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Coronary Artery Disease–Associated Locus on Chromosome 9p21 and Early Markers of Atherosclerosis


Background—Genome-wide association studies have recently identified a locus on chromosome 9p21 that influences risk of coronary artery disease (CAD). The effect of the locus on early markers of atherosclerosis is unknown. We examined its association with carotid intima-media thickness (CIMT) and brachial flow-mediated dilatation (FMD).

Methods and Results—We genotyped 2277 individuals, age 24 to 39 years, from the Cardiovascular Risk in Young Finns Study with CIMT and FMD measurements and 1295 individuals, age 46 to 76 years, from the Health 2000 Survey with CIMT for rs1333049, the chromosome 9p21 variant showing the strongest association with CAD. Both mean and maximum CIMT were significantly higher (P<0.001) in the older subjects of the Health 2000 Survey compared with the Young Finns Study. However, there was no association of the rs1333049 genotype with either mean or maximum CIMT at either age (P=0.959 and 0.971 for the 2 phenotypes in the Young Finns Study and P=0.714 and 0.725 in the Health 2000 Survey). Similarly, there was no association of the locus with variation in FMD in the Young Finns cohort (P=0.521).

Conclusions—The chromosome 9p21 locus does not influence CAD risk through a mechanism that also affects CIMT or induces early changes in FMD. (Arterioscler Thromb Vasc Biol. 2008;28:1679-1683)

Key Words: genetics ● coronary artery diseases ● atherosclerosis ● carotid-intima media thickness ● endothelial dysfunction

Coronary artery disease (CAD) has a significant genetic determination that has hitherto been poorly characterized. However, recent genome-wide association studies have identified several novel loci that are strongly associated with CAD. Specifically, a common variant located in a region adjacent to the cyclin dependent kinase inhibitors, CDKN2A (encoding p16INK4a) and CDKN2B (p15INK4b) on chromosome 9p21.3 has been associated with increased risk in 4 separate genome-wide association and follow-up studies.

Clinical manifestations of CAD represent the end stage of a chronic process. As genetic variants are present from birth, their effects on markers of atherosclerosis may be discernible at an earlier stage. Carotid intima-media thickness (CIMT) is an accurately quantifiable and reproducible marker of atherosclerotic risk and predicts future cardiovascular events. Similarly, impaired brachial artery flow-mediated dilatation (FMD) is another marker of atherosclerotic risk and predicts cardiovascular events. In this study, we investigated the association of the CAD associated variant on chromosome 9p21.3 on CIMT in 2 population based cohorts of different ages, in combination spanning the age range from 24 to 76 years. We also examined its association with variation in FMD in young healthy subjects.

Materials and Methods

Subjects

We studied subjects from 2 population based cohorts—the Cardiovascular Risk in Young Finns Study and the Health 2000 Survey.

The Cardiovascular Risk in Young Finns Study is a multi-center study of atherosclerotic risk factors of children and young adults (http://vanha.med.utu.fi/cardio/youngfinnssurvey/). The first cross-
sectional study was conducted in 1980 and included 3596 healthy
children and adolescents, age 3, 6, 9, 12, 15, and 18 years. Details of
the study design have been presented elsewhere. Thereafter, these
subjects have been followed with periodic examinations. In 2001,
2620 individuals, who had then reached the age of between 24 to 39
years, were studied. In addition to detailed risk factor assessments,
ultrasound examination of CIMT and brachial endothelial function
were carried out. The Health 2000 Survey was a large Finnish cross-sectional health
examination survey carried out in 2000 to 2001. The overall study
cohort was a 2-stage stratified cluster sample (8028 persons) represent-
ing the entire Finnish population age 30 years and above. To study cardiovascular disease risk factors and diabetes more thor-
oughly, a supplemental study was carried out (sample size 1867 and
participation rate 82%). The subjects in the supplemental study were
45 years and older, and the study was executed in the catchment
areas of the 5 Finnish University Hospitals because specialized
equipment was required. Carotid ultrasound examination was part of
this supplemental study. There were 1295 subjects (595 men and
700 women; mean age, 58 years; range, 46 to 76 years) with available carotid ultrasound data. These individuals were selected to
be our study group for the present analysis.

Clinical Characteristics
Body mass index (BMI) was calculated as weight in kilograms
divided by the square of height in meters. Blood pressure (BP) was
measured using a random zero sphygmomanometer in the Young
Fins Study and the automatic Omron M4 sphygmomanometer
(Omron Healthcare Europe B.V.) in the supplemental Health 2000
Study. Values for systolic and diastolic blood pressure were defined
by Korotkoff phases I and V, respectively. The averages of 3
measurements obtained after 5 minutes of sitting with 1 to 2 minutes
between readings were used in analyses. Smoking habits were
inquired with a questionnaire.

Laboratory Tests
In both studies, venous blood samples were taken after an overnight
fast. Total cholesterol, HDL cholesterol, triglyceride, and glucose
conzentrations were determined enzymatically (Roche Diagnostics,
GmbH for HDL; Olympus System Reagent for total cholesterol,
triglyceride, and glucose) with a clinical chemistry analyser (Olym-
pus, AU400). LDL cholesterol was calculated with the Friedewald
formula.

Ultrasound Imaging
In the Young Fins Study, carotid ultrasound studies were performed
using a high-resolution ultrasound system (Sequoia 512, Acuson) with
13.0 MHz linear array transducer. CIMT was measured about
10 mm from the bifurcation on the left common carotid artery
focusing the image on the posterior wall and recording images from the
angle showing the greatest distance between the lumen-intima
interface and the media-adventitia interface. At least 4 measure-
ments were taken at each scan of the common carotid artery incident
with the R-wave of the continuously monitored ECG to derive mean
and maximum CIMT. The scans were analyzed by 1 reader blinded
to subjects’ details. The between visit (2 visits 3 months apart)
coefficient of variation of mean CIMT measurements for a subset of
the subjects was 6.4%. To assess brachial artery FMD, the left brachial artery
was measured both at rest and during the reactive hyperemia. Increased
flow was induced by inflation of a pneumatic tourniquet placed around the arm to a pressure of 250 mm Hg for 4.5
minutes, followed by release. Three measurements of arterial diam-
eter were performed at end-diastole at a fixed distance from an
anatomic marker at rest and at 40, 60, and 80 seconds after cuff
release. The vessel diameter after reactive hyperemia was expressed
as the percentage relative to the resting scan. The between-visit CV
for brachial diameter was 3.2% and for FMD 26.0%.

In the Health 2000 supplemental study carotid ultrasound exami-
nation of the right carotid artery was performed according to a
standardized protocol using a 7.5 MHz linear array transducer. The examinations were performed by centrally trained and certified
sonographers at 5 study locations around Finland. CIMT measure-
ments were performed off-line with the use of automated imaging
processing software. One reader was responsible for reading all ultrasound images. Mean and maximum CIMT were again calcu-
lated. The intrareader reproducibility of the CIMT measurements
was assessed by calculation of the CIMT twice from 571 randomly
selected images of 108 study subjects several weeks apart. The mean
difference of the 2 measurements was 0.001 mm (SD 0.123), and the
intraclss correlation was 0.934 (P<0.001).

Genotyping
Genomic DNA was extracted from peripheral blood leukocytes using
a commercially available kit (Qiagen Inc). rs1333049 was genotyped
by allelic discrimination using a standard TaqMan assay (further
details available on request). Fluorescence was detected post poly-
merase chain reaction (PCR) using the ABI Prism 7900HT Sequence
Detector System and genotypes called using ABI Prism SDS
software version 2.1 (ABI). For reference, in the genome-wide association studies, the CAD associated (risk) allele for rs133309
was C.

Statistical Analysis
Univariate data comparisons between genotype groups (and between
subjects in the 2 studies) were based on analysis of variance for
continuous variables and Chi-square test for categorical variables.
Because of skewed distributions, the values for triglycerides were
log transformed. Multiple logistic regression analysis was used to
identify clinical and laboratory variables that were independently
associated with mean and maximum CIMT and to assess the effect
of genotype on CIMT taking these variables into account. The statistical tests were performed with SPSS (version 14.00) for the
Young Fins Study and SAS (version 8.1) for the Health 2000
cohort. 95% CI for allele frequencies were calculated using confi-
dence interval calculation program, CIA (version 2.1.2). Power
calculations were undertaken using the PS (Power and Sample size
calculator) program.

Results
The age range of the subjects was 24 to 39 years (55% female) in the Young Fins Study and 46 to 76 years (55% female) in the Health 2000 cohort. The frequency of the C allele for rs1333049 was 0.41 (95% CI: 0.40 to 0.43) in the Young Fins Study and 0.42 (95% CI: 0.40 to 0.44) in the
Health 2000 subjects and the genotypes were in Hardy Weinberg equilibrium in both cohorts. The characteristics of the
subjects in each study partitioned by genotype for rs1333049 are shown in Table 1. Overall, the older subjects
from the Health 2000 survey had higher BMI, LDL, and HDL
cholesterol levels and higher average systolic and diastolic blood pressures compared with the subjects in the Young
Fins Study (P<0.001). However, there was no significant difference in any of these traits according to rs1333049
genotype in either age group (Table 1).

Both mean and maximum CIMT were higher in the Health
2000 cohort compared with the Young Fins subjects (Table 1). However, there was no effect of the rs1333049 genotype
on either phenotype in either the Young Fins study (P=0.959 and P=0.977, respectively) or in the Health 2000
cohort (P=0.714 and P=0.729). Specifically we did not see a higher CIMT in subjects carrying the CAD-risk associated
allele (C) in either cohort. Brachial FMD responses (available
in Young Fins) were likewise similar across the genotypes
(Table 1, P=0.521).
The results of multivariate logistic regression analysis of mean CIMT in the 2 studies are shown in Table 2. In the Young Finns Study, there were highly significant independent effects of age, gender, BMI, and BP on CIMT and borderline significant effect of smoking. In the Health 2000 cohort, there were similarly significant independent effects of age, gender, and SBP on CIMT. HDL-cholesterol, and smoking but not BMI were also independently associated with CIMT in this cohort. Taking these factors into account there was no independent effect of the rs1333049 genotype on mean CIMT (Table 2). The results for maximum CIMT were similar (not shown).

Discussion
Within a short period of its identification, the chromosome 9p21 locus has been shown to have a robust association with CAD in a wide range of populations.1–5,15 The risk-associated allele, defined by the C allele of rs1333049, or alleles of other SNPs in strong linkage disequilibrium with it examined in some studies, have consistently shown an increased risk of 25% to 40% per copy of allele. The region of association with CAD on chromosome 9p21 spans 50 to 60 kb and is located adjacent to genes coding for the cyclin-dependent kinases p16/CDKN2A and p15/CDKN2B as well as p14/ARF. These genes play a central role in the regulation of the cell cycle and may be implicated in the pathogenesis of atherosclerosis through their role in transforming growth factor (TGF)-β–induced growth inhibition.16,17 Interestingly, although the 9p21 locus itself does not contain a protein coding gene, recent studies have shown that it codes a large noncoding RNA, ANRIL, which is expressed in atherosclerotic tissue.15,18 Furthermore, expression of ANRIL is coordinated with that of p14/ARF and possibly also p16/CDKN2A and p15/CDKN2B, in both physiological and pathological conditions,18 suggesting that it may regulate the expression of these genes. Further studies are required, but this could provide a potential mechanism by which the locus affects CAD risk.

In this context, it is relevant to examine the association of the 9p21 locus with other forms of atherosclerotic and vascular disease as well as markers of atherosclerosis. Indeed, a recent study has also shown an association of the locus with abdominal aortic aneurysms as well as with intracranial aneurysms.19 Among atherosclerosis-related phenotypes, CIMT has gained particular prominence, both because of the ease, accuracy, and reproducibility of its measurement.

Table 1. Demographic and Phenotypic Characteristics of Subjects in the Cardiovascular Risk in Young Finns Study and in the Health 2000 Cohort Partitioned by Genotype for the Coronary Artery Disease Risk Variant on Chromosomes 9p21.3

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Cardiovascular Risk in Young Finns</th>
<th>Health 2000 Cohort</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>GG</td>
<td>GC</td>
</tr>
<tr>
<td>Age, y</td>
<td>31.6 (5.0)</td>
<td>31.8 (5.0)</td>
</tr>
<tr>
<td>Males, %</td>
<td>790</td>
<td>1093</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25.1 (4.3)</td>
<td>25.0 (4.4)</td>
</tr>
<tr>
<td>LDL chol, mmol/l</td>
<td>3.27 (0.83)</td>
<td>3.30 (0.86)</td>
</tr>
<tr>
<td>HDL chol, mmol/l</td>
<td>1.30 (0.32)</td>
<td>1.30 (0.32)</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>1.31 (0.73)</td>
<td>1.34 (0.92)</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>116.7 (12.8)</td>
<td>116.8 (13.4)</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>70.7 (10.3)</td>
<td>70.8 (11.1)</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>25</td>
<td>23</td>
</tr>
<tr>
<td>Mean CIMT, mm</td>
<td>0.58 (0.09)</td>
<td>0.58 (0.09)</td>
</tr>
<tr>
<td>Max CIMT, mm</td>
<td>0.62 (0.10)</td>
<td>0.62 (0.10)</td>
</tr>
<tr>
<td>FMD, %</td>
<td>7.84 (4.34)</td>
<td>8.04 (4.47)</td>
</tr>
</tbody>
</table>

Values are mean (SD) or prevalence in %. The numbers below each value is the No. of subjects for which the data were available. CIMT indicates carotid intima media thickness; FMD, Brachial artery flow-mediated dilatation; NA, not available. P values are unadjusted values based on analysis of variance for continuous variables and Chi-square test for smoking and gender. The CAD-risk associated allele is the C allele.
to 29%) increased risk of coronary artery disease.5 These results demonstrate.1–5 Similarly, it is unlikely that the lack of association of the chromosome 9p21 locus in CIMT in 2 cohorts, including a cohort of young adult subjects as well as a cohort with an age-range similar to cohorts which demonstrate the association of the locus with CAD. Somewhat surprisingly, we found no evidence of an association of the locus with either mean or maximum CIMT in either age group.

A number of possible explanations for the lack of association of the 9p21 locus with CIMT need to be considered. It is unlikely that selection bias is a factor. Both cohorts were population based, ethnically homogeneous, and of Caucasian origin where the association with 9p21 has been robustly demonstrated.1–5 Similarly, it is unlikely that the lack of association reflects either imprecision of measurement of CIMT or adequate power to detect an effect. Prospective studies have shown that every 0.1-mm increase in CIMT is associated with a 20% to 30% higher risk of subsequent CAD.7,22,23 In a recent meta-analysis of the association between rs1333049 and coronary artery disease, each copy of the risk allele (C) was associated with a 24% (95% CI: 20% to 29%) increased risk of coronary artery disease.5 These estimates suggest that the expected effect of the rs1333049 on CIMT would be approximately 0.1 mm per allele if the association with CAD was mediated through a similar mechanism. Posthoc power calculations in our data showed that we had 80% power at an alpha of 0.05 to detect a 0.02-mm difference in mean CIMT between CC and GG subjects in both cohorts. Furthermore, we easily detected several previously reported effects of other cardiovascular risk factors on CIMT in both cohorts. Therefore, a plausible, and perhaps mechanistically more interesting, explanation is that the chromosome 9p21 locus affects risk of CAD through mechanisms that are not manifested in the carotid wall and reflected by changes in CIMT.

Endothelial dysfunction is believed to be an early event in atherosclerosis and may predate the development of clinical disease by several decades.6–24,25 Reduction in FMD is a validated marker of endothelial dysfunction and predicts future cardiovascular events, at least in older adults.6 Several traditional risk factors for atherosclerosis such as hypercholesterolaemia, diabetes, and hypertension correlate with reductions in FMD.25 Although the lack of a significant association between the 9p21 locus and FMD in the Young Finns study does not exclude the possibility that such an effect will be observed in older subjects, our finding again suggests that this genetic locus does not enhance risk of CAD by itself primarily causing endothelial dysfunction at a young age.

The recent finding that the 9p21 locus is also associated with the development of intracranial aneurysms19 suggests that the mechanism of its effect on the vascular wall is perhaps more complex than simply promoting the development of atherosclerosis. If the mechanism relates to cell growth and turnover as discussed earlier, it is possible that this affects coronary plaque stability or vulnerability rather than its development per se. Further studies are necessary to understand the mechanism(s) by which the chromosome 9p21 locus affects risk of CAD. In this regard, our finding of a lack of association of the locus with CIMT and with FMD at a young age provides valuable information in directing this search.

Sources of Funding
The Young Finns Study has been financially supported by the Academy of Finland (grants no. 77841, 210283, 34316, 117941), the

<table>
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<tr>
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<th>Young Finns Study</th>
<th>Health 2000 Cohort</th>
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<tbody>
<tr>
<td></td>
<td>Beta (error)</td>
<td>P</td>
</tr>
<tr>
<td>Gender</td>
<td>0.0101 (0.0043)</td>
<td>0.017</td>
</tr>
<tr>
<td>Age</td>
<td>0.0050 (0.0004)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>BMI</td>
<td>0.0024 (0.0005)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>SBP</td>
<td>0.0054 (0.0020)</td>
<td>0.010</td>
</tr>
<tr>
<td>DBP</td>
<td>0.0060 (0.0025)</td>
<td>0.015</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>0.0036 (0.0023)</td>
<td>0.121</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.0004 (0.0066)</td>
<td>0.947</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>−0.0062 (0.0045)</td>
<td>0.161</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.0086 (0.0048)</td>
<td>0.050</td>
</tr>
<tr>
<td>rs1333049 GG/GC vs CC</td>
<td>−0.0009 (0.0048)</td>
<td>0.845</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure. The beta values are based on the following: age, per year increase; BMI, per 1 kg/m² increase; for SBP and DBP, per 10 mm Hg increase; for LDL, HDL, and triglycerides, per 1 mmol/l increase. The beta for triglycerides is for log-transformed values. The beta coefficients for the comparison of GG/GC vs CC genotypes is shown. The results were similar when modelling GG vs GC vs CC or GG vs CC. The CAD risk associated allele is the C allele.
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Disclosures
None.

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1. The Wellcome Trust Case Control Consortium. Genomewide association study of 14,000 cases of seven common diseases and 3,000 shared controls. Nature. 2007;447:661–678.