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Journal of the American College of Cardiology
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Published by Elsevier Inc.

Vol. 58, No. 4, 2011
ISSN 0735-1097/$36.00
doi:10.1016/j.jacc.2010.11.075

Genetics/Genomics

Influence of 9p21.3 Genetic Variants on Clinical and Angiographic Outcomes in Early-Onset Myocardial Infarction

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Objectives The purpose of this study was to test whether the 9p21.3 variant *rs1333040* influences the occurrence of new cardiovascular events and coronary atherosclerosis progression after early-onset myocardial infarction.

Background 9p21.3 genetic variants are associated with ischemic heart disease, but it is not known whether they influence prognosis after an acute coronary event.

Methods Within the Italian Genetic Study of Early-onset Myocardial Infarction, we genotyped *rs1333040* in 1,508 patients hospitalized for a first myocardial infarction before the age of 45 years who underwent coronary angiography without index event coronary revascularization. They were followed up for major cardiovascular events and angiographic coronary atherosclerosis progression.

Results Over 16,599 person-years, there were 683 cardiovascular events and 492 primary endpoints: 77 cardiovascular deaths, 223 reoccurrences of myocardial infarction, and 383 coronary artery revascularizations. The *rs1333040* genotype had a significant influence ($p = 0.01$) on the primary endpoint, with an adjusted hazard ratio of 1.19 (95% confidence interval [CI]: 1.08 to 1.37) for heterozygous carriers and 1.41 (95% CI: 1.06 to 1.87) for homozygous carriers. Analysis of the individual components of the primary endpoints provided no significant evidence that the *rs1333040* genotype influenced the hazard of cardiovascular death ($p = 0.24$) or the reoccurrence of myocardial infarction ($p = 0.57$), but did provide significant evidence that it influenced on the hazard of coronary revascularization, with adjusted heterozygous and homozygous ratios of 1.38 (95% CI: 1.17 to 1.63) and 1.90 (95% CI: 1.36 to 2.65) ($p = 0.00015$), respectively. It also significantly influenced the angiographic endpoint of coronary atherosclerosis progression ($p = 0.002$).

Conclusions In early-onset myocardial infarction, the 9p21.3 variant *rs1333040* affects the progression of coronary atherosclerosis and the probability of coronary artery revascularization during long-term follow-up. (J Am Coll Cardiol 2011;58:426–34) © 2011 by the American College of Cardiology Foundation

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Epidemiological and family data suggest that genetic factors have an impact on the occurrence of myocardial infarction, particularly at an early age (1,2). Genome-wide association studies have revealed an association between common genetic variants in chromosomal region 9p21.3 and ischemic heart disease (3–7), a finding that has been replicated in a number of case-control studies of populations of different ethnic origin (8–16).

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The mechanism by which 9p21.3 genetic variants influence ischemic heart disease is still unknown. Indirect data suggest that they may affect the development and progression of coronary atherosclerosis (17–19), but no clinical results are available currently concerning their influence on prospectively observed cardiovascular events or the progression of coronary atherosclerosis.

The Italian Genetic Study of Early-onset Myocardial Infarction was designed to study the genetics of susceptibility to myocardial infarction. It was a prospective, nationwide project involving patients who were hospitalized because of a first myocardial infarction before the age of 45 years and who were followed up for the occurrence of cardiovascular events. We assessed the influence of the 9p21.3 variant tagged by *rs1333040* on the occurrence of major adverse cardiovascular events and coronary atherosclerosis progression during long-term follow-up.

Methods

The Italian Genetic Study of Early-onset Myocardial Infarction involved 125 coronary care units. It was divided into 2 parts: a case-control study and a prospective follow-up of the cases. The cases and controls were enrolled between 1988 and 2002. Cases were considered eligible if they were hospitalized for a first myocardial infarction before the age of 45 years and underwent coronary angiography without coronary revascularization for the index event; controls were eligible if they were blood donors without a history of thromboembolic disease and were unrelated to the patients, but were matched individually with the patients by age, gender, and geographic origin.

Abbreviations and Acronyms

CI = confidence interval

Study protocol. The protocol was approved by the institutional review boards of all of the participating centers, and all of the patients gave their written informed consent. After identifying the patients suitable for enrollment, the investigators collected their written informed consent, completed a standardized case report form covering their family history of cardiovascular diseases, cardiovascular risk factors, lifestyles, and medications and obtained a blood sample for plasma separation and deoxyribonucleic acid extraction. The patients were followed up by means of standardized telephone contacts, outpatient visits, and subsequent hospital admissions for the occurrence of endpoints. An attempt was made to telephone all of the patients (or their families or primary care physicians if they could not be contacted); if they reported an event, their medical records were obtained.

Blood collection, processing, and storage. Blood was drawn from the antecubital vein into 3 tubes containing 0.106 M trisodium citrate and was separated into plasma and red cells by means of centrifugation. The plasma was divided into 5 aliquots and was stored at −80°C. Deoxyribonucleic acid was isolated from the white blood cells using the salting-out method and was stored in alcohol at −20°C.

Clinical endpoints. The primary endpoint was the composite of cardiovascular death, the reoccurrence of myocardial infarction, and coronary artery revascularization by means of a percutaneous coronary intervention or coronary artery bypass surgery. Only the event that occurred first was counted.

All deaths were investigated using the death certificate specifying the cause of death; all other events were investigated by means of source data verification. Cardiovascular death was defined as any death attributed to a cardiovascular cause on the death certificate. The reoccurrence of myocardial infarction was defined as subsequent hospitalization ending with a discharge diagnosis of myocardial infarction. Rehospitalization for revascularization was defined as any period of hospitalization during which the patient underwent coronary revascularization percutaneously or by means of coronary artery bypass surgery. Other thromboembolic events were defined as any thromboembolic event requiring hospitalization.

All of the events were adjudicated by an events committee of 2 cardiologists who were unaware of the genotyping results. In the case of disagreement, the opinion of a third cardiologist was required.

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This study was supported by the Italian charitable foundation "Associazione per lo Studio della Trombosi in Cardiologia" and the Atherosclerosis, Thrombosis, and Vascular Biology Italian Study Group. The Italian foundation "Associazione per lo Studio della Trombosi in Cardiologia" and the Atherosclerosis, Thrombosis, and Vascular Biology Italian Study Group were not involved in designing or conducting the study; in data collection, management, analysis, or interpretation; or in preparing, reviewing, or approving the manuscript. Dr. Galli is employed by Mundipharma Italy. Dr. Kathiresan has received research grants from Pfizer, Merck, and Almylam. All other authors have reported that they have no relationships to disclose.

Manuscript received November 29, 2009; revised manuscript received October 28, 2010, accepted November 23, 2010.

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Assessment of angiographic coronary artery disease. The absence of any narrowing in coronary diameter was considered evidence of normal coronary arteries; a narrowing of <70% (50% in the case of the left main coronary artery) was considered nonsignificant coronary artery stenosis, and a narrowing of 70% or more (50% or more in the case of the left main coronary artery) was considered significant coronary artery stenosis. A significant coronary artery stenosis involving 1 of the 3 major coronary arteries was defined as single-vessel disease; if significant coronary artery stenoses affected 2 or 3 of the major coronary arteries, it was defined as multivessel disease.

The extent of coronary artery disease was evaluated using the Duke Coronary Artery Disease Index (20), which was established in all patients on the basis of the coronary angiography performed during hospitalization for the index myocardial infarction. In the subset of patients who underwent coronary angiography during follow-up, the progression of coronary artery disease was evaluated by comparing the Duke Coronary Artery Disease Index of the first coronary angiography with that of the follow-up coronary angiography. If the patient underwent more than one coronary angiography during follow-up, the last one was used. Angiographic progression of coronary atherosclerosis was defined as the need for angiographic assessment during follow-up accompanied by an increase in the Duke Coronary Artery Disease Index of more than the median value of the observed changes.

Genotyping. Genotyping was performed using matrix-assisted laser desorption ionization time-of-flight mass spectrometry and a Sequenome MassARRAY platform (San Diego, California). The genotyping success rate was 99.8% (range 99.6% to 99.9%); the genotyping error rate estimated using 15 samples genotyped in quadruplicate was <0.7% (21).

Statistical analysis. This study focused on a single locus (the single nucleotide polymorphism *rs1333040*) and used it as a tag for 9p21.3 genetic variants that may influence the occurrence of cardiovascular events after early-onset myocardial infarction. The locus was chosen on the basis of the results of an earlier case-control study (also part of the Italian Genetic Study of Early-onset Myocardial Infarction) in which 5 9p21.3 genetic variants that have shown a consistent signal of association with ischemic heart disease in previous genome-wide association studies were tested for their association with early-onset myocardial infarction. These variants were: *rs10116277*, *rs1333040*, *rs2383206*, *rs10757278*, and *rs1333049*. The single nucleotide polymorphism *rs1333040* showed the strongest association with early-onset myocardial infarction, with no residual association being found at any of the remaining 4 loci, conditional on *rs1333040*. These findings prompted the present investigation of the effect of *rs1333040* on prospectively observed cardiovascular events after early-onset myocardial infarction.

The primary endpoint of the analysis was the composite of cardiovascular death, the reoccurrence of myocardial infarction, and coronary artery revascularization by means of a percutaneous coronary intervention or coronary artery bypass surgery. The primary endpoint-free time was defined as the time between the index myocardial infarction and the earliest of the above events, or the time of censoring.

The angiographic endpoint of progression of coronary atherosclerosis was considered to have been met when the increase in the Duke Coronary Artery Disease Index from baseline to follow-up coronary angiography was more than the median value of the observed changes. A first assessment of the Duke Coronary Artery Disease Index was made in all patients at the time of the baseline coronary angiography examination, and a subsequent assessment was performed only in those patients who repeated coronary angiography during follow-up. The progression of coronary atherosclerosis was defined as the need for an angiographic assessment during follow-up accompanied by an increase in the Duke Coronary Artery Disease Index of at least 19 (the median increase in our sample) over baseline.

The dependence of primary endpoint-free time on *rs1333040* was analyzed using Cox's regression model for censored failure time data, adjusting for the extent of coronary artery disease at the time of the coronary angiography performed during the index hospitalization and the main risk factors under a proportional hazard assumption.

Step-wise variable selection was used to isolate minimally adequate models. This involved the interleaving of short sequences of backward variable elimination and short sequences of forward inclusion. In the case of a significant association between *rs1333040* and the primary endpoint, subsequent Cox analyses were made to test the association with each component of the primary endpoint. The corresponding estimates of heterozygous and homozygous relative risk were obtained from each of the analyses. Kaplan-Meier estimates of the genotype-specific cumulative curves for the various endpoints were also calculated. The dependence of the number of significant coronary artery stenoses on the number of copies of the *rs1333040* risk allele was analyzed by means of logistic regression. The association between the 9p21.3 variant *rs1333040* and coronary artery disease, as measured at the time of the first infarction, was tested by means of the Cochran-Armitage test of trend and the chi-square test of association.

Results

Demographic characteristics and traditional risk factors. The case sample consisted of 1,508 patients (1,334 men and 174 women) whose median age at the time of their first myocardial infarction was 41 years (interquartile range: 37 to 43 years). The genotype distribution of *rs1333040* in the study population was: 141 patients had no risk allele (CC), 587 patients had 1 risk allele (CT), and 780 patients had 2 risk alleles (TT). There was no evidence of any departure from Hardy-Weinberg equilibrium ($p > 0.05$). Table 1 shows the demographic and angiographic characteristics of

Table 1 Demographic and Angiographic Characteristics and Traditional Risk Factors by *rs1333040* Genotype

Variable	All Patients (n = 1,508)	Patients With C/C Genotype (n = 141)	Patients With C/T Genotype (n = 587)	Patients With T/T Genotype (n = 780)	p Value
Age (yrs)	41 (37–43)	35 (41–43)	37 (41–43)	37 (40–43)	0.45
Sex					0.65
Male	1,334	122 (86.52)	518 (88.25)	694 (88.97)	
Female	174	19 (13.48)	69 (11.75)	86 (11.03)	
Family history of CAD					0.36
Yes	1,240	115 (81.56)	479 (81.60)	646 (82.82)	
No	268	26 (18.44)	108 (18.40)	134 (17.18)	
Diabetes					0.9
Yes	117	12 (8.70)	41 (7.02)	64 (8.22)	
No	1,384	126 (91.30)	543 (92.98)	715 (91.78)	
Smoking					0.16
Yes	1,312	115 (82.14)	515 (88.03)	682 (87.55)	
No	192	25 (17.86)	70 (11.97)	97 (12.45)	
Hypertension					0.23
Yes	414	35 (24.82)	155 (26.72)	224 (28.9)	
No	1,082	106 (75.18)	425 (73.28)	551 (71.10)	
Body mass index (kg/m^2)					0.37
Normal	536	62 (43.97)	196 (33.97)	278 (36.10)	
Pre-obese	651	52 (36.88)	258 (44.71)	341 (44.29)	
Obese	301	27 (19.15)	123 (21.32)	151 (19.61)	
Hypercholesterolemia					0.77
Yes	842	78 (58.65)	326 (60.93)	438 (60.41)	
No	551	55 (41.35)	209 (39.07)	287 (39.59)	
Coronary artery disease					0.001
Present	1,226	>101	463	662	
Absent	282	36	127	119	
Duke Coronary Artery Disease index	31 (23–37)	26 (19–37)	30 (23–37)	33 (23–37)	1e-06

Values are median (interquartile range), n, or n (%). The frequency totals may be less than the sample size of 1,508 because of missing values.
CAD = coronary artery disease.

the patients and the frequency distribution of the traditional risk factors by genotype.

rs1333040 genotype and long-term clinical outcomes. The patients were followed up for a median of 9.95 years (interquartile range: 8 to 11.8 years), a total of 16,599 person-years. The vital status of 1,497 patients (99%) was ascertained, and 1,434 (95%) completed the follow-up. During the follow-up, there were 683 cardiovascular events and 492 primary endpoints: 77 patients died of cardiovascular death, 223 experienced reoccurrences of myocardial infarction, and 383 underwent coronary artery revascularization (230 by means of a percutaneous coronary intervention and 153 by means of coronary artery bypass surgery). During the follow-up, 53 patients experienced more than 1 myocardial infarction, 85 patients underwent more than 1 percutaneous coronary revascularization, and 2 patients underwent more than 1 coronary artery bypass intervention.

The *rs1333040* genotype had a significant (p = 0.01) influence on the primary endpoint, with an adjusted heterozygous hazard ratio of 1.19 (95% confidence interval [CI]: 1.08 to 1.37) and homozygous hazard ratio of 1.41 (95% CI: 1.06 to 1.87). Table 2 shows the estimated hazard ratios, 95% CIs, and p values of the independent predictors of the primary endpoint.

Table 2 Influence of *rs1333040* Genotype on the Primary Endpoint and on the Individual Components of Primary Endpoint

Endpoint	Genotype	Hazard Ratio	95% Confidence Interval	p Value
Primary endpoint	Heterozygous	1.19	1.08–1.37	0.01
	Homozygous	1.41	1.06–1.87	
Cardiovascular death	Heterozygous	1.10	0.80–1.40	0.24
	Homozygous	1.20	0.60–2.50	
Reoccurrence of myocardial infarction	Heterozygous	1.05	0.86–1.28	0.47
	Homozygous	1.10	0.74–1.64	
Coronary artery revascularization	Heterozygous	1.38	1.17–1.63	0.00015
	Homozygous	1.90	1.36–2.65	

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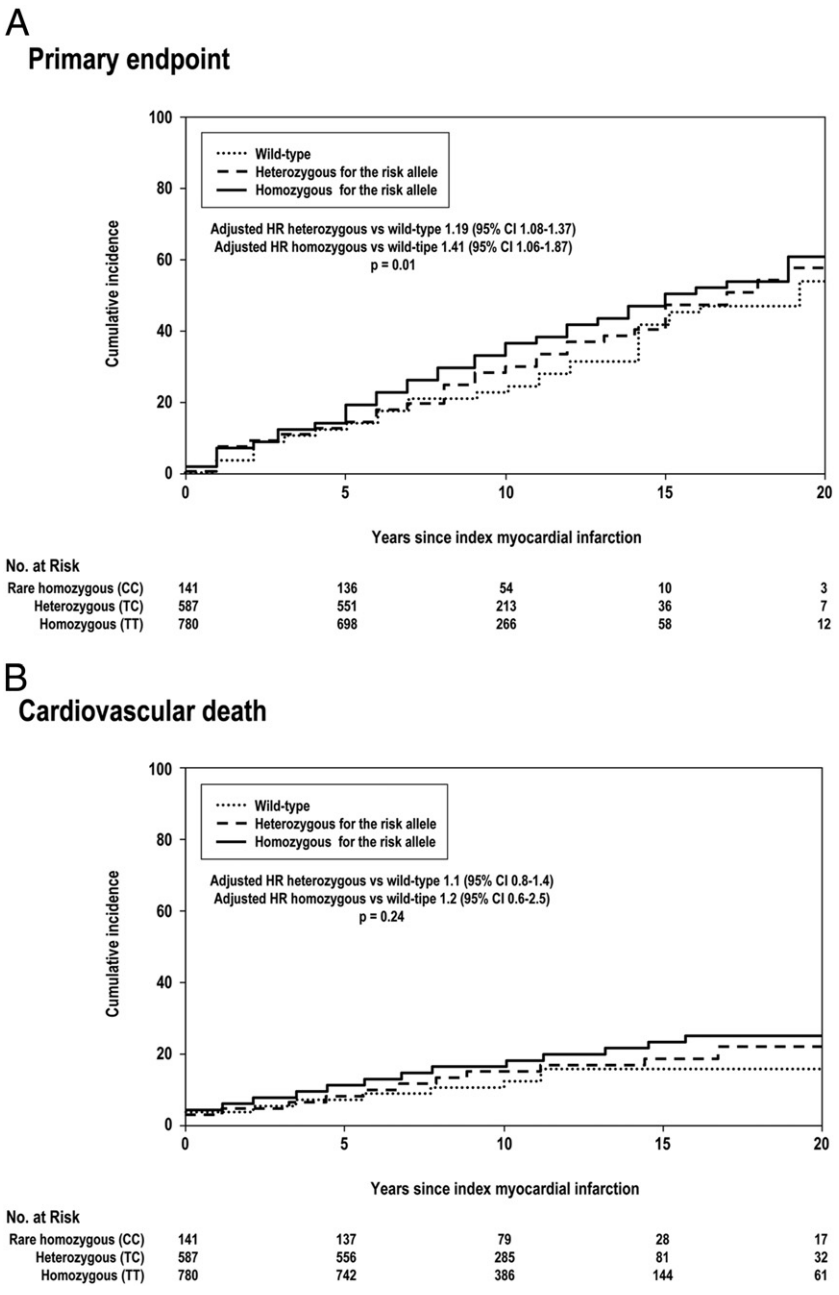

Figure 1 **Cumulative Incidence of Cardiovascular Events After Early-Onset Myocardial Infarction by *rs1333040* Genotype**

Kaplan-Meier cumulative incidence curves of **(A)** the primary endpoint and the individual components of the primary endpoint: **(B)** cardiovascular death, **(C)** reoccurrence of myocardial infarction, and **(D)** coronary artery revascularization. CI = confidence interval; HR = hazard ratio.

The analyses of the individual components of the primary endpoint provided no significant evidence of an influence of the *rs1333040* genotype on the hazard of cardiovascular death (p = 0.24) or the reoccurrence of myocardial infarction (p = 0.47). The adjusted hazard ratio for cardiovascular death was 1.1 (95% CI: 0.8 to 1.4) for heterozygous carriers of the risk allele and 1.2 (95% CI: 0.6 to 2.5) for homozygous carriers of the risk allele; the adjusted hazard ratio for the reoccurrence of myocardial infarction was 1.05 for heterozygous carriers of the risk allele (95% CI: 0.86 to 1.28) and 1.10 for homozygous carriers of the risk allele (95% CI: 0.74 to 1.64), respectively. However, there was significant evidence (p = 0.00015) that the *rs1333040* genotype influenced the hazard of undergoing coronary artery revascularization during follow-up, with an adjusted heterozygous hazard ratio of 1.38 (95% CI: 1.17 to 1.63) and a homozygous hazard ratio of 1.90 (95% CI: 1.36 to 2.65). Kaplan-Meier curves of the cumulative incidence of the pri-

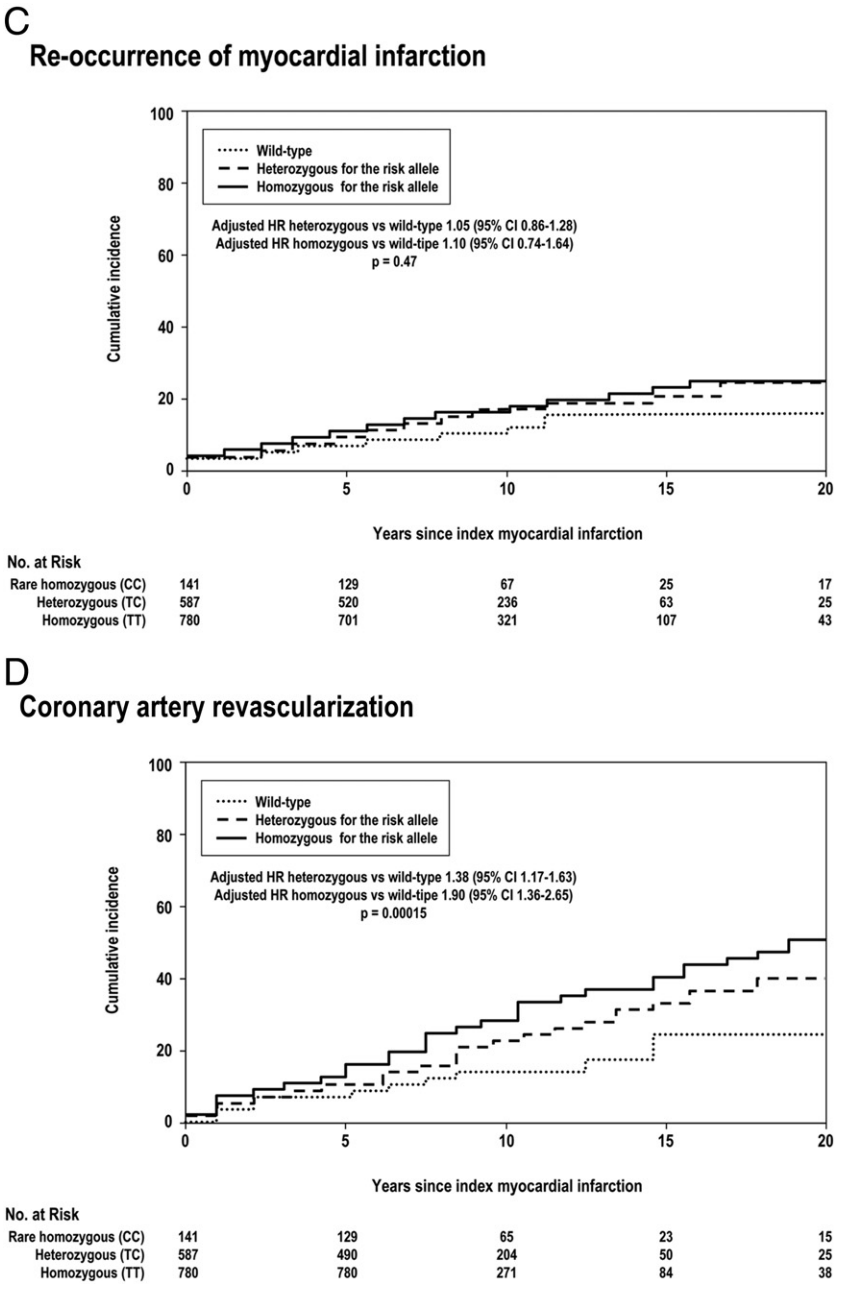

Figure 1 (continued)

mary endpoint and the individual components of the primary endpoint are shown in Figure 1 by *rs1333040* genotype. Table 3 shows estimated hazard ratios, 95% CIs, and p values for the independent predictors of the primary endpoint.

rs1333040 genotype and coronary artery disease. Coronary angiography at the time of the index myocardial infarction showed normal coronary arteries or coronary atherosclerosis without significant stenoses in 282 patients and significant coronary artery disease in 1,226 patients.

There was significant evidence of an association between the *rs1333040* genotype and the presence of significant coronary artery stenoses ($p = 0.014$) and the Duke Coronary Artery Disease Index ($p = 1\ e^{-06}$) at the time of the index coronary angiography (Table 1).

During the follow-up, 405 patients underwent at least 1 repeat coronary angiography. The median time between the coronary angiography performed during the index hospitalization and the coronary angiography performed during follow-up was 6.6 years (interquartile range: 4 to 9 years), for a total of 2,625 person-years of angiographic follow-up. The median change in the Duke Coronary Artery Disease Index was 19 (interquartile range: 0 to 24). Individual and

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Table 3 Independent Predictors of the Primary Endpoint: Estimated Hazard Ratios, 95% Confidence Intervals, and p Values

Explanatory Variable	Hazard Ratio	95% Confidence Interval	p Value
Diabetes mellitus (yes vs. no)	1.30	1.0–1.79	0.046
rs1333040 (per risk allele)	1.19	1.08–1.37	0.01
Body mass index (per U)	1.022	1.0012–1.044	0.038
Coronary artery disease (diseased vessels vs. normal)	1.60	1.4–1.8	0.0000000000004

median changes in the Duke Coronary Artery Disease Index by *rs1333040* genotype are shown in Figure 2.

The *rs133340* genotype had a significant influence (p = 0.002) on the angiographic endpoint of coronary atherosclerosis progression. The adjusted heterozygous hazard ratio was 1.5 (95% CI: 1.17 to 2.02), and the adjusted homozygous hazard ratio was 2.2 (95% CI: 1.3 to 2.7). The Kaplan-Meier curves of the cumulative incidence of the angiographic endpoint of coronary atherosclerosis progression by *rs1333040* genotype are shown in Figure 3.

Discussion

This study assessed the influence of 9p21.3 *rs1333040* on the risk of incident cardiovascular events and the progression of coronary atherosclerosis in individuals who experienced a first myocardial infarction at a young age. Its major finding is that 9p21.3 *rs1333040* influences the probability of undergoing subsequent coronary artery revascularization after a first myocardial infarction, even when accounting for the extent of coronary artery disease at the time of the initial acute coronary event. We observed that the variant was also associated with the progression of coronary atherosclerosis, thus suggesting a mechanism for the increased future revascularization rate in carriers of the risk allele.

These results confirm the findings of genome-wide association studies (3–7) suggesting that genetic variations in the 9p21.3 chromosomal region represent a novel risk factor for coronary artery disease. Multiple single nucleotide polymorphisms in strong linkage disequilibrium have been identified in this genomic region of approximately 50,000 bases, but they probably represent the same genetic signal (5,6). The functional effect of this signal remains unknown, because there are no annotated genes in this region.

The prospective nature of our study allows a true assessment of the risk of cardiovascular events after early-onset myocardial infarction related to the 9p21.3 variation. The *rs1333040* genotype significantly influenced the hazard of the combined endpoint of cardiovascular death, myocardial infarction, or the need for coronary artery revascularization, with an estimated increased risk of 19% for heterozygous and 41% for homozygous carriers of the risk allele. Despite the strong influence of the genotype on the need for subsequent coronary artery revascularization, no evidence was found to support its influence on the risk of cardiovascular death or the reoccurrence of myocardial infarction.

Why the 9p21.3 genetic variant *rs1333040* influences the need for coronary artery revascularization, but does not affect cardiovascular death or the reoccurrence of myocardial infarction, remains unknown. It can be surmised that this genetic variant influences only the development and progression of coronary atherosclerosis, and not plaque instability or coronary thrombosis. A similar observation was made by Horne et al. (17), who found that the 9p21.3 genetic variant was not associated with incident events or

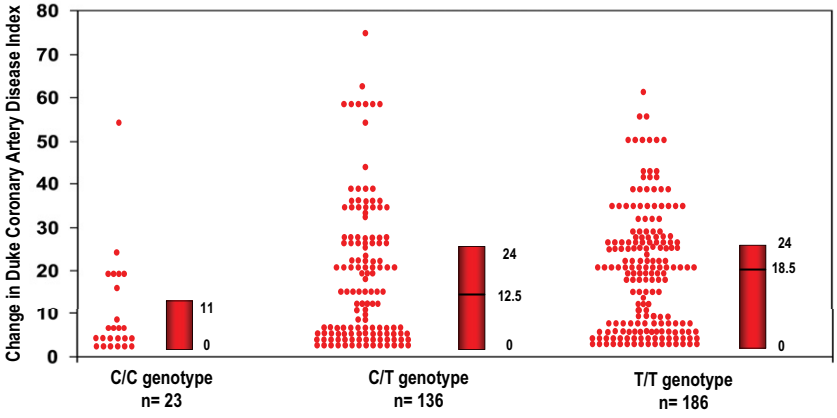

Figure 2 Changes in the Duke Coronary Artery Disease Index by *rs1333040* Genotype

Individual and median changes in the Duke Coronary Artery Disease Index by *rs1333040* genotype.

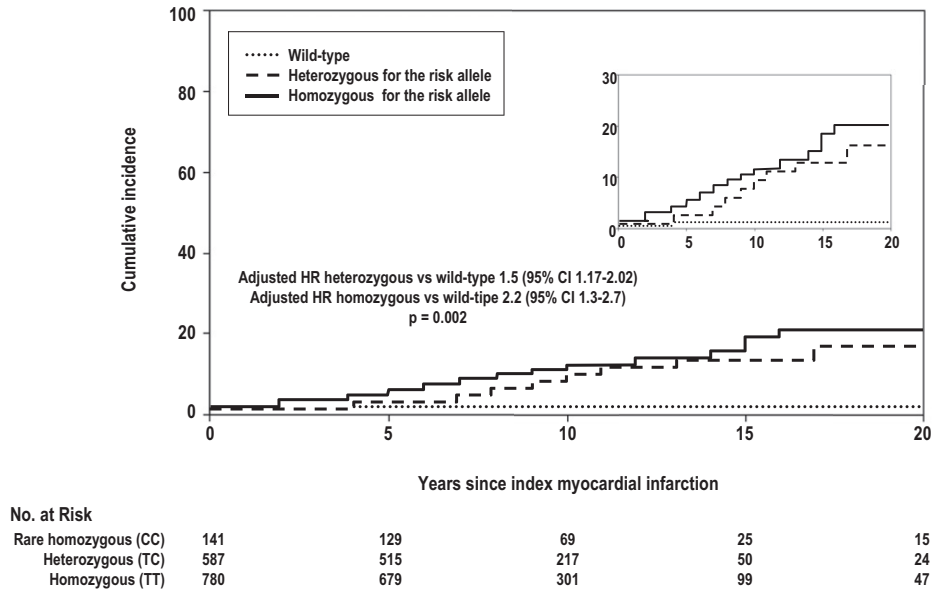

Figure 3 **Cumulative Incidence of the Angiographic Endpoint of Coronary Atherosclerosis Progression by *rs1333040* Genotype**

Kaplan-Meier cumulative incidence curves of the angiographic endpoint. The **inset** shows the same data on an enlarged y-axis and a condensed x-axis. Abbreviations as in Figure 1.

prevalent myocardial infarction in a population of patients undergoing coronary angiography, although it did predict a diagnosis of coronary artery disease.

The 9p21.3 region has been associated with the calcium score (5,14), severe premature atherosclerosis (5), the prevalence of angiographic coronary artery disease (18,22), and the progression of carotid atherosclerosis (19). In keeping with these findings, we observed an association between the 9p21.3 genetic variant *rs1333040* and the prevalence of coronary artery disease at the time of the coronary angiography performed during the index hospitalization. In addition, the variant influenced the progression of coronary atherosclerosis as measured by changes in the Duke Coronary Artery Disease Index. Carriers of 2 risk alleles showed more rapid progression of coronary atherosclerosis, whereas carriers of 1 risk allele showed intermediate progression between that of wild-type and homozygous carriers, thus indicating a gene gradient for the progression of coronary atherosclerosis.

Study limitations. One possible limitation of this study is the use of early-onset myocardial infarction as a clinical model to study the influence of 9p21.3 sequence variants on cardiovascular events and the progression of coronary atherosclerosis. It is known that early-onset myocardial infarction is greatly influenced by genetics (1,2), and so the effect of genetic factors may be overestimated in this population. However, we focused on the narrow phenotype of early-onset myocardial infarction because its degree of inheritability makes it very promising for fruitful genetic mapping, although future studies are clearly needed to assess the real influence of 9p21.3 genetic variants in broader populations of patients with all of the manifestations of ischemic heart disease.

Another possible limitation may be related to the selection of patients with early-onset myocardial infarction who did not undergo coronary revascularization at the time of the index event. We did this to be able to assess the influence of the 9p21.3 genetic variant *rs1333040* on the cumulative probability of cardiovascular events and the progression of atherosclerosis by eliminating the interference of coronary revascularization. However, because it may have had an impact on the incidence of cardiovascular events, our conclusions apply only to patients with early-onset myocardial infarction not undergoing coronary revascularization for the index event.

Moreover, the data relating to the progression of atherosclerosis came only from the patients who underwent repeat coronary angiography for clinical reasons. This selection bias may have confounding potential, but performing coronary angiography for scientific reasons has ethical implications that are difficult to overcome.

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Key Words: early-onset myocardial infarction ■ outcomes ■ *rs1333040* ■ 9p21.3 genetic variants.