Original Research

Analysis of the Composition of Plasma Lipoproteins in Patients with Extensive Coronary Heart Disease Using ^{1}H NMR Spectroscopy

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Introduction: Alterations in the lipid composition and overall structure of plasma lipoproteins have been correlated with pathological situations such as dyslipidaemia, coronary heart disease (CHD), hypertension, and renal disease. In the present study ^{1}H NMR spectroscopy was used to analyse the lipid composition of HDL and nonHDL lipoproteins in patients with triple vessel CHD and in patients with normal coronary arteries.

Methods: Serum samples were collected from 50 patients with triple vessel CHD and 41 patients with normal coronary vessels, both documented angiographically. The classical risk factors for CHD were recorded and each patient's standard lipid profile was determined. HDL and nonHDL lipoprotein particles were separated by precipitation with Dextran Sulphate/$MgCl_2$. HDL and nonHDL lipid fractions were extracted with chloroform:methanol (1:2, v/v). ^{1}H NMR spectra were recorded on a Bruker DRX-600 spectrometer.

Results: In the HDL fraction of patients with triple vessel disease the percentage of triglycerides was significantly higher than in those with normal coronary arteries, whereas the percentages of cholesterol esters, phosphatidylcholine and sphingomyelin, as well as polyunsaturated fatty acids, such as linoleic, arachidonic, and eicosapentaenoic, were significantly lower. In the nonHDL fraction significantly higher levels of triglycerides and lower levels of polyunsaturated fatty acids were observed.

Conclusions: Patients with established CHD show significant alterations in the composition of plasma lipoproteins compared to those with normal coronary arteries. Further study of plasma lipoprotein composition might be able to identify components as indexes for the existence of CHD.

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Alterations in the lipid composition and the overall structure of plasma lipoprotein particles have been correlated with pathological conditions such as dyslipidaemia, hypertension, and renal disease,[1-3] as well as with the presence and degree of severity of coronary heart disease (CHD).[4-7] Although the standard techniques for the analysis of the composition of lipoprotein particles allow the simultaneous detection and quantification of lipids, they are not used widely in clinical practice because they are time-consuming and require sample preparation at many stages. As an alternative, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy is a non-invasive analytical technique for the study of the lipid composition and molecular structure of plasma lipoproteins,[8] as well as for the measurement of lipoprotein subfractions.[9] This method needs little sample preparation and, without the need for chemical modification, provides a rapid and comprehensive lipid analysis, in which lipid components are represented by a signal that is characteristic of their structure, and whose

intensity is proportional to their concentrations. In a recent study, the analysis of overall plasma lipoprotein composition by NMR spectroscopy in patients with CHD showed that the method is able to detect the presence and the severity of the disease with a sensitivity and specificity >90%.[10]

The aim of the present study was to evaluate the contribution of ^{1}H NMR spectroscopy to the detailed analysis of the lipid composition of HDL and nonHDL lipoproteins in patients with triple vessel CHD and in those with normal coronary arteries.

Material and methods

Study population

The study included 50 consecutive patients (42 men [84%], mean age 67.7 ± 9.9 years) with mild dyslipidaemia (total serum cholesterol levels <250 mg/dl), no history of cardiovascular disease, but a confirmed acute coronary syndrome (onset of symptoms within 12 hours prior to hospital admission), who had an uncomplicated clinical course and angiographically proven extensive CHD (at least one stenosis >50% of the lumen in at least three epicardial coronary arteries). The diagnosis of acute coronary syndrome was based on the clinical picture (typical chest pain lasting >20 minutes) and the presence of electrocardiographic changes, with or without increased biochemical indexes of myocardial necrosis (total creatinine kinase [CK] or its myocardial fraction [CK-MB] double the normal values, or positive troponin I values >0.3 ng/ml).

The control group consisted of 41 consecutive patients (25 men [61%], mean age 61.2 ± 9.6 years) who underwent coronary angiography for the investigation of atypical clinical symptoms. These patients had had a non-diagnostic stress test and showed angiographically "normal" coronary vessels (smooth coronary vessels with normal luminal diameter).

Patients who were under hypolipidaemic medication, had a history of diabetes mellitus, liver or kidney disease, or malignancy, or had any disease of rheumatic aetiology, were excluded from the study. Patients who underwent diagnostic coronary angiography as part of the primary treatment of the acute coronary episode, patients whose coronary angiogram showed less extensive CHD, i.e. ≤2 vessels, and patients with triglyceride levels >400 mg/dl were also excluded.

All patients gave informed, written consent before their inclusion in the study.

Collection of blood samples and measurement of biochemical parameters

In the patients who had a confirmed acute coronary episode blood samples were obtained within the first 12 hours from the onset of symptoms. In the patients of the control group, samples were collected in the morning before angiography. Analysis of serum lipid parameters (total cholesterol, HDL-cholesterol, triglycerides) were measured using an Olympus AU600 analyser (Olympus Diagnostica, Hamburg, Germany). Apolipoproteins (ApoAI, ApoB, ApoE) and lipoprotein(a) [Lp(a)] were measured on a Behring Nephelometer BN (Dade-Behring, Liebernach, Germany). LDL-cholesterol was calculated by the Friedewald formula.

Lipid extraction

Separation of HDL lipoproteins from nonHDL particles was performed by precipitation with Dextran Sulphate/$MgCl_2$[11] and the lipids of the two fractions were extracted with a chloroform:methanol mixture, according to a modification of the Bligh and Dyer method.[12]

Recording of ^{1}H NMR spectrum

The ^{1}H NMR spectra were recorded at 298°K on a Bruker Avance DRX-600 NMR spectrometer operating at a field strength of 14.1 Tesla (600 MHz). The identification of the chemical shifts, δ, of the peaks was based on the international literature.[13-15]

^{1}H NMR analysis of the total lipid extract of HDL lipoproteins

Figure 1 shows a representative ^{1}H NMR spectrum of the lipid extract of HDL lipoproteins. In this spectrum appeared all the peaks that correspond to cholesterol, free (FC) and esterified (EC), phospholipids, phosphatidylcholine (PC), sphingomyelin (SM), the glycerol backbone of triglycerides, and fatty acids (Table 1, Figure 1).

Cholesterol

Cholesterol is identified and quantified by the characteristic signal of the C-18 methyl group at 0.70 ppm, from which the esterified/free cholesterol ratio is calculated (Figure 1A).

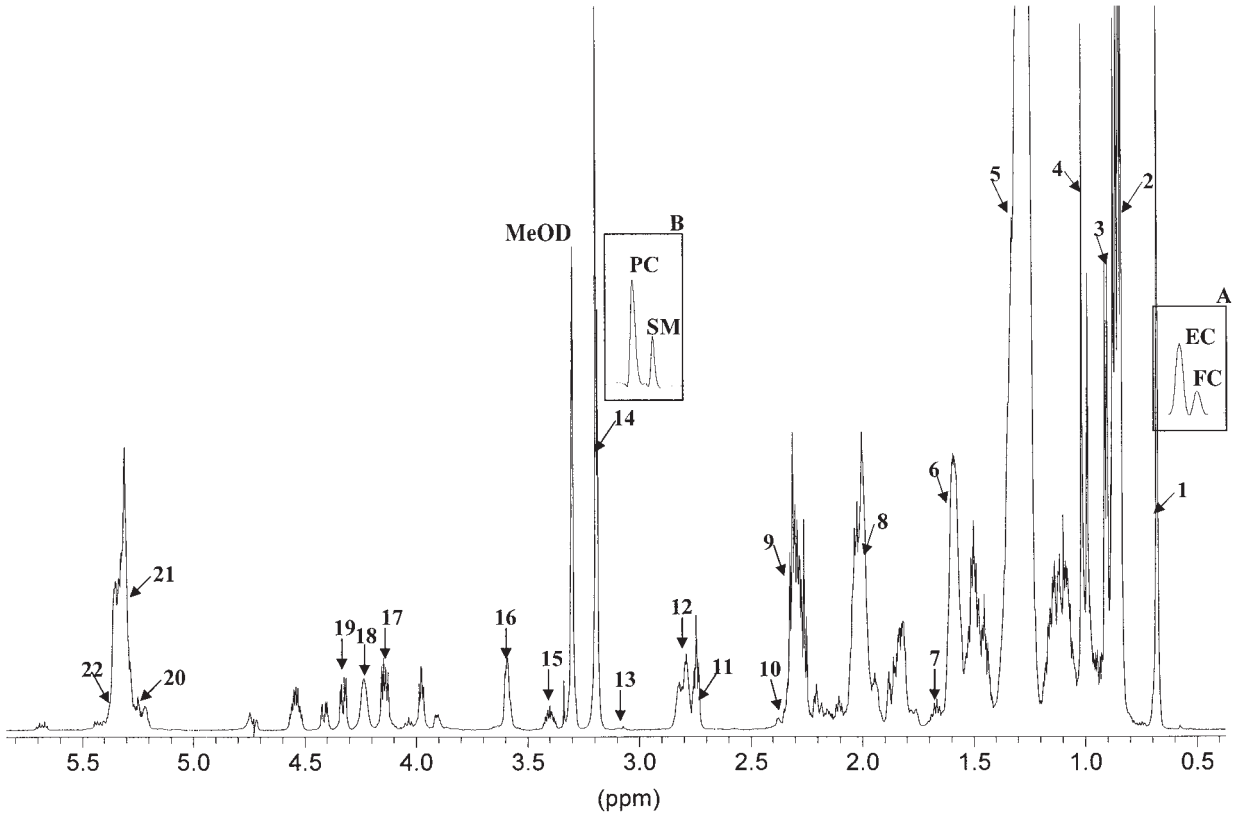

Figure 1. ^{1}H NMR spectrum of an HDL lipid extract.

Table 1. ^{1}H NMR resonances of HDL lipids.

Lipid	Assignments	Chemical shift (ppm)
Cholesterol	1. $C_{18}\underline{H}_3$	0.70
	3. $C_{26}\underline{H}_3$, $C_{27}\underline{H}_3$, $C_{21}\underline{H}_3$	0.87
	4. $C_{19}\underline{H}_3$	1.00
	15. $C_3\underline{H}$	3.40
	22. $C_6\underline{H}$	5.36
Triglycerides	17. $C_1\underline{H}^u$ and $C_3\underline{H}^u$ of glycerol	4.16
	19. $C_1\underline{H}^d$ and $C_3\underline{H}^d$ of glycerol	4.32
	20. $C_2\underline{H}$ of glycerol	5.22
Phospholipids	14. $N^+(C\underline{H}_3)_3$ of PC & SM	3.20
	16. $C\underline{H}_2$-$N^+(CH_3)_3$ of PC & SM	3.59
	18. –O-$C\underline{H}_2$-CH_2-$N^+(CH_3)_3$ of PC & SM	4.24
	13. $C\underline{H}_2$-NH_2 of PE	3.10
Fatty acids	2. ω-$C\underline{H}_3$	0.88
	5. $(C\underline{H}_2)$n	1.30
	6. CO-CH_2-$C\underline{H}_2$	1.59
	7. β-$C\underline{H}_2$ of ARA+EPA	1.67
	8. $C\underline{H}$-CH=	2.04
	9. -CO-$C\underline{H}_2$	2.30
	10. α and β $C\underline{H}_2$ of DHA	2.38
	11. -CH=CH-$C\underline{H}_2$-CH=CH- of linoleic	2.75
	12. $(CH{=}CH\text{-}C\underline{H}_2\text{-}CH{=}CH)_n$, n>1	2.80
	21. $C\underline{H}{=}C\underline{H}$	5.36

ARA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; SM, sphingomyelin.

Phospholipids

The two main phospholipids of plasma lipoproteins, phosphatidylcholine (PC) and sphingomyelin (SM), are identified and quantified by the peak of the N-trimethyl group and from this peak their ratio is calculated (Figure 1B).

Triglycerides

The quantification of triglycerides is made from the signal at 4.32 ppm.

Fatty acids

The methyl- and methylene-protons are shown in total at positions 0.88 and 1.30 ppm, while the α- and β-methylene protons for the carboxyl group are at 2.30 and 1.59 ppm, respectively. Unsaturated fatty acids are detected from the signal of the allylic protons at 2.04 ppm, the diallylic at 2.80 ppm, and the olefinic protons at 5.36 ppm. Of the specific polyunsaturated fatty acids in the NMR spectrum, we can distinguish linoleic acid at 2.75 ppm, docosahexaenoic acid (DHA) at 2.38 ppm, and the sum of arachidonic and eicosapentaenoic acids (ARA + EPA) at 1.65 ppm.

Statistical analysis

The statistical analysis was performed with the Statistica 6.0 program (StatSoft, Inc., Tulsa OK, USA). Parameters were compared using Student's t-test or the Mann-Whitney test, as necessary. The existence of markers for the existence of CHD among the study parameters, the relative risk (odds ratio) and the 95% confidence intervals (95%CI) were evaluated using multivariate logistic regression analysis. A p-value <0.05 was the criterion of statistical significance throughout.

Results

The main clinical and biochemical data from the two groups studied are shown in Table 2. The patients with extensive CHD were older than those with normal coronary arteries and had significantly lower levels of serum HDL-cholesterol and ApoAI, as well as significantly higher triglyceride levels. There were no differences between the groups in body mass index, incidence of arterial hypertension, smoking, or family history of early cardiovascular disease. In addition, total cholesterol, LDL-cholesterol, nonHDL-cholesterol, ApoB and Lp(a) did not differ significantly between the two groups.

Table 2. Clinical and biochemical data of the study population.

	Patients with triple vessel disease (n=50)	Patients with normal coronary arteries (n=41)
Age (years)	67.7 ± 9.9*	61.2 ± 9.6
Men/women	42/8	25/16
Risk factors for coronary heart disease:		
Hypertension (n,%)	31 (62.0%)	22 (53.7%)
Current smokers (n,%)	18 (36.0%)	14 (34.1%)
Family history of early cardiovascular disease (n,%)	3 (6.0%)	5 (12.1%)
Body mass index (kg/m^2)	26.9 ± 2.37	27.8 ± 2.24
Lipid parameters:		
Total cholesterol (mmol/L)	5.20 ± 1.20	5.53 ± 1.39
LDL-cholesterol (mmol/L)	3.33 ± 1.04	3.64 ± 1.12
HDL-cholesterol (mmol/L)	1.04 ± 0.22*	1.25 ± 0.31
nonHDL-cholesterol (mmol/L)	4.16 ± 1.05	4.28 ± 1.17
Triglycerides (mmol/L)	1.77 ± 0.65†	1.38 ± 0.51
Apolipoprotein AI (mg/dl)	103.32 ± 26. 41†	134.72 ± 24.28
Apolipoprotein B (mg/dl)	93.19 ± 27.31	99.43 ± 26.76
Apolipoprotein E (mg/dl)	32.39 ± 10.47	37.88 ± 13.53
Lipoprotein (a) (mg/dl) mean (range)	27.6 (2.5-102)	34.6 (9.5-167)

*p<0.01, †p<0.001 compared to patients with normal coronary arteries.

Table 3 shows a comparison of the composition of HDL particles in the two groups studied. The lipid components calculated from the NMR spectrum were cholesterol (total, free, esterified), triglycerides and phospholipids (total, phosphatidylcholine, sphingomyelin). In addition, the degree of unsaturation and the average chain length of fatty acids, total polyunsaturated fatty acids, as well as linoleic, docosahexaenoic acid (DHA), and the sum of arachidonic and eicosapentaenoic acid (ARA+EPA) were calculated. Patients with triple vessel disease showed a 17% lower percentage of HDL-cholesterol, mainly esterified, than did patients with normal coronary arteries, whereas the proportion of triglycerides in the HDL particles was 19% greater. Total phospholipids were about 20% lower in patients with triple vessel disease, due to a 19% reduction in phosphatidylcholine, while the decrease of sphingomyelin was similar to that seen for cholesterol content. The proportion of polyunsaturated fatty acids was significantly lower in patients with triple vessel disease than in patients with normal coronary arteries, as were linoleic and the sum of ARA+ EPA fatty acids.

Table 4 shows a comparison of the composition

Table 3. Lipid composition of HDL lipoproteins in the study population.

Parameter (mmol/L)	Patients with triple vessel disease (n=50)	Patients with normal coronary arteries (n=41)	p	%
Total cholesterol	1.04 ± 0.22	1.25 ± 0.31	<0.01	↓ 17
Free	0.25 ± 0.06	0.29 ± 0.08	<0.05	↓ 14
Esterified	0.79 ± 0.18	0.95 ± 0.24	<0.01	↓ 17
Triglycerides	0.31 ± 0.10	0.26 ± 0.09	<0.05	↑ 19
Total phospholipids	1.11 ± 0.27	1.39 ± 0.27	<0.05	↓ 20
Phosphatidylcholine	0.90 ± 0.23	1.11 ± 0.25	<0.05	↓ 19
Sphingomyelin	0.21 ± 0.04	0.25 ± 0.06	<0.01	↓ 16
Fatty acids:				
Degree of unsaturation	1.35 ± 0.13	1.36 ± 0.08	NS	↓ 1
Average chain length	18.59 ± 1.93	18.43 ± 1.40	NS	↑ 1
Polyunsaturated	2.96 ± 0.69	3.39 ± 0.69	<0.05	↓ 13
Linoleic acid	0.93 ± 0.21	1.03 ± 0.20	<0.05	↓ 19
Docosahexaenoic acid	0.17 ± 0.06	0.16 ± 0.05	NS	↑ 1
ARA+EPA	0.49 ± 0.15	0.58 ± 0.16	<0.05	↓ 16

ARA+EPA – arachidonic and eicosapentaenoic acid.

Table 4. Lipid composition of nonHDL lipoproteins in the study population.

Parameter (mmol/L)	Patients with triple vessel disease (n=50)	Patients with normal coronary arteries (n=41)	p	%
Total cholesterol	4.16 ± 1.05	4.28 ± 1.17	NS	↓ 3
Free	1.33 ± 0.33	1.34 ± 0.34	NS	↓ 1
Esterified	2.82 ± 0.74	2.94 ± 0.84	NS	↓ 4
Triglycerides	2.83 ± 1.22	2.21 ± 0.90	<0.05	↑ 28
Total phospholipids	1.73 ± 0.42	1.68 ± 0.42	NS	↑ 3
Phosphatidylcholine	1.17 ± 0.32	1.11 ± 0.29	NS	↑ 5
Sphingomyelin	0.56 ± 0.14	0.57 ± 0.15	NS	↓ 2
Fatty acids:				
Degree of unsaturation	1.24 ± 0.07	1.26 ± 0.07	NS	↓ 2
Average chain length	16.28 ± 0.59	16.47 ± 0.91	NS	↓ 1
Polyunsaturated	5.87 ± 1.39	5.93 ± 1.58	NS	↓ 1
Linoleic acid	2.47 ± 0.79	2.46 ± 0.64	NS	–
Docosahexaenoic acid	0.16 ± 0.08	0.19 ± 0.11	<0.05	↓16
ARA+EPA	0.80 ± 0.26	1.01 ± 0.35	<0.01	↓21

ARA+EPA – arachidonic and eicosapentaenoic acid.

of nonHDL particles, which did not exhibit many differences between the two groups studied. The main differences seen were a significantly higher proportion of triglycerides (28%) and a lower proportion of docosahexaenoic acid and the sum of ARA+EPA fatty acids in the patients with triple vessel disease compared to those with normal coronary arteries.

Table 5 shows the results of the multivariate analysis after correction for all risk factors. The main markers of the presence of extensive CHD were the esterified cholesterol of HDL lipoproteins and the percentage of the sum of ARA+EPA in nonHDL particles.

Discussion

Clinical studies have shown that specific alterations in the lipid composition of lipoprotein particles are associated with the presence and severity of CHD.[4-7,16-19] Established techniques for the analysis of lipoprotein composition are laborious and time-consuming, and therefore unsuitable for large clinical studies. In the present study, we used NMR spectroscopy for the rapid and overall investigation of the lipid composition of HDL and nonHDL lipoproteins of patients with triple vessel CHD compared to patients with normal coronary arteries.

The main differences between the two groups in the composition of HDL lipoproteins were the higher triglyceride content and the lower proportion of cholesterol esters, phosphatidylcholine, sphingomyelin and polyunsaturated fatty acids such as linoleic and the sum of ARA+EPA in patients with triple vessel disease compared to those with normal coronary arteries. Studies in reconstituted HDL particles have shown that increased triglyceride content in the particles' core has a negative effect on their stability, while stability is enhanced when the nucleus is enriched with cholesterol esters. In addition, core neutral lipids affect the surface charge and structure of the particle. Triglycerides reduce the stability of the α-helix of ApoAI and increase its tendency to detach from the particle and be removed from the plasma, whereas esterified cholesterol increases the helix's stability.[1,20]

The reduced proportion of phospholipids, sphingomyelin and phosphatidylcholine seen in the present study is in agreement with recent clinical studies showing that the proportion of phospholipids in the HDL lipoproteins of patients with CHD is lower than in those with normal coronary arteries, and is more strongly correlated with the severity of the disease than is HDL-cholesterol.[4-7] In addition, experimental studies have noted the important role of phospholipids in lipoprotein metabolism. Sphingomyelin affects the structure and the stability of discoidal and spherical HDL particles[21] and enrichment with sphingomyelin increases the cholesterol efflux capacity.[22,23] Furthermore, the ability of HDL particles to inhibit the adhesion of monocytes to the surface of endothelial cells by reducing the expression of the adhesion molecules E-selectin, ICAM-1, and VCAM-1 is due to the presence of phosphatidylcholine that has polyunsaturated fatty acids in its molecule.[24,25]

The reduced levels of polyunsaturated fatty acids seen in this study, in both the HDL and nonHDL fractions, are in agreement with the findings of large epidemiological studies that noted the beneficial role of polyunsaturated fatty acids in reducing the risk of coronary heart disease.[26-28]

Study limitations

This study had certain limitations. It included a rather small number of patients in each study group. In addition, the comparisons were between patients with normal coronary arteries and those with triple vessel CHD, while patients with disease of moderate extent were excluded. This choice was made in order to accentuate inter-group differences and was based on the hypothesis that variations in lipid composition probably depend not only on the existence but also on the extent of CHD. The investigation of patients with moderate disease will require future studies.

Table 5. Results of multivariate analysis.

Factor	Odds ratio	95%CI	p
HDL:			
Esterified cholesterol	0.009	0.001-0.138	0.001
NonHDL:			
ARA+EPA	0.730	0.614-0.867	0.0001

ARA+EPA – arachidonic and eicosapentaenoic acid.

Conclusions

Lipid analysis by NMR spectroscopy is a rapid technique that could contribute to a better understanding of the alterations in the composition of plasma lipoproteins as a result of pathological or genetic factors, as well as to the investigation of the efficacy of diet and medication. Measurement of plasma lipoprotein constituents could provide new atherogenic indexes that cannot easily be determined using standard biochemical methods. Apart from cholesterol, this technique offers a way of monitoring the effect of hypolipidaemic therapy. Collection of data from a larger number of patients could lead to the creation of a reliable database relating to CHD.

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