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HDL Sphingomyelin Determinations in Normal Individuals and Patients with Type IV-Hyperlipoproteinaemia

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Summary: In 138 men and 79 women sphingomyelin values in the supernatant of sera obtained by precipitation of apolipoprotein B-containing lipoproteins (= high density lipoprotein-sphingomyelin, HDL sphingomyelin) were determined using an enzymatic method. In men, lower HDL sphingomyelin values were obtained (0.22 ± 0.06 mmol/l) than for women (0.24 ± 0.05 mmol/l) ($p < 0.05$). In both sexes HDL sphingomyelin values were independent of age, with the exception that in men ≥ 55 years, HDL sphingomyelin concentrations were lower.

The correlation coefficients calculated for HDL sphingomyelin and hypertriglyceridaemia and relative body weight were lower than the correlation coefficients calculated for the corresponding HDL cholesterol values and both risk factors.

In women with hypertriglyceridaemia (≥ 2.28 mmol/l) HDL sphingomyelin values were lower than those for normotriglyceridaemic women within the same age group, while in male hypertriglyceridaemic patients within the same age group there were no differences in HDL sphingomyelin values in comparison to normotriglyceridaemic male subjects. In both sexes the differences measured between hypertriglyceridaemic and normotriglyceridaemic subjects for HDL sphingomyelin were lower than for the corresponding HDL cholesterol values.

The results obtained show that HDL sphingomyelin values are not simply a reflection of HDL cholesterol values.

HDL-Sphingomyelinwerte von normalen Individuen und von Patienten mit Hyperlipoproteinämie Typ IV

Zusammenfassung: Bei 138 Männern und 79 Frauen wurde im Überstand nach Präzipitation Apolipoprotein B-haltiger Lipoproteine mit Phosphorwolframsäure/MgCl₂ der Sphingomyelingehalt mit einer enzymatischen Methode gemessen. Männer wiesen im Vergleich zu Frauen gering niedrigere HDL-Sphingomyelingehalte auf (Männer: $0,22 \pm 0,06$ mmol/l, Frauen: $0,24 \pm 0,05$ mmol/l, $p < 0,05$). Bei beiden Geschlechtern bestand keine Altersabhängigkeit mit Ausnahme gering erniedrigter HDL-Sphingomyelinwerte bei Männern ≥ 55 Jahren. Die Korrelationsanalyse ergab eine deutlich geringere Beziehung von HDL-Sphingomyelinwerten zu den Risikofaktoren Hypertriglyceridämie und relatives Körpergewicht als von den gleichzeitig bestimmten HDL-Cholesterinwerten zu den genannten Risikofaktoren.

Bei Patienten mit einer Hypertriglyceridämie $\geq 2,28$ mmol/l wurden bei Frauen im Vergleich zu gleichaltrigen Normotriglyceridämikerinnen erniedrigte HDL-Sphingomyelinwerte gemessen, während bei gleichaltrigen Männern keine Unterschiede zwischen Hypertriglyceridämikern und Normotriglyceridämikern beobachtet wurden. Die gemessenen HDL-Sphingomyelinunterschiede zwischen Hypertriglyceridämikern und Normotriglyceridämikern waren geringer als die Unterschiede der entsprechenden HDL-Cholesterinwerte.

Die Ergebnisse deuten darauf hin, daß HDL-Sphingomyelinwerte nicht einfach HDL-Cholesterinwerte reflektieren.

Introduction

In addition to the determination of cholesterol and triglycerides as a basic part of the diagnostic program for disorders of the lipid metabolism, analysis of HDL cholesterol as an indicator of the risk of coronary heart disease has recently gained increased importance (1, 2). Although the phospholipid content of HDL is greater than its cholesterol content, the determination of HDL phospholipids as well as determination of the individual phospholipid fractions of HDL (phosphatidyl choline, sphingomyelin, lysophosphatidyl choline) has until now not become routine, due to the elaborate nature of the analysis. Thus, in particular, the possible relationship of HDL phospholipid fractions to the risk factors of coronary heart diseases has remained unclear.

It is now possible through the use of phospholipases with specific activity, which have recently become commercially available, as well as recently developed sensitive assays for choline, to conduct relatively rapid and simple analysis of various phospholipid fractions. Recently we have developed an accurate and precise method for determination of HDL sphingomyelin (3). The present study reports initial findings regarding the distribution of HDL sphingomyelin in normal individuals, the correlation of HDL sphingomyelin to HDL cholesterol as well as the relationship of HDL sphingomyelin to various risk factors of coronary heart disease. Furthermore, the concentrations of HDL sphingomyelin were also studied in patients with type IV-hyperlipoproteinaemia and these results were compared with the normal population.

Materials and Methods

138 men and 79 women were selected from a company in Westfalia ("Prospective epidemiologic study of company employees in Westfalia"). Blood was collected in a specially outfitted bus after 12 h of fasting and was allowed to clot for 30 minutes at room temperature. After removal of cells by centrifugation at 3000 min⁻¹, the serum was stored at +4 °C and transported to our laboratory within a maximum of 3 days.

Blood samples of patients (39 men, 26 women) with type IV hyperlipoproteinaemia (triglycerides ≥ 2.28 mmol/l, cholesterol < 6.7 mmol/l) were prepared in a similar manner. Experiments were performed within 25 hours after receiving the samples. Serum levels of cholesterol, triglycerides, glucose and uric acid were determined with the SMAC Autoanalyzer (Technicon GmbH., Bad Vilbel, FRG) as described elsewhere (4). The HDL fractions were analysed in the supernatant following precipitation of apolipoprotein B-containing lipoproteins using phosphotungstic acid/MgCl₂ (2). HDL cholesterol was determined enzymatically using the cholesterol oxidase/4-aminoantipyrine method (Boehringer Mannheim). The determination of HDL sphingomyelin has been described elsewhere in detail (3). LDL cholesterol was calculated from total cholesterol, HDL cholesterol and triglycerides according to the formula of Friedewald (5).

Results

Distribution of HDL sphingomyelin values in normal individuals

Table 1 shows the values for HDL sphingomyelin determined for the group of company employees. The HDL phospholipid fraction investigated exhibited nearly normal distribution in both sexes. In women slightly higher HDL sphingomyelin values could be obtained in comparison to men ($p < 0.01$). Defining the range of normal values as the average value $\pm 2s$, normal values of 0.10 to 0.34 mmol/l (men) and 0.14 to 0.34 mmol/l (women) were calculated for HDL sphingomyelin. For comparison the distribution of HDL cholesterol is also shown in table 1. In both sexes HDL cholesterol values were closely correlated with the corresponding HDL sphingomyelin values (men: $r = 0.54$ $p < 0.001$, women: $r = 0.44$ $p < 0.001$).

Tab. 1. Distribution of HDL sphingomyelin and of the corresponding HDL cholesterol values (mmol/l) (15–64 years) (prospective epidemiological study in company employees in Westfalia)

	HDL sphingomyelin		HDL cholesterol	
	men n = 138	women ¹⁾ n = 79	men n = 138	women ²⁾ n = 79
Mean	0.22	0.24	1.15	1.42
Standard deviation	0.06	0.05	0.33	0.31
Median	0.22	0.23	1.10	1.42
Minimum	0.08	0.11	0.65	0.72
Maximum	0.44	0.38	2.79	2.14
5% percentile	0.11	0.16	0.74	0.96
25% percentile	0.18	0.21	0.88	1.14
75% percentile	0.25	0.27	1.30	1.55
95% percentile	0.30	0.32	1.81	1.94

¹⁾ $p < 0.01$ in comparison to men

²⁾ $p < 0.001$ in comparison to men

As table 2 shows there was no age dependency of HDL sphingomyelin in either sex with the exception that in men ≥ 55 years HDL sphingomyelin values were approximately 0.04 mmol/l lower than in the total group studied ($p < 0.05$).

Relationship between HDL sphingomyelin and risk factors for atherosclerosis

Analysis of the correlation between HDL sphingomyelin and various risk factors for coronary heart disease yielded the results compiled in table 3. A significantly negative correlation was determined between HDL sphingomyelin and triglycerides ($r = -0.19$, $p < 0.05$). In women, though not in men, HDL sphingomyelin showed a significantly

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In order to integrate the large quantities of information available regarding the chemistry and immunology of insulin as well as the clinical aspects of diabetes and its control, it is necessary to establish interdisciplinary lines of communication between specialists in these various fields. It was the intention of a Symposium held in Konstanz, FRG, from September 28 to October 1, 1980, to bring together clinicians, immunologists and chemists active in insulin research, to provide an open forum to exchange ideas and experience, to establish contacts and to intensify cooperation between these groups.

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Tab. 2. HDL sphingomyelin values (mmol/l ($\bar{x} \pm S. D.$)) in different age classes (prospective epidemiological study in company employees in Westfalia)

	HDL sphingomyelin men	women
15-24 years	0.21 $\pm$ 0.05 n = 12	0.24 $\pm$ 0.07 n = 14
25-34 years	0.21 $\pm$ 0.05 n = 32	0.24 $\pm$ 0.04 n = 20
35-44 years	0.23 $\pm$ 0.06 n = 35	0.24 $\pm$ 0.06 n = 18
45-54 years	0.23 $\pm$ 0.05 n = 35	0.24 $\pm$ 0.05 n = 18
≥ 55 years (- 64 years)	0.18 $\pm$ 0.06 ¹⁾ n = 15	0.25 $\pm$ 0.05 n = 9

¹⁾ p < 0.05 in comparison to the total group

negative correlation with the LDL cholesterol level ($r = -0.27$, $p < 0.01$). For comparison the coefficients of correlation between the corresponding values of HDL cholesterol and various risk factors were also calculated. In men and women the coefficients of correlation for HDL cholesterol and triglycerides and for HDL cholesterol and body weight respectively were higher than the corresponding correlation coefficients for the HDL sphingomyelin and the risk factors.

HDL sphingomyelin values in patients with type IV hyperlipoproteinaemia

In hypertriglyceridaemic women HDL sphingomyelin values were significantly lower in comparison with normal individuals of similar age while in male patients with type IV hyperlipoproteinaemia no differences in comparison to normals of similar age could be observed (table 4). However, in male and

Tab. 3. Coefficients of correlation between HDL sphingomyelin and risk factors and the corresponding HDL cholesterol values and risk factors (prospective epidemiological study in company employees in Westfalia)

	HDL sphingomyelin		HDL cholesterol	
	men (n = 138)	women (n = 79)	men (n = 138)	women (n = 80)
Age	-0.08	0.02	0.09	0.35)
Total cholesterol	0.11	-0.11	0.14	0.38 ³⁾
LDL cholesterol	0.01	-0.27 ¹⁾	-0.02	0.13
Triglycerides	-0.19 ¹⁾	0.04	-0.43 ³⁾	-0.16
Glucose	-0.11	-0.11	-0.03	-0.05
Uric acid	-0.06	0.003	-0.11	0.05
Relative body weight	-0.06	0.09	-0.19 ¹⁾	-0.12
Systolic blood pressure	0.11	0.06	0.25 ²⁾	-0.06
Diastolic blood pressure	0.13	0.11	0.16	0.02
Cigarette smoking	0.06 (n = 66)	0.06 (n = 23)	0.04 (n = 66)	-0.39 (n = 23)

¹⁾ p < 0.05 ²⁾ p < 0.01 ³⁾ p < 0.001Tab. 4. Mean $\pm$ standard deviation of HDL sphingomyelin, HDL cholesterol, total cholesterol and triglyceride values in patients with type IV hypertriglyceridaemia (triglycerides ≥ 2.28 mmol/l)

	Age (years)	HDL sphingomyelin	HDL cholesterol	Total cholesterol	Triglycerides
Men					
Normals (n = 39)	46.1 $\pm$ 12.4	0.20 $\pm$ 0.06	1.18 $\pm$ 0.44	5.01 $\pm$ 0.84	1.35 $\pm$ 0.49
Type IV (n = 39)	46.7 $\pm$ 15.7	0.18 $\pm$ 0.06	0.80 $\pm$ 0.26 ²⁾	4.87 $\pm$ 1.16	3.60 $\pm$ 0.74 ²⁾
Women					
Normals (n = 26)	45.7 $\pm$ 13.6	0.24 $\pm$ 0.06	1.47 $\pm$ 0.33	5.45 $\pm$ 0.75	1.08 $\pm$ 0.47
Type IV (n = 26)	48.5 $\pm$ 17.3	0.17 $\pm$ 0.08 ²⁾	0.72 $\pm$ 0.36 ²⁾	4.71 $\pm$ 0.98 ¹⁾	4.04 $\pm$ 1.23 ²⁾

¹⁾ p < 0.01 ²⁾ p < 0.001

female patients with type IV hyperlipoproteinaemia the corresponding HDL cholesterol values were significantly lower than the corresponding values in normal subjects. In both sexes the difference of HDL sphingomyelin values between hypertriglyceridaemic patients and normal individuals were lower than the difference of the corresponding HDL cholesterol concentrations.

Discussion

Epidemiological (6–10) and clinical studies (11, 12) have shown that HDL cholesterol is an indicator of the risk of coronary heart disease. However, HDL do not represent a single substance, but consist of a heterogeneous mixture of particles of varying composition and physicochemical properties (13). Thus, the HDL cholesterol values cannot be equated with the HDL mass and consequently, the findings obtained with HDL cholesterol cannot simply be applied to other HDL components.

In contrast to HDL cholesterol, little is known about the significance of other HDL constituents with regard to their possible relationship to the risk of coronary heart disease. However, attention has recently focused on additional quantification of HDL apolipoproteins. It has been demonstrated that the analysis of HDL apolipoproteins and HDL cholesterol gives different results with regard to coronary risk (14).

Analysis of HDL phospholipids has been conducted to date only to a limited degree on account of the elaborate analysis involved (15, 16) and has been limited for the most part to HDL total choline containing phospholipids (17). However, the analysis of total choline containing phospholipids in the supernatant obtained after precipitation of apolipoprotein B-containing lipoproteins does not take account of the fact that a main part of the lysophosphatidyl choline moiety recovered in the supernatant is bound to albumin. In contrast to the analysis of total choline containing phospholipids the determination of the individual phospholipids, e. g. phosphatidyl choline or sphingomyelin, in the apolipoprotein B-free supernatant avoids this problem.

Through the introduction of commercially available phospholipases and the use of sensitive assays for choline it has now been possible to conduct relatively rapid and simple analysis of the individual phospholipids. Recently we have developed an accurate and precise method for determination of HDL sphingomyelin using the supernatant after precipitation of apolipoprotein B-containing lipoproteins with phosphotungstate/MgCl₂ (3). The principle of this method

is based on the specific cleavage of sphingomyelin by purified sphingomyelinase from *B. cereus*. The phosphoryl choline released is hydrolysed to phosphate and choline in the presence of alkaline phosphatase. Choline is analysed spectrophotometrically using choline kinase. As described elsewhere in detail (3), in comparison to the conventional chemical method, the enzymatic method provided consistent HDL sphingomyelin values. Furthermore, consistent values were obtained with the enzymatic method for the HDL fraction isolated after ultracentrifugation (1.063–1.21 kg/l) and after precipitation of the apolipoprotein B-containing lipoproteins. A value of 5.7% was determined as the variation coefficient for the precision in series and of 5.3% for the precision from day to day (3).

As our findings show HDL sphingomyelin and HDL cholesterol values were closely correlated. On the other hand, HDL sphingomyelin values are not simply a reflection of the corresponding HDL cholesterol values. In contrast to the clearly higher HDL cholesterol values for women, HDL sphingomyelin values in women were only slightly higher than in men. Furthermore, for the HDL sphingomyelin values examined and the risk factors of triglycerides and relative body weight, coefficients of correlation were calculated which were obviously lower than for the corresponding HDL cholesterol values and both risk factors. It is possible that the relationship between HDL sphingomyelin and triglycerides or between HDL sphingomyelin and relative body weight may be lower than the known negative correlation between HDL cholesterol and both risk factors (18). These findings obtained from a relatively small population should be expanded in the framework of a larger study. In patients with type IV hyperlipoproteinaemia, low HDL cholesterol concentrations in blood serum are often observed (19). The pathobiochemical relationship between hypertriglyceridaemia and low levels of HDL cholesterol is discussed elsewhere in detail (19). According to our findings in female patients with type IV hyperlipoproteinaemia, low HDL cholesterol concentrations were observed in parallel with low concentrations of HDL sphingomyelin, whereas in male patients, low HDL cholesterol concentrations were also observed, but HDL sphingomyelin values did not differ significantly from those measured in normal individuals. The difference between men and women is obviously linked to our observation that normal men have lower HDL sphingomyelin values compared to normal women, while in male and female hypertriglyceridaemic patients nearly the same HDL sphingomyelin values were observed. On the other hand, in both sexes the difference between hypertriglyceridaemic

patients and normal individuals was obviously lower for HDL sphingomyelin values (men: -10.0%, women: -29.2%, on average) than for HDL cholesterol values (men: -32.2%, women: -51.0%, on average). Evidently, particularly in male hypertriglyceridaemic patients, the sphingomyelin moiety of HDL becomes relatively increased in comparison to the cholesterol moiety of HDL. The metabolic origin of this change in the HDL lipid composition, possibly due to impaired lipolysis of the triglyceride-rich lipoproteins, deserves further investigation.

In view of increased interest in the determination of HDL sphingomyelin, clinical laboratories need suitable material for quality control. As shown from findings of Warnick et al. (20) and from own results (2) conventional lyophilized sera are not suitable for the precipitation of apolipoprotein B-containing lipoproteins. The use of sera pools stored at $<-15^{\circ}\text{C}$ may be an alternative. In our experience, HDL sphingomyelin values are stable for more than 12 weeks in frozen sera.

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