

Familial hypercholesterolaemia: cholesterol efflux and coronary disease

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ABSTRACT

Background Coronary heart disease (CHD) risk inversely associates with levels of high-density lipoprotein cholesterol (HDL-C). The protective effect of HDL is thought to depend on its functionality, such as its ability to induce cholesterol efflux.

Materials and methods We compared plasma cholesterol efflux capacity between male familial hypercholesterolaemia (FH) patients with and without CHD relative to their non-FH brothers, and examined HDL constituents including sphingosine-1-phosphate (S1P) and its carrier apolipoprotein M (apoM).

Results Seven FH patients were asymptomatic and six had experienced a cardiac event at a mean age of 39 years. Compared to their non-FH brothers, cholesterol efflux from macrophages to plasma from the FH patients without CHD was $16 \pm 22\%$ (mean \pm SD) higher and to plasma from the FH patients with CHD was $7 \pm 8\%$ lower ($P = 0.03$, CHD vs. non-CHD). Compared to their non-FH brothers, FH patients without CHD displayed significantly higher levels of HDL-cholesterol, HDL-S1P and apoM, while FH patients with CHD displayed lower levels than their non-FH brothers.

Conclusions A higher plasma cholesterol efflux capacity and higher S1P and apoM content of HDL in asymptomatic FH patients may play a role in their apparent protection from premature CHD.

Keywords Apolipoprotein M, cholesterol efflux, familial hypercholesterolaemia, high-density lipoprotein, sphingolipids, sphingosine-phosphate.

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Introduction

Familial hypercholesterolaemia (FH), predominantly caused by mutations in the low-density lipoprotein (LDL) receptor gene, is characterized by a severely increased risk of coronary heart disease (CHD) [1]. FH patients with high levels of high-density lipoprotein cholesterol (HDL-C) are relatively protected from early development of CHD comparable to the effect of high HDL-C in the general population [2,3]. It is known that the atheroprotective effect of HDL is not simply related to absolute HDL-C levels. For instance, genetic variants or drugs that raise plasma HDL-C levels not always lead to CHD risk reduction [4,5]. One of the key roles of HDL is in reverse cholesterol transport by facilitating cholesterol

efflux from macrophages and returning cholesterol to the liver for clearance [6,7]. In two large population-based studies with around 3000 participants each, cholesterol efflux capacity was inversely associated with the incidence of cardiovascular events [8,9]. Evidence from epidemiological studies suggests that the protective role of HDL in the development of atherosclerosis might be due to its content of the bioactive sphingolipid sphingosine-1-phosphate (S1P) [10–15]. S1P as well as its carrier apolipoprotein M (apoM) mediate many of the beneficial effects of HDL [10,14,16–18]. ApoM is important for the formation of nascent HDL and for HDL-mediated cholesterol efflux [19,20]. Both HDL S1P levels and polymorphisms in *APOM* have been correlated with CHD risk [19–21].

In the present study, we investigated whether the capacity of plasma to induce cholesterol efflux from macrophages is associated with residual CHD risk in FH patients. To minimize variability of genetic and environmental factors, we selected sib-pairs, consisting of an FH patient and his non-FH brother. In addition, we investigated whether differences in HDL composition are associated with different CHD risk among these FH patients.

Materials and methods

Study population

Heterozygous male FH patients with a known mutation in the *LDLR* or *APOB-100* gene, not participating in an intervention study and having at least one brother without FH were selected for this study. Twenty FH patients were approached, of whom five patients and two brothers refused to participate because of logistic reasons. A total of 13 sib-pairs were included, of whom seven FH patients had no symptoms of CHD while the other six had developed symptomatic CHD. Therefore, the final selection was as follows: seven sib-pairs consisted of an FH patient without CHD and a brother with neither FH nor CHD; the other six pairs consisted of an FH patient with CHD and a brother with neither FH nor CHD (Fig. 1). To reduce the influence of environmental factors, we asked all participants to quit their medication six weeks prior to blood sampling and to refrain from smoking in the week before blood sampling [22]. Blood was sampled after fasting overnight and was placed on ice immediately; plasma was prepared as soon as possible and stored at -80°C until further experiments.

The medical ethical committee of the Erasmus MC approved the protocol and all participants gave written informed consent.

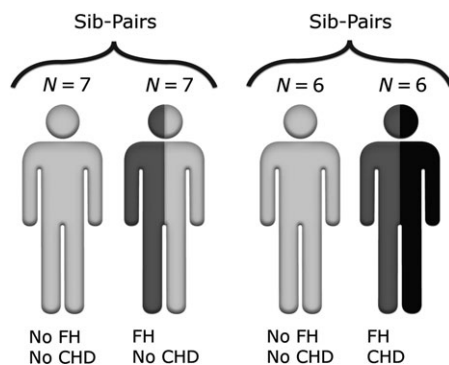


Figure 1 Study design. Seven sib-pairs consisted of one FH and one non-FH brother, both without CHD. Six pairs consisted of one brother with FH and CHD, the other without FH and CHD. FH, familial hypercholesterolaemia; CHD, coronary heart disease.

Cholesterol efflux experiments

Cholesterol efflux experiments were performed as described previously [23]. THP-1 cells, a human monocyte-cell line, were cultured in RPMI 1640 medium supplemented with 10% foetal calf serum, 2 mmol/L glutamine and 100 IU/L penicillin/streptomycin at 37°C and 5% CO_2 in air. Cells were plated into 24-wells plates (250 000 cells/well) and differentiation into macrophages was induced by treatment for 72 h with 50 ng/mL phorbol 12-myristate 13-acetate. Subsequently, cells were incubated for 24 h with 0.5 $\mu\text{Ci}/\text{mL}$ [^3H]cholesterol in RPMI medium supplemented with 0.2% fatty acid free bovine serum albumin (BSA). Then, cells were washed three times with 0.3 mL phosphate-buffered saline supplemented with 0.2% BSA. [^3H]cholesterol efflux was determined by incubating cells for 4 h at 37°C in acceptor medium, which consisted of RPMI supplemented with heparin (1.25 units/mL) and 2% (v/v) plasma. Plasma from an unrelated healthy volunteer without FH and CHD was used as reference, and medium supplemented with 0.2% (w/v) BSA was used as a control for basal efflux. At the end of the incubation, cell-free medium was collected and cells were dissolved in 0.1 M NaOH. Radioactivity in medium and cells was counted by liquid scintillation spectrometry. The ratio between radioactivity in medium and radioactivity in cells plus medium was taken as cholesterol efflux capacity. The mean of four wells treated with the same plasma or control medium was taken. All experiments were repeated three times.

Cholesterol, triglyceride and pre- β HDL levels

Plasma cholesterol, HDL-C and triglyceride levels were measured using a COBAS Mira analyzer (Roche Diagnostics, Indianapolis, IN, USA). LDL-C was calculated using the Friedewald formula. Pre- β HDL levels were measured using the commercially available ELISA kit by Daiichi Pure Chemicals (Tokyo, Japan).

Lipoprotein profiles and apolipoprotein levels

Lipoprotein profiles were obtained using density gradient ultracentrifugation [24]. KBr (0.35 g/mL plasma) was added to plasma to obtain a density of 1.26 g/mL. Of this plasma, 1 mL was placed in an ultracentrifuge tube and 1.9 mL of KBr solutions of 1.21, 1.10, 1.063, 1.04 and 1.02 g/mL in physiological salt were layered successively on top, followed by 1 mL of water. Samples were centrifuged at 207 000 g for 18 h at 4°C using a SW41 rotor in a L-70 Beckman ultracentrifuge (Beckman Instruments, Indianapolis, IN, USA). Thereafter, the density gradient was fractionated from the bottom into 250 μL fractions. Fractions with densities ranging from 1.125–1.21 g/mL and 1.062–1.125 g/mL were considered to constitute HDL3 and HDL2, respectively [25]. Cholesterol, triglyceride, apoA-I and apoA-II in the fractions were measured using a Selectra E (DDS

Diagnostic system). ApoM was measured by a specific human apoM ELISA [26].

Sphingosine-1-phosphate levels

Sphingosine-1-phosphate levels in the density gradient fractions were quantified by a modified LC-MS/MS method described in detail previously [27]. In brief, 50 μ L methanol containing C17-S1P (113 nmol/L; Avanti Polar Lipids, Alabaster, AL, USA) was added to 25 μ L fraction (from the 250 μ L fractions derived from ultracentrifugation as described above), or to S1P standards (Avanti Polar Lipids) in KBr solution (density of 1.02 and 1.21 g/mL). The mixture was incubated on ice for 30 min and centrifuged for 30 min at 18 000 g at 4 °C. Of the clear supernatant, 15 μ L was injected onto an Agilent 1200SL system (Agilent Technology, Amstelveen, the Netherlands) and run through a Xterra C18 column (2.1 \times 10 mm, 3.5 μ m, Waters Chromatography, Etten-Leur, the Netherlands) at 40 °C. The elution started with 1 min of 50% mobile phase B (10% water, 0.25% formic acid, 2.5 mM ammoniumformate in methanol) in mobile phase A (10% methanol, 0.25% formic acid, 2.5 mM ammonium formate in water), followed by a linear gradient from 50% to 90% B in A for 6 min, and finally 100% B for 3 min. The flow rate was set at 0.25 mL/min and total run time was 14 min.

The effluent was directed to an Agilent 6410 triple quadruple mass spectrometer and analysed in positive ion mode following electrospray ionization. The MS/MS transitions of S1P m/z 380 \rightarrow 264, and of C17-S1P m/z 366 \rightarrow 250 were quantified by taking the ratios of the integrated peaks.

Statistical analyses

General characteristics were analysed using ANOVA and chi square test. Cholesterol efflux experiments were normalized according to results with plasma from the unrelated healthy volunteer. The percentage difference between the FH patient and his non-FH brother was compared between sib-pairs with and without CHD using an independent *t*-test. The differences in lipid levels, sphingolipid levels and apolipoprotein levels between FH patients with and without CHD were analysed by linear regression adjusted for the value in the brother without FH.

Results

All brothers with FH had a confirmed pathogenic LDL-receptor or apoB100 gene mutation while all brothers without FH tested negative (Table S1). At the moment of blood sampling, the average age of the FH patients without CHD was 41 years (range 23–65) and of FH patients with CHD was 51 years (range 49–63). However, the average age of the FH patients without CHD was similar to the mean age at which the FH patients with CHD had their first cardiac event (39 years; range 27–52; $P = 0.85$). Six out of seven FH patients without CHD

were current or former smokers; three of them had refrained from smoking for at least 10 years at the time of sampling (Table 1). Five out of the six FH patients with CHD smoked at the time of event and all but one quitted at least 10 years before sampling. All current smokers admitted that they had smoked a few cigarettes the week prior to blood sampling even though they were asked not to. The FH patients used cholesterol-lowering medication ranging from simvastatin 40 mg to atorvastatin 80 mg with ezetimibe 10 mg. This medication was stopped for 6 weeks prior to blood sampling.

As expected, the FH patients had significantly higher total and LDL-cholesterol (LDL-C) levels than their non-FH sibs (Table 1). Levels of HDL-C and triglyceride were not significantly different between the FH subjects and their respective non-FH sibs. There was a trend towards higher HDL-C levels in FH patients without CHD than in FH patients with CHD compared to their respective non-FH brothers (paired analysis $P = 0.076$). The mean pre- β HDL level in FH subjects was 49 μ g/mL (SD 15) and in non-FH subjects 40 μ g/mL (SD 13; $P = 0.13$). In paired analyses, the difference between FH patients without and FH patients with CHD was not significant ($P = 0.20$).

Cholesterol efflux experiments

Plasma from six out of seven FH patients without CHD induced more cholesterol efflux from cholesterol-loaded macrophages than plasma from their non-FH sibs. In contrast, plasma from four out of six FH patients with CHD induced less efflux than that of their non-FH sibs (Fig. 2a). The mean paired difference in cholesterol efflux between FH patient and his non-FH brother was +16% (SD 22%) for the pairs without CHD and -7% (SD 8%) in the pairs with an affected FH patient (analysis of pairs without vs. with CHD, $P = 0.03$; Fig. 2b). These differences in efflux were independent of baseline values and within-pair differences of HDL-C, LDL-C levels, as these differences remained statistically significant when these co-variables were included in a multiple linear regression analysis (data not shown). Overall cholesterol efflux was slightly, but non-significantly, higher in the pairs with CHD in comparison with those without.

Lipoprotein profiles

Analyses of the full lipoprotein profiles revealed differences in the distribution of cholesterol within the HDL subfractions and within the LDL subfractions (full profiles shown in Fig. S1a).

In comparison with their non-FH brothers, the FH subjects without CHD had significantly higher HDL-C in the HDL fractions of medium and low density (Fig. 3a). Levels of apoA-I were highest in HDL of all FH patients without CHD (FH patients without CHD 1.2 g/L, their non-FH brothers as well as the FH patients with CHD 0.9 g/L, their brothers without FH 1.0 g/L), while levels of apoA-II were not different between FH

Table 1 General characteristics

	Sib-pair of FH patient without CHD (n = 7)		Sib-pair of FH patient with CHD (n = 6)	
	FH–	FH+CHD–	FH–	FH+CHD+
Age	41 ± 13	41 ± 16	46 ± 8	51 ± 8
Age at event				39 ± 10
Smoking ever	2	6	4	5
Current smoking	1	2	1	1
Total cholesterol [mmol/L]	5.5 ± 0.9 [†]	7.7 ± 2.0 [†]	5.9 ± 0.7*	8.3 ± 1.8*
LDL-C [mmol/L]	3.9 ± 0.8 [†]	6.0 ± 2.1 [†]	4.5 ± 0.7*	6.9 ± 1.9*
HDL-C [mmol/L]	1.39 ± 0.33	1.42 ± 0.38	1.19 ± 0.26	1.15 ± 0.12
Triglycerides [mmol/L]	1.12 ± 0.53	1.24 ± 0.45	1.35 ± 0.32	1.05 ± 0.38
Pre-β HDL [μg/mL]	43 ± 1.9	51 ± 2.1	38 ± 4.5 [‡]	47 ± 4.7 [‡]
HDL ₃ -C (mmol/L)	1.05 ± 0.14	0.87 ± 0.13	0.70 ± 0.09	0.74 ± 0.09
HDL ₂ -C (mmol/L)	0.47 ± 0.06	0.43 ± 0.04	0.25 ± 0.02	0.34 ± 0.02
HDL ₃ S1P (AU/L)	7.17 ± 1.06	8.27 ± 1.77	7.33 ± 0.56	6.90 ± 0.83
HDL ₂ S1P (AU/L)	2.17 ± 0.97	2.76 ± 1.05	1.13 ± 0.21	1.26 ± 0.43

FH, familial hypercholesterolaemia; CHD, coronary heart disease; AU, arbitrary units.

All FH patients were 'off' statin treatment.

Values are mean ± SD.

[†]P = 0.035, [‡]P = 0.008, *P = 0.025, [‡]P = 0.006.

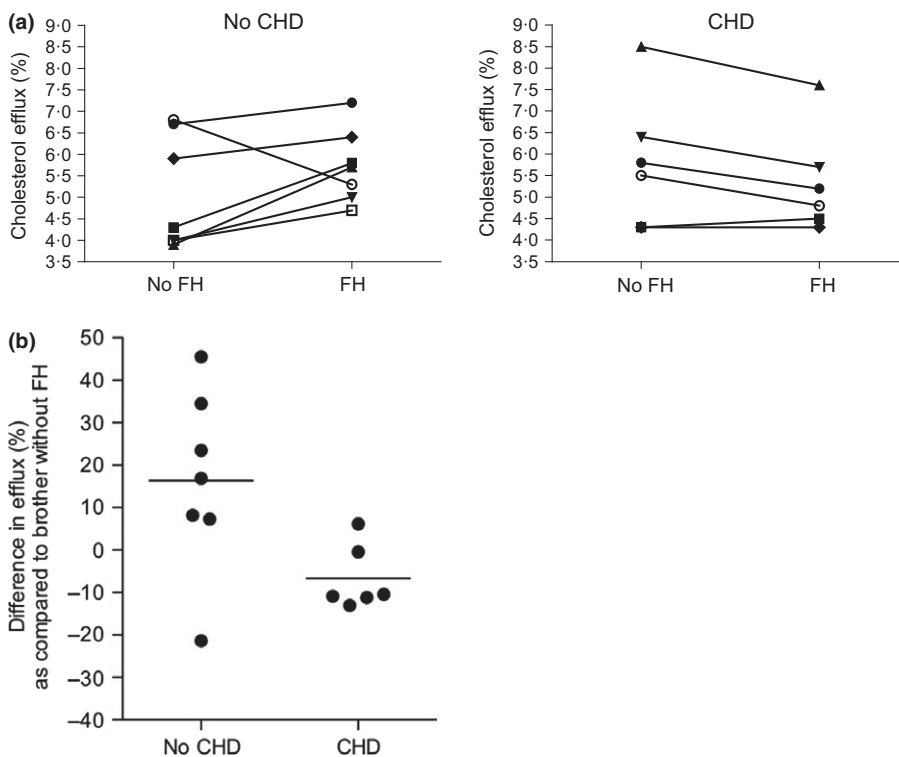


Figure 2 Difference in cholesterol efflux from cholesterol-labelled macrophages to plasma. (a) Individual differences in cholesterol efflux to plasma from an FH patient and his non-FH brother as a percentage of the efflux to plasma of the non-FH sib taken as 100%. Some lines (No CHD two, CHD four) almost completely overlap. (b) Percentages difference in cholesterol efflux when compared to the brother without FH. FH, familial hypercholesterolaemia; CHD, coronary heart disease.

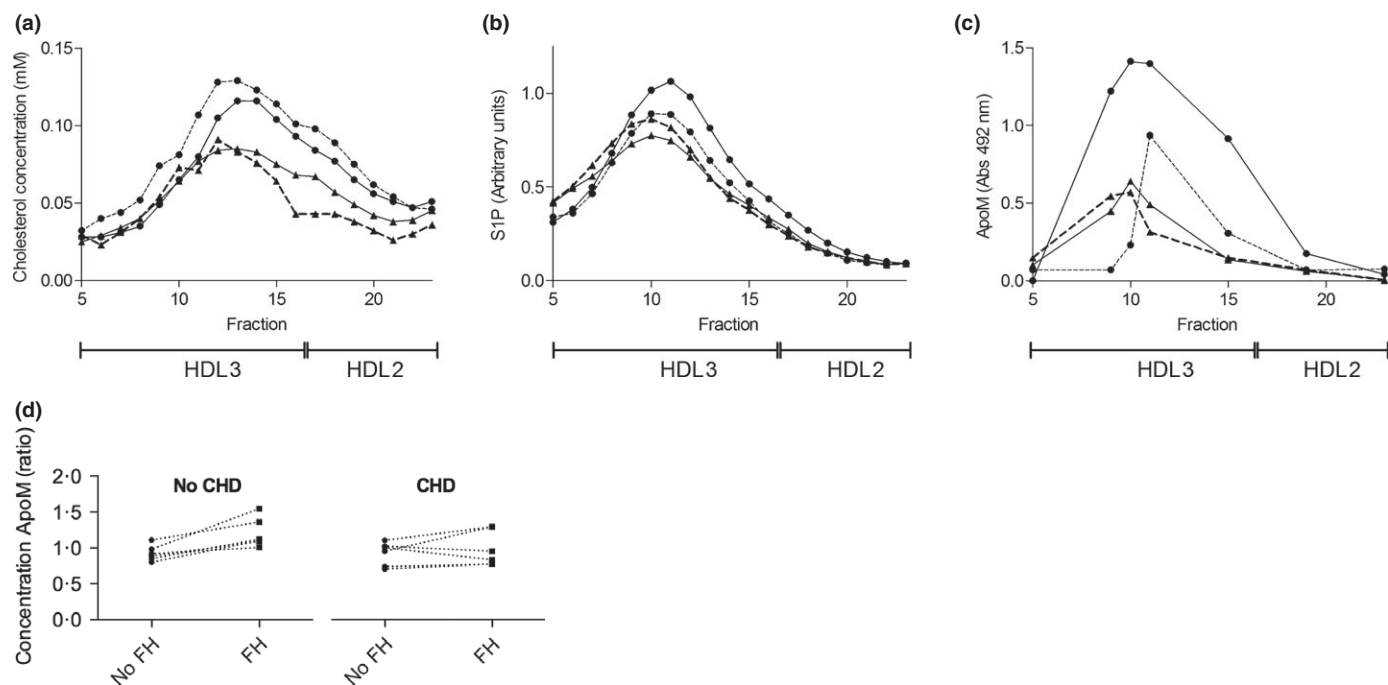


Figure 3 Levels of cholesterol (a), S1P in high-density lipoprotein (HDL) (b) and apoM (c), and apoM in plasma (d). In c, apoM was measured in all separate HDL density fractions that were pooled from all individuals in each group. In d, apoM plasma concentrations were measured in each individual, and values in each familial hypercholesterolaemia patient were connected to his respective brother. Data are expressed as % of an unrelated control pool plasma.

patients without CHD and their non-FH sibs (since fractions were pooled per group, no *P*-value for paired analysis available; pooled data shown in Fig. S1b,c).

S1P and apoM

Sphingosine-1-phosphate levels in plasma were not different between the groups. Upon density gradient centrifugation, S1P was predominantly present in the HDL3 subfractions (Fig. 3b). Among the four groups, HDL3-S1P levels were highest in the FH patients without CHD; HDL3-S1P levels were comparable in the other three groups. Plasma apoM levels were 30% higher in FH patients without CHD than in their non-FH sibs ($P = 0.015$; Fig. 3d), whereas there was no significant difference between FH patient with CHD and their brothers. When measured in the HDL3 subfractions from the density gradient with pooled fractions per group, apoM was much higher in FH patients without than with CHD (Fig. 3c); HDL3-apoM was comparable among the three other groups.

Correlation of HDL composition and cholesterol efflux capacity

Cholesterol efflux capacity was not significantly correlated with pre- β HDL levels (Pearson correlation coefficient -0.4 , $P = 0.2$). Similarly, no correlation was found between cholesterol efflux

capacity and HDL-C, HDL2-C, HDL3-C or apoA-I levels, nor with plasma apoM levels or HDL3-S1P levels.

Discussion

Compared to their non-FH brothers, the cholesterol efflux capacity of plasma from FH patients without CHD was higher than that of patients who already had experienced a cardiac event. In addition, HDL composition differed with more S1P and apoM in HDL3 of the FH patients without CHD. Our data suggest that FH patients with relatively higher cholesterol efflux capacity of plasma and higher levels of HDL3-bound S1P and apoM are relatively protected from a cardiac event.

Two recent large population-based studies in 2924 participants of the Dallas Heart Study and 3494 participants of the EPIC-Norfolk study [8,9] reported cholesterol efflux capacity being an independent risk indicator of cardiovascular events. Earlier smaller studies did not all confirm this association [28–30]. Differences in outcome may be explained by the methods used. In the first two large studies, J774 macrophages were used with stimulated ABCA1 expression, while the other studies used other cell types with or without regulated ABCA1 expression. Because we used THP-1 cells with a low ABCA1 expression, we cannot directly compare our data with these studies.

In our study, differences in cholesterol efflux capacity among all individuals did not correlate with differences in HDL-C, HDL-apoA-I or pre- β -HDL levels. This is in line with earlier studies showing that cholesterol efflux capacity of plasma not merely depends on the amount of HDL cholesterol [5,8,31,32], but may be due to additional differences in composition of HDL. A previous study in normocholesterolaemic subjects undergoing coronary angiography suggested that HDL2 is most important for cholesterol efflux capacity of plasma. Interestingly, in a study comparing young (13–29 years) FH patients without symptomatic CHD with healthy individuals, HDL2 from FH patients appeared to be less efficient in inducing cholesterol efflux [33,34]. One could argue that the higher amount of HDL2 in the FH patients without CHD in our study might compensate for this lower functionality. We chose to use whole plasma in order to reflect the *in vivo* situation as closely as possible while net cholesterol efflux from tissues also depends on the presence of apoB-containing lipoproteins. We cannot exclude the possibility that differences in cholesterol efflux we identified were due to differences in cholesterol efflux to LDL or very-low-density lipoprotein VLDL.

The higher, although nonsignificantly, levels of apoB-containing lipoproteins may explain the higher overall cholesterol efflux capacity of the brother from the families with CHD [35]. However, additional experiments on a limited number of samples using apoB-depleted plasma showed similar results as using whole plasma (data not shown).

Evidence from epidemiological studies indicates that the protective role of HDL in the development of atherosclerosis is largely due to its content of the bioactive sphingolipid, S1P [13–18]. HDL of FH patients without CHD contained higher amounts of S1P and its carrier molecule apoM, particularly in HDL3. In a recent study in gestational diabetes mellitus, cholesterol efflux capacity was shown to be decreased in parallel with plasma apoM [36]. This is in line with our finding and together suggests a link between apoM and cholesterol efflux capacity and CHD risk. However, we did not find a significant correlation of cholesterol efflux capacity and differences in levels of HDL-bound S1P and apoM between FH patients without and with CHD.

Differences in HDL-S1P as well as apoM might also reflect functional differences not related to the cholesterol efflux pathway. S1P and apoM mediate many of the beneficial cardiovascular effects attributed to HDL such as vascular integrity and vasorelaxation [10,14,16–18,21]. We did not study these additional pathways.

The strength of the current study design is that by including non-FH brothers, molecular and genetic heterogeneity were diminished as far as possible. Comparable HDL-C levels between brothers suggest we succeeded in this aim [37].

Limitations of this study include, firstly, the limited number of subjects, thereby limiting statistical power. However, this also led to the advantage that all samples could be tested in a single efflux experiment, eliminating inter-experiment variability. Secondly, two of the FH patients in the non-symptomatic group may have been too young to have had a cardiac event. However, they were in the age range in which one of the symptomatic FH patients developed his first event. Moreover, as a group the nonsymptomatic FH patients were at the mean age of first event in the symptomatic group. Lastly, due to recent developments, we chose to focus on S1P and apoM, out of all components and apolipoproteins present in HDL. To further unravel mechanisms underlying the differences in cholesterol efflux reflecting differences in HDL dynamics, it would be interesting to measure activity of, for example, cholesteryl ester transfer protein (CETP), ATP-binding cassette transporter A1 (ABCA1) and scavenger receptor class B member 1 (SR-BI) in the subfractions. We measured CETP levels in serum but did not find significant differences (data not shown). However, it would be of interest to separate HDL2 and HDL3 here as well.

In conclusion, we have shown that plasma from FH patients without CHD has a higher cholesterol efflux capacity than plasma from FH patients with CHD, in comparison to their respective non-FH brothers. This may explain why these FH patients appear to be protected against CHD despite their high LDL-cholesterol levels. The observed higher efflux capacity may involve differences in composition of HDL including cholesterol, S1P and apoM levels. Increased S1P levels in HDL might also point at differences in HDL functionality other than cholesterol efflux capacity. These data suggest that CHD risk in FH patients is modulated by familial factors not related to the LDL-receptor locus that modulate the composition of HDL and the effectiveness of reverse cholesterol transport.

Disclosures

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All authors declare that they have no conflicts of interest.

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Authors' contributions

JV contributed to the design of the work, performed part of the experiments and wrote the manuscript. RV, RY, LvV, JB, DW, TR and CC performed part of the experiments: RV and RY cholesterol efflux essays and lipoprotein files, LvV and JB lipoprotein profiles including apolipoprotein measurements; DW and TR LC-MS/-MS; CC apoM measurements. JV and JBvdN approached potential participants and got informed consent. JV and JB performed blood sampling. JBvdN, CRP, JPK, MM and EJGS contributed to the design of the work. JV, RV, RY, JBvdN, CRP, CC, BD, JPK, MM and EJGS participated in the analyses. CRP and JPK supervised JV during a part of the experiments performed at UCSF in San Francisco. All authors reviewed and edited the paper.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1. Complete lipoprotein density profiles and apoA-I and apoA-II content of HDL. On the X-axis fraction number, on the Y axis concentration of cholesterol (a), apoA-I (b) and apoA-II (c).

Table S1. Age and mutation for each brother.