Association Analysis of Allelic Variants of USF1 in Coronary Atherosclerosis

Kati Kristiansson, Erkki Ilveskoski, Terho Lehtimäki, Leena Peltonen, Markus Perola, Pekka J. Karhunen

Objective—USF1 regulates the transcription of more than 40 cardiovascular related genes and is well established as a gene associated with familial combined hyperlipidemia, a condition increasing the risk for coronary heart disease. No detailed data, however, exists on the impact of this gene to the critical outcome at the tissue level: different types of atherosclerotic lesions.

Methods and Results—We analyzed the USF1 in 2 autopsy series of altogether 700 middle-aged men (the Helsinki Sudden Death Study) with quantitative morphometric measurements of coronary atherosclerosis. SNP rs2516839, tagging common USF1 haplotypes, associated with the presence of several types of atherosclerotic lesions, particularly with the proportion of advanced atherosclerotic plaques (P=0.02) and area of calcified lesions (P<0.001) of the coronary arteries. Importantly, carriers of risk alleles of rs2516839 also showed a 2-fold risk for sudden cardiac death (genotype TT versus CC; OR 2.10, 95% CI 1.17 to 3.75, P=0.04). The risk effect of rs2516839 was present also in aorta samples of the men.

Conclusions—Our findings in this unique study sample suggest that USF1 contributes to atherosclerosis, the pathological arterial wall phenotype resulting in coronary heart disease and in its most dramatic consequence—sudden cardiac death.

(Key Words: atherosclerosis ■ coronary ■ genes ■ genetics ■ death, sudden)

In addition to established risk factors such as elevated total cholesterol levels, hypertension, smoking, and obesity, the progression of coronary heart disease (CHD) is modified by a still unknown composition of risk alleles of genes interacting with lifestyle risks. We initially identified USF1 as the first major gene associated with familial combined hyperlipidemia (FCHL),1 a phenotype characterized by elevated levels of serum total cholesterol, or triglycerides, or both. Later, several other studies also implied an association between the USF1 gene and severe common aberrations in lipid and glucose metabolism.2–12 A feature common to these aberrations is their contribution to the risk for premature CHD and myocardial infarction (MI). We have recently shown that allelic variants of the upstream transcription factor 1 (USF1) gene affect the prospective risk for cardiovascular disease (CVD) in two large population-based studies.13 Thus the USF1 gene is a promising candidate to contribute to the complex genetic background of CHD. The gene is biologically highly relevant, it encodes a ubiquitously expressed transcriptional regulator of several genes that are functionally important for the lipid accumulation, inflammation, and thrombotic complications of the coronary plaque.14–17 However, limited data so far exists on the association of the USF1 gene and the critical biological outcome: the extent and severity of coronary atherosclerosis. To study whether allelic variants of the USF1 relate to the progression of coronary atherosclerosis, we used an autopsy series of middle-aged Finnish men with detailed quantitative measurements of various types of atherosclerotic lesions in coronary arteries. Our data suggests that specific USF1 alleles contribute to coronary atherosclerosis.

Methods

The Prospective Autopsy Series of Middle-Aged Men

The Helsinki Sudden Death Study (HSDS) comprised 2 prospective series of altogether 700 men, aged 33 to 70 (mean age 53; Table 1), who underwent a medicolegal autopsy.16,19 The 2 series were collected at 10-year intervals; the first series (n=400) during 1981 to 1982 and the second series (n=300) during 1991 to 1992.

Protocols of 2 international studies were used to define atherosclerosis.20,21 The areas of different types of lesions were expressed...
in percentages by dividing the lesion area by the total area of the artery sample and multiplying it by 100%.

**USF1 Polymorphisms**
To comprehensively analyze the allelic diversity of the USF1 gene locus, htSNPs rs10908821, rs2774276, rs2073658, rs2516839, rs1556259, and rs2774279 (Figure 1) were first genotyped in the 1991 to 1992 autopsy series. SNPs rs2073658, rs2516839, and rs2774279 capturing the 2 most common haplotypes of USF1 (Figure 1) were genotyped in the older 1981 to 1982 series.

**Statistical Analysis**
The linkage disequilibrium analysis and estimation of haplotype frequencies were performed for the HSDS 1991 to 1992 series with the publicly available Haploview software version 3.2.22 The distributions of the continuous atherosclerosis variables were highly skewed (Table 1). We therefore modeled the risk of atherosclerosis with age, BMI, and series-adjusted ordinal regression model (cumulative logit model) where quartiles of the atherosclerotic variable were the ordinal outcome (supplemental Table I, available online at http://atvb.ahajournals.org).

**Results**
The cause of death for one third of the study subjects was sudden cardiac death (SCD) attributable to coronary heart disease (CHD) with or without myocardial infarction (MI; Table 1). In detailed computer-assisted morphometric measurement of different types of atherosclerotic plaques, over 90% of the study subjects had signs of early atherosclerosis and as many as 40% had areas of complicated atherosclerotic lesions in their coronary arteries (Table 1).

We genotyped a total of 6 SNPs covering the 5.7 kb USF1 gene and defining a total of 5 haplotypes in the Finnish population because of the high extent of linkage disequilibrium between the SNPs in this population (Figure 1). These haplotype-tagging SNPs facilitated the monitoring of most of the allelic diversity of USF1 in Finns.

**Association of USF1 Variants With Atherosclerosis**

**Coronary and Aortic Atherosclerosis**
In both autopsy series, collected with a 10-year interval, a significant risk effect of the T-allele of SNP rs2516839 was observed in ordinal regression analysis: Carriers of 2 risk alleles were 2.4 times as likely to have more severely calcified coronary arteries in the 1981 to 1982 series (OR 2.39, 95% CI 1.30 to 4.40) and 3.2 times as likely in the 1991 to 1992 series (OR 3.20, 95% CI 1.68 to 6.09) than noncarriers (Figure 2). Although the risk effect of rs2516839 was in general more evident in the later autopsy series (1991 to 1992), in both series the carriers of the risk allele (T) had greater odds of having larger advanced atherosclerotic lesion areas as the noncarriers both in their coronary arteries and in abdominal aorta.

In the combined data set of the 2 autopsy series, with increased power to detect significant risk effects of the USF1 alleles, we observed htSNP rs2516839 to significantly associate with areas of several types of advanced atherosclerotic lesions with age, BMI, and series-adjusted ordinal regression model (cumulative logit model) where quartiles of the atherosclerotic variable were the ordinal outcome (supplemental Table I, available online at http://atvb.ahajournals.org).

For further details, please see supplemental materials, available online at http://atvb.ahajournals.org.

### Table 1. Characteristics of Study Series of Helsinki Sudden Death Study

<table>
<thead>
<tr>
<th></th>
<th>1981 to 1982 Series (n=400)</th>
<th>1991 to 1992 Series (n=300)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years±SD</td>
<td>53.8±9.5</td>
<td>52.1±9.6</td>
</tr>
<tr>
<td>Body mass index, kg/m²±SD</td>
<td>24.2±4.6</td>
<td>25.1±5.0</td>
</tr>
<tr>
<td>Diabetes*</td>
<td>91/332</td>
<td>22/154</td>
</tr>
<tr>
<td>Hypertension*</td>
<td>57/400</td>
<td>50/300</td>
</tr>
<tr>
<td>Smoking*</td>
<td>283/335</td>
<td>129/165</td>
</tr>
<tr>
<td>Cause of death, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sudden cardiac death</td>
<td>150 (37.5)</td>
<td>80 (27)</td>
</tr>
<tr>
<td>Other disease</td>
<td>98 (24.5)</td>
<td>100 (33)</td>
</tr>
<tr>
<td>Non-natural deaths (accidents, suicides)</td>
<td>152 (38)</td>
<td>120 (40)</td>
</tr>
<tr>
<td>Presence of coronary atherosclerotic changes, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty streaks</td>
<td>381 (95)</td>
<td>282 (94)</td>
</tr>
<tr>
<td>Fibrous lesions</td>
<td>346 (87)</td>
<td>232 (77)</td>
</tr>
<tr>
<td>Complicated lesions</td>
<td>195 (49)</td>
<td>101 (34)</td>
</tr>
<tr>
<td>Calcified lesions</td>
<td>317 (79)</td>
<td>265 (68)</td>
</tr>
<tr>
<td>&gt;50% coronary narrowing in more than one coronary artery</td>
<td>120 (30)</td>
<td>38 (17)</td>
</tr>
</tbody>
</table>

*Number of study subjects with this characteristic/number of study subjects with interview data on this risk factor available.

Figure 1. USF1 haplotypes. The genotyped USF1 polymorphisms tag 5 haplotypes. The T allele of the rs2516839 is present in the 2 most common haplotypes of USF1, and the G allele of the rs2774279 is present in the 2 least common haplotypes of USF1. The minor alleles of all other SNPs tag 1 haplotype of USF1. $R^2$ values between USF1 polymorphisms range from 0.03 to 0.51.

[984 Arterioscler Thromb Vasc Biol May 2008](http://atvb.ahajournals.org/ Downloaded from)
lesions (Table 2). T-allele of the SNP correlated with a higher probability of more severe atherosclerosis. Conversely, carrying 2 C-alleles of this SNP seemed to protect from advanced atherosclerotic lesions. Although this protective effect was statistically significant in ordinal regression analysis for several atherosclerotic lesion types in the coronary arteries, and for the area of advanced (characterized by fibrous or complicated area) and calcified atherosclerotic lesions both in coronary arteries and abdominal aorta, the trend of this protective effect was detectable for all the lesion types studied (excluding coronary fatty streaks; Table 2).

Further, although SNPs rs2073658 and rs2774279 both showed association with calcification of the coronary arteries, their risk effects were nonsignificant at the presence of the risk effect of rs2516839 (supplemental Table II). Carriers of the risk allele (T) of rs2516839, the SNP associated with coronary and aortic atherosclerosis, also had a suggestive increased risk for having >50% coronary narrowing in more than one coronary artery (for details please see supplemental materials).

This risk-associated T allele of the SNP does not tag a single haplotype of USF1, but is present in the 2 most common (61%) haplotypes of USF1 (CTCTAG and CCCTAA, Figure 1). In our study sample, CTCTAG associated with the proportion of advanced lesion area out of the total lesion area and CCCTAA associated with the extent of coronary calcification. These associations were, however, weaker than those obtained from analysis of rs2516839 genotypes (for details please see supplemental materials).

**USF1 Variants and Sudden Cardiac Death**

In the combined data set the carriers of the rs2516839 risk (TT) genotype had a 2-fold risk for SCD when compared to that of the carriers of the protective genotype (TT versus CC; OR 2.10, 95% CI 1.17 to 3.75, \(P=0.01\), \(P\) adjusted for multiple comparisons=0.04, and CT versus CC; OR 1.92, 95% CI 1.09 to 3.39, \(P=0.02\), \(P\) adjusted for multiple comparisons=0.10). In the 1991 to 1992 series, increased risk for SCD was associated with the rs2516839 risk allele (T) containing genotypes (TT versus CC; OR 2.95, 95% CI 1.11 to 7.85, \(P=0.03\), \(P\) adjusted for multiple comparisons=0.12, and CT versus CC; OR 2.94, 95% CI 1.12 to 7.72, \(P=0.03\), \(P\) adjusted for multiple comparisons=0.25). The risk effect of SNP rs2516839 on SCD in the older 1981 to 1982 series

![Figure 2. USF1 Rs2516839 genotype and atherosclerosis in coronary arteries and abdominal aorta in the 2 autopsy series. The figure shows odds ratios and their confidence intervals from ordinal logistic regression analysis which compared the CT and TT genotypes to the CC genotype. Advanced atherosclerosis=plaque area covered either by fibrotic or complicated lesion. * \(P<0.05\), † \(P<0.01\), ‡ \(P<0.001\), §Multiple testing adjusted \(P<0.05\).]("http://atvb.ahajournals.org/attachment.php?sid=35e3b8a3-c1ac-46f5-9b1c-76f5e9a8885b")

**Figure 2.** USF1 Rs2516839 genotype and atherosclerosis in coronary arteries and abdominal aorta in the 2 autopsy series. The figure shows odds ratios and their confidence intervals from ordinal logistic regression analysis which compared the CT and TT genotypes to the CC genotype. Advanced atherosclerosis=plaque area covered either by fibrotic or complicated lesion. * \(P<0.05\), † \(P<0.01\), ‡ \(P<0.001\), §Multiple testing adjusted \(P<0.05\).
failed to reach statistical significance, although we observed an OR suggesting a trend for risk (OR 1.71, 95% CI 0.82 to 3.58, \(P=0.15\)). In the older 1981 to 1982 series increased risk for SCD was, however, attributed to the closely linked AA-genotype of rs2774279 (OR 2.39, 95% CI 1.09 to 5.24, \(P=0.03\), \(P\) adjusted for multiple comparisons=0.45, when compared to that of the GG genotype).

We further tested whether the association of rs2516839 with SCD was independent of its association with coronary calcification. Fitting the calcification covariate in the model reduced the risk associated with rs2516839 from odds ratio 2.10 to 1.59 (genotype TT versus CC). Fitting other lesions types, however, led to more modest reduction in the risk associated with rs2516839 (supplemental Table III and supplemental results). These result suggested that part of the increased SCD risk associated with rs2516839 was attributable to the contribution of rs2516839 to the development of severe coronary artery disease characterized by coronary calcification.

**Discussion**

Here we have studied the impact of allelic variants of USF1 gene on the biological outcome of disturbed lipid metabolism and vessel wall endothelial function; the atherosclerosis of coronary arteries. We observed an association of USF1 variants with coronary narrowing and several atherosclerotic lesion phenotypes, measured with computer-assisted planimetry, both in coronary arteries as well as in abdominal aorta. The risk effect of USF1 was evident in two distinct autopsy series, and we observed consistent and significant associations in the data after adjusting for multiple comparisons.

The risk effect of USF1 was more evident in the later autopsy series (1991 to 1992). There the proportion of study subjects affected with advanced atherosclerotic lesions was smaller than in the 1981 to 1982 series (Table 1). This decrease is likely to be explained by reduced clustering of lifestyle risk factors, which can increase the environmental “noise” and complicate genetic analyses in the 1981 to 1982 series compared to the 1991 to 1992 series. Nevertheless, indication of the risk effect of USF1 was present in both series.

Importantly, the atherosclerosis-associated USF1 variant significantly increased the risk for the most severe consequence of atherosclerosis: prehospital sudden cardiac death. SCD is nowadays the most significant contributor to mortality from CHD, in particular in middle age, where up to 80%
of deaths attributable to CHD belong to this category.\textsuperscript{23–25} Previous studies have suggested a role for USF1 in the etiology of CHD; we initially identified USF1 as the first major gene associating with familial combined hyperlipidemia (FCHL) in families who were ascertained through a proband with premature CHD. Subsequently, we observed USF1 variants to contribute to the risk of incident cardiovascular disease among women.\textsuperscript{13} Although we observed an association between USF1 variants and CHD also at our present study, our results were not directly comparable with the previous findings as our study sample consisted of men only. Interestingly, in our study, a part of the increased risk for SCD associated with USF1 was attributable to the contribution of the gene to coronary calcification. The gene, however, also seemed to have a risk effect independent from the effect of atherosclerotic lesions.

In a recent population-based cohort study, Reiner and colleagues observed a USF1 variant (rs3737787, which strongly correlates with rs2073658 of our study) to associate with risk of coronary artery calcium.\textsuperscript{12} In line with this, the amount of coronary calcification was significantly associated with variation at the USF1 locus in our study, although rs2073658 was not the most significantly associated variant. While we observed USF1 to associate also with other types of atherosclerotic lesions, all of which were significantly correlated with the amount of calcification, the association with calcification remained most substantial. Some evidence already exists on how USF1 could participate in the build up of calcified lesions in the arteries; USF1 is involved in the regulation of osteopontin expression in arterial smooth muscle cells.\textsuperscript{26,27} Osteopontin is an inhibitor of vascular calcification,\textsuperscript{28} and as a transcription factor USF1 could regulate the expression of other important genes involved in similar processes as well.

Most of the studies on the USF1 gene have examined only a few allelic variants of the gene, instead of comprehensively using the genetic variation at the USF1 locus.\textsuperscript{2–4,8,9,11,29} Here, with knowledge of the linkage disequilibrium structure at the USF1 locus in our study population, we genotyped 6 hSNPs to capture the full allelic diversity of the USF1 gene in Finns, known to show wider linkage disequilibrium intervals between the SNPs.\textsuperscript{30} The SNP showing the strongest evidence of an association with atherosclerosis and SCD was rs2516839, tagging the 2 most common haplotypes of USF1.

SNP rs2516839, located in an untranslated exon of USF1, did not consistently associate with serum triglyceride levels in Finnish familial combined hyperlipidemia families, although a suggestive association was observed in one of the analyses.\textsuperscript{1} The common allele (T) of the SNP, the risk allele in our data, associated with higher cholesterol and triglyceride levels also in Utah families ascertained for type 2 diabetes.\textsuperscript{7} In another study, our protective genotype (C) of SNP rs2516839 showed suggestive association with lower risk of metabolic syndrome in Chinese hospital cases.\textsuperscript{6} However, in a population based data set from the Finns the C-allele of rs2516839 was associated with higher lipid values among study subjects with CVD.\textsuperscript{13} Unfortunately, we do not have data on the cholesterol levels of our study sample and thus could not directly relate atherosclerotic lesion areas to cholesterol levels, and the exceptional ascertainment of our study population prevents direct comparisons with previous studies. What emerges from this study is evidence of association of USF1 with quantitative atherosclerotic phenotypes of arterial wall, the tissue level biological end state resulting in CHD.

The previous studies underline the general influence of USF1 in regulating the expression of critical genes of lipid and glucose metabolism. As a transcription factor regulating the expression of more than 40 cardiovascular related genes\textsuperscript{29} involved in lipid metabolism, hemostasis, inflammation, and endothelial function, USF1 could contribute to the development of atherosclerosis and its complications through various pathways. Evidence of these contributions already exists for lipolysis,\textsuperscript{4,8} inflammation,\textsuperscript{1,12,29} and now for calcification from our study and Reiner and colleagues.\textsuperscript{12} Variation at the USF1 locus could thus contribute to the increased risk of CHD among FCHL patients by affecting lipid metabolism related pathways, which increase the levels of serum lipids. Another separate pathway leading to an increased risk of CHD could stem from the effect of USF1 on coronary calcification, and suggestive evidence for such separate effect already exists.\textsuperscript{12} This separate effect could involve inflammation pathways with markers such as CRP,\textsuperscript{14,31} USF1 variants could also contribute to SCD via factors affecting plaque stability. How exactly the effect of USF1 is related in these different pathways remains to be assessed with further studies, for instance with studies addressing the relationship between the USF1 gene variants and arterial transcript profiles.

In addition to the role of USF1 in various atherosclerosis pathways, its genetic analysis is possibly complicated by other genetic and environmental factors. The effect of USF1 could be cell type– or tissue-specific, involve interaction with other genes, and depend on different hormonal/environmental cues. Some evidence of sex- or age-specific effects of USF1 already exists.\textsuperscript{1,10–13} Further, the influence of genetic variation in USF1 could be more readily observed in subjects with certain pathophysiological condition, such as CHD, diabetes, or obesity. These gene–gene or gene-environmental interactions may explain some of the heterogeneity between previous USF1 studies.

The number of functional genetic variants at the USF1 locus remains also unsolved. The locus could harbor several rare or common variants with distinct effects. Some evidence of the functional role of USF1 variants on the transcript levels of downstream genes already exists from fat biopsy samples.\textsuperscript{1,29} Sequencing of the complete USF1 and neighboring region in large study samples and detailed functional studies would facilitate the identification of the variants of USF1 with functional relevance.

The variant showing the strongest evidence of an association with atherosclerosis and SCD in our study, rs2516839, is located within the 5′ untranslated region of the first exon of USF1. Although untranslated variants do not alter the amino acid sequence of a protein, they can for instance affect the transcription level of mRNA, splicing patterns, and other posttranscriptional modifications of mRNA, or stability or localization of mRNA in the cell. The effect of rs2516839
and other USF1 variants on such features should be assessed in future functional studies. Rs2516839 could also be a marker for another yet unrecognized functional marker or domain.

Given the high linkage disequilibrium at the USF1 locus and at its surroundings, we cannot exclude the potential contribution of other genetic variants neighboring USF1 to the association signal we observed. Future studies, preferably in populations with reduced linkage disequilibrium at the locus, should assess the relative impact of a large number of variants in an extended chromosomal region. Accordingly, Reiner and colleagues suggested in their USF1 study that “the true susceptibility effect on mortality may be attributable, at least in part, to neighboring polymorphisms in other genes.”

To conclude, our data provide genetic evidence that specific USF1 alleles would contribute to coronary atherosclerosis. The significance of the role of USF1 in atherosclerotic process was evidenced by data from 2, similarly ascertained, independent autopsy series as well as by the observed association also with aortic lesions. The USF1 gene variant contributing to advanced atherosclerosis also associated with a 2-fold increased risk for SCD, which accounts nowadays for most of the mortality associated with coronary heart disease in the Western world.

Acknowledgments
We thank Mervi Alanne, Kirsia Auro, Pekka Ellonen, Minttu Jussila, Kaisa Silander, and Minna Suvela for their excellent technical assistance. Seppo Tyynela is acknowledged for morphometric measurements of the coronary and aortic artery samples and coronary casts.

Sources of Funding
This study has been supported by the Center of Excellence in Complex Disease Genetics of the Academy of Finland, the Jenny and Antti Wihuri Foundation, Aarne Koskelo Foundation, the Finnish Foundation for Cardiovascular Research, and GenomEUtwin supported by the European Commission under the program “Quality of Life and Management of the Living Resources” of 5th Framework Programme (no. QLG2-CT-2002-01254), and by the Emil Aaltonen Foundation, Medical Research Fund of Tampere University Hospital, the Pirkanmaa Regional Fund of the Finnish Cultural Foundation, and the Yrjö Jahnsson Foundation.

Disclosures
L.P. is a member of the board of directors of Orion Corporation.

References


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Arterioscler Thromb Vasc Biol. 2008;28:983-989; originally published online February 14, 2008;
doi: 10.1161/ATVBAHA.107.156463
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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SUPPLEMENTAL METHODS

The Prospective Autopsy Series of Middle-Aged Men

The Helsinki Sudden Death study was approved by the Ethics Committee of the Department of Forensic Medicine, University of Helsinki.

The Helsinki Sudden Death Study (HSDS) was designed to study the lifestyle and genetic factors predisposing to sudden death in Finnish middle-aged men who died in Helsinki City area during a calendar-year period 1,2. The study population comprised two prospective series of altogether 700 men, aged 33 to 70 (mean age 53), who underwent a medicolegal autopsy at the Department of Forensic Medicine, University of Helsinki. The two series were collected at 10-year intervals; the first series (n=400) during 1981-1982 and the second series (n=300) during 1991-1992. Of the 700 deaths in the study sample, 230 (32.9%) were prehospital sudden cardiac deaths (SCD) due to coronary heart disease without valvular disease, cardiomyopathy or other cardiac diseases, 198 (28.3%) deaths due to other diseases, and 272 (38.9%) non-natural deaths (accidents, suicides). Of the SCDs, 194 (84.3%) were due myocardial infarction (MI), of whom 114 (49.6%) were acute MIs and 80 (34.8%) were related to presumed arrhythmia due to old MI scar in the absence of acute histological changes. The rest 36 (15.7%) SCDs were due to CHD without acute or old MI. The presence of recent MI and old MI were confirmed by macroscopic and histopathological examination of the myocardium 1-3.

Measuring the Atherosclerotic Lesion Areas and Coronary Narrowings

The stenosis of the proximal, medial and distal thirds of the main trunks of the left anterior descending coronary artery, left circumflex coronary artery, and right coronary artery were measured from rubber casts as described earlier 1,2. The most severely narrowed arterial segment from these nine measurements was chosen to represent the severity of the coronary narrowing of that study subject. The proximal parts of the three main epicardial coronary arteries and abdominal aortas were collected for the computer-assisted measurements of atherosclerotic changes 1,2. The most severely affected artery of the three coronary arteries from each individual was chosen to represent the atherosclerosis of the coronary tree. Protocols of two international studies were used to define atherosclerosis 4,5. We analyzed the areas stained red with Sudan IV and unaffected with other types of changes as fatty streaks. Elevated plaque areas that exhibited no ulceration or thrombosis were analyzed as fibrotic lesions, and plaque areas with ulceration or thrombosis were considered as complicated lesions. An advanced plaque area variable for the analyses was formed by combining elevated plaque areas and complicated lesion areas. Total atherosclerotic plaque area in the coronary arteries or abdominal aorta comprised of vessel-wall covered with any of the following lesion types: fatty streaks, elevated plaques or complicated
lesions. The areas of different types of lesions were expressed in percentages by dividing the lesion area by the total area of the artery sample and multiplying it by 100%. Calcification of the arteries was similarly measured in x-rays of the artery samples.

Assessment of the atherosclerotic plaques was performed similarly in the two autopsy series and although the proportion of study subjects affected with advanced lesions was smaller in the 1991-92 series than in the 1981-82 series, in both series the distinct majority showed advanced atherosclerosis.

Genotyping

In the HSDS 1991-2 series, DNA from frozen (-70°C) cardiac samples was isolated by the standard phenol-chloroform method \(^6\). In the 1981-2 series, DNA was extracted from paraffin-embedded samples of cardiac muscle with a modification of the method by Isola and colleagues \(^7\).

SeattleSNPs Variation Discovery Resource (SeattleSNPs. NHLBI Program for Genomic Applications, SeattleSNPs, Seattle, WA (URL: \url{http://pga.gs.washington.edu})) was utilized in the SNP selection and the selected SNPs covered all common (average minor allele frequency >4%) linkage disequilibrium select bins for the European descent. The genotyping of the \textit{USF1} polymorphisms was done either by allele-specific primer extension on microarrays (\textit{rs2073658}, \textit{rs2516839}, and \textit{rs2774279}) or MassARRAY System (Sequenom, San Diego, CA) (\textit{rs10908821}, \textit{rs2774276}, \textit{rs1556259})\(^8,9\). To comprehensively analyze the allelic diversity of the \textit{USF1} gene locus, \textit{htSNPs} \textit{rs10908821}, \textit{rs2774276}, \textit{rs2073658}, \textit{rs2516839}, \textit{rs1556259}, and \textit{rs2774279} were first genotyped in the 1991-2 autopsy series. SNPs \textit{rs2073658}, \textit{rs2516839}, and \textit{rs2774279} capturing the two most common haplotypes of \textit{USF1} were genotyped in the older 1981-2 series. Due to non-significant association with atherosclerosis in the haplotype analysis of 1991-92 series and technical issues, the remaining three SNPs were not genotyped in the older 1981-2 series (see Figure 1 and haplotype analyses).

The genotyping success rate was 99% for \textit{rs10908821}, 98% for \textit{rs2774276}, 92% for \textit{rs2073658}, 82% for \textit{rs1556259}, 94% for \textit{rs2516839}, and 97% for \textit{rs2774279}. The distribution of SNP genotypes followed Hardy-Weinberg equilibrium in the study sample \((p > 0.01)\).

Minor allele frequencies of the genotyped \textit{USF1} SNPs were 0.14 (\textit{rs10908821 G}-allele), 0.35 (\textit{rs2073658 T}-allele), 0.24 (\textit{rs2774276 G}-allele), 0.38 (\textit{rs2516839 C}-allele), 0.14 (\textit{rs1556259 G}-allele), and 0.27 (\textit{rs2774279 A}-allele) in the HSDS 1991-2 study sample. These frequencies were consistent with others thus far published \(^{10}\).

The linkage disequilibrium parameter D’ was 1 between all SNP pairs. \textit{Rs2516839} was the SNP with most linkage disequilibrium with other SNPs: The average \(r^2\) for SNP pairs including \textit{rs2516839} was 0.31, when it was 0.10 - 0.27 for pairs including other SNPs (Figure 1).
**Statistical Analysis**

Allele and genotype frequencies were determined and deviation from the Hardy-Weinberg equilibrium was tested with Pearson's chi-square. The linkage disequilibrium analysis and estimation of haplotype frequencies were performed for the HSDS 1991-2 series with the publicly available Haploview software version 3.2.

To examine the relationship between *USF1* genetic variation and the extent of the atherosclerotic changes of the arteries or the risk for SCD, genotype-based and haplotype-based analyses were performed in a combined data set of HSDS 1981-2 series and 1991-2 series. The haplotype carrier statuses for the study subjects were determined from the haplotype-tagging properties of the SNP alleles. The two HSDS series were also analyzed independently to establish how the associations observed in the combined data replicate in the two series, although, the decreased number of study subjects in these analyses would reduce the power to detect associations.

The distributions of the continuous atherosclerosis variables were highly skewed: Most (95%) of the study subjects had coronary fatty streaks, whereas 17% lacked any fibrous lesions, and 58% were without complicated lesions (Table 1). We therefore modeled the risk of atherosclerosis with multivariable ordinal regression model (cumulative logit model) where quartiles of the atherosclerotic variable were the ordinal outcome (Table I, please see www.ahajournals.org).

The risks for SCD and for >50% narrowing in more than one coronary artery were modeled with standard multivariable logistic regression models. In all regression models, age, BMI, and series (when applicable) were covariates (BMI was not a covariate in the model where the risk for SCD was analyzed). The genotypes were analyzed under a co-dominant model.

The relationship between coronary calcification and other coronary atherosclerosis measures in the study sample was tested with Spearman correlation statistics.

Statistical analyses were carried out with the SAS statistical software (versions 8.2. and 9.1 for Windows) (SAS Institute Inc., SAS OnlineDoc®, Version 8, Cary, NC: SAS Institute Inc., 1999). The logistic regression analyses were performed with the SAS LOGISTIC procedure. Statistical significance was set at 0.05. The $p$-values were adjusted for multiple comparisons with false discovery rate (FDR) implemented in the SAS MULTTEST procedure. Each adjustment procedure included one SNP or a haplotype, and all tested phenotypes.
REFERENCES


SUPPLEMENTAL RESULTS

Rs2073658 and Rs2774279 and Coronary and Aortic Atherosclerosis

A suggestive association \((p = 0.04)\), which did not persist after adjusting for multiple testing \((p = 0.46)\), between rs2073658 and the proportion of advanced lesion area out of the total lesion area in coronary arteries was observed. SNPs rs2073658 and rs2774279 failed to associate with any other of the atherosclerotic variables tested (data not shown).

Narrowing of the Coronary Arteries

In the combined data set of both series, the carriers of the risk allele \((T)\) of the rs2516839 also had a suggestive increased risk for having \(>50\%\) coronary narrowing in more than one coronary artery \((TT; \text{OR} 1.86, 95\% \text{CI} 0.93-3.69, p = 0.08, p \text{ adjusted for multiple comparisons} = 0.18 \) and \(CT; \text{OR} 1.90, 95\% \text{CI} 0.97-3.73, p = 0.06, p \text{ adjusted for multiple comparisons} = 0.09)\) when compared to that of the \(CC\) genotype. SNPs rs2073658 and rs2774279 failed to significantly associate with the presence of coronary narrowing.

USF1 Haplotypes and Atherosclerotic Lesions

Rs2516839, the SNP associated with coronary and aortic atherosclerosis (Table 2 and Figure 2), does not tag a single haplotype of USF1. Instead, the risk-associated \(T\) allele of the SNP is present in the two most common (61\%) haplotypes of USF1 (CTCTAG and CCCTAA, Figure 1). In a haplotype-based analysis of coronary and aortic atherosclerosis, the carriership of the second most common haplotype of USF1 (CCCTAA) was associated with a greater probability of being in a higher category of coronary calcification \((\text{OR} 1.53, 95\% \text{CI} 1.16-2.03, p = 0.003, \text{multiple testing adjusted} p = 0.05)\). CTCTAG showed suggestive association with higher proportion of advanced lesion area out of the total lesion area \((\text{OR} 1.40, 95\% \text{CI} 1.05-1.87, p = 0.02, \text{multiple testing adjusted} p = 0.40)\). In addition, haplotype GCGCAG initially associated with the proportion of advanced lesion area out of the total lesion area \((\text{OR} 0.59, 95\% \text{CI} 0.36-0.97, p = 0.04)\), and calcification of the coronary arteries \((\text{OR} 0.59, 95\% \text{CI} 0.36-0.97, p = 0.04)\), however these associations were not significant after adjusting the \(p\)-values for multiple comparisons.

Rs2516839, calcification and risk for sudden cardiac death

In the combined data set the carriers of the rs2516839 risk \((TT)\) genotype had a 2–fold risk for SCD when compared to that of the carriers of the protective genotype \((TT \text{ vs. } CC; \text{OR} 2.10, 95\% \text{CI} 1.17-3.75, p = 0.01, p \text{ adjusted for multiple comparisons} = 0.04, \text{and } CT \text{ vs. } CC; \text{OR} 1.92, 95\% \text{CI} 1.09-3.39, p = 0.02, p \text{ adjusted for multiple comparisons} = 0.10)\). We further tested
whether the association of rs2516839 with SCD was independent of its association with coronary calcification by including calcification in the logistic regression model as a covariate (Table III). Fitting the calcification covariate in the model reduced the risk associated with rs2516839 from odds ratio 2.10 to 1.59 (genotype TT vs. CC). Fitting other lesions types, however, led to more modest reduction in the risk associated with rs2516839 (Table III). The result suggested that part of the increased SCD risk associated with rs2516839 was due to rs2516839’s contribution to the development of severe coronary artery disease characterized by coronary calcification. Coronary calcification significantly increased the risk for SCD (OR 1.89, 95 % CI 1.57-2.27) (Table III). SCD risk associated with calcification was, however, similar to the risk for SCD associated with other lesion types, such as fibrous lesions (OR 1.47, 95 % CI 1.25-1.74), complicated lesions (OR 1.89, 95 % CI 1.64-2.18), total lesion area (OR 1.88, 95 % CI 1.58-2.24), advanced atherosclerosis (OR 1.89, 95 % CI 1.58-2.24), and proportion of advanced lesion area out of the total lesion area (OR 1.54, 95 % CI 1.30-1.81).

The relationship between coronary calcification, the phenotype most significantly associated with rs2516839, and other coronary atherosclerosis measures in the study sample was tested with Spearman correlation statistics. Significant correlation between the amount of calcification and other lesion types was observed; the spearman correlation coefficients varied from 0.41 to 0.50 (p<0.0001).
**SUPPLEMENTAL TABLES**

**Table I.** Atherosclerosis Variables as Quartiles in the Ordinal Logistic Regression Analysis.

<table>
<thead>
<tr>
<th></th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Coronary arteries</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty streaks</td>
<td>0-3.4</td>
<td>3.5-6.3</td>
<td>6.4-11.4</td>
<td>&gt;11.4</td>
</tr>
<tr>
<td>Fibrous lesions</td>
<td>0-2.1</td>
<td>2.2-5.8</td>
<td>5.9-10.5</td>
<td>&gt;10.5</td>
</tr>
<tr>
<td>Complicated lesions</td>
<td>0</td>
<td>0</td>
<td>0.1-2.7</td>
<td>&gt;2.7</td>
</tr>
<tr>
<td>Total lesion area</td>
<td>0-8.4</td>
<td>8.5-13.9</td>
<td>14.0-23.5</td>
<td>&gt;23.5</td>
</tr>
<tr>
<td>Advanced atherosclerosis†</td>
<td>0-2.7</td>
<td>2.8-7.1</td>
<td>7.2-12.9</td>
<td>&gt;12.9</td>
</tr>
<tr>
<td>Proportion of advanced lesion area out of the total lesion area</td>
<td>0-0.4</td>
<td>0.5-0.6</td>
<td>0.7-0.8</td>
<td>&gt;0.8</td>
</tr>
<tr>
<td>Calcified lesions</td>
<td>0-0.1</td>
<td>0.2-1.5</td>
<td>1.6-5.8</td>
<td>&gt;5.8</td>
</tr>
<tr>
<td><strong>Abdominal aorta</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fatty streaks</td>
<td>0-5.9</td>
<td>6.0-10.2</td>
<td>10.3-17.8</td>
<td>&gt;17.8</td>
</tr>
<tr>
<td>Fibrous lesions</td>
<td>0-2.1</td>
<td>2.2-5.0</td>
<td>5.1-9.5</td>
<td>&gt;9.5</td>
</tr>
<tr>
<td>Complicated lesions</td>
<td>0</td>
<td>0.1-2.2</td>
<td>2.3-8.6</td>
<td>&gt;8.6</td>
</tr>
<tr>
<td>Total lesion area</td>
<td>0-13.8</td>
<td>13.9-23.3</td>
<td>23.4-34.4</td>
<td>&gt;34.4</td>
</tr>
<tr>
<td>Advanced atherosclerosis†</td>
<td>0-2.8</td>
<td>2.9-10.0</td>
<td>10.1-18.4</td>
<td>&gt;18.4</td>
</tr>
<tr>
<td>Proportion of advanced lesion area out of the total lesion area</td>
<td>0-0.2</td>
<td>0.3-0.5</td>
<td>0.6-0.6</td>
<td>&gt;0.6</td>
</tr>
<tr>
<td>Calcified lesions</td>
<td>0-0.2</td>
<td>0.3-1.6</td>
<td>1.7-5.2</td>
<td>&gt;5.2</td>
</tr>
</tbody>
</table>

* The areas of different types of lesions are expressed in percentages by dividing the lesion area by the total area of the artery sample and multiplying it by 100%.

† Plaque area covered either by fibrotic or complicated lesion
Table II. Association of the USF1 rs2774279 and rs2073658 Genotypes with Calcification in Coronary Arteries in the Presence of SNP rs2516839.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotypes compared</th>
<th>OR (95% CI)*</th>
<th>p-value†</th>
<th>Covariates in the model</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2516839</td>
<td>CT vs. CC</td>
<td>1.48 (0.97-2.26)</td>
<td>0.07 (0.18)</td>
<td>age, BMI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.65 (1.04-2.64)</td>
<td>0.04 (0.09)</td>
<td>age, BMI, rs2073658</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.34 (0.85-2.12)</td>
<td>0.20 (0.32)</td>
<td>age, BMI, rs2774279</td>
</tr>
<tr>
<td></td>
<td>TT vs. CC</td>
<td>2.80 (1.80-4.35)</td>
<td>&lt;0.001 (&lt;0.001)</td>
<td>age, BMI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.24 (1.91-5.48)</td>
<td>&lt;0.001 (&lt;0.001)</td>
<td>age, BMI, rs2073658</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.50 (1.52-4.13)</td>
<td>&lt;0.001 (0.001)</td>
<td>age, BMI, rs2774279</td>
</tr>
<tr>
<td>rs2073658</td>
<td>CT vs. CC</td>
<td>1.06 (0.78-1.44)</td>
<td>0.71 (0.77)</td>
<td>age, BMI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.76 (0.54-1.08)</td>
<td>0.13 (0.26)</td>
<td>age, BMI, rs2516839</td>
</tr>
<tr>
<td></td>
<td>TT vs. CC</td>
<td>1.99 (1.25-3.18)</td>
<td>0.004 (0.07)</td>
<td>age, BMI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.03 (0.59-1.81)</td>
<td>0.91 (0.91)</td>
<td>age, BMI, rs2516839</td>
</tr>
<tr>
<td>rs2774279</td>
<td>AG vs. GG</td>
<td>1.47 (1.09-1.98)</td>
<td>0.01 (0.19)</td>
<td>age, BMI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.20 (0.86-1.67)</td>
<td>0.28 (0.38)</td>
<td>age, BMI, rs2516839</td>
</tr>
<tr>
<td></td>
<td>AA vs. GG</td>
<td>1.82 (1.09-3.03)</td>
<td>0.02 (0.36)</td>
<td>age, BMI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.17 (0.65-2.12)</td>
<td>0.60 (0.69)</td>
<td>age, BMI, rs2516839</td>
</tr>
</tbody>
</table>

SNP: Single nucleotide polymorphism; OR: Odds ratio; CI: Confidence interval; BMI: Body mass index

* Odds ratio and 95% confidence interval from ordinal logistic regression analysis (adjusted for age and BMI)
† p-value adjusted for multiple comparisons in parenthesis
Table III. Association of the *USF1* Rs2516839 and Calcification with Sudden Cardiac Death in Models Adjusted for Atherosclerosis Variables

<table>
<thead>
<tr>
<th>Model*</th>
<th>rs2516839</th>
<th>calcification</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CC vs. TT</td>
<td>CT vs. TT</td>
</tr>
<tr>
<td>SCD = <em>USF1</em></td>
<td>2.10 (1.17-3.75)</td>
<td>1.92 (1.09-3.39)</td>
</tr>
<tr>
<td>SCD = calcification</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>SCD = <em>USF1</em> + calcification</td>
<td>1.59 (0.86-2.93)</td>
<td>1.74 (0.96-3.16)</td>
</tr>
<tr>
<td>SCD = <em>USF1</em> + calcification + all other lesions</td>
<td>1.71 (0.88-3.31)</td>
<td>1.92 (1.01-3.66)</td>
</tr>
<tr>
<td>SCD = calcification + all other lesions</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>SCD = <em>USF1</em> + fibrous lesions</td>
<td>1.96 (1.06-3.63)</td>
<td>1.92 (1.05-3.51)</td>
</tr>
<tr>
<td>SCD = <em>USF1</em> + complicated lesions</td>
<td>2.01 (1.05-3.83)</td>
<td>2.09 (1.11-3.94)</td>
</tr>
<tr>
<td>SCD = <em>USF1</em> + total lesion area</td>
<td>2.08 (1.11-3.93)</td>
<td>1.93 (1.04-3.58)</td>
</tr>
<tr>
<td>SCD = <em>USF1</em> + advanced atherosclerosis</td>
<td>1.88 (1.00-3.54)</td>
<td>1.89 (1.02-3.51)</td>
</tr>
<tr>
<td>SCD = <em>USF1</em> + proportion of advanced lesion area out of the total lesion area</td>
<td>1.92 (1.04-3.55)</td>
<td>1.93 (1.06-3.52)</td>
</tr>
</tbody>
</table>

*all models are adjusted for age and HSDS series
Na= not applicable