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Measurement of Fractional Esterification Rate of Cholesterol in Plasma Depleted of Apoprotein B Containing Lipoprotein: Methods and Normal Values

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Summary

The distribution of differently sized HDL particles in the plasma can be assessed by measurement of the fractional rate of cholesterol esterification (FER_{HDL}). We have characterized the isotopic assay and compared it to the enzymatic measurement of the decrease in HDL free cholesterol (mass assay). The normal values of FER_{HDL} were established in 116 apparently healthy individuals. The isotopic assay is particularly sensitive to changes in the incubation temperature above 37 °C. The reproducibility of the assay in aliquots of plasma stored at −20 °C and −70 °C for 3 months and even up to 2 years was high. Intraindividual variability of FER_{HDL} is low. In the subjects in whom FER_{HDL} was measured over a 3-month and 2–5 years' period, FER_{HDL} showed a low variability (97.5±2.6 % and 101±6.0 % respectively in a paired t-test). Comparison of the isotopic assay and the mass assay revealed that the isotopic assay was much more reproducible. Normal values of FER_{HDL} and the HDL subspecies distribution (using gradient gel electrophoresis) were established in 63 men and 56 women. The average values of FER_{HDL} were significantly higher in men (16.8±4.5 %/h) than in women (10.6±3.6 %/h) and correlated well with the distribution of the HDL subspecies. FER_{HDL} radioassay as a highly reproducible method for the assessment of HDL subspecies distribution which may be suitable for both retrospective and prospective studies of diseases of atherogenous origin.

Key words

HDL subclasses – Cholesterol esterification rate – Lecithin: cholesterol acyltransferase – Radioassay – Electrophoresis – Gradient gel

Introduction

We have previously demonstrated that the rate of cholesterol esterification in apoB lipoprotein-depleted plasma is directly related to the composition of the HDL subspecies (Dobiášová *et al.* 1991, 1992, Dobiášová and Frohlich 1994). The rate is increased in individuals with a high relative concentration of small $HDL_{3b,c}$ particles and it is decreased by the large HDL_{2b} particles.

Thus, the estimation of FER_{HDL} reflects the ratio between the small and large HDL particles in the plasma. We have suggested that this measurement can serve as a functional test of the quality of plasma high density lipoproteins. As the antiatherogenic potential of the small and large HDL differs (Dobiášová *et al.* 1992, Dobiášová and Frohlich 1994, Hamsten *et al.* 1990, Drexel *et al.* 1992), with the small particles having less and the large ones a more protective potential, we believe that FER_{HDL} may indicate the subject's susceptibility to atherosclerosis. In our previous work we demonstrated that high FER_{HDL} reflected increased risk of atherosclerosis and that it may be a better predictive parameter than the measurement of some other plasma lipoproteins (Dobiášová and Frohlich 1994).

However, the introduction of this test to clinical practice requires its further characterization and better understanding of factors that influence the accuracy and reproducibility of the measurement. In this paper we further describe the factors that influence the reproducibility of the current isotopic method for

measurement of FER_{HDL}, its short- and longer-term variability as well as the comparison of the isotopic method with enzymatic measurements of the decrease in the mass of HDL-free cholesterol. Finally, we discuss the normal values of FER_{HDL} in men and women.

Material and Methods

[7(n)-^{3}H]-cholesterol (specific activity 5Ci/mmol) and [1a, 2a(n)-^{3}H] cholesteryl linoleate (specific activity 40 mCi/mmol) were purchased from Amersham Corp. England. Reagent kits for determination of total cholesterol and triglycerides were purchased from Boehringer (Mannheim, FRG) and the kit for assay of unesterified cholesterol was purchased from Wako Chemicals (Richmond, VA, USA). All chemicals were of analytical grade.

Subjects

Sixty-three apparently healthy men (age range 23–85 years) and 53 women (age range 22–61 years) from the Institute of Physiology of the Czech Academy of Sciences in Prague and the Atherosclerosis Specialty Laboratory, UBC in Vancouver whose plasma lipids were within desirable limits volunteered for the determination of normal values. To study the reproducibility of the method, we used sequential blood samples from patients of Vancouver St. Paul's Hospital Lipid Clinic (n=56) and from volunteers from the Institute of Physiology in Prague (n=32). The study was approved by the Institutional Review Boards of UBC and St. Paul's Hospital.

Lipid analysis

Blood was collected into EDTA containing tubes after a 12 h overnight fast, placed on ice and centrifuged within 2 h at 1750 x *g* for 10 min to separate plasma. Plasma was analyzed within 48 h if kept in a refrigerator, or within 3 months if stored at −20 °C or within 5 years when stored at −70 °C. VLDL/LDL depleted plasma (HDL-plasma) was prepared by precipitation of apolipoprotein B containing lipoproteins with phosphotungstate-$MgCl_2$ (Burstein *et al.* 1970). Fifty microlitres of the phosphotungstate solution (4 g phosphotungstic acid, 16 ml of 1 M NaOH in 100 ml deionized water) was added to 500 μl plasma (in 1 ml plastic centrifugation vials), stirred and followed by addition of 12.5 μl $MgCl_2$ solution (2 M). After the solution was mixed and allowed to stand for 20 min at 4 °C, the suspension was centrifuged at 12 000 rpm at 4 °C. This precipitation method does not interfere with the enzymatic analysis of HDL cholesterol (total or free) or the estimation of the esterification rate.

Precise SMI digital adjust micro/petters (SMI Liquid Handling Products, American Dade, Miami, FL, U.S.A.) were used to decrease the plasma sample volume to 100 μl.

Determination of esterification rate (FER_{HDL})

The improvement of the isotopic method that was used in our studies (Dobiášová *et al.* 1991, 1992, Dobiášová and Frohlich 1994) is described. The essential step of the method consists of transferring a trace amount of ^{3}H- cholesterol from a paper disc to lipoproteins in a sample of HDL plasma. Spontaneous transfer of the label proceeds at low temperature and labelling homogeneity is attained after 18 hours.

The paper discs of approximately 5 mm in diameter are cut out from Whatman 1 filter paper using a letter punch and transferred with a pair of tweezers on thin hypodermic needles fixed to a stand. Three μCi of [7(n)-^{3}H]-cholesterol (specific activity 5 Ci/mmol) in 3 μl of ethanol is evenly spread onto each disc. After evaporation of the solvent, the discs are placed separately in individual stoppered 5 ml glass vials and kept in a refrigerator; they can be used for at least 3 months.

Labelling of the lipoprotein sample at low temperature and the processing of the labelled sample proceeds as follows: to 75 μl of Tris buffer (Tris 10 mmol/l, NaCl 150 mmol/l, EDTA 0.01 %, NaN_3 0.03 %) are added 30 μl of precooled (4 °C) HDL plasma and the ^{3}H-cholesterol labelled paper disc is immersed into each diluted solution, the tubes are stoppered and incubated on ice overnight. The discs are then discarded and the test tubes with labelled HDL plasma are placed in a shaking water bath and incubated at 37 °C for 30 min. The tubes are then placed on ice, and the contents mixed immediately with 1.5 ml of 98 % ethanol. The mixture is stirred and left standing for 2 h at room temperature. Samples are then centrifuged at 2000 rpm for 10 min and the supernatants are dried out by evaporation in a stream of air at 4 °C. The dry residue is dissolved in 100 μl of chloroform containing standards of unesterified and esterified cholesterol as carriers (15 mg of cholesterol and 10 mg of cholesteryl esters in 1 ml of chloroform) to make visualization of labelled components in the diluted sample possible. Detection of the lipids by iodine vapours follows their separation on TLC plates (Merck, FRG) using petroleum ether – diethylether acetic acid (105:18:1.5 v/v/v) as solvents. The contours of the resulting spots are outlined with pencil and any remaining traces of iodine are allowed to sublimate. The spots are then cut out, put into vials with a scintillation cocktail (PPO, POPOP in toluene), shaken and left standing for at least 3 h. Radioactivity of the samples is determined in a liquid scintillation counter. FER_{HDL} is calculated as the difference between the percentage of labelled esterified cholesterol before and after incubation. In human HDL plasma, the percentage of cholesterol esterified before the

incubation was always below 0.3 %. FER_{HDL} is calculated as follows:

$$FER_{HDL} = (\%\ \text{cholesteryl ester}_{(30\ min)} \times 2) - 0.32$$

Effect of temperature

Fig. 1 shows the effect on the reaction rate of increasing temperature (between 35 °C and 39 °C) in four different plasma samples. The rate of esterification increases by 38–59 % between 37 °C and 39 °C but only by about 20–22 % between 35 °C and 37 °C.

Time course of the reaction

The esterification of cholesterol in HDL plasma is linear for up to 60 min in all samples (Fig. 2). It is evident from Fig. 3 that 80–90 % of the free cholesterol in the sample is esterified within 24 h.

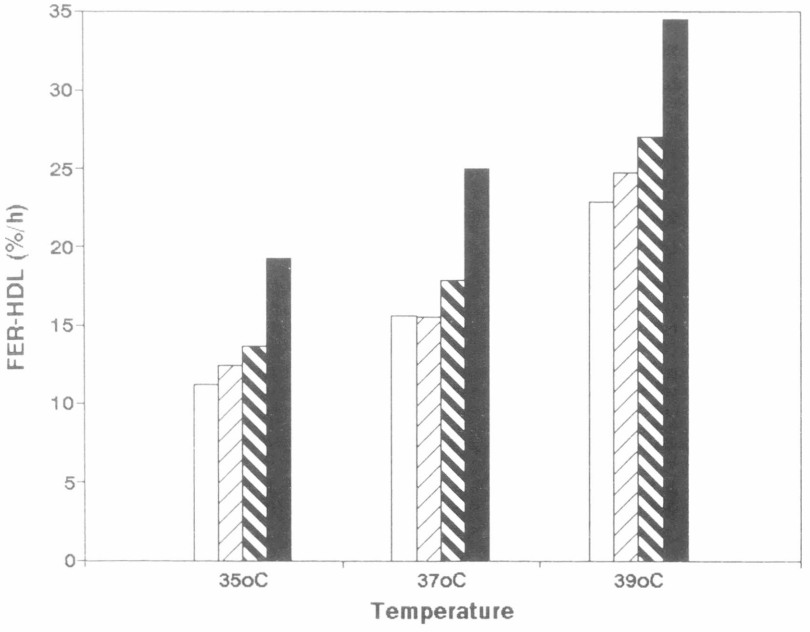

Fig. 1
Effect of temperature on FER_{HDL} assay in four different samples of HDL plasma.

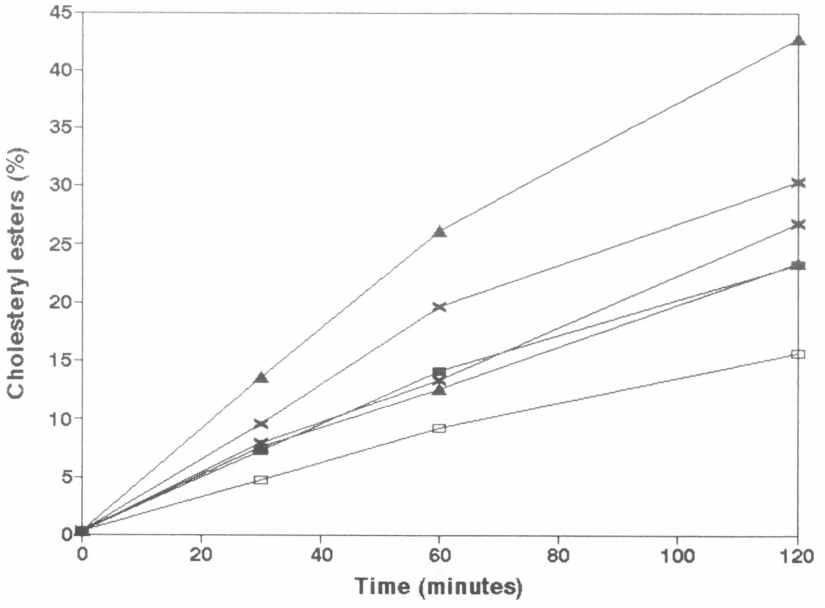

Fig. 2
Time course of cholesterol esterification in six different HDL plasma samples over 120 min.

Measurement of the decrease in the mass of free cholesterol

We used Wako free cholesterol kit (Wako Chemicals USA, Inc., Richmond, VA). Free cholesterol is determined after reaction with cholesterol oxidase and the generated hydrogen peroxide is determined colorimetrically after reaction with 4-aminoantipyrine (Allain *et al.* 1974).

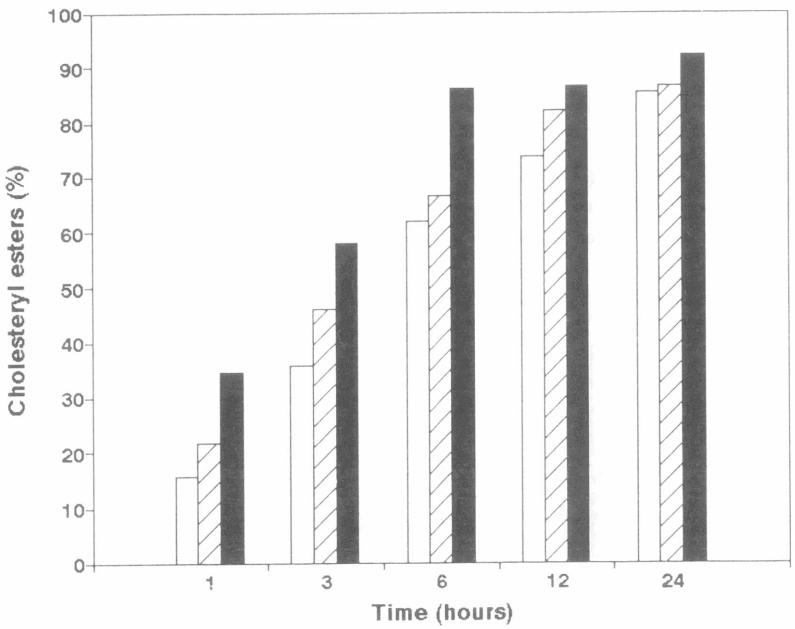

Fig. 3
Time course of cholesterol esterification in three different HDL plasma samples over 24 h.

The precision of the assay varies from coefficient of variation of 0.65 % at cholesterol concentration of 2.6 mmol/l to 1.4 % at 1.2 mmol/l. At lower concentrations of free cholesterol, as used in our FER_{HDL} assay (typically between 0.2 and 0.3 mmol/l), the coefficient of variation of the assay was always less than 5 %.

Gradient gel electrophoresis of HDL

Plasma lipoproteins were isolated by ultracentrifugation at final plasma density of 1.21 g/ml in 65Ti rotor in a Beckman L7 ultracentrifuge at 40 000 rpm for 24 h at 15 °C. The non-dialysed lipoprotein fraction was mixed with the sampling buffer containing 40 % sucrose, and 5 μl of the sample were applied to each lane of a 4–30 % polyacrylamide gradient gel (Pharmacia, Sweden). The samples were electrophorezed in Tris/borate/EDTA buffer, pH 8.3, as described by Nichols *et al.* (1986) for 21 h at 125 V and stained for proteins by Commassie Brilliant Blue. A mixture of globular proteins (HMW Calibration Kit, Pharmacia) was run concurrently as particle size markers. Three of the HDL subclasses are distinctly resolved in the gel: HDL_{2b} (9.5-12.9 nm), HDL_{3a} (possibly with HDL_{2a}, 8.2–9.5 nm) and HDL_{3b} (with HDL_{3c}, 7.0–8.2 nm). These particle sizes are similar to those previously reported (Williams *et al.* 1990). The relative content of HDL subpopulations is estimated by determining the areas under the peaks of the laser densitometer scan of the gel (LKB Ultroscan XL-LKB, Sweden).

Statistical analyses

Student's t-test (paired and unpaired) was used to establish significant differences between the mean values of each group (or pairs). The correlation coefficient r was calculated by linear regression analysis.

Results

1. *Reproducibility of the method*

a) The interassay variability was assessed in three HDL-plasma samples with FER_{HDL} values of 8.5, 18.7 and 26.8 %/h. The measurement of FER_{HDL} was repeated 12 times over a period of one month with three fresh samples analyze initially and frozen aliquots used for the repeated estimations. The coefficient of variation for the 12 measurements ranged from 4.5 to 7.3 % .

b) The FER_{HDL} assay was repeated in 23 samples that were kept at −20 °C for two months. The average values of FER_{HDL} in the initial fresh and in the stored frozen plasma samples were very close: 21.39±6.86 before and 21.79±6.41 %/h after the storage. The difference between the two estimations (tested by the paired t-test) was not significant at the 10 % level. The correlation between the first and second measurements was high (r=0.923). The concentrations of free HDL cholesterol were similar in both estimations.

c) Eighteen plasma samples were stored at −70 °C for two years. Average values of FER_{HDL} were 23.3±4.9 %/h and 21.6±5.9 %/h in the fresh and stored samples, respectively (r=0.895). Again there was no significant difference at the 10 % level between pairs. However, the concentration of free cholesterol in HDL plasma decreased with storage from 230±50 μmol/l to 180±40 μmol/l over the 2 years of storage ($p<0.05$).

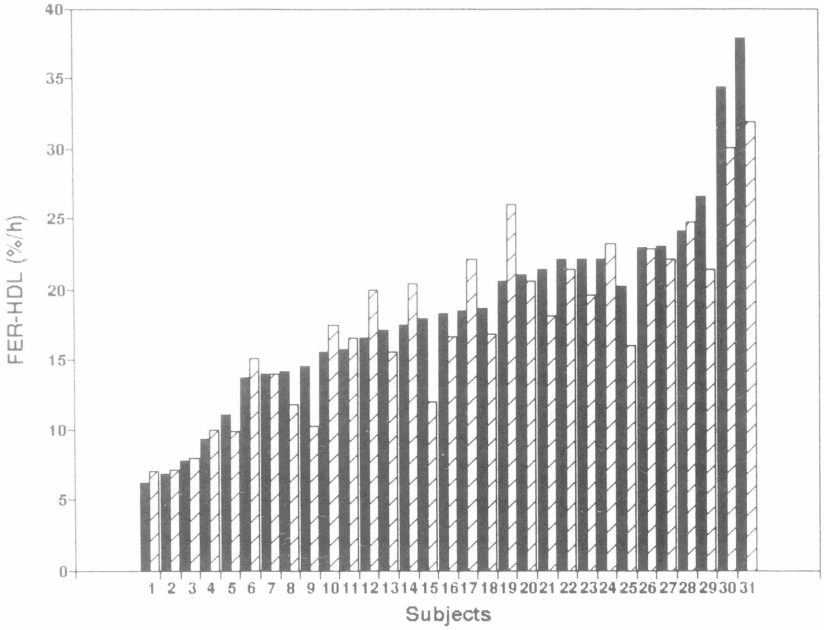

Fig. 4
Intraindividual variation in FER_{HDL} within 3 months. Volunteers (1–31) had FER_{HDL} measured at baseline (full columns) and at 3 months (hatched columns).

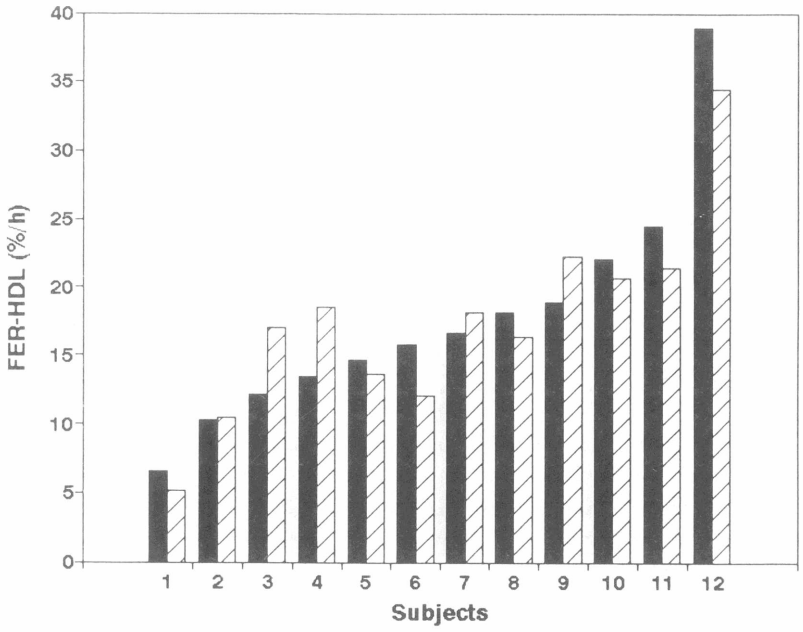

Fig. 5
Long-term intraindividual variation in FER_{HDL}. Volunteers (1–12) had FER_{HDL} measured at baseline (full columns) and within 2-5 years (hatched columns).

2. *Variability of FER_{HDL} in individual subjects*

a) *Short-term (3 months)* FER_{HDL} was measured twice 3 months apart in 31 subjects. As is shown in Fig. 4, there was no significant difference between the first and second estimation (FER_{HDL} 1/FER_{HDL} 2 = 0.975±0.026 in a paired t-test). The values of FER_{HDL} as well as of plasma cholesterol, HDL total and free cholesterol and the relative composition of HDL subspecies showed little change while the triglyceride concentration, as expected, were more variable.

b) Long-term (within 5 years). Fig. 5 shows the changes in FER_{HDL} in 12 subjects without major risk factors for coronary artery disease over a period of 2–5 years. As shown in Fig. 5, there were no significant differences (at the 10 % level) between the first and second measurements (FER_{HDL} 1/FER_{HDL} 2 = 1.01±0.06 in the paired t-test).

3. Comparison of the isotopic and mass method for estimation of FER_{HDL}

FER_{HDL} was also estimated by the enzymatic determination of free cholesterol in HDL plasma before and after incubation at 37 °C in 23 patients of the Lipid Clinic (see Methods). The values were always lower than those estimated using the isotopic assay although the correlation between the methods was highly significant ($r=0.84$, $p<0.001$). Table 1 illustrates the differences between the course of cholesterol esterification determined by the two methods. It is

evident that the percentage of free cholesterol which is esterified is lower at all the time intervals (in mass assay) although the degree of this difference varies among the individual samples.

Table 1
Time course of cholesterol esterification by radioassay and by mass assay

Time (h)	1	3	6	12	24
Radioassay					
Sample 1	34.6	57.8	86.0	86.8	92.3
Sample 2	21.9	45.9	66.6	82.1	86.7
Sample 3	15.7	35.9	61.8	73.7	85.6
Mass assay					
Sample 1	27.3	56.5	67.3	79.8	80.1
Sample 2	15.9	37.4	59.2	77.8	82.1
Sample 3	5.6	25.0	44.8	64.0	76.4

Radioassay, increase of cholesterol ester in HDL-plasma (%); Mass assay, decrease of free cholesterol in HDL-plasma (%)

Table 2
Summary of subject data: Women

	Units	All women	Premenopausal	Postmenopausal
n		53	33	20
Age	Years	44 ± 14	36 ± 9	59 ± 5**
BI		99 ± 15	98 ± 15	100 ± 15
TC	mmol/l	5.11 ± 1.01	4.70 ± 0.83	5.77 ± 0.94**
TG	mmol/l	0.92 ± 0.43	0.88 ± 0.39	0.99 ± 0.48
HDL-TC	mmol/l	1.29 ± 0.47	1.30 ± 0.53	1.28 ± 0.32
HDL-FC	μmol/l	308 ± 70	306 ± 73	311 ± 66
FER_{HDL}	%/h	10.57 ± 3.55	10.53 ± 3.47	10.62 ± 3.68
HDL_{2b}	%	26.6 ± 11.1	25.9 ± 10.3	27.3 ± 11.8
HDL_{3a}	%	58.6 ± 6.7	59.1 ± 6.2	58.1 ± 7.3
HDL_{3b}	%	14.9 ± 7.1	15.0 ± 7.9	14.7 ± 6.0

*BI, Brocca index determined as {wt ± kg/height ± cm − 100}x100; TC, total plasma cholesterol; FC, free plasma cholesterol; TG, triglycerides; HDL-TC, HDL-FC total and free cholesterol; HDL_{2b}, HDL_{3a}, $HDL_{3b,c}$ subclasses of HDL. Data are presented as means ± S.D. Significant differences between the means: * $P < 0.05$, ** $P < 0.001$*

4. Normal values of FER_{HDL}

Tables 2 and 3 summarize the data on FER_{HDL} and the relative proportion of HDL subspecies in 63 men and 56 women (see Methods for description of the subjects). As expected, there were differences in HDL composition between men and women, the latter having a relatively higher content of HDL_{2b}, higher HDL free cholesterol and lower relative content of $HDL_{3b,c}$. There were no differences in the relative content of HDL_{3a}. As reported previously, there was a marked difference in FER_{HDL} between men (16.84 ± 4.50 %/h) and women (10.57 ± 3.55 %/h). Correlation analysis showed a direct relation of FER_{HDL} to the relative concentration of $HDL_{3b,c}$ and an inverse relation to HDL_{2b} (Table 4).

Table 3
Summary of subject data: Men

	Units	All men	Middle aged	Septuagenarians
n		63	48	15
Age	Years	51±18	42±12	76±6**
BI		101±10	101±10	103±12
TC	mmol/l	5.31±0.97	5.03±0.83	6.07±0.95**
TG	mmol/l	1.3±0.6	1.3±0.6	1.4±0.7
HDL-TC	mmol/l	1.06±0.24	1.11±0.25	0.94±0.15*
HDL-FC	μmol/l	236±54	241±55	212±45
FER_{HDL}	%/h	16.84±4.50	16.47±4.17	17.73±5.32
HDL_{2b}	%	15.8±8.4	14.6±8.1	18.4±8.5
HDL_{3a}	%	61.9±9.9	62.7±10.0	60.1±9.5
HDL_{3b}	%	22.3±11.4	22.5±11.5	21.8±11.3

*BI, Brocca index determined as {wt±kg/height±cm−100}x100; TC, total plasma cholesterol; FC, free plasma cholesterol; TG, triglycerides; HDL-TC, HDL-FC total and free cholesterol; HDL_{2b}, HDL_{3a}, $HDL_{3b,c}$ subclasses of HDL. Data are presented as means ±S.D. Significant differences between the mean values, * P<0.01, ** P<0.001*

Table 4
Correlation ±r of FER_{HDL} with other parameters.

	MEN (n=63)	WOMEN (n=53)
Age	N.S.	N.S.
BI	0.380**	0.470**
TC	N.S.	N.S.
TG	0.370**	0.330*
HDL-TC	−0.384**	−0.445**
HDL-FC	−0.296*	−0.579***
HDL_{2b}	−0.503***	−0.627***
HDL_{3a}	−0.468**	0.355**
$HDL_{3b,c}$	0.708***	0.620***

*Significance of correlation: * P<0.05, ** P<0.01, *** P<0.001*

Subgroup analysis

When the group of women was divided into those pre- (n=33) and postmenopausal (n=20), the only difference, in addition to age, was higher plasma total cholesterol in the postmenopausal group (p<0.001). However, all the other measured parameters including FER_{HDL} and relative composition of HDL subfractions were similar (Table 2).

On the other hand, when the group of men was separated by age into those "middle aged" (42±12 years) and old (76±6 years), there was a similar increase in total plasma cholesterol in the older group (p<0.001); in addition the HDL cholesterol was lower in the older men (p<0.01). However, neither FER_{HDL} nor the relative content of HDL subfractions differed in the two groups (Table 3).

Discussion

Our data indicate that both the relative composition of HDL subfractions and the value of FER_{HDL} are stabile in the individual subject's plasma. There were no major differences in this parameter in apparently healthy individuals without risk factors for atherosclerosis either 3 months or 2–5 years after the

first assessment. The reproducibility of the isotopic method (using repeated assays of the frozen aliquots of plasma) was good with coefficient of variation ranging between 5 and 8 %. The most important factor influencing the reproducibility of the method is the temperature particularly above 37 °C. This is not surprising when we consider the fact that the action of lecithin cholesterol acyltransferase (LCAT) depends on the fluidity of its lipid substrate. Of interest is the finding that despite the observed decrease in the content of free cholesterol in HDL of stored plasma samples the rate of esterification (FER_{HDL}) was unchanged. This suggests that the activity of LCAT is not limited by the amount of the substrate (free cholesterol) but by the presence of small HDL particles which are the preferred substrate for the enzyme. If there is no change in the amount of large HDL_{2b} particles, the known inhibitors of the reaction (Barter *et al.* 1984), the esterification rate is unchanged. Thus, FER_{HDL} is reproducible even in samples of plasma that have been stored for longer periods of time where, we assume, free cholesterol exchange takes place between HDL particles of various sizes.

Our previous studies have shown that FER_{HDL} discriminates well between patients who have developed coronary artery disease and risk factor-free controls (Dobiášová *et al.* 1991, 1992, Dobiášová and Frohlich 1994). Its predictive value appears to be higher than that of conventional plasma lipid parameters such as cholesterol, triglycerides, HDL cholesterol and the HDL_2 and HDL_3 ratio. Comparison of this method with analysis of LpAI and LpAI/AII particles in the plasma was also favourable (Dobiášová and Frohlich 1994).

Because of the potential of this test for assessing the risk of coronary artery disease we tried to establish a mass assay that could be automated and that would not require handling of radioisotopes. While both the isotopic and mass assays of FER_{HDL} yielded similar results and were fairly well correlated ($r = 0.84$), the reproducibility of the mass method was much poorer than that of the radioassay. In addition, the average values of FER_{HDL} were lower than those of the mass assay. Explanations of the observed differences are a) the possibility of significant error in the measurement of the concentration of HDL-free cholesterol before and after incubation (because the summation of error of both estimations can occur at a low concentration of free cholesterol)), b) the longer lag phase for the mass method (the rate of esterification was highest between the second and third hour of incubation whereas in the isotope assay the reaction was linear up to 30 min), and c) the possibility of isotope dilution. In both assays of FER_{HDL} the esterification of HDL free cholesterol proceeds until 80–90 % is esterified. The reaction continues to completion because there is apparently no feedback regulation in the plasma depleted of apoB containing lipoproteins and no hydrolysis of cholesteryl esters occurs (this was confirmed by incubation of HDL plasma with labelled cholesteryl linoleate – data not shown). Furthermore, the lysophosphatidylcholine generated by the reaction is bound by albumin present in the HDL plasma.

The normal values presented in this study confirm the previously noted differences in FER_{HDL} values between men and women and their correlation with the distribution of subspecies of HDL. The relative amount of the large protective HDL_{2b} particles was about twice as high in women compared to men while men had values of the small $HDL_{3b,c}$ particles about 30 % higher than women. The role of medium sized HDL_{3a} particles, which represent about 60 % of all HDL particles, is not clear. Its relation to FER_{HDL} was inverse in men while a direct relation to FER_{HDL} was found in women. However, this correlation was weak although statistically significant (Table 4).

The measurement of FER_{HDL} using the isotopic assay represents a highly reproducible "functional" method for assessing of the pattern of HDL subclasses in the plasma. The FER_{HDL} values remain stable over a long period of storage (up to 5 years) at −70 °C and thus the assay can be used for both long-term retrospective and prospective studies.

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References

ALLAIN C.C., POON L.S., CHAN C.S., RICHMOND W.: Enzymatic determination of total serum cholesterol. *Clin. Chem.* **29:** 470–475, 1974.

BARTER P.J., HOPKINS G.J., GORJATSCHKO L, JONES M.E.: Competitive inhibition of plasma cholesterol esterification by human high density lipoprotein-subfraction 2. *Biochim. Biophys. Acta* **793:** 260–268, 1984.

BURSTEIN M., SCHOLNICK H.R., MORFIN R.: Rapid method for isolation of lipoproteins from human serum precipitation with polyanions. *J. Lipid Res.* **11:** 583–595, 1970.

DOBIÁŠOVÁ M., FROHLICH J.J.: Structural and functional assessment of high-density lipoprotein heterogeneity. *Clin. Chem.* **40:** 1554–1558, 1994.

DOBIÁŠOVÁ M., STŘÍBRNÁ J., SPARKS D.L., PRITCHARD P.H., FROHLICH J.J.: Cholesterol esterification rates in very low density lipoprotein and low density lipoprotein depleted plasma: relation to high density lipoprotein subspecies, sex, hyperlipidemia, and coronary artery disease. *Arterioscler. Thromb.* **11:** 64–70, 1991.

DOBIÁŠOVÁ M., STŘÍBRNÁ J., PRITCHARD P.H., FROHLICH J.J.: Cholesterol esterification rate in plasma depleted of very low and low density lipoprotein is controlled by the proportion of HDL_2 and HDL_3 subclasses: study in hypertensive and normal middle aged and septuagenarian men. *J. Lipid Res.* **33:** 1411–1418, 1992.

DREXEL H., AMAN F.W., RENTSCH K., NEUNSCHWANDER C., LEUTHY A., KHAN S.I., FOLIATH F.: Relation of the level of high-density lipoprotein subfractions to the presence and extent of coronary artery disease. *Am. J. Cardiol.* **70:** 436–440, 1992.

HAMSTEN A., JOHANSSON J., NILSSON-EHLA P., CARLSON L.A. Plasma high density lipoprotein subclasses and coronary atherosclerosis. In: *Disorders of HDL.* L. CARLSON (ed.), Smith Gordon, London, 1990, pp. 155–162.

NICHOLS A.V., KRAUSS R.M., MUSLINER T.A.: Nondenaturing polyacrylamide gradient gel electrophoresis. *Meth. Enzymol.* **128:** 417–431, 1986.

WILLIAMS P.T., KRAUSS R.M., NICHOLS A.V., VRANIZAN K.M., WOOD P.D.S.: Identifying the predominant peak diameter of high-density and low-density lipoproteins by diameter of high-density and low-density lipoproteins by electrophoresis. *J. Lipid Res.* **31:** 1131–1139, 1990.

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