LETTER TO JMG

Association of genetic variants in the HDL receptor, SR-B1, with abnormal lipids in women with coronary artery disease

J J McCarthy, Lehner T, C Reeves, D J Moliterno, L K Newby, W J Rogers, E J Topol, for the GeneQuest investigators*

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Plasma lipid concentrations are complex traits having both environmental and genetic determinants. About half of the variation in HDL-C and TG may be genetic[1–3] with the same genes possibly accounting for the correlated traits of LDL particle size, TG, and HDL-C.[4] A number of variants in candidate genes have been implicated in the regulation of plasma lipid levels.[5] In addition, genome scans have identified chromosomal regions with suggestive linkage to the TG:HDL-C ratio and other lipid parameters.[6 7] Nonetheless, much of the genetic variability in HDL-C and TG levels remains unexplained.

Decreased HDL cholesterol (HDL-C) and raised triglyceride (TG) levels are well known risk factors for the development of coronary artery disease (CAD) and atherosclerosis.[8] Plasma TG and HDL-C levels are highly correlated, but there is some evidence that they may exert independent effects on coronary disease risk.[9] Indeed, the simultaneous use of both may more accurately predict risk of coronary disease. Among patients with low HDL-C, risk of CAD was more than doubled in those with concurrent high TG compared to those with low TG levels in both the PROCAM[10] and Helsinki Heart studies.[11] Furthermore, the ratio of TG:HDL-C has been shown to correlate with LDL particle size[12] and is a powerful predictor of future myocardial infarction.[13]

The scavenger receptor class B type 1, SR-B1, is a key component in the reverse cholesterol transport pathway where it binds HDL-C with high affinity and is involved in the selective transfer of lipids from HDL-C.[14 15] It is expressed primarily in liver and non-placental steroidogenic tissues and mediates selective cholesterol uptake by a mechanism distinct from the classical low density lipoprotein cholesterol (LDL-C) receptor pathway.[16] Previous studies[17 18] have found single nucleotide polymorphisms (SNPs) in the gene for SR-B1, *SCARB1*, associated with plasma lipids. In one of these studies by our group,[18] three populations of diabetic kindred showed an association between combinations of SNPs in *SCARB1* and HDL-C that appeared to be modified by gender. We sought to confirm and extend these findings in a case series of patients with premature, familial CAD and therefore enriched for low HDL-C. The most common cause of low HDL-C associated with CAD is hypertriglyceridaemia.[19] Accordingly, we chose to examine associations between *SCARB1* SNPs and HDL-C, TG, and the TG:HDL-C ratio.

METHODS
Study populations
White subjects were drawn from the GeneQuest study, a collection of small nuclear families ascertained for premature

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*Listed in the Appendix

Key points

- Decreased HDL cholesterol and raised triglyceride levels are well known risk factors for atherosclerosis whose genetic basis remains largely unexplained. We examined polymorphisms in the gene for the HDL receptor, SR-B1 (*SCARB1*), to determine their association with plasma lipids. The association of the four most common polymorphisms in *SCARB1* with HDL cholesterol, triglyceride levels, and their ratio (TG:HDL-C) was evaluated in 371 white coronary artery disease patients.

- Combined genotypes at exon 8 (silent) and intron 5 were associated with triglyceride levels (p=0.001), the TG:HDL-C ratio (p=0.0003), and HDL cholesterol (p=0.07) in women. In our study, this combination of genotypes accounted for 19% of the variance in triglycerides, 22% of the variance in the TG:HDL-C ratio, and 10% of the variance in HDL cholesterol in women. These same associations were not found in men (p>0.60 for all).

- Women heterozygous for both polymorphisms had mean HDL cholesterol=0.65 mmol/l, mean triglycerides=8.87 mmol/l, and TG:HDL-C ratio=16.93. This combination was far less frequent in women (4%) than in men (15%, p=0.03), suggesting a selective disadvantage.

- Genetic variants in the HDL receptor, SR-B1, may be an important determinant of abnormal lipoproteins in women and confer particular susceptibility to coronary artery disease.

CAD at 15 US medical centres. Each proband was required to have expressed CAD by the age of 45 if male or 50 if female, and have at least one living sib also meeting this criterion. The institutional review board at each participating institution approved the protocol, and all patients gave informed consent to participate. At enrolment, clinical data and non-fasting blood samples, obtained by drawing into tubes containing EDTA, were collected. Plasma HDL-C, total cholesterol, and TG levels were measured using standard procedures at a central laboratory at the Cleveland Clinic. Genomic DNA was isolated from peripheral blood lymphocytes using the Puregene kit (Gentra Systems Inc) according to the manufacturer's suggested protocol at a commercial laboratory. For the purpose of the current study, a case series of unrelated subjects with CAD was selected such that only one from each family was represented, giving preference to the sib with the earlier age of onset.
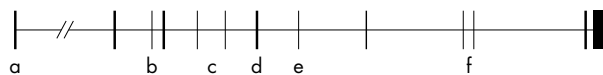
Figure 1 Diagram of *SCARB1* gene showing position of SNPs examined in this study. Vertical lines are exons, horizontal lines are introns. Average exon size is ~200 bp. Average intron size is ~3 kb. Intron 1 is ~50 kb. SNPs are as follows: a=EX1, exon 1 (G→A) missense change from glycine to serine; b=EX3, exon 3 (G→A) missense change from valine to isoleucine; c=IVS5, intron 5 (C→T); d=EX7, exon 7 (C→T) silent; e=EX8, exon 8 (C→T) silent; f=IVS10, intron 10 (C→G).

To uncover possible selective pressures on subjects with certain *SCARB1* genotypes, we estimated SNP genotype frequencies within a general US white population sample, comparing frequencies between men and women. The 46 men and 79 women over the age of 50 years used in this analysis were identified through random digit dialling in Atlanta, Georgia. The local institutional review board approved this collection.

Genotyping *SCARB1* variants
The six *SCARB1* SNPs evaluated in this study are shown in fig 1. Genotypes were obtained using the 5' nuclease assay with allele specific *Taq*Man probes.[20] PCR conditions, oligonucleotides primers and probes are available from the authors upon request.

Statistical analysis
Linkage disequilibrium was assessed with the normalised disequilibrium parameter,[21] D', using the EM algorithm.[22] All other analyses were performed using the SAS statistical package version 6.12 (SAS Institute Inc). The association of *SCARB1* genotypes with HDL-C, TG, and the TG:HDL-C ratio was assessed using the general linear model procedure (PROC GLM). Since TG levels are not normally distributed, both TG and the ratio of TG:HDL-C were log transformed before analysis. Regression diagnostics were run on both variables to ensure normality of distribution and equal variance. Each SNP was defined as a "class" variable with three levels representing the three possible genotypes (homozygous variant, heterozygous, and homozygous wild type). Because the homozygous variant genotype for IVS10 was found in only one person, this genotype was grouped with heterozygotes for analysis. Hardy-Weinberg equilibrium was assessed with a chi-square goodness of fit.

To determine the effect of *SCARB1* variants on lipid levels, we ran linear regression models with the following variables: individual SNPs (EX1, IVS5, EX8, and IVS10) and pairwise combinations of SNPs showing significant linkage disequilibrium (D'>0.30 and p<0.05); possible confounders including NIDDM status, BMI, age, gender, and lipid lowering drug use; and interaction terms for genotype combinations and gender to assess effect modification. Combinations were evaluated as interaction terms, as an indirect means of assessing the effect of haplotypes. Three way SNP interactions were not evaluated owing to small sample size. The full model was run using a backwards stepwise elimination procedure, with care taken to keep main effects in the model in cases where significant interaction was present.
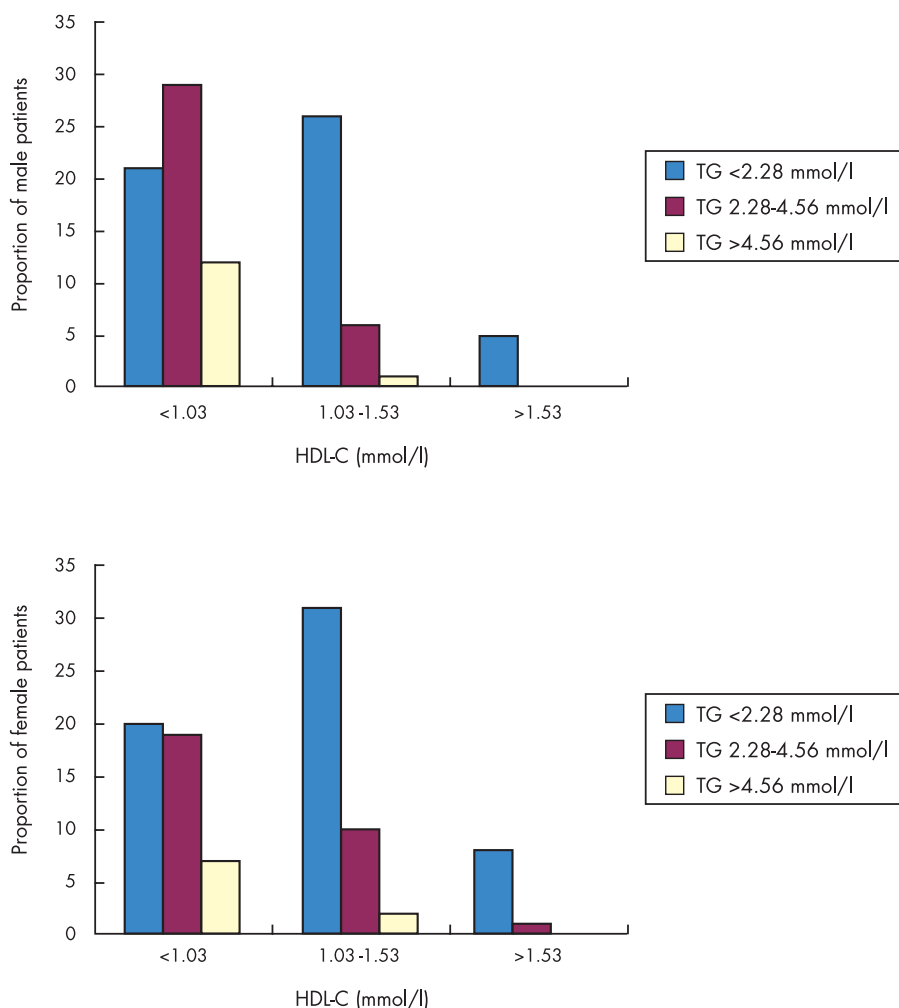

Figure 2 Proportion of patients from the GeneQuest study by HDL-C and TG category. Top panel, men. Bottom panel, women.

Table 1 Mean levels (SD) of HDL-C, TG, and TG:HDL-C ratio by combined genotypes at IVS5 and EX8 for women. Units of HDL-C and TG are mmol/l; p values for TG and TG:HDL ratio are for log transformed data

IVS5	EX8	No	Mean HDL-C	Mean TG	Mean TG:HDL-C
CT	CT	3	0.65 (0.27)	8.87 (6.50)	16.93 (13.74)
CT	TT	1	0.93	9.09	9.76
CC	CC	20	1.10 (0.29)	2.87 (2.32)	2.96 (2.68)
CC	CT	44	1.11 (0.30)	2.43 (1.59)	2.50 (2.10)
CC	TT	22	1.06 (0.27)	2.00 (0.85)	1.98 (0.91)
CT	CC	10	1.25 (0.30)	2.04 (1.26)	1.71 (1.15)
			p=0.07	p=0.001	p=0.0003

When significant genotype by gender interaction was detected, men and women were then examined separately in order to assess the magnitude of effect, including proportion of variance explained, for each gender. Differences in mean lipid levels by genotype combination were determined using a general linear model containing only those SNPs or SNP combinations which were significant (p<0.05). Significance was determined using the F statistic for the model. The proportion of variance in lipid levels owing to genetic variation at the *SCARB1* locus was estimated using the R square statistic.

RESULTS

The study population included 113 female and 258 male CAD patients. At enrollment, subjects ranged in age from 29 to 72 years with a mean of 47.3 years for women and 47.7 years for men. Patients were retrospectively ascertained with an average time from their qualifying event to enrollment of 6.8 years (range 0-30 years) for women and 9.3 years (range 0-42 years) for men. Because all subjects were chosen from families originally ascertained for CAD, they are enriched for CAD risk factors including low HDL-C, high TG, high BMI, and type 2 diabetes mellitus (NIDDM). The mean BMI for women was 29.1 (range 16-53) and for men 29.7 (range 19-61). Fifteen percent of women and 8% of men had NIDDM. The distribution of HDL-C and TG levels in the population are shown in fig 2 for men and women. Forty-seven percent of women and 63% of men had low HDL-C, below 1.03 mmol/l (40 mg/dl). Among those with low HDL-C, 57% of women and 66% of men also had raised TG levels, above 2.28 mmol/l (200 mg/dl). Fifty-five percent of women were postmenopausal and 59% of postmenopausal women were taking hormone therapy.

Variant allele frequencies were 0.12 for EX1_A, 0.006 for EX3_A, 0.08 for IVS5_T, 0.01 for EX7_T, 0.51 for EX8_C, and 0.06 for IVS10_G. All SNPs were in Hardy-Weinberg equilibrium (p>0.10). Because EX3_A and EX7_T were rare, they were not included in statistical analyses. No evidence was found for linkage disequilibrium (a non-random association or correlation) between the EX1 polymorphism and any of the other three common polymorphisms (D'=0.21, 0.23, and 0.36 for linkage disequilibrium with IVS5, EX8, and IVS10, respectively). Strong, significant linkage disequilibrium was found between three pairs: EX8 and IVS5 (D'=0.68, p<0.0001), EX8 and IVS10 (D'=0.65, p=0.006), and IVS5 and IVS10 (D'=0.99, p=0.05), suggesting that these three variants may be inherited together as a haplotype. The combined effects of these SNP pairs were evaluated further.

Results of linear regression analysis showed that HDL-C levels were significantly associated with BMI (p<0.0001)and combinations of genotypes at two sites in *SCARB1*, the effect of which was modified by gender. Significant interaction was found for gender, the IVS5 SNP, and the EX8 SNP (gender*IVS5*EX8, p=0.009). We carried out similar analyses

examining TG as the dependent variable and again found BMI (p=0.0102) and the interaction term gender*IVS5*EX8 (p=0.0009) to be significant. Finally, to account for the combined effect of low HDL-C and high TG levels, we examined the ratio of TG:HDL-C and once more found significant association for BMI (p=0.0005) and the interaction term gender*IVS5*EX8 (p=0.0003). Variables not significantly associated with lipid levels in all three models included lipid lowering drug use, NIDDM, age, the IVS10 SNP, and the EX1 SNP.

General linear models run for men and women separately showed striking differences. Significant interaction between the IVS5 and EX8 SNPs (IVS5*EX8) was found for HDL-C (p=0.016), TG (p=0.0004), and for the TG:HDL-C ratio (p<0.0001) in women. These results remained virtually unchanged (<10% difference in p values) with BMI in the model, suggesting that BMI is not a confounder of the observed associations. In men, no significant interactions in the association of HDL-C (p=0.224), TG (p=0.445), or TG:HDL-C (p=0.274) were found for IVS5*EX8 or any other combination of SNPs. No individual *SCARB1* SNPs were associated with lipid levels either.

Mean levels of HDL-C, TG, and the TG:HDL-C ratio in women by combined IVS5 and EX8 genotypes are presented in table 1. Significant differences were found between genotype combinations of IVS5 and EX8 for TG (p=0.001) and TG:HDL-C ratio (p=0.0003). The association with HDL-C was borderline significant (p=0.07). The amount of variance in serum lipid levels explained by the combined genotypes of EX8 and IVS5 in women was 19.4% for TG, 21.9% for the ratio of TG:HDL-C, and 10.1% for HDL-C. The two most infrequent combinations of genotypes gave the lowest mean level of HDL-C, highest mean level of TG, and the highest TG:HDL-C ratio. The one woman who was heterozygous at IVS5 (CT genotype) and homozygous at EX8 (TT genotype) had an HDL-C of 0.93 mmol/l (36 mg/dl), TG of 9.09 mmol/l (797 mg/dl), and TG:HDL-C ratio of 9.76. Three women heterozygous at both IVS5 (CT) and EX8 (CT) had the lowest mean level of HDL-C, 0.65 mmol/l (25.0 mg/dl), high mean level of TG, 8.87 mmol/l (778 mg/dl), and highest TG:HDL-C ratio (16.93). Other characteristics of these four women are shown in table 2. When the data were reanalysed removing the four women with the "risky" genotype combinations, the variance in the TG:HDL-C ratio explained by *SCARB1* fell to 2.5%.

Because the serum lipid levels of the three women heterozygous at both IVS5 and EX8 were extreme, we hypothesised that women with this combination (3% in the GeneQuest series) may experience reduced fitness and therefore be subject to negative selection. To test this indirectly, we compared the frequency of the combination between men and women from GeneQuest and found a higher frequency in men, which was borderline significant (9%, p=0.07). We also compared the frequency between men

Table 2 Additional characteristics of the four women with IVS5-EX8 combinations associated with extreme TG:HDL-C ratios

	Patient A	Patient B	Patient C	Patient D
IVS5 genotype	CT	CT	CT	CT
EX8 genotype	TT	CT	CT	CT
HDL-C (mmol/l)	0.93	0.96	0.47	0.52
TG (mmol/l)	9.09	2.71	8.23	15.66
BMI	33	29	33	34
Diabetes	NIDDM	No	NIDDM	IDDM
Age at enrolment	44	45	52	49
Qualifying event	MI	MI	MI	MI
Age at event	32	45	34	39
Hypertension	Yes	No	No	Yes

and women >50 years old from a general US white population sample. The frequency in women, 0.038 (95% confidence interval 0.008 to 0.107), was significantly lower than in men, 0.152 (95% confidence interval 0.063 to 0.289), Fisher's two tailed p value 0.038.

To determine if the observed associations could have been detected by examining anything less than a three way interaction, we tested the association of each of the SNPs individually and in pairs and found no significant associations (p values ranged from 0.18 to 0.95). When we tested the interaction between each individual SNP and gender, we found IVS5*gender significant for TG (p=0.05) and borderline significant for the TG:HDL-C ratio (p=0.07).

Two additional variants were genotyped in this population including a silent SNP in exon 7 (EX7) and a missense SNP in exon 3 (EX3). No women carried the EX3 variant and only two carried the EX7 variant. One had an HDL-C of 0.72 mmol/l, TG of 1.88 mmol/l and the other had an HDL-C of 1.01 mmol/l, TG of 1.50 mmol/l.

DISCUSSION

We have found evidence for an association between a combination of two common variants in the *SCARB1* gene and plasma lipid levels in female patients with coronary artery disease. Combined genotypes for a silent variant in exon 8 and the intron 5 SNP were strongly associated with the ratio of TG:HDL-C, an important risk factor for atherosclerosis and a component of the metabolic syndrome. These two variants accounted for nearly a quarter of the variability in this trait in women from our study, almost exclusively owing to the presence of one of two relatively uncommon combinations of genotypes that were associated with extreme lipid levels. Interestingly, the same associations were not found in men, suggesting that the genetic basis of dyslipidaemias and the attendant liability for coronary disease may differ between men and women.

Dyslipidaemia is a common and heterogeneous manifestation of premature, familial CAD. Among patients from the GeneQuest study, nearly half of all women and two-thirds of men had low HDL-C, often but not always accompanied by raised TG levels. This represents a substantial increase relative to an age and race comparable group from NHANES III, where only 14.5% of women and 40.5% of men had HDL-C<1.03 mmol/l. By examining a population enriched for lipid abnormalities, we were able to identify a specific combination of genotypes (IVS5 CT and EX8 CT) occurring in three women, which was associated with extremely low HDL-C and extremely high TG in women.

Subjects with genetic variants associated with deleterious phenotypes, such as extreme lipid profiles, may experience increased mortality relative to those subjects without the genetic variants. Therefore, we are likely to see a reduced frequency of these genetic variants in the population. In our study, the SR-BI variants were associated with extreme lipid abnormalities in women, but not in men. When we examine the frequency of the SR-BI genotype combination (IVS5 CT and EX8 CT) within an older general population, we in fact see a lower frequency in women (4%) compared to men (15%), suggesting that women with these variants may be experiencing greater mortality than men.

The association of *SCARB1* polymorphisms with low HDL-C in our study remained significant after controlling for NIDDM status, BMI, age, and lipid lowering medication use suggesting a lack of confounding by these variables. Other variables known to be associated with serum lipids such as alcohol and smoking were not included in our analysis because of the retrospective nature of our study. While it is unlikely that they would be confounding factors, they may modify the genetic effect through interaction. Future prospective studies should address their effects.

This is not the first study to consider an association between genetic variants in *SCARB1* and serum lipids. One previous study among a diabetic kindred by our group[18] also found the combined genotypes at EX8 and IVS5 to be associated with HDL-C in women and much less strongly associated in men. The other study that examined variants in *SCARB1* with serum lipids[16] was the first to describe the interaction between genotype and gender. However, in their cohort of Spanish white subjects, no associations were found between SNPs in EX8 or IVS5 and serum lipids in women. As this population comprised healthy subjects, an association with extreme dyslipidaemia would have been unlikely. Furthermore, in their analysis of haplotypes, double heterozygous subjects (IVS5 CT and EX8 CT, those very subjects showing an association with HDL-C in our population) were eliminated.

Because the IVS5 and EX8 SNPs are in linkage disequilibrium, their combined effect on lipid levels may reflect an underlying haplotype(s), or segment of DNA defined by several variants inherited together as a unit, that is the basis of the observed association. Haplotypes can be unequivocally assigned from all combinations of genotypes except for those subjects heterozygous at both IVS5 and EX8. For this reason, the critical haplotype associated with the lowest level of HDL-C and highest TG could not be precisely defined.

It is unclear whether the IVS5 and EX8 SNPs themselves play a causative role in the observed associations. The IVS5 SNP is in an intron, with no known or inferred effect on splicing or gene regulation. The exon 8 SNP is silent, resulting in no change in the amino acid sequence of the protein product. Nonetheless, the combined IVS5 and EX8 genotypes appear to be an excellent marker of an underlying genetic variant(s) in *SCARB1* leading to a raised TG:HDL-C ratio. In this study, we genotyped all known SNPs uncovered in a systematic screen of the coding region of *SCARB1* in white subjects.[17] Further exploration of intronic and regulatory regions to identify the true causative variant(s) and elucidation of their function via the binding and uptake of cholesterol esters from HDL would provide additional compelling evidence for a role of *SCARB1* genetic variants in lipid metabolism.

SCARB1 variants associated with serum lipids in our study appear to act in a gender dependent manner. Significant gene by gender interaction was found, showing an underlying association in women, but not in men. While these gender differences are remarkable, they are not unexpected. It is well known that HDL-C levels are affected by sex hormone status. Furthermore, the expression of *SCARB1* is known to be regulated by oestrogen. Oestrogen treatment of rats has been shown to downregulate the SR-B1 isoform of *SCARB1*[23][24] and upregulate the splice variant, SR-BII, in the

liver.[25] Moreover, overexpression of SR-B1 in the liver has been shown to result in a pronounced fall in plasma HDL-C.[26] It is possible that the regulation of *SCARB1* by oestrogen is influenced by genetic variants in *SCARB1*, which may have implications for the treatment of postmenopausal women with hormone replacement therapy (HRT). A recent study found the differential response of HDL-C to HRT to be dependent on presence of genetic variants in the oestrogen receptor gene.[27] In an analogous manner, *SCARB1* variants may be shown to modulate the effect of HRT on plasma lipid levels in women. We were unable to test this hypothesis directly in our study population owing to small numbers and incomplete data on HRT, but will address this topic in future studies.

None of the individual variants in *SCARB1* alone showed an association with serum lipids in our study. In fact, an association was only apparent when combinations of SNPs were examined, taking into account an interaction with gender. Once the interaction was taken into account, the association was strong and highly significant. This underscores the complex nature of dyslipidaemias and illustrates the challenges encountered in uncovering and reproducing genetic associations.

The results of our study pertain to a group of white women with a high prevalence of dyslipidaemia as well as other metabolic abnormalities and risk factors for premature CAD. It is unclear whether these same associations would be found in women without these other risk factors or in different ethnic groups. Nonetheless, our study confirms previously reported associations between variants in *SCARB1* and HDL-C in diabetic kindred and extends these findings to the TG:HDL-C ratio in women with premature coronary disease. Combinations of common SNPs in *SCARB1* may be an important determinant of high TG:HDL-C ratio among white women with CAD.

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APPENDIX

GENEQUEST INVESTIGATORS

Eric J Topol (Study Chairperson), *Cleveland Clinic Foundation.* Study Investigators and Coordinators (United States): David J Moliterno, Patricia Welsh, Monique Rosenthal, *Cleveland Clinic Foundation, Cleveland, Ohio*; Spencer B King III, William Anderson, Joe Jean Borowski, Kris Anderberg, *Emory University Hospital, Atlanta, Georgia*; David R Holmes Jr, Charanjit Rihal, Sharon McIntire-Langworthy, *Mayo Clinic, Rochester, Minnesota*; William Rogers, Ann Snider, *University of Alabama Medical Center, Birmingham, Alabama*; L Kristin Newby, Laura Drew, *Duke University Medical Center, Durham, North Carolina*; Dean Kereiakes, Eli Roth, Louise Wohlford, *The Lindner Center for Clinical Cardiovascular Research, Cincinnati, Ohio*; Anthony De Franco, Teresa Schrader, *LeBauer Cardiovascular Research Foundation, Greensboro, North Carolina*; Phillip Gainey, Sandra Arsenault, *St Joseph Hospital, Savannah, Georgia*; Paul Casale, Joann Tuzi, *Lancaster Heart Foundation, Lancaster, Pennsylvania*; Jeffrey Anderson, Juli Jerman, Rob Pearson, Ann Allen, *Latter Day Saints Hospital, Salt Lake City, Utah*; Sherwyn Schwartz, Sue Beasie, *Diabetes and Glandular Associates, San Antonio, Texas*; Frank Aguirre, Sandra Aubuchon, Kristin Weisbrod, *St Louis University Hospital, St Louis, Missouri*; Jeffrey Carney, Muriel Harris, *The Heart Group, Saginaw, Michigan*; Jim Bengtson, Mary Adolphson, *Michigan Heart and Vascular Institute, Ypsilanti, Michigan*; John Rudoff, Sue Williams, *Oregon Cardiology Clinic, PC, Portland, Oregon*; Jeanette McCarthy, Laura Carleu, *Millennium Pharmaceuticals, Cambridge, MA, USA.*

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Authors' affiliations

J J McCarthy, School of Public Health, San Diego State University, San Diego, CA, USA
T Lehner, Rockefeller University, New York, NY, USA
C Reeves, Millennium Pharmaceuticals Inc, Cambridge, MA, USA
D J Moliterno, E J Topol, Cleveland Clinic Foundation, Cleveland, OH, USA
L K Newby, Duke University Medical Center, Durham, NC, USA
W J Rogers, University of Alabama Medical Center, Birmingham, AL, USA

Correspondence to: Dr J J McCarthy, School of Public Health, San Diego State University, 5500 Campanile Drive, San Diego, CA 92182-4162, USA; mccarthy@mail.sdsu.edu

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