

## Increased Coronary Heart Disease in Japanese-American Men with Mutation in the Cholesteryl Ester Transfer Protein Gene Despite Increased HDL Levels

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### Abstract

Plasma high density lipoprotein (HDL) levels are strongly genetically determined and show a general inverse relationship with coronary heart disease (CHD). The cholesteryl ester transfer protein (CETP) mediates the transfer of cholesteryl esters from HDL to other lipoproteins and is a key participant in the reverse transport of cholesterol from the periphery to the liver. A high prevalence of two different CETP gene mutations (D442G, 5.1%; intron 14G:A, 0.5%), was found in 3,469 men of Japanese ancestry in the Honolulu Heart Program and mutations were associated with decreased CETP (−35%) and increased HDL chol levels (+10% for D442G). However, the overall prevalence of definite CHD was 21% in men with mutations and 16% in men without mutations. The relative risk (RR) of CHD was 1.43 in men with mutations ( $P < .05$ ); after adjustment for CHD risk factors, the RR was 1.55 ( $P = .02$ ); after additional adjustment for HDL levels, the RR was 1.68 ( $P = .008$ ). Similar RR values were obtained for the D442G mutation alone. Increased CHD in men with mutations was primarily observed for HDL chol 41–60 mg/dl; for HDL chol > 60 mg/dl men with and without mutations had low CHD prevalence. Thus, genetic CETP deficiency appears to be an independent risk factor for CHD, primarily due to increased CHD prevalence in men with the D442G mutation and HDL cholesterol between 41 and 60 mg/dl. The findings suggest that both HDL concentration and the dynamics of cholesterol transport through HDL (i.e., reverse cholesterol transport) determine the anti-atherogenicity of the HDL fraction. (*J. Clin. Invest.* 1996. 97:2917–2923.) Key words: HDL • CETP • atherosclerosis • coronary heart disease • mutation

### Introduction

There is a strong inverse relationship between plasma HDL

cholesterol concentration and coronary heart disease (CHD)<sup>1</sup> (1–4). The mechanisms underlying this relationship are poorly understood. One possible explanation is related to the role of HDL in reverse cholesterol transport, i.e., the transfer of cholesterol from peripheral tissues into HDL and then to the liver (5–10). However, HDL has a number of other potentially important anti-atherogenic properties including anti-oxidant effects (11, 12) and the ability to inhibit aggregation of atherogenic lipoproteins (13, 14), an early essential event in atherogenesis (14). Recent studies in transgenic mice with increased HDL levels due to overexpression of the main HDL protein, apoA-I, strongly support a direct anti-atherogenic effect of HDL (15, 16).

Twin and family studies indicate that 40–60% of the variation of HDL cholesterol between individuals is determined by genetic factors (17, 18). However, the common genetic determinants of altered HDL levels have not been well defined. Sibpair linkage studies have suggested that variation in HDL cholesterol between individuals is related to the inheritance of alleles at or near the CETP and apoA-II genes (19) or the hepatic lipase and apoA-I/CIII/A-IV loci (20). The cholesteryl ester transfer protein (CETP) mediates the transfer of cholesteryl esters from HDL or LDL into triglyceride-rich lipoproteins, and thereby stimulates reverse cholesterol transport (21). Genetic deficiency of CETP results in marked increases in HDL levels in homozygotes and moderate increases in heterozygotes (21–24). In the Japanese two common CETP gene mutations (an intron 14 splicing defect and an exon 15 missense mutation [aspartate 442:glycine]) explain about 10% of the variance of HDL cholesterol in the general population (25). While CETP deficiency might be an anti-atherogenic state, due to HDL elevation, the role of CETP in reverse cholesterol transport suggests the opposite conclusion (21). The high prevalence of CETP gene mutations provided the opportunity to examine the relationship between CETP deficiency, increased HDL levels and CHD.

### Methods

**Subjects.** The current study is a cross-sectional survey of the relationship between CETP gene mutations and CHD, based on examination

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1. Abbreviations used in this paper: C.I., confidence interval; CETP, cholesteryl ester transfer protein; CHD, coronary heart disease; HHP, Honolulu Heart Program; LCAT, lecithin:cholesterol acyltransferase; OR, odds ratio; RR, relative risk.

4 of the Honolulu Heart Program (HHP) Cohort. A baseline examination of 8,006 men who were living on the island of Oahu and born between 1900 and 1919 was conducted between 1965 and 1968 (26, 27). All subjects were identified as being Issei (born in Japan), Nisei (born of Issei parents in the United States) or Kibei (born of Issei parents in the United States and returned to Japan for a significant portion of their education) (27). Thus, all men derive from pure Japanese ancestry without admixture of non-Japanese heritage. Of these 8,006 men, there were 4,678 survivors who were eligible for participation in Examination 4. A total of 3,741 examinations were conducted among survivors between February 1991 and January 1994; complete information, including an unambiguous assessment of CETP gene mutation status was available on 3,469 of them.

Participants fasted for 12 h before the clinic visit. Resting seated blood pressure measurements were obtained prior to collecting a fasting blood sample. Subsequent examinations included a resting 12-lead EKG, anthropometric measures, and a physical examination. Questionnaire data included assessment of smoking, alcohol intake, and prescription medications.

**Prevalent CHD.** Two levels of coronary heart disease prevalence were assessed (definite and possible). These classifications were made by a surveillance committee, which periodically reviews all clinical information collected from hospital admissions, past examinations, and death certificates (28). Definite CHD endpoints included (a) prior myocardial infarction (MI) detected by hospital surveillance, (b) silent MIs from EKG data, (c) acute coronary insufficiency, or angina pectoris resulting in surgical intervention, and (d) temporal changes in EKG diagnostic of myocardial infarction. Possible CHD indicated less certain clinical evidence for coronary conditions (vague anginal complaints not requiring surgical intervention, equivocal EKG and cardiac enzyme findings) suggesting they could not be classified unequivocally as free from CHD.

**Laboratory analyses.** Total cholesterol, high density lipoprotein (HDL) cholesterol, and triglycerides were determined (29). Fasting lipids were measured on an Olympus Demand system (Olympus Corp., Lake Success, NY) which had been standardized in the CDC Lipid Standardization program. LDL-cholesterol was estimated using the Friedewald formula for all men with measured triglycerides < 400 mg/dl (30).

Buffy coat samples were extracted to obtain DNA. The presence of CETP gene mutations were assessed by PCR amplification and restriction enzyme digestion, as described previously (25). The procedure yields product of two different sizes for wild type or mutant DNA. When no product was obtained by PCR, the sample was classified as indeterminate. Plasma CETP concentration was determined using an established solid phase competitive RIA (31). Plasma CETP concentrations were determined on all men with HDL cholesterol > 75 mg/dl, and < 30 mg/dl, as well as on a random sample of 500 men with HDL cholesterol between 30 and 75 mg/dl.

**Statistical analyses.** Analyses were conducted using SAS software program. Descriptive statistics such as means and standard deviations were calculated and compared using PROC TTEST and PROC GLM, and frequency statistics were done using PROC FREQ. Measures of association relating the prevalence of CHD and the presence of a CETP gene mutation were assessed using risk differences and odds ratios. Odds ratios (or relative risk) were calculated using logistic regression methods, and adjusted for covariates as indicated by the sequence of analyses presented in the Results. Statistical significance of differences in the distribution of plasma CETP concentration between men having any CETP mutation and men having no mutation was assessed among independent strata of HDL cholesterol concentration using the nonparametric Wilcoxon 2-sample test.

Homogeneity of associations relating CHD prevalence and the prevalence of men with a CETP mutation across strata of HDL cholesterol were assessed using the methods described by Fleiss (32). Significance of Type I statistical error was chosen as  $P < .05$ , or 95% confidence intervals were determined.

## Results

An unambiguous assignment of mutation status was obtained on 3,469 men, or 93% of the 3,741 men for whom complete information was obtained at exam 4 (see Methods). 170 men were heterozygous and 6 were homozygous for the exon 15 missense mutation (Table I). Only 17 men were heterozygous for the intron 14 splicing defect. The overall prevalence of

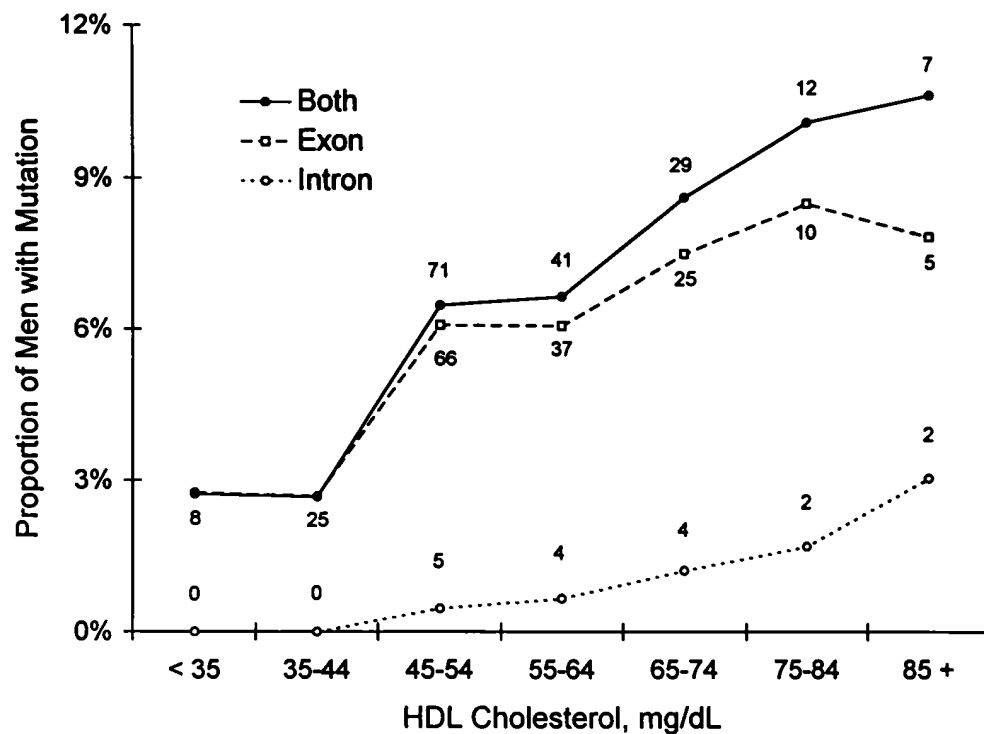


Figure 1. Prevalence of CETP gene mutations in strata of HDL-cholesterol for 3469 men in the HHP cohort. The data are shown for an exon 15 missense mutation (D442:G) and for an intron 14 splicing defect.

Table I. Prevalence of Men with CETP Gene Mutations Among 3,469 Subjects

Mutation	Prevalence	Allele frequency	n
Intron 14		0.0025	
Heterozygote	0.49%		17
Homozygote	0%		0
Exon 15		0.0262	
Heterozygote	4.9%		170
Homozygote	0.17%		6
Any CETP mutation	5.6%		193

n, Number of subjects

CETP gene mutations in this population sample was 5.6%. There was a pronounced increase in the prevalence of both mutations within strata of increasing HDL levels (Fig. 1,  $P < .001$ ). However, for both mutations the largest numbers of men with the mutation were found in the HDL chol 45–54 mg/dl stratum.

Mean HDL cholesterol concentrations were significantly higher in men with intron 14 or exon 15 mutations, compared to men with no CETP gene mutation (both  $P < .001$ , Table II). HDL chol values were significantly higher for the int 14 compared with the exon 15 mutation ( $P < .001$ ). Triglyceride concentrations were significantly lower in men heterozygous for

the exon 15 mutation, as was serum glucose. Compared with men with no evidence of CHD, men identified as definite CHD cases had, on average, significantly lower HDL cholesterol, higher triglycerides and higher serum glucose; blood pressure was also lower among cases, possibly reflecting treatment. Marked and statistically significant relationships were noted between CHD status and alcohol, cigarette or hypertension medication use (not shown). There was no relation between alcohol or cigarette use and mutation status.

At every level of HDL cholesterol concentration, the plasma CETP concentration was lower in men with mutations, compared with men with no mutation (Fig. 2); the difference was significant ( $P < .05$ ) for each HDL stratum. Among men with no mutation, the concentration of CETP was similar for all strata of HDL, except at the highest ( $> 85$  mg/dl), where it fell slightly.

In men without mutations, the overall prevalence of definite CHD was 16% versus 21% in men with mutations. The number of men and the prevalence of CHD (possible or definite) is shown for each mutation in Table III. Although the number of men with the int 14 mutation was small, the CHD prevalence data appeared similar for both mutations. For the exon 15 mutation the increase in CHD was more evident for the firmer diagnostic category definite CHD than for possible CHD. Since the same trend in the data was seen for both mutations, and since the primary biological effect of both mutations is to decrease plasma CETP levels and activity (25), we further analyzed the data with both mutations combined or for the D442G mutation alone.

Table II. Mean and Standard Deviation (SD) of Age, Total Cholesterol, HDL Cholesterol, LDL Cholesterol, Triglycerides, Serum Glucose, and Systolic and Diastolic Blood Pressures Among Groupings of Men with Various CETP Mutations and Among Groupings of Men with Prevalent Coronary Heart Disease

Variable	Mutation Status				CHD Status		
	Intron 14		Exon 15		None	Possible	Definite
	None	Heterozygote	Heterozygote	Homozygote			
Sample Size (N) <sup>‡</sup>	3276	17	170	6	2460	455	554
Age, yrs	77.7	78.5	77.6	76.3	77.6	78.4**	77.4
SD	4.6	4.5	4.4	4.1	4.6	5.0	4.3
HDL Cholesterol, mg/dL <sup>§</sup>	50.5	66.9***	55.4***	54.5	51.5	51.3	47.5***
SD	13.1	19.3	14.0	16.0	13.2	14.2	12.1
LDL <sup>‡</sup> cholesterol, mg/dL <sup>§</sup>	110.3	97.0	109.5	105.3	110.6	107.4	110.9
SD	31.1	25.3	29.1	35.7	31.0	30.4	31.0
Triglycerides, mg/dL <sup>  </sup>	148.5	123.3	135.0*	169.3	145.3	149.2	157.2**
SD	87.7	56.7	84.9	79.6	82.2	89.0	106.4
Total Cholesterol, mg/dL <sup>§</sup>	189.6	188.6	191.8	193.7	190.5	187.8	187.8
SD	33.0	39.8	32.9	44.0	33.3	31.5	33.1
Glucose, mg/dL	113.5	108.2	108.5*	114.5	111.5	114.0	120.3***
SD	29.9	18.4	22.2	19.8	26.6	34.0	36.2
Sys BP, mmHg	149.4	143.4	148.8	153.3	149.5	152.0*	146.5**
SD	23.5	17.3	24.9	22.2	23.0	25.6	24.0
Dia BP, mmHg	79.9	80.1	79.2	78.2	80.3	80.4	77.8***
SD	11.4	10.2	10.9	8.6	11.1	12.2	11.7

<sup>‡</sup>Men with triglycerides  $> 400$  mg/dL excluded. Thus, N = 3,203; 17; 168; 6; and N = 2,414; 444; 536, respectively.

Sys, systolic; Dia, diastolic; BP, blood pressure.

<sup>§</sup>To convert to mmol/L multiply by 0.02586.

<sup>||</sup>To convert to mmol/L multiply by 0.01129.

\* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ : Statistical significance of difference between status category and "none."

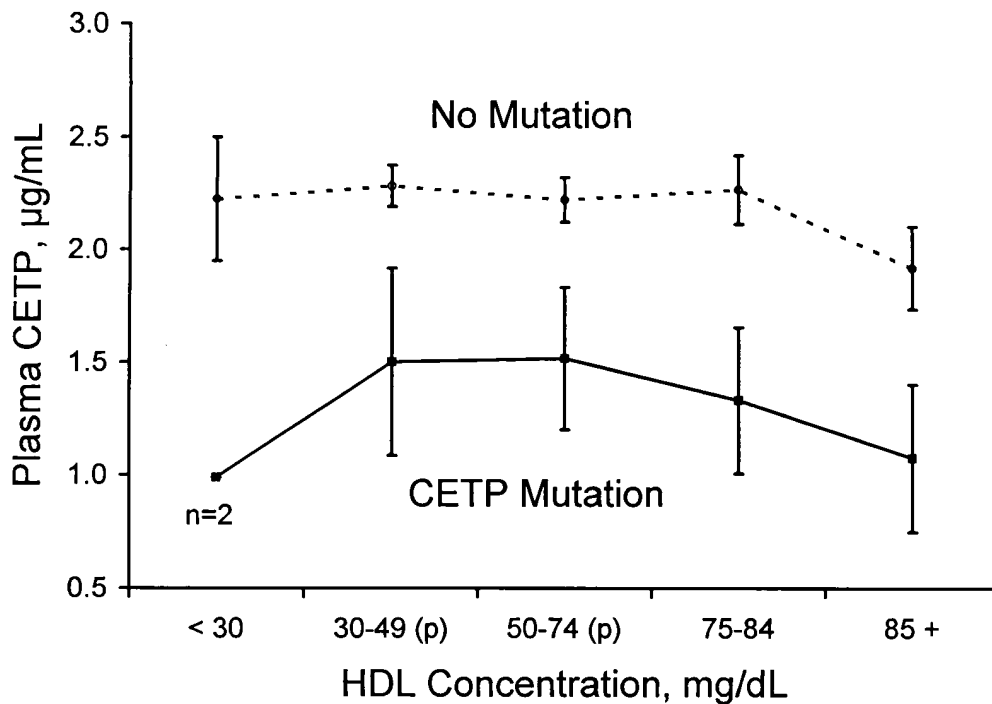


Figure 2. Average plasma concentration of CETP among men having a CETP gene mutation and men having no mutation, stratified by concentration of HDL cholesterol. HDL strata with a designation (p) represent men derived from a 14% probability sampling of the population (see Methods). The 95% confidence intervals of estimated means are also illustrated. Among men with no mutation, the average CETP concentration was significantly lower ( $P < .001$ ) at the highest HDL stratum,  $\geq 85$  mg/dl, compared with the next lower stratum, 75–84 mg/dl.

Using the relative risk or odds ratio (OR) as estimated by logistic regression modeling, a significant association was found between prevalence of definite CHD and CETP mutation status. The combined data for both mutations is shown in Table IV. With no adjustment for any co-variate, the OR was 1.43 ( $P < .05$ ). Adjusting for known risk factors for CHD (see Table IV), increased the OR to 1.55 ( $P = .024$ ). Adjusting only for HDL cholesterol produced an OR of 1.63 ( $P = .011$ ), and adjusting for the other risk factors plus HDL produced the highest OR of 1.68 ( $P = .008$ ). Adjustment for the treatment of hypertension exclusively by non-diuretics decreased the measure of association to 1.54.

When analyzed for the D442G mutation alone, similar results were obtained. The OR values were as follows: no adjustment, 1.39 ( $P = .10$ ); group 1.49 ( $P = .056$ ); HDL, 1.56 ( $P = .029$ ); group + HDL 1.61 ( $P = .024$ ), group + HDL + nondiuretic antihypertensives, 1.51 ( $P = .054$ ).

There was no significant association between the diagnostic category possible CHD and CETP mutation status (OR 1.23,

95% CI 0.80–1.88). There was also no relationship of possible CHD to HDL concentration.

The relationship between definite CHD prevalence and mutation status is shown for different levels (approximately quartiles) of HDL cholesterol concentration in Fig. 3. In men without mutations there was a systematic decrease in CHD prevalence with increasing HDL cholesterol concentrations ( $P < .001$ ). However, in men with CETP gene mutations, the prevalence of CHD remained similar to that in the lowest stratum of HDL cholesterol until the highest stratum,  $> 60$  mg/dl, where it fell precipitously to a value comparable to men with no mutation. The differences in prevalence of CHD between men with and without mutations in the middle region of the HDL cholesterol distribution were significantly larger than differences at either low or high HDL cholesterol strata ( $\chi^2$  test of homogeneity = 17.0,  $df = 3$ ,  $P < .001$ ).

The age of onset of definite CHD was estimated from surveillance or examination records. Those who had no mutation

Table III. Number of Men with Possible and Definite Prevalent Coronary Heart Disease (CHD) According to CETP Mutation Status

CETP Mutation	Number of men	CHD Status at Exam 4 (prevalence)	
		Possible	Definite
None	3276	427 (13.0%)	515 (15.7%)
Intron 14 heterozygotes	17	3 (17.6%)	3 (17.6%)
Exon 15 heterozygotes	170	24 (14.1%)	34 (20.0%)
Exon 15 homozygotes	6	1 (16.7%)	2 (33.3%)

Table IV. Odds Ratio (OR) and 95% Confidence Interval (C.I.) Relating the Prevalence of Definite Coronary Heart Disease to the Prevalence of Men Having a CETP Mutation

Covariates	Definite coronary heart disease		
	OR	95% C.I.	P value
None	1.43	1.00–2.03	0.049
Group*	1.55	1.06–2.26	0.024
HDL	1.63	1.12–2.38	0.011
Group* + HDL	1.68	1.15–2.47	0.008
Group* + HDL + nondiuretics	1.54	1.04–2.28	0.029

Each row depicts a new model that includes the listed covariates as potential confounders.

\*Age, alcohol use, smoking status, blood pressure, LDL cholesterol, triglycerides, serum glucose, use of diuretics for hypertension.

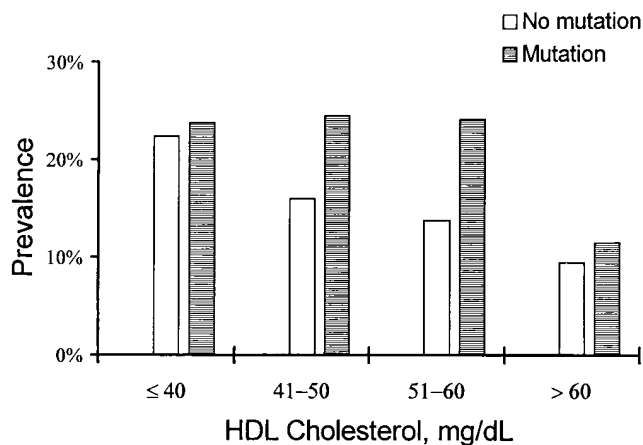


Figure 3. Prevalence of men with definite coronary heart disease among four strata of HDL cholesterol for two groupings of men with and without a CETP mutation. HDL strata cut-points are approximately equal to quartiles.

were  $68.7 \pm 8.8$  (mean  $\pm$  SD) years old when they were recognized as having CHD, while those with a mutation were  $68.8 \pm 11.2$  years. There appears to be no difference in age of onset of CHD in men with and without mutations among survivors to exam 4.

## Discussion

This study was undertaken to test the hypothesis that genetic deficiency of cholesteryl ester transfer protein (CETP) is associated with a decreased prevalence of coronary heart disease (CHD) (21). This hypothesis was not supported by the data. In fact, genetic CETP deficiency was associated with increased CHD prevalence and was a significant, independent risk factor for CHD. This result is consistent with recent studies in CETP transgenic mice demonstrating an anti-atherogenic effect of CETP (33), and a report describing premature CHD and corneal arcus in subjects with genetic CETP deficiency and low levels of hepatic lipase activity (34).

The most likely explanation for the excess CHD associated with genetic CETP deficiency is related to the role of CETP in reverse cholesterol transport. CETP promotes exchange of HDL cholesteryl ester for triglycerides of triglyceride-rich lipoproteins. The subsequent actions of hepatic lipase on triglyceride-rich HDL promotes formation of smaller HDL particles which may be both optimal effectors of cellular cholesterol efflux and substrates for the LCAT reaction (6, 7, 21). In CETP transgenic mice there is enhanced plasma CE formation (35), whereas in human CETP deficiency plasma CE formation is impaired (36). Thus, CETP action in vivo appears to enhance cholesterol esterification. Increased CHD associated with combined CETP and hepatic lipase deficiency is also consistent with this explanation (34).

The Honolulu Heart Program is a true population-based survey of Japanese-American men. Thus, it provides a more objective picture of the impact of genetic CETP deficiency in a general population than earlier reports based on small clinic samples (22–25). Thus, the D442 mutation was associated with only about a 10% mean increase in HDL chol, and the int14A change with a larger 32% change. Heterozygous mutations

were associated with decreased triglyceride and glucose levels (Table II), an observation not previously appreciated (22–25). Heterozygous CETP deficiency was associated with a substantial (35%) decrease in plasma CETP at all HDL levels (Fig. 2). Since CETP may influence HDL speciation and LCAT activity (21), the impact of genetic deficiency on reverse cholesterol transport may be more pronounced than simple changes in HDL-chol suggest.

An important aspect of our findings was that the increased risk of CHD in subjects with CETP gene mutations was largely present in individuals with HDL cholesterol 40–60 mg/dl. Those with HDL cholesterol > 60 mg/dl had a low CHD prevalence, comparable to non-mutants (Fig. 3). This is consistent with earlier reports of low CHD prevalence in subjects with CETP deficiency and high HDL (23), including the recent report (34) of combined hepatic lipase and CETP deficiency where CHD prevalence was only 9% in males and 2% in females with marked hyperalphalipoproteinemia and genetic CETP deficiency even though selected from a hospital based population.

The subjects with no or small HDL increases who experienced excess CHD nonetheless had substantially decreased plasma CETP levels, on average 35% (Fig. 2). Heterozygous CETP deficiency, while causing modest change in HDL cholesterol, is associated with twofold increases in the ratio of HDL-2 and HDL-3 and an absolute deficiency of smaller HDL species (23) which have been implicated in cellular cholesterol efflux (7). The defects in plasma cholesterol esterification seen in subjects with genetic CETP deficiency (36) are also observed in D442G heterozygotes. (H. Oliveira, A. Inazu, H. Mabuchi, and A. Tall, unpublished observation). Thus, a defect in reverse cholesterol transport is plausible as the explanation for excess CHD in heterozygous CETP deficiency. The lower CHD prevalence at HDL-chol > 60 mg/dl could indicate that a sufficient increase in HDL particle number overcomes a qualitative defect in the ability of HDL to participate in reverse cholesterol transport. Alternatively, there may be a separate anti-atherogenic effect of HDL, such as anti-oxidant (11, 12) or anti-aggregatory effects (13, 14) which overcomes the defect in reverse cholesterol transport at very high HDL levels.

The recent results in CETP transgenic mice, showing an anti-atherogenic effect, support the present findings, but indicate that the effects of CETP on atherogenesis depend on the metabolic context. Overexpression of the CETP transgene by itself resulted in decreased HDL cholesterol levels (35) and an increase in early atherosclerotic lesions of the proximal mouse aorta (33, 37). However, in humans VLDL and LDL levels are much higher than in mice and the cholesteryl ester transfer process is driven by hypertriglyceridemia (21). To mimic the human physiology (38, 39), the CETP transgene was bred into a background of hypertriglyceridemia due to apo CIII transgene overexpression (33, 40). In this setting CETP gene expression was anti-atherogenic (33). In hypertriglyceridemic mice with CETP expression the formation of small potentially anti-atherogenic HDL particles is markedly increased (40). Thus, the specific interactions of hypertriglyceridemia with CETP expression in mice is consistent with an anti-atherogenic effect of CETP related to reverse cholesterol transport.

There are a number of limitations of the present study. First, the numbers of men with mutations was relatively small and are significant only for the D442G mutation. Although it is

unlikely the findings arose by chance, they could reflect the effect on CHD of a confounding variable. For example, there could be unknown genes influencing CHD risk and associated with the D442G mutation as a result of genetic admixture or linkage disequilibrium. Second, the subjects are elderly men, and represent the survivors of the original 8,006 men in the Honolulu Heart Program cohort. Conceivably, CETP gene mutation could delay the onset of CHD, and then cause an increased prevalence of CHD in surviving older mutants. However, the mean age of onset of CHD was similar in men with and without mutations. Also, the prevalence of CETP mutations was lower in the elderly men in this study (5.6%) compared to a younger group of Japanese men [9% (25)]. Thus a delayed onset of CHD due to the mutation seems unlikely. Moreover, the general inverse relationship between HDL levels and CHD is observed in the elderly (4), and was seen in this study (Fig. 3). Clearly, genetic CETP deficiency, while increasing HDL, results in a distinctive relationship between HDL and CHD (Fig. 3). Overall, it will be important to confirm these findings in a younger age group, with other and larger numbers of CETP mutations, and preferably in a prospective study design.

If confirmed, there will be practical implications. CETP inhibition by drugs has been suggested as a therapeutic approach for increasing HDL levels (22). The results (Fig. 3) suggest that for this to be effective, HDL cholesterol > 60 mg/dl would have to be achieved. In subjects with genetic CETP deficiency and CHD, it may be desirable to try to increase HDL levels to > 60 mg/dl, for example, by weight loss, exercise, moderate alcohol intake, cessation of cigarette smoking, or by administration of appropriate drugs. Finally, the paradoxical relationship between mutation, HDL levels, and CHD suggests that an elucidation of the genetic determinants of HDL levels will provide novel information on CHD risk.

Glomset (5) first proposed that HDL, LCAT and cholesteryl ester transfer in plasma are involved in a process of reverse cholesterol transport with anti-atherogenic consequences. However, LCAT deficiency is not sufficiently common to allow an objective, population based assessment of its impact on CHD. The present findings of excess CHD associated with genetic CETP deficiency are consistent with the general concept that a defect in reverse cholesterol transport results in an increase in CHD. This conclusion is the more striking since an increased CHD prevalence in men with genetic CETP deficiency is observed despite higher HDL cholesterol concentrations.

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