The Pre-beta, lipoprotein phenomenon in relation to serum cholesterol and triglyceride levels, the Lp(a) lipoprotein and coronary heart disease
The Pre-beta\textsubscript{4} lipoprotein phenomenon in relation to serum cholesterol and triglyceride levels, the Lp(a) lipoprotein and coronary heart disease.

BY

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To my wife Annalisa
and my children Catharina
   Ann-Charlotte
      Per
    Hans
The present publication is based on the following papers:


These papers will be referred to in the text by their Roman numerals.
Pre-beta lipoprotein and Lp(a) lipoprotein in patients with sustained myocardial infarction and in presumably healthy middle-aged males in relation to serum fractionation with ultracentrifugation.
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INTRODUCTION

Cholesterol (CH), triglycerides (TG) and phospholipids (PL) are insoluble in water. In serum these lipids are transported mainly together with specific proteins as particles soluble in water named lipoproteins (LP).

Structural and soluble lipoproteins.

It is generally recognized that there are two different forms of lipoproteins; the structural which constitutes the membrane lipoproteins and the soluble lipoproteins of extracellular fluids (70).

Membranes play an important role in almost all cellular phenomena. The understanding of their molecular structure is, however, still rudimentary. The lipids probably constitute the matrix with the major portion of the PL in bilayer form. The polar head groups are spaced about 40-45 Å apart (51, 87). The proteins are predominant and are of two categories termed peripheral and integral (86). Peripheral proteins, held to the membrane by weak noncovalent interactions, are not strongly associated with membrane lipids. The major portion of the proteins are the grossly heterogenous, largely globular, integral proteins. They may be partly embedded in the phospholipid interior or penetrate the entire membrane with polar regions in contact with the aqueous solvent on both sides of the membrane. A small fraction of the lipid may be specific and more tightly coupled to integral protein.

Protein-lipid interactions may be important for membrane functions as many membrane-bound antigens and enzymes require specific phospholipids for the expression of their activities (90). Some experiments indicate that integral protein distribution is essentially random and that there is a free diffusion and intermixing of lipid, protein and lipoprotein within a fluid membrane matrix (65, 60, 43).

According to the hypothesis of Singer and Nicolson (87) cell membranes then are oriented viscous solutions of amphipatic proteins and lipids in instantaneous termodynamic equilibrium. Hydrogen bonding and electrostatic interactions may be of secondary importance for the gross structure. The polarly oriented molecules would undergo only translational diffusion in the plane of the membrane, permitting an asymmetry in composition between the two surfaces of membranes.

A number of critical metabolic functions performed by cell membranes may require the translational mobility of some important integral proteins (87).

Transport through the membrane may include single diffusion as well as specific protein adsorption sites on the membrane surface as well as other processes (93).

The role of cholesterol, when present in membranes, remains uncertain. Formation of the cholesterol-phospholipid complex supposed to occur leads to tighter packing, or compaction of the hydrocarbon
chains in the interior of the membrane, responsible for many of the properties of the plasma membrane. Mammalian plasma membrane lipids contain a significant quantity of cholesterol, up to 30%, PL constituting most of the remainder (99).

It has been shown that increased free cholesterol "solubility" in plasma results in loss of red cell membrane cholesterol and increased red cell osmotic fragility in the rat (78).

As a consequence of mainly variations in the ratios between lipid and protein content, soluble lipoproteins show a great difference in both size and hydrated density. A widely accepted classification system, based on density, divides the lipoproteins into four major classes; Chylomicrones, very low density lipoproteins (VLDL), low density lipoproteins (LDL) and high density lipoproteins (HDL). These density classes roughly correspond to four distinguished bands on cellulose acetate or agarose gel electrophoresis named chylomicrones, beta-lipoproteins, pre-beta lipoproteins and alfa-lipoproteins.

The differences in electrophoretic mobility are primarily a consequence of variations in distribution of at least four specific proteins called apolipoprotein A, B, C and D. These apolipoproteins are essential compounds for the structure, lipid composition and metabolism of lipoproteins. A new and more adequate classification system, based on apolipoproteins, has been proposed by Alaupovic (3). Apolipoprotein A consists of two nonidentical peptides A_1 and A_2. The A_1 peptide acts as a cofactor for the enzyme lecithin: cholesterol acyltransferase (LCAT) (68). Apolipoprotein C promotes the action of the enzyme lipoprotein lipase (LPL). It is a mixture of three different polypeptides, CI, CII and CIII. How these different terminologies roughly correspond to each other (42) is shown in fig. 1.

Serum lipoproteins are specialized products of mainly two types of cells, the absorptive cells of the small intestine and the hepatic parenycymal cells. Apart from the transport of TG and CH, their functions are not especially well known.

Even soluble LP have only recently become the subject of intensive structural studies. They are unlikely to be structurally static since it is well known that some of their components exchange continously. Some common principles may apply to both structural and soluble LP with a concept of a fluid or dynamic structure in which all or many of the lipoprotein components are undergoing continued reorganization (79).

Size and composition of VLDL and chylomicrones are influenced by the sites of origin, dietary intake and metabolic state of individuals. VLDL contain a spectrum of particles that may represent a continuous or discontinuous group of lipoproteins (79).

From a structural point of view chylomicrones and VLDL appear to represent members of the same family, having the same constituents although in different proportions. They have few polar components which have the ability to undergo interchange with similar constituents of the other serum lipoproteins leading to surface modifications.
The concept of a surface made up of polar components, and a predominantly hydrophobic interior may appear to receive some experimental support (101). On the basis of equilibration studies, one may surmise that phospholipids and free cholesterol, being readily exchangeable, may have a surface location. The same would apply for the protein moiety shown to be readily attacked by proteolytic enzymes (79).

Experimental evidence appears to suggest that these constituents are organized in a monolayer film which, although unable to cover the whole surface will provide chylomicrones and VLDL with sufficient polarity to ensure solubility in water.

Even in the smaller VLDL, significantly richer in free cholesterol, phospholipids and protein the polar surface constituents may not completely cover the core of triglycerides and cholesterol esters.

The LDL-particles may be a set of globular subunits about 50 Å in diameter. The proposed structures are at the moment to be considered as useful working hypotheses in need of additional experimental evidence. They are compatible with the general structural concept of the polar groups located totally or predominantly at the surface with the non-polar groups in the core (63, 2).

The specific properties and relative proportions of the two major apolipoproteins A_I and A_II in HDL as well as their specific affinity for lipids would be the main determinants in modulating the structure of these particles (79). Apolipoprotein C with three main distinct peptides may represent an "extrinsic" constituent of HDL secondary to the exchange and transfer processes occurring between HDL and VLDL (79).

Several studies (23, 79) have suggested that a large percentage of the HDL protein and the phospholipids are located at the surface, as well as the existence of a basic organizational pattern that allows, in some degree, translational motion. A metastable structure is consistent with the known property of HDL to exchange lipids with other lipoprotein complexes.

The TG transported by Chylomicrones and VLDL in serum serve as energy source. The removal of these TG seems to be rather complex, involving lipoprotein lipase and hepatic lipase. Lipoprotein lipase is probably activated by the apolipoprotein CII polypeptide (49). Chylomicrones are removed very fast and serum normally contains no measurable amount after a 12 hours' fast. During the catabolism of VLDL a part of the TG is transferred to HDL in exchange for cholesterol esters. This transfer is stimulated by the action of the enzyme LCAT. LCAT is synthesized in the liver and secreted together with HDL and transfers unsaturated fatty acids in 2-position of lecithin into 3-position of cholesterol. This reaction probably accounts for all esterified cholesterol found in serum after a 12 hours' fast and also promotes transfer of free cholesterol and lecithin from VLDL and plasma cell membranes to HDL which is the substrate for LCAT (47, 48).
Even LDL has a comparatively short half life, reported to be of about 1-3 days (68, 49). Its apolipoprotein B is probably degraded in the liver without being reused (68). Extrahepatic tissue seems to be able to metabolize LDL as well. One of the main functions of LDL is probably to transport cholesterol in esterified form from peripheral tissues to the liver where the cholesterolesters again are hydrolyzed. According to Goldstein et al (20) another, hypothetical function of LDL, would be to inhibit the cellular synthesis of cholesterol, through interaction with the cell membranes and inhibition of the rate limiting enzyme HMG-CoA reductase by LDL cholesterol.

HDL in serum are rather heterogenous in size and chemical composition as a result of the metabolic transformations catalyzed by LCAT. The abnormalities in LP metabolism in the rare disease of LCAT deficiency (46) indicates the importance of that reaction for the normal LP, cholesterol and TG metabolism. It seems as if free cholesterol needed to be esterified before it can be taken care of and metabolized in a normal way. Cholesterol esters are probably also important structural components for the stability of HDL (79, 40).

The Lp(a) lipoprotein.

Using anti-sera obtained by immunization of rabbits, a new human LP polymorphism was discovered in 1963 by Berg, the Lp(a) antigen. The Lp(a)-antigen revealed by rabbit-immune serum with the technique used was inherited in an autosomal dominant fashion (12). Further studies have strongly supported the hypothesis of an autosomal dominant inheritance of the Lp(a) antigen (74).

The Lp(a) lipoprotein seems to be a variant of human beta-lipoproteins (13). The lipoprotein particles have a spherical structure like LDL but they have a larger diameter and a higher molecular weight (85). The frequency of Lp(a+) individuals in Caucasian populations is about 0.35 (13). The LP migrates slower than the bulk of beta-lipoprotein in disc electrophoresis in polyacrylamide gel and has pre-beta mobility in the common agarose gel or paper electrophoresis (85, 73, 4). It is present in the 1.050 - 1.080 (1.090) g/ml density class upon ultracentrifugation (50, 4). Rider, Levy & Fredrickson (73) found that the atypical pre-beta-lipoprotein that did not float at the density 1,006 g/ml, called "sinking" pre-beta-Lp was the Lp(a) antigen.

The Lp(a) lipoprotein is very similar to LDL in its lipid composition but the composition of its aminoacids is different from both LDL and HDL (85). Several authors (35, 94, 34) have observed that the Lp(a) lipoprotein disintegrates upon storage or during purification. When stored at 0°C, purified homogenous Lp(a) lipoprotein dissociated into components and gave 4 bands in polyacrylamide gel electrophoresis. Electrophoretic, immunochemical and density characterisation suggested that the 4 bands corresponded to whole Lp(a) LP, LDL, Lp(a) protein and albumin. The last 2 fractions did not stain with lipid stain (Sudan Black) (34). However, Albers et al (4) found that the Lp(a) lipoprotein concentration did not change significantly when plasma was stored at -20°C or 4°C during a 4 week period.
The homogenous Lp(a) lipoprotein reacted with both anti-LDL and anti-Lp(a) serum but not with albumin antiserum, indicating that albumin is an integral part of the Lp(a) lipoprotein (34). The Lp(a) lipoprotein has also been found to react with antiserum to apolipoprotein C (81). The protein moiety of the Lp(a) lipoprotein contains about 65% of LDL apoprotein, 20% of Lp(a) protein and less than 15% of albumin (34, 94).

The carbohydrate content of the Lp(a) lipoprotein is 0.26 mg/mg protein which is higher than for any other soluble lipoprotein, the content of sialic acid being about six times as high as in plasma LDL (34). High contents of mono- and diglycerides have also been reported (91).

Albers et al (4) recently reported that 81% of 340 unrelated fasting subjects tested had levels of Lp(a) lipoprotein exceeding 1.5 mg/100 ml which was the lower limit of sensitivity of their radial immunoassay used. After concentration of the Lp(a-) plasmas about four-fold as many as 92% of the total population sampled had detectable levels of Lp(a) lipoprotein. No significant correlation with age, sex, CH or TG concentrations was found. The authors present the arbitrary 95th percentile upper cut off for the normal Lp(a) level to be 48 mg/100 ml but state that such a level would have no clinical utility since Lp(a) concentrations have yet to be correlated with any disease condition. The Lp(a) lipoprotein has also previously been suggested to be a quantitative genetic trait present in all individuals (53) rather than a qualitative genetic marker (12).

**Coronary heart disease (CHD) and atherosclerosis in relation to hyperlipidemia, hyperlipoproteinemia and other risk factors.**

Risk factors are characteristics, signs, or symptoms of a CHD-free individual, which are statistically associated with an increased incidence of subsequent CHD. The association between CHD and risk factors is only statistical and causality has not been proven for any of them. Nor is it known whether some of the risk factors act through a common denominator.

A large amount of evidence has been accumulated through the years showing that hyperlipemia is associated with coronary heart disease (CHD). In the vast majority of these studies, CHD refers strictly to the clinical entities myocardial infarction (MI), angina pectoris and sudden death of no apparent cause other than CHD (84). The earliest recognized and most incriminated disturbance with increased risk for the development of CHD is hypercholesterolaemia. This risk is indisputed in the homozygous and probably also the heterozygous forms of hereditary hypercholesterolemia of Muller & Harbitz (52, 64). These data and those continuously accumulating have stressed the importance of genetic factors in serum lipid and lipoprotein disorders (50).

In the prospective population study of Framingham (56) the risk of CHD proved proportional to the antecedant serum cholesterol level
in men of all ages studied, as well as in younger women. The net effect declined with age in both sexes, especially in women beyond 50 years of age. Nothing suggested that a particular level was "critical".

The use of both cholesterol and Sf 20-400 pre-beta lipoprotein was little better in discriminating potential CHD cases than cholesterol alone in men and younger women.

Modest elevations of serum cholesterol between 250-350 mg/100 ml were found to be a common and potent factor contributing to risk of CHD, depending on age and sex between 2 to 5 times higher than was noted with values below the average of about 220 mg/100 ml. Only 6 out of 5,127 individuals investigated had xanthomas and a cholesterol value above 400 mg/100 ml. All six died of CHD before their fiftieth birthdays. The authors concluded (56) that even moderately elevated CH values, regardless of how CH is partitioned among the LP, are associated with increased risk of CHD, and that elevated endogenous triglyceride values appear to be significant only when accompanied by high CH values.

Several other studies reviewed by Simborg (84) have all demonstrated an association between high values of serum cholesterol and a high incidence of CHD.

However, both retrospective (24) and prospective (21) studies suggest that elevated triglycerides alone, or in combination with other hyperlipoproteinemias also represent an increased risk of CHD.

The fact that none of the major serum lipids circulate in a simple state and that cholesterol and triglycerides are present in all lipoprotein classes makes it desirable to evaluate which of the LP may be involved in hyperlipemia. It is also probable that the LP more accurately mirror the nature of the metabolic defect.

In line with this the lipoprotein typing system (41) was introduced to identify different and presumably genetic disorders in lipoproteins (41, 42). This classification, recently slightly modified (9), is shown in fig. 1 as it applies to the schematic LP spectrum (49).

Knowledge of the LP-type is important in order to select more specific therapy in some subjects with hyperlipemia (50). Its contribution to predict risk of CHD, however, remains to be determined. The LP-typing system (41) has been too recently introduced to allow conclusions from prospective studies (56).

Besides cholesterol, the literature contains studies implicating over 35 individual risk factors relating to CHD (84).

There is overwhelming evidence that CHD increases with age (27, 69).

Sex is also important as the incidence of CHD in women is roughly one-fifth that of men in the age groups 40-60 years (84).
The statistical evidence associating smoking with an increased probability of developing CHD is impressive. Evidences indicate that the risk associated with cigarette smoking is most pronounced in the age groups under 65 and does increase with the number of cigarettes smoked (84). The combination of elevated cholesterol values and smoking may be especially dangerous (27). Apparently "smoking", including many variables, is complicated to define, being also a psychological and social phenomenon.

There is no significant evidence against the association of increased risk of CHD and elevated blood pressure (BP), being one of the most securely established risk factors (38). The risk of CHD increases gradually from low to high BP, including increases through the range commonly considered as "normal" (84).

According to the official definition, "atherosclerosis is a variable combination of changes of the intima of arteries (as distinct from arterioles) consisting of the focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous-tissue and calcium deposits, and associated with medial changes" (97).

The arterial wall reacts to different noxious agents i.e. CH in the same way, with swollen endothelial cells and oedema in the intima (88). If the stress persists, proliferation of intimal smooth muscle cells leads to a fibrous plaque. If the endothelium becomes defect, foam cells often appear and an advanced atherosclerotic lesion may ultimately develop. These advanced changes (ateromas) with retention of i.e. lipoproteins may be secondary to cell proliferation which leads to hypoxia and disturbance of the normal cell metabolism (88).

Even if the atherosclerotic process increases with increasing age (27, 69), it can start early in life, and atherosclerosis may persist long before symptoms of CHD develop. Autopsies of soldiers (average age 22.1) killed in action during the Korean war showed that 15.3 per cent had coronary plaques occluding more than 1/2 of a lumen (37).

Böttcher (22) showed that the lipids, as dry weight of human aorta, rose by 344 % between the ages of 6-56. Phospholipids increased by 145 % and cholesterol esters by 5,800 %. The source of phospholipid may primarily be synthesis in situ (102).

Kinetic studies of cholesterol-fed animals suggest that during atherogenesis there is a gradual increase in permeability of the arterial wall which promotes the influx and retention of serum LP cholesterol and not of intact LP. Apparently even a removal mechanism exists, presumably an efflux of cholesterol back into the blood stream. The proposed increase in permeability of the arterial wall does not appear to be restricted to a certain age as arterial CH accumulated rapidly even in newborn rabbits on a high cholesterol intake (102). These studies indicate that defective endothelium with increased permeability is a key factor for this type of atherogenesis.
After experimental induction of arterial lesions with defective reendothelialization in rabbits Björkerud et al (18) found increased transfer, esterification and deposition of cholesterol into the vessel wall. After the formation of an endothelial lining, cholesterol deposits were, however, rapidly eliminated, suggesting an endothelial function also in the mobilization of CH deposits from the arterial wall. In arterial lesions with rapid reendothelialization no deposition of cholesterol was observed. The authors suggest that the structural correlate of a hypothetical barrier against excessive CH influx and deposition is the continuous arterial endothelium.

"Normal aging" leads to a thicker intima, especially at arterial branching points and in the aortic arch, i.e. regions where increased hemodynamic strain may be expected (88). These regions are also predilected sites for atherosclerosis, (89), and have an increased endothelial turn-over. The application of cell viability tests have indicated that in these regions areas with defective endothelium are common even in normal rats and rabbits (17).

It seems probable that cooperation between risk factors that damage the endothelium of the vessel's wall, variable in the individual case, may be of primary importance in atherogenesis. Among those are probably hypercholesterolaemia, hypertension and smoking.

MATERIAL AND METHODS.

Investigated individuals.

Principles of selection for participation in the studies have been described in paper I-XI.

Blood sampling.

In a part of the epidemiological investigation (44) described in paper II, all specimens were taken in the afternoon. The subjects were asked not to eat within 4-6 hours before the examination. Except for the test meals, all the other specimens were collected between 08.00 - 08.30 a.m. after a 12-hour fast.

Serum has been used in all investigations and the blood samples are collected directly into glass tubes with no preservative added. After clotting and centrifugation all the serum samples were stored at 4°C until used for analysis. Extraction of lipids was performed the same day as soon as possible.

Procedure for the test meal.

The subjects came to the laboratory at seven o'clock in the morning after a 12-hour fast. After blood sampling, a breakfast was given them consisting of 2 eggs (135 g), 2 dl of ordinary milk, 2 slices of white bread (40 g) and butter (15 g). Further details are described in paper IV.
Electrophoretic procedures.

The method used for electrophoresis of lipoproteins on cellulose acetate membranes (Sephaphore III, Gelman instruments Co) is described in paper I. The lipoprotein bands are stained according to Kohn (58).

Agarose gel electrophoresis was performed as previously described (29, 32) in 0.5% agarose (Behringwerke) in Veronal buffer pH 8.6 with an ionic strength of 0.045, using the apparatus and technical principles as described by Laurell & Johansson (55) and Noble et al (66). The agarose solution is spread on a 1 mm glass plate placed on a levelling table. Gel bridges were used as for protein electrophoresis. Slits are produced with 11 mm broad strips of filterpaper in the 1.5 mm layer of agarose and are filled with 5 µl serum. One serum control is diluted with half its volume of an 0.1%(w/v) solution of bromphenol blue. The albumin front is then allowed to move 4.2 cm at a constant voltage of 260 V (About 50 min). During the electrophoresis the plate is cooled with circulating tap water.

The same reproducible results are obtained with the LKB 2117 multiphor electrophoresis cell (LKB-Beckman, Sweden) using 5-double filter papers (I.H. Munktell, No 10) as bridges instead of the cloths supplied with the equipment. The buffer solution is renewed for each electrophoretic run.

The procedure is then carried out as previously described (29, 32). The lipoprotein fractions are stained with Sudan Black B.

Classification of the Pre-β\textsubscript{1}-lipoprotein.

The principles for pre-β\textsubscript{1}-classification on cellulose acetate is described in paper I. On cellulose acetate the β-lipoprotein fraction is sometimes split into two peaks. None of these cases has been typed as pre-β\textsubscript{1} positive.

With the agarose gel electrophoretic method used a pre-β\textsubscript{1} lipoprotein fraction is classified as a well separated fraction between the usual β and pre-β lipoprotein fractions, clearly detectable with the eye. With that technique the pre-β lipoprotein fraction sometimes shows a tendency to split into two very close but not clearly separated fractions. These cases are not classified as pre-β\textsubscript{1} positive. The pre-β\textsubscript{1} lipoprotein fraction is, however, on both cellulose acetate and in agarose gel electrophoresis often situated closer to the pre-β lipoprotein fraction than to the β-lipoprotein fraction. The serum samples have not been stored more than 30 hours at 4°C before electrophoretic separation.

Determination of triglycerides and total and free cholesterol.

Before analysis 0.5 ml serum is added to test tubes containing 2 g of silicic acid mixed with 9.5 ml redistilled isopropanol. Mixing is performed during a 25 min period.
Serum triglycerides are in all studies determined according to the method of Cramp & Robertson (28). Unless otherwise indicated, total cholesterol is determined according to a modification of the procedure described by Levine & Zak (61). The Technicon Auto-Analyzer I (Technicon Instruments Corporation, Tarrytown, N. Y.) is used for both determinations as outlined (8).

A human control serum (Hyland lab, Calif) is analysed with each series of determinations for both cholesterol and triglycerides. Routine calculations of these control values show a mean value for triglycerides of 1.19 mmol/l and a standard deviation of 0.10 mmol/l (CV = 8.4%). The corresponding values for cholesterol is 158.1 mg/100 ml and 5.70 mg/100 ml (CV = 3.6%).

Two different methods have been used for the determination of both total and free cholesterol from the same serum sample. For both methods details are given in paper VI.

One is the spectrophotometric method of Webster (96). All analysis with this method were done in triplicate. The precision, calculated from two selected determinations on each serum sample from 50 consecutive individuals gave a CV of 0.9 % within the range of 174-446 mg/100 ml for total cholesterol and CV of 1.2 % within the range of 118-290 mg/100 ml for free cholesterol.

In the second method used serum was extracted according to Abell et al (1). However, a gas chromatographic technique was used instead of the photometric evaluation (Paper VI). From duplicate analysis of different serum samples, a CV of 2.3 % was calculated within the range of 164-304 mg/100 ml for total cholesterol. The corresponding value for free cholesterol within the range of 48-97 mg/100 ml was 3.7 %.

Liponorm reference serum (Nyegaard & Co., Oslo, Norway) was used as a control.

Lp(a)-typing.
The immunological Lp(a) testing has been conducted in Oslo as described previously (16).

Ultracentrifugation.
In one series of presumably healthy males investigated (Paper XI) the serum samples were fractionated by ultracentrifugation using an MSE superspeed 65 preparative ultracentrifuge equipped with a Titanium Angle Rotor (Cat. No. 59119) for 8 x 35 ml fitted with adapters to carry 8 x 10 ml polycarbonate tubes.

ECG and selective coronary angiography.
ECG recordings and coding at rest and during exercise tests are described in paper II and V, and in paper VIII. Selective coronary angiography is done by the Judkins technique (Paper VIII).
Statistical methods.

Statistical analysis were done using Student's t-test (two-tailed) for independent means (5). The $X^2$-test is used for testing the significance of differences (5). The Fisher test of exact probability is used in some cases (82).

RESULTS

Pre-beta\textsubscript{1} lipoprotein on cellulose acetate.

Lipoprotein electrophoresis on only cellulose acetate (Sepraphore III, Gelman Instrument CO) was used in the studies reported in paper I-V. As described in paper II, the 56-60 year old males were investigated in the afternoon about 6 hours after a meal. In 39 of the 325 lipoprotein electrophoresis the presence or absence of a pre-β\textsubscript{1} fraction was not possible to decide, and the results with them were omitted in the further analysis. 30 of these individuals had hyperlipidemia and a broad pre-β band.

In the remaining 286 males a significant correlation was found between the statement of precordial pain according to two different kinds of questions in a questionnaire and the occurrence of a pre-β\textsubscript{1} fraction upon lipoprotein electrophoresis (p < 0.02 and p < 0.01).

A highly significant correlation was found between the clinical diagnosis of typical or suspected angina pectoris, with and without the addition of cases with atypical chest pain, and the occurrence of a pre-β\textsubscript{1} fraction (p < 0.001) (Table I, paper II).

The limited family data presented in paper III from 2 investigated families suggest that the pre-β\textsubscript{1} lipoprotein fraction found on cellulose acetate is governed by autosomal, dominant inheritance.

In paper IV it is shown that a pre-β\textsubscript{1} fraction on cellulose acetate is found in some selected individuals (2 of 5 presented cases) only some hours after the test meal. In most cases, however, a pre-β\textsubscript{1} fraction was reproduced even after a 12 hours' fast. In preliminary studies no extra fractions appeared within 8 hours after a glucose-load (150 g sugar).

The frequency of the pre-β\textsubscript{1} lipoprotein in 20 patients with sustained myocardial infarction was compared with that in 8 selected normal controls, as described in detail in paper V. After a 12-hour fast 10 out of 18 individuals with a lipoprotein electrophoresis possible to interprete had a detectable pre-β\textsubscript{1} fraction, i.e. 56 %, in contrast to 1 of the eight controls (13 %). Although this material is small the tendency is the same as that found for the correlation between angina pectoris and pre-β\textsubscript{1} as described in paper II.

Out of 1229 males in the epidemiological investigation between the age of 41-60 years with interpretable lipoprotein electrophoresis (Paper X) 278, i.e. 23 %, were found to have a pre-β\textsubscript{1} lipoprotein fraction. As shown in table I significantly higher mean values for triglycerides and cholesterol are found in the pre-β\textsubscript{1} positive group.
In the oldest 286 individuals presented above, a significant difference between positive and negative groups is found only for triglycerides.

The mean values for cholesterol in age subgroups are constantly higher in subject revealing a pre-β\(^{-1}\) fraction, up to the age of 60 years (Fig. 5 in paper X). In pre-β\(^{-1}\) positive subjects the mean values for triglycerides increase according to age to a maximum at 52-55 years. For older males mean values tend to decrease. In pre-β\(^{-1}\) negative subjects the mean values for triglycerides are almost constant between the age of 41-60 years.

Pre-beta\(^{-1}\) lipoprotein on agarose gel and/or on cellulose acetate in young healthy males and in male patients in relation to total and free cholesterol and triglycerides.

Paper VI reports the results of analysis of free and total cholesterol and triglycerides in 96 consecutive male patients, mean age 58 years, visiting the laboratory for routine blood sampling, and in 68 apparently healthy schoolboys, 16-17 years old. Classification of pre-β\(^{-1}\) positive subjects was done on agarose gel in the male patients and on both agarose gel and cellulose acetate in the young healthy males. In both series significantly higher mean values for total and free cholesterol were found in pre-β\(^{-1}\) positive than in pre-β\(^{-1}\) negative individuals (p < 0.01). In the series of male patients the mean percentage of esterified cholesterol was lower in the pre-β\(^{-1}\) positive group (66.6 %) than in the negative group (68.9 %). Among the young healthy males the mean percentage of esterified cholesterol was about equal in both groups (Table I and II in paper VI).

Clinical diagnosis were possible to collect retrospectively in 79 of the 96 male patients. There was a positive accumulation of pre-β\(^{-1}\) positives in those patients with a diagnosis suggesting atherosclerosis (Fig. 1 in Paper VI).

Pre-beta\(^{1}\) lipoprotein on agarose gel and Lp(a) antigen in young adults.

The series of 16-17 year old school-boys investigated as reported in paper VII includes the 68 young school-boys studied in paper VI. In addition a series of 53 females, 24-30 years old, was studied.

In both series a highly significant, positive correlation between Lp(a) phenotype and presence or absence of pre-β\(^{-1}\)-lipoprotein was observed (p < 0.0001). In the young males a visual scoring of the intensity of the pre-β\(^{-1}\)-lipoprotein zone was performed, and 6 samples were scored as strongly positive. These six samples were also positive, and exhibited strong reactions, with respect to the Lp(a) antigen (Table I in paper VII).

The pre-β\(^{-1}\) lipoprotein could be demonstrated 2.6 times more frequently, and the Lp(a) frequency was 2.8 times higher in females with a positive family history of CHD than in those with a negative one. However, these differences were not statistically significant. The tendency to a higher frequency of positives, for the pre-β\(^{-1}\)-lipoprotein as well as the Lp(a) antigen was more pronounced in the group of females who had a first-degree relative with CHD, but compared to
those with a negative family history the difference was still not significant (Table II in paper VII).

No association was found between presence of pre-β₄-lipoprotein or LP phenotype and smoking in the two series. This was true whether persons smoking 1 or more cigarettes per day or those smoking 10 or more cigarettes per day were scored as smokers and those smoking less than 1 or less than 10 were scored as non-smokers (Table IV in paper VII).

Pre-beta lipoprotein in relation to Lp(a) lipoprotein and coronary atherosclerosis documented by angiography.

The characteristics of the series investigated are documented in paper VIII. Twenty-five of the 46 Finnish patients studied had normal coronary arteries and 21 coronary atherosclerosis documented by angiography. The presence of hyperlipidemia was roughly equal in the two angiographically different groups (Fig. 1 in paper VIII). Except for the occurrence of pre-β₄-lipoprotein the sole significant difference between the two groups was a more frequent positive family history (p < 0.05) in patients with CAD (Table II in paper VIII). Six of the 25 patients with NCA (24%) had a pre-β₄-lipoprotein fraction in contrast to 11 of 21 patients with CAD (52%). This difference is of borderline significance (p = 0.046).

A family history of CHD in first degree relatives was found more frequently in individuals with pre-β₄-lipoprotein (p = 0.035). In 13 of the 14 (93%) pre-β₄ positives who knew their family history it was positive for CHD. Of the 10 patients in the group with normal coronaries who had a negative family history, however, 9 (90%) also had a negative finding for pre-β₄. Pre-β₄ positivity was also correlated to smoking (p < 0.005) and hyperlipidemia (p < 0.02).

Lp(a) lipoprotein typing was also conducted, without knowledge of the result of the electrophoretic analysis, in the above related series of 46 Finnish patients. As for pre-β₄ classification, Lp(a) phenotypes were also scored without knowledge of the angiographic data.

As shown in paper IX there was a highly significant, positive correlation between the phenotype Lp(a+) and presence of pre-β₄ lipoprotein (p < 0.001)

A significant difference (p < 0.01) was found in the frequency of the phenotype Lp(a+) in this series of 46 patients (59%) when compared with that in 61 healthy Finns tested previously (31%). Lp(a) phenotype was, however, not associated with atherosclerotic lesions, demonstrable with angiography (0.70 < p < 0.80).

As mentioned there was a highly significant, positive correlation between pre-β₄ and smoking in the total series; this was mostly due to a strong association (p < 0.005) in those patients who had angiographically abnormal coronary arteries. For the Lp(a) trait there was no statistically significant association in the total series of patients.
Among those patients who exhibited abnormal coronaries on radiological examination, however, there was an association: 9 out of 10 smokers with abnormal coronary arteries were Lp(a+) \((p < 0.02)\).

Results of cholesterol and triglyceride analysis in different categories of the 46 Finnish patients are presented in table II and III.

In the whole series the mean cholesterol value in people exhibiting pre-\(\beta^1\) lipoprotein, 252 mg/100 ml was higher than in those not possessing this component, 221 mg/100 ml \((p < 0.02)\). The difference was also significant when comparison was conducted within the group of patients with abnormalities of coronary arteries upon radiological examination \((p < 0.05)\).

There was no statistical significance for a tendency towards a higher cholesterol value in Lp(a+) than in Lp(a-) individuals. Those who were Lp(a+) without having the pre-\(\beta^1\) lipoprotein had a mean cholesterol value which was practically the same as the one for Lp(a-) individuals (Table II).

For triglycerides statistically significant differences in mean values were found only between pre-\(\beta^1\) positive and negative patients in the whole series \((p < 0.02)\) and between pre-\(\beta^1\) positive and negative patients with normal coronary arteries upon radiological examination. Those 11 patients who were Lp(a+) without having the pre-\(\beta^1\) lipoprotein had a low mean value for triglycerides (Table III).

Pre-beta\(^1\) lipoprotein and Lp(a) lipoprotein in patients with sustained myocardial infarction and in presumably healthy middle aged males in relation to serum fractionation with ultracentrifugation.

The series of patients consisted of 58 individuals from the Boden area who had suffered MI fulfilling the WHO criteria (100) for the diagnosis of definite acute MI. All blood samples were obtained after a 12 hours' fast, at least 3 months after the acute MI. Fifty-one of the 58 patients were males. The mean age was 59 years and the range 36-74 years. The distribution of the 58 patients with respect to Lp(a) phenotype and presence or absence of pre-beta\(^1\) lipoprotein is shown in Table IV. A highly significant positive correlation was observed \((p < 0.0001)\).

In a series of 107 presumably healthy 50-52 year old males from the same area a lot of analysis were performed including total cholesterol, triglycerides and lipoprotein electrophoresis in 0.5 % agarose gel with the method described. In addition each serum sample with a detectable pre-\(\beta^1\) lipoprotein fraction was fractionated by ultracentrifugation at the density 1.050 g/ml and both supernatant and infranatant were subjected to electrophoretic analysis. Lp(a) typing was performed in Oslo as described previously. All serum samples were taken after a 12-hour fast.

The distribution of the 107 middle-aged males with respect to Lp(a) type and presence or absence of pre-\(\beta^1\) lipoprotein is shown in table V. A highly significant positive correlation between the two phenomena was observed \((p < 0.0001)\).
Nineteen of the 25 pre-β₁ positive subjects were found to have a pre-β₁ fraction with a density above 1.050 g/ml in contrast to the remaining six subjects who were found to have a fraction with a density below 1.050 g/ml. These six subjects were all found among those seven pre-β₁ positive who were typed as Lp(a) negative.

These six males were later reinvestigated after a new blood sampling (12-hour fast). Serum was fractionated at density 1.006 g/ml using ultracentrifugation. Four of them were found to have a pre-β₁ lipoprotein fraction of very low density. The other two had no detectable pre-β₁ lipoprotein fraction upon reinvestigation. None of them had a pre-β₁ fraction of density above 1.006 g/ml.

The prevalence of relevant variables in the two series is shown in Table VI. The sole significant differences were a higher frequency of both Lp(a) antigen and pre-β₁-lipoprotein in patients with sustained MI.

DISCUSSION

The extra lipoprotein fraction that we first detected between the "normal beta and pre-beta-lipoprotein fractions with the cellulose acetate electrophoretic method described (Paper I) had not previously been used in the classification of lipoprotein abnormalities.

As the relation to or identity with i.e. the genetic variant of beta lipoprotein, the Lp(a) lipoprotein disclosed by Berg (12) 1963 was not known, we named it the pre-beta₁ lipoprotein fraction.

Serum was used in all studies. For lipid analysis and especially for lipoprotein electrophoresis, EDTA-plasma from sterile vacutainers, may be preferred. By binding divalent cations, EDTA (1 mg/ml blood) prevents the oxidation of lipoprotein lipids (9, 62). This may be of special importance in Lp(a) typing since oxidative cleavage of Lp(a) with perjodate was shown to result in a complete loss of the Lp(a) lipoprotein ability to form precipitates with anti-Lp(a) antiserum, possibly indicating a carbohydrate nature of the Lp(a) antigen determinant (92).

Storage of the sample is, however, of great importance for detection of the pre-β₁ lipoprotein upon electrophoretic separation, either serum or EDTA plasma is used. The pre-β₁ lipoprotein is not detectable in serum samples stored for more than 72 hours at 4°C and for shorter periods at room temperature with both electrophoretic methods used. This seems mainly or only to depend on a decreased pre-β mobility in stored specimens which results in coalescence of the pre-β and pre-β₁ fractions upon electrophoresis (Fig. 2 and 3). After storage of pre-β₁ positive serum samples at 4°C during 6 days lipoprotein fractions with pre-β₁ mobility were still found in the fractions with density above 1.006 g/ml after ultracentrifugation of the samples (Fig. 3).

In frozen specimens, thawed only once, the pre-β₁ lipoprotein is, however, reproducibly detectable upon electrophoresis after storage for periods of up to 2 to 3 weeks at -20°C. As freezing has been reported not to affect either CH or TG analysis (9) it can be recommended if samples can not be analyzed immediately.
Comparison of lipids and LP between different groups seems to be justified if the groups represent an extraction of the population with the same age and sex and living in the same geographical area. In addition the specimens ought to be taken after a fixed period of fasting and preferably at the same time in the year to avoid seasonal variations.

For the pre-β classification and probably also for the Lp(a) typing, specimens must be stored under the same conditions and investigated as soon as possible at equal times after sampling.

These conditions are fulfilled in the groups compared in paper I-VII, and in paper XI. The fasting period in the study reported in paper II was, for practical reasons, about 6-8 hours. No difference in diet habits was, however, found between the compared pre-β positive and negative groups. Nor was there any difference in medication between these two groups.

In paper VIII more females than males were found in the group of patients with normal coronaries than in the group with coronary artery disease. As only one pre-β positive patient (with normal coronaries) in this series was found to be Lp(a-) and a high association (P < 0.001) was found between the phenotype Lp(a+) and presence of pre-β lipoprotein, it may still be justified to compare pre-β positive and negative groups. In several investigations the Lp(a) lipoprotein was found in approximately 35% of healthy people of Western European extraction and has not been found to be significantly correlated with age or sex (13). For the same reasons it may be justified to compare the frequency of Lp(a+) patients in the series of 46 Finnish patients with the one previously found in 61 healthy Finns as described in paper VIII.

The comparison made between pre-β frequency in the series of 46 Finnish patients and the one found in the epidemiological investigation in Northern Sweden as described in paper VIII may not be quite justified as the fasting periods were different in the two investigations. The pre-β frequency (52%) found in the Finnish patients with coronary artery disease is, however, considerably higher than the one found (on cellulose acetate as used in the epidemiological screening) in 75 healthy school-boys (27%) in the Boden area (32). These 27% also represent the highest frequency of pre-β positive individuals found in a presumably healthy extraction of the population with the cellulose acetate method used.

Except for the determinations of free and total cholesterol with a modified method using gas chromatography, the methods used are routine laboratory procedures performed as outlined (28, 61, 8, 96).

The method for free and total cholesterol determinations using a gas-chromatographic technique for evaluation as described in paper VI is similar to that described by Blomhoff (19), and the coefficients of variation are also of the same order of magnitude. Accuracy has always been within 2% of the "true" value given for gas-chromatographic analysis of total cholesterol for the reference serum used (Liponorm, Nyegaard & Co, Oslo, Norway). Repeated standard determination has not given indications for a significant cholesterol
absorption on the solid supporting material during analysis of the samples in the investigated series, using the same column.

The procedure for cellulose acetate electrophoresis on Sephraphore III membranes is described in paper I. As shown in paper I, fig. 3, the pre-β-lipoprotein fraction is very sharp and well separated from the beta-lipoprotein-fraction.

In accordance with others using cellulose acetate (26, 11, 25) we have found that chylomicrones remain at the point of application. After a test meal lipoprotein fractions may, however, appear in some individuals between the pre-β and β-lipoprotein fractions on cellulose acetate. These lipoprotein-fractions are not found on agarose gel electrophoresis. At present it is not known whether they represent degradation products of chylomicrones (26) or some other lipoprotein phenomena.

During the first period using cellulose acetate membranes (Sepraphore III) we had no technical problems as reported in paper I-V. Later on, however, we found that new lots of Sephraphore III membranes gave variations in the electrophoretic pattern. Such a variation was sometimes found in membranes within the same lot.

We have not been able to find any apparent cause for this difference in lipoprotein pattern except for those probably related to the material and procedure used in the manufacture of the cellulose acetate membranes. The same findings have been reported by other authors (10, 77) and may in part be explained by the considerable endo-osmotic flow found in cellulose acetate membranes during electrophoresis (31). Besides, electrophoresis of serum lipoproteins on cellulose acetate is technically more difficult than electrophoresis in agarose gel.

In spite of the sharp separation of the lipoprotein fractions on cellulose acetate and their correspondence with the density classes of the ultracentrifuge (33), I do not recommend this method as a routine laboratory procedure. It may, however, be used for special purposes under well controlled conditions with a routine check of each membrane.

The method used for agarose gel electrophoresis is described in this paper and is the same as reported previously (29, 32). It is based mainly on the technique and equipment used for protein electrophoresis (55) and the findings reported by Noble et al (66, 67). The high degree of agreement between agarose gel electrophoresis and ultracentrifugation previously reported (67) seems to be valid also for the method used in these investigations. The pattern closely resembles the one found on cellulose acetate. In agreement with Noble (66) we have found that a decreased gel concentration increases the distance between the beta and pre-beta peaks and that a less concentrated buffer increases the extent of the migration and of the separation of serum lipoproteins. In contrast to Noble (66), however, we have found no practical difficulties in using 0.5% agarose, which seems to produce sharper lipoprotein bands with better resolution than in agarose-agar gel. In almost all sera from presumably healthy individuals we have found one sharp β-zone, one or two (seldom 3) distinctly separated pre-beta bands and two alpha bands. Chylomicrones remain at the origin. Sometimes the "fast moving" pre-β band tend to split into two zones.
In addition and in agreement with Noble et al (67), we have frequently found two fractions upon agarose gel electrophoresis of the VLDL fraction isolated by preparative ultracentrifugation. There is excellent reproducibility between duplicate samples, and generally the relative mobility of different fractions has not changed. The same batch of agarose (Behringwerke) was used for several years. The method is recommended as a routine laboratory procedure as well as a tool for the study of lipoprotein properties.

The extent to which different types of probable "pre-ß variants" contributed to the pre-ß positive group reported in paper II can not be stated definitely as neither lipoprotein electrophoresis on agarose gel, nor ultracentrifugation nor Lp(a) testing were performed in that epidemiological study.

Although the pre-ß positive group of individuals defined in paper II on cellulose acetate electrophoresis in the non-fasting state probably is genetically inhomogenous it is interesting in many respects. It defines a group of individuals with higher mean CH and, especially with increasing age, higher mean TG levels than the rest of the population. Most of the individuals classified as pre-ß positive in this series probably have a pre-ß lipoprotein of density above 1.050 g/ml, identical to the Lp(a) lipoprotein. Other results suggest that this lipoprotein may be associated with early atherosclerosis (Paper IX, XI). Also the pre-ß positive group of individuals defined in paper II probably includes some individuals with high triglyceride levels, at least after a meal, supposed to aggravate or precipitate acute arterial insufficiency, as anginal attacks, in atherosclerotic subjects (59). All this facts may explain the higher frequency of angina found in the pre-ß positive group in this study (Paper II).

The reasons for the discrepancies found between the two electrophoretic methods used is under investigation. They may depend on methodological (31) as well as other reasons. The variations found in the Lipoprotein pattern on some cellulose acetate membranes may contribute to the discrepancies between the two electrophoretic methods in spite of the efforts made to control them.

The lipoprotein pattern on agarose gel electrophoresis with the method used has been reproducible when the same samples were analyzed in duplicate on different glass plates. No general variation in the lipoprotein pattern has been found.

In all series investigated a highly significant, positive correlation has been found between the presence of a pre-ß lipoprotein on agarose gel electrophoresis and the phenotype Lp(a+), tested blindly and independently in the same serum samples as described previously (Paper IX, Table I (P< 0.001), Paper VII, Table I (P< 0.0001), Tables IV and V).

However, LP fractions of very low density with pre-ß mobility have been found in some presumably healthy Lp(a) negative individuals on agarose gel electrophoresis after a 12-hour fast. In these individuals the lipoprotein often migrates slower and is found nearer the beta-
lipoprotein fraction than usually is the case. The nature of this type of lipoprotein with pre-ß mobility has to be investigated further. Some of them may represent type III heterozygotes.

One would then sometimes expect to find individuals with two extra lipoprotein fractions between the ß and pre-ß fractions in Lp(a+) individuals. In the series mentioned above, one of the Lp(a+) middle-aged males was also found to have two extra pre-ß lipoprotein fractions of different mobility.

The results of the present investigations (Paper VI, VIII, IX) indicate that the pre-beta lipoprotein found after a 12-hour fast on agarose gel electrophoresis is associated with early atherosclerosis (paper VIII) and/or with symptoms or signs of CHD (Paper VI, VIII, XI). It also seems reasonable to suppose that this association is mostly due to the pre-beta lipoprotein of density above 1.050 g/ml, identical, as it seems, with relatively high amounts of Lp(a) lipoprotein. A need for a reliable method to quantitate the Lp(a) lipoprotein apparently exists.

As far as the present investigations go the pre-beta lipoprotein of density above 1.050 g/ml / Lp(a) antigen may then represent a genetic risk factor with respect to early atherosclerosis and CAD.

As the pre-ß lipoprotein with density above 1.050 g/ml is not detected or does not separate from the common pre-beta VLDL fraction in some (94) or all cases (36) using conventional routine methods for lipoprotein electrophoresis on paper or in agarose gel, the use of the described method is also recommended to avoiderronious results in lipoprotein typing.

It seems as if the basic pathogenetic mechanisms responsible for an atherogenic effect of tobacco smoke remain to be elucidated. With exception for the possible, direct effects of nicotine, through catecholamines, on the myocardium, it has never been possible to produce lesions in the vasculature through the action of nicotine accepted as atherosclerotic in nature (7).

However, by exposing rabbits fed with cholesterol to carbon monoxide, giving carboxyhemoglobin values of 11-20 %, not uncommon in heavy smokers, or to oxygen-deficient atmosphere (10 % oxygen) Astrup et al (7) found the developement of a deep transmural lipid infiltration and a tissue cholesterol, 2.5 times greater than in the controls. Hyperoxia seemed to prevent lipid tissue deposition. Small concentrations of carbon monoxide in rabbits on a normal diet produced vascular damage of the early atherosclerotic type. Especially in the groups exposed to carbon monoxide exudate in the serous cavities occurred frequently, suggesting an increased permeability of the endo- and mesothelial membranes caused by carbon monoxide. Increased vascular permeability for albumin (83) in human subjects after being exposed to carbon monoxide and hypoxia and a significant decrease of plasma volume and accumulation of fluid in various tissues (6) has also been shown. Robertson (76) exposed intimal cells cultivated in vitro to low concentrations of oxygen and found an uptake of cholesterol increased 6 to 7 times. Then Astrup et al (7) suggest that carbon
monoxide in tobacco smoke might be a major factor in the increase of atherosclerotic diseases found in smokers.

This effect of hypoxia in smokers is interesting in relation to the association found between pre-\(\beta\), lipoprotein/Lp(a) antigen among 75 young healthy males studied (32) had a significantly higher mean value of total CO\(_2\) (\(P < 0.001\)) and hematocrit (\(P < 0.05\)) and a lower mean value of chloride (\(P < 0.05\)) than the pre-\(\beta\) negative group. A higher mean value of total CO\(_2\) (\(P < 0.02\)) and a lower mean value of chloride (\(P < 0.05\)) was also found in this series when all pre-\(\beta\), positive were compared with the pre-beta\(\_\) negative group. No difference in smoking habits was found between the compared negative and positive groups in these two series of presumably healthy individuals investigated. These findings, if further confirmed, may explain, at least in part, why smoking could be particularly harmful in individuals possessing the pre-beta\(\_\)/Lp(a) lipoprotein.

As a possible correlation between the presence of the pre-beta, lipoprotein fraction and angina pectoris was found (30), a series of studies, as described, were undertaken to investigate this further. The statistically significant correlation between the occurrence of pre-beta\(\_\) lipoprotein and angina of effort, described in paper II, had not been reported earlier. Noble (66) had found the occurrence of double pre-beta peaks in some individuals upon agarose gel electrophoresis using low agarose gel concentrations. During our studies the findings of extra lipoprotein fractions on different supporting media were, however, reported by several authors (35, 11, 98, 75, 45).

Rider, Levy and Fredrickson reported in an abstract (73) that 11% of over 3500 investigated subjects had pre-beta material with density from 1.050-1.080, dubbed "sinking" pre-beta. No linkage to other dyslipoproteinemias was found. Antiserum prepared to "sinking" pre-beta reacted with all plasmas containing "sinking" pre-beta and in addition with 20% of plasmas with no "sinking" pre-beta visible upon electrophoresis. An antiserum to Berg's Lp antigen reacted with all plasmas containing "sinking" pre-beta and it was concluded that "sinking" pre-beta is the Lp antigen. This conclusion is in agreement with our findings.

No association between extra lipoprotein fractions or Lp(a) lipoprotein and disease was, however, found in these studies.

By electrophoresis on paper of the whole plasma or serum and of VLDL and low density - high density lipoprotein fractions after ultracentrifugation Ellefson et al (36), during a 3-year period, investigated 14,988 patients and 1,662 apparently healthy subjects. They frequently found a pre-beta lipoprotein of density greater than 1.063 g/ml, referred to as pre-beta\(\_\) HDL, which was not
characteristic of types IV and V hyperlipidemia. On paper electro­
phoresis, pre-beta HDL had the same mobility as pre-beta VLDL of type IV hyperlipidaemias and it was stated that pre-beta HDL "cannot be distinguished from the characteristic pre-beta VLDL by methods of electrophoretic analysis which usually are used routinely in clinical laboratories". Through polyacrylamide gel, however, pre­
beta HDL migrated behind the beta LDL.

Strongly defined bands of pre-beta HDL were found by Ellefson et
al (36) in 12 % of the lipoprotein electrophoregrams of all the subjects
studied; in 13 % the pre-beta HDL bands were only barely discernible.
No association of pre-beta HDL with a specific disorder was recog­
nized. A search for such an association was, however, suggested as
approximately 90 % of the subjects with a pre-beta HDL were patients
with a variety of metabolic disorders.

Except for Papadopoulos et al who found two pre-beta bands on
agarose gel electrophoresis in a higher frequency in serum from
patients with myocardial infarction (71) and coronary atherosclerosis
(72) than in presumably normal subjects, suggesting a pathogenetic
relationship, none had found a correlation between the occurrence of
an extra pre-beta lipoprotein fraction, "sinking" pre-beta, the Lp(a)
antigen and a specific disease.

Recently, however, reports have been published on a correlation of
dual pre-beta lipoprotein peaks with a family history of CHD (57) and
of a significant correlation (P < 0.001) between a pre-beta^1 lipoprotein
subfraction on agarose gel electrophoresis and moderate to severe
coronary artery disease (54). A higher frequency of "sinking" pre­
beta lipoprotein in patients with myocardial infarction (38 %) and
intermittent claudication (41 %) than in healthy men (26 %) has also
been reported (39). All these findings are in agreement with those
reported in this paper.

It seems justified to suggest that the pre-beta^1 lipoprotein of density
above 1.050 g/ml, the pre-beta HDL (36), the "sinking" pre-beta (73)
and the Lp(a) antigen are synonyms for the same lipoprotein phenome­
non. However, extra pre-beta lipoprotein fractions of different nature
and density may sometimes be found upon agarose and cellulose acetate
electrophoresis with the methods used in these studies.

The differences found (32) in mean total CO2, chloride and hemato­
crite levels between strong pre-beta^1/Lp(a) positive and pre-beta^1
negative young, healthy males may indicate a difference in membrane
composition or function between the two groups. Needless to say,
further studies are required to support this suggestion. This also
applies to the clarification of the significance of the relationship found
(paper VIII, IX) between pre-beta^1/Lp(a) lipoprotein and smoking.

It does not seem to be convincingly proven that the Lp(a) antigen, as
suggested (53) is a quantitative genetic trait present in all individuals.
Until the genesis and metabolism of the enigmatic Lp(a) lipoprotein
will have been further clarified, the bimodality observed in Lp(a)
quantitation (80) and the extensive family data previously referred to,
strongly argue against control by more than one locus. Even if all Lp(a-)
individuals should have trace amounts of Lp(a) lipoprotein in their
serum it is still completely compatible with a simple Mendelian
inheritance. The problems of specificity and standardization of the
Lp(a) antisera (15) must also be taken into consideration.

So far the source of the Lp(a) lipoprotein does not seem to be disclosed.
However, the findings by Enholm et al (34) that the purified Lp(a) lipo-
protein dissociates into apparently normal LDL, albumin and the "Lp(a)
protein" may suggest that it is formed in the circulation during the VLDL
metabolism.

Berg found a high degree of similarity between LDL and Lp(a) protein
and the histocompatibility antigens, closest to the Lp(a) lipoprotein,
suggesting a relationship between the Lp(a) lipoprotein and antigens
on cell membranes (14). Human skin grafts were also found to survive
longer when donors and recipients were of the same Lp(a) type, rather
than of different ones (15).

Lipoprotein lipase seems to be present even in the larger arteries.
This suggests that the concentration of soluble lipoproteins at the
interface of the blood-artery may greatly exceed the concentration
in the circulating blood (103).

A defect binding of VLDL to the membrane during the proposed
formation of the Lp(a) LP could, to some extent, alter transport
processes, i.e. transport of cholesterol through, or enzyme
activities localized to, the cell membrane.

The hypothesis of a variance in soluble very low density lipoprotein-
membrane interaction, leading to production of the Lp(a) lipoprotein
as a soluble variant of LDL in some individuals suggests further
fascinating and more intricate studies.

The existence of comparatively big and cholesterol-rich soluble Lp(a)
lipoprotein particles in serum may also be of importance for the
"secondary" processes in the development of atherosclerotic lesions.
These big particles can perhaps be more easily precipitated by acid
mucopolyaccharides in the arterial walls than LDL. The Lp(a) lipo-
protein has also been found in atherosclerotic lesions in Lp(a) positive
individuals (95).
ABSTRACT

The main purpose of the studies summarized in this paper was to investigate the relationship between the occurrence of an extra pre-beta lipoprotein fraction named the pre-beta lipoprotein, first found in some individuals upon lipoprotein electrophoresis on cellulose acetate, and early atherosclerosis and symptoms and signs of CHD as well as the relationship of the pre-beta lipoprotein to the genetically determined variant of LDL, the Lp(a) lipoprotein. Later lipoprotein electrophoresis in low concentration (0.5%) of agarose gel has mainly been used.

The experience and results of the two methods used for lipoprotein electrophoresis in connection with other findings are:

A. Both methods used give a separation between the beta and pre-beta lipoprotein zones, good enough to detect extra lipoprotein fractions.

B. The agarose gel electrophoretic method has always been found to be reproducible and is easy to use. The cellulose acetate (Sephracore III) method is more difficult to perform as a routine procedure and has to be well controlled as sometimes variations in the lipoprotein pattern, probably due to the membrane manufacturing procedure, has been found between different membranes. The agarose gel electrophoretic method used is then recommended as a routine laboratory procedure.

C. With the agarose gel electrophoretic method used a highly significant positive correlation has been found (P<0.001) between presence of pre-beta lipoprotein and the phenotype Lp(a+) in several series of subjects studied after a 12-hour fast.

D. The results of contemporary ultracentrifugation analysis are consistent with the interpretation that the pre-beta lipoprotein with density between 1.050-1.080 g/ml is identical with or closely related to the Lp(a) lipoprotein. In a few cases, however, VLDL fractions with pre-beta mobility, not associated with the Lp(a) lipoprotein, have been found in presumably healthy middle-aged males with the agarose gel electrophoretic technique used.

E. In a series of Finnish patients studied a pre-beta lipoprotein was found more frequently in patients with CAD documented by angiography than in individuals with NCA (P=0.046). A frequency of Lp(a+) which was significantly higher (P<0.02) than in previously investigated, healthy subjects, was also found. In a series of patients with sustained MI we also found a higher frequency of both Lp(a) antigen (P=0.036) and pre-beta lipoprotein (P=0.038) than in a series of presumably healthy middle-aged males from the same area.

F. In the non-fasting state and after test meals, fractions classified as pre-beta lipoprotein, probably VLDL in nature, appear in some individuals on cellulose acetate but not on agarose gel electrophoresis. These fractions may have contributed to the highly significant positive association found (P<0.001) between the occurrence of a pre-beta lipoprotein and clinical diagnosis of typical or suspected angina.
pectoris in a series of 56-60 year old males investigated after a 6-8 hours' fast using lipoprotein electrophoresis on cellulose acetate (Sepraphore III).

G. No association has been found between either pre-beta lipoprotein and Lp(a) lipoprotein and smoking in series of apparently healthy individuals. A positive association was, however, found between both pre-ß lipoprotein and smoking (P* 0.005) and Lp(a) lipoprotein and smoking (P < 0.02) in the small series of 46 Finnish patients with angiographically proven CAD.

H. A family history of CHD was found more often (P = 0.035) in first degree relatives to pre-beta positive than to pre-beta negative patients in the series of Finnish patients. In a series of young females a 3:1 relation was found for the frequency of presence of pre-beta lipoprotein on agarose gel electrophoresis as well as of Lp(a) positivity in those with one parent with CHD compared with those with a completely negative family history.

Since the pre-beta lipoprotein and the Lp(a) antigen can be determined early in life, the present studies might have some importance for the preventive medicine of coronary heart disease.
Fig. 1.: "Lipoprotein spectrum" showing the two lipoprotein terminologies and density intervals and major flotation rates (Sf values) of lipoprotein classes. Applied to the spectrum are also the distribution of the apolipoproteins (apo-LP) the lipoprotein Lp(a), and the distribution of the characteristic lipoprotein elevations as they occur in the six types of hyperlipoproteinemia according to Fredrickson and Lees (42). (CH = Cholesterol, TG = Triglycerides. From A. Gustafson with kind permission.)

Fig. 2.: Electrophoresis of lipoproteins in agarose gel (0.5 %). A pre-beta_1 lipoprotein fraction is found in the serum from subject 3.

Fig. 3.: Electrophoresis of lipoproteins in agarose gel (0.5 %) of whole serum and the fraction with density above 1.006 g/ml after storage of the pre-beta_1 positive sample 3 in fig. 2 for 6 days at +4°C.
## Table I

Cholesterol and triglyceride analysis in pre-beta\(_1\) positive and negative groups of 1229 investigated males.

a) all subjects investigated.
b) eldest age-groups only.

<table>
<thead>
<tr>
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<th>Pre-(\beta) positive</th>
<th>Pre-(\beta) negative</th>
<th>t-test</th>
<th>p</th>
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<tbody>
<tr>
<td><strong>Men born 1910 - 1919 (n=1229)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.6 ± 1.3</td>
<td>2.2 ± 1.2</td>
<td>4.64</td>
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<td>Cholesterol (mg/100 ml)</td>
<td>265.3 ± 42.1</td>
<td>255.0 ± 42.5</td>
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<td>&lt;0.001</td>
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<tr>
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<th>Pre-(\beta) positive</th>
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</thead>
<tbody>
<tr>
<td><strong>Men born 1910 - 1913 (n=286)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>2.9 ± 1.2</td>
<td>2.3 ± 1.3</td>
<td>2.95</td>
<td>0.01</td>
</tr>
<tr>
<td>Cholesterol (mg/100 ml)</td>
<td>257.3 ± 39.2</td>
<td>255.4 ± 41.6</td>
<td>0.30</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
Table II
Total serum cholesterol (mg/100 ml) in different categories of 46 Finnish patients.

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>$M \pm SD$</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-β₁⁺</td>
<td>17</td>
<td>252.1 ± 35.8</td>
<td>2.68</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Pre-β₁⁻</td>
<td>29</td>
<td>221.2 ± 38.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lp(a⁺)</td>
<td>27</td>
<td>240.8 ± 43.3</td>
<td>1.68</td>
<td>N.S.</td>
</tr>
<tr>
<td>Lp(a⁻)</td>
<td>19</td>
<td>221.0 ± 33.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angio⁺</td>
<td>21</td>
<td>245.0 ± 5.0</td>
<td>1.96</td>
<td>N.S.</td>
</tr>
<tr>
<td>Angio⁻</td>
<td>25</td>
<td>222.2 ± 42.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-β₁⁺, angio⁺</td>
<td>11</td>
<td>259.6 ± 29.5</td>
<td>1.18</td>
<td>N.S.</td>
</tr>
<tr>
<td>Pre-β₁⁺, angio⁻</td>
<td>6</td>
<td>238.3 ± 44.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-β₁⁻, angio⁺</td>
<td>10</td>
<td>227.4 ± 36.4</td>
<td>0.58</td>
<td>N.S.</td>
</tr>
<tr>
<td>Pre-β₁⁻, angio⁻</td>
<td>19</td>
<td>218.4 ± 40.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-β₁⁺, angio⁺</td>
<td>11</td>
<td>259.6 ± 29.5</td>
<td>2.18</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Pre-β₁⁻, angio⁺</td>
<td>10</td>
<td>227.4 ± 36.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-β₁⁺, angio⁻</td>
<td>6</td>
<td>238.3 ± 44.7</td>
<td>1.04</td>
<td>N.S.</td>
</tr>
<tr>
<td>Pre-β₁⁻, angio⁻</td>
<td>19</td>
<td>218.4 ± 40.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lp(a⁺), pre-β₁⁻</td>
<td>11</td>
<td>223.1 ± 47.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pre-β₁⁺ = Pre-β₁⁺-lipoprotein present
Pre-β₁⁻ = Pre-β₁⁻-lipoprotein absent
Angio⁺ = Abnormal coronary arteries upon radiological examination
Angio⁻ = Normal coronary arteries upon radiological examination.
Table III
Serum triglycerides mmol/l in different categories of 46 Finnish patients.

<table>
<thead>
<tr>
<th>Category</th>
<th>n</th>
<th>M ± SD</th>
<th>t</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-(\beta_1) +</td>
<td>17</td>
<td>2.02 ± 0.91</td>
<td>2.89</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Pre-(\beta_1) -</td>
<td>29</td>
<td>1.44 ± 0.46</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lp(a+)</td>
<td>27</td>
<td>1.68 ± 0.52</td>
<td>0.24</td>
<td>N.S.</td>
</tr>
<tr>
<td>Lp(a-)</td>
<td>19</td>
<td>1.63 ± 0.94</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Angio+</td>
<td>21</td>
<td>1.64 ± 0.47</td>
<td>0.12</td>
<td>N.S.</td>
</tr>
<tr>
<td>Angio-</td>
<td>25</td>
<td>1.67 ± 0.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-(\beta_1) +, angio+</td>
<td>11</td>
<td>1.80 ± 0.52</td>
<td>1.42</td>
<td>N.S.</td>
</tr>
<tr>
<td>Pre-(\beta_1) +, angio-</td>
<td>6</td>
<td>2.43 ± 1.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-(\beta_1) -, angio+</td>
<td>10</td>
<td>1.49 ± 0.38</td>
<td>0.37</td>
<td>N.S.</td>
</tr>
<tr>
<td>Pre-(\beta_1) -, angio-</td>
<td>19</td>
<td>1.42 ± 0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-(\beta_1) +, angio+</td>
<td>11</td>
<td>1.80 ± 0.52</td>
<td>1.50</td>
<td>N.S.</td>
</tr>
<tr>
<td>Pre-(\beta_1) -, angio+</td>
<td>10</td>
<td>1.49 ± 0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-(\beta_1) +, angio-</td>
<td>6</td>
<td>2.43 ± 1.34</td>
<td>2.88</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Pre-(\beta_1) -, angio-</td>
<td>19</td>
<td>1.42 ± 0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lp(a+), pre-(\beta)</td>
<td>11</td>
<td>1.43 ± 0.39</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Pre-\(\beta_1\) + = Pre-\(\beta_1\) -lipoprotein present
Pre-\(\beta_1\) - = Pre-\(\beta_1\) -lipoprotein absent
Angio+ = Abnormal coronary arteries upon radiological examination.
Angio- = Normal coronary arteries upon radiological examination.
Table IV

Pre-β\textsubscript{1} lipoprotein and Lp(a) antigen in 58 Swedish patients with sustained myocardial infarction.

<table>
<thead>
<tr>
<th>Number of individuals</th>
<th>Pre-β\textsubscript{1} - lipoprotein present</th>
<th>Pre-β\textsubscript{1} - lipoprotein absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lp(a+)</td>
<td>19</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>Lp(a-)</td>
<td>3</td>
<td>31</td>
<td>34</td>
</tr>
<tr>
<td>Total</td>
<td>22</td>
<td>36</td>
<td>58</td>
</tr>
</tbody>
</table>

X\textsuperscript{2} = 26.66, 1 D.F., P < 0.0001

Table V

Pre-β\textsubscript{1} lipoprotein and Lp(a) antigen in 107 Swedish males, 50 - 52 years old.

<table>
<thead>
<tr>
<th>Number of individuals</th>
<th>Pre-β\textsubscript{1} - lipoprotein present</th>
<th>Pre-β\textsubscript{1} - lipoprotein absent</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lp(a+)</td>
<td>18</td>
<td>10</td>
<td>28</td>
</tr>
<tr>
<td>Lp(a-)</td>
<td>7</td>
<td>72</td>
<td>79</td>
</tr>
<tr>
<td>Total</td>
<td>25</td>
<td>82</td>
<td>107</td>
</tr>
</tbody>
</table>

X\textsuperscript{2} = 32.44, 1 D.F., P < 0.0001
Several variables in 103 healthy males (HM), 50 - 52 years old, and in 58 patients (MI) with sustained myocardial infarction (all from Northern Sweden).

<table>
<thead>
<tr>
<th>Variable</th>
<th>HM</th>
<th>MI</th>
<th>Analysis of difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive family history</td>
<td>39/103 (38 %)</td>
<td>17/58 (29 %)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Smoking</td>
<td>51/103 (50 %)</td>
<td>32/58 (55 %)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Never smoked</td>
<td>27/103 (26 %)</td>
<td>13/58 (22 %)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Pre-β₁ lipoprotein present</td>
<td>24/103 (23 %)</td>
<td>22/58 (38 %)</td>
<td>P = 0.038 (^x)</td>
</tr>
<tr>
<td>Lp(a+)</td>
<td>27/103 (26 %)</td>
<td>24/58 (41 %)</td>
<td>P = 0.036 (^x)</td>
</tr>
<tr>
<td>Hypercholesterolemia or hypertriglyceridemia</td>
<td>50/103 (49 %)</td>
<td>38/58 (66 %)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Both hypercholesterolemia and hypertriglyceridemia</td>
<td>15/103 (15 %)</td>
<td>16/58 (28 %)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>35/103 (34 %)</td>
<td>24/58 (41 %)</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

\(^x\) Fisher exact test
REFERENCES.


100. Working Group on Ischemic Heart Disease Registers. WHO, Copenhagen 1968.


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