(a) values in -20°C sera were on average 25% lower compared to sera stored at -70°C (r = 0.992), while Mercodia results differed by 10% (r = 0.996). In two and a half year old sera Lp(a) values were diminished by approximately 5% in aliquots stored at -20°C compared to aliquots stored at -70°C by either method (r= 0.999). Serumpools, stored at -70°C, were stable with either method up to two years. With the ELISA test kit average serum Lp(a) degradation was -7% in aliquots that were stored two and a half years at -70°C; the IRMA data could not be compared between time points. In sera sampled four years apart a 6% reduction was found with the IRMA method in specimens stored at -20°C for one year, compared to specimens stored at -70°C during five years ($P < 0.05$).
We conclude that either method is sensitive to Lp(a) degradation during long-term storage, especially at -20°C. Reliable Lp(a) determinations can be performed with both methods in sera that are stored for up to two years at -70°C.

INTRODUCTION

Today, there is broad agreement that a high lipoprotein(a) (Lp(a)) level is a significant genetic risk factor for coronary heart disease (CHD) (1). Notwithstanding, some negative clinical case-control studies appeared that failed to detect the association between elevated Lp(a) and CHD (2). Common denominator in those studies was the fact that Lp(a) was measured retrospectively in stored sera, using commercial Lp(a) test kits for which the impact of storage upon serum Lp(a) levels was unclear. Consequently, it became a controversial issue whether Lp(a) can be correctly measured in frozen sera (3). To date, variable changes have been documented in Lp(a) concentrations after storage, depending on the assay type. First, Lp(a) concentrations have been shown to fall significantly at one, three and

six months of storage with a radial immunodiffusion kit, mean Lp(a) degradation, for the -20°C and -70°C data combined, being 46% after six months. Second, when measured by ELISA the changes in concentrations after storage were less marked (4, 5-6), except for the Macra Lp(a) kit from Terumo (7). Third, when measured by agarose electrophoresis, Lp(a) could be isolated if sera were stored less than two weeks at -20°C (8). Finally, repeated freezing and thawing has also been shown to diminish Lp(a) concentrations, and this was more marked when Lp(a) concentrations were determined by ELISA compared to an immunoturbidimetric assay (9).

We recently completed a large angiographic lipid intervention trial, the Regression Growth Evaluation Statin Study (REGRESS) (10). The REGRESS database provided us with the opportunity to determine retrospectively the role of lipoprotein(a) on progression of coronary atherosclerosis in stored sera. To overcome potential methodological confounding of the Lp(a) results by sample storage it was decided to examine the impact of storage time and storage temperature on two candidate Lp(a) assays.

METHODS

Lipoprotein(a) assays

Enzyme immunoassay:

Lp(a) was measured by a non-competitive sandwich ELISA technique using polyclonal goat antibody raised against purified human Lp(a) (Cat. Nr. 610220; Biopool, AB, Sweden). One antibody was coated on the micro-test wells and the other was conjugated with the peroxidase enzyme. The final dilution of the samples for the assay was 1:2601. The assay is calibrated to total Lp(a) mass and results are expressed in mg/l Lp(a) (11). The assay range is 10-650 mg/l. The interassay coefficients of variation in home-made serumpools were 7.2% at 67 mg/l (N = 23); 6.6% at 214 mg/l (N = 26) and 7.6% at 387 mg/l (N = 26).

In long-term stored specimens lot number 1261066 was used; for evaluating the effect of overnight storage at +4°C, -20°C and -70°C lot number 1261067 was used. All Lp(a) analyses were done in duplicate, according to the manufacturer's instruction.

Two-site immunoradiometric assay for apolipoprotein(a):

Lp(a) was measured as apolipoprotein(a) (apo(a)) by the Mercodia apo(a) RIA 100 test kit (Mercodia AB, Uppsala, Sweden). This test kit was previously distributed by Pharmacia. The assay is a solid-phase two-site immunoradiometric assay which uses two monoclonal antibodies in excess that are directed towards different epitopes of apo(a), but not against apo(a) kringle IV (11). Sera are pretreated with a "pretreatment solution" (1 hour incubation at room temperature), and subsequently diluted with a stabilizing, Kathon CG-containing sample diluent, prior to analysis. It is suggested by Berg that the Lp(a) polypeptide chain is detached from the Lp(a) particle after pretreating the samples (3). During incubation apo(a) reacted with a ^{125}I-labelled monoclonal antibody and with an antibody attached to spherical micro Sepharose particles. The apo(a)-antibody complex was separated from the excess ^{125}I-antibody with decanting solution, followed by centrifugation and decanting. The pellet was counted for radioactivity with a gamma counter. The final dilution of the samples for the assay is 42-fold. Results are reported in U/l apolipoprotein(a). According to the manufacturer, U/l should very approximately correspond to 0.7 mg/l Lp(a) mass. The assay range is 17 - 840 U/l. The interassay coefficients of variation of the IRMA method in the same serumpools as those used for the ELISA assay, were 5.1% at 111 U/l (N = 43), 5.0% at 358 U/l (N = 50) and 3.7% at 629 U/l (N = 50).

Specimens

Stored sera were obtained from the serumbanks of the Departments of Epidemiology and Clinical Chemistry from the Rotterdam University Hospital, Rotterdam, the Netherlands. First, split aliquots that were stored for respectively two and a half (N = 31) and six (N = 32) years at both -20°C and -70°C were randomly taken from the Rotterdam Elderly Study serumbank (12) and analyzed with either Lp(a) method. Second, a selection of sera from Belgian schoolchildren that were previously analyzed with the Biopool Lp(a) kit (13), and of which split serum aliquots were kept frozen at -70°C, were reassessed two and a half year later. Sample selection was made in such a way that the previously determined serum Lp(a) values had a Gaussian distribution across the Lp(a) measuring range of the kit (13). Third, sera from the Rotterdam Elderly Study (12), which were

stored for five years at -70°C, were analyzed with the Mercodia kit and compared to sera which were sampled four years later and stored for one year at -20°C (N = 85). Finally, fresh sera from the hospital routine were used for evaluation of the effect of overnight storage at 4°C, -20°C and -70°C (N = 43). All frozen sera were thawed at room temperature and determined the same day. Paired aliquots, stored at +4°C and/or -20°C and -70°C, were analyzed in duplicate in the same run, in order to omit between-run variation. None of the sera had previously been thawed. Home-made serumpools at low, medium and high levels which were stored at -70°C, were used for internal quality control and for checking long-term stability of the calibration of either Lp(a) method. Besides, the stability of single-donor citrate plasma at three levels was examined.

Statistical methods

Comparability between Lp(a) concentrations, obtained by the IRMA and/or ELISA test kits at different storage times and temperatures, were examined by Passing and Bablok regression analysis (14). Throughout the article, a significance level of $\alpha = 0.05$ was adopted.

RESULTS

In figures 1a (Mercodia) and 1b (Biopool) quality control data, obtained in frozen serumpools over a two year period, are presented. From figures 1a and 1b it is obvious that measured Lp(a) levels in serumpools stored at -70°C were stable during two years with either method; after two years of storage at -70°C a monotonic decline was observed, especially for the Biopool kit. Besides, these figures insure long-term stability of the calibration of the assays examined. Note that citrate plasma was not suitable for long-term quality control as severe Lp(a) degradation occurred already within three months of storage at -70°C, the ELISA kit being most sensitive.

The impact of storage time and temperature on Lp(a) measurements, as determined by the Biopool and the Mercodia test kits, respectively, is illustrated in table 1. Overnight storage at -20°C respectively -70°C did not affect Lp(a) IRMA

or ELISA values, signifying that freezing by itself was not detoriating. In contrast, in two and a half year old sera Lp(a) values were on average, for either method, reduced by circa 5% in aliquots stored at -20°C, compared to aliquots stored at -70°C. In six year old sera Biopool Lp(a) values were on average 25% lower in aliquots stored at -20°C compared to those stored at -70°C, while Mercodia results differed by 10%. Biases at -20°C compared to -70°C were not specimen related and were proportional across the Lp(a) measuring range. Notwithstanding the enhanced rate of Lp(a) degradation upon long-term storage at -20°C, within-method correlation coefficients between -20°C and -70°C data were excellent (table 1; $r \geq 0.992$). Moreover, with the Mercodia kit a significant negative bias was found between Lp(a) values measured in sera that were resampled and stored during one year at -20°C, compared to sera that were kept frozen during five years at -70°C, suggesting accelerated Lp(a) degradation at -20°C already within 12 months of storage (table 1). For the Biopool kit mean absolute Lp(a) degradation was -7% after two and a half years of storage at -70°C; the IRMA data could not be compared between time points.

The impact of storage conditions on the Biopool (Y) / Mercodia (X) intermethod comparison is illustrated in table 2. In fresh unfrozen sera, the slope of the regression equation depicts that U/l approximates 0.54 mg/l Lp(a) rather than 0.70 mg/l. Overnight freezing did not significantly affect the method means or the magnitude of the slopes of the regression equations. In case of sera stored for six years at -20°C compared to -70°C, the slopes of the regression equations diminished from 0.654 to 0.539, illustrating the more pronounced sensitivity of the Biopool assay to Lp(a) degradation with time at -20°C.

DISCUSSION

The opportunity for analyzing Lp(a) in stored serum samples from REGRESS (10, chapter 9 of this thesis) prompted us to consider the effects of storage on serum concentrations of Lp(a) for two candidate Lp(a) test kits. After all, Berg suggested that serious methodological problems with commercial Lp(a)

Table 1. Effect of storage time and temperature on Lp(a) measurements as determined by the Biopool ELISA and the Mercodia IRMA test kits for Lp(a). Regression analyses were performed according to Passing and Bablok (14).

Storage conditions/ Y versus X	Lp(a) assay	N	Slope	Intercept	Correlation Coefficient	Mean X	Mean Y
Analyzing split aliquots out of the same specimens:							
24 hrs at -20°C	Biopool	43	1.000	0.20	0.998	147	148
versus 24 hrs at 4°C	Mercodia	43	1.001	-1.03	1.000	270	269
24 hrs at -70°C	Biopool	43	0.974	0.79	0.996	147	143
versus 24 hrs at 4°C	Mercodia	43	0.993	-0.63	1.000	270	270
2.5 yrs at -70°C versus fresh frozen	Biopool	49	0.916[a]	2.45	0.995	255	237
2.5 yrs at -20°C	Biopool	31	0.962[a]	0.76	0.999	147	141
versus 2.5 yrs at -70°C	Mercodia	29	0.951[a]	-2.44	0.999	248	233
6 yrs at -20°C	Biopool	32	0.743[a]	1.33	0.992	152	112
versus 6 yrs at -70°C	Mercodia	26	0.898[a]	-20[b]	0.996	297	253
Analyzing paired specimens from different blood samplings:							
1 yr at -20°C versus 5 yrs at -70°C	Mercodia	85	0.941[a]	-3.06	0.979	364	344

[a] Slope is significantly different from one at $\alpha = 0.05$.
[b] Intercept is significantly different from zero at $\alpha = 0.05$.

kits and failure to consider the Lp(a) lability during sample storage might explain the failure of some clinical studies to detect an association between high levels of Lp(a) and CHD (3). Moreover, Berg criticized the shortcomings of manufacturers who market test kits for Lp(a) that are not scientifically validated, and are not monitored against research-level systems for quantitative determination of Lp(a) lipoprotein where any immunological reaction not involving Lp(a) would be detected. Also, the use of undisclosed test procedures or contents of test reagents, like e.g. "pretreatment solutions", were denounced as unfortunate practices.

Table 2. Effect of storage time and temperature on Lp(a) intermethod comparison. Serum Lp(a) levels were determined using the Biopool ELISA and the Mercodia IRMA test kit. Regression analyses were performed according to Passing and Bablok (14).

Y versus X	Storage conditions	N	Slope	Intercept	Correlation Coefficient	Mean X	Mean Y
Biopool versus Mercodia	24 h, 4°C[a]	43	0.544[c]	+0.19	0.996	270	147
	24 h, -20°C[a]	43	0.551[c]	+0.27	0.995	269	148
	24 h, -70°C[a]	43	0.525[c]	+0.26	0.993	270	143
	fresh frozen versus 2.5 yrs at -70°C[b]	46	0.641[c]	-4.79	0.994	399	249
	2.5 yrs at -70°C[b]	46	0.580[c]	-0.99	0.997	399	231
	2.5 yrs at -20°C[b]	29	0.638[c]	+2.26	0.996	233	150
	2.5 yrs at -70°C[b]	29	0.636[c]	+1.67	0.995	248	157
	6 yrs at -20°C[b]	25	0.539[c]	+8.07	0.993	246	138
	6 yrs at -70°C[b]	29	0.654[c]	+1.33	0.999	270	161

[a] Lotnumbers of the Biopool and Mercodia kits used were 1261067 and 882, respectively.
[b] Lotnumbers of the Biopool and Mercodia kits used were 1261066 and 479, respectively.
[c] Slope is significantly different from one at $\alpha = 0.05$.

Consequently, users of test kits themselve must ascertain whether the reactivity of the corresponding epitopes of Lp(a) remain constant upon freezing and storage of the samples, and whether suitable conditions and duration of storage can be identified.

The Lp(a) test kits examined in this study were: the Mercodia apo(a) IRMA assay, which uses indeed a "pretreatment solution" of unknown composition, and the Biopool ELISA assay. Our data display that freezing by itself, represented by 24 hour freezing, did not affect measured Lp(a) values by either method (table 1). Also, correct Lp(a) measurements can be performed with both Lp(a) test kits in serumpools that were stored at -70°C up to two years (figures 1a and 1b). After two years of storage, both test kits were affected by Lp(a) degradation, resulting in decreased Lp(a) values that fell below the "mean - 1 S.D." limit. Analogously, a

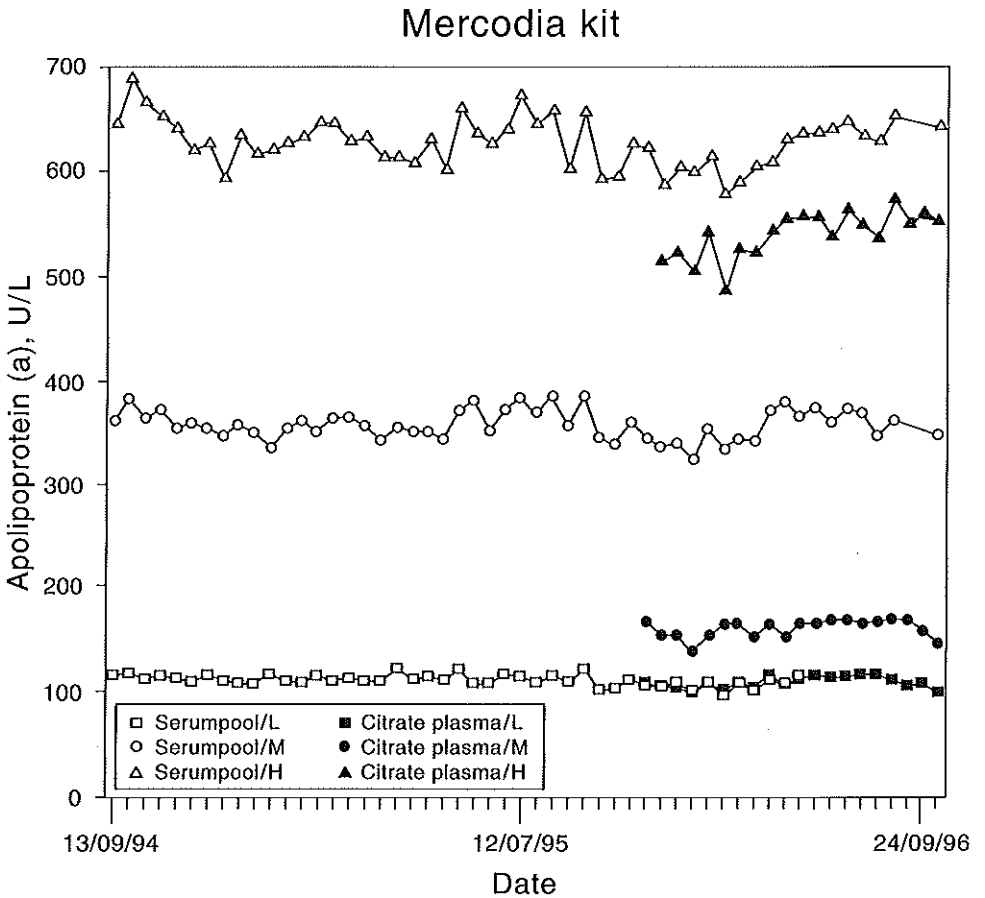

Figure 1a. Monitoring of apolipoprotein(a) levels as measured by the Mercodia IRMA assay in serumpools (low (L), medium (M), high (H) level) and in single donor citrate plasma (low, medium, high level). Serumpools and citrate plasma were aliquoted and stored at -70°C. Each aliquot was thawed only once. Storage time covered the period from August 30th, 1994 to December 12th, 1996.

significant negative bias was found with the ELISA test kit between Lp(a) values measured in paired serum aliquots which were stored for two and a half year at -70°C, compared to Lp(a) values determined in at that time fresh frozen aliquots (table 1). From these data it can be assumed that Lp(a) levels in individual serum specimens can be measured adequately with either technique, if sera are stored during maximal two years at -70°C.

Besides time in storage, storage temperature also had an effect: in case that

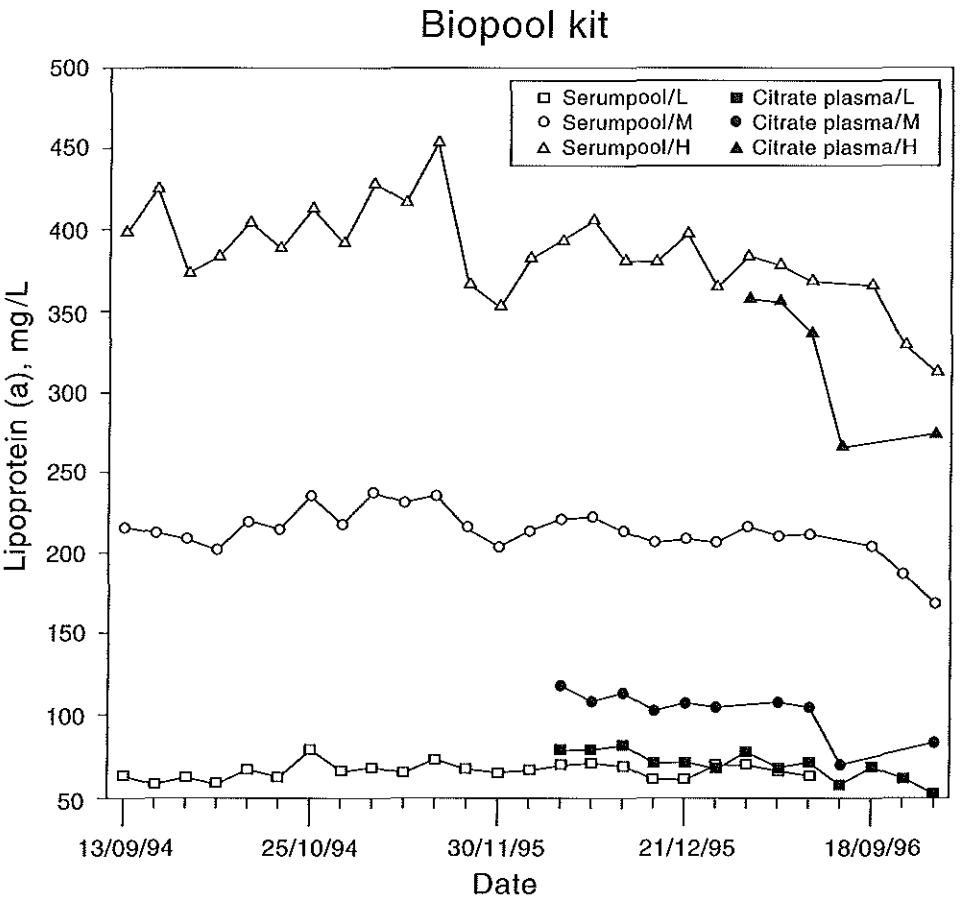

Figure 1b. Monitoring of lipoprotein(a) levels as measured by the Biopool ELISA assay in serumpools (low (L), medium (M), high (H) level) and in single donor citrate plasma (low, medium, high level). Serumpools and citrate plasma were aliquoted and stored at -70°C. Each aliquot was thawed only once. Storage time covered the period from September 13th, 1994 to December 12th, 1996.

split serum aliquots were stored two and a half year at -20°C and -70°C, the average reduction in Lp(a) levels at -20°C compared to -70°C was of the order of the assay coefficient of variation. In case that paired serum aliquots were stored for six years at both -20°C and -70°C, average biases at the -20°C compared to the -70°C storage condition, were -25% for the ELISA and -10% for the IRMA method, i.e. exceeding more than twice the interassay coefficients of variation of either method (table 1). These 6 data illustrate that the rate of Lp(a) degradation is accelerated

upon long-term storage at -20°C compared to storage at -70°C. Degradation was detected by both Lp(a) test kits, but was more pronounced for the ELISA method.

The finding that storage affects the observed Lp(a) values probably results from altered exposure of antigenic sites. For instance, oxidative modification of Lp(a) upon storage may produce significant changes in Lp(a) conformation, affecting the immunoreactivity of the antibodies used (15). Different sensitivities of the Lp(a) test kits to Lp(a) degradation can be explained by different reactivities with different fragments. The magnitude of the *serum* Lp(a) decline noted with the Biopool ELISA kit was similar to the decline demonstrated by Kronenberg et al. who found a mean *plasma* Lp(a) decrease of 7% after 24 months of storage at -80°C, using another ELISA kit (5). Furthermore, our Biopool data extend the findings of Craig et al. who documented that storage time, but not temperature, was an important determinant of Lp(a) degradation over a six-month period (4).

In this study, i.e. over longer storage periods, storage temperature also became a determinant of Lp(a) degradation as determined by both test kits. Finally, we demonstrated that the IRMA method is also affected by Lp(a) degradation during long-term storage, though less than the ELISA method. Therefore, we can not underscore the assumption of Berg that the IRMA kit fails to detect the lability of Lp(a) lipoprotein particles upon storage, making it a questionable tool to examine human sera with respect to levels of Lp(a) (3). In fact, we believe that measuring free Lp(a) polypeptide chain in "pretreated" samples, rather than measuring intact Lp(a) lipoprotein particles, makes the IRMA assay less vulnerable to Lp(a) degradation than the ELISA assay. To the best of our knowledge, the IRMA data presented here are the first examining the effect of long-term storage on serum Lp(a) levels. In view of the excellent intermethod correlations (table 2), it seems that both the IRMA and the ELISA method will produce reliable Lp(a) results in sera stored less than two years at -70°C.

We conclude that Lp(a) levels should preferentially be determined in sera stored at -70°C, because long-term storage at -20°C is much more damaging to Lp(a) lipoprotein particles than long-term storage at -70°C. Correct Lp(a) measurements can be performed with either the Biopool ELISA or the Mercodia IRMA test kit for Lp(a) if sera are stored less than two years at -70°C. Finally, it is especially important to analyze all samples from patients and/or controls in clinical

studies by the same standardized technique, preferentially using fresh samples, or if that is impossible, using frozen samples stored at or below -70°C for about the same length of time and thawed only once. This implicates that Lp(a) studies should be carefully planned in advance.

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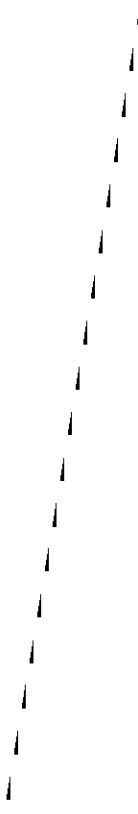

Chapter 3

SIGNIFICANCE OF VARIOUS PARAMETERS DERIVED FROM BIOLOGICAL VARIABILITY OF LIPOPROTEIN(a), HOMOCYSTEINE, CYSTEINE AND TOTAL ANTI-OXIDANT STATUS

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ABSTRACT

Analytical and biological components of variability, and various derived indices have been determined for lipoprotein(a) (Lp(a)), homocysteine (Hcy), cysteine (Cys) and Total Anti-Oxidant Status (TAOS) in ostensibly healthy adult Caucasians, and in stable outpatients with an elevated serum Lp(a). In healthy Caucasians average intra-individual biological coefficients of variation (CV_b) were 20.0% for Lp(a), 9.4% for Hcy, 5.9% for Cys and 2.8% for TAOS, CV_b's being similar in men and women. In the outpatient group CV_b's were comparable for Hcy, Cys and TAOS, but significantly lower for Lp(a) (7.5% versus 20.0%; $P < 0.0001$). Moreover, a significant inverse relationship between biological and analytical coefficient of variation (CV_a), respectively, and serum Lp(a) levels was demonstrated. We conclude that average CV_a and CV_b values, and hence average derived indices, are adequate for Hcy, Cys and TAOS, whereas individual values should be used for Lp(a).

ABBREVIATIONS:

Lp(a): lipoprotein(a); apo(a): apolipoprotein(a); Hcy: total homocysteine; Cys: cysteine; TAOS: total anti-oxidant status; CV_a: analytical coefficient of variation; CV_b: within-subject or intra-individual biological coefficient of variation; CV_g: between subject or interindividual biological coefficient of variation; AG CV_a: analytical goal for imprecision; AG bias: analytical goal for bias; RCV: reference change value; NS: number of specimens; df: degrees of freedom.

INTRODUCTION

In addition to the traditional lipid and lipoprotein risk factors for atherosclerotic disease, serum lipoprotein(a) (Lp(a)), plasma homocysteine (Hcy) and serum anti-oxidant levels are increasingly recognized as independent risk factors for atherosclerosis (1-3). Lp(a) is a lipoprotein particle that resembles LDL with a disulfide-linked apolipoprotein(a) (apo(a)) side chain. Apo(a) is structurally related to plasminogen, although it has no plasminogen activity and, so far, its physiolo-

gical function has not been unravelled. Lp(a) is mainly synthesized by the liver, its levels being mostly genetically determined and fairly constant throughout an individual's life (1, 4-5). Across populations, mean and median Lp(a) concentrations are lower in Caucasians than in people whose ancestors originated in Africa (6) or the Indian subcontinent (7). However, high average concentrations of serum Lp(a) are observed in Caucasians with a family history of premature ischaemic heart disease (1). Although its evolutionary conservation suggests some selective advantage during some stage of man's evolution, it is hypothesized that in our present condition Lp(a) may increase the likelihood of thrombosis occuring on atheromatous plaques, due to its plasminogen resemblance.

Hcy, which is derived from the intracellular metabolism of methionine, is exported into plasma where it circulates primarily in oxidized form (i.e. homocyst(e)ine and cysteine-homocysteine disulfide) and bound to proteins. Concentrations of total homocysteine (Hcy) are increased in 15-40% of patients with coronary, cerebral or peripheral arterial diseases (2, 8). Mechanisms that may relate to the pathogenesis of atherothrombosis in hyperhomocyst(e)inemia are the change in hemostatic condition from antithrombotic to thrombogenic, the increased incorporation of Lp(a) into fibrin and the increased oxidation of low-density lipoprotein (LDL) (2, 8).

The *oxidation hypothesis* of atherosclerotic disease emphasizes the causal role of oxidized lipoproteins in atherogenesis (9). If decreased anti-oxidant levels accelerate lipoprotein oxidation and hence atherosclerotic disease, detection of a decreased Total Anti-Oxidant Status, as measured by anti-oxidant mediated quenching of the absorbance of a radical cation (10), may prove to be a valuable test.

An aspect of introduction and evaluation of new procedures that should receive enough attention is the significant amount of information that can be obtained by generation and application of data on intra- and interindividual biological variation (11, 12). To date, data on biological variation are absent for Hcy (and Cys) and TAOS, and abundant but conflicting for Lp(a) (13-19). In case of Lp(a), earlier estimates of the biological intra-individual coefficient of variation (CV_b) showed a 7% week-to-week variation (13) while in the ARIC study (14) the CV_b was estimated to be as low as 2.9%. More recent studies (15-17) reported

average CV_b's of 7.6%, 10% and 18%, whereas Marcovina et al. (18, 19) found the estimated CV_b to be highly variable (range 3 - 51%) and to have a systematic inverse relation with the Lp(a) concentration. Possibly, the highly skewed Lp(a) distribution and the 1000-fold interindividual spread in blood Lp(a) concentrations in Caucasians, in combination with the investigation of rather limited numbers of individuals, may have caused apparently conflicting data on intra-individual biological variation of Lp(a).

In the present study, a comprehensive biological variability study for these analytes was carried out in a large group of healthy sex- and age-matched Caucasians. In order to ensure inclusion of an adequate number of individuals with high serum Lp(a), stable outpatients from the Lipid Clinic who repeatedly had Lp(a) mass levels > 300 mg/l were enrolled. An experimental protocol that minimized preanalytical and analytical sources of variability was used. The aims were: 1) to estimate, in healthy and in chronically diseased but stable Caucasians, the biological variation of Lp(a), Hcy, Cys and TAOS around the intra-individual homeostatic setpoints as well as the relation between the biological intra-individual variation and the analyte level; 2) to determine desirable analytical goals for these new or potential risk factors, based on intra- and interindividual biological variation (11); 3) to gain a clear understanding of the value of conventional population based reference values for these analytes (12); 4) to gain a clear insight into significant and insignificant analyte changes in serial specimens (12, 20); and 5) to calculate the minimum number of serial specimens needed to determine the 'true' analyte concentration (21).

MATERIALS AND METHODS

Study subjects

Healthy volunteers

Fifty-four physically healthy Caucasians who had a stable lifestyle and diet, and who were not on lipid lowering medication (27 men and 27 women; age range: 21 to 46 years) were enrolled. Ongoing intake of supplementary vitamins, minor tranquillizers or oral contraceptives throughout the study period was allowed, as well as occasional intake of an over-the-counter drug such as aspirin or parace-

tamol. All subjects were free of endocrine, metabolic and immune disorders. None of the women with childbearing capability became pregnant during the study.

Outpatients with hyper-Lp(a)-lipoproteinaemia

Twelve Caucasian outpatients (5 males and 7 females; age range: 22 to 69 years) from the Lipid Clinic of the University Hospital Rotterdam, with an Lp(a) mass larger than 300 mg/l, were included. All patients were on lipid lowering diet for at least three months before enrollment.

Study protocol

Blood was collected bi-weekly at each of four visits per individual. Subjects were seen in standardized format at each occasion, i.e., they were asked to fast for 10 to 12 hours before each visit, and to maintain their diet, lifestyle and possible medication throughout the evaluation period. The design and intention of the study were thoroughly explained to all subjects and informed consent was obtained. All study subjects were interrogated on each of the four visits by one of two physicians who checked, by means of a predefined questionnaire, whether diet, lifestyle, smoking and drinking habits, and possible medication were maintained throughout the study. Height and weight were measured at the first visit, whereas body weight was checked at each subsequent visit. Height was measured to the nearest 0.5 cm, while weight was recorded without shoes and outerwear to the nearest 0.5 kg. Fertile women were questionned about possible new pregnancies.

Venous blood was collected in the upright sitting position, immediately after individuals had been seated. Sampling was done between 8:00 and 10:00 a.m. by a single phlebotomist. Whole blood was collected for Lp(a) and TAOS (22), whereas EDTA blood was collected for Hcy and Cys analyses (23). Except for serum TAOS, which has limited stability according to the manufacturer, all four samples from one individual were analyzed in one run at the end of the study, to omit between-run analytical variation. The study protocol was approved by the Medical Ethical Committee of the University Hospital.

Specimen handling and storage

A strictly predefined protocol was used for specimen preparation: EDTA

(1.5 g/l) blood tubes were put on crushed ice immediately after blood collection (23). Whole blood tubes were kept at room temperature till clotting took place. Both whole blood and EDTA tubes were centrifuged at 4°C (10 min., 1500 g) within one hour after blood drawing (23). Serum and EDTA plasma were separated from the cells immediately after centrifugation. TAOS determinations were performed the same day, whereas the other aliquots were stored at -70°C for combined analysis of all samples from one individual at the end of the study. Blood specimens for this study were gathered during a 3 month period.

Lipoprotein(a), homocysteine, cysteine and TAOS measurements

Lp(a) was measured in serum using an anti-apo(a) polyclonal capture ELISA from Biopool (TintElize lipoprotein(a), Cat. No. 610220, Biopool AB, Umeå, Sweden) (4-6). Total Hcy and Cys were measured in EDTA plasma using a rapid, isocratic HPLC method (24, 25). Serum TAOS was applicated on a Hitachi 911 analyzer (Boehringer Inc, Mannheim, Germany) using the Randox kit and calibrator respectively control material (Cat. No. NX2332 and Cat. No. NX2331, Randox Ltd, Ardmore, United Kingdom). The TAOS assay measures the anti-oxidant mediated quenching of the absorbance of a radical cation (10).

TAOS analyses were done in duplicate at the day of sample collection, whereas Lp(a), Hcy and Cys analyses from one individual were performed in duplicate within one run at the end of the study. The maximum sample storage time for frozen aliquots was five months. To further minimize analytical variation, a single technician performed all the assays and single lots of reagents were used. The between-run CV for the TAOS control material was 4.3% (N = 15 runs), corresponding to a between-run variance of 0.0025.

Statistics

If a quantity X exhibits biological variation such that the standard deviation in an individual is proportional to the homeostatic setpoint of the individual, then the quantity X is said to have a constant CV. The CV is a parameter expressing the proportionality of the standard deviation to the homeostatic setpoint. It can be defined similarly for the analytical variation around the true value of a specimen. When the CV of X is small, then it is well approximated by the standard deviation

(σ) of ln X, with few assumptions required regarding the distribution of X or ln X. A proof of this is given in the Appendix.

The smaller the CV, the better the approximation will be. From a statistical point of view, it is better to directly estimate the CV by estimating the σ of ln X as a single parameter (be it an approximation), than as a ratio of an estimated standard deviation to an estimated mean. For CV's smaller than 0.4 (i.e. < 40%) the approximation by σ of ln X is good enough for practical purposes, considering the efficiency gained by estimating it as a single parameter. In summary, analytical CV (CV_a) and CV_b can be estimated as σ_a respectively σ_b after log transformation of the measured analyte values.

Hitherto, means, variances (σ^2) and CV's were estimated by using standard formulas. In case of TAOS, serial specimens were analyzed in separate runs, and thus σ^2_b was an estimator for the total of within-subject and between-run variance. To obtain a proper estimate of the within-subject variance for TAOS, the between-run variance derived from the TAOS control material was subtracted from σ^2_b.

Indices were derived from CV_a and CV_b data (11-12, 20-21, 26) as follows: analytical goal for imprecision (AG CV_a) = $\leq$ 1/2 CV_b; analytical goal for bias (AG bias) = $\leq$ 1/4 $(CV_b^2 + CV_g^2)^{1/2}$; index of individuality = $(CV_b^2 + CV_a^2)^{1/2}/ CV_g$; reference change value (RCV) or critical difference = 2.77* $(CV_a^2 + CV_b^2)^{1/2}$; number of specimens required to ensure with 95% confidence that the mean result is within ± 5% of the individual's homeostatic setpoint (NS (± 5%)) = 1.96^2 $[(CV_a^2 + CV_b^2)/25]$; number of specimens required to ensure with 95% confidence that the mean result is within ± 10% of the individual's homeostatic setpoint (NS (± 10%)) = $1.96^2[(CV_a^2 + CV_b^2)/100]$.

Data analysis

Data analysis was done separately for healthy subjects and outpatients after removing one sample from the TAOS data set because of *in vitro* hemolysis. All analyte results were transformed using natural logarithms. Variances calculated from the logarithmically transformed data were multiplied by 10000 to convert the estimated standard deviations (σ) to the coefficients of variation, expressed in percent.

Differences in biological variation between men and women in each group

were tested by calculating ratios of the pooled variance of analytical and within-subject variance from one gender, to the other gender. These calculated F-ratios were compared to the critical F-values ($\alpha = 0.05$). Differences in biological variability between the healthy subject and patient groups were calculated in a similar way. Contribution of analytical variability to total test variability was calculated as: $[((CV_a^2/CV_b^2) + 1)^{1/2} - 1]* 100\%$ (11). Concentration dependency of CV_b and CV_a versus the average analyte concentration was studied using linear regression analysis: ln CV = ln α - ß ln mean + residual. The null-hypothesis checked was that the slope ß would be equal to zero. 95% Confidence intervals for serum Lp(a) were calculated as $\pm$ 1.96 $[(\text{analyte level} * 0.01 * CV_a)^2 + (\text{analyte level} * 0.01 * CV_b)^2]^{1/2}$ / number of specimens$^{1/2}$. Overall, a significance level of $P \leq 0.05$ was adopted.

RESULTS

Table 1 summarizes the baseline characteristics of the study subjects as well as the analyte levels at the first visit. Mean intra-individual weight changes varied between -0.06 kg and +0.98 kg between subsequent visits (data not shown). Table 2 summarizes mean CV_a, CV_b and CV_g, and percentile distributions of CV_b for Lp(a), Hcy and Cys, while for TAOS only mean CV_b and CV_g are presented. In outpatients similar average CV_b estimates were found, except for Lp(a) (7.5% versus 20.0%; $F = 7.07$; df1 = 162; df2 = 36; $P < 0.0001$). CV_b did not differ between men and women for any of the analytes, either in healthy subjects ($F < 1.44$ at $\alpha = 0.05$; df1 = 81; df2 = 81) or in outpatients ($F < 2.18$ at $\alpha = 0.05$; df1 = 15; df2 = 21 or $F < 2.37$ at $\alpha = 0.05$; df1 = 21; df2 = 15) (data not shown). Based on average CV_a and CV_b values we found that less than 10% of the total test variability was analytical for Lp(a), Hcy and Cys, while for TAOS up to 98% of the observed test variability was analytical. Table 3 displays the average derived indices, for each analyte studied. Analytical goals for imprecision, based on average CV_b, were achieved for all analytes, except for TAOS (11-12, 26). All analytes had marked individuality (index < 0.6) (12), demonstrating that the use of population based reference values is inadequate for their interpretation. In the healthy subject

group average applicable differences required for two results to be significantly different at $\alpha = 0.05$ were 60% for Lp(a), 28% for Hcy, 17% for Cys and 9% for TAOS (20). Further, table 3 reveals that for proper assessment of coronary artery disease risk in the population by means of the assays used, multiple serial specimens are needed if the observed value should be within e.g. 5% of the true value.

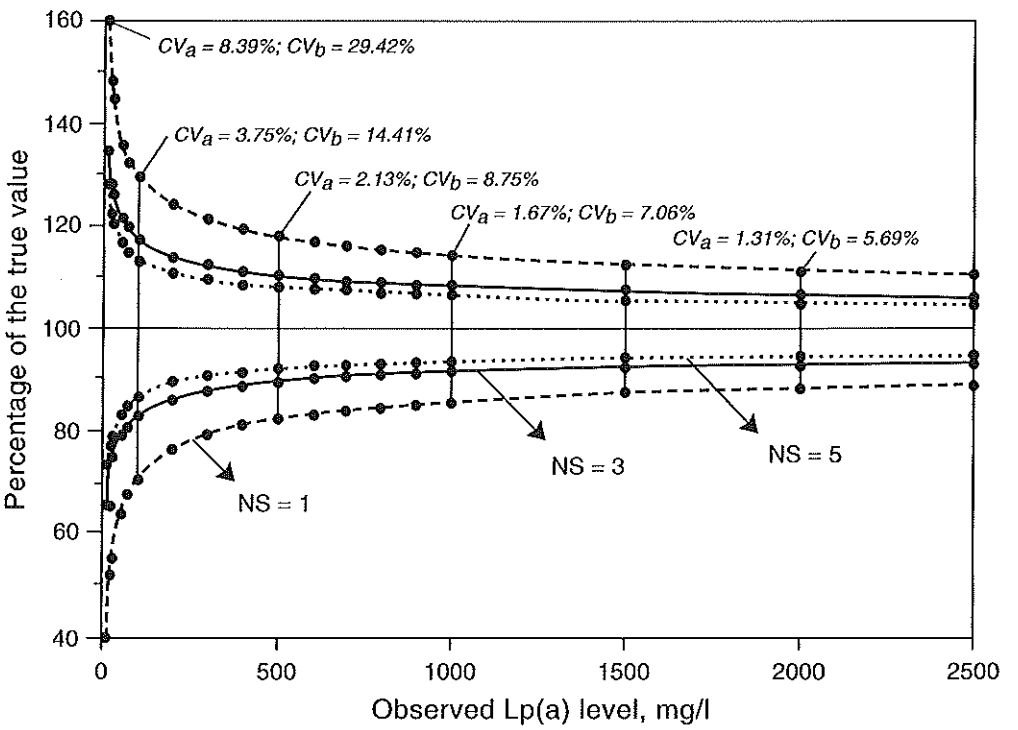

Figure 1. Confidence intervals (95%) for the subject's true value of Lp(a) as percentage of the observed serum Lp(a) value for one, three and five serial specimens. CV_a: analytical coefficient of variation; CV_b: biological coefficient of variation; NS: number of serial specimens.

Concentration dependency of $Ln(CV_a)$ and $Ln(CV_b)$ was studied in the healthy subject group for all analytes (data not shown). None of the parameters showed concentration dependency, except Ln(Lp(a)), the slopes being (borderline) significantly different from zero ($P = 0.04$ for CV_a versus subject mean per visit; $P = 0.07$ for CV_b versus overall subject mean). After taking the anti-logarithm the equations were: $CV_b = [(42.9/Lp(a)^{0.31}) * \exp((0.82)^2/2)]$ and $CV_a = [(12.2/$

$Lp(a)^{0.35}$) * exp((0.93)^2/2)]. Notable is that the slopes were comparable, whereas the intercept with the Y-axis was 3.5 times higher for CV_b compared to CV_a. Figure 1 illustrates the impact of changing CV_a respectively CV_b values across the Lp(a) concentration range on the Lp(a) test variability, analyzing one, three respectively five serial specimens. If at least three serial specimens are analyzed per individual the observed Lp(a) result is within ± 10% of the true value if the Lp(a) concentration is above 500 mg/l. Below 500 mg/l the test uncertainty runs up quickly, due to increasing biological and analytical CV's. If only one specimen is analyzed, the observed Lp(a) value is within ± 15-20% of the true value, even at Lp(a) levels larger than 500 mg/l. Below 500 mg/l the confidence limits increase even more dramatically. At 300 mg/l, an internationally recognized though arbitrarily defined cutpoint for Lp(a) (1), the confidence intervals range between

Table 1. Baseline Characteristics of the Study Subjects

Mean ± S.D. (median) or percentage	Healthy participants N = 54	Outpatients with Lp(a) > 300 mg/l N = 12
Male/female ratio (%)	50/50	42/58
Smoking behaviour:		
- non-smokers (%)	61	58
- ex-smokers (%)	20	33
- smokers (%)	19	8
Alcohol use, no/yes (%)	19/81	0/100
Regular physical exercise, no/yes (%)	46/54	67/33
Lipid lowering medication, no/yes (%)	100/0	17/83
Age (years)	32.7 ± 6.6	47.0 ± 13.8
Height (cm)	174.9 ± 8.7	169.0 ± 9.3
Weight (kg)	74.4 ± 12.4	73.5 ± 14.4
Body mass index (kg/m^2)	24.4 ± 3.9	25.6 ± 3.8
Ln Lp(a) (mg/l)[a]	4.491 ± 1.385 (4.411)	6.578 ± 0.590 (6.686)
Homocysteine (µmol/l)	11.55 ± 3.65 (10.78)	15.37 ± 8.35 (13.03)
Cysteine (µmol/l)	235 ± 36 (232)	260 ± 32 (257)
TAOS (mmol/l)	1.334 ± 0.082 (1.334)	1.245 ± 0.077 (1.231)

[a]: natural logarithm values for Lp(a)

Table 2. Overall within-run analytical and intra-and interindividual variation of Lp(a), homocysteine, cysteine and Total Anti-Oxidant Status in Healthy Subjects and in Stable Outpatients with hyper-Lp(a)-lipoproteinaemia.

Parameter	Analyte concentration range at first visit	Mean CV, %			CV_b, % Percentiles					Range		Contribution of mean CV_a to total test variation, %
		CV_a	CV_b	CV_g	10	25	50	75	90	Min.	Max.	
Healthy Subjects (N = 54)												
Lp(a), mg/l	2 - 1105	8.6	20.0	ND	1.9	6.6	10.8	23.4	30.7	0.0	58.7	8.9
Hcy, µmol/l	7.6 - 30.4	3.7	9.4	23.9	2.4	3.8	7.9	11.4	14.4	0.0	26.1	7.3
Cys, µmol/l	140 - 334	1.7	5.9	12.3	1.9	3.3	4.7	7.1	9.2	0.0	11.1	4.1
TAOS, mmol/l	1.174 - 1.498	4.3	2.8	4.5	-	-	-	-	-	-	-	97.8
Outpatients (N = 12)												
Lp(a), mg/l	201 - 1715	3.1	7.5	ND	1.3	3.8	6.1	9.5	13.9	1.1	14.8	8.3
Hcy, µmol/l	8.4 - 37.1	4.0	9.3	ND	3.1	5.7	6.3	9.9	17.0	2.9	17.9	8.8
Cys, µmol/l	214 - 335	2.0	6.0	12.9	1.1	2.9	5.5	8.2	9.0	0.0	9.0	5.4
TAOS, mmol/l	1.149 - 1.416	4.3	1.0	3.5	-	-	-	-	-	-	-	-

Coefficients of variation were calculated from duplicate measurements at four bi-weekly visits, after logarithmic transformation of the analyte concentrations. Lp(a), Hcy and Cys analyses being performed in one run at the end of the collection period, and TAOS analyses, for reasons of limited stability, being performed at the day of sample collection, i.e. in 4 different runs. For TAOS the CV_a represents the between-day CV of the assay, calculated from the TAOS control material. For TAOS only average CV_b and CV_g were estimated, by correcting for the average between-day variance of the TAOS control.

CV_a: analytical coefficient of variation; CV_b: intra-individual biological coefficient of variation; CV_g: interindividual biological coefficient of variation, Lp(a): lipoprotein(a); Hcy: homocysteine, Cys: cysteine, TAOS: total anti-oxidant status, ND: no data ($\sigma > 0.40$).

± 21%, ± 12% and ± 9%, respectively, depending on whether one, three or five serial specimens were analyzed.

DISCUSSION

This study reports comprehensive data about biological variability and certain indices derived from it for Lp(a), Hcy, Cys and TAOS. So far, data on biological variation are absent for Hcy, Cys and TAOS, and conflicting for Lp(a) (13-19). According to Fraser, estimates of within-subject biological variation should be independent of [1]) the population examined, [2]) the age and the number of the

Table 3. Indices derived from biological variation data for Lp(a), homocysteine, cysteine and Total Anti-Oxidant Status in Healthy Subjects and in Stable Outpatients with hyper-Lp(a)-lipoproteinaemia.

Parameter	AG CV_a (%)	AG bias (%)	Index of individuality	RCV (%)	NS (± 5%)	NS (± 10%)
Healthy Subjects (N = 54)						
Lp(a), mg/l	(10.0)	ND	(0.15)	(60)	(73)	(18)
Hcy, µmol/l	4.7	6.4	0.42	28	16	4
Cys, µmol/l	2.9	3.4	0.50	17	6	1
TAOS, mmol/l	1.4	1.3	0.62	9	5	1
Outpatients (N = 12)						
Lp(a), mg/l	(3.8)	ND	NA	(23)	(10)	(3)
Hcy, µmol/l	4.6	ND	NA	28	16	4
Cys, µmol/l	3.0	3.6	NA	18	6	2
TAOS, mmol/l	0.5	0.9	NA	5	4	1

Average indices were calculated based on average CV_a, CV_b and CV_g values, using the formulas presented in Statistics. Such an approach is valid for Hcy, Cys and TAOS, but not for Lp(a) due to concentration dependency of CV_a and CV_b. Therefore, average indices derived for Lp(a) are placed between brackets and should be interpreted with caution.
AG CV_a: analytical goal for imprecision; AG bias: analytical goal for bias; RCV: reference change value; NS (± 5%) respectively NS (± 10%): number of serial specimens required to reduce uncertainty to within ± 5% respectively ± 10% of the true value. Lp(a): lipoprotein(a); Hcy: homocysteine; Cys: cysteine; TAOS: Total Anti-Oxidant Status, ND: no data; NA: not applicable.

subjects studied, 3) the locale where the study was conducted, 4) the health of the subjects, 5) the time scale and 6) the analytical variability of the methodology used (11-12). However, as a result of the wide variation of published average CV_b estimates for Lp(a), Fraser's points of departures do not seem to be universally valid across different Lp(a) populations (18-19). Therefore it was decided to investigate biological variation both in a large randomized sample of healthy Caucasians and in a selected hyper-Lp(a)-lipoproteinaemic patient group, enabling to study eventual heterogeneity of within-subject variation.

In healthy Caucasians average CV_b's were 20.0% for Lp(a), 9.4% for Hcy, 5.9% for Cys and 2.8% for TAOS (table 2), mean CV_b's being similar in men and women for all analytes studied. In the outpatient group comparable CV_b estimates were found for Hcy, Cys and TAOS but not for Lp(a) (7.5% in outpatients versus 20.0% in healthy controls). Moreover, in accordance with Marcovina et al. (18-19) a systematic inverse relation was demonstrated between CV_a respectively CV_b and Lp(a) level. For the other analytes, no concentration dependency was found. Our data 1) illustrate the inadequacy of using average CV_b and CV_a values for Lp(a); 2) explain the controversy in literature regarding intra-individual biological variability of Lp(a) (13-17) and corroborate the findings of Marcovina et al. (18-19); and c) underscore the fact that the intra-individual biological variability of Lp(a) is greater than previously believed, especially in the low concentration range (1).

Data on interindividual biological variation (CV_g) are presented if meaningful (table 2). In general, interperson variability is determined by age, sex, diet and genetics. In case of Lp(a) interperson variability is mainly determined by genetics (1), whereas diet and genetics may influence plasma Hcy levels (2, 8, 23). Because the outpatient group represents a selected high risk group, including individuals with both elevated Lp(a) and Hcy levels (table 1), CV_g data can not be extrapolated from one study to another. Consequently, CV_g data are specific for the population studied and therefore are of limited value.

Several indices have been derived from the biological variability study. First, analytical goals for imprecision, having been the subject of a variety of approaches (11-12), were calculated. In this study the approach of Harris et al. (26) was used, which states that maximum allowable analytical imprecision should be $\leq 1/2\ CV_b$.

After all, the more closely the parameter is controlled by homeostatic mechanisms, the more stringent the analytical requirements should be. Average goals for CV_a were met for Hcy and Cys, but not for TAOS (table 3), average goals for CV_a being similar for Hcy and Cys in either study group. In contrast, for Lp(a) a single mean CV_a goal was not ubiquitously valid due to concentration dependency of the CV_b estimates. From the estimated regression lines that described the relationship between CV_a respectively CV_b and Lp(a) concentrations a fairly constant, 3.5-fold difference between CV_a and CV_b could be demonstrated across the entire Lp(a) concentration range, signifying that the imprecision of the Lp(a) Biopool kit used is adequate at all Lp(a) levels. Therefore, we disagree with Pagani and Panteghini (15, 21) who claimed that in practice the analytical goal for Lp(a) cannot be achieved with current assays. Notwithstanding the lack of international Lp(a) standardization and the fact that a different ELISA was used by these authors, the discrepancy with their data can be explained by the inappropriate use of average CV_b and CV_a estimates. Finally, although the CV_a goals were met for Lp(a) and Hcy (and Cys), one may object that the assays were performed under optimal conditions of variance as between-day variation was omitted. Yet, between-day analytical CV's from routine practice in our laboratory of 7.1% at 67 mg/l, 4.2% at 213 mg/l and 5.1% at 379 mg/l were achieved for Lp(a), whereas between-day CV's of 4.0% at 19.5 µmol/l and 3.2% at 52.2 µmol/l were obtained for Hcy, confirming the practical attainment of the analytical goals.

Secondly, desirable goals for average analytical bias were calculated (table 3) (11-12). Documenting bias of routine assays necessitates the development of reference and/or definitive methods and standardization programs for the analytes studied. So far, no international standardization has been reached.

Thirdly, the utility of conventional population-based reference values was assessed by calculating an *index of individuality* in the healthy subject group (table 3). The index gives a philosophical view on the interpretation of analyte data measured in healthy individuals and pathological changes in relation to reference intervals (12). If the index is < 0.6, the use of reference intervals is of limited value in the detection of unusual individual results; if the index is > 1.4, reference values are of significant utility. In this study, all analytes had marked individuality, demonstrating that the use of population based reference values is inadequate for

their interpretation. This favours the adoption of cutpoints based upon relative risk of coronary artery disease.

Fourthly, biological, in addition to analytical variation data, are also used for the critical evaluation of the significance of changes in results obtained from analysis of serial specimens (12, 20). To interpret serial results objectively it is necessary to know the change that must occur before significance can be claimed. This *reference change value* (RCV) depends on both analytical and intra-individual biological variation, and holds only if all individuals have the same within-subject variation and if the analytical variation is constant across the concentration range. For Hcy, Cys and TAOS average RCV's for detecting significant changes in 50% of the individuals are presented in table 3. In view of the concentration dependency of CV_a and CV_b for Lp(a), i.e. the observed variance reduction with increasing Lp(a) levels, mean CV_a and CV_b can not be the basis for calculating the critical difference that is generally applicable in all individuals. So far, critical differences for Lp(a) were reported by one group (15), the RCV being estimated as 29%, based on a mean CV_a of 7.4% and a mean CV_b of 7.6%. According to our findings critical differences for Lp(a) should be calculated based on individual CV_a and CV_b values.

Finally, from the variation data obtained in this study one can estimate the number of specimens required to determine the individual's true homeostatic set-point value (21). Again, simple recommendations regarding the average number of specimens needed can be made for Hcy, Cys and TAOS (table 3) while such an approach is not valid for Lp(a) (figure 1). However, in the light of the enormous interindividual concentration differences we agree with Marcovina et al. (18-19) that the CV_b of Lp(a) is not likely to be an important contributor to the misclassification of an individual's risk, unless the value is near the cutpoint of enhanced coronary artery disease risk.

Overall, the findings in the present study demonstrate that the understanding of the magnitude of the physiological variations that occur in Lp(a), Hcy, Cys and TAOS concentrations in serum or plasma is indispensable for proper use of these laboratory data for risk classification of patients with coronary artery disease. In essence, we demonstrated that average CV_a and CV_b estimates and mean derived indices are valid for Hcy, Cys and TAOS, whereas individual values should be used

for Lp(a). Secondly, the analytical performance of the Lp(a), Hcy and Cys assays used is acceptable taking into consideration the biological variation of these parameters, whereas the performance of the TAOS assay was insufficient.

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APPENDIX

A measured value X is supposed to have an expectation E(X) = T, the unknown true value of X, and a constant coefficient of variation CV(X). A simple model that implies these conditions is: $X = T * U$, with U a random error term with mean E(U) = 1 and standard deviation SD(U) = σ. This standard deviation is assumed to be small enough (e.g. $\sigma < 0.4$) that the probability of negative or zero values of U must be zero. No further assumptions about the distribution of U are made.

The coefficient of variation of X equals: CV(X) = SD(X) / E(X) = [(T * σ) / T] = σ, which is a constant single parameter. An approximate estimator for σ can be obtained by first taking the natural logarithm: ln X = ln T + ln U, and then applying the so-called "delta method" (27) to the variance of ln U:

$$\text{var}(\ln U) \approx \left(\frac{d(\ln U)}{dU}\right)^2_{U=1} * \text{var}(U)$$

so that var (ln U) $\approx \sigma^2$, because $(d(\ln U)/ dU)^2$ developed for E(U) = 1, equals unity (27). Hence, CV(X) can approximately be estimated by SD (ln X) within the same subject or within the same specimen under very general conditions.

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Chapter 4

SERUM LP(a) LEVELS IN AFRICAN ABORIGINAL PYGMIES AND BANTUS, COMPARED TO CAUCASIAN AND ASIAN POPULATION SAMPLES

Based upon:

Serum Lipoprotein(a) levels in Racially Different Populations.
Am J Epidemiol 1992; 136: 441-9.

Serum Lp(a) levels in African Aboriginal Pygmies and Bantus,
compared to Caucasian and Asian Population Samples.
J Clin Epidemiol; accepted

ABSTRACT

Serum lipoprotein(a) (Lp(a)) and its correlates were studied in African Aboriginal Pygmies (N = 146) and Bantus (N = 208) from Cameroon. Geometric mean Lp(a) levels were 274 and 289 mg/l in Bantu males and females respectively, and 220 and 299 mg/l in Pygmy males and females, the gender difference being significant in Pygmies (P = 0.024). In Pygmies 41% and 52% of the males respectively females had Lp(a) levels above 300 mg/l, compared to 47% and 55% in Bantus. Overall, Lp(a) levels did not significantly differ between Pygmies and Bantus, and did not correlate with age, body mass index (BMI), systolic and diastolic blood pressure. Compared to healthy Asian and Caucasian population samples, age- and BMI-adjusted geometric Lp(a) means were 2.3 to 5.0-fold higher in Pygmy and Bantu males, and 2.9 to 3.6-fold higher in Pygmy and Bantu females ($P \leq 0.05$). Across the population samples studied ethnicity predicted 12% and 17% of serum Lp(a) variance in males respectively females.

INTRODUCTION

Lipoprotein(a) (Lp(a)) is a cholesterol-rich complex lipoprotein macromolecule, consisting of a Low Density Lipoprotein (LDL)-like particle attached by a disulfide bond to a unique glycoprotein, i.e. apolipoprotein(a) (apo(a)) (1). Apo(a) is unique because of its structural analogy with plasminogen (2), and its size and functional polymorphism (3, 4). Most studies reported that serum Lp(a) levels are mainly genetically determined -variation at the apo(a) locus contributing to this heritability- and are hardly influenced by lifestyle, diet or drugs (1, 5-9). On the other hand, a few studies have shown that the serum Lp(a) level is correlated with other variables, such as age (10-12), sex (11), smoking (10), blood pressure (13), waist-to-hip ratio (10), impaired glucose tolerance (14-16), proteinuria (17), fibrinogen levels (13, 18, 19) and use of medication (20, 21).

So far, and despite the fact that Lp(a) had an attraction upon researchers like a

"femme fatale", its physiological function is, 34 years after its discovery, still not unraveled. From experimental observations it is hypothesized that the cholesterol-rich Lp(a) lipoprotein particle probably exports cholesterol out of the liver providing a continuous peripheral supply of such Lp(a)-cholesterol, which is independent from diet and other factors and which is reflected by the highly stable Lp(a) concentration over long-time periods (22). The most likely targets of Lp(a)-cholesterol are endocrine organs with high steroid hormone production. More is known about its pathophysiogical role. First, numerous epidemiological and clinical studies, mainly in Caucasians, demonstrated that Lp(a) was an independent risk factor for coronary heart disease (CHD) (6, 23), stroke (24) and preclinical atherosclerosis (25). Second, Rath et al. (26) demonstrated Lp(a) depositions in the vessel wall of grafted arteries of coronary bypass patients, and found an association with serum Lp(a) levels. Third, it is postulated that elevated Lp(a) levels might interfere with fibrinolysis, due to its structural analogy with plasminogen (27, 28). Therefore, an elevated Lp(a) level is considered to be a risk factor for both atherogenesis and thrombogenesis, at least in Caucasians (1, 6, 23).

Lipoprotein(a) is an inherited CHD risk factor which, to our knowledge, has not been investigated in Aboriginal African Pygmies yet (1, 6). Besides, knowledge of the genetic susceptibility of traditional populations may be valuable in order to anticipate *future* cardiovascular risks, especially if these populations would move from traditional lifestyles to more sedentary lifestyles (29-31). After all, considerable excess of coronary heart disease mortality and greatly increased prevalence of type 2 diabetes mellitus have been reported among expatriate 'Westernized' Asian communities (29, 30) as well as in Aborigines from Western Australian in transition to urbanization and Westernization (31). As only a few hunter-gatherer populations remain throughout the World, African Pygmies being one of them (32-34), and as urbanization may occur in the future, it was decided to investigate the genetic susceptibility, i.e. serum Lp(a) levels, and lifestyle factors of traditional African Aboriginal Pygmy samples from South-West Cameroon, compared to Bantu samples from the same region. Because of the lack of international Lp(a) standardization (35), and to enable relative comparability across populations, serum Lp(a) levels in Pygmies and Bantus were compared to those of Caucasian and Asian population samples.

The objectives of this cross-sectional study were, firstly, to provide descriptive data upon serum Lp(a) levels in population-based samples of apparently healthy African Aboriginal Pygmies and neighboring Bantus, and to describe its relationships to other cardiovascular risk factors. Secondly, to compare serum Lp(a) concentrations in African Pygmies and Bantus to those in randomized Belgian, Hungarian and Philippine population samples, displaying varying serum cholesterol levels and prevalences of CHD. Finally, to compare serum Lp(a) and serum cholesterol levels in Pygmies and Bantus to those in healthy respectively diseased Caucasians with over CHD.

MATERIALS AND METHODS

Study populations

This study was part of a wider survey of health in Pygmy and Bantu populations in Cameroon, and was carried out in January through February 1994, and in May through June 1994 (33, 34). Non fasting sera from Bantus and aboriginal Pygmies living in South-West Cameroon were obtained as described by Kesteloot et al. (33, 34). Pygmies examined live in the tropical forests 3° to 4° north of the Equator in small communities of 60 to 100 individuals, including children. Two distinct Pygmy populations were investigated: one in the Mecasse region in the Dja reservation, consisting of three distinct communities spreaded out over a 30 km distance. The second Pygmy population was living in the region of Lolodorf, at a distance of about 80 km. The participation rate was estimated to be about 100% in three of the four Pygmy communities, whereas it was only 50% in the fourth Pygmy community due to a conflict of authority of part of the community to the tribal chief. Besides, three communities of neighboring Bantus were examined: one living in close contact with the Pygmies in the Mecasse region, one living in the Lolodorf region, and a separate community living in the village of Bengbis. In the Bantu population participation was essentially on a first-come, first-served basis due to time and blood sample collecting material restraints. Many more Bantus volunteered to participate but could not be accomodated. Consequently, it was impossible to examine a random sample of the population.

The Pygmies studied are pure hunter-gatherers, whereas the Bantus are agriculturers cultivating corn, manioc and plantain. Since Pygmies do not know their age, the latter was estimated with the help of a Bantu teacher, by referral to important past events. After specimen collection and clot separation with a hand centrifuge, sera were frozen within 8 hours and stored for maximum one month at -20°C, i.e. during the expedition period in Cameroon. Thereafter sera were shipped to Belgium and the Netherlands, stored at -70°C, and analyzed for serum Lp(a) within 8 months after sample collection.

Serum Lp(a) levels in Pygmies and Bantus were compared with fasting serum Lp(a) levels in Caucasian and Asian population samples (Belgians, N = 905; Hungarians, N = 400 and Philippines, N = 195). All Caucasian and Asian specimens investigated were from unrelated, apparently healthy and randomly selected subjects. Firstly, Belgian sera were obtained from age- and sex-matched 20 to 39 year old employees from each Belgian province. Recruitment was done in the context of a cross-sectional study investigating the contribution of environmental and genetically determined cardiovascular risk factors to regional serum cholesterol and cardiovascular mortality differences in Belgium (chapter 6 of this thesis). To that, employees of different socio-economic status were invited to participate in this study, prior to a scheduled medical check-up. Previous randomization and subsequent invitation, and medical check-up were conducted by the Flemings IDEWE and the Wallonian CeSI, two 'Centres de Services Interenterprises-Médecine du Travail'. Informed consent was obtained from all participants. Individual questionnaires were distributed during the medical check-up; volunteers were invited to fill in their identity, home address, nationality, and to indicate their smoking, drinking, dietary and life-style habits, education and profession, use of hormonal contraception, consumption of lipid lowering or other medication, etc.... The filled-in questionnaire was checked by the physician who did the medical check-up, and completed if necessary. Besides, height and weight, as well as blood pressure were measured by the physician. As the Belgian study group is well documented, it was used as a reference population sample in this comparative Lp(a) study. Randomized Hungarian samples were obtained via the National Institute of Food Hygiene and Nutrition, Budapest, Hungary (36), and were from adults living in Budapest, and in three different Hungarian regions, i.e. Fejér,

Békés, Komárom-Esztergom. Philippine sera were from free living individuals on Cebu Island and were randomized and obtained via the Philippine Heart Center in Quezon City.

Sera from diseased Caucasians were from unrelated Belgian male patients who underwent elected coronary artery bypass grafting (CABG) (N = 100) as described previously (37), or presented with an acute myocardial infarction (N = 50) as diagnosed by WHO criteria. In the diseased group serum Lp(a) was determined the day before bypass in the CABG patient group, and within 6 hours after onset of chest pain in the AMI patient group. All CABG and AMI patients included were free of insulin-dependent diabetes, renal insufficiency, cerebrovascular accidents and liver disease. Notable is that in the CABG study group, only patients with preoperative serum cholesterol values between 4.66 and 7.24 mmol/l were included (37). These highly selected, diseased Caucasian males were included to enable comparison of serum Lp(a) values in free-living Pygmies and Bantus and in healthy Caucasians to those in diseased Caucasians, using the same Lp(a) methodology.

Laboratory methods

Serum Lp(a) was determined using an anti-apo(a) polyclonal capture ELISA from Biopool (TintElize lipoprotein(a), Cat. No. 610220; Biopool AB, Umeå, Sweden). The capture polyclonal anti-apo(a) antibody does not cross-react with plasminogen up to 1000 mg/l. Lp(a) determinations in all population samples were performed using the TintElize Biopool kit, the assay being calibrated to total Lp(a) mass. Considering the lability of the Lp(a) lipoprotein particle (35), a maximum serum storage time of one year at -70°C was respected. At our experimental conditions, it was documented by means of frozen human serum pools that storage did not affect Lp(a) levels up to two years.

Cholesterol was determined enzymatically using CHOD-PAP reagents. Lipid analyses in the Belgian and Philippine population samples were performed at the University Hospital Leuven, Belgium, while all other determinations were performed at the Lipid Reference Laboratory of the University Hospital Rotterdam, the Netherlands. The Lipid Reference Laboratory (LRL) Rotterdam maintains total

cholesterol standardization through the Lipid Standardization Panel of the Centers for Disease Control (CDC)- National Heart Lung and Blood Institute, Atlanta, Georgia, U.S.A. The LRL Rotterdam is also a permanent member of the Cholesterol Reference Method Laboratory Network (CRMLN) established and coordinated by CDC (38). The cholesterol values determined in the Leuven University Hospital were also found to be traceable to the Abell-Kendall reference method: in subsets of the samples it was documented that the mean average cholesterol bias was in accordance with current NCEP performance guidelines (bias $\leq 3\%$ versus the Abell-Kendall reference method) (39).

Statistical methods

Basic statistical analysis, analysis of variance (ANOVA) and Pearson correlation analyses were performed using the SPSS/PC+ package (version 5.0.2). Lp(a) and cholesterol data were logarithmically (natural) transformed in all population samples, and geometric means were calculated. Gender differences within populations were evaluated using Student's T-test. Lp(a) differences among population samples were evaluated by one-way ANOVA in either gender. The multiple range test of Student-Newman-Keuls was used for multiple-comparison of sample means. Age and body mass index (BMI)- adjustments of geometric Lp(a) and cholesterol means were performed by means of multiple linear regression analysis (MLR). BMI was defined as weight (in kg) / height^2 (in m^2). On the aggregated population level a weighted least squares MLR analysis was performed to investigate whether mean BMI and mean age could predict male respectively female mean serum cholesterol and Lp(a) levels across populations. A statistical significance level of $\alpha = 0.05$ was adopted.

RESULTS

Table 1 displays mean ($\pm$ S.D.) age, BMI and cholesterol per gender for the population samples under study. Significant age, BMI and cholesterol differences existed among all five population samples (ANOVA, $P < 0.0001$). Table 2 presents the unadjusted serum Lp(a) levels in African Pygmies and Bantus, compared to

Caucasian and Asian population samples. Geometric mean Lp(a) levels were 274 and 289 mg/l in respectively Bantu males (N = 93) and females (N = 115), and 220 and 299 mg/l in Pygmy males (N = 63) and females (N = 83). The gender difference was significant in Pygmies (P = 0.024) and not in Bantus. Likewise, Philippine females had higher Lp(a) values than males (P = 0.001). Lp(a) frequency distributions in Pygmy and Bantu were less skewed to the low concentration end, compared to Caucasian and Asian distributions (data not shown). In Pygmy males and females respectively 41 and 52% of the participants had Lp(a) levels above 300 mg/l, compared to 47 and 55% of the Bantus. Serum Lp(a) levels did not significantly differ between Pygmies and Bantus. Multiple-comparison of LnLp(a) means demonstrated that Lp(a) levels in Pygmies and Bantus were in either gender significantly higher compared to those measured in any other population sample (SNK-test; $P \leq 0.05$).

Overall upper reference ranges for men and women combined, defined as 75th percentiles, were lowest in Asians (162 mg/l), intermediate in Caucasians (204 mg/l), and highest in African Pygmies and Bantus (479 mg/l). The 75th percentile in the diseased Caucasian group was 349 mg/l (data not shown).

Lp(a) levels in Bantu males and females, and in Pygmy females were found to correlate with total cholesterol, LDL-cholesterol (LDL-c) and apolipoprotein B (apo B) but not with age, BMI, systolic and diastolic blood pressure, HDL-cholesterol and apolipoprotein A-I (table 3). Serum cholesterol was associated with age in Bantu males (r = 0.25; P = 0.02), while borderline significant in Pygmy males (r = 0.22; P = 0.08). In contrast, serum cholesterol was not significantly associated with age in Pygmy respectively Bantu females (data not shown).

Table 4 depicts age- and BMI-adjusted geometric Lp(a) and cholesterol means per gender for the population samples studied. P-values are given versus the Belgian population sample, the latter being the reference population sample in the MLR analysis. Pygmy and Bantu concentrations differed significantly from Belgian levels ($P < 0.0001$), Pygmies and Bantus having the lowest serum cholesterol, and the highest serum Lp(a) levels among the population samples studied. Philippine males had Lp(a) levels that were significantly lower compared to those in Belgian males, while Hungarian females displayed significantly higher Lp(a) levels compared to Belgian females. Both Hungarian males and females had

significantly higher cholesterol values compared to Belgians. Age and BMI-adjusted geometric Lp(a) means were 2.8 and 4.3-fold higher in male respectively female Pygmies compared to Belgian males and females, and 3.5 and 3.9-fold higher in male and female Bantus ($P < 0.0001$). Finally, age- and BMI-adjusted serum Lp(a) levels in diseased Belgian males were significantly higher than in apparently healthy Belgian males: 131 mg/l in CABG patients and 114 mg/l in AMI patients, compared to 72 mg/l in healthy male participants.

Multiple linear regression (MLR) analysis pointed out that BMI, age and ethnicity explained 12 and 17% of serum Lp(a) variance in respectively males and females, compared to 60 and 51% of serum cholesterol variance. BMI and age alone explained maximum 3% of Lp(a) variance. After adjusting for ethnicity BMI remained a borderline significant predictor in either gender (BMI in males: $P = 0.08$; BMI in females: $P = 0.09$), in contrast with age. Moreover, table 5 demonstrates that at the aggregated level, i.e. across the population samples studied, overall mean BMI was negatively correlated with overall mean Lp(a) levels ($P = 0.05$) if men and women were grouped.

DISCUSSION

The Pygmies studied are pure hunter-gatherers and are the Aborigines in Africa south of the Sahara. They were over-run by the Bantu who, from 1200-1400 A.D. gradually spread from the upper Nile Valley and the region of Lake Chad southward and westward over the continent (32). Up to now, Pygmies live in isolated groups among the Bantus and along the Equator. As described by Kesteloot et al. (33, 40), the characteristic diminutive Pygmy stature together with the consistent Pygmy body measurements, lifestyle factors and serum lipid levels compared to previous reports (32), are a reflection of the homogeneity of the Pygmy race and of the fact that a representative sample was taken.

The Pygmy sample investigated, though partly consisting of related individuals as children were included, was derived from four different communities living in and nearby the Dja reservation in South-West Cameroon. Due to the high participation rate the Pygmy sample is a good representation of the current Pygmy

cohabitation in those specific regions. However, the representativeness for the total African Pygmy population is unknown. The same holds for the Bantu sample investigated: volunteers from three neighboring communities, mainly entered on a first-come, first-served basis and partly containing relatives, were studied. Notwithstanding the fact that the Bantu population sample was not randomly gathered, it reflects the Bantu cultures from the Mecasse and the Lolodorf region, and the city of Bengbis.

In this study we focused upon the genetically determined Lp(a) lipoprotein risk factor, in relation to the modifiable serum cholesterol risk factor and to ethnicity. Notable features of our study are the exceptionally low mean serum cholesterol levels in Pygmies, the absent or minor cholesterol increase with estimated age, as well as the two- to five-fold higher mean Lp(a) levels (tables 2 and 4) compared to Asians and Caucasians. Bantus had the same mean Lp(a) level as Pygmies, while age- and BMI-adjusted serum cholesterol levels were approximately 5% higher both in males and females, and increased significantly with age in Bantu males. From table 2 it can be seen that 41% to 55% of Pygmies and Bantus had serum Lp(a) levels above 300 mg/l, the universally accepted upper reference limit in Caucasians. As presence of CHD is very unlikely in African Pygmies (32) and Bantus, the high serum Lp(a) concentrations samples must either be counteracted by other factors, or be in itself an insufficient cause for developing atherosclerosis. Consequently, we agree with others (41, 42) that for identifying subjects at increased risk of coronary heart disease, ethnicity-related cutoff values, based on 75th percentiles, should be used for Lp(a).

Using MLR, serum Lp(a) variances could be explained up to 12% and 17% in males and females, respectively across the healthy population samples studied, and were predicted exclusively by ethnicity and barely by age or BMI. However, at the aggregated level mean BMI was negatively correlated with mean Lp(a) if men and women were combined, while no significant correlation was observed with mean age (table 5). Finally, the tight univariate correlations demonstrated with total cholesterol, apo B and LDL-c in Pygmies and Bantus could be explained by the high relative percentage of Lp(a)-cholesterol and Lp(a)-apo B compared to Asians and Caucasians (table 3).

The large differences in serum Lp(a) between African Pygmies and Bantus

Table 1. Age, body mass index and serum cholesterol levels in African Pygmies and Bantus, stratified by sex, compared to Asian and Caucasian population samples

MEN	N	Age (years) Mean ± S.D.	BMI (kg/m^2) Mean ± S.D.	Cholesterol (mmol/l) Mean ± S.D.
Apparently healthy population samples:				
Africans				
Pygmies	63	33.8 ± 16.6	0.0 ± 2.4	2.885 ± 0.693
Bantus	93	41.2 ± 20.0	20.6 ± 2.6	3.119 ± 0.725
Caucasians				
Belgians	413	31.4 ± 5.2	24.1 ± 3.3	5.279 ± 1.073
Hungarians	200	42.2 ± 12.3	26.8 ± 3.9	6.017 ± 1.223
Asians				
Philippines	96	43.7 ± 10.7	23.8 ± 3.4	5.301 ± 0.981
Diseased Caucasians (Belgians):				
CABG patients	100	53.8 ± 4.8	25.8 ± 2.6	5.589 ± 0.891
AMI patients	50	59.5 ± 11.6	25.5 ± 3.1	5.551 ± 1.025
WOMEN	N	Age (years) Mean ± S.D.	BMI (kg/m^2) Mean ± S.D.	Cholesterol (mmol/l) Mean ± S.D.
Apparently healthy population samples:				
Africans				
Pygmies	83	29.4 ± 15.3	19.9 ± 3.0	3.174 ± 0.673
Bantus	115	44.8 ± 17.4	20.9 ± 2.9	3.559 ± 0.714
Caucasians				
Belgians	492	30.2 ± 5.3	22.2 ± 3.1	5.117 ± 0.971
Hungarians	200	40.3 ± 10.5	26.0 ± 5.6	5.702 ± 1.162
Asians				
Philippines	99	46.9 ± 10.4	23.1 ± 3.9	5.433 ± 1.155

AMI: acute myocardial infarction; CABG: coronary artery bypass grafting.

compared to Asians (43, 44) and Caucasians (10-13, 41, 43, 45, 46), and the absence of association with many other variables except serum lipids, are consistent with previous reports which state that Lp(a) levels are largely genetically determined (1, 5, 6). Also, the height and distribution of the serum Lp(a) levels in

Table 2. Serum lipoprotein(a) levels in African Pygmies and Bantus, stratified by sex, compared with randomly selected Caucasian and Asian population samples

MEN			Lipoprotein(a) mass (mg/l) Percentile							> 300 mg/l
Population	N	Geometric mean	5	10	25	50	75	90	95	(%)
Apparently healthy population samples:										
Africans										
Pygmies	63	220*	37	62	141	234	424	530	758	41
Bantus	93	274	50	78	180	259	525	826	972	47
Caucasians										
Belgians	413	70	6	10	30	71	216	455	566	20
Hungarians	200	85	11	17	39	73	249	464	645	21
Asians										
Philippines	96	52*	9	12	24	52	127	269	323	7
Diseased Caucasians (Belgians):										
CABG pts.	100	130	10	17	61	135	409	576	824	34
AMI pts.	50	113	14	35	58	97	249	599	651	19

WOMEN			Lipoprotein(a) mass (mg/l) Percentile							> 300 mg/l
Population	N	Geometric mean	5	10	25	50	75	90	95	(%)
Apparently healthy population samples:										
Africans										
Pygmies	83	299	86	125	192	331	557	669	928	52
Bantus	115	289	58	74	179	317	538	888	1111	55
Caucasians										
Belgians	492	67	7	11	27	67	186	411	553	16
Hungarians	200	86	12	19	34	74	237	566	776	22
Asians										
Philippines	99	86	18	21	44	88	176	294	399	9

*$P \leq 0.05$ for gender differences within population samples (Student's T-test).
AMI: acute myocardial infarction; CABG: coronary artery bypass grafting.

Pygmies and Bantus seem to be comparable or even higher than those described in Nigerians (43), Congolese (47), Sudanese (48) and American blacks (19, 25, 49-53).

Table 3. Pearson correlations of LnLp(a) with anthropometric data and serum lipids in African Pygmies and Bantus, with respect to gender

Variable	Bantu males N = 92	Bantu females N = 113	Pygmy males N = 63	Pygmy females N = 83
Age	-0.06	0.06	-0.10	0.16
BMI	0.12	-0.15	0.06	0.10
SBP	-0.07	-0.09	-0.01	0.09
DBP	-0.06	0.15	-0.04	0.02
Heart rate	-0.03	-0.04	0.13	0.08
Apo A-I	0.11	-0.00	-0.00	-0.05
HDL-c	0.06	-0.02	-0.04	0.12
Apo B	0.38***	0.31**	0.19	0.31**
LDL-c	0.50***	0.39***	0.03	0.35**
Cholesterol	0.35***	0.28**	0.06	0.28*
Triglycerides	-0.26*	-0.02	0.13	-0.13

*P ≤ 0.05; **: P ≤ 0.01; ***: P ≤ 0.001

Apo A-I: apolipoprotein A-I; apo B: apolipoprotein B; BMI: body mass index; HDL-c: HDL- cholesterol; LDL-c: LDL-cholesterol; DBP: diastolic blood pressure; SBP: systolic blood pressure.

The high Lp(a) levels in blacks, without corresponding high prevalence of CHD, suggest that race and gender differences in apo(a) phenotypes, hemostatic activity or other unrelated factors may contribute to this paradox. However, from twin studies it became clear that neither behavioral or environmental correlates, nor variation in the apo(a) size phenotype appeared to explain the higher mean Lp(a) levels among blacks compared to whites (7). Rather these findings corroborate the hypothesis that Lp(a) is a continuous supplier of liver cholesterol to peripheral endocrine organs independent of triglycerides or dietary cholesterol intake, being preferentially preserved during human evolution in black African populations of which the food supply was not as regulated as that of Caucasian and Asian populations. In contrast, in civilized human populations Lp(a) may have lost its phylogenetical importance since other cholesterol-rich lipoproteins, notably LDL, are highly abundant. Moreover, potential adverse effects of high Lp(a) levels should become overt especially in these civilized populations. This may be in line with the significant shift-to-the-right of the Lp(a) distributions (table 2) and of the

Table 4. Age- and BMI-adjusted geometric mean values of serum lipoprotein(a) and serum cholesterol in African Pygmies and Bantus, stratified by sex, compared with randomly selected Caucasian and Asian population samples

Men Population	N	Cholesterol (mmol/l)	P-value versus Belgian males	Lipoprotein(a) (mg/l)	P-value versus Belgian males
Apparently healthy population samples:					
Africans					
Pygmies	63	3.017	< 0.0001	204	< 0.0001
Bantus	93	3.159	< 0.0001	253	< 0.0001
Caucasians					
Belgians	413	5.336	-	72	-
Hungarians	200	5.659	0.0012	88	NS
Asians					
Philippines	96	5.159	NS	51	0.0243
Diseased Caucasians (Belgians):					
CABG patients	100	5.159	NS	131	0.0003
AMI patients	50	4.895	0.0164	114	0.0545

Women Population	N	Cholesterol (mmol/l)	P-value versus Belgian females	Lipoprotein(a) (mg/l)	P-value versus Belgian females
Apparently healthy population samples:					
Africans					
Pygmies	83	3.241	< 0.0001	289	< 0.0001
Bantus	115	3.445	< 0.0001	264	< 0.0001
Caucasians					
Belgians	492	5.104	-	67	-
Hungarians	200	5.296	0.0360	90	0.0129
Asians					
Philippines	99	5.111	NS	81	NS

AMI: acute myocardial infarction; CABG: coronary artery bypass grafting; NS: not significant.

geometric means (table 4) in Caucasian CABG respectively AMI patients compared to healthy Caucasians, notwithstanding similar (CABG group) or even

Table 5. Weighted least squares multiple linear regression analysis in aggregated Caucasian, Asian and African population samples (N = 5), stratified by sex, with mean serum lipoprotein(a) and mean serum cholesterol as dependent variables, and with mean age and mean BMI as independent variables

Dependent variable	Predictors	ß-coefficient	S.E.	P-value
Men				
Mean Ln Lp(a)	mean BMI (kg/m^2)	-0.1873	0.1174	NS
(Ln mg/l)	mean age (yr)	0.0316	0.0447	NS
	constant	7.8378	2.9511	NS
Mean Ln chol.	mean BMI (kg/m^2)	0.1148	0.0250	0.04
(Ln mmol/l)	mean age (yr)	-0.0085	0.0095	NS
	constant	-0.8720	0.6284	NS
Women				
Mean Ln Lp(a)	mean BMI (kg/m^2)	-0.2071	0.1610	NS
(Ln mg/l)	mean age (yr)	0.0510	0.0445	NS
	constant	7.4687	3.4060	NS
Mean Ln chol.	mean BMI (kg/m^2)	0.0885	0.0445	NS
(Ln mmol/l)	mean age (yr)	-0.0087	0.0123	NS
	constant	-0.1421	0.9421	NS
Men and Women				
Mean Ln Lp(a)	sex (female)	-0.1272	0.3170	NS
(Ln mg/l)	mean BMI (kg/m^2)	-0.1954	0.0812	0.05
	mean age (yr)	0.0435	0.0258	NS
	constant	7.5961	1.9049	0.007

BMI: body mass index; Chol.: cholesterol; Ln: natural logarithm; NS: not significant; S.E.: standard error.

lower (AMI group) mean cholesterol levels in the diseased Caucasian groups (table 4). The shift-to-the-right is also in accordance with the findings of numerous other authors (6, 22). Although CHD is a multifactorial disease, the 1.6- respectively 1.8-fold higher adjusted Lp(a) means in diseased compared to healthy Caucasians may be elements aggravating the atherosclerosis risk of these subjects and contributing to their disease. In contrast, the high Lp(a) levels in Pygmies and

Bantus without concomittant high prevalence of CHD are difficult to understand. Yet, according to Maher et al. (54) Lp(a) is especially atherogenic in combination with high (LDL-) cholesterol levels or other acquired risk factors, e.g. due to Westernization (29-31), that can nurture Lp(a) into a more potent risk factor. In order to draw firm conclusions regarding the Lp(a) pathogenicity in Pygmies, Bantus and other population samples, prospective studies are warranted that document Lp(a) atherogenicity and its determinants in different populations.

Major strengths of this study are the fact that serum Lp(a) levels were obtained using one and the same method in all population samples, and were determined in frozen sera that were adequately stored for less than one year. Therefore, storage time and temperature did not confound the measured Lp(a) results (35). Limitations of this study are related to the cross-sectional study design and the relatively small sample sizes. Moreover, Pygmy and Bantu samples were not completely randomized, in contrast with the Asian and Caucasian samples. Consequently, extrapolation of these data to whole populations should be done with caution. Also, the diseased Caucasians represent two strongly selected groups that were, with the exception of BMI and age, not matched or adjusted for other cardiovascular risk factors.

We conclude that African Aboriginal Pygmies and Bantus have serum Lp(a) levels that are comparable, with adjusted Lp(a) means being up to fivefold higher compared to Asian and Caucasian means. In contrast, adjusted serum cholesterol means were 0.57 to 0.67-fold the Caucasian and Asian means. In the light of the virtual absence of CHD in Aboriginal African Pygmies and Bantus, high serum Lp(a) levels do not *seem* to be very deleterious in these population samples. Consequently, ethnicity-related upper reference values should be used for Lp(a). Also, longitudinal studies are warranted to determine the pathogenicity of Lp(a) in different populations with variable interrelationships between *nature* and *nurture*.

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Chapter 5

SERUM LIPOPROTEIN(a) LEVELS AND SEXUAL MATURATION: MODULATION BY SEX HORMONES?

Based upon:

Pubertal Serum Lipoprotein (a) and its Correlates in Belgian Schoolchildren.
Int J Epidemiol 1995; 24: 78-87.

ABSTRACT

Background

Serum lipoprotein (a) (Lp(a)) is an independent risk factor for premature coronary artery disease in Caucasians. Lp(a) serum levels generally remain fairly constant throughout an individual's life, but are presumably modulated by sex hormones. This study documents the distribution and correlates of serum Lp(a) during sexual maturation in Belgian children, and compares Lp(a) levels to those in Belgian adults.

Methods

Serum Lp(a), lipid and apolipoprotein A-I and B levels were determined cross-sectionally in 266 Belgian schoolchildren and adolescents, in relation to sexual maturation, anthropometrics and socio-economic status. Sexual maturity was scored according to Tanner's classification.

Results

Median Lp(a) levels were 82, 117, 110, 100 and 73 mg/l at the five subsequent genital development stages in boys (ANOVA, P = 0.816), and 73, 78, 204, 110 and 114 mg/l at the five breast development stages in girls (ANOVA, P = 0.087). Lp(a) distributions in boys and girls were skewed to the right, overall medians being 82 and 94 mg/l (P = 0.2537). The 90th and 95th percentiles were 515 respectively 712 mg/l. The geometric Lp(a) mean in children was significantly higher compared to that in 683 sex-matched Belgian adults (89 mg/l versus 69 mg/l, P = 0.006). Multiple linear regression pointed out that developmental age, chronologic age, body mass index and/or systolic blood pressure predicted serum lipid and apolipoproteins levels, but none of the Lp(a) variance.

Conclusion

Pubertal stage was not correlated with Lp(a) levels in Belgian schoolchildren, supporting the contention that serum Lp(a) is predominantly genetically controlled.

INTRODUCTION

Lipoprotein (a) is a major, independent risk factor for the development of premature coronary heart disease in Caucasians (1-3). Quantitative genetic studies provided evidence that serum Lp(a) levels are largely genetically determined (4,5). Yet, several investigators have demonstrated recently that Lp(a) levels are modulated by endogenous and exogenous sex hormones. In males with prostatic carcinoma Lp(a) levels were found to decrease upon estrogen treatment, and to increase after orchidectomy (7). In post-menopausal women placed on estrogen, progestagen, or estrogen-progestagen treatment, 16 to 50% decreases in Lp(a) levels occurred (8), and a 65% reduction in Lp(a) levels was reported after treatment with stanozolol, an anabolic steroid structurally related to testosterone (9). In pre-menopausal women a 78% decrease in Lp(a) levels was reported with danazol (10). In pregnant women Lp(a) levels were reported to fluctuate and to return to basal values post-partum (11, 12). It was even reported that Lp(a) levels varied within the normal female menstrual cycle (13). From these studies it seems that administration of natural or artificial sex hormones suppresses serum Lp(a) levels, and that shortage increases serum Lp(a) levels. So far, it has been clearly documented that significant lipid changes occur during sexual maturation in normal puberty (14-16). Whether physiological sexual maturation and serum lipoprotein (a) (Lp(a)) levels are associated, has not been investigated yet. As there appears to be a positive relationship between the height of the serum Lp(a) levels and the amount of Lp(a) deposited in the arterial wall (17), and as the PDAY study (18) demonstrated that coronary artery fatty streak formation starts already during childhood, it is important to investigate serum Lp(a) levels in pediatric populations, in relation to other cardiovascular risk factors.

This cross-sectional study was set up, first, to determine serum Lp(a) levels and upper reference limits (URL's) in pubertal children, in comparison with those in Belgian adults; second, to study the possible relationship between sexual maturation and serum Lp(a) levels; and third, to determine possible determinants of serum Lp(a) levels during puberty, in comparison with those of serum lipids and apolipoproteins.

METHODS

Study populations

Healthy children from schoolclasses selected out of nine different schools in Sint-Niklaas, Belgium, in whom a medical check-up was scheduled by the Medical School Health Service of Sint-Jozef, Sint-Niklaas, were invited to participate in this study. Sint-Niklaas is a city located in Flanders, i.e. the northern Dutch-speaking part of Belgium where the population has lower serum cholesterol levels compared to Wallonians, living in the southern French-speaking part of Belgium (19). Informed consent was obtained from the parents or guardians of each child.

Of the 289 participants 23 were excluded from the final data analysis because [1]) they did not have Belgian nationality, [2]) their parents were immigrants, [3]) they smoked, or [4]) they used hormonal contraception. Exclusion was done to maintain homogeneity among dietary habits and genetic effects. Consequently, 266 Belgian schoolchildren (136 boys, 130 girls) were included. Of the examined children 54.5% went to primary schools, 35.0% to grammar schools and 10.5% to technical and vocational training schools. In the same period serum Lp(a) was determined, using the same methodology, in 683 healthy adults, aged 20-39 years, from Flanders, Belgium.

Physical examination

Each child underwent a physical examination by a school physician (N = 3) who measured height, weight, systolic and diastolic blood pressure, and who asked, if relevant, for use of hormonal contraception, smoking habits and intake of medication. Height was measured to the nearest 0.5 cm, while weight was recorded to the nearest 0.1 kg. Systolic and diastolic blood pressure (SBP and DBP) were measured to the nearest 2 mmHg. None of the children was taking any cholesterol- or lipid-lowering medication. Other medication taken could be grouped into five categories: anti-allergica (0.75%), anti-tussiva (0.75%), antibiotics (1.1%), anti-asthmatica (1.5%), and vitamins and minerals (4.5%).

School physicians scored the children for pubic hair (P1 to P6), male genitalia (G1 to G5), and female breast (M1 to M5) development, according to Tanner (20).

The ratings for sexual maturation ranged from prepubertal (P1G1 in boys, P1M1 in girls) to post-pubertal (P5G5 or P6G5 in boys, P5M5 or P6M5 in girls). According to sexual maturation, 34.2% of the children were prepubertal, 35.0% pubertal and 30.8% post-pubertal.

Addition of respective M (girls) or G (boys) ratings to P ratings (boys and girls), resulted in a combined "maturity index" per individual that ranged between 2 (immature) and 10 or 11 (mature).

Questionnaire and socio-economic status of the family

A questionnaire was sent to all parents of the children that gave informed consent. Questions were raised regarding the parents education level, the age at which the parents graduated, and their profession. Parents were also inquired whether their child was using any medication lately.

The socio-economic status (SES) of the family was scored as described by Black (21) by means of six categories that are primarily based on occupation of the parents: three non-manual higher categories (classes I, II, IIIN) and three manual lower categories (classes IIIM, IV, V). The highest score of the family was used to determine the SES of the child. In this study group 74.5% of the children were from higher socio-economic classes, while 21.5% were from lower socio-economic classes. Although 4.0% of the scores was missing, the children involved were included in the data analysis.

Sampling

Children fasted overnight before venipuncture. Samples were gathered during a one-month period. Serum was aliquotted within four hours after venipuncture, and stored frozen at -30°C until analysis took place. The maximum storage time was two months.

Serum lipoprotein (a), serum lipid and apolipoprotein analyses

Serum total cholesterol and triglycerides were determined on a Chem I analyzer (Technicon Instruments Cooperation, Tarrytown, New York, USA) with enzymatic assays from Technicon (Method Nr. SA4-0305L90 and SA4-0324L90). The Chem I enzymatic cholesterol method was standardized against the Abell-

Kendall Reference Method. Lp(a)-adjusted serum cholesterol was calculated as mmol/l total cholesterol - (mg/l Lp(a) mass * (0.45/386.7)) (22). HDL-cholesterol (HDL-c) was determined in the supernatant with enzymatic reagents (Cat. Nr. 236691, Boehringer, Mannheim, Germany), after precipitation of apo B-containing lipoproteins with phosphotungstic acid/$MgCl_2$ (Cat. Nr. 14210, Merck, Darmstadt, Germany). LDL-cholesterol (LDL-c) was calculated with the Friedewald formula. Apolipoprotein A-I and B (apo A-I and B) were determined by rate immunonephelometry on a Beckman Array analyzer (Beckman Instruments, Inc., Brea, California) with apo A-I and apo B reagents (Cat. Nr. P/N 449300 and Cat. Nr. P/N 449310, Beckman) in combination with apolipoprotein calibrator and apolipoprotein diluent, Cat. Nr.449370 respectively 449380, Beckman).

Serum lipoprotein (a) was determined using an anti-apo(a) polyclonal capture ELISA from Biopool (TintElize lipoprotein(a), Cat. Nr. 610220, Biopool AB, Umeå, Sweden). The capture polyclonal anti-apo(a) antibody does not cross-react with plasminogen up to 1000 mg/l. The assay is calibrated to total Lp(a) mass.

Statistical analyses

Basic statistics and analysis of variance (ANOVA) were performed using the Statistical Analysis System (SAS) (23). A statistical significance level of $\alpha = 0.05$ was adopted. Body mass index (BMI) was defined as weight (in kg)/height^2 (in m^2). In case of (log)normal frequency distributions, statistically significant differences between subgroup means or means from different maturation stages were evaluated primarily by Students' T-test and by one- or two-way ANOVA (PROC ANOVA and GLM ANOVA). GLM (general linear models) ANOVA was used because it corrects for varying numbers of subjects between maturation stages. In case the (log) data did not display a Gaussian distribution, differences between means were tested by Mann-Whitney or Kruskal-Wallis non-parametric tests.

Backward multiple linear regression (MLR) was performed for boys and girls separately ($p_{out} = 0.10$). The dependent variables investigated were: Ln Lp(a), Ln cholesterol, Ln HDL-c, Ln apo A-I, LDL-c, apo B, and triglycerides. The independent variables were: age, BMI, maturity index, maturity index^2, SBP, DBP, SES and SES^2. Squared independent variables were introduced for sexual maturity and SES in order to identify curvilinear relationships: in case of ordinally scaled

variables distances between X-categories do not necessarily reflect equal Y-distances over the range of the X-variable.

RESULTS

Median Lp(a) levels and mean values of measured variables according to sex and sexual maturation stage

In tables 1 and 2 the anthropometric characteristics of the children are stratified according to developmental age. Mean age, BMI and SBP differ significantly across the Tanner stages in boys ($P < 0.0001$) and in girls ($P < 0.05$). Tables 3 and 4 illustrate serum Lp(a), lipid and apolipoprotein levels for the same strata. Because the Lp(a) frequency distributions were highly skewed to the right, median and LnLp(a) data were included. In boys two-way ANOVA demonstrated a significant mean difference across the Tanner stages for serum HDL-c and apo A-I ($P < 0.0001$), and for cholesterol ($P = 0.0292$). In girls, triglyceride means differed significantly between the sexual maturation stages ($P = 0.0031$). Neither in girls, nor in boys a significant mean LnLp(a) difference could be demonstrated between Tanner stages, despite the fact that mean LnLp(a) and median Lp(a) levels displayed a tendency towards higher values during puberty, compared to the levels in the pre- and post-pubertal stages.

Overall median Lp(a) levels were 94 mg/l in girls versus 82 mg/l in boys ($P = 0.2537$). The Lp(a) frequency distribution histogram for both sexes combined was highly skewed to the right, with Lp(a) levels ranging from 4 to 2025 mg/l, with an overall median Lp(a) level of 87 mg/l, and with 56% and 80% of the school-children having an Lp(a) level below 100 and 300 mg/l respectively. Median pre-pubertal, pubertal, and post-pubertal Lp(a) levels for boys and girls combined were 82, 110, and 89 mg/l respectively ($P = 0.3978$).

Median Lp(a) levels and mean values of measured variables according to sex and age quartiles

In table 5 lipid variables are stratified in age quartiles per sex. From table 5 it is obvious that [1]) mean cholesterol and HDL-c differences in boys between the first

and last quartile are less pronounced than the mean differences in table 3, illustrating the stronger association between serum lipid changes and developmental age, compared to chronologic age; [2]) mean Ln Lp(a) levels also did not differ significantly between age quartiles; [3]) girls mature at a younger age than boys: in comparable age quartiles the maturity indices are higher in girls than in boys.

Serum Lp(a) medians, interquartile ranges (IQR) and upper reference limits in Belgian schoolchildren, compared to adults

The data are presented in table 6. The parametric upper normal 97.5% limit was calculated as LnLp(a) mean + 2 SD and back-transformed, while the parametric IQR was calculated as LnLp(a) mean ± 0.67 SD. Table 6 illustrates that Lp(a) levels are higher in Belgian children compared to adults, the difference in geometric mean being significant (P = 0.006).

Predictors of serum Lp(a), serum lipid and serum apolipoproteins levels during sexual maturation

The results of the backward multiple regression analysis are summarized in table 7. Developmental age and BMI were the most important predictors of serum lipids and apolipoproteins. The selected independent variables explained 2.9-33.4% of the serum lipid and apolipoprotein variance in boys, while none of them was significantly associated with serum Lp(a) levels. In girls, 5.1-15.0% of serum apo A-I, apo B, triglyceride and HDL-c variance could be predicted, but none of the cholesterol, LDL-c or Lp(a) variance.

A non-linear relationship, described by a quadratic term, was found for girls' triglycerides. Consequently, the maturity index partially predicted the triglyceride level by means of both a linear and a quadratic term ($Y = aX_1 + bX_1^2 + cX_2 + ...$; with Y = triglyceride level, X_1 = maturity index, X_2 = another predictor variable from the defined model, a and b = regression coefficients from respectively the linear and quadratic predictor variable). The point of inflection, calculated as "-a/2b", was at a maturity index of 5.7, being a maximum. In a simple linear model Lp(a) variance was explained for 5.2% by cholesterol. When using Lp(a)-adjusted cholesterol, Lp(a) variance was no longer explained (data not shown).

Table 1. Anthropometric characteristics of Belgian schoolboys (N = 136) stratified by Tanner's sexual maturation stages for genitalia (G1-G5) and pubic hair (P1-P6) development

Tanner stage	N	Age (years)	Height (cm)	Weight (kg)	BMI[a] (kg/m^2)	SBP[b] (mmHg)	DBP[c] (mmHg)
		Mean ± S.D.					
G1	51	9.7 ± 1.7	138.2 ± 9.3	31.4 ± 6.6	16.33 ± 2.19	109.8 ± 10.0	70.8 ± 9.1
G2	15	12.0 ± 1.3	153.0 ± 8.2	40.0 ± 4.0	17.14 ± 1.78	117.5 ± 8.2	70.3 ± 6.8
G3 + G4[d]	25	15.1 ± 1.1	170.6 ± 6.8	56.3 ± 7.8	19.28 ± 1.98	126.0 ± 10.3	68.0 ± 9.2
G5	45	16.0 ± 1.7	176.1 ± 6.9	65.1 ± 10.3	20.96 ± 2.92	132.1 ± 15.3	71.8 ± 9.1
Kruskal-Wallis or GLM anova, P-value		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.5077
P1	54	9.8 ± 1.9	139.2 ± 10.4	32.1 ± 7.1	16.42 ± 2.19	109.8 ± 10.0	70.5 ± 9.0
P2	11	11.6 ± 0.5	152.1 ± 6.5	39.2 ± 3.6	17.02 ± 1.84	120.2 ± 5.8	71.8 ± 7.1
P3	9	14.4 ± 1.7	165.1 ± 9.2	50.6 ± 9.9	18.38 ± 1.72	119.9 ± 5.8	70.9 ± 9.2
P4	19	15.3 ± 0.4	172.3 ± 5.8	57.3 ± 6.8	19.30 ± 2.10	126.7 ± 12.6	65.7 ± 8.7
P5	19	15.3 ± 0.3	175.7 ± 7.7	61.8 ± 9.2	19.96 ± 2.20	134.9 ± 19.2	73.4 ± 9.8
P6	24	16.5 ± 2.2	176.7 ± 6.3	69.1 ± 9.8	22.16 ± 3.06	131.6 ± 9.6	71.5 ± 8.1
Kruskal-Wallis or GLM anova, P-value		< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.1557
TOTAL	136	13.0 ± 3.2	158.0 ± 18.8	47.8 ± 16.9	18.45 ± 3.11	120.9 ± 15.2	70.5 ± 8.9

[a]body mass index; [b]systolic blood pressure; [c]diastolic blood pressure; [d]G3 was added to G4 because of the small number of observations in G3.

Table 2. Anthropometric characteristics of Belgian schoolgirls (N = 130) stratified by Tanner's sexual maturation stages for breast (M1-M5) and pubic hair (P1-P6) development

Tanner stage	N	Age (years)	Height (cm)	Weight (kg)	BMI[a] (kg/m^2)	SBP[b] (mmHg)	DBP[c] (mmHg)
		Mean ± S.D.					
M1	41	8.7 ± 1.0	132.1 ± 6.6	27.6 ± 4.6	15.73 ± 1.65	112.8 ± 9.1	67.6 ± 8.7
M2	28	11.1 ± 0.6	146.6 ± 5.6	38.5 ± 6.9	17.91 ± 2.88	119.3 ± 10.2	74.1 ± 9.7
M3	12	12.3 ± 1.6	156.8 ± 6.3	48.0 ± 8.8	19.43 ± 2.87	121.1 ± 11.1	69.3 ± 8.7
M4	11	14.9 ± 1.3	163.9 ± 6.5	54.7 ± 8.5	20.38 ± 3.07	112.2 ± 7.4	67.3 ± 10.0
M5	39	16.2 ± 1.5	167.0 ± 5.8	57.6 ± 7.1	20.65 ± 2.10	115.8 ± 8.5	67.6 ± 7.1
Kruskal-Wallis or GLM anova, P-value		< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0074	0.0143
P1	41	8.7 ± 1.1	132.2 ± 6.9	27.8 ± 5.1	15.78 ± 1.75	113.4 ± 9.4	68.1 ± 8.8
P2	26	11.1 ± 0.8	146.4 ± 5.8	38.6 ± 6.9	18.00 ± 2.89	118.4 ± 10.6	73.2 ± 10.4
P3	12	11.4 ± 0.5	154.2 ± 6.6	46.4 ± 10.5	19.35 ± 3.23	121.2 ± 9.9	70.0 ± 8.8
P4	12	15.1 ± 0.9	164.0 ± 6.2	51.9 ± 8.5	19.22 ± 2.50	114.1 ± 8.7	68.4 ± 9.7
P5	33	15.6 ± 0.5	166.5 ± 5.8	57.3 ± 7.4	20.67 ± 2.33	114.5 ± 9.0	67.5 ± 7.2
P6	7	18.9 ± 1.8	167.9 ± 6.5	59.9 ± 5.1	21.27 ± 1.71	119.4 ± 6.6	66.7 ± 7.1
Kruskal-Wallis or GLM anova, P-value		< 0.0001	< 0.0001	< 0.0001	< 0.0001	0.0498	0.1342
TOTAL	130	12.3 ± 3.3	150.4 ± 15.7	42.9 ± 14.1	18.34 ± 3.07	115.8 ± 9.6	69.1 ± 8.9

[a]body mass index; [b]systolic blood pressure; [c]diastolic blood pressure.

Table 3. Serum lipoprotein (a), serum lipid and apolipoprotein levels in 136 Belgian schoolboys, stratified by Tanner's sexual maturation stages for genitalia (G1-G5) and pubic hair (P1-P6) development

Tanner stage	N	Lp(a) (mg/l)	Lp(a) (mg/l)	Ln Lp(a) (Ln mg/l)	Cholesterol (mmol/l)	HDL-c[a] (mmol/l)	LDL-c[b] (mmol/l)	Apo A-I[c] (g/l)	Apo B[d] (g/l)	Triglycerides (mmol/l)
		Median	Mean ± S.D.							
G1	51	82	166 ± 314	4.29 ± 1.25	4.30 ± 0.65	1.46 ± 0.27	2.48 ± 0.61	1.58 ± 0.21	0.91 ± 0.18	0.77 ± 0.26
G2	15	117	191 ± 229	4.74 ± 1.06	4.55 ± 0.91	1.50 ± 0.27	2.67 ± 0.91	1.71 ± 0.21	0.94 ± 0.25	0.85 ± 0.17
G3 + G4[e]	25	110	182 ± 263	4.44 ± 1.33	3.95 ± 0.60	1.12 ± 0.27	2.44 ± 0.60	1.36 ± 0.17	0.82 ± 0.20	0.85 ± 0.33
G5	45	73	167 ± 209	4.45 ± 1.19	4.10 ± 0.85	1.10 ± 0.21	2.61 ± 0.83	1.33 ± 0.16	0.90 ± 0.25	0.84 ± 0.23
One-way GLM ANOVA, P-value		-	-	0.8160	0.0485	< 0.0001	0.6889	< 0.0001	0.0820	0.2037
P1	54	82	164 ± 305	4.32 ± 1.23	4.30 ± 0.66	1.46 ± 0.27	2.49 ± 0.63	1.59 ± 0.21	0.91 ± 0.18	0.78 ± 0.25
P2	11	94	166 ± 225	4.57 ± 1.08	4.52 ± 0.94	1.60 ± 0.23	2.55 ± 0.87	1.76 ± 0.22	0.94 ± 0.27	0.82 ± 0.17
P3	9	110	169 ± 189	4.74 ± 0.88	3.69 ± 0.84	1.06 ± 0.35	2.30 ± 0.83	1.33 ± 0.20	0.76 ± 0.21	0.72 ± 0.14
P4	19	115	215 ± 297	4.48 ± 1.52	4.13 ± 0.56	1.16 ± 0.20	2.56 ± 0.59	1.39 ± 0.15	0.85 ± 0.19	0.90 ± 0.36
P5	19	59	176 ± 209	4.36 ± 1.40	4.17 ± 1.18	1.13 ± 0.25	2.67 ± 1.18	1.34 ± 0.17	0.90 ± 0.35	0.83 ± 0.23
P6	24	71	157 ± 219	4.46 ± 1.06	4.08 ± 0.48	1.07 ± 0.18	2.61 ± 0.43	1.31 ± 0.15	0.91 ± 0.14	0.87 ± 0.24
One-way GLM ANOVA, P-value		-	-	0.7027	0.0964	0.0880	0.1434	0.3140	0.6338	0.4193
Two-way GLM ANOVA, P-value		-	-	0.8691	0.0292	< 0.0001	0.3097	< 0.0001	0.2306	0.2842
TOTAL	136	82	172 ± 262	4.42 ± 1.22	4.20 ± 0.76	1.29 ± 0.31	2.54 ± 0.72	1.47 ± 0.23	0.89 ± 0.22	0.82 ± 0.26

[a]High density lipoprotein cholesterol; [b]Low density lipoprotein cholesterol; [c]apolipoprotein A-I; [d]apolipoprotein B; [e]G3 was added to G4 because of the small number of observations in G3.

Table 4. Serum lipoprotein (a), serum lipid and apolipoprotein levels in 130 Belgian schoolgirls, stratified by Tanner's sexual maturation stages for breast (M1-M5) and pubic hair (P1-P6) development

Tanner stage	N	Lp(a) (mg/l)	Lp(a) (mg/l)	Ln Lp(a) (Ln mg/l)	Cholesterol (mmol/l)	HDL-c[a] (mmol/l)	LDL-c[b] (mmol/l)	Apo A-I[c] (g/l)	Apo B[d] (g/l)	Triglycerides (mmol/l)
		Median	Mean ± S.D.							
M1	41	73	189 ± 213	4.48 ± 1.40	4.48 ± 0.73	1.46 ± 0.31	2.69 ± 0.62	1.53 ± 0.21	1.01 ± 0.23	0.73 ± 0.23
M2	28	78	111 ± 115	4.20 ± 1.14	4.43 ± 0.75	1.34 ± 0.22	2.68 ± 0.74	1.45 ± 0.17	0.97 ± 0.24	0.90 ± 0.36
M3	12	204	312 ± 247	5.38 ± 0.97	4.61 ± 0.81	1.39 ± 0.34	2.71 ± 0.71	1.51 ± 0.23	1.03 ± 0.21	1.11 ± 0.48
M4	11	110	214 ± 262	4.74 ± 1.24	4.42 ± 0.57	1.38 ± 0.17	2.67 ± 0.45	1.47 ± 0.14	1.00 ± 0.20	0.82 ± 0.17
M5	38	114	214 ± 242	4.62 ± 1.38	4.35 ± 0.80	1.38 ± 0.30	2.59 ± 0.75	1.44 ± 0.20	0.92 ± 0.23	0.84 ± 0.21
One-way GLM ANOVA, P-value		-	-	0.0870	0.8288	0.5218	0.8795	0.2276	0.2631	0.0015
P1	41	73	188 ± 214	4.48 ± 1.40	4.51 ± 0.73	1.47 ± 0.30	2.71 ± 0.63	1.54 ± 0.20	1.01 ± 0.23	0.73 ± 0.23
P2	26	78	98 ± 79	4.16 ± 1.09	4.44 ± 0.74	1.31 ± 0.22	2.71 ± 0.72	1.42 ± 0.18	1.00 ± 0.23	0.91 ± 0.36
P3	12	182	242 ± 196	5.10 ± 1.00	4.43 ± 0.86	1.45 ± 0.30	2.55 ± 0.79	1.52 ± 0.20	0.95 ± 0.27	0.96 ± 0.44
P4	12	153	260 ± 296	4.90 ± 1.30	4.47 ± 0.61	1.39 ± 0.21	2.68 ± 0.47	1.49 ± 0.17	1.00 ± 0.17	0.86 ± 0.24
P5	32	114	222 ± 233	4.74 ± 1.30	4.34 ± 0.78	1.34 ± 0.24	2.59 ± 0.72	1.43 ± 0.19	0.93 ± 0.22	0.90 ± 0.27
P6	7	156	239 ± 336	4.32 ± 1.87	4.41 ± 0.90	1.49 ± 0.51	2.60 ± 0.91	1.43 ± 0.24	0.90 ± 0.27	0.71 ± 0.12
One-way GLM ANOVA, P-value		-	-	0.3100	0.5697	0.1922	0.5489	0.3227	0.1815	0.1591
Two-way GLM ANOVA, P-value		-	-	0.1236	0.7988	0.3029	0.8125	0.2480	0.1753	0.0031
TOTAL	130	94	193 ± 218	4.57 ± 1.31	4.44 ± 0.74	1.40 ± 0.28	2.66 ± 0.68	1.48 ± 0.20	0.98 ± 0.23	0.84 ± 0.30

[a]High density lipoprotein cholesterol; [b]Low density lipoprotein cholesterol; [c]apolipoprotein A-I; [d]apolipoprotein B.

Table 5. Serum lipoprotein (a), serum lipid and apolipoprotein levels of Belgian schoolchildren (136 boys, 130 girls) stratified by age quartiles

Age	N	Combined maturity index	Lp(a) (mg/l)	Lp(a) (mg/l)	Ln Lp(a) (Ln mg/l)	Cholesterol (mmol/l)	HDL-c[a] (mmol/l)	LDL-c[b] (mmol/l)	Apo A-I[c] (g/l)	Apo B[d] (g/l)	Triglycerides (mmol/l)
Years		Mean ± SD	Median	Mean ± S.D.							
Boys											
< 11.1	34	2.1 ± 0.3	79	146 ± 189	4.31 ± 1.24	4.14 ± 0.59	1.42 ± 0.23	2.38 ± 0.55	1.52 ± 0.18	0.90 ± 0.17	0.74 ± 0.29
11.1 - 14.5	32	3.1 ± 1.3	102	201 ± 377	4.51 ± 1.21	4.51 ± 0.85	1.50 ± 0.32	2.63 ± 0.83	1.67 ± 0.25	0.93 ± 0.23	0.83 ± 0.16
14.6 - 15.4	34	9.3 ± 1.9	90	170 ± 233	4.50 ± 1.15	4.04 ± 0.65	1.10 ± 0.27	2.56 ± 0.62	1.36 ± 0.19	0.88 ± 0.20	0.85 ± 0.27
> 15.4	36	9.3 ± 1.9	65	173 ± 226	4.38 ± 1.32	4.12 ± 0.86	1.14 ± 0.22	2.59 ± 0.86	1.35 ± 0.16	0.87 ± 0.27	0.84 ± 0.27
ANOVA, P-value			-	-	0.8906	0.0576	< 0.0001	0.5339	< 0.0001	0.6741	0.0662
Girls											
< 8.7	32	2.0 ± 0.2	78	204 ± 230	4.50 ± 1.49	4.46 ± 0.78	1.44 ± 0.33	2.69 ± 0.65	1.53 ± 0.22	1.02 ± 0.24	0.70 ± 0.24
8.7 - 11.4	34	3.9 ± 1.2	77	136 ± 145	4.34 ± 1.18	4.45 ± 0.66	1.35 ± 0.24	2.67 ± 0.67	1.46 ± 0.18	0.98 ± 0.23	0.95 ± 0.39
11.5 - 15.3	32	7.3 ± 2.6	152	233 ± 223	4.96 ± 1.10	4.53 ± 0.81	1.39 ± 0.30	2.73 ± 0.72	1.46 ± 0.22	1.00 ± 0.23	0.91 ± 0.27
> 15.3	32	9.9 ± 0.9	107	203 ± 261	4.47 ± 1.44	4.29 ± 0.75	1.41 ± 0.27	2.52 ± 0.70	1.45 ± 0.16	0.90 ± 0.21	0.80 ± 0.21
ANOVA, P-value			-	-	0.2456	0.6083	0.5848	0.5225	0.3979	0.1494	0.0008

[a]High density lipoprotein cholesterol; [b]Low density lipoprotein cholesterol; [c]apolipoprotein A-I; [d]apolipoprotein B.

Table 6. Serum Lp(a) means, medians, interquartile ranges and upper reference limits in Belgian schoolchildren and adults

Lp(a), mg/l	Children (N = 266)	Adults (N = 683)
Non-parametric:		
Mean ± S.D.	182 ± 241	144 ± 179
Median	87	67
25 - 75 percentile range	41 - 204	30 - 186
2.5 - 97.5 percentile range	6 - 801	5 - 640
Upper reference limits:		
90th percentile	515	403
95th percentile	712	536
Parametric:		
Geometric mean	89*	69*
Mean ± 0.67 S.D.	38 - 208	28 - 167
Mean ± 2 S.D.	7 - 1125	5 - 981

*Student's T-test: P = 0.006

DISCUSSION

As Lp(a) is an independent risk factor for premature coronary heart disease (1-3), and as efforts aimed at the primary prevention of coronary artery disease have to be initiated during childhood, it is essential to investigate cardiovascular risk factors, including serum Lp(a), in pediatric populations. Increased serum Lp(a) levels possibly interfere with fibrinolytic degradation of blood clots, promoting progression of atherosclerotic lesions (24). Consequently, it is important to consider upper reference limits for this lipoprotein. In this population sample the non-parametric 90th and 95th percentiles for Lp(a) mass, determined with the TintElize Biopool kit, are 515 and 712 mg/l, being substantially higher than the generally accepted 300 mg/l cutoff value which is related with an increased cardiovascular risk. Therefore, longitudinal studies are warranted to evaluate the future risk attributable to Lp(a) levels at these upper reference limits.

Studies involving adolescent populations need to take into account developmental age (14-16, 25) because physiological endpoints, i.e. lipid levels,

generally correlate better with developmental age than with chronological age. Tanner's classification system was used to score developmental age in this population sample (20). Tanner's system is based upon biologic maturation during adolescence using the developmental stages of secondary sex characteristics. Separate ratings, and not average ratings, are recommmended for the Tanner stages because disparities of two to three ordinal ranks between the two ratings have been reported, and as within-age correlations between these two ratings are low (16). In agreement with other investigators (14-16, 25), cross-classification of this study population by age and sexual maturity revealed broad distributions of developmental stages within adolescents with identical ages. Such variation is reported to have multiple causes, the rapid evolving changes in sex hormones, which have a broad range of normal onset, being a key factor (15, 26).

Serum lipid changes during sexual maturation generally corresponded with previously published data. Post-pubertal HDL-c levels decreased by 25% in boys (-0.36 mmol/l) and by 5.5% (-0.08 mmol/l) in girls, compared to pre-pubertal levels. The apo A-I trends co-varied with the HDL-c changes in boys and girls. These findings are concordant with the mean α-lipoprotein cholesterol decreases of 25% and 8.1% respectively, in the large-scale Bogalusa Heart Study (14). In mature compared to immature boys respectively girls, mean total cholesterol decreased by 4.7% and 3.1%, while Berenson et al. (14) found decreases of 11% and 5.1% respectively. The smaller cholesterol decrease in boys in this study is related to the LDL-c increase (+5.2%) during maturation, suggesting a more unfavourable diet in Belgians (19).

Statistical analysis using ANOVA could not demonstrate a significant difference between mean Ln Lp(a) levels of different Tanner stages, neither in boys nor in girls. Nevertheless, median Lp(a) and mean Ln Lp(a) levels tended to be higher during puberty, suggesting similarity with the 'post-pubertal cholesterol dip' (27). Although these findings appear to contradict those studies showing a significant Lp(a) decrease after a rise or administration of sex hormones (7-12), they are in line with the data of the Framingham Offspring Population study (28) where endogeneous sex hormone deficiency in post-menopausal women resulted in a modest 8% increase of age-adjusted serum Lp(a), also being statistically insignificant. Besides, Lp(a) levels in Belgian children were significantly higher than in Belgian

Table 7. Backward multiple linear regression analysis on Belgian schoolchildren with serum Lp(a), serum apolipoproteins, and serum lipids as dependent variables, and with age, BMI, SBP, DBP, maturity index, maturity index^2, SES, and SES^2 as independent variables

Dependent variable	Predictors in the equation	ß	SE ß	P-Value	Adjusted R^2
Boys (N = 136)					
Ln cholesterol	maturity index	-0.0158	0.0057	0.0060	
(Ln mmol/l)	BMI	0.0139	0.0067	0.0421	
	SES	-0.0238	0.0133	0.0751	
					0.075
Ln HDL-c[a]	maturity index	-0.0444	0.0063	0.0000	
(Ln mmol/l)	SBP	0.0029	0.0015	0.0596	
	SES	-0.0271	0.0153	0.0787	
					0.334
Ln apo A-I[b]	maturity index	-0.0434	0.0069	0.0000	
(Ln g/l)	age	0.0217	0.0074	0.0040	
	SBP	0.0023	0.0010	0.0205	
					0.322
LDL-c[c]	BMI	0.0451	0.0211	0.0340	
(mmol/l)					
					0.029
Apo B[d]	BMI	0.0219	0.0085	0.0116	
(g/l)	maturity index	-0.0154	0.0073	0.0371	
					0.039
Triglycerides	age	0.0181	0.0074	0.0163	
(mmol/l)					
					0.040
Ln Lp(a)	no predictors				
(Ln mg/l)					0.000
Girls (N = 130)					
Ln cholesterol	no predictors				
(Ln mmol/l)					0.000
Ln HDL-c	BMI	-0.0345	0.0073	0.0000	
(Ln mmol/l)	age	0.0176	0.0064	0.0072	
					0.144
Ln apo A-I	BMI	-0.0174	0.0040	0.0000	
(Ln g/l)					0.131

Table 7. Cont'd

Dependent variable	Predictors in the equation	ß	SE ß	P-Value	Adjusted R^2
LDL-c (mmol/l)	no predictors				
					0.000
Apo B	maturity index	-0.0220	0.0076	0.0046	
(g/l)	BMI	0.0186	0.0086	0.0323	
					0.051
Triglycerides	BMI	0.0375	0.0113	0.0012	
(mmol/l)	maturity index^2	-0.0107	0.0038	0.0050	
	maturity index	0.1222	0.0485	0.0131	
					0.150
Ln Lp(a) (Ln mg/l)	no predictors				0.000

Age in years; BMI: body mass index calculated as [weight (kg) / height^2 (m^2)]; DBP: diastolic blood pressure in mmHg; maturity index: sum of Tanner pubic hair and genitalia development scores in boys, respectively pubic hair and breast development scores in girls; SBP: systolic blood pressure in mmHg; SES: socio-economic status, categorized as described by Black (21). [a]High density lipoprotein cholesterol; [b]apolipoprotein A-I; [c]Low density lipoprotein cholesterol; [d]apolipoprotein B.

sex-matched adults, aged 20-39 years. We hypothesize that hormonal changes during puberty might account for differences in serum Lp(a) between adolescents and adults. Of course, these findings have to be interpreted with caution because just a cross-sectional examination was performed where only internal relationships at a single point in time could be observed, whereas almost all findings in literature were based upon before- and after-treatment measurement of serum Lp(a) in the same individuals. Second, longitudinal studies are still required for an ideal evaluation of Lp(a) changes over time.

Backward multiple linear regression demonstrated that developmental age is a better predictor of serum lipid and apolipoprotein levels than chronologic age, and that knowledge of developmental age, BMI, SBP, chronological age and SES accounted for up to 15.0% respectively 33.4% of the variability in the levels of serum lipids or apolipoproteins in girls and boys. Total cholesterol and triglyceride

variances were explained by 7.5% respectively 4% in boys, and by 0% respectively 15% in girls, the mean being close to the 4% and 11% reported by Frerichs et al. (25) for the two genders pooled. HDL-c variance was explained by 33.4% in boys and by 14.4% in girls, which is clearly more than the 6% found by Frerichs et al. (25), suggesting that the model used in this study is superior. Ommitting the quadratic term from the model predicting girls' triglyceride variance, the residual plot displayed a curvilinear relationship versus the maturity index, and meanwhile the explained variance was lowered. This finding too has to be interpreted with caution as predictions and extrapollations may be less accurate, especially there where only limited observations were available per maturity index score.

The lack of association between serum Lp(a) and BMI is in accordance with previously published data in healthy adolescents (22) and adults (29-31), and contradicts others (32, 33). Further, Vella et al. (22) also could not demonstrate a relation between serum Lp(a) and age in 11- to 19-year-old adolescents. The lack of correlation between SBP and serum Lp(a) is concurrent with the findings of Slunga et al. in a middle-aged normotensive population (30). The lack of a significant effect of puberty on serum Lp(a) is in accordance with the lack of association between *in vivo* sex hormones and Lp(a) concentrations in middle-aged men (34), and with the lack of a significant effect of menopausal status in women (28).

An innovative aspect of this cross-sectional Lp(a) study in schoolchildren was the inclusion of a measure of developmental age. As the mean age of onset of puberty varies between populations, and changes over time within populations, the inclusion of developmental age enables one to compare adolescent Lp(a), lipid and apolipoprotein data between populations. So far, due to the lack of Lp(a) standardization, and because many investigators only report Lp(a) means, and no medians, geometric means or percentiles, comparability of Lp(a) data between different investigators has been hampered. From these cross-sectional data it can be concluded first, that serum Lp(a) levels in Belgian peri-pubertal children are significantly higher than those in Belgian adults, aged 20-39 years; second, that serum Lp(a) levels are not significantly associated with the Tanner maturity stages; and third, that developmental age, BMI, SBP, chronologic age and SES predict serum lipid and apolipoprotein levels, but not serum Lp(a) levels, hence supporting the hypothesis that Lp(a) levels are primarily genetically controlled.

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Chapter 6

REGIONAL SERUM CHOLESTEROL DIFFERENCES IN BELGIUM: DO GENETICALLY DETERMINED CARDIOVASCULAR RISK FACTORS CONTRIBUTE?

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ABSTRACT

Background

Differences in serum lipid distribution and mortality from ischaemic heart disease have repeatedly been reported between Belgian northerners and southerners. We aimed to investigate whether, besides differences in lifestyle factors, genetically determined risk factors such as lipoprotein(a) (Lp(a)) and apo E polymorphism were involved.

Methods

Fasting serum lipids, apolipoprotein (apo) A-I and B, and Lp(a) levels were examined in randomly selected, 20-39 year old Belgian males and females from the north (Flanders) and the south (Wallonia) of Belgium (N = 900). Apo E phenotype distribution was investigated in equally sized random subsamples from either region (N = 249).

Results

Mean serum cholesterol, LDL-cholesterol (LDL-c), apo B and triglyceride levels were higher in southerners compared to northerners within each gender, the difference being significant in 30-39 year old males. Analogously, average HDL-cholesterol and apo A-I levels were significantly lower in 30-39 year old male southerners compared to their northern counterparts. Median Lp(a) was 67 mg/l in northerners and 75 mg/l in southerners, the difference not being significant.
The apo E phenotype distribution was similar in both regions ($\chi^2 = 7.213$; df = 5; P = 0.2053), whereas the average effects of the apo E alleles differed between the two regions. In southerners the ε4 effect upon adjusted apo B and LDL-c levels was ≈ +12% and the ε2 effect was ≈-15%; in northerners the ε4 and ε2 effects were ≈ +5% and ≈ -25%, respectively. The apo E polymorphism did not affect serum Lp(a) levels.

Conclusion

Regional cholesterol differences between Belgian northerners and southerners can not be explained by differences in serum Lp(a) or apo E phenotype distribution

and seem entirely attributable to differences in lifestyle factors. The less favourable ε2 and ε4 effects in southerners compared to northerners reflect modulation of the apo E gene by particular environments.

INTRODUCTION

Regional differences in serum lipid distribution and mortality from ischaemic heart disease have repeatedly been described for Belgium, a small industrialized country of only 11781 square miles with the pecularity of two cultural communities, i.e. a Dutch-speaking community in the north (Flanders and Campine), and a French-speaking community in the south (Wallonia). The linguistic difference has created a cultural frontier, cutting horizontally through Belgium, hampering the transmission of information from one side to the other, and leading to newspapers, radio and television addressing themselves separately to one or other community.

It was first discovered at the end of the sixties, during an epidemiological survey in the Belgian army that important regional serum cholesterol differences existed in males in all 5-year age classes between the age of 15 and 55 years (1). The higher serum cholesterol in the southerners, coupled to a higher morbidity from ischaemic heart disease and peripheral vascular disease, was confirmed in the seventies by surveys in male postal (2) and factory workers (3). In the eighties the Belgian Interuniversity Research on Nutrition and Health (BIRNH) documented similar cholesterol differences between Flemings and Walloons in all age categories, both in males and females, and found differences of 20% in coronary mortality between the two regions (4). As to the origin of these serum cholesterol differences, the less favourable food pattern in Walloons in terms of saturated and polyunsaturated fat intake as well as nutritional cholesterol intake have been hold responsible (4). Whether, besides differences in lifestyle factors, variations in genetically determined cardiovascular risk factors might contribute to the observed north-south differences in serum cholesterol has not been investigated so far. Two potential candidates, co-determining serum lipid and serum cholesterol levels, that might be involved are the type and blood levels of apolipoprotein E (apo E) (5) and

lipoprotein(a) (Lp(a)) (6).

First, apolipoprotein E (apo E) is a normal constituent of triglyceride-rich lipoproteins and high density lipoproteins (HDL). The primary function presently known for apo E is the clearance of triglyceride-rich lipoprotein remnants, due to its recognition by the LDL-receptor and the LDL-receptor-related protein on hepatic cells (7). Besides, apo E plays a crucial role in transport and redistribution of lipids in peripheral tissues such as brain, peripheral nerves and the arterial wall (8). In humans, the structural gene locus of apo E is genetically polymorphic: three common alleles (ε2, ε3 and ε4) code for three isoforms (the predominant E3 isoform and its mutant forms, E2 and E4), resulting in three homozygous phenotypes (E2/2, E3/3 and E4/4) and three heterozygous phenotypes (E2/4, E3/4, E2/4). The E4 isoform exhibits similar or higher binding activity to the LDL-receptor than E3, while the E2 isoform displays a lower affinity. The polymorphism of the apo E gene locus has important consequences for the serum lipid profile, since it accounts for up to 7% of the genetic variance and 16% of the total serum cholesterol in the normal population (9). The EARS study (10) depicted a clear-cut gradient for the ε4 allele frequency in Europe, decreasing from 0.18 in Finland to 0.11 in Mediterranean countries, following the gradient of coronary heart disease mortality. The study also provided a body of evidence that the apo E polymorphism strongly contributes to the development of coronary heart disease and that ε4 is a major risk factor responsible for familial predisposition to this disease. Hence, apo E4 carriers suffer more frequently from myocardial infarction, attributed to the cholesterol-raising effect of ε4 due to down-regulation of the LDL-receptor (11). So far, no other gene has been identified that contributes as much to normal cholesterol variability as the apo E gene, i.e., the ε2 allele predisposing to lower LDL-cholesterol (LDL-c) and apolipoprotein B (apo B) levels and the ε4 allele being associated with higher LDL-c and apo B levels (5, 10).

Second, Lp(a) is an atherogenic LDL-like lipoprotein particle, that contains apo B disulfide-bonded to a unique apo(a) apolipoprotein, and consists of approximately one third of cholesterol (6, 12). So far, its physiological function is not unravelled (12). In most human populations the serum concentration of Lp(a) is extremely variable, ranging from 0 to 2000 mg/l (13, 14); the same holds for its cholesterol content (6). Lp(a) concentrations in Caucasians are not normally

distributed and concentrations above 200 to 300 mg/l have been found to be associated with increased risk of atherosclerosis (15). The apparent molecular weight of apo(a) appears to range from 250-900 kDa (12, 15). The basis for this variation lies in the peculiar structure of this protein, which is strikingly similar to plasminogen, comprising several distinct protein domains (signal, tail, kringles I to V and protease domain). Apo(a) contains a signal domain, a variable number of kringle IV repeats, a unique kringle V and a mutated protease domain, but lacks the tail and kringles I, II and III. Apo(a) is found to be extremely heterogeneous, the heterogeneity being caused by the highly heritable and variable number of kringle IV repeats at the apo(a) gene locus (12, 15). The large interindividual differences in serum Lp(a) are inversely associated with the number of kringle IV repeats (15). Allelic variation of the apolipoprotein(a) gene on levels of Lp(a) determines as much as 90% of the interindividual variation in Lp(a) (12,15).

This investigation was undertaken to determine whether genetically determined cardiovascular risk factors (apo E4, Lp(a)) might contribute to regional cholesterol differences in Belgium. To that end, apo E polymorphism and serum Lp(a) levels were examined in randomly selected 20-39 year old males and females from either region, in relation to serum lipid and apolipoprotein levels. Besides, it was investigated whether apo E polymorphism affected serum Lp(a) levels.

METHODS

Population description and sample collection

Sera were obtained from healthy, unrelated, 20 to 39 year old employees from Flanders (N = 683) and Wallonia (N = 217). For that purpose, employees working at companies spreaded throughout the two regions (32 companies in Flanders; 15 companies in Wallonia) were, prior to a scheduled medical check-up, invited to participate in this study. Previous selection and subsequent invitation, and medical check-up were conducted by the Flemish IDEWE and the Wallonian CeSI, two 'Centres de Services Inter-enterprises - Médecine du Travail'. Informed consent was obtained from all participants.

Blood was collected in the morning at the point of medical check-up into

whole blood tubes after overnight fasting (including abstinence from alcohol). The same day, the whole blood tubes were transported to the Central Laboratory of the Leuven University Hospital, Belgium. No special precautions were taken. Serum was harvested at the day of sample collection by centrifugation at 1500 g for 10 minutes at room temperature. Subsequently, the serum was divided into five aliquots: [1]) for determination of serum lipids and γ-glutamyltransferase activity (GGT); [2]) for apolipoprotein A-I and B analyses; [3]) for Lp(a) analyses; [4]) for apo E phenotyping and [5]) a left-over serum aliquot for long-term storage at -70°C. Serum lipids and GGT were determined freshly, whereas aliquots for apolipoprotein A-I and B, and Lp(a) analyses were stored frozen at -20°C during maximum two months. Before freezing, aliquots for apo E phenotyping (250 µl) were preserved with an anti-proteolytic cocktail (5 µl) containing aprotinin (1 mg/ml), lima bean trypsin inhibitor (2 mg/ml), soybean trypsin inhibitor (2 mg/ml), benzamidin (3.132 mg/ml), glutathione (20 mg/ml), D-phenylalanyl-L-propyl-L-arginine chloromethyl keton (50 µg/ml), NaN_3 (10 mg/ml), Tritriplex (0.65 mg/ml), streptomycin sulfate (8 mg/ml), and sodium benzylpenicillin (8 mg/ml).

Questionnaire

Questionnaires were distributed at the time of the medical check-up. Participants were invited to fill in their identity, home address and postcode, nationality, ethnicity (Caucasian/ Asian/ African), date of birth, type of education, profession and age at graduation. Moreover, participants were questionned about the current use (and type) of hormonal contraceptives and possible pregnancies or hysterectomies (for females), about smoking, drinking, and sporting habits, and about the use of lipid-lowering medication. To that end, the number and type of alcoholic beverages (wine, beer, liqueur) drank per day and per week were scored, as well as the number of cigarettes, cigars or pipes smoked per day, and the hours of physical exercise taken per week.

The filled-in questionnaires were checked upon completeness by the physicians who conducted the medical check-up. Additional questions were raised whenever appropriate. Non-Caucasians, pregnant and hysterectomized women, and individuals taking any lipid-lowering medication were excluded from the study at this stage.

Socio-economic status

Socio-economic status was essentially scored as described by Black (16), by means of six categories that are primarily based on occupation: three non-manual higher categories (classes I, II, IIIN; categorized as 1, 2, and 3 respectively) and three manual lower categories (IIIM, IV, V; categorized as 4, 5, and 6 respectively). For classification, the type of education and the age at graduation were also taken into account. Scoring was done by two independent observers, who were blinded to all study results. Inconsistent scores were verified; in case of remaining disagreement definitive categorization was done by a third observer.

Anthropometric and blood pressure measurements

Body height was measured to the nearest 0.5 cm. Body weight was measured to the nearest 0.1 kg. Body mass index (BMI) was calculated as weight (in kilograms) divided by height (in meters squared). Systolic (SBP) and diastolic (DBP) blood pressure were measured to the nearest mmHg. All measurements were performed by the physicians in charge of the medical check-up.

Serum GGT, serum lipid and apolipoprotein analyses

Cholesterol was determined enzymatically, using CHOD-PAP reagent. HDL- and LDL-cholesterol were determined with CHOD-PAP reagents, after precipitation with $MgCl_2$ / phosphotungstic acid and polyvinyl sulfate, respectively. Besides, LDL-c was estimated by the original Friedewald formula (17), as well as by a modified Friedewald formula that corrects for the Lp(a)-cholesterol contribution (6). Total triglycerides were determined using a GPO-PAP reagent; no free glycerol correction was made. Apolipoprotein (apo) A-I and B were determined by immunoturbidimetry (reference values: 0.80-1.50 g/l for apo A-I and 0.60-0.95 g/l for apo B). γ-Glutamyltransferase (GGT) activity was determined using γ-glutamyl-carboxynitroanilide and glycylglycine substrates (reference values: 5-28 IU/l in males; 4-18 IU/l in females). All reagents were purchased from Boehringer (Boehringer Mannheim, Mannheim, Germany).

Accuracy of the routine cholesterol and HDL-c assay methods used was checked retrospectively by the Lipid Reference Laboratory of the University Hospital, Rotterdam, the Netherlands, versus the Abell-Kendall Reference Method

and the CDC HDL-c Designated Comparison Method, respectively (18, 19). Throughout the study period (i.e. at 5 checkpoints) average biases of all six samples analyzed per checkpoint were ≤ 3% for total cholesterol and ≤ 5% for HDL-c, fulfilling the recommended 1998 NCEP performance guidelines for these analytes (18, 19).

Serum lipoprotein(a) quantification and apo E phenotyping

Lipoprotein(a) was determined using an anti-apo(a) polyclonal capture ELISA from Biopool (TintElize lipoprotein(a), Cat. No. 610220; Biopool AB, Umeå, Sweden) (20). Apo E phenotyping was performed by a micro-method based on isoelectric focusing (pH 4-7) of delipidated serum samples, followed by immunoblotting on a nitrocellulose filter and use of polyclonal rabbit anti-apo E antiserum as the first antibody (21). Lp(a) mass measurements were performed in all volunteers (N = 900), whereas apo E phenotyping was done in a subgroup (N = 249), matched for region (Flemings: N = 125, Walloons: N = 124). After laboratory analysis, lipid and lipoprotein data were reported by letter to each participant, together with reference and/or consensus values of the investigated parameters and an interpretation of the lipid profile (normal/abnormal).

Statistical analysis

Regional differences between baseline characteristics and serum lipid and (apo)lipoprotein data were determined with Students' T-test, Mann-Whitney rank-sum test, or chi-square test where appropriate. Lp(a) data were logarithmically transformed (natural logarithm), due to extreme skewness of its distribution.

Multiple linear regression models controlling for a number of covariables (ANCOVA) were used for examining the differences in serum lipid and (apo)lipoprotein levels between Flanders and Wallonia. To this end, cholesterol, LDL-c, apo B and Lp(a) data were logarithmically transformed, whereas this was not required for HDL-c and apo A-I. A first model controlled for the covariables age, sex and BMI. A second model controlled for age, sex, BMI, SBP, DBP, γ-glutamyltransferase activity, oral contraceptive use, smoking, physical excercise, and socio-economic status. A third model controlled for apo E phenotypes, in addition to the variables controlled for in model 2. The average effects of the apo E

alleles (α_i with i = 2, 3, 4) on the adjusted serum lipid and (apo)lipoprotein concentrations and the variances of these effects attributable to genotypic differences were estimated according to the method of Sing and Davignon (5). Significance of each estimated α_i (i = 2, 3, 4) was also tested ($Z = (\alpha'_i - 0) / \sqrt{(var(\alpha'_i))}$, with $\alpha'_i = \alpha_i - \alpha_{mean}$, the null hypothesis ($H_0$: $\alpha'_i = 0$) being rejected if $| Z | > 2$ at $\alpha = 0.05$).

A χ^2 goodness-of-fit test was used to test the genetic Hardy-Weinberg equilibrium for the apo E polymorphism. Apo E allele frequencies were estimated using the gene-counting method. The difference in apo E allele frequencies between the two regions was tested using a χ^2-association test. Differences in mean lipid and (apo)lipoprotein levels between apo E phenotypic groups were tested parametrically using one-way analysis of variance with the Student-Newman-Keuls multiple range test or Kruskal-Wallis non-parametric test. A significance level of $\alpha = 0.05$ was adopted throughout this article.

RESULTS

Description of the studied population sample

Anthropometric and other characteristics of the Belgian population sample, stratified by region, sex and 10-year age classes, are presented in table 1. Mean body mass index (BMI) was significantly higher in French-speaking males than in Dutch-speaking males, in both age classes. BMI also tended to be higher in French-speaking females than in Dutch-speaking females. The socio-economic status and the age at graduation was generally lower in Walloon participants compared to Flemish participants, reaching statistical significance in nearly all strata.

Serum lipids and (apo)lipoprotein parameters

Average serum lipid, Lp(a) and apolipoprotein A-I and B levels by region, sex and age class are presented in table 2. Cholesterol and LDL-cholesterol levels were higher in Walloons, irrespective of gender and age class. The difference was significant in 30-39 year old males. Analogously, the apo B level was significantly

higher in 30-39 year old male Walloons compared to their Flemish counterparts. Other adverse characteristics of the lipid profile in Walloon males were the significantly lower HDL-c and apo A-I levels in 30-39 year old participants.

Serum Lp(a) levels (table 3) were similar in Walloons and Flemings, median Lp(a) being 67 mg/l in Flemings and 75 mg/l in Walloons. The overall median Lp(a) level in the 900 Belgians was 68 mg/l.

Factors influencing lipid, apolipoprotein and Lp(a) parameters

Oral contraceptives:

The impact of oral contraceptives on the serum lipid profile of Dutch- and French- speaking females is shown in table 4. Oral contraceptives increased average serum triglyceride and apo A-I levels in all strata. Analogously, HDL-c levels showed a tendency to higher levels in females on oral contraceptives. Lp(a) levels were not affected by oral contraceptive use, nor by smoking or physical exercise (data not shown).

Apo E polymorphism:

The apo E phenotype distribution, as determined in a subgroup (N = 249) that was matched for region, is presented in table 5. The observed phenotype distribution was in Hardy-Weinberg equilibrium within each region. Apo E phenotype distributions were similar in Flemings and Walloons. Consequently, the data were pooled. The overall relative apo E allele frequencies in the studied sample were 0.092 for ε2, 0.767 for ε3 and 0.141 for ε4.

The average impact of the apo E polymorphism on apo B-containing lipoprotein levels appeared large in this population sample, cholesterol, LDL-c and apo B levels being lowest in E2-carriers and highest in E4-carriers. The trend was obvious, already by univariate analysis using unadjusted values (table 6). On the contrary, no significant effect of the apo E polymorphism could be demonstrated upon serum apo A-I, HDL-c, triglyceride and Lp(a) levels.

Multivariable models explaining phenotypic variance

The simplest MLR model (table 7), controlling for age, gender and BMI, explained 4% to 6.4% of the variances in cholesterol, LDL-c and apo B levels,

but 29.7% and 33.6% of the apo A-I and HDL-c variances, respectively. Model 2, which additionally adjusted for multiple lifestyle factors, explained 14.1% to 18.8% of the variances in cholesterol, LDL-c and apo B levels, and 31.9%, 33.9% and 39.9% of triglyceride, apo A-I and HDL-c variances, respectively. The third MLR model controlling for apo E phenotypes in addition to the parameters controlled for in model 2, is obviously the best, explaining 25% to 41% of the observed phenotypic variation of all lipid parameters, except for Lp(a). Common predictors of total cholesterol, LDL-c and apo B levels were smoking, linear age, GGT (ln) and apo E2-containing phenotypes. None of the models predicted Lp(a) variances (probability F statistics: NS).

Applying the second best MLR model to the entire Belgian population sample (N = 900) smoking and body mass index were major predictors associated with all lipoprotein and apolipoprotein levels, except with Lp(a) (data not shown). The activity of γ-glutamyltransferase, an indicator of alcohol (ab)use, was positively associated with triglycerides, total and LDL-cholesterol, and apo B levels. As expected, apo A-I and HDL-c were associated positively with use of contraceptive hormones, and negatively with smoking and BMI.

Average effects of the three common apo E alleles on adjusted serum lipid and (apo)lipoprotein levels

Estimates and significancies of the average effects of the common apo E alleles on adjusted serum lipid, apolipoprotein and lipoprotein(a) levels are presented in table 8. Striking are the regional differences in apo E allelic effects on adjusted serum lipid levels: in southerners the ε4 and ε2 effects upon adjusted apo B and LDL-c levels were approximately +12% respectively -15% ($P < 0.05$), whereas halved (≈ +5%; NS) respectively doubled (≈ -25%; $P < 0.05$) in northerners.

Overall, presence of the ε2 allele reduced serum cholesterol levels on average by 11%, and LDL-c and apo B levels by 20 and 19%, respectively ($P < 0.05$). Presence of the ε4 allele increased cholesterol levels by 5% (NS), and LDL-c and apo B levels by 11% ($P < 0.05$). In case of the ε3 allele serum triglyceride levels were significantly lower ($P < 0.05$).

Contribution of the apo E locus to the total variability of serum lipids and (apo)lipoproteins in the Belgian population sample

Using the third MLR model, the apo E locus explained 8.8% of the total cholesterol variance, 13.5% of the LDL-c variance and 16.9% of the apo B variance. Less than 4% of the HDL-c, apo A-I, triglyceride and Lp(a) variances could be attributed to the apo E locus (data not shown).

DISCUSSION

It has been established that besides lifestyle factors, heridity attributes to about 50% of interindividual serum cholesterol variation (22). The involvement of many candidate genes in determining phenotypic variation in serum cholesterol and other serum lipids is supported by research reviewed by e.g. Ferrell (22). In general, polymorphic variation in several genes influences variation in a particular trait -e.g. serum cholesterol- and each gene influences variation in more than one trait. As a rule, a particular gene explains only a fraction of variation in a particular trait. The apo E polymorphism e.g. explains 1% of the variation in total serum cholesterol in males, but 10% in females (23). The gene coding for the apo(a) molecule is an exception, allelic variation in the apo(a) gene determining as much as 90% of the interindividual variation in Lp(a) mass, and hence Lp(a)-cholesterol, in the population at large (24).

Although, with respect to the Belgian regional cholesterol differences, intake of a diet rich in saturated fat has been frequently invoked to explain the more adverse serum lipid distribution and higher mortality rate due to cardiovascular disease in Wallonia than in Flanders (3, 4), we aimed to examine whether differences in genetic stock between Belgian northerners and southerners might exist and contribute to the observed regional cholesterol differences. To that, Lp(a) levels and apo E polymorphism, two genetically determined cardiovascular risk factors that affect serum cholesterol, were assessed.

Table 1. Anthropometric and other characteristics in Flemings and Walloons, as stratified by sex and age category

Sex = male

Variable		20 - 29 years			30 - 39 years		
Mean ± S.D. or %	Units	Flanders N = 116	Wallonia N = 41	P-value	Flanders N = 164	Wallonia N = 91	P-value
Age	years	25.6 ± 2.7	26.2 ± 2.4	0.227	34.7 ± 2.7	35.0 ± 2.9	0.422
Height	cm	178.2 ± 6.0	176.9 ± 5.8	0.246	177.5 ± 6.6	175.6 ± 6.1	0.029
Weight	kg	72.3 ± 9.9	77.4 ± 12.3	0.008	75.7 ± 10.5	79.3 ± 12.0	0.014
Body mass index	kg/m^2	22.7 ± 2.7	24.7 ± 3.6	0.002	24.0 ± 3.0	25.7 ± 3.7	< 0.0005
Systolic blood pressure	mmHg	123 ± 11	126 ± 12	0.101	123 ± 11	130 ± 13	< 0.0005
Diastolic blood pressure	mmHg	76 ± 9	76 ± 9	0.811	76 ± 8	79 ± 10	0.034
GGT[a]	U/l	11.6 ± 6.3	16.2 ± 18.8	0.433	16.0 ± 12.9	8.8 ± 3.5	0.105
Age at graduation	years	21.3 ± 2.3	19.8 ± 2.5	0.001	21.3 ± 2.6	19.6 ± 2.8	< 0.0005
Current smokers	%	23.3	41.5	0.043	21.3	38.5	0.005
Alcohol users	%	89.7	78.0	0.107	89.6	85.5	0.330
Socio-economic class	%			< 0.0005			< 0.0005
I		13.9	12.2		18.9	4.4	
II		45.3	9.8		41.5	18.7	
IIIN		28.7	12.2		26.8	8.8	
IIIM		11.3	48.8		9.8	42.9	
IV		0.9	17.1		3.0	23.1	
V		-	-		-	2.2	

[a]γ-glutamyltransferase activity

Table 1. Cont'd.

Sex = female

Variable Mean ± S.D. or %	Units	20 - 29 years Flanders N = 211	20 - 29 years Wallonia N = 32	P-value	30 - 39 years Flanders N = 196	30 - 39 years Wallonia N = 53	P-value
Age	years	25.6 ± 2.6	25.7 ± 2.7	0.891	34.6 ± 2.8	34.8 ± 3.0	0.724
Height	cm	166.1 ± 6.0	164.0 ± 5.5	0.055	164.8 ± 5.6	161.8 ± 7.5	0.008
Weight	kg	60.2 ± 9.4	60.9 ± 9.3	0.692	60.2 ± 7.9	61.1 ± 11.2	0.579
Body mass index	kg/m^2	21.8 ± 3.0	22.6 ± 3.1	0.143	22.2 ± 2.7	23.4 ± 4.5	0.057
Systolic blood pressure	mmHg	115 ± 10	119 ± 14	0.163	116 ± 11	120 ± 15	0.113
Diastolic blood pressure	mmHg	73 ± 8	71 ± 8	0.333	74 ± 8	74 ± 11	0.717
GGT[a]	U/l	8.3 ± 4.2	8.9 ± 7.0	0.791	8.8 ± 3.5	11.0 ± 6.9	0.164
Age at graduation	years	20.5 ± 2.1	19.5 ± 1.9	0.014	19.6 ± 2.8	18.8 ± 2.6	0.067
Current smokers	%	23.2	28.1	0.701	27.0	37.7	0.178
Alcohol users	%	64.0	50.0	0.186	63.8	58.5	0.585
Oral contraceptive use	%	67.3	65.6	1.000	39.3	50.9	0.171
Socio-economic class	%			0.002			0.009
I		5.2	-		4.1	-	
II		42.2	28.1		36.7	28.3	
IIIN		35.5	25.0		31.1	20.8	
IIIM		10.4	31.3		7.7	20.8	
IV		6.2	15.6		6.3	20.8	
V		0.5	-		4.1	9.4	

[a]γ-glutamyltransferase activity

Table 2. Mean (± S.D.) serum lipid, lipoprotein and apolipoprotein levels in Belgians, by region, sex and age category

Sex = male

Variable Mean ± S.D.	Units	20 - 29 years			30 - 39 years		
		Flanders N = 116	Wallonia N = 41	P-value	Flanders N = 164	Wallonia N = 91	P-value
Triglycerides	mmol/l	1.032 ± 0.438	1.213 ± 0.711	0.408	1.381 ± 1.212	2.088 ± 2.105	0.002
Cholesterol	mmol/l	4.834 ± 0.767	5.087 ± 0.815	0.076	5.297 ± 0.892	5.900 ± 1.455	< 0.001
LDL-cholesterol (PVS)[a]	mmol/l	3.126 ± 0.837	3.248 ± 0.733	0.410	3.533 ± 0.898	3.936 ± 1.260	0.009
LDL-cholesterol (Friedewald)[b]	mmol/l	3.034 ± 0.737	3.243 ± 0.639	0.108	3.400 ± 0.836	3.786 ± 1.247	0.009
LDL-cholesterol (Friedewald, corrected for Lp(a)-cholesterol)[c]	mmol/l	2.937 ± 0.725	3.097 ± 0.607	0.207	3.276 ± 0.800	3.651 ± 1.241	0.010
HDL-cholesterol	mmol/l	1.326 ± 0.310	1.292 ± 0.282	0.539	1.284 ± 0.290	1.164 ± 0.290	0.002
Lp(a)	mg/l	125 ± 169	188 ± 207	-	165 ± 204	174 ± 221	-
Ln Lp(a)[d]	Ln mg/l	4.082 ± 1.340	4.300 ± 1.672	0.406	4.312 ± 1.419	4.354 ± 1.402	0.822
Apolipoprotein A-I	g/l	1.24 ± 0.21	1.21 ± 0.20	0.495	1.27 ± 0.22	1.16 ± 0.21	< 0.001
Apolipoprotein B	g/l	0.70 ± 0.16	0.72 ± 0.16	0.523	0.79 ± 0.18	0.87 ± 0.25	0.010

[a]LDL-cholesterol as determined after polyvinyl sulfate (PVS) precipitation of LDL and Lp(a), by subtracting supernatant cholesterol from total cholesterol.

[b]LDL-cholesterol as estimated by the Friedewald formula, i.e. LDL-c (mmol/l) = total cholesterol - HDL-cholesterol - triglycerides/2.2.

[c]LDL-cholesterol as estimated by the Friedewald formula, corrected for Lp(a)-cholesterol, i.e. LDL-c (mmol/l) = total cholesterol - HDL-cholesterol - triglycerides/2.2 - (0.3*Lp(a)/386.7) (6).

[d]Ln Lp(a) = naturally logarithmically transformed Lp(a).

Table 2. Cont'd.

Sex = female

Variable Mean ± S.D.	Units	20 - 29 years Flanders N = 112	20 - 29 years Wallonia N = 32	P-value	30 - 39 years Flanders N = 196	30 - 39 years Wallonia N = 53	P-value
Triglycerides	mmol/l	1.090 ± 0.404	1.109 ± 0.465	0.725	1.005 ± 0.464	1.148 ± 0.579	0.136
Cholesterol	mmol/l	5.111 ± 0.960	5.438 ± 1.100	0.080	5.045 ± 0.897	5.212 ± 1.160	0.333
LDL-cholesterol (PVS)[a]	mmol/l	3.101 ± 1.015	3.424 ± 1.124	0.099	3.057 ± 0.946	3.179 ± 1.188	0.490
LDL-cholesterol (Friedewald)[b]	mmol/l	2.963 ± 0.894	3.284 ± 1.016	0.064	2.954 ± 0.874	3.107 ± 1.100	0.352
LDL-cholesterol (Friedewald, corrected for Lp(a)-cholesterol)[c]	mmol/l	2.856 ± 0.885	3.136 ± 0.964	0.101	2.841 ± 0.862	3.001 ± 1.050	0.253
HDL-cholesterol	mmol/l	1.653 ± 0.366	1.649 ± 0.392	0.961	1.634 ± 0.343	1.583 ± 0.357	0.340
Lp(a)	mg/l	136 ± 177	192 ± 269	-	146 ± 166	137 ± 183	-
Ln Lp(a)[d]	Ln mg/l	4.188 ± 1.291	4.144 ± 1.708	0.888	4.283 ± 1.295	4.013 ± 1.524	0.197
Apolipoprotein A-I	g/l	1.56 ± 0.32	1.52 ± 0.29	0.537	1.55 ± 0.30	1.50 ± 0.31	0.221
Apolipoprotein B	g/l	0.71 ± 0.19	0.73 ± 0.18	0.726	0.70 ± 0.18	0.70 ± 0.23	0.926

[a]LDL-cholesterol as determined after polyvinyl sulfate (PVS) precipitation of LDL and Lp(a), by subtracting supernatant cholesterol from total cholesterol.

[b]LDL-cholesterol as estimated by the Friedewald formula, i.e. LDL-c (mmol/l) = total cholesterol - HDL-cholesterol - triglycerides/2.2.

[c]LDL-cholesterol as estimated by the Friedewald formula, corrected for Lp(a)-cholesterol, i.e. LDL-c (mmol/l) = total cholesterol - HDL-cholesterol - triglycerides/2.2 - (0.3*Lp(a)/386.7) (6).

[d]Ln Lp(a) = naturally logarithmically transformed Lp(a).

Table 3. Serum Lp(a) levels in 900 randomly recruited Belgian employees, by region, sex and age class

Region	Age category	Sex	Number of subjects	Lp(a) mean (mg/l)	Lp(a) percentiles (mg/l)						
					5th	10th	25th	50th	75th	90th	95th
Flanders	20 - 29 yr	Male	115	125	6	10	31	64	131	355	497
		Female	210	136	8	11	30	63	171	366	530
	30 - 39 yr	Male	162	165	8	11	26	77	243	475	549
		Female	196	146	8	13	27	72	213	437	539
	Pooled		683	144	8	12	30	67	186	403	536
Wallonia	20 - 29 yr	Male	41	188	3	4	24	81	351	508	634
		Female	32	192	4	4	15	70	251	689	834
	30 - 39 yr	Male	91	174	5	13	34	69	240	496	698
		Female	53	137	4	6	21	61	141	415	571
	Pooled		217	170	4	8	26	75	247	513	664
Overall			900	150	6	11	28	68	192	426	561

After natural log transformation, average Lp(a) was not significantly different between Walloons and Flemings (P = 0.983).

Table 4. Impact of oral contraceptive use on serum triglycerides, apolipoprotein A-I, HDL-cholesterol and Lp(a) levels in Belgian females, stratified by region and age class

Parameter Mean ± S.D.	Units	Flemish females, 20 - 29 yr Oral contraceptives			Walloon females, 20 - 29 yr Oral contraceptives		
		No N = 69	Yes N = 169	P-value	No N = 11	Yes N = 21	P-value
Ln triglycerides[a]	Ln mmol/l	- 0.0985 ± 0.410	0.0771 ± 0.335	0.002	- 0.0992 ± 0.272	0.0905 ± 0.437	0.200
Apolipoprotein A-I	g/l	1.41 ± 0.32	1.63 ± 0.29	< 0.001	1.38 ± 0.23	1.60 ± 0.29	0.041
HDL-cholesterol	mmol/l	1.571 ± 0.396	1.693 ± 0.345	0.023	1.552 ± 0.281	1.701 ± 0.437	0.315
Ln Lp(a)	Ln mg/l	4.386 ± 1.307	4.093 ± 1.278	0.125	3.743 ± 2.128	4.353 ± 1.458	0.346
		Flemish females, 30 - 39 yr Oral contraceptives			Walloon females, 30 - 39 yr Oral contraceptives		
		No N = 119	Yes N = 77	P-value N = 26	No N = 27	Yes	P-value
Ln triglycerides	Ln mmol/l	- 0.214 ± 0.373	0.125 ± 0.368	< 0.001	- 0.189 ± 0.449	0.233 ± 0.414	0.001
Apolipoprotein A-I	g/l	1.51 ± 0.25	1.63 ± 0.36	0.011	1.36 ± 0.26	1.63 ± 0.30	0.001
HDL-cholesterol	mmol/l	1.625 ± 0.306	1.648 ± 0.396	0.667	1.527 ± 0.363	1.637 ± 0.350	0.266
Ln Lp(a)	Ln mg/l	4.313 ± 1.277	4.237 ± 1.328	0.687	3.913 ± 1.476	4.110 ± 1.590	0.644

[a]Ln triglycerides: naturally logarthmically transformed triglyceride levels.

Table 5. Apolipoprotein E phenotype distribution in 249 apparently healthy Belgians, stratified by region

Apo E phenotype	Flanders*	Wallonia*	Pooled
E2/2	1	0	1
E2/3	18	21	39
E3/3	77	69	146
E4/2	1	4	5
E4/3	27	24	51
E4/4	1	6	7
All phenotypes	125	124	249

*The apo E phenotype distribution is similar in Flemings and Walloons ($\chi^2 = 7.213$; df = 5; P = 0.2053).

The study was designed using an apparently healthy working population from the northern (Flanders) and the southern (Wallonia) region of Belgium. The study population has several advantages. First, the population is homogeneous with regard to ethnic origin as all participants were Caucasians. Second, the population is considered to be representative for the northern respectively the southern region of Belgium because no special exclusion criteria were used -with the exception of the exclusion of non-Caucasians, and pregnant or hysterectomized women- and because participants were recruited from 32 worksites located in the northern part of Belgium, and 15 worksites in the southern part of Belgium. Third, the study population contains both males and females at a reproductive age (20 to 39 years), excluding effects of menopause on serum lipid profiles. Fourth, information was collected on various factors that relate to lifestyle, such as smoking, physical exercise, oral contraceptive use, alcohol use and GGT activity, or that reflect both environmental and genetic factors, such as body mass index. Fifth, fasting sera were collected, enabling valid determinations of HDL-c, LDL-c and triglycerides (18, 19, 25).

Major outcomes of this study were the following. First, the more adverse serum lipid profile in southerners compared to northerners (table 2) was reconfirmed in this study, the difference being significant in 30 to 39 year old males.

Table 6. Unadjusted serum lipid, apolipoprotein and lipoprotein(a) levels by apolipoprotein E phenotype in 249 apparently healthy Belgian males and females.

Mean ± S.D.	Units	Apo E phenotype						One-way ANOVA (SNK - KW)[b]
		E2/2	E2/3	E3/3	E4/2	E4/3	E4/4	Probability F ratio
Number	-	1	39	146	5	51	7	-
Relative frequency[a]	%	0.4	15.7	58.6	2.0	20.5	2.8	-
Variable:								
Triglycerides	mmol/l	1.184	1.552 ± 1.226	1.191 ± 0.829	1.431 ± 0.408	1.499 ± 1.888	2.993 ± 4.317	NS[c]
Cholesterol	mmol/l	3.501	4.878 ± 1.001	5.454 ± 1.063	5.767 ± 1.061	5.633 ± 1.198	6.709 ± 1.981	0.0011
LDL-cholesterol[d]	mmol/l	1.821	2.867 ± 0.885	3.573 ± 1.100	3.713 ± 1.105	3.816 ± 1.143	4.310 ± 1.571	0.0001
(HDL-cholesterol	mmol/l	1.19	1.382 ± 0.397	1.431 ± 0.372	1.360 ± 0.499	1.370 ± 0.426	1.304 ± 0.503	NS
Apolipoprotein A-I	g/l	1.15	1.27 ± 0.26	1.27 ± 0.24	1.24 ± 0.23	1.24 ± 0.23	1.25 ± 0.45	NS
Apolipoprotein B	g/l	0.32	0.67 ± 0.22	0.80 ± 0.21	0.86 ± 0.17	0.86 ± 0.24	0.97 ± 0.30	< 0.0001
Ln Lp(a)[e]	Ln mg/l	4.852	3.965 ± 1.602	4.397 ± 1.419	5.109 ± 1.161	4.215 ± 1.646	3.565 ± 0.558	NS
Median Ln Lp(a)	Ln mg/l	-	3.714	4.477	5.124	4.331	3.526	-

[a]The phenotype distribution is in Hardy-Weinberg equilibrium (Goodness-of-fit test; $\chi^2 = 2.334$; df = 5; P = 0.801). [b] P-value indicating the significance level of the difference among phenotypic means of the lipid traits, calculated by one-way ANOVA and using a Student-Newman-Keuls (SNK) parametric test or a Kruskal-Wallis (KW) non-parametric test. [c] NS: not significant at $\alpha = 0.05$. [d] LDL-cholesterol as determined after polyvinyl sulfate (PVS) precipitation of LDL and Lp(a), by subtracting supernatant cholesterol from total cholesterol. [e]Ln Lp(a): naturally logarithmically transformed Lp(a).

Table 7. Percentage total phenotypic variance explained by different multivariable models in a randomly selected Belgian population sample (N = 249).

Dependent variable	Multivariate regression analysis / Adjusted R^2 (%)		
	Model 1	Model 2	Model 3
Cholesterol (ln)	4.0%	18.8%	25.5%
LDL-cholesterol (ln)[a]	6.0%	14.1%	25.6%
Apolipoprotein B (ln)	6.4%	18.8%	31.8%
Lipoprotein(a) (ln)	0.0%	2.1%	3.5%
Triglycerides (ln)	15.3%	31.9%	34.0%
Apolipoprotein A-I	29.7%	33.9%	33.7%
HDL-cholesterol	33.6%	39.9%	40.5%

Model 1 includes the covariables age, sex and body mass index. Model 2 includes the covariables age, sex, body mass index, systolic and diastolic blood pressure, smoking and sporting habits, use of oral contraceptives, γ-glutamyltransferase activity and socio-economic status.
Model 3 controls for apo E phenotypes in addition to the parameters controlled for in model 2.
The probability of the F ratios was highly significant for all models, except for Lp(a).
[a]LDL-cholesterol as determined after polyvinyl sulfate (PVS) precipitation of LDL and Lp(a), by subtracting supernatant cholesterol from total cholesterol.

This finding is in accordance with previous publications examining north-south differences in cardiovascular risk factor distribution, and implies that regional differences in serum lipid distribution in Belgium, first observed in the sixties, still persist in the nineties (1-4).

Second, similar median and log mean Lp(a) levels were found in Belgian northerners and southerners (table 3). Accordingly, mean estimated LDL-c levels remained 5 to 11% lower in Flemings compared to Walloons within each age and gender class, also after correcting for Lp(a)-cholesterol content (table 2). After all, the average contribution of Lp(a)-cholesterol to estimated LDL-c was similar in both regions and amounted maximally 4.5% within each category. Consequently Lp(a), and hence Lp(a)-cholesterol, do not contribute to the observed regional cholesterol differences.

Third, the apo E phenotype distribution was found to be similar in both regions (table 5). Besides, the overall apo E allele frequencies counted in this study

Table 8. Estimates and significancies of the average effects of the three common apo E alleles on adjusted serum lipid, apolipoprotein and lipoprotein(a) levels in a random sample of unrelated, healthy Belgian employees (N = 249).

Parameter		Estimated average effect of the apo E alleles								
		Flanders			Wallonia			Pooled		
	Units	α_2	α_3	α_4	α_2	α_3	α_4	α_2	α_3	α_4
Cholesterol	mmol/l	x 0.85*	x 1.02	x 1.02	x 0.92*	x 1.00	x 1.06	x 0.89*	x 1.00	x 1.05
LDL-cholesterol (PVS)[a]	mmol/l	x 0.73*	x 1.03*	x 1.04	x 0.85*	x 1.00	x 1.12*	x 0.80*	x 1.01	x 1.11*
Apolipoprotein B	g/l	x 0.77*	x 1.02	x 1.06	x 0.85*	x 1.00	x 1.13*	x 0.81*	x 1.01	x 1.11*
Triglycerides	mmol/l	x 1.12	x 0.98	x 1.05	x 1.07	x 0.97	x 1.12	x 1.14	x 0.97*	x 1.11
Lp(a)	mg/l	x 0.75	x 1.01	x 1.13	x 1.05	x 1.07	x 0.68	x 0.96	x 1.03	x 0.86
HDL-cholesterol	mmol/l	- 0.046	+ 0.001	+ 0.023	+ 0.003	+ 0.016	- 0.091	- 0.036	+ 0.014	- 0.051
Apolipoprotein A-I	g/l	+ 0.026	- 0.004	+ 0.002	+ 0.006	+ 0.007	- 0.040	+ 0.010	+ 0.004	- 0.027

Serum lipid, apolipoprotein and Lp(a) data were adjusted for age, sex, body mass index, systolic and diastolic blood pressure, smoking, physical exercise, use of oral contraceptives, γ-glutamyltransferase activity and socio-economic status. Apo E allelic effects were calculated according to the method of Sing and Davignon (5). *: Apo E allelic effects significant at $\alpha = 0.05$. See statistical analysis for calculating significancies of the apo E allelic effects.

[a]LDL-cholesterol as determined after polyvinyl sulfate (PVS) precipitation of LDL and Lp(a), by subtracting supernatant cholesterol from total cholesterol.

were similar to the apo E allele frequencies reported by Braeckman and coworkers in 30 to 59 year old Flemish males ($f_{\varepsilon 2}$, $f_{\varepsilon 3}$ and $f_{\varepsilon 4}$: 0.092, 0.767 and 0.141 (this study) versus 0.072, 0.765 and 0.163 (26)). Also, the associations of apo E polymorphism with the lipids and apolipoproteins analyzed (tables 6 and 8) were consistent with the well identified effects of apo E, the average effect of the ε2 allele being to lower LDL-c and apo B, and of the ε4 allele being to increase LDL-c and apo B (5, 7, 10, 23). Whereas the ε effect on serum (LDL-) cholesterol and apo B levels is consistent across most population studies, there are controversial results about the ε effect on triglycerides, since these vary widely within and among individuals, masking a clear effect of apo E phenotype. In this study ε2 and ε4 alleles tended to increase the level of triglycerides (NS), in accordance with the meta-analysis of Dallongeville and coworkers (27), and the EARS study results (10).

Strikingly, the magnitudes of the apo E allelic effects upon adjusted LDL-c and apo B levels differed between Belgian northerners and southerners (table 8). In northerners, the LDL-c and apo B increasing effect attributable to ε4 was minor and insignificant (≈ +5%), whereas the decreasing effect by ε2 was hudge and significant (≈ -25%); in southerners however, ε4 increased adjusted LDL-c and apo B levels by ≈ +12% ($P < 0.05$), while ε2 decreased these lipid parameters to a similar extent ($P < 0.05$). The less favourable apo E allelic effects in southerners compared to northerners cause the more adverse lipid profile in southerners. Although the association of ε2 with lower (LDL-) cholesterol and apo B, and of ε4 with higher (LDL-)cholesterol and apo B is well established in the general population, considerable heterogeneity with respect to the magnitude of the allelic effects estimated in different populations has been reported (7). Several studies have shown a lowering effect of ε2 on cholesterol levels double to fourfold the ε4 increasing effect (Flemings in this study), whereas similar effects of both have been reported elsewhere (Walloons in this study, 28, 29). Eco-genetic interactions have been hold responsible, at least in homogenous population samples (30), whereas confounding might have occurred in case of heterogenous samples due to different apo E allele frequencies (29). As to the origin of the regional differences in ε effects between Belgian northerners and southerners, a gene-environment interaction could cause different allelic effects under different environmental

conditions in the same population. In view of the well-documented higher intake of saturated fat and dietary cholesterol in the south of Belgium compared to the north (3-4), we hypothesize that the less favourable saturated fat intake in southerners explains the differences in ε effects. Support is given by a recent meta-analysis, showing that apo E genotype effects are modulated via alterations of amount and type of dietary fat (30), and by others (31). In contrast to dietary saturated fat, the apo E gene loci do not have a major effect on the response of lipid levels to increased dietary cholesterol (32).

Overall, the apo E locus contributed substantially to the variances of serum total cholesterol, LDL-c and apo B (8.8, 13.5 and 16.9% respectively), far exceeding the 0.9 and 2.8% reported for total cholesterol respectively apo B by Braeckman et al. in Flemish males (26). In view of the reported differences between men and women with regard to the impact of the apo E genotype on both the means and variances of the distributions of serum lipids and apolipoproteins (36), the large contribution of the apo E locus to the variances of cholesterol, LDL-c and apo B is probably related to the inclusion of women in the sample, and especially of women taking exogenous hormones (57% of the studied females). After all, the apo E polymorphism is reported to explain up to 10% of the adjusted interindividual serum cholesterol variation in females, compared to only 1% in males (23). Furthermore, if women are included the increasing effect of ε4 is smaller and the decreasing effect of ε2 is larger, as one can expect from the protective effect of estrogen through upregulation of the hepatic LDL receptor (33). Also, women having an ε4 allele and taking exogenous hormones showed a greater cholesterol-elevating effect of this allele than women not taking hormones (36). In accordance with others (5, 7, 23, 27) the contribution of the apo E locus to the triglyceride, apo A-I and HDL-c variances was minor (3.5%, 1.0% and 2.0% respectively).

Fourth, the apo E polymorphism did not affect serum Lp(a) levels, supporting the contention that the LDL-receptor is not a major contributor to the Lp(a) catabolism (12, 24). The lack of relationship between apo E phenotype and Lp(a) was also observed by Schaefer et al. in the Framingham Offspring Study (33) and Muros et al. in a Spanish working population of Tenerife (34), but is dissimilar to the findings of Tiret and coworkers in the EARS study (10), where ε2 had a

lowering effect on Lp(a), and to the findings of de Knijff et al. (35), who showed in a Dutch population sample a 25% increasing effect on serum Lp(a) level by the ε4 allele, equal and opposite to the ε2 effect.

To the best of our knowledge, this study is the first examining the contribution of Lp(a) and apo E polymorphism to regional cholesterol differences in Flanders and Wallonia. Drawbacks of this study may be related to the limited sample size and the fact that apo E polymorphism was checked in a subgroup only. Second, no dietary survey was performed, and hence the hypothesis regarding a diet - apo E genotype interaction being responsible for the regional differences in ε effects, remains to be proven. Third, other candidate gene loci which have not been investigated here might also be involved and co-determine responsiveness of serum lipids to dietary fat alterations (30).

In conclusion, the more adverse lipid profile in Belgian southerners compared to northerners is reconfirmed in this study. As to the origin of the regional cholesterol differences, no differences could be demonstrated in serum Lp(a) or apo E phenotype distribution between northerners and southerners, supporting a similar genetic background. On the contrary, the average effects of the apo E alleles upon adjusted LDL-c and apo B levels differed between the regions, the adverse ε4 effect being doubled and the protective ε2 effect being halved in southerners compared to northerners. These findings suggest that similar genetic information variably affects intermediate traits in Flanders and Wallonia in particular environments.

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Chapter 7

POSTOPERATIVE TIME COURSES OF SERUM LIPOPROTEIN(a), SERUM LIPIDS AND SERUM APOLIPOPROTEINS IN PLACEBO AND PRAVASTATIN TREATED CAUCASIAN MALES UNDERGOING CORONARY ARTERY BYPASS GRAFTING

Based upon:

Time Course of Serum Lipids and Lipoprotein Levels after Coronary Bypass Surgery: Modifications by Pravastatin.
Acta Cardiologica 1992; XLVII: 519-28.

Time Course of Serum Lp(a) in Men after Coronary Artery Bypass Grafting.
Acta Cardiologica 1992; XLVII: 529-48.

ABSTRACT

The serum Lp(a) time course was studied in 100 Caucasian male patients who underwent coronary artery bypass grafting (CABG). The patients were randomized in a placebo (N = 50) and a pravastatin treated (N = 50) group. The pravastatin regimen was 10 mg daily from the third post-operative day on and 20 mg daily after 1 week during 11 weeks. Lp(a) levels and serum lipids were analyzed at baseline, at 3 and 10 days, and at 4 and 12 weeks post-CABG.

Serum Lp(a) levels were decreased at the third post-operative day, parallelling the changes observed for the other serum lipids and apolipoproteins when using extracorporeal circulation. The mean Lp(a) decline at the third post-operative day was 60.8% in the placebo group and 58.2% in the pravastatin group. In contrast with the other serum lipids, a slight but significant Lp(a) overshooting was noticed at day 10, followed by a decrease of the serum Lp(a) levels to pre-operative levels one month after the acute event. The Lp(a) time courses were equal in respectively placebo and pravastatin treated patients, underscoring that pravastatin treatment did not exert a statistically significant effect on serum Lp(a) levels at any time point.

It is concluded that significant time-dependent changes of serum Lp(a) occur post-CABG, and that reliable post-operative Lp(a) measurements can be made at earliest one month post-CABG.

INTRODUCTION

Serum lipids are known to decrease after coronary bypass grafting (CABG) (1, 2), aortafemoral bypass (3), abdominal surgery (4) and acute myocardial infarction (5, 6). As there is emerging evidence at the moment that lipoprotein Lp(a) is a new independent atherothrombotic risk factor (7-15) and as nothing is known about the Lp(a) time course after coronary bypass surgery, we studied the serum Lp(a) changes in 100 male patients who underwent CABG.

Lipoprotein(a) can be defined as a genetic variant of LDL, having as a protein

moiety apo B-100 disulfide-linked to apo(a), the distinctive glycoprotein of Lp(a) that is structurally related to plasminogen. Epidemiological studies and studies at the level of the arterial wall pointed out that Lp(a) is an independent cardiovascular pathogen (16, 17). Serum Lp(a) is also a marker for saphenous vein graft occlusion after CABG (7). The exact mechanism of its atherogenicity is not known, however, because of its structural similarities with plasminogen (18), Lp(a) has been considered a lipoprotein particle with both thrombogenic and atherogenic potential. In the general population Lp(a) levels vary widely among individuals -on the order of 1000 fold-, in spite of the rather limited intraindividual changes. Serum Lp(a) levels are mainly (± 60%) determined by alleles at the hypervariable apo(a) gene locus, but other genetic, hormonal and environmental factors also affect serum Lp(a) levels (19, 20). In addition, Lp(a) has been hypothesized to be an acute phase reactant (21, 22). The serum Lp(a) distribution in healthy Caucasians is highly skewed to the right, with a median Lp(a) value lower than the population mean. After natural Ln transformation the serum Lp(a) distribution becomes almost Gaussian (23). Consequently, either median or Ln transformed Lp(a) data will be discussed further on. Aside from several promising leads, there are no universally accepted ways to lower high plasma Lp(a) levels (12). At this time, lipidologists target efforts towards the modifiable risk factors by using the appropriate diet and, whenever necessary, drug therapy. Nevertheless, assessment of the Lp(a) lipid status is very valuable in cases of premature cardiovascular disease in normolipidemic individuals.

The aims of this study were multiple: 1) to document the serum Lp(a) changes during the 12 week follow-up period after CABG, both in a placebo group and in a treatment group where pravastatin, a HMG-Coenzyme A reductase inhibitor, was given from the third post-operative day on; 2) to compare the Lp(a) time course post-CABG with the time course of the other lipids, again in treatment and placebo group; 3) to compare the Lp(a) changes in comparison with other surgical interventions reported in literature; 4) to evaluate in what time-frame after CABG a reliable Lp(a) lipid status assessment can be performed.

MATERIALS AND METHODS

Study population

The study population consisted of 100 male subjects (age class 39-61 years) in whom CABG was planned. In order to enter the study the serum cholesterol level at intake had to be between 180 and 280 mg/dl; subjects with a cholesterol higher than 280 mg/dl were not included as drug treatment was considered to be a necessity in these cases. Patients with insulin-dependent diabetes, renal insufficiency (serum creatinine > 1.5 mg/dl), cerebro-vascular accidents, liver disease and other debilitating diseases were excluded from the study. The selection of the participants occurred between January 1991 and March 1992. The patients were randomized (table 1) to the placebo (N = 50; mean age = 54.5 years, S.D. = 4.4 years) or the treatment group (N = 50; mean age = 53.1 years; S.D. = 5.1 years). The daily treatment in the latter consisted of 10 mg pravastatin from the third day post-CABG during the first week, followed by 20 mg during the next 11 weeks. All operations were performed with assistance of the heart-lung machine. Informed consent was obtained from all participants and the study protocol was approved by the ethical committee of the University Hospital Leuven.

Patient samples

Venous blood samples were taken after an overnight fast the morning before surgery (D0), the third (D3) and tenth (D10) day post-CABG, and after 4 (W4) and 12 weeks (W12). Blood was collected into SST Vacutainer tubes (Becton-Dickenson) for the serum lipid and creatinine assessment, into heparin Vacutainer tubes for ASAT, ALAT and GGT determinations and into EDTA Vacutainer tubes for haemoglobin and haematocrit determinations. The serum lipids were evaluated at every sampling time while the control parameters were only evaluated at D0, W4 and W12 after coronary bypass surgery.

Laboratory Methods

Lipoprotein (a) was measured using a commercial anti-apo (a) polyclonal sandwich-type ELISA immunoassay, named TintElize™ lipoprotein(a), Cat. Nr.

610220 (Biopool AB, Umeå, Sweden) according to the instruction sheet of the manufacturer. Using the above described method of analysis for Lp(a), a between-day coefficient of variation of 7.7% was obtained at an Lp(a) mass level of 184 mg/l (N = 23). Cross-reactivity with plasminogen was checked and could not be demonstrated up to 1000 mg/l.

Cholesterol on the one hand and HDL- and LDL-cholesterol on the other hand were determined enzymatically on a Hitachi 737 (Boehringer) respectively RA 1000 analyser (Technicon) with CHOD-PAP reagents (Cat. Nr. 237574, Boehringer, Mannheim, Germany); HDL-cholesterol was determined in the supernatant after precipitation of all apo B-containing lipoproteins with $MgCl_2$/ phosphotungstic acid (Cat. Nr. 400971, Boehringer, Mannheim, Germany). LDL-cholesterol was determined indirectly: LDL was precipitated with polyvinyl sulfate in an imidazole buffer (Cat. Nr. 726290, Boehringer, Mannheim, Germany), non-LDL-cholesterol was determined in the supernatant, LDL-c was calculated by subtracting the non-LDL-cholesterol from the total cholesterol. Apo A-I and apo B were analyzed by immunoturbidimetry on a Hitachi 705 analyser using polyclonal antisera (Cat. Nr. 1174371 and 1174380 for apo A-I and apo B respectively, Boehringer, Mannheim, Germany). Control parameters were determined using standard procedures.

Statistical methods

For the statistical analysis the placebo group was compared with the pravastatin treated group. Calculations were done with the package Statistical Analysis System (SAS). The time course of the lipid parameters was evaluated using a repeated measurements model including all dropouts. This model assumes normally distributed data at all time points (this is satisfied for all lipid parameters and after Ln tranformation for the Lp(a) data) and also that the dropout reason is not related to the value of the lipid parameters at the time of dropout. It was found that these assumptions could be made. The calculations were done with the BMDP program (BMDP). This analysis provides also corrected means of the lipid parameters at the different time points based on the fitted statistical model. Hereafter these means are called "model based" means. The Pearson correlation coefficients for Lp(a) versus the other lipid parameters were calculated after natural Ln

transformation of serum Lp(a) levels. Generally, a significance level of $\alpha = 0.05$ was adopted.

RESULTS

The baseline characteristics of the placebo and the pravastatin treatment groups are given in table 1. The matching was good as no statistically significant differences existed between the two groups (T-test and Wilcoxon test). Table 2 demonstrates the mean, median and mean log Lp(a) time course after CABG. An important decrease in the median Lp(a) level from 127.5 at D0 to 59 mg/l at D3 in the placebo group and from 141 at D0 to 74 mg/l at D3 in the treatment group can be seen. The fall parallels the one noted for the other serum lipids (table 3).
A strong positive correlation ($P < 0.001$) was found between the absolute Lp(a) decline -calculated as D0 - D3- and the pre-operative Lp(a) values at D0 ($r = 0.983$; decline = -22 + 0.70 X in the placebo group and $r = 0.959$; decline = -0.37 + 0.569 X in the pravastatin group). The mean Lp(a) decline at D3 was 60.8% in the placebo group and 58.2% in the pravastatin group. Repeated measurements analysis, taking dropouts into account, showed no statistically significant treatment effect on serum Lp(a) levels ($P = 0.61$) at all time points.

At D10 after CABG the median Lp(a) level had run up to 141 mg/l in the placebo group and to 150 mg/l in the treatment group. At 4 and 12 weeks median Lp(a) levels were respectively 150.5 and 158 mg/l in the placebo group and 172.5 and 171 mg/l in the pravastatin group. At the tenth post-operative day the median Lp(a) (table 2) and the mean log Lp(a) levels (figure 1, table 2) were even higher than the respectively pre-operative median Lp(a) and mean log Lp(a) values. This contrasts with the time course of the other serum lipids where baseline values of the lipid means in the placebo group were reached not until 12 weeks after coronary artery bypass surgery, according to this sampling scheme (figure 1, table 3). Repeated measurements analysis of variance, taking dropouts into account, demonstrated a statistically significant time effect in both the placebo and pravastatin treated groups ($P < 0.0001$) between the log Lp(a) levels at D3 versus D0 ($P < 0.0001$) and at D10 versus D0 ($P < 0.005$). However, no significant

difference could be shown for the log Lp(a) levels at W4 and W12 versus the pre-operative log Lp(a) values. Also, repeated measurements analysis showed no combined time and treatment effect (P = 0.82).

In the treatment group the cholesterol, LDL-c and apo B levels remained 20% - 30% lower compared to the placebo group due to the effect of the HMG-CoA reductase inhibitor (table 3). Pravastatin was well tolerated and no statistical evidence was found of a different number of adverse events under the two treatment regimens (two-tailed Fisher exact test). The control parameters ASAT, ALAT and GGT (table 1) were identical at the end of week 12 compared to preoperative levels (Wilcoxon test).

Statistically significant Pearson correlation coefficients arose at the third post-operative day between log Lp(a) serum levels and total cholesterol, HDL-c, LDL-c and apo A-I levels in the placebo group and with total cholesterol, LDL-c and apo B in the pravastatin group (table 4). In contrast, at D0 -with one exception- no statistically significant correlations for log Lp(a) with any of the lipids could be demonstrated. As pravastatin treatment was initiated only at D3, all patient data at D0 and D3 were pooled. In this case all correlations became highly significant at D3 and were not significant at D0 (table 4). Absence of significant correlations for log Lp(a) versus the serum lipids was also true at D10, W4 and W12.

Looking at the Lp(a) distributions at D0, D3, D10, W4 and W12 least skewness and kurtosis was seen at D10 and not at D3 (data not shown). At D3 the skewness to the right of the frequency distribution curve was comparable to that in the pre-operative state in both treatment and placebo group.

DISCUSSION

The decrease of mean and "model based" mean log serum Lp(a) levels (figure 1) at the third post-operative day parallels the changes noted with the other serum lipids when using extracorporeal circulation (1, 2). However, slight but significant Lp(a) overshooting at day 10 followed by a decrease of the serum Lp(a) levels to pre-operative levels 1 month after the acute event, contrasts with the 3 month time period needed for the other serum lipids in the placebo group in order

to reach baseline values. The reasons for the decline in serum lipids are multifactorial and incompletely understood: there is a pump time related removal of lipoproteins during extracorporeal circulation and different lipoprotein classes seem to be differentially affected (1).

Maeda et al. (22) have described serum Lp(a) as an acute phase protein as transient Lp(a) increases up to 221% of the baseline value 11 days after acute myocardial infarction, and up to 227% of the initial values 7 days after surgical operations occurred.

The reported Lp(a) increases follow the same time course as the CRP, α_1-antitrypsin, haptoglobin, interleukin 1 and 6, and orosomucoid increases (21, 22, 24). According to Maeda and co-authors, gradual increases in serum Lp(a) were observed during the first few days both in the acute myocardial infarct patient group as well as in the patient group that underwent surgical intervention, with return to baseline Lp(a) values more than 1 month after the acute episode. The authors concluded that Lp(a) is one of the acute phase proteins which play important functional roles in tissue recovery after infarction and surgical trauma.

From figure 1 it can be seen that in this cardiovascular surgery group the so-called "gradual Lp(a) increase" from the first days on, is either masked or absent. However, the four week time period needed for normalization of Lp(a) values after CABG fits with the report of Maeda et al. (22). In contrast to Maeda's study population, we have a larger and more strictly defined patient group: the ages ranged only from 39 to 61 years and all 100 male patients underwent one type of cardiovascular surgery. We hypothesize that the steep decline of serum Lp(a) (nadir at day 3) on the one hand and the overshooting at day 10 on the other hand are the net result of removal of Lp(a) particles during extracorporeal circulation on the one hand and acute phase reaction on the other hand.

The broad, flat Lp(a) distribution curves 10 days post-CABG corroborate with our hypothesis and with the data of Maeda et al.: a variable acute phase reaction makes the frequency distribution curve more Gaussian and less skewed. This effect is not present at the third post-operative day because at that moment we mainly see the effect of removal of Lp(a) due to extracorporeal circulation.

Cholesterol lowering drug therapy in CABG patients (25) was used in this study as it is a sound strategy for optimizing graft patency, slowing the progression

Table 1. Anthropometric data and control parameters of patients undergoing coronary artery bypass grafting, at baseline and in the post-operative period

		Placebo group			Pravastatin group			T-test or Wilcoxon test
		Day 0	Week 4	Week 12	Day 0	Week 4	Week 12	
Parameter	Units	Mean ± S.D.			Mean ± S.D.			P-value
Age	years	54.5 ± 4.4			53.1 ± 5.1			N.S.
Weight	kg	76.3 ± 9.1	75.1 ± 8.6	76.5 ± 8.8	78.0 ± 9.4	75.8 ± 8.7	76.2 ± 8.0	N.S.
Height	cm	172.3 ± 5.6			173.2 ± 5.5			N.S.
SBP	mmHg	122.2 ± 17.8	118.4 ± 12.0	122.7 ± 14.4	122.4 ± 14.9	116.2 ± 13.4	119.0 ± 13.6	N.S.
DBP	mmHg	76.0 ± 11.0	74.4 ± 9.4	79.9 ± 9.2	72.2 ± 9.6	74.8 ± 9.2	76.9 ± 10.5	N.S.
Heart rate	bpm	66.1 ± 8.8	77.5 ± 11.7	71.3 ± 8.8	66.5 ± 7.6	76.0 ± 8.5	71.3 ± 8.3	N.S.
Creatinine	mg/dl	1.14 ± 0.16	1.12 ± 0.16	1.14 ± 0.17	1.12 ± 0.17	1.13 ± 0.24	1.11 ± 0.18	N.S.
Haemoglobin	g/dl	14.7 ± 1.21			14.7 ± 1.12			N.S.
Haematocrit	l/l	0.44 ± 0.04			0.44 ± 0.04			N.S.
ASAT	IU/l	12.0 ± 10.7	9.4 ± 2.7	10.9 ± 4.7	12.1 ± 9.4	10.7 ± 5.3	10.7 ± 3.5	N.S.
ALAT	IU/l	16.1 ± 13.2	13.3 ± 11.3	12.1 ± 6.6	19.7 ± 22.6	16.2 ± 13.7	12.4 ± 5.4	N.S.
GGT	IU/l	16.8 ± 11.8	20.7 ± 11.3	18.0 ± 12.9	18.0 ± 14.8	22.8 ± 26.0	16.0 ± 8.3	N.S.

Table 2. Time course of mean, median and Ln Lp(a) levels after coronary artery bypass surgery in both placebo and treatment (pravastatin) group and in the total patient group

	Placebo	Pravastatin	Total patient group	P-value
Lp(a) (mg/l)	mean ± S.D. mean Ln ± S.D. median (min-max) N	mean ± S.D. mean Ln ± S.D. median (min-max) N	mean ± S.D. mean Ln ± S.D. median (min-max) N	T-test* logtrans-formed Lp(a)
Day 0	239 ± 268 4.85 ± 1.30 128 (2-1377) 50	280 ± 296 4.89 ± 1.47 141 (2-1272) 48	259 ± 281 4.87 ± 1.38 135 (2-1377) 98	0.88
Day 3	94 ± 88 4.14 ± 0.94 59 (7-462) 50	117 ± 133 4.24 ± 1.07 74 (5-660) 47	105 ± 112 4.19 ± 1.00 67 (5-660) 97	0.65
Day 10	207 ± 172 4.92 ± 1.03 141 (10-728) 48	264 ± 240 5.06 ± 1.14 150 (11-948) 45	234 ± 209 4.99 ± 1.08 148 (10-948) 93	0.52
Week 4	231 ± 230 4.90 ± 1.20 151 (3-945) 50	315 ± 334 5.09 ± 1.40 173 (1-1623) 48	272 ± 287 4.99 ± 1.29 151 (1-1623) 98	0.47
Week 12	229 ± 232 4.82 ± 1.31 158 (3-1044) 48	294 ± 300 4.94 ± 1.45 171 (4-1172) 43	260 ± 267 4.88 ± 1.37 164 (3-1172) 91	0.70

*The T-test checks for statistically significant differences of mean Ln Lp(a) levels between placebo and pravastatin group. Repeated measurements analysis, taking dropouts into account: treatment effect: $P = 0.61$; time effect: $P < 0.0001$; treatment x time effect: $P = 0.82$.

Table 3. Time courses of serum lipids and serum apolipoproteins after coronary artery bypass surgery in placebo and pravastatin treated patients

Parameter	Units	Preoperative	Day 3	Day 10	Week 4	Week 12
		Mean ± S.D.				
Placebo group						
Cholesterol	mg/dl	215 ± 37	120 ± 24	153 ± 25	193 ± 30	223 ± 34
LDL-cholesterol	mg/dl	150 ± 38	73 ± 22	104 ± 27	132 ± 28	151 ± 29
Apo B	mg/dl	97 ± 20	56 ± 13	79 ± 15	88 ± 17	96 ± 17
HDL-cholesterol	mg/dl	36.6 ± 10.3	23.3 ± 8.4	22.6 ± 4.9	32.5 ± 7.6	37.8 ± 9.2
Apo A-I	mg/dl	99 ± 20	54 ± 17	69 ± 11	92 ± 15	105 ± 16
Treatment group						
Cholesterol	mg/dl	218 ± 32	118 ± 23	139 ± 22[b]	159 ± 22[c]	175 ± 28[c]
LDL-cholesterol	mg/dl	158 ± 36	72 ± 20	92 ± 21[a]	99 ± 23[c]	109 ± 27[c]
Apo B	mg/dl	100 ± 18	56 ± 12	73 ± 13[a]	74 ± 13[c]	75 ± 15[c]
HDL-cholesterol	mg/dl	34.0 ± 7.8	22.4 ± 8.5	22.3 ± 4.2	31.0 ± 5.7	35.9 ± 7.3
Apo A-I	mg/dl	99 ± 17	53 ± 16	67 ± 11	92 ± 13	102 ± 16

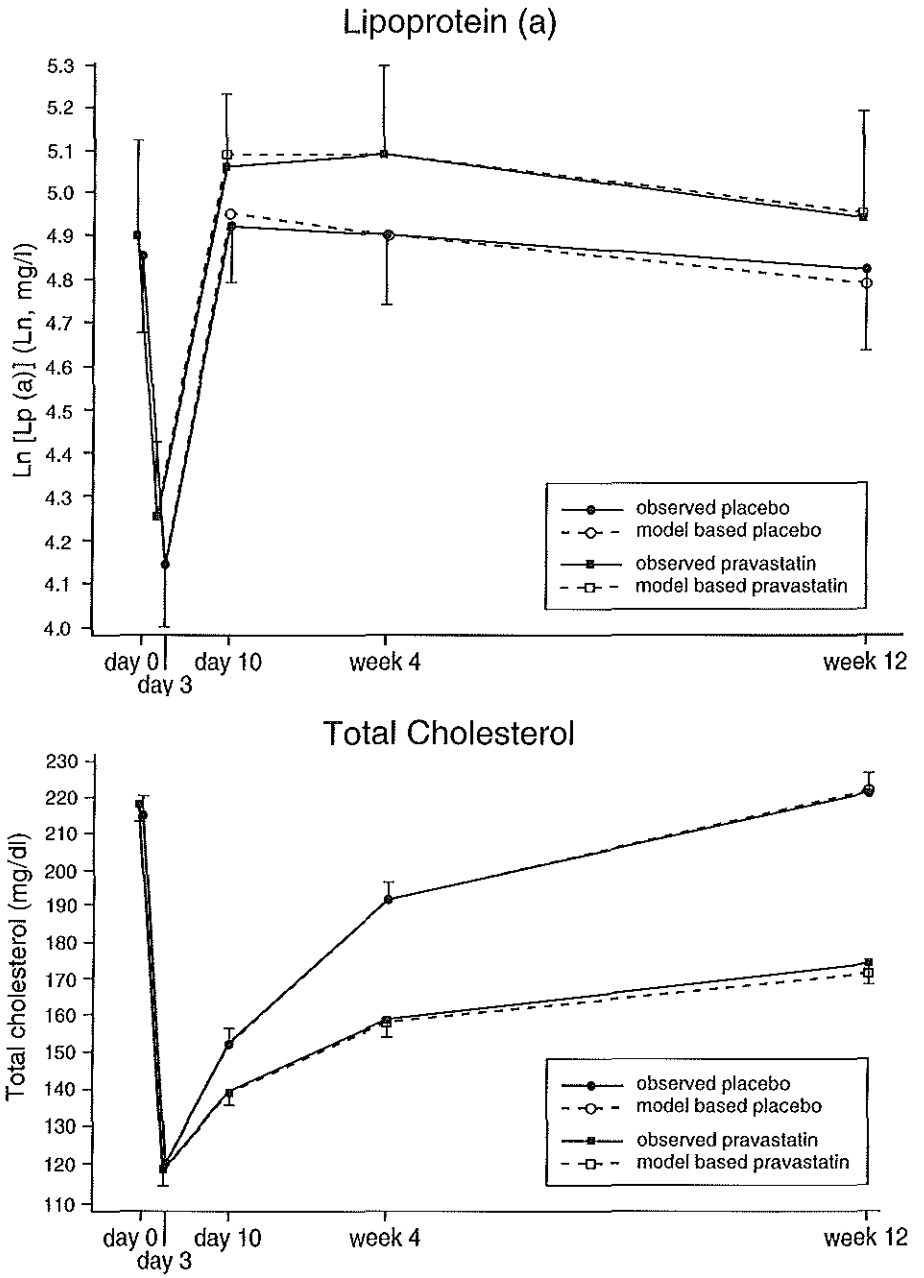
[a]$P < 0.05$; [b]$P < 0.001$; [c]$P < 0.0001$ represent the statistically significant differences in time course of serum lipids between placebo and treatment group.

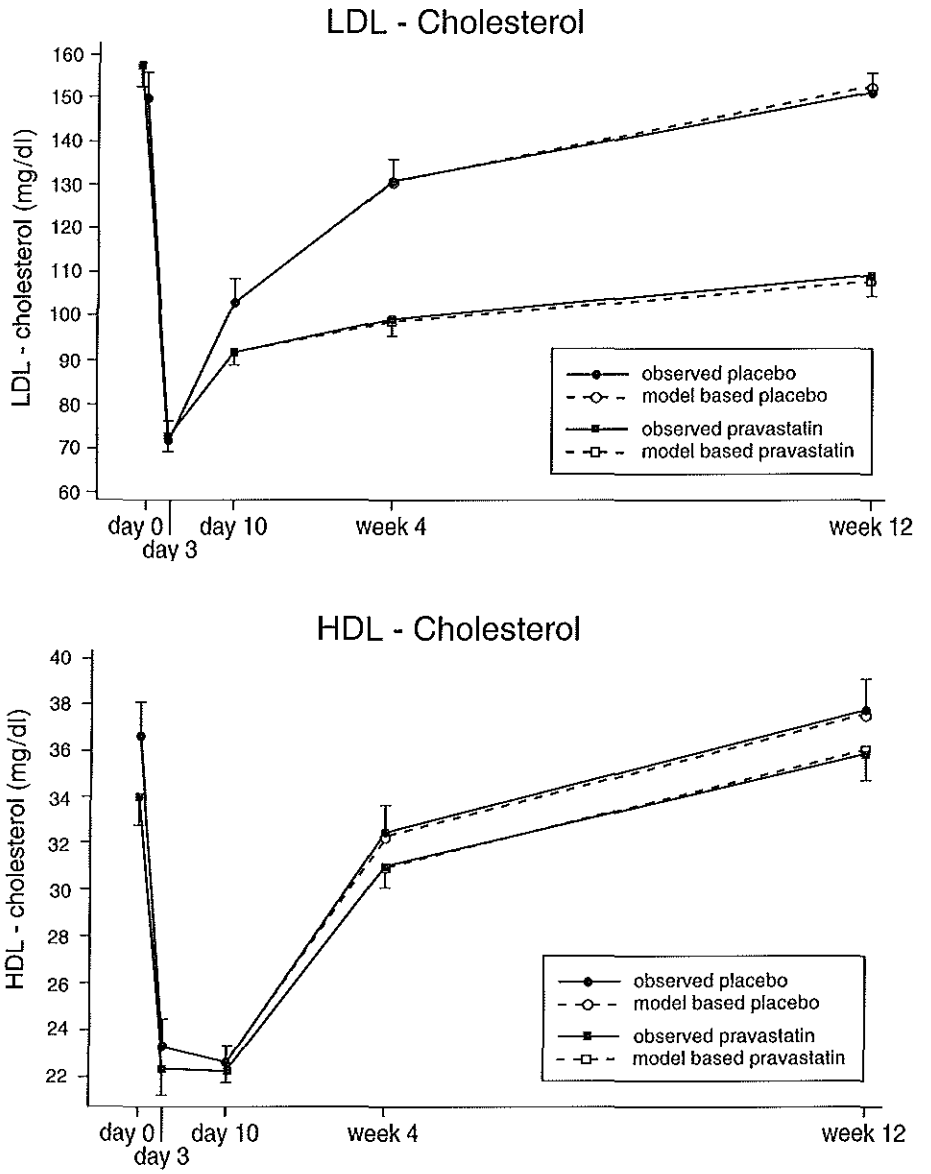
Table 4. Pearson correlation coefficients and probabilities for Ln Lp(a) versus cholesterol, LDL-cholesterol, apolipoprotein B, HDL- cholesterol and apolipoprotein A-I before coronary artery bypass surgery and at the third post-operative day.

Pre-operative	Placebo group			Treatment group			Total patient group		
	N	r	p	N	r	p	N	r	p
Ln Lp(a) versus cholesterol	50	0.199	0.167*	48	0.068	0.645*	98	0.135	0.185*
Ln Lp(a) versus LDL-c	48	0.075	0.610*	47	0.200	0.178*	95	0.137	0.184*
Ln Lp(a) versus apo B	50	0.115	0.426*	47	0.079	0.596*	97	0.098	0.338*
Ln Lp(a) versus HDL-c	48	0.276	0.057*	47	-0.079	0.598*	95	0.110	0.290*
Ln Lp(a) versus apo A-I	50	0.298	0.036	47	-0.156	0.296*	97	0.078	0.450*
At the third post-operative day	Placebo group			Treatment group			Total patient group		
	N	r	p	N	r	p	N	r	p
Ln Lp(a) versus cholesterol	50	0.342	0.015	47	0.422	0.003	97	0.360	0.0003
Ln Lp(a) versus LDL-c	50	0.334	0.018	47	0.448	0.002	97	0.388	0.0001
Ln Lp(a) versus apo B	50	0.229	0.111*	46	0.350	0.017	96	0.287	0.0046
Ln Lp(a) versus HDL-c	50	0.333	0.018	47	0.113	0.448*	97	0.216	0.0339
Ln Lp(a) versus apo A-I	50	0.404	0.004	46	0.013	0.933*	96	0.204	0.0462

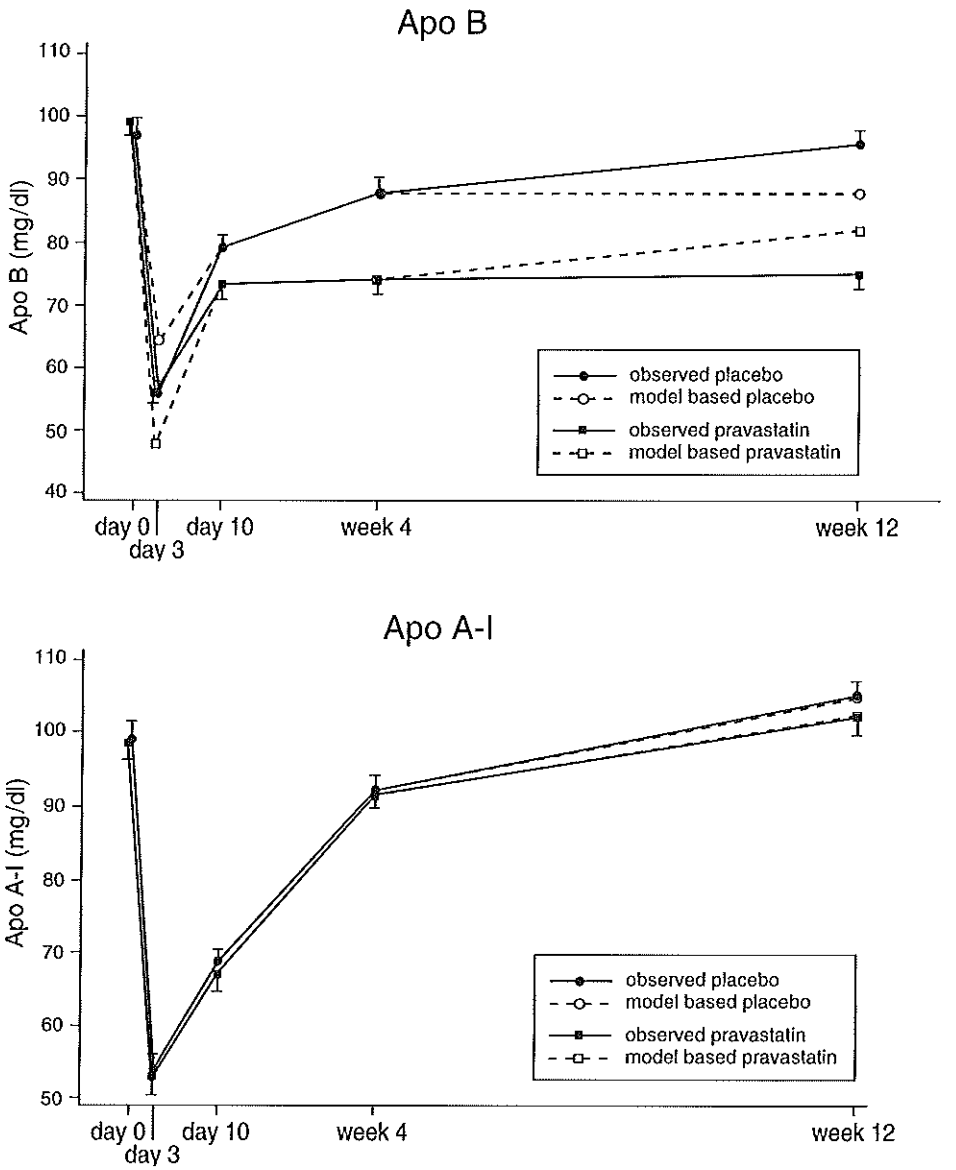
*No statistically significant correlation at $\alpha = 0.05$.

Figure 1. Time course of mean Ln Lp(a) ± SEM and "model based" mean Ln Lp(a) concentrations after coronary artery bypass grafting for placebo and pravastatin treated patient groups, as compared to the time courses of serum lipids and serum apolipoproteins. Mean values ± standard error of the mean (SEM) are given.





of atherosclerosis and reducing the need for repeat surgery. No statistically significant effect of pravastatin, a HMG-CoA reductase inhibitor, on the serum Lp(a) levels could be shown as there was no significant difference neither between the Lp(a) time course nor between the mean log Lp(a) levels in both treatment and placebo group. The lack of effect of the current pravastatin dosage on serum Lp(a)



levels confirms the report of Berg and Leren (26) and of Jacob et al. (27). It contrasts with the findings of Jurgens et al. (28) and with the report of Kostner et al. (29). Kostner and coworkers (29) reported significant increases in serum Lp(a) in half of the hypercholesterolemic patients after treatment with lovastatin (27.0% and 33.7% change in serum Lp(a) levels after daily administration of respectively

20 and 80 mg lovastatin). In the other half of his patients no significant change was seen. Although there is some controverse in literature, the conference chairmen of the Second International Symposium on Lp(a), New Orleans, Louisiana, United States (November 1992), firmly concluded that HMG-Coenzyme A reductase inhibitors do not have any effect on serum Lp(a) levels (Dr. Gotto, personal communication). The data from this study corroborate these conclusions, as pravastatin did not exert an unfavorable effect on serum Lp(a) levels.

It is concluded that the Lp(a) time-course after CABG is characterized by a nadir Lp(a) level at the third post-operative day, by a slight but significant overshooting at the tenth post-operative day and by rapid normalization to baseline values at the fourth post-operative week. The nadir for serum Lp(a) at day 3 post-CABG is also present in the other serum lipid time patterns. The serum Lp(a) decline the first three days after CABG contrasts with the Lp(a) increase seen after acute myocardial infarction or other surgical operations not using the heart-lung pump. Within our cardiovascular surgery (CABG) patient group, from the third post-CABG day onwards, the Lp(a) time course diverged from the time course of the other serum lipids as the serum lipids did not overshoot their baseline values at day 10 and displayed a gradual increase to baseline values not earlier than three months post-CABG. We hypothesize that the Lp(a) overshooting at the tenth post-operative day and the more rapid normalization of serum Lp(a) (after 1 month) compared to the other serum lipids, are explained by the acute phase behaviour of serum Lp(a).

In summary, this study clearly depicts 1) that there is a significant time-dependent effect on the serum Lp(a) levels post-CABG, 2) that there is no effect of treatment (pravastatin) neither on the Lp(a) time course nor on the overall Lp(a) levels, 3) that there is no combined time x treatment effect, and 4) that reliable post-operative Lp(a) measurements can be made at earliest one month post-CABG.

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Chapter 8

RAPID LIPOPROTEIN(a) CHANGES DURING CORONARY ARTERY BYPASS GRAFTING: AN EPIPHENOMENON?

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ABSTRACT

The lipoprotein(a) (Lp(a)) time course during and after coronay artery bypass grafting was examined in 20 male Caucasians, in relation to the time courses of serum cholesterol and serum triglycerides. Samples were taken at 8 different time points. Baseline geometric means for Lp(a), cholesterol and triglycerides were 115 mg/l, 5.73 mmol/l and 1.73 mmol/l, respectively. Up to 10 min after cardiopulmonary bypass (CPB) and after correction for haemodilution, no observable effect of CPB on serum concentrations of Lp(a) could be demonstrated, whereas serum concentrations of total cholesterol and triglycerides showed a progressive and significant decline. Ten minutes after stopping CPB, geometric means for cholesterol and triglyceride were 3.90 and 0.90 mmol/l, respectively. At the third post-operative day geometric Lp(a) and cholesterol means further decreased to 62 mg/l and 2.97 mmol/l, respectively, while triglycerides increased again. It is concluded that Lp(a) levels remain constant during CPB, but mimic total cholesterol changes in the post-CABG period.

INTRODUCTION

Serum lipids, lipoprotein and apolipoprotein levels are generally known to be affected by major surgery and trauma (1). Whereas literature upon peri-operative time courses of serum lipid levels after coronary artery bypass grafting (CABG) is quite unanimous (2), it is conflicting with respect to serum lipoprotein(a) (Lp(a)) changes (3-5), adding to the reputation that Lp(a) continues to confuse and mystify. CABG surgery is special in that it involves both major surgery and the use of extracorporeal circulation and a cardiopulmonary bypass pump, which necessitates hemodilution to prime the pump. Some authors showed a profound Lp(a) decline after coronary surgery and a non-significant trend to overshoot above baseline values at the end of the first post-operative week, followed by normalization after one month (3, 4). In contrast, Sgoutas et al. (5) reported that Lp(a) levels doubled within minutes of cardiopulmonary bypass (CPB), i.e. hours before any rise in

C-reactive protein, and persisted at a similar level up to at least the third post-operative day. As CPB is associated with acute activation of the complement system and with changes in fibrinolytic parameters (6), Sgoutas suggested that Lp(a) might respond to events associated with immunological activation. Moreover, the authors hypothesized that the Lp(a) changes in the acute setting of CABG might provide a model for understanding control of its production and function.

In order to shed more light upon the validity of the published data, serum Lp(a) and serum lipid changes during and after CABG were re-examined, using multiple timed venous blood samples, standardised anaesthesia and documented CPB procedures in all patients.

SUBJECTS AND METHODS

Twenty Caucasian males, aged 43 to 72 years, undergoing elective CABG, were included. None of them had done a myocardial infarction up to three months before coronary surgery. All cardiac medications were continued into the peri-operative period. Venous blood was collected for haemoglobin, haematocrit, serum Lp(a) and serum lipids. Blood samples were procured the day before coronary surgery, before and after induction of anaesthesia, before and after intravenous heparin administration, and before, during and after CABG (tables 1-3). Before beginning CPB, lidoflazine (1 mg/kg) was administrated intravenously. Anaesthesia was induced by etomidate (0.3 mg/kg), sufentanil (50 μg) and a relaxant, pancuronium or vecuronium (0.1 mg/kg), and maintained by midazolam (10 mg) and supplemental sufentanil, in such a way that hemodynamic stability was preserved. Heparinization was initiated by administrating 300 IU of heparin per kg body weight. During CPB, additional heparin was possibly given in order to maintain the Activated Clotting Time between 400 and 600 seconds. In the CPB circuit different types of membrane oxygenators (Dideco Δ703, Macchi, Maxima 1300 or Scimed Ultrox) were employed. The circuit was primed with Ringer's-lactate solution, containing human albumin, mannitol and heparin (5000 IU). Aorta unclamping was done intermittently. No cell-saver was used. After initiation of

extracorporeal circulation patients were systematically cooled to 28°C. No patient was transfused with heterologous plasma during CPB, and no patient showed post-operative complications. Research procedures followed were in accordance with the ethical standards of the University Hospital internal review board.

Haemoglobin and haematocrit were determined in fresh EDTA blood, using standard procedures, while serum was stored at -20°C for batch Lp(a) and lipid analyses. Maximum storage time was two months. Serum Lp(a) concentrations were determined by ELISA (Cat. Nr. 610220; Biopool AB, Umeå, Sweden). According to Biopool, the Lp(a) test kit utilizes sheep polyclonal antibodies against purified human Lp(a) which are adsorbed against immobilized low density lipoprotein (LDL) and immobilized human plasminogen, and affinity purified on immobilized Lp(a). Lp(a) is measured by using Lp(a) specific antibodies immobilized on micro-test plates as catch antibodies and Lp(a) specific antibodies conjugated to horse radish peroxidase as tag antibodies. Serum cholesterol was measured by an enzymatic CHOD-PAP method. Total serum triglycerides were measured by a GPO-PAP method, and no correction was made for free glycerol. Typical CVs for the Lp(a), cholesterol and triglyceride assays used were 6.7%, 2.1% and 2.8%, respectively.

Per patient, sera were measured in duplicate within one run. Lp(a) and serum lipids levels were corrected for hemodilution by multiplying measured values by the ratio of baseline haematocrit to haematocrit at the time of sampling. Corrected Lp(a) and serum lipid values were logarithmically transformed and geometric means were calculated at each sampling time. Differences between sampling points larger than three times the between-day CV were considered significant.

RESULTS

In all subjects, haemoglobin, haematocrit, serum Lp(a) and serum lipids decreased before and during CPB (data not shown). Ten minutes after heparinization the haematocrit was on average at 92% of the average baseline value; 20 minutes after starting CPB the haematocrit was at 67.5% and 10 minutes after stopping CPB it was at 71%. After correction for hemodilution (table 1), serum

cholesterol levels still showed a linear decline across all sampling points, leading to a geometric mean cholesterol value at the third post-operative day of 2.97 mmol/l, i.e. 52% of the baseline value. Triglycerides levels (table 2) also displayed a gradual decline during and immediately after stopping CPB, but were either normalized or increased above baseline at the third post-operative day. In contrast, serum Lp(a) levels (table 3) did not significantly change during and up to 10 minutes after CABG, whereas at the third post-operative day geometric mean Lp(a) was only 62 mg/l, i.e. 54% of the baseline geometric mean.

DISCUSSION

Surgery, just like infection and inflammation, stimulates cytokine production which results in marked changes in the blood concentrations of specific proteins, the so-called "acute phase proteins" (1). The acute phase response of the host to various stimuli is believed to be advantageous, acute phase proteins playing an important homeostatic role in the injured or diseased patient. To this end, C-reactive protein and complement 3, for example, help in the opsonization of bacteria, immune complexes and foreign particles. Likewise, cytokine-induced changes in serum lipid and lipoprotein levels are also considered to be opportune and part of the acute phase response. It has been documented in primates that the cytokine-mediated acute phase response increases VLDL, and hence serum triglyceride levels, decreases HDL- and LDL-cholesterol levels and increases Lp(a) levels (1). The increase in serum triglycerides is believed to be beneficial as, firstly, elevated VLDL levels enhance delivery of lipids to cells that are involved in tissue repair and to cells that are activated during the immune response, and secondly, represent a detoxifying mechanism by binding endotoxins and a variety of viruses (1). The decrease in serum cholesterol partially results from decreased LDL production due to reduced cholesterol and apo B secretion, and increased LDL-receptor activity. The latter also reflects enhanced cholesterol delivery to cells. So far, the significance of the concomittant Lp(a) increase is incompletely understood (1).

In the present study the Lp(a) time course during and after CABG was

examined, in relation to the time courses of the other serum lipids. Up to 10 minutes after CPB and after correction for hemodilution, no observable effect of CPB on serum concentrations of Lp(a) could be demonstrated, whereas serum concentrations of total cholesterol and triglycerides showed a progressive and significant decline. The peri-operative triglyceride decline likely reflected increased lipoprotein lipase activity and ensuing intravascular lipolysis caused by massive heparin cofactor administration during CPB. At the third post-operative day, the acute phase response became obvious, triglyceride levels being either normalized or increased compared to baseline levels, and cholesterol levels being profoundly decreased. Notably, Lp(a) levels were similarly decreased as serum cholesterol levels at the third post-operative day. In case of serum lipids, our observations underscore previous work upon changes of serum lipids following major surgical operations, either without or with extracorporeal circulation and hemodilution (2, 7). With respect to Lp(a), we reconfirm that Lp(a) mimics total cholesterol changes in the immediate post-CABG period, as evidenced by the similar percent decrease at the third post-operative day for both parameters, confirming previous work from us (3) and others (4), but frustrating Sgoutas' findings (5). Although differences between anesthetic agents and CPB systems employed may partially explain the dissimilarity, it is likely that basal Lp(a) levels, as measured by Sgoutas et al., have been underestimated due to the fact that baseline samples were drawn after induction of anaesthesia and prior to CABG, neglecting expansion of circulating volume, commonly practiced by anaesthetists prior to coronary surgery. Hence, we hypothesize that invalid basal values formed the basis for the acute phase behaviour of Lp(a) *in minutes* after CPB, and for masking the Lp(a) invariability and the modest serum lipid decline during CPB. Moreover, although the Lp(a) nadir at the third post-operative day apparently conflicts with the acute phase behaviour of Lp(a), this is not the case.

After all, it was previously demonstrated that the Lp(a) time course deviates from the cholesterol time course only after the third post-operative day, and displays an increase compared to baseline Lp(a) levels at the tenth post-operative day (2, 3).

Finally, we hypothesize that one of the mechanisms responsible for reducing cholesterol levels after major surgery, i.e. diminished LDL production due to

Table 1. Time-dependent serum cholesterol concentrations* in 20 Caucasian males undergoing cardiopulmonary bypass for coronary artery bypass grafting

Subject	Time[a]							
	1	2	3	4	5	6	7	8
Cholesterol levels, mmol/l								
1	5.7	6.1	5.8	5.4	5.9	4.9	4.6	2.5
2	6.1		4.9	4.8	5.1	4.6	4.1	3.4
3	6.6	7.0	6.4	5.5	6.0	5.4	5.0	4.7
4	5.1	3.4	4.1	4.2	3.3	3.5	3.3	2.2
5	5.8		4.8	4.3	4.5	4.3	4.0	2.7
6	6.6		4.8	5.5	5.7	4.6	4.7	4.1
7	4.6		3.5	3.7	3.4	3.0	2.7	2.1
8	4.3		3.5	3.3	3.2	2.8	2.7	1.7
9	6.9	6.3	5.8	6.0	6.0	5.0	5.0	2.5
10	5.6		4.5	4.5	5.0	4.1	4.5	3.1
11	8.2	6.6	7.0	7.1	7.3	4.3	3.9	3.1
12	5.6		4.3	4.3	3.5	3.3	2.8	4.3
13	4.6	4.3	3.8	4.3	3.2	3.2	3.3	2.0
14	7.4	6.5	6.5	6.4	6.3	5.3	4.7	3.7
15	4.3	4.6	3.6	3.5	3.7	3.3	3.6	3.0
16	5.3	4.8	4.6	4.4	4.4	3.7	2.9	4.3
17	6.9	5.8	5.8	5.8	6.0	4.9	4.9	3.5
18	4.4	4.0	3.9	3.8	3.9	3.6	3.6	2.5
19	6.7	6.5	5.9	4.9	4.8	4.7	4.9	3.0
20	5.8		5.2	5.2	4.2	4.4	4.4	3.1
Mean	5.83	(5.49)	4.94	4.85	4.77	4.15	3.98	3.08
SD	1.10	(1.20)	1.07	1.01	1.23	0.79	0.82	0.84
Geometric mean	5.73	(5.36)	4.83	4.75	4.62	4.07	3.90	2.97

*Serum cholesterol, triglyceride and lipoprotein (a) concentrations are corrected for changes in plasma volume, whenever appropriate: plasma volume corrections were always made for the samples collected at time point 6 and 7, and eventually for samples drawn at sample points 5 and 8.

[a]Key to time points: 1, baseline, i.e. the day before coronary surgery; 2, before anaesthesia; 3, after anaesthesia; 4, before heparinization; 5, 10 min after heparinization; 6, 20 min after starting cardiopulmonary bypass; 7, 10 min after stopping cardiopulmonary bypass; 8, 72 h after starting cardiopulmonary bypass.

Table 2. Time-dependent serum triglyceride concentrations* in 20 Caucasian males undergoing cardiopulmonary bypass for coronary artery bypass grafting

Subject	Time[a]							
	1	2	3	4	5	6	7	8
Triglyceride levels, mmol/l								
1	1.85	1.25	1.16	1.18	1.11	1.03	1.23	3.31
2	6.48		4.25	4.37	4.07	3.48	2.83	5.62
3	1.35	1.18	1.02	1.06	0.93	0.82	0.73	1.90
4	1.81	1.45	1.57	1.52	1.34	1.19	1.11	2.94
5	1.68		1.44	1.43	1.63	2.06	2.02	1.78
6	3.41		2.15	1.19	1.92	2.05	1.78	1.51
7	2.42		1.19	1.48	1.12	1.20	0.86	0.80
8	1.12		0.69	0.65	0.53	0.45	0.45	0.92
9	1.10	0.93	0.60	0.79	0.58	0.70	0.64	1.20
10	1.32		1.67	1.85	1.65	1.27	0.97	1.36
11	1.26	0.78	0.81	0.84	0.82	0.60	0.51	0.87
12	2.25		0.96	1.07	0.64	0.56	0.51	1.56
13	2.10	2.31	2.00	2.09	1.47	1.30	0.97	1.75
14	1.71	1.03	0.92	0.95	0.83	0.76	0.86	1.57
15	1.24	1.16	0.95	1.14	1.25	1.05	0.71	1.14
16	1.45	1.83	1.47	1.58	1.35	1.11	0.81	2.44
17	1.36	1.13	0.89	0.88	0.78	0.75	0.79	1.61
18	1.51	1.25	1.13	1.14	1.14	0.99	0.90	1.28
19	1.95	1.92	1.56	1.49	1.16	1.08	0.89	1.62
20	1.19		1.00	0.81	0.78	0.66	0.59	1.64
Mean	1.93	(1.35)	1.37	1.38	1.26	1.16	1.01	1.84
SD	1.21	(0.45)	0.80	0.80	0.76	0.69	0.58	1.09
Geometric mean	1.73	(1.29)	1.23	1.24	1.11	1.02	0.90	1.64

[a]See table 1 for key to footnotes.

decreased cholesterol and apo B secretion, might also explain the initial decline in Lp(a) levels in the first post-operative days (1).

In conclusion, the paradoxical Lp(a) increase noted *in minutes* after starting CPB was an artefact. In our effort to unravel the role of the enigmatic Lp(a) particle we should be careful not to make the mystery deeper.

Table 3. Time-dependent serum lipoprotein(a) concentrations* in 20 Caucasian males undergoing cardiopulmonary bypass for coronary artery bypass grafting

Subject	Time[a]							
	1	2	3	4	5	6	7	8
Lp(a) levels, mg/l								
1	62	83	120	109	156	176	194	16
2	25		24	29	28	26	21	11
3	283	343	358	289	465	392	369	112
4	911	883	1059	1043	691	692	720	186
5	85		96	81	122	183	98	47
6	29		44	36	57	45	51	36
7	485		506	492	538	504	390	150
8	88		83	83	103	98	94	37
9	1303	1492	1138	1286	1284	1028	1066	350
10	343		557	525	641	558	604	209
11	291	279	266	289	320	191	181	101
12	97		132	87	147	134	117	68
13	13	13	15	16	18	18	20	13
14	540	535	545	524	548	459	376	237
15	111	84	74	50	104	171	171	67
16	11	37	7	14	7	9	11	47
17	24	18	17	14	17	20	14	36
18	114	85	74	92	137	143	165	44
19	133	142	249	140	205	203	190	75
20	106		124	140	107	110	111	34
Mean	253	(333)	274	267	285	258	248	94
SD 336	(447)	333	353	323	268	274	90	
Geometric mean	115	(139)	124	118	139	138	130	62

[a]See table 1 for key to footnotes.

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Chapter 9

MODULATION OF LIPOPROTEIN(a) ATHEROGENICITY BY HDL CHOLESTEROL LEVELS IN MIDDLE-AGED MEN WITH SYMPTOMATIC CORONARY ARTERY DISEASE AND NORMAL TO MODERATELY ELEVATED SERUM CHOLESTEROL

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STRUCTURED ABSTRACT

Objective

This study sought to examine whether lipoprotein(a) levels predict coronary artery lumen changes in patients with symptomatic coronary artery disease (CAD) and normal to moderate hypercholesterolaemia.

Background

Conflicting reports have been published lately, confirming or refuting the association of lipoprotein(a) with clinical events or angiographically verified disease progression.

Methods

The association between serum lipoprotein(a) and coronary artery lumen changes was studied in 704 males who entered REGRESS, a double-blind, placebo controlled, quantitative angiographical study which assessed the effect of two year of pravastatin treatment. The primary endpoints were change in average mean segment diameter (MSD) and change in average minimum obstruction diameter (MOD). Cases and controls were classified into progressors, regressors and stable patients, and median lipoprotein(a) concentrations were compared. Bi- and multivariate regression analyses were performed in the overall patient group and in high-risk subgroups.

Results

Pravastatin treatment did not affect serum apolipoprotein(a) levels. In placebo treated patients median *in-trial* apolipoprotein(a) levels in regressing, stable and progressing patients were 130, 162 and 251 U/l respectively, while 143, 224 and 306 U/l in pravastatin treated patients. Predictors of MSD (MOD) changes were baseline MSD (MOD), *in-trial* apolipoprotein(a), *in-trial* HDL cholesterol and baseline use of long acting nitrates. The multivariate models explained 14% and 12% of respectively MSD and MOD changes; apolipoprotein(a) explained only 2.6% and 4.8%. Yet, in patients with *in-trial* HDL cholesterol levels below 0.7 mmol/l, apolipoprotein(a) explained up to 37% of the arteriographic changes.

Conclusion

Serum lipoprotein(a) predicts coronary artery lumen changes in normal to moderately hypercholesterolaemic Caucasian males with CAD, its atherogenicity being marked in case of concomitant hypoalphalipoproteinaemia.

CONDENSED ABSTRACT

Lipoprotein(a) has a limited prognostic value concerning progression of the disease in the total group of patients with symptomatic CAD and average serum lipid levels. However, lipoprotein(a) has great prognostic value in a subgroup of patients with concomitant hypoalphalipoproteinemia (< 0.7 mmol/l). Therefore, measurement of lipoprotein(a) should not be restricted to hypercholesterolaemic individuals, as reported previously, but should be extended to other high-risk patients, especially those with low HDL cholesterol. Finally, future treatment therapies should aim to increase serum HDL levels in hypoalphalipoproteinemic patients, in order to offset the adverse effects of apolipoprotein(a).

INTRODUCTION

Lipoprotein(a) represents a family of LDL-like lipoproteins that contain apolipoprotein B_{100} linked by a disulfide bridge to a highly polymorphic glycoprotein, called apolipoprotein(a). In healthy individuals serum lipoprotein(a) levels are known to be fairly constant throughout life, the levels being mainly genetically determined (1). Lipoprotein(a) is characterized by an important degree of structural and functional heterogeneity, and is postulated to be both atherogenic and thrombogenic. The former because of its high cholesterol content, the latter because of its molecular mimicricy with plasminogen (1, 2). So far, its physiological function is still unknown. Since the discovery of lipoprotein(a) by Berg in 1963, numerous studies (3-24) have appeared which examined the relationship between lipoprotein(a) and coronary artery disease (CAD), including

epidemiologic and clinical case-control studies that investigated the association of lipoprotein(a) levels with presence and severity of CAD, myocardial infarction, restenosis after angioplasty (16), and vein-graft occlusion after coronary artery bypass grafting (17). In most studies (3-17) it was concluded from multivariate analysis including age, body mass index, blood pressure, cigarette smoking, HDL cholesterol and LDL cholesterol levels, that lipoprotein(a) is an independent risk factor for development of CAD. However, the role of lipoprotein(a) in CAD has also been questioned (18-24). Some of the conflicting results may be explained by methodological confounders (25). Presently, evidence is accumulating that the challenge of elevated lipoprotein(a) may not be as deleterious as previously thought since it appears that whether or not lipoprotein(a) causes CAD may depend on interaction of lipoprotein(a) with environmental or genetic factors. First, serum lipoprotein(a) levels differ between populations, median lipoprotein(a) levels being approximately two- to threefold higher in Blacks than in Caucasians (26, 27). Yet, in the absence of atherogenic diets Blacks exhibit low morbidity and mortality rates due to CAD. Analogously, transgenic mice possessing the apolipoprotein(a) gene developed atherosclerosis only when fed with an atherogenic diet, but not on their normal chow diet (28). Second, in a human angiographic study Armstrong and coworkers (3) reported that increased LDL concentrations markedly increased the risk of CAD due to elevated lipoprotein(a) levels, indicating modulation of lipoprotein(a) atherogenicity by LDL cholesterol. Third, several case-control studies demonstrated 'positive' associations between lipoprotein(a) levels and presence or severity of CAD only in specific patient subsets such as patients with a parental history of myocardial infarction (6), young males with premature CAD (8, 12), and patients with hyperlipidemia (10, 11). Fourth, more conflicting reports have been published lately, confirming or refuting the association of lipoprotein(a) with angiographically verified disease progression or clinical events. Watts et al. (29) and Marburger et al. (30) found that lipoprotein(a) correlated poorly with arteriographic changes over time in patients who underwent a diet and exercise program, or received lipid lowering medication. In contrast, Terres et al. (31) demonstrated that lipoprotein(a) correlated strongly with rapid disease progression in untreated patients. Finally, Thompson and colleagues (32) showed that lowering both lipoprotein(a) and LDL cholesterol levels in patients with familial hyper-

cholesterolaemia produced no greater angiographic benefit than lowering LDL cholesterol levels alone. Consequently, the latter observations appear to detract from the causal importance of lipoprotein(a) relative to LDL in coronary atherosclerosis.

We recently completed a large angiographic lipid intervention trial, the Regression Growth Evaluation Statin Study (REGRESS) (33). The REGRESS database provided us with the opportunity to determine the role of lipoprotein(a) on progression of coronary atherosclerosis in a prospective manner in patients with normal to moderately elevated serum cholesterol levels. We also compared lipoprotein(a) levels of REGRESS patients with those of healthy controls. Furthermore, we aimed to delineate in men with normal to moderately elevated serum cholesterol the lipoprotein milieus in which lipoprotein(a) mostly exerts its adverse effects.

METHODS

Study design and subjects

REGRESS is a prospective, double-blind, placebo-controlled, multicenter study that assessed the effect of treatment with pravastatin (40 mg/day) on progression and regression of angiographically documented CAD (33). Dutch male patients (N = 885) suffering from symptomatic coronary atherosclerosis, who had normal to moderately elevated cholesterol (4 - 8 mmol/l) and triglycerides (< 4 mmol/l), and underwent various forms of primary treatment (PTCA, CABG, medical treatment) were included. All were Caucasian. Details of the enrollment procedure, treatment and follow-up and primary treatment blocks are described elsewhere (33).

Lipoprotein(a), quantified by its apolipoprotein(a) content, was determined in 704 patients who completed the REGRESS study and from whom left-over serum was available both at baseline and at 24 months. Ninety-five cases could not be included in the lipoprotein(a) substudy because of adverse events, premature study discontinuation and death; in another 86 cases left-over serum was not available either at baseline or at 24 months. Baseline characteristics of cases not included in this substudy (N = 181) were not significantly different from those included (table1).

Evaluable baseline and final coronary angiograms were availabe in 81% of the patients (N = 567), a similar percentage as in the main trial (33).

For comparison, apolipoprotein(a) levels were analyzed in unrelated, apparently healthy male controls (N = 274) who had no clinical symptoms of CAD. Entry criteria were: Caucasian origin; cholesterol between 4 and 8 mmol/l; 20 kg/m^2 ≤ body mass index ≤ 30 kg/m^2; no diabetes; no previous history of coronary or cerebral infarction, PTCA, revascularization and cancer; no intake of oral anticoagulants, antihypertensive or cholesterol lowering medication; and an intake of less than three glasses of alcoholic beverage per day.

Quantitative coronary arteriography and clinical events

Coronary arteriograms were analyzed by quantitative computer analysis (Cardiovascular Measurement System) as described elsewhere (33). To standardize vasomotor tone 5-10 mg isosorbide dinitrate was administered sublingually 5-10 minutes prior to coronary angiography. Primary endpoints were: 1) change in average Mean Segment Diameter (MSD) per patient, and 2) change in average Minimum Obstruction Diameter (MOD) per patient. If a segment or lesion was adequately visualized in two (preferably orthogonal) projections and free of significant foreshortening in both views, the average values of the parameters in both projections were calculated. To calculate average MSD and MOD per patient the MSDs and MODs of all qualifying segments or obstructions were added and divided by the number of contributing segments or obstructions. Changes of MSD (N = 562) and MOD (N = 567) were computed using those segments considered not influenced by PTCA or CABG during the trial (33).

Analogous to the main angiographic trial (33), patients were categorized with regard to MOD and clinical events (i.e. nonfatal myocardial infarction and coronary artery disease death) as regressors, stable patients and progressors according to the following definitions: 1) a progressor is a patient with at least one lesion worsening by ≥ 0.4 mm or developing a lesion that reduces the lumen diameter by ≥ 0.4 mm; 2) a regressor is a patient with at least one lesion improving by ≥ 0.4 mm and no lesions worsening ≥ 0.4 mm; 3) a stable patient is a patient with no lesions worsening or improving by ≥ 0.4 mm. Patients with a mixture of regressing and progressing lesions were considered to be progressors, as well as

patients who suffered from new clinical events, irrespective of their angiographic outcome.

Specimen collection and storage

The REGRESS main study was ongoing between January 1990 and December 1993. Blood specimens were taken from fasting patients. Serum and plasma were harvested locally after centrifugation. Sera for the core Lipid Reference Laboratory, Rotterdam, the Netherlands, were immediately aliquotted, stored at -20 °C and weekly shipped on dry ice. In the week of arrival sera were thawed for regular serum lipid analyses. Aliquots for serum apolipoprotein(a) analysis were stored frozen at -70°C for maximally two years.

Fresh frozen EDTA-plasma was obtained from healthy controls. Sampling and randomization was done by the Dutch Institute of Public Health (RIVM, Bilthoven, the Netherlands). Plasma apolipoprotein(a) values were recalculated to serum apolipoprotein(a) values through multiplying by 1.03.

Laboratory measurements

Apolipoprotein(a) was measured with the Pharmacia apolipoprotein(a) RIA kit (Kabi Pharmacia Diagnostics, Uppsala, Sweden, Cat. Nr. 10-6497-01). Results are expressed in U liter-1 apolipoprotein(a). According to the manufacturer, 1 unit apolipoprotein(a) is very approximately equivalent to 0.7 mg lipoprotein(a) mass. Serum apolipoprotein(a) determinations were performed at baseline and after 24 months, paired analyses being available from 704 REGRESS patients (80% of patients included in the main study). Throughout the article the follow-up apolipoprotein(a) values, as determined in the blood specimens sampled at 24 months, are indicated as *in-trial* apolipoprotein(a) concentrations. Interassay coefficients of variation varied between 3.5% and 7.3% for low level controls (130-200 U/l), and between 3.3% and 5.6% for high level controls (350-500 U/l). As international standardization is lacking, overlapping lots of frozen serum pools at three different levels (low, medium and high) were analyzed throughout this substudy, to ensure 'traceability' among different reagent lots. Besides, we documented that freezing sera at -70°C up to two years did not impact apolipoprotein(a) measurements.

Serum cholesterol, HDL cholesterol and triglycerides were measured on (semi-) automated analyzers, using standard enzymic techniques. Cholesterol determinations were performed using a cholesteroloxidase/phenol/aminophenazone method (Boehringer, Mannheim, Germany) (34) which was standardized by calibration on a human reference serum pool with target values established by the Abell-Kendall Reference Method (35). HDL cholesterol was measured in the supernatant after phosphotungstic acid / $MgCl_2$ precipitation of apolipoprotein B-containing lipoproteins (Merck, Darmstadt, Germany), using Preciset™ (Boehringer, Mannheim, Germany) as a calibrator. Triglycerides were determined using a glycerol-phosphate oxidase/phenol/aminophenazon method without glycerol blank correction (Technicon Inc., Tarrytown, New York, USA). LDL cholesterol was calculated using the Friedewald formula (36). Since the Friedewald formula does not account for cholesterol associated with lipoprotein(a), estimated LDL cholesterol (mmol/l) was corrected by subtracting lipoprotein(a)-cholesterol, calculated as [0.30 x lipoprotein(a) mass / 386.65] (37). Serum lipids were analyzed at baseline, and after 2, 4, 6, 12, 18 and 24 months of treatment or placebo. *In-trial* serum lipid concentrations were calculated by averaging serum lipid values obtained per patient during the entire treatment/placebo phase.

The Lipid Reference Laboratory maintains cholesterol and HDL cholesterol standardization through the Lipid Standardization Program of the Centers for Disease Control (CDC) - National Heart Lung and Blood Institute, and fulfills its criteria concerning accuracy and precision. The laboratory also is a member of the Cholesterol Reference Method Laboratory Network established by CDC (38).

Blood glucose was measured with standard technology in the local hospital laboratories at baseline, and at 12 and 24 months. *In-trial* glucose concentrations were derived by averaging 12 and 24 month glucose values. *Baseline* plasma fibrinogen was measured centrally with an enzyme immunoassay method that used a monoclonal antibody against the carboxyl-terminal end of the fibrinogen Aα-chain as the capture antibody (G8), and a monoclonal antibody against the amino-terminal end of the Aα-chain (Y18) as the tagging antibody (39, 40).

Statistical Analysis

Student's t-test, Mann-Whitney or Pearson's chi-square test were used to

compare group means and/or medians. ANOVA or Kruskall-Wallis tests were used to compare different patient groups. The natural logs of triglyceride, fibrinogen, apolipoprotein(a) and LDL cholesterol/HDL cholesterol ratio were used to normalize distributions, while baseline MOD and MOD changes were ranked because of their skewness.

Bivariate relations were quantified with Pearson's or Spearmans rank correlation coefficients. Multiple linear regression (MLR) analyses were employed to estimate effects of variates independent of others: partial correlations were used to quantify the relation between two variables, independent of others. Squared partial correlation coefficients, multiplied by 100, were calculated to estimate the percentages of arteriographic changes explained by individual variates. Stepwise forward selection was used to build up the MLR model. The criterion for a variable to enter and to remain in the model was that its initial probability value in the presence of other variables should not exceed 0.05. F-statistics were used for the selection process. As a last step interactions between the variables remaining finally in the model were tested. Throughout the study the adopted significance level was $\alpha = 0.05$.

RESULTS

Baseline Characteristics

Baseline characteristics of the REGRESS patients included (N = 704) and not included (N = 181) are listed in table 1. No significant difference could be demonstrated between the two groups. In the studied patients (N = 704) overall mean age (± S.D.) was 56 (± 8) years, mean (± S.D.) body mass index was 26.0 (± 2.7) kg/m^2, mean (± S.D.) systolic blood pressure was 135 (± 18) mmHg and mean diastolic blood pressure (± S.D.) was 82 (± 10) mmHg. About 89% of the participants had previously smoked while 28% were current smokers. Of the studied patients, 51% were randomized to pravastatin treatment. For 702 patients baseline coronary scores could be computed. Two baseline angiograms were lost. The average MSD was 2.55 mm, and the average MOD was 1.89 mm. At baseline overall median apolipoprotein(a) was 236 U/l, the 25-75th interval being 91-665 U/l.

Serum apolipoprotein(a) was ≥ 286 U/l in 45%, and ≥ 430 U/l in 38% of the patients, 286 respectively 430 U/l being very approximately equal to 200 and 300 mg/l lipoprotein(a) mass, the generally accepted cut-off values for elevated CAD risk. Baseline median apolipoprotein(a) levels did not significantly differ between patients randomized to pravastatin or placebo (P = 0.84). Mean baseline cholesterol and HDL cholesterol levels were respectively 6.04 and 0.93 mmol/l.

The male control group (N = 274) had a mean (± S.D.) age of 50 (± 7) years, a mean (± S.D.) body mass index of 25.0 (± 2.4) kg/m^2, a mean (± S.D.) systolic blood pressure of 122 (± 13) mmHg and a mean diastolic blood pressure (± S.D.) of 77 (± 9) mmHg. About 77% of the participants had previously smoked while 45% were current smokers. The mean alcohol intake was 1.14 beverages per day. Median apolipoprotein(a) was 136 U/l and the 25-75th interval was 65-485 U/l. Mean cholesterol and HDL cholesterol levels were 5.66 and 1.11 mmol/l, respectively. After adjusting for age, body mass index, systolic and diastolic blood pressure, alcohol intake and smoking habits apolipoprotein(a) means were 300 U/l in the control group, versus 418 U/l in the REGRESS patient group (P = 0.0004). Adjusted mean cholesterol and HDL cholesterol were respectively 5.71 and 1.13 mmol/l in the control group, and 6.07 and 0.92 mmol/l in the patient group. P-values for the differences between adjusted means were 0.0044 and < 0.0001 for respectively cholesterol and HDL cholesterol (data not shown).

In-trial Serum Apolipoprotein(a) levels in Placebo and Pravastatin treated patients

Median baseline apolipoprotein(a) levels were 238 and 236 U/l in the placebo respectively pravastatin group; at study end median apolipoprotein(a) levels in placebo and pravastatin treated patients were 217 and 219 U/l (NS; data not shown). Spearman rank correlation between baseline and follow-up apolipoprotein(a) levels was 0.96. Whereas pravastatin did not significantly influence median apolipoprotein(a) levels, it significantly reduced mean cholesterol, LDL cholesterol and triglyceride levels, and increased mean HDL cholesterol compared to placebo treated patients (P < 0.001), the maximum pravastatin effect being reached three to four weeks after starting therapy (33).

In-trial Serum Apolipoprotein(a) and Metabolic Parameters in patients showing Progression, No Change, or Regression during the two year follow-up period

Table 2 demonstrates median *in-trial* serum apolipoprotein(a) levels in regressing, stable and progressing patients. In placebo treated patients apolipoprotein(a) levels differed significantly among categories, median apolipoprotein(a) levels being 1.2-fold higher in stable patients and 1.9-fold higher in progressors, compared to regressors (P = 0.0067). A similar trend was present in the pravastatin treated group, median apolipoprotein(a) levels being 1.6-fold higher in stable patients and 2.1-fold higher in progressors (NS). Overall, median apolipoprotein(a) levels were 143, 177 and 259 U/l in respectively regressing, stable and progressing patients (P = 0.0075; data not shown).

In placebo treated patients, median *in-trial* serum lipid levels did not differ among categories, whereas fibrinogen did (P = 0.038). In pravastatin treated patients, total and LDL cholesterol differed among groups. However, after correction for lipoprotein(a)-cholesterol, differences in median LDL cholesterol became insignificant.

Correlates and predictors of Coronary Score Changes

Bivariate correlation analysis (table 3) demonstrated that baseline MOD, baseline MSD, baseline use of long-acting nitrates and allocation to pravastatin were significant correlates to coronary score changes. Baseline and *in-trial* apolipoprotein(a) levels correlated significantly with MOD changes, but not with MSD changes. From table 3 it becomes obvious that *in-trial* serum lipid levels were more consistently and more closely related to arteriographic changes than baseline serum lipid levels. Table 4 demonstrates that after adjusting for the variates mentioned in the table, apolipoprotein(a) became significantly correlated with both MOD and MSD changes. Other predictors were *in-trial* HDL cholesterol, baseline MSD, baseline MOD and baseline use of long acting nitrates. Overall the MLR models predicted 14% of MSD changes and 12% of MOD changes; by itself, *in-trial* apolipoprotein(a) explained 2.6% of MSD changes and 4.8% of MOD changes (P < 0.01). From table 4 it becomes obvious that because of entering *in-trial* serum lipid concentrations into the model, allocation to pravastatin

no longer predicted MOD respectively MSD changes. In table 5 the magnitude of the effect of *in-trial* apolipoprotein(a) on arteriographic changes is demonstrated after adjusting for significant covariates only. From the ß-coefficients of the MLR equation it can be estimated that the mean MSD decrease per patient is 0.022 mm per Ln apolipoprotein(a) increment, and 0.060 mm per 0.5 mmol/l HDL cholesterol decrease. Analogously, mean MOD decrease per patient is estimated to be 0.025 mm per Ln apolipoprotein(a) increment, and 0.038 mm per 0.5 mmol/l HDL cholesterol decrease (derived from unranked MOD data which are not shown).

Modulation of apolipoprotein(a) atherogenicity by the lipoprotein milieu?

Apolipoprotein(a) atherogenicity was investigated in high-risk subgroups (table 6). Stratifications were made using the 10th percentile value of *in-trial* HDL cholesterol, and the 90th percentile values of *in-trial* LDL cholesterol (corrected), triglycerides, and LDL cholesterol/HDL cholesterol (N = 704). Table 6 demonstrates that *in-trial* serum apolipoprotein(a) correlates much stronger with adjusted MSD and MOD changes in patients with *in-trial* HDL cholesterol below 0.7 mmol/l, explaining 30% respectively 37% of their variances ($P < 0.05$). In contrast, in the subgroup with *in-trial* LDL cholesterol ≥ 4.96 mmol/l, only 1% and 2% of the MSD respectively MOD changes could be explained by serum apolipoprotein(a) (NS).

Modulation of apolipoprotein(a) atherogenicity by *in-trial* HDL cholesterol levels is presented in figure 1. Scattergrams display adjusted MSD respectively MOD reductions versus Ln apolipoprotein(a) for the two HDL cholesterol strata. The steep slope of the regression line in the low HDL subgroup (< 0.7 mmol/l) compared to the moderate slope in the higher HDL subgroup (≥ 0.7 mmol/l) reflects enhanced progression of CAD at similar apolipoprotein(a) levels in the low HDL subgroup. In the low HDL subgroup, the intersection of the regression lines with the line of no progression or regression coincides, for both MOD and MSD changes, with Ln apolipoprotein(a) of approximately 2, i.e. as low as 7 U/l apolipoprotein(a). In the lower risk stratum similar albeit weaker trends were found across the measured serum apolipoprotein(a) range. Yet, no clear cut-off apolipoprotein(a) above which most patients progressed could be demonstrated.

Table 1. Baseline Characteristics of the studied REGRESS patients (N = 704)

	Cases, no apo(a)	Cases, with apo(a)				P-Value[d]
Mean ± S.D. N (%)	N = 181	Overall N = 704	Placebo N = 346	Pravastatin N = 358	P-value[c]	
Patient records:						
Age, y	57 ± 8	56 ± 8	55 ± 8	57 ± 8	0.03	0.10
Systolic BP, mmHg	133 ± 20	135 ± 18	135 ± 19	135 ± 17	0.98	0.98
Diastolic BP, mmHg	80 ± 11	82 ± 10	82 ± 10	81 ± 9	0.14	0.18
BP ≥ 160/95 mmHg, n (%)	30 (17%)	123 (18%)	64 (19%)	59 (17%)	0.48	0.79
Hypertension by history, n (%)	53 (29%)	193 (27%)	103 (30%)	90 (25%)	0.17	0.59
Current Smokers, n (%)	51 (28%)	194 (28%)	94 (27%)	100 (28%)	0.82	0.84
Previous Smokers, n (%)	154 (86%)	624 (89%)	301 (87%)	323 (90%)	0.18	0.26
Body mass index, kg/m^2	26.1 ± 2.5	26.0 ± 2.7	26.2 ± 2.7	25.8 ± 2.8	0.10	0.59
Body mass index ≥ 30 kg/m^2, n (%)	14 (8%)	60 (9%)	33 (10%)	27 (8%)	0.32	0.72
Long acting nitrates, n (%)	100 (56%)	391 (56%)	191 (55%)	200 (56%)	0.86	0.99
ß-blocking agents, n (%)	141 (78%)	509 (72%)	253 (73%)	256 (72%)	0.63	0.10
Calcium Channel Blockers, n (%)	111 (62%)	425 (60%)	207 (60%)	218 (61%)	0.77	0.75
Familial heart disease, %	85 (48%)	345 (49%)	168 (49%)	177 (49%)	0.81	0.77
History of myocardial infarction, %	92 (51%)	327 (46%)	154 (45%)	173 (48%)	0.31	0.26
History of PTCA, %	8 (4.4%)	44 (6.3%)	22 (6.0%)	22 (6.0%)	0.91	0.36
Angiographic data:						
Vessel score, n (%)					0.51	0.13
1	61 (34%)	300 (43%)	154 (45%)	146 (41%)		
2	66 (37%)	234 (33%)	114 (33%)	120 (34%)		
3	50 (28%)	168 (24%)	77 (22%)	91 (26%)		

Table 1. Cont'd

	Cases, no apo(a)	Cases, with apo(a)				P-Value[d]
Mean ± S.D. N (%)	N = 181	Overall N = 704	Placebo N = 346	Pravastatin N = 358	P-value[c]	
MSD, mm	2.55 ± 0.42	2.55 ± 0.40	2.56 ± 0.41	2.54 ± 0.38	0.47	0.85
MOD, mm	1.88 ± 0.38	1.89 ± 0.34	1.91 ± 0.34	1.88 ± 0.34	0.27	0.68
Laboratory data:						
Apolipoprotein(a), U/l[a]	N.A	415 ± 422 (236)	430 ± 442 (238)	400 ± 402 (236)	0.86	-
Cholesterol, mmol/l	6.03 ± 0.81	6.04 ± 0.87	6.06 ± 0.86	6.02 ± 0.88	0.56	0.86
HDL cholesterol, mmol/l	0.93 ± 0.25	0.93 ± 0.23	0.92 ± 0.22	0.94 ± 0.23	0.28	0.92
LDL cholesterol, mmol/l	4.30 ± 0.75	4.31 ± 0.79	4.32 ± 0.79	4.30 ± 0.79	0.61	0.87
Corrected LDL cholesterol, mmol/l[b]	N.A.	4.09 ± 0.79	4.11 ± 0.77	4.07 ± 0.80	0.57	-
Triglycerides, mmol/l	1.96 ± 0.69	1.94 ± 0.71	1.97 ± 0.72	1.92 ± 0.69	0.47	0.70
LDL cholesterol/HDL cholesterol ratio	4.90 ± 1.37	4.88 ± 1.40	4.94 ± 1.43	4.83 ± 1.38	0.54	0.88
Blood glucose, mmol/l	5.24 ± 1.20	5.36 ± 1.31	5.27 ± 1.15	5.44 ± 1.45	0.08	0.31
Fibrinogen, g/l	3.15 ± 1.28	3.36 ± 1.42	3.45 ± 1.43	3.28 ± 1.42	0.21	0.14

HDL: high-density lipoprotein; LDL: low-density lipoprotein; MOD: minimum obstruction diameter; MSD: mean segment diameter; N.A.: not available; PTCA: percutaneous transluminal coronary angioplasty. See "Methods" for definitions of coronary scores. Vessel score: number of diseased coronary vessels. [a]Apolipoprotein(a) values are expressed as mean ± S.D. (median). [b]LDL cholesterol (mmol/l) corrected for lipoprotein(a)-cholesterol and calculated as: Cholesterol (mmol/l) - HDL cholesterol (mmol/l) - [triglycerides (mmol/l)/2.2] - [(apolipoprotein(a) (U/l) * 0.7 * 0.3) / 386.65]. [c]P-values for differences in means between pravastatin and placebo treated cases in which apo(a) was measured. [d]P-values for differences in means between cases with apo(a) measured respectively not measured. P-values were derived from Student's t-test, Pearson's chi-square test, or Mann-Whitney test, where appropriate.

Table 2. Serum Apolipoprotein(a) and other Laboratory Findings in Regressing, Stable, and Progressing patients (N = 567)

	Placebo treated patients			
In-trial concentration: Median (minimum - maximum range)	Regressing (N = 27)	Stable (N = 95)	Progressing (N = 164)	P-value (Kruskall-Wallis test)
Apolipoprotein(a), U/l	130 (6-1236)	162 (5-1677)	251 (5-2143)	0.0067
Total cholesterol, mmol/l	6.30 (3.43-8.52)	6.14 (3.77-8.43)	6.06 (2.99-8.79)	0.58
LDL cholesterol, mmol/l	4.34 (1.84-6.86)	4.39 (2.46-6.51)	4.37 (1.78-6.74)	0.68
Corrected LDL cholesterol, mmol/l[a]	4.17 (1.82-6.76)	4.22 (2.20-6.41)	4.11 (1.69-6.67)	0.42
HDL cholesterol, mmol/l	0.94 (0.58-1.82)	0.88 (0.56-1.58)	0.86 (0.42-2.14)	0.61
LDL cholesterol/HDL cholesterol	4.62 (1.59-8.30)	4.93 (2.59-8.86)	5.05 (1.67-11.64)	0.55
Triglycerides, mmol/l	1.74 (0.45-5.90)	1.57 (0.57-7.10)	1.37 (0.38-7.60)	0.10
Blood glucose, mmol/l	5.1 (4.1-7.1)	5.1 (3.6-10.2)	5.2 (3.4-9.0)	0.97
Fibrinogen, g/l[b]	2.68 (1.28-6.61)	3.19 (1.03-7.79)	3.29 (1.27-7.73)	0.038

Table 2. Cont'd

In-trial concentration: Median (minimum - maximum range)	Pravastatin treated patients: Regressing (N = 45)	Stable (N = 108)	Progressing (N = 45)	P-value (Kruskall-Wallis test)
Apolipoprotein(a), U/l	143 (8-1573)	224 (10-2295)	306 (5-1627)	0.34
Total cholesterol, mmol/l	4.46 (3.23-7.06)	4.88 (2.78-7.31)	4.87 (3.11-9.91)	0.04
LDL cholesterol, mmol/l	2.98 (1.83-4.46)	3.13 (1.56-5.52)	3.28 (1.56-5.97)	0.03
Corrected LDL cholesterol, mmol/l[a]	2.69 (1.70-4.39)	2.87 (1.33-5.51)	3.12 (1.21-5.72)	0.09
HDL cholesterol, mmol/l	0.99 (0.55-1.59)	0.95 (0.55-1.91)	1.03 (0.55-1.91)	0.54
LDL cholesterol/HDL cholesterol	2.99 (1.55-6.64)	3.15 (1.09-8.49)	3.32 (1.37-10.85)	0.06
Triglycerides, mmol/l	1.34 (0.48-4.32)	1.31 (0.44-5.65)	1.30 (0.45-7.10)	0.70
Blood glucose, mmol/l	5.3 (4.2-11.1)	5.0 (3.4-12.6)	5.20 (3.30-11.0)	0.36
Fibrinogen, g/l[b]	3.12 (2.04-5.91)	3.02 (1.30-8.40)	2.98 (1.16-8.42)	0.10

See "Methods" for definitions of progression, no change and regression. [a]LDL cholesterol (mmol/l) corrected for lipoprotein(a)-cholesterol and calculated as: Cholesterol (mmol/l) - HDL cholesterol (mmol/l) - [triglycerides (mmol/l)/2.2] - [(apolipoprotein(a) (U/l) * 0.7 * 0.3) / 386.65]. [b]*baseline* fibrinogen concentration.

Table 3. Relations between Changes of Coronary Scores with Clinical and Laboratory Findings. Results of bivariate correlation analysis.

	Change of MSD N = 562	Change of MOD N = 567
Baseline MSD, mm	0.31[c]	0.17[c]
Baseline MOD, mm	0.27[c]	0.24[c]
Age, y	-0.05	-0.01
Body mass index, kg/m^2	0.02	0.01
Current smoking	0.03	0.02
Previous smoking	0.04	0.01
Systolic blood pressure, mmHg	0.03	0.08[a]
Diastolic blood pressure, mmHg	0.05	0.12[b]
Long acting nitrate medication	0.14[c]	0.09[a]
ß-blocking medication	0.08[a]	0.08[a]
Ca channel blocking medication	0.02	-0.04
Pravastatin medication	-0.12[b]	-0.16[c]
Baseline concentration:		
Apolipoprotein(a), U/l (ln)	0.06	0.09[a]
Total cholesterol, mmol/l	0.06	0.06
HDL cholesterol, mmol/l	-0.05	-0.02
LDL cholesterol, mmol/l	0.07[a]	0.06
LDL cholesterol/HDL cholesterol ratio (ln)	0.09[a]	0.07[a]
Corrected LDL cholesterol, mmol/l[d]	0.08[a]	0.06
Triglycerides, mmol/l (ln)	0.05	0.02
Blood glucose, mmol/l	0.05	-0.04
Fibrinogen, g/l (ln)	0.00	0.03
In-trial concentration:		
Apolipoprotein(a), U/l (ln)	0.05	0.11[b]
Total cholesterol, mmol/l	0.06	0.11[b]
HDL cholesterol, mmol/l	-0.08[a]	-0.10[b]
LDL cholesterol, mmol/l	0.09[a]	0.13[c]
LDL cholesterol/HDL cholesterol ratio (ln)	0.13[c]	0.18[c]
Corrected LDL cholesterol, mmol/l[d]	0.08[a]	0.11[b]
Triglycerides, mmol/l (ln)	-0.05	-0.00
Blood glucose, mmol/l	-0.01	0.02

ln = natural logarithm; MOD = minimum obstruction diameter; MSD = mean segment diameter. See "Methods" for definitions of coronary scores. Results are reported as Pearson correlation coefficients with change of MSD respectively ranked MOD.
[a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$. [d]LDL cholesterol corrected for lipoprotein(a)-cholesterol and calculated as: Cholesterol (mmol/l) - HDL cholesterol (mmol/l) - [triglycerides (mmol/l)/2.2] - [(apolipoprotein(a) (U/l) * 0.7 * 0.3) / 386.65].

Table 4. Relations between Adjusted Coronary Score Changes and Clinical and Laboratory Findings. Results of Multiple Regression Analyses.

	Change of	
	MSD N = 562	MOD N = 567
Baseline MSD	0.28[c]	-
Baseline MOD	-	0.27[c]
Age	0.00	0.13[a]
Body mass index	-0.02	-0.01
Current smoking	0.08	0.05
Previous smoking	0.08	0.08
Systolic blood pressure	-0.08	-0.09
Diastolic blood pressure	0.08	0.10
Long acting nitrate medication	0.22[c]	0.12
Pravastatin medication	-0.03	-0.07
In-trial apolipoprotein(a)	0.16[b]	0.22[c]
In-trial LDL cholesterol (corrected)[d]	0.05	0.02
In-trial HDL cholesterol	-0.17[b]	-0.14[a]
In-trial triglycerides	-0.08	-0.01
In-trial blood glucose	0.08	0.09
Baseline fibrinogen	-0.03	0.00
Multiple R	0.43	0.41
Adjusted R^2	14%	12%

HDL: high-density lipoprotein; LDL: low-density lipoprotein; ln = natural logarithm; MSD = mean segment diameter; MOD: minimum obstruction diameter. Results are reported as partial correlation coefficients, adjusted for all other variates in the table, for change of MSD respectively change of ranked MOD.
[a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$. [d]LDL cholesterol (mmol/l) corrected for Lp(a)-cholesterol and calculated as: Cholesterol (mmol/l) - HDL cholesterol (mmol/l) - [triglycerides (mmol/l)/2.2] - [(apolipoprotein(a) (U/l) * 0.7 * 0.3) / 386.65].

DISCUSSION

The consistent clinical lesson from current arteriographic trials is that patients with documented CAD benefit from aggressive lipoprotein manipulations with regard to both coronary artery lumen change and clinical events (41), irrespective

Table 5. Predictors of arteriographic changes (MOD, MSD) in the studied REGRESS patients (N = 567). Results of Stepwise forward Multiple Linear Regression analysis.

Predictors in the equation	ß	SE ß	P-value	Adjusted R^2 (%)
Dependent variable = change of MSD:				
Baseline MSD, mm	0.112	0.024	< 0.0001	6.5
Long acting nitrate medication	0.080	0.022	0.0003	10.1
In-trial HDL cholesterol, mmol/l	-0.120	0.043	0.0057	12.0
In-trial apolipoprotein(a), U/l (ln)	0.022	0.008	0.0070	13.9
(Constant)	0.278	0.095	0.0036	-
Dependent variable = change of MOD (ranked):				
Baseline MOD, mm	0.247	0.055	< 0.0001	5.6
In-trial apolipoprotein(a), U/l (ln)	22.47	6.41	0.0005	8.9
In-trial HDL cholesterol, mmol/l	-82.48	33.11	0.0133	10.4
Long acting nitrate medication	37.81	17.56	0.0321	11.5
(Constant)	153.0	50.47	0.0027	-

ß = regression coefficient; ln = natural logarithm; MOD = minimum obstruction diameter; MSD = mean segment diameter; SE = standard error. See "Methods" for definitions of coronary scores. Baseline MOD, baseline MSD, age, body mass index, current and previous smoking, systolic and diastolic blood pressure, use of long acting nitrates, allocation to pravastatin, baseline fibrinogen levels, and *in-trial* serum levels of apolipoprotein(a), LDL cholesterol, HDL cholesterol, triglycerides and blood glucose were the variables entered into the model.

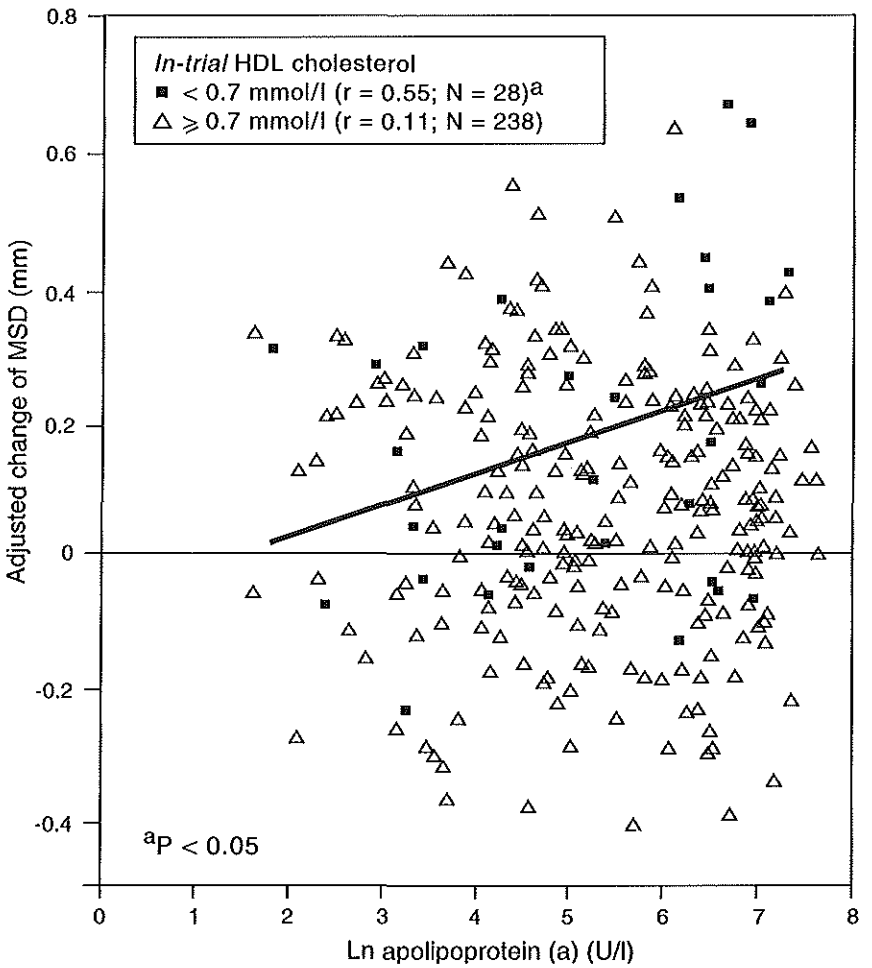
of the baseline cholesterol level. Subgroup analysis of the Scandinavian Simvastatin Survival Study (4S) (42) showed that percentage reductions in LDL cholesterol and decreases in relative risk of coronary heart disease in patients on simvastatin were comparable and constant across all quartiles of baseline LDL cholesterol, suggesting that the percentage reduction of LDL cholesterol rather than its absolute level on treatment was the determinant of clinical benefit. Thompson et al. (43) further tested this hypothesis across eleven quantitative angiographic trials and found a significant relationship between percent change in LDL cholesterol and change in percent diameter stenosis. Yet, this precept was tempered by e.g. the results of HARP in which lipid-lowering appeared ineffectual

Table 6. Correlations between *in-trial* Serum Apolipoprotein(a) levels and Changes of Coronary Scores in selected strata. Results of bi- and multivariate correlation analyses.

	Change of MSD N = 562				Change of MOD N = 567			
Stratification*	N	Pearson correlation coefficient	N	Partial correlation coefficient**	N	Pearson correlation coefficient	N	Partial correlation coefficient**
HDL cholesterol < 0.70 mmol/l	51	0.27[a]	28	0.55[a]	52	0.31[b]	30	0.61[b]
HDL cholesterol ≥ 0.70 mmol/l	501	0.04	238	0.11	505	0.10[a]	242	0.18[b]
Triglycerides ≥ 2.74 mmol/l	53	0.24	20	0.44	57	0.37[b]	22	0.27
Triglycerides < 2.74 mmol/l	499	0.04	248	0.14[a]	500	0.08	250	0.20[b]
LDL cholesterol ≥ 4.96 mmol/l***	60	-0.06	32	0.01	61	-0.01	33	0.14
LDL cholesterol < 4.96 mmol/l***	482	0.09	236	0.17[a]	486	0.13[b]	39	0.23[c]
LDL cholesterol/HDL cholesterol ≥ 6.11***	55	0.28[a]	33	0.40	56	0.12	34	0.34
LDL cholesterol/HDL cholesterol < 6.11***	487	0.04	237	0.15[a]	491	0.11[a]	240	0.21[b]

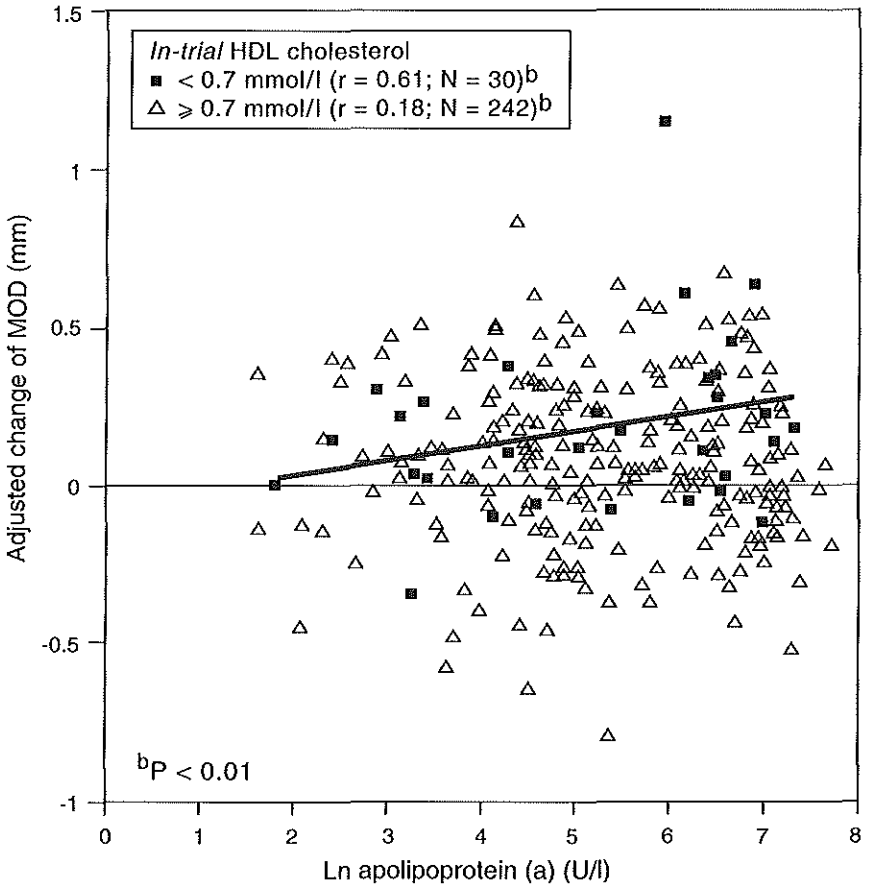
*Cut-off values for risk stratification were based upon the 10th percentile *in-trial* value for HDL cholesterol, and the 90th percentile *in-trial* values for LDL cholesterol, triglycerides and LDL cholesterol/HDL cholesterol ratio. **The model parameters controlled for are: baseline MSD, baseline MOD, age, body mass index, smoking habits, systolic and diastolic blood pressure, pravastatin treatment, use of long acting nitrates, baseline fibrinogen, and *in-trial* blood glucose, serum LDL cholesterol, HDL cholesterol, and triglyceride levels. ***LDL cholesterol was corrected for lipoprotein(a)-cholesterol and calculated as: Cholesterol (mmol/l) - HDL cholesterol (mmol/l) - [triglycerides (mmol/l)/2.2] - (apolipoprotein(a) (U/l) * 0.7 * 0.3) / 386.65]. [a]$p < 0.05$; [b]$p < 0.01$; [c]$p < 0.001$.

Figure 1. Partial correlation between *in-trial* serum apolipoprotein(a) levels and changes of mean segment diameter respectively minimum obstruction diameter in selected HDL cholesterol strata (HDL cholesterol cut-off = 0.7 mmol/l). The model parameters controlled for are: baseline MSD, baseline MOD, age, body mass index, smoking status, systolic and diastolic blood pressure, pravastatin treatment, use of long acting nitrates, baseline fibrinogen, *in-trial* blood glucose, and *in-trial* serum LDL cholesterol, HDL cholesterol, and triglyceride levels. For the low HDL cholesterol subgroup the regression lines are given.



in patients whose baseline values of LDL cholesterol were in the normal range (44). Taken together, the 4S data and analyses of quantitative coronary angiographic trials suggest that although lowering LDL cholesterol by 35% often

Figure 1. Cont'd.



helps to slow or halt progression of atherosclerosis, it does not always do so (43). This presumably reflects the importance of other risk factors in promoting progression of lesions, for which lipoprotein(a) is a candidate. As only few prospective angiographic studies have hitherto examined the association of serum lipoprotein(a) with the course of CAD (29-32, 45), we aimed to define, in a broader range of patients with documented CAD, in which patients lipoprotein(a) assessments have the highest predictive value. To this end, apolipoprotein(a) levels were determined in adequately stored left-over sera from REGRESS patients (33).

Results of this substudy are threefold. Firstly, median baseline apolipoprotein(a) levels were significantly elevated in REGRESS patients, who all had symptomatic CAD, compared to healthy control subjects (236 U/l versus 136 U/l;

$P < 0.001$); furthermore, median *in-trial* apolipoprotein(a) levels were significantly higher in progressors (259 U/l) and stable patients (177 U/l) than they were in regressors (143 U/l). Secondly, *in-trial* apolipoprotein(a) and *in-trial* HDL cholesterol, but not *in-trial* LDL cholesterol, predicted the course of CAD in normal to moderately hypercholesterolaemic males. Thirdly, apolipoprotein(a) atherogenicity was far more daunting in case of pathologic partnerships with concomitant low HDL cholesterol levels (< 10^{th} percentile).

The right-shift of the serum apolipoprotein(a) distribution in REGRESS patients compared to apparently healthy controls is in accordance with the findings of numerous population and clinical studies (3-17). The significantly higher apolipoprotein(a) levels in patients categorized as progressors or stable compared to regressors (table 2) are in agreement with the data of Terres et al. (31) but in contrast with the data of Watts et al. (29) and Marburger et al. (30). The finding that, when entering *in-trial* serum lipid levels instead of baseline lipid levels into the MLR models, both *in-trial* apolipoprotein(a) and *in-trial* HDL cholesterol, but not *in-trial* LDL cholesterol, predicted arteriographic changes (tables 4 and 5) in this patient group is not surprising when one takes into account the constellation of serum lipids in the REGRESS population. After all, decreased HDL cholesterol levels (< 0.90 mmol/l), pre-existing in approximately 50% of the REGRESS population (table 1), and elevated apolipoprotein(a) levels were major characteristics of its risk profile, besides moderate baseline hypercholesterolaemia. Moreover, the cholesterol-lowering intervention with pravastatin in half of the patients, mainly affecting LDL cholesterol levels, further diminished the causal role of LDL as a risk factor. Although apolipoprotein(a) was a predictor of MSD and MOD changes in the studied REGRESS patients, it explained only 2.6% respectively 4.8% of coronary score changes (table 4). In case of concomitant presence of *in-trial* HDL cholesterol below 0.7 mmol/l, the strength of the association increased tremendously, explaining up to 30 and 37% of adjusted MSD respectively MOD changes (figure 1, table 6). By delineating other adverse lipoprotein milieus, our results extend the findings of Armstrong and coworkers (3), and Maher and coworkers (46) in selected hyper-cholesterolaemic patients in whom dependence of apolipoprotein(a) atherogenicity on serum LDL cholesterol levels was described. Although modulation of apolipoprotein(a) atherogenicity by

in-trial LDL cholesterol levels was present in the REGRESS patient group it was less prominent than the modulation by *in-trial* HDL cholesterol (table 6). Moreover, our data suggest that differences in lipoprotein milieus and other covariates may explain discrepancies between studies investigating the association between lipoprotein(a) levels and CAD course (29-32). Also, whereas Maher et al. (46) documented that arterial benefits of substantial (> 10%) LDL cholesterol reductions were only significant in patients with lipoprotein(a) levels in at least the 90th percentile, we found, even at low apolipoprotein(a) levels, a deleterious effect of the lipoprotein(a) level on coronary lumen changes in the low HDL subgroup.

Throughout the article *in-trial* serum lipid concentrations were used to examine the association between apolipoprotein(a) levels and CAD course. This was decided because *in-trial* serum lipid levels correlated more closely with changes of MOD and MSD than baseline serum lipid levels (table 3). Also, patient data were pooled to a single collective because serum apolipoprotein(a) levels were not affected by pravastatin treatment (median levels after two years of follow-up: 217 U/l in controls versus 219 U/l in cases), and because allocation to pravastatin no longer predicted arteriographic changes in multivariate models in which *in-trial* serum lipid levels were entered as covariates (tables 4 and 5). Yet, in order not to ignore any direct effect of pravastatin treatment upon the CAD course (47), adjustments for allocation to pravastatin were made. The finding that baseline use of long-acting nitrates seemed independently associated with the course of CAD probably reflected less favourable clinical outcome in more severely diseased patients.

To our knowledge, this is the first prospective angiographic study of this size in a wide range of patients with manifest CAD and normal to moderately elevated cholesterol, which delineates the lipoprotein milieus that restrain or reinforce apolipoprotein(a) atherogenicity. Another advantage is related to the fact that LDL cholesterol was corrected for lipoprotein(a)-cholesterol, allowing to distinguish the differential effects of these lipoproteins on the course of CAD. Furthermore, since all apolipoprotein(a) measurements were performed within two years, apolipoprotein(a) results were not confounded by lipoprotein(a) degradation due to long-term storage (25). Limitations of this substudy are related to the fact that so far we do not know whether the atherothrombogenicity of lipoprotein(a) is

adequately measured by total lipoprotein(a) or apolipoprotein(a) concentrations alone. Future studies should investigate the genetically determined structural and/or functional polymorphism of lipoprotein(a). Secondly, the number of major clinical events was too small (N =12) to examine the association of apolipoprotein(a) levels with patient outcome [4/358 (1.1%) in the pravastatin treated group and 8/346 (2.3%) in the placebo treated group; data not shown]. However, as angiographic changes strongly correlate with future coronary events (48-50), it is realistic to anticipate also in this population an effect of apolipoprotein(a) levels on clinical events with longer follow-up.

We conclude that lipoprotein(a) is a predictor of coronary artery lumen changes in normal to moderately hypercholesterolaemic male Caucasians, its atherogenicity being very pronounced in case of concomitant hypoalphalipoproteinemia.

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Chapter 10

GENERAL DISCUSSION

CONCLUDING REMARKS

This thesis deals with documenting serum Lp(a) and its correlates across Asian, African and Caucasian populations, and with delineating the lipoprotein milieus in Caucasians in which serum lipoprotein(a) (Lp(a)) truly predicts angiographically documented progression of coronary artery disease. Besides, the effects of sexual maturation, apo E polymorphism, clinical state of the patient and pravastatin treatment on its levels were examined.

To this end, Lp(a) was measured with either an enzyme-linked immunosorbent assay (ELISA) (Biopool Ltd, Umeå, Sweden) or, in the case of the REGRESS study, with an immunoradiometric (IRMA) kit (Mercodia, Upssala, Sweden). The first method reports in mg/l Lp(a) mass; the latter in U/l apo(a) mass. According to Mercodia, 1 U/l corresponds very approximately to 0.7 mg/l Lp(a) mass. Yet, from the method comparisons displayed in chapter 2, it is obvious that methodological differences exist (ELISA = 0.19 + 0.544 IRMA; r = 0.996), underscoring the need for international standardization of Lp(a) (1, 2). Moreover, it was documented that either Lp(a) test method was sensitive to Lp(a) degradation upon long-term storage of sera, especially at -20°C, and that valid Lp(a) determinations could be conducted only if sera were stored both at -70°C and for less than two years. Finally, our stability data proved that the conclusions of some of the 'negative' Lp(a) studies, that failed to find an association between elevated Lp(a) levels and clinical events (3-5), were at least questionable as the data were confounded by long-term storage of specimens causing Lp(a) degradation (6).

In chapter 3 it is demonstrated that, due to the marked skewness of the serum Lp(a) distribution in Caucasians and the 1000-fold interindividual differences in serum levels, the average intra-individual biological coefficient of variation differed among groups, i.e. among ostensibly healthy volunteers and stable outpatients with hyper-Lp(a)-lipoproteinemia. In healthy Caucasian men and women the average intra-individual biological coefficient of variation (CV_b) was 20.0%, and no significant difference was found between men and women. In a stable outpatient group the CV_b was significantly lower (7.5% versus 20.0%;

$P < 0.0001$). Besides, a significant, inverse relationship existed between biological and analytical coefficient of variation (CV_a), respectively, and serum Lp(a) levels. It was concluded that in the case of Lp(a) the general approach of using average CV_b (and hence average CV_a) values is not valid. Instead, individual CV_b values should be used because a significant and inverse concentration dependency was observed. The latter finding also explains discrepant CV_bs reported in literature so far (7-13).

In the epidemiological part, Lp(a) was firstly studied across populations characterized by different prevalences of coronary heart disease. In chapter 4 serum Lp(a) and its correlates were studied in unique sera of African Aboriginal Pygmies ($N = 146$) and Bantus ($N = 208$) from Cameroon, and compared to Asian and Caucasian population samples, using the same Lp(a) methodology. Geometric mean Lp(a) levels were 274 and 289 mg/l in Bantu males and females respectively, and 220 and 299 mg/l in Pygmy males and females, the gender difference being significant in Pygmies ($P = 0.024$). In Pygmies 41 and 52% of the males and females, respectively, had Lp(a) levels above 300 mg/l, compared to 47 and 55% in Bantus. Overall, Lp(a) levels did not significantly differ between Pygmies and Bantus, and did not correlate with age, body mass index (BMI), systolic and diastolic blood pressure. Compared to healthy Asian and Caucasian population samples, age- and BMI-adjusted geometric Lp(a) means were 2.3 to 5.0-fold higher in Pygmy and Bantu males, and 2.9 to 3.6-fold higher in Pygmy and Bantu females ($P \leq 0.05$), notwithstanding the virtual absence of overt coronary heart disease. From these comparative population studies it is concluded that ethnicity is a significant determinant of serum Lp(a) levels, and that elevated serum levels of Lp(a) are not necessarily associated with an increased prevalence of atherosclerosis. Moreover, these data suggest that the arbitrary cut-off of 300 mg/l, indicating enhanced risk for coronary heart disease in Caucasians (14), does not apply to the African population samples studied. After all, Lp(a) excess, being prevalent in Africans, does not seem to be as challenging in Africans as in Caucasians.

Secondly, Lp(a) and its correlates were investigated in a Caucasian population sample from a typical Western industrialized country, having a relatively high

prevalence of coronary heart disease (chapters 5 and 6). To this end, unrelated and ostensibly healthy Belgian children and adults were examined. In chapter 5 serum Lp(a) levels and the impact of sexual maturation upon serum Lp(a) were investigated in pubertal schoolchildren, using a cross-sectional design. Median Lp(a) levels were 82, 117, 110, 100 and 73 mg/l at the five subsequent genital development stages in boys (ANOVA, $P = 0.816$), and 73, 78, 204, 110 and 114 mg/l at the five breast development stages in girls (ANOVA, $P = 0.087$). Lp(a) distributions in boys and girls were skewed to the low concentration end, overall medians being 82 and 94 mg/l ($P = 0.254$). The 90^{th} and 95^{th} percentiles were 515 respectively 712 mg/l. Multiple linear regression pointed out that developmental age, chronologic age, body mass index and/or systolic blood pressure predicted serum lipid and apolipoproteins levels, but could not explain the Lp(a) variance. It was concluded that pubertal stage was not significantly associated with Lp(a) levels in Belgian schoolchildren, supporting the contention that serum Lp(a) is predominantly under genetic control (14). On the other hand, the overall geometric Lp(a) mean in schoolchildren was found to be significantly higher compared to that in 683 sex-matched, 20 to 39 year old adults from the same region (89 mg/l versus 69 mg/l; $P = 0.006$), suggesting an effect of age and/or of sexual maturation and puberty on serum Lp(a) levels, similar to the effect observed on the serum cholesterol levels (15).

In chapter 6 it was investigated whether genetically determined risk factors, i.e. lipoprotein(a) and apo E polymorphism, contributed to the repeatedly observed north-south differences in serum lipid -mainly cholesterol- distribution and mortality from ischaemic heart disease in Belgium (16-19). Besides, the impact of the apo E polymorphism on the serum Lp(a) levels was examined. Median Lp(a) was found to be 67 mg/l in northerners and 75 mg/l in southerners, the difference not being significant. The apo E phenotype distribution was similar in both regions, the overall relative apo E allele frequency being 0.092 for ε2, 0.767 for ε3 and 0.141 for ε4. Strikingly, the average effects of the apo E alleles on adjusted serum lipid levels differed among regions: in southerners the ε4 and the ε2 effects upon adjusted apo B and LDL-c levels were $\approx +12\%$ and $\approx -15\%$, respectively ($P < 0.05$); in northerners the ε4 and ε2 effects were $\approx +5\%$ (NS) and $\approx -25\%$ ($P < 0.05$), respectively. On the contrary, the apo E polymorphism did not at all

affect the serum Lp(a) levels. It is concluded from this study that Lp(a) levels and apo E phenotype distribution do not significantly differ between Belgian northerners (Flemings) and southerners (Walloons), supporting a similar genetic background.

The more pronounced LDL-c- and apo B-raising effect of the ε4 allele and the less marked LDL-c- and apo B-decreasing effect of the ε2 allele in southerners compared to northerners, suggest an apo E gene - environment interaction that variably affects LDL-c and apo B levels among the two regions. In view of the well-documented differences in saturated fat intake between Belgian northerners and southerners (18, 19), it is hypothesized that the higher saturated fat intake in southerners likely explains the differences in observed apo E allelic effects.

In the clinical part, the clinical relevance of the enigmatic Lp(a) was studied in patients with documented coronary artery disease, either from Belgian (chapters 7 and 8) or from Dutch ancestry (chapter 9). Analogously, serum Lp(a) and its correlates were studied, now in the context of two placebo-controlled clinical intervention trials. In the first clinical study the serum Lp(a) time course was studied in 100 male patients undergoing elective coronary artery bypass grafting (CABG), using a cross-sectional design. In this study, baseline median Lp(a) was approximately twice the median Lp(a) value measured in apparently healthy Belgian volunteers (chapter 6, 20), indicating a high prevalence of Lp(a) excess in atherosclerotic Belgian males. Patients were randomized in a placebo (N = 50) and a pravastatin treated (N = 50) group. The pravastatin regimen was 10 mg daily from the third post-operative day on, and 20 mg daily after 1 week during 11 weeks. Lp(a) levels and serum lipids were analyzed at baseline, at 3 and 10 days, and at 4 and 12 weeks post-CABG. Irrespective of treatment, a similar and significant decline of serum Lp(a) was observed in all patients at the third post-operative day, which paralleled the changes noted for the other serum lipids in the post-CABG period and which could not be explained by hemodilution effects (chapter 7). Overall, median Lp(a) decreased from 135 mg/l at baseline to 67 mg/l at the third post-operative day, apparently conflicting with the paradoxical increase described by Sgoutas et al. (21). At the 10th post-operative day a slight but significant overshooting was noticed for Lp(a) but not for the other serum lipids, followed

by a decrease of the serum Lp(a) levels to pre-operative levels one month after the acute event. The "overshooting" at day 10 accorded with the acute phase behaviour of Lp(a) as documented by Maeda and coworkers (22), and with the presence of multiple interleukin-6 responsive elements in the apo(a) promotor gene (23).

From this study it is concluded that there is a significant time-dependency of the serum Lp(a) levels in the post-CABG period and that there is no significant effect of pravastatin treatment upon serum Lp(a). The data also reveal that reliable post-operative Lp(a) measurements can be made at earliest 1 month post-CABG, illustrating that a stable clinical state in the patient is a prerequisite for getting a valid Lp(a) level. Moreover, in an additional study (chapter 8) Lp(a) time courses were documented during cardiopulmonary bypass, using serial specimens. It was suggested that the paradoxical Lp(a) increase noted within minutes after starting cardiopulmonary bypass by Sgoutas and coworkers (21) is an artefact, caused by taking inappropriate baseline Lp(a) values as reference points.

Finally, in the context of a prospective arteriographic study (24) we examined retrospectively whether lipoprotein(a) levels predicted coronary artery lumen changes in patients with symptomatic coronary artery disease and normal to moderate hypercholesterolaemia. To this end, the association between serum lipoprotein(a) and coronary artery lumen changes was studied in 704 males who entered REGRESS, a double-blind, placebo controlled, quantitative angiographical study which assessed the effect of two year of pravastatin treatment. Primary endpoints were change in average mean segment diameter (MSD) and change in average minimum obstruction diameter (MOD). In this study the Mercodia test kit for Lp(a) was used. Median serum apolipoprotein(a) (apo(a)) levels were 1.74-fold higher in Dutchmen with symptomatic coronary heart disease compared to healthy male controls (236 U/l versus 136 U/l), again pointing to a high prevalence of Lp(a) excess in atherosclerotic Caucasian males. In placebo treated patients median *in-trial* apo(a) levels in regressing, stable and progressing patients were 130, 162 and 251 U/l respectively, while 143, 224 and 306 U/l in pravastatin treated patients. Pravastatin treatment during 24 months did not significantly affect serum apolipoprotein(a) levels. Overall, median *in-trial* apo(a) levels differed significantly between progressors, regressors and stable patients. Predictors of MSD (MOD) changes were baseline MSD (MOD), *in-trial* apolipoprotein(a), *in-*

trial HDL cholesterol and baseline use of long acting nitrates. The multivariate models explained 14% and 12% of respectively MSD and MOD changes; apolipoprotein(a) by itself explained only 2.6% and 4.8%. In a subgroup of patients having *in-trial* HDL cholesterol levels below 0.7 mmol/l, apolipoprotein(a) explained up to 37% of arteriographic changes. A novel finding in this study is related to the fact that serum lipoprotein(a) predicted coronary artery lumen changes also in normal to moderately hypercholesterolaemic Caucasian males with coronary artery disease, and not only in hypercholesterolemic men (25). Besides, the Lp(a) atherogenicity was shown to be especially pronounced in case of concomitant hypoalphalipoproteinaemia. Therefore, these findings provide an original extension to current literature upon Lp(a) and coronary artery disease in Caucasians, by delineating other lipoprotein milieus -besides LDL excess- that augment the adverse effects of Lp(a) (25). Finally, these data shed some light on the conflicting reports that had been published lately, confirming or refuting the association of lipoprotein(a) with clinical events or angiographically verified disease progression (3-5, 14).

In conclusion, the epidemiological studies in African, Asian and Caucasian populations suggest that the challenge of elevated Lp(a) levels is not as daunting in Africans as it is in Caucasians. The higher serum Lp(a) levels in blacks must either be counteracted by other factors or be in itself an insufficient cause for developing atherosclerosis, since the blacks studied have virtually no coronary heart disease. From the clinical studies in atherosclerotic Caucasian males it was established that median Lp(a) levels were significantly higher in patients with coronary artery disease compared to healthy controls, indicating a high prevalence of Lp(a) excess in the atherosclerotic patient, in agreement with others (26-29). Finally, Lp(a) was shown to predict coronary artery lumen changes also in normal to moderately hypercholesterolemic middle-aged Caucasian males, especially in the case of concomitant hypoalphalipoproteinemia, underscoring its role as an independent and premature risk factor for coronary artery disease in Caucasian males, and pointing to the tangled web of coronary risk factors and the multifactorial origin of coronary artery disease.

LIPOPROTEIN(a) DETERMINATIONS IN PERSPECTIVE

The relationship between serum Lp(a) and the presence and severity of coronary artery disease is supported by growing clinical evidence (14, 23, this thesis), so that at this stage it has become irrefutable. Yet, several unresolved issues necessitate further research:

- *Basic researchers* need to concentrate on developing a better understanding of the normal physiological functions of Lp(a) *in vivo*.

- *Epidemiologists* should elucidate the role of Lp(a) lipoprotein excess in the development of atherosclerotic vascular disease for both genders in all racial groups, and define valid, population-dependent thresholds for enhanced CHD risk.

- *Clinical chemists* should implement routine LDL-cholesterol methods that distinguish between LDL-cholesterol and Lp(a)-cholesterol, two cardiovascular pathogens with different (patho)physiological roles. Moreover, they should develop a reference or a 'designated comparison method' which shows no cross-reactivity with plasminogen or apo B_{100}, and is insensitive to the apo(a) size polymorphism and apo(a) sequence polymorphism. This accuracy base should subsequently be used to target calibrators and controls, and to standardize and validate commercial Lp(a) test kits.

- The emerging notion that the pathogenicity of Lp(a) is increased in the presence of other risk factors, should invite *clinicians* to develop approaches for their correction, particularly in subjects with personal and/or family history of atherosclerotic vascular disease.

- Once the biological function of Lp(a) is unraveled, *pharmacologists* should develop specific Lp(a) lowering medications.

- Recently, an *Lp(a)-cholesterol method* has been described (30). Its clinical usefulness should be determined and compared to that of current Lp(a) test kits.

BEYOND LP(a) MASS DETERMINATIONS

The molecular basis of diversity in Lp(a) serum levels has been under scrutiny for some time (31, 32). Kraft et al. documented that the intraindividual variability in Lp(a) levels is almost entirely explained by variation at the apo(a) locus; nevertheless, only 46% of the Lp(a) variability is explained by the apo(a) size polymorphism in Caucasians (33). This suggests further heterogeneity relating to Lp(a) levels in the apo(a) gene. Also, apo(a) alleles of the same size have been associated with up to 200-fold differences in Lp(a) concentrations (34, 35). Thus additional factors, besides the apo(a) size polymorphism, must affect serum Lp(a) levels.

Recently, apo(a) sequence polymorphisms, eventually leading to functional polymorphisms in terms of lysine and fibrinogen binding, have been found (36-38). After all, human apo(a), like plasminogen, has lysine binding functions which may be relevant for interference with the fibrinolytic process *in vivo* and relate to its athero-thrombogenicity. For plasminogen, the lysine binding function is localized in kringles 1 and 4. In the case of apo(a) in Lp(a), two lysine-dependent binding sites have been identified: 1) one represented by the lysine binding pocket in kringle 4 type 10 (or kringle 4-37) which is mainly responsible for the binding of Lp(a) to a lysine-Sepharose column (36-38), and 2) another one represented by the "lysine-proline (Lys-Pro) sensitive domain", localized between kringles 5 and 9, which has the capacity to bind to both plasmin modified fibrinogen and lysine-Sepharose, and which is for ≈70% masked by attachment of apo(a) to $apoB_{100}$ in Lp(a) (39, 40). As human apo(a) is demonstrated to be mutable in kringle 4 type 10, and as one of the mutations identified thus far decreases the *in vitro* lysine binding, it was suggested that any decrease in the lysine binding capacity, regardless of its cause, should decrease the cardiovascular pathogenicity of Lp(a). Consequently, enthusiastic researchers recommended that the lysine binding polymorphism in kringle 4 type 10 should be determined when assessing the cardiovascular pathogenicity of this LDL variant, along with measurements of serum Lp(a) and assessment of the apo(a) size polymorphism (38). To date however, a critical role of kringle 4 type 10 in the binding of apo(a) to fibrinogen is

no longer supported (40). At this moment it is also unclear whether the assessment of the actual fibrin(ogen) binding potential of Lp(a), related to its athero-thrombogenicity, should include the determination of free apo(a), characterized by a completely unmasked "Lys-Pro sensitive domain" which may enable a better complexation with fibrin(ogen) as well as an enhanced contribution to the formation of the atherosclerotic plaque (40).

Additionally, 5' flanking region polymorphisms for the apo(a) gene have been identified. They include four alleles whose frequency appears to vary with the presence or absence of overt atherosclerosis among two distinct ethnic groups (41), and a pentanucleotide repeat polymorphism which accounts for up to 14% of Lp(a) concentration variability among European Caucasians (42). Finally, a C/T polymorphism in the 5' flanking region has been identified that appears to reduce translational efficiency by 60% (43). Thus, allelic variation in transcriptional control may contribute significantly to heritable differences in serum Lp(a) levels. Consequently, to critically establish the pathological role of Lp(a), future clinical trials should investigate apo(a) genotype, lysine-binding function of Lp(a), and possibly the other polymorphisms, along with serum Lp(a).

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SUMMARY

In this dissertation, serum lipoprotein(a) (Lp(a)) and serum lipid levels are described as determined in healthy Caucasian, Asian, and African populations, and in Caucasians with clinical symptoms of coronary heart disease. Also considered is whether serum Lp(a) predicts further progression of coronary artery disease in middle-aged, moderately hypercholesterolaemic Caucasian males with documented coronary artery disease.

Chapter 1 consists of a short consideration of the epidemiology of coronary heart disease in Western industrialized countries. In addition to hypercholesterolaemia, the high prevalence of hyper-Lp(a)-lipoproteinaemia in Caucasian men and women with *premature* coronary heart disease is discussed, and the possible causal role of Lp(a) in the development of coronary heart disease is postulated.The Lp(a) structure and the pathophysiological mechanism of Lp(a), as presently understood, are described. Because of its unique structural characteristics, Lp(a) is a Janus-faced compound, both faces being ugly: a cholesterol-rich, Low-Density Lipoprotein part that increases the supply of cholesterol to the vascular wall (atherogenesis) and a plasminogen-like apo(a) part that retards fibrinolysis and accelerates the thickening of the vascular wall (thrombogenesis).

Besides, the pitfalls of the current generation of Lp(a) test kits are reviewed.

In **chapter 2**, the stability of Lp(a) in human samples frozen at -20°C and -70°C is documented. A storage temperature of -70°C turned out to assure better stability than a storage temperature of -20°C. Yet, even at -70°C Lp(a) degradation occurred if *sera* were stored longer than two years. I.e., with both the ELISA method of Biopool and the IRMA method of Mercodia, a storage period of more than two years led to significant underestimation of the measured Lp(a) concentrations.

In **chapter 3** the biological variation of Lp(a) in healthy volunteers (N = 54), and in patients from the lipid policlinic with an Lp(a) excess (N = 12), is investi-

gated. In view of the thousand-fold interindividual spread in serum Lp(a) levels in healthy individuals, a significant and inverse relation emerged between the biological coefficients of variation and the serum Lp(a) concentrations. Therefore, the general practice of using average biological coefficients of variation must, for Lp(a), be advised against. Instead, individual, concentration-dependent biological coefficients of variation are recommended. The same applies for the analytical coefficient of variation and the derived indices.

In **chapter 4**, serum Lp(a) levels are documented in apparently healthy Africans [Pygmies (N = 146) and Bantus (N = 208)] and compared with those in healthy Caucasians [Belgians (N = 905) and Hungarians (N = 400)] and Asians [Philippinos (N = 195)]. The age and body mass index-adjusted geometric Lp(a) means turned out to be 2.3 to 5 times higher in the Pygmies and the Bantus with respect to the other population samples. To our current knowledge, coronary heart disease does not occur among Pygmies and Bantus; among Westerners, however, the prevalence of coronary heart disease is very high. In middle-aged, symptomatic Caucasian males (N = 100) who underwent elective coronary artery bypass surgery, the median pre-operative Lp(a) level was 135 mg/l as opposed to 71 mg/l in healthy Caucasian males (N = 413). The doubling of the median Lp(a) level in Caucasians with overt coronary heart disease as compared with apparently healthy individuals, reflects the shift to the right of the Lp(a) frequency distribution curve in the patients.

It is concluded from this comparative, cross-sectional study that high Lp(a) levels in African blacks do not of themselves constitute a precipitating factor for the development of coronary heart disease.

In **chapter 5**, the effect of sexual maturity on the serum Lp(a) levels is investigated in Caucasian (Belgian) schoolchildren (N = 266) using a cross-sectional study design. Sexual maturity was scored by Tanner's method (with pubic hair appearance in boys and girls and breast development and genital development in girls and boys, respectively). A significant effect of sexual maturity or of chronological age could not be demonstrated with this study design. Nevertheless, the overall geometric Lp(a) mean was significantly higher in the Belgian school-

children than in young Belgian adults (N = 683) (89 versus 69 mg/l; P = 0.006), parallel to the well-defined cholesterol increase during puberty.

In **chapter 6**, healthy Flemings (N = 683) and Walloons (N = 217) are examined to determine whether genetically determined risk factors, namely Lp(a) and apo E polymorphism, contribute in part to the north/south (Flanders/Wallonia) differences in serum cholesterol distribution and mortality in Belgium reported in the literature. The impact of the apo E polymorphism on serum Lp(a) levels is also studied. Median Lp(a) values were 67 mg/l in Flemings and 75 mg/l in Walloons, which difference is not significant. The apo E phenotype distribution was identical in the two groups, although the effects of the apo E alleles differed: the apo B and LDL-cholesterol increasing effect of the ε4 allele was +12% in the Walloons and +5% in the Flemings; the apo B and LDL-cholesterol reducing effect of the ε2 allele was -15% in the Walloons and -25% in the Flemings. The interaction of the apo E gene with specific regional lifestyle factors, such as the ingestion of saturated fats, can probably account for this. No significant effect of the apo E polymorphism on the serum Lp(a) level could be demonstrated.

This study shows that there are no genetically determined differences between Flemings and Walloons as regards the cardiovascular risk factors investigated.

In **chapter 7**, in a placebo-controlled intervention study of 100 Caucasian men who underwent elective coronary artery bypass grafting (CABG), the Lp(a) time courses *after* coronary artery bypass and the influence of pravastatin are studied. On the third post-operative day, the median Lp(a) level was halved with respect to the pre-operative level (from 135 to 67 mg/l), independent of pravastatin administration. The acute phase behaviour of Lp(a) became observable on day 10, and the Lp(a) levels were again normalized after one month. No demonstratable effect of pravastatin on serum Lp(a) was observed.

Chapter 8 gives the results of the monitoring of the Lp(a) time courses *during* coronary artery bypass surgery in 20 additional Caucasian patients. Preoperatively, the geometric Lp(a) averaged 115 mg/l (versus 130 mg/l in the 100 CABG patients described in chapter 7). The paradoxical increase in Lp(a) a few minutes after the

starting of the pump, as reported by Sgoutas et al., could not be confirmed. Incorrect -underestimated- pre-operative Lp(a) measurements might be the cause of this controversy.

In **chapter 9**, Lp(a) is measured retrospectively in the context of the Regression Growth Evaluation Statin Study (REGRESS), a double-blind, placebo-controlled, prospective, arteriographic study in moderately hypercholesterolaemic Caucasian (Dutch) males (N = 704), in which the effect of cholesterol-reducing treatment with pravastatin, an HMG-CoA reductase inhibitor, on progression of coronary artery disease was examined. In the REGRESS patient group, the median serum apo(a) level turned out to be 1.74 times higher than the median level in healthy Dutch males (N = 274) (236 U/l and 136 U/l in REGRESS patients and healthy controls, respectively). It did turn out that the patients who showed progression had significantly higher *in-trial* apo(a) levels than the stable patients and the regressors (259 U/l versus 177 U/l and 143 U/l; P = 0.0075). Multivariate analysis showed that the apo(a) levels explained at most 5% of the angio-graphically quantified progression of coronary sclerosis ($P < 0.05$). In the patient subgroup with low *in-trial* HDL cholesterol (< 0.7 mmol/l), serum apo(a) levels explained 30% and 37% of the diffuse and the focal coronary sclerosis, respectively ($P < 0.05$).

From the REGRESS Lp(a) study it is concluded that Lp(a) is an indepen-dent predictor of coronary artery disease in middle-aged Caucasian males, and that severe and concomitant hypoalphalipoproteinemia increases the atherothrom-bogenicity of Lp(a) substantially.

SAMENVATTING

In deze dissertatie worden serum lipoproteïne(a) (Lp(a)) en serum lipiden spiegels beschreven zoals bepaald in gezonde Kaukasische, Aziatische en Afrikaanse populaties, alsook in Kaukasiërs met klinische symptomen van coronaire hartziekten. Tevens wordt onderzocht of de serum Lp(a) spiegels progressie van coronair lijden voorspellen in matig hypercholesterolemische, Kaukasische mannen van middelbare leeftijd met coronaire sclerose.

Hoofdstuk 1 bevat een korte beschouwing omtrent het epidemisch voorkomen van coronaire hartziekten in de Westerse geïndustrialiseerde wereld. Naast hypercholesterolemie wordt de hoge prevalentie van hyper-Lp(a)-lipoproteïnemie in Kaukasische mannen en vrouwen met *premature* coronaire hartziekten besproken, en wordt de mogelijk oorzakelijke rol van Lp(a) bij het ontstaan van coronaire hartziekten gepostuleerd.

De Lp(a) structuur en het tot dusver opgehelderde pathofysiologische werkingsmechanisme van Lp(a) worden beschreven. Omwille van zijn unieke structurele kenmerken wordt Lp(a) beschouwd als een Janus met twee lelijke gezichten: een cholesterolrijk "Low Density Lipoproteïne"-deel dat de toevoer van cholesterol naar de vaatwand vergroot (atherogeen), en een plasminogeen-achtig apo(a) deel dat de fibrinolyse vertraagt en de verdikking van de vaatwand versnelt (thrombogeen).

Daarnaast worden de tekortkomingen van de huidige generatie Lp(a) bepalingsmethoden vermeld.

In **hoofdstuk 2** wordt de stabiliteit van Lp(a) in ingevroren humane monsters bij -20°C en -70°C beschreven. Een bewaartemperatuur van -70°C bleek een betere stabiliteit te garanderen dan een bewaartemperatuur van -20°C. Maar ook bij -70°C trad er, bij bewaring van humane *sera* gedurende meer dan twee jaar, Lp(a) degradatie op. D.w.z.: zowel bij de ELISA bepalingsmethode van Biopool als bij de IRMA bepalingsmethode van Mercodia leidde een bewaartermijn van meer dan twee jaar tot een significante onderschatting van de gemeten Lp(a)-concentraties.

In **hoofdstuk 3** wordt de biologische variatie van Lp(a) in gezonde vrijwilligers (N = 54), en in patiënten van de lipidenpolikliniek met Lp(a) excess (N = 12), onderzocht. Gezien de 1000-voudige interindividuele spreiding in Lp(a) spiegels bij gezonden, bleek een significante, inverse relatie te bestaan tussen de biologische variatiecoëfficiënten enerzijds en de Lp(a) spiegels anderzijds. In tegenstelling tot wat gangbaar is dient het gebruik van een gemiddelde biologische variatiecoëfficiënt voor Lp(a) daarom afgeraden te worden. Het gebruik van individuele, concentratie-afhankelijke biologische variatiecoëfficiënten verdient aanbeveling. Hetzelfde geldt voor de analytische variatiecoëfficiënt en de afgeleide indices.

In **hoofdstuk 4** worden serum Lp(a) spiegels gedocumenteerd in ogenschijnlijk gezonde Afrikanen [Pygmeeën (N = 146) en Bantoes (N = 208)], en vergeleken met deze in gezonde Kaukasiërs [Belgen (N = 905) en Hongaren (N = 400)] en Aziaten [Filippino's (N = 195)]. Leeftijd- en "body mass index"-geadjusteerde geometrische Lp(a) gemiddelden bleken 2.3 tot 5.0 x hoger in Pygmeeën en Bantoes t.o.v. de andere populaties. Voor zover bekend komen coronaire hartziekten bij Pygmeeën en Bantoes niet voor; bij Westerlingen daarentegen is de prevalentie van coronaire hartziekten zeer hoog. In symptomatische Kaukasische mannen (N = 100) van middelbare leeftijd die een geplande coronaire bypass operatie ondergingen, was de mediane Lp(a) spiegel pre- operatief 135 mg/l, t.o.v. 71 mg/l in gezonde Kaukasische mannen (N = 413). De verdubbeling van de mediane Lp(a)-spiegel in Kaukasiërs mét coronaire sclerose, t.o.v. ogenschijnlijk gezonden, weerspiegelt de rechtsverschuiving van de Lp(a)-frequentiedistributiecurve bij de patiënten.

Uit dit vergelijkend, cross-sectioneel onderzoek blijkt dat hoge Lp(a) spiegels in zwarte Afrikaners op zich geen precipiterende factor vormen voor het ontstaan van coronaire hartziekten.

In **hoofdstuk 5** wordt in Kaukasische (Belgische) schoolkinderen (N = 266) het effect van seksuele maturatie op de serum Lp(a) bloedspiegels onderzocht met een cross-sectioneel studieopzet. Seksuele maturatie werd gescoord volgens Tanner (aan de hand van pubisbeharing bij jongens én meisjes, en aan de hand van borstontwikkeling en genitaliënontwikkeling bij respectievelijk meisjes en jon-

gens). Een significant effect van seksuele maturatie of van chronologische leeftijd kon met dit studieopzet niet aangetoond worden. Toch bleek het globaal geometrisch Lp(a) gemiddelde significant hoger in Belgische schoolkinderen t.o.v. Belgische jongvolwassenen (N = 683) (89 versus 69 mg/l; P = 0.006), parallel aan de welomschreven cholesteroltoename gedurende de puberteit.

In **hoofdstuk 6** wordt in gezonde Vlamingen (N = 683) en Walen (N = 217) onderzocht of genetisch bepaalde risicofactoren, met name Lp(a) en het apo E polymorfisme, partieel bijdragen tot de in de literatuur beschreven Noord (Vlaanderen) - Zuid (Wallonië) verschillen in serum cholesteroldistributie en mortaliteit in België. Tevens wordt de impact van het apo E polymorfisme op de serum Lp(a) spiegels onderzocht. Mediane Lp(a) waarden waren 67 mg/l in Vlamingen en 75 mg/l in Walen; de verschillen waren niet significant. De apo E fenotype distributie was identiek in Vlaanderen en Wallonië. Toch verschilden de effecten van de apo E allelen tussen Vlamingen en Walen: het apo B en LDL-cholesterol verhogend effect van het ε4 alleel was +12% in Walen t.o.v. +5% in Vlamingen; het apo B en LDL-cholesterol verlagend effect van het ε2 alleel was -15% in Walen t.o.v. -25% in Vlamingen. Dit is waarschijnlijk te wijten aan de interactie van het apo E gen met specifieke, regiogebonden leefstijlfactoren, bijvoorbeeld de verzadigd vet-inname. Er kon geen significant effect aangetoond worden van het apo E polymorfisme op de serum Lp(a) spiegel.

Uit dit onderzoek blijkt dat er geen genetisch bepaalde verschillen bestaan tussen Vlamingen en Walen m.b.t. de onderzochte cardiovasculaire risicofactoren.

In **hoofdstuk 7** wordt het Lp(a) tijdsverloop *na* coronaire bypass chirurgie bestudeerd in een placebo gecontroleerde interventiestudie bij 100 Kaukasische mannen. De beïnvloeding van serum Lp(a) spiegels door pravastatine wordt eveneens getoetst. Op de derde post-operatieve dag bleek de mediane Lp(a) spiegel, onafhankelijk van pravastatine toediening, gehalveerd t.o.v. de pre-operatieve Lp(a) spiegel (van 135 naar 67 mg/l). Op dag 10 werd het acute fase gedrag van Lp(a) waarneembaar. Na één maand waren de Lp(a) spiegels opnieuw genormaliseerd. Er was geen beïnvloeding van serum Lp(a) door pravastatine aantoonbaar.

Daarnaast wordt in **hoofdstuk 8** het Lp(a) tijdsverloop *gedurende* coronaire bypass chirurgie gevolgd van 20 additionele Kaukasische mannelijke patiënten. Pre-operatief was het geometrisch Lp(a) gemiddelde 115 mg/l (versus 130 mg/l in de 100 CABG patiënten beschreven in hoofdstuk 7). Een eerder door Sgoutas et al. beschreven paradoxale Lp(a) toename enkele minuten na het starten van de pomp, kon door ons niet bevestigd worden. Onjuiste -onderschatte- pre-operatieve Lp(a) metingen zijn mogelijk de oorzaak van deze schijnbare controverse.

In **hoofdstuk 9** wordt retrospectief Lp(a) gemeten in het kader van de Regression Growth Evaluation Statin Study (REGRESS), een dubbel-blinde, placebo-gecontroleerde, prospectieve, arteriografische studie in matig hypercholesterolemische Kaukasische (Nederlandse) mannen (N = 704) waarin het effect onderzocht werd van cholesterolverlagende behandeling met de HMG-CoA reductaseremmer pravastatine op progressie van coronaire sclerose. In de REGRESS patiëntengroep bleek de mediane serum apo(a) spiegel 1.74 x hoger t.o.v. de mediane spiegel in gezonde Nederlandse mannen (N = 274) (236 U/l en 136 U/l in REGRESS patiënten en gezonde controles, respectievelijk). Patiënten die progressie vertoonden, hadden significant hogere *in-trial* apo(a) waarden dan stabiele patiënten en regressoren (259 U/l versus 177 U/l en 143 U/l, respectievelijk; P = 0.0075). Multivariate analyse toonde aan dat de serum apo(a) spiegels hooguit 5% van de angiografisch gekwantificeerde progressie van coronaire sclerose verklaarden (P < 0.05). In de patiëntensubgroep met laag *in-trial* HDL-cholesterol (< 0.7 mmol/l), verklaarden de serum apo(a) spiegels respectievelijk 30% en 37% van de voortschrijdende diffuse en focale coronaire sclerose (P < 0.05).

Uit de REGRESS Lp(a) studie blijkt dat serum Lp(a) in matig hypercholesterolemische Kaukasische mannen van middelbare leeftijd een onafhankelijke voorspeller is voor coronaire sclerose, en dat ernstige én gelijktijdige hypoalfalipoproteïnemie in belangrijke mate de atherothrombogeniciteit van Lp(a) verhoogt.

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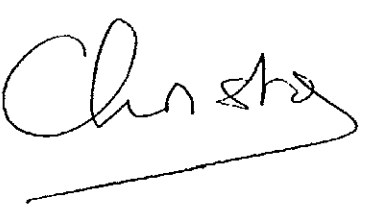

CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 29 maart 1959 in Beernem, België. Het diploma Moderne Humaniora werd gehaald aan het Sint-Jozef Instituut te Brugge in 1977. In datzelfde jaar werd aangevangen met de studie Farmacie aan de Katholieke Universiteit Leuven, België. Het apothekersdiploma werd behaald in 1982. Aansluitend werd de opleiding tot klinisch bioloog gestart. Het diploma van Apotheker Specialist in de Biologische Ontledingen werd behaald in 1985. In de periode augustus 1985 - juni 1992 is zij werkzaam geweest als staflid op het Centraal Klinisch Chemisch Laboratorium van de Universitaire Ziekenhuizen te Leuven. In 1989 werd het additionele diploma behaald voor het gebruik van radioisotopen voor in vitro diagnostiek. Sinds juli 1992 is zij werkzaam als staflid op het Centraal Klinisch Chemisch Laboratorium van het Academisch Ziekenhuis Rotterdam en als hoofd van het Lipiden Referentie Laboratorium. Per 1 maart 1993 is zij in Nederland ingeschreven in het Register van Erkend Klinisch Chemici.

Zij is sinds mei 1992 getrouwd met Ids H. Boersma en samen zijn zij de ouders van Aukje en Harmen (†).

LIST OF ABBREVIATIONS

Apo(a):	apolipoprotein(a)
Apo A-I:	apolipoprotein A-I
Apo B:	apolipoprotein B
BMI:	body mass index
CABG:	coronary artery bypass grafting
CAD:	coronary artery disease
CDC:	Centers for Disease Control
CHD:	coronary heart disease
CPB:	cardiopulmonary bypass
CRMLN:	Cholesterol Reference Method Laboratory Network
CV_a:	analytical coefficient of variation
CV_b:	biological coefficient of variation
Cys:	cysteine
DBP:	diastolic blood pressure
(GLM) ANOVA:	(general linear models) analysis of variance
Hcy:	homocysteine
HDL:	High Density Lipoprotein
HDL-c:	High Density Lipoprotein cholesterol
IQR:	interquartile range
LDL:	Low Density Lipoprotein
LDL-c:	Low Density Lipoprotein cholesterol
Lp(a):	lipoprotein (a)
LRL:	Lipid Reference Laboratory
MLR:	multiple linear regression
MSD:	mean segment diameter
MOD:	minimum obstruction diameter
NA:	not applicable
NCEP:	National Cholesterol Education Program

NS:	not significant (except in chapter 3 where it stands for number of specimens)
PTCA:	percutaneous transluminal coronary angioplasty
REGRESS:	Regression Growth Evaluation Statin Study
SAS:	statistical analysis system
SBP:	systolic blood pressure
SD:	standard deviation
SE:	standard error
SEM:	standard error of the mean
SES:	socio-economic status
TAOS:	Total Anti-Oxidant Status