G20210A Prothrombin Gene Polymorphism and Prothrombin Activity in Subjects With or Without Angiographically Documented Coronary Artery Disease

Carla Russo, MD; Domenico Girelli, MD, PhD; Oliviero Olivieri, MD; Patrizia Guarini, BD; Franco Manzato, MD; Francesca Pizzolo, MD; Barbara Zaia, MD; Alessandro Mazzucco, MD; Roberto Corrocher, MD

Background—G20210A prothrombin mutation has been associated with high prothrombin levels and an increased risk of venous thrombosis. The role of this common polymorphism, as well as that of prothrombin levels, in determining the risk of arterial disease is still somewhat controversial.

Methods and Results—We determined the prevalence of the G20210A mutation and prothrombin activity in 660 individuals, of whom 436 had angiographically documented severe coronary artery disease (CAD patients) and 224 had normal coronary angiography (CAD-free control subjects). Heterozygosity for the 20210A allele was found in 5.3% of the CAD patients versus 3.1% of the CAD-free subjects (P = 0.21). Similarly, no statistically significant difference was found between CAD patients with or without previous myocardial infarction (4.5% versus 5.3%, respectively; P = 0.73). The genotype-phenotype correlation study showed a significant influence of the 20210A allele on prothrombin activity, with higher levels in carriers compared with noncarriers (153.2% versus 122.2%, respectively; P < 0.001). Prothrombin activity was significantly higher in CAD patients than in CAD-free subjects (132.8% versus 123.3%, respectively; P < 0.005). By multiple logistic regression, prothrombin activity in the upper tertile of the control distribution was significantly associated with CAD compared with prothrombin activity in the lower tertile (adjusted odds ratio 1.86, 95% CI 1.01 to 3.4).

Conclusions—In a population with a clear-cut definition of the phenotype, the G20210A prothrombin mutation was not significantly associated, per se, with either angiographically documented CAD or myocardial infarction, whereas it significantly influenced prothrombin activity. In our population, high prothrombin activity itself was independently associated with CAD but not with the presence or absence of previous myocardial infarction. (Circulation. 2001;103: 2436-2440.)

Key Words: coagulation ■ risk factors ■ coronary disease ■ atherosclerosis ■ genetics
confer an increased risk of arterial disease. To date, however, studies attempting to answer this question have yielded conflicting results. In some reports, being a carrier of the mutation was associated with an increased risk of myocardial infarction (MI).\textsuperscript{12–14} Especially through interaction with other major risk factors.\textsuperscript{15–17} Nevertheless, the only prospective study published so far failed to establish any association between the 20210A allele and MI.\textsuperscript{18}

We examined the prevalence of the G20210A prothrombin mutation in a population of subjects with angiographic documentation of the condition of their coronary vessels. We studied a group of patients with severe coronary atherosclerosis, with or without a documented history of MI, and a control group with normal coronary arteries. Our aims were as follows: (1) to assess the association of the G20210A mutation per se with CAD and/or MI, (2) to evaluate the relationship between the G20210A mutation and plasma prothrombin levels, and (3) to examine whether elevated prothrombin levels themselves were associated with CAD.

Methods

Study Population

The selection criteria have already been described in detail\textsuperscript{19} and are outlined briefly below. We studied 660 consecutive unrelated adult patients of both sexes recruited from those referred to the Verona University Institute of Cardiovascular Surgery. Four hundred thirty-six were candidates for coronary artery bypass grafting, with angiographically documented severe multivessel CAD; 224 subjects, examined for reasons other than suspected CAD (mainly valvular disease), with angiographically documented normal coronary arteries were included as a control group. They also had neither a history nor clinical or instrumental evidence of atherosclerosis in other vascular districts. Though clinical history and a complete examination excluded the presence of any acute or chronic disease; moreover, as a further control, the most commonly used markers of inflammation were within the limits of the normal range: C-reactive protein (1.75±1.6 mg/dL), fibrinogen (300±70 mg/dL), and blood leukocytes (6650±1700 mm\textsuperscript{3}).

Patients and control subjects came from the same geographical area (Northern Italy) and had a similar socioeconomic background. A complete clinical history, including cardiovascular risk factors such as smoking and hypertension, was taken for all participants. Prothrombin activity of subjects taking anticoagulant drugs at the time of blood sampling or during the previous week was not included in the statistical analyses.

Subdivision of CAD patients into MI and non-MI groups was performed by combining the history data with a thorough review of medical records showing diagnostic ECG and enzyme changes and/or the typical sequelae of MI at ventriculographic angiography. Appropriate documentation was obtained from 396 (91%) of 436 (244 MI and 152 non-MI) CAD patients. CAD patients did not suffer from acute MI or unstable angina (which might cause transient changes in prothrombin activity), at least during the 40 days preceding blood sampling.

The severity of CAD was determined by the number of significantly stenosed coronary arteries, ie, lesions with >50% luminal stenosis. The angiograms were assessed by 2 cardiologists who were unaware that the patients were to be included in the study. Most of the patients (76%) had severe CAD involving all 3 major coronary arteries, 18% had 2 stenosed vessels, and 6% had 1 stenosed vessel. Informed consent was obtained from all subjects after a full explanation of the study.

Biochemical Analyses

Samples of venous blood were drawn from each subject in the free-living state, after an overnight fast, at a scheduled ambulatory evaluation a few days before surgery. Serum lipids and other risk factors were determined as previously described.\textsuperscript{19}

Prothrombin Assay

The determination of coagulation factor II activity was performed on a Behring coagulation timer (BCT, Dade-Behring) by modification of the 1-stage prothrombin time with the use of factor II–deficient plasma (Dade-Behring) and Thromborel-S (Dade-Behring). Coagulation time (by BCT) was calibrated with standard human plasma (Dade-Behring). Results were expressed in terms of factor activity (%).

Mutation Analysis

DNA was extracted from peripheral blood lymphocytes by using the phenol/chloroform protocol. Dr Christian Oberkanins (ViennaLab Labordiagnostika, GmbH, Vienna, Austria) kindly supplied the prothrombin gene mutation assay for the in vitro amplification of prothrombin gene sequences and the subsequent detection of prothrombin gene G20210A mutation by allele-specific hybridization in microwells.

Statistical Analysis

All computations were performed by using the SPSS 7.5.21 statistical package (SPSS Inc). Distributions of continuous variables were expressed as mean±SD. Logarithmic transformation was performed on skewed variables, including prothrombin. Statistical significance for differences in quantitative variables was tested by the Student unpaired t test. Genotype frequencies for prothrombin gene polymorphism were compared by χ\textsuperscript{2} analysis in patients and control subjects, with the values predicted by assumption of a Hardy-Weinberg equilibrium. To assess the association between genotype and CAD or MI, odds ratios (ORs) with 95% CIs were calculated.

Simple correlations between prothrombin activity and the other variables were determined by means of the Pearson coefficient; the independence of these associations was evaluated by stepwise multiple linear regression analysis.

After dividing the study sample into tertiles of prothrombin levels (based on the distribution of the control values), we used logistic regression analysis to assess the association of CAD with the highest levels of prothrombin, in relation to the lowest reference level. Risk estimates were obtained with multivariate models that additionally adjusted for age, sex, body mass index, hypertension, smoking, and plasma levels of cholesterol, triglycerides, homocysteine, fibrinogen, and glucose. All probability values were 2-tailed, and the CI was computed at the 95% level.

Results

Table 1 shows the baseline characteristics of all participants. As expected, CAD patients had more conventional risk factors than did control subjects. Regarding the factor V Leiden mutation, no homozygous subjects were found. The prevalence of heterozygosity for this mutation was no different either between CAD patients and control subjects or between the MI and non-MI groups.

Prothrombin activity was significantly higher in CAD patients than in control subjects (P<0.005). No differences were found in prothrombin activity of the MI versus non-MI groups. No homozygous carriers of G20210A prothrombin mutation were found either in patients or in control subjects. Although we found a higher prevalence of individuals with the 20210A allele among CAD patients than among control subjects (5.3% versus 3.1%, respectively), this difference did not reach statistical significance (OR 1.72, 95% CI 0.72 to 4.08). No significant difference was observed when individuals with or without previously documented MI were compared (4.5% versus 5.3%, respectively; OR 0.85, 95% CI 0.33 to 2.16) (Table 2).
The genotype-phenotype correlation study, which focused on the effects of G20210A mutation on prothrombin plasma activity, was performed by using combined data from the whole population, after exclusion of those individuals taking anticoagulant drugs at the time of blood sampling or during the previous week. Prothrombin activity was significantly influenced by the presence of the mutant allele (Table 3), inasmuch as heterozygous carriers had higher prothrombin activity (almost 25% higher, on average) than did noncarriers (P<0.001).

A proportion of prothrombin activity variability was explained by stepwise multiple linear regression analysis (R=0.392, R²=0.154), showing positive associations with fibrinogen (P<0.01, β-coefficient=0.129), homocysteine (P<0.001, β-coefficient=0.201), and total cholesterol (P<0.001, β-coefficient=0.237) and an inverse association with age (P=0.001, β-coefficient=−0.183). Women had higher prothrombin activity than did men (P<0.05).

It has been suggested that the risk of CAD may be amplified by the combination of the 20210A allele with other major risk factors. Thus, we tested the distribution of the heterozygosity for the 20210A allele between CAD patients and control subjects belonging to high-risk categories, such as smokers, those aged >55 years, and those with hypercholesterolemia, hypertension, or obesity. In these categories, too, statistical significance was not reached.

Similarly, to emphasize the role of G20210A polymorphism, the distribution of heterozygosity for the 20210A allele between CAD patients and control subjects was assessed in subgroups with a low-risk profile, such as individuals aged <55 years and individuals without conventional risk factors of CAD, but again, the results were not statistically significant. Prothrombin activity was divided into tertiles (based on the distribution of values in control subjects), and the OR for CAD was calculated for the upper tertile (values ranging from 130% to 280%) compared with the lower tertile (68% to 105%). The crude OR was 2.11, with 95% CI from 1.35 to 3.27. After including several putative confounding variables (age, sex, body mass index, hypertension, smoking, and plasma levels of cholesterol, triglycerides, homocysteine, fibrinogen, and glucose) in the multivariate logistic model, the adjusted OR was 1.86, with 95% CI from 1.35 to 3.27.
age-matched control subjects. Such discrepancies may be precisely for this reason, on the other hand, we cannot rule out the possibility that in more selected CAD populations, the bias was avoided. Moreover, inasmuch as our CAD patients were appropriate for ensuring the quality of controls. Furthermore, because of the high heterogeneity of the population observed, these results probably cannot be generalized, inasmuch as the 20210A allele seemed to be relevant for arterial thrombosis in selected cases. Indeed, others have found a significantly increased prevalence of the prothrombin gene 20210A variant in patients with arterial disease compared with newborns or age-matched control subjects. Such discrepancies may be partly accounted for by the substantial variation in the 20210A allele frequency among the populations. Other reasons for such discrepancies may be differences in the prevalence of other genetic and environmental risk factors in different populations and/or in different selection criteria. A key point in genetic studies is the rigorous definition of phenotypes in cases and controls. This seems particularly true of CAD association studies, because individuals with substantial, though not yet clinically manifested, CAD may be erroneously included as control subjects, yielding an increased probability of null results. Having included only individuals with an objective angiographic documentation of coronary artery status, we feel confident that this potential bias was avoided. Moreover, inasmuch as our CAD patients had a substantial burden of conventional risk factors (Table 1), our results may reasonably hold well for the general population of CAD patients seen in clinical practice. Precisely for this reason, on the other hand, we cannot rule out the possibility that in more selected CAD populations, the presence of the prothrombin 20210A allele may be relevant for atherothrombosis. Indeed, we conducted a separate assessment of particular subgroups with a low-risk profile (i.e., young subjects and/or subjects without traditional risk factors), as is often the case in studies seeking to establish the role of genetic risk factors. Again, our findings were consistent with the null hypothesis, but any conclusion must be viewed with caution, given the very small sample sizes. A similar interpretation might explain the lack of association between the 20210A allele and CAD in certain subgroups of our population with the concomitant presence of other major risk factors (e.g., smoking, hypertension, and obesity), at variance with data previously reported by others.

Worthy of note is the fact that the present study confirms the significant variability of prothrombin as a function of G20210A genotypes, inasmuch as heterozygous carriers had significantly higher prothrombin activity than did non-carriers. Consistent with this finding is a very recent report that strongly supports the view that G20210A represents a functional polymorphism.

High prothrombin levels have already been identified as a risk factor for venous thrombosis, but little is known regarding high prothrombin levels in relation to arterial disease. Interestingly, we found significantly higher prothrombin activity in CAD patients than in control subjects, suggesting that elevated prothrombin activity itself may be associated with CAD.

A number of the environmental factors examined were found to be associated with prothrombin activity in the present study. Women had higher prothrombin activity than did men, which is not surprising, because female sex hormones may shorten prothrombin time by modulating several coagulative parameters. Prothrombin activity was also positively associated with plasma cholesterol, fibrinogen, and homocysteine and inversely associated with age. However, the exact mechanisms of these associations still need to be fully clarified; therefore, further studies are necessary for a better understanding of this aspect.

On the other hand, the OR for MI for the upper tertile of prothrombin, compared with the lower tertile, did not prove to be statistically significant.

## Discussion

Although the prevalence of G20210A prothrombin gene mutation was higher among our CAD patients than among control subjects, this difference did not reach statistical significance. Similarly, we found no difference in distribution of genotypes between CAD patients with or without previous MI. These results provide evidence against a major role for G20210A mutation as a risk factor for either CAD or MI, at least in the present study conditions.

Previous data are controversial. Some authors have reported observations consistent with ours; in particular, the only prospective study published so far found no association between the 20210A allele and the risk of MI. However, caution in the interpretation of the results of that study has been suggested, mainly because of the procedures used for end-point validation, which were considered inappropriate for ensuring the quality of controls. Furthermore, because of the high heterogeneity of the population observed, these results probably cannot be generalized, inasmuch as the 20210A allele seemed to be relevant for arterial thrombosis in selected cases. Indeed, others have found a significantly increased prevalence of the prothrombin gene 20210A variant in patients with arterial disease compared with newborns or age-matched control subjects. Such discrepancies may be partly accounted for by the substantial variation in the 20210A allele frequency among the populations. Other reasons for such discrepancies may be differences in the prevalence of other genetic and environmental risk factors in different populations and/or in different selection criteria. A key point in genetic studies is the rigorous definition of phenotypes in cases and controls. This seems particularly true of CAD association studies, because individuals with substantial, though not yet clinically manifested, CAD may be erroneously included as control subjects, yielding an increased probability of null results. Having included only individuals with an objective angiographic documentation of coronary artery status, we feel confident that this potential bias was avoided. Moreover, inasmuch as our CAD patients had a substantial burden of conventional risk factors (Table 1), our results may reasonably hold well for the general population of CAD patients seen in clinical practice. Precisely for this reason, on the other hand, we cannot rule out the possibility that in more selected CAD populations, the presence of the prothrombin 20210A allele may be relevant for atherothrombosis. Indeed, we conducted a separate assessment of particular subgroups with a low-risk profile (i.e., young subjects and/or subjects without traditional risk factors), as is often the case in studies seeking to establish the role of genetic risk factors. Again, our findings were consistent with the null hypothesis, but any conclusion must be viewed with caution, given the very small sample sizes. A similar interpretation might explain the lack of association between the 20210A allele and CAD in certain subgroups of our population with the concomitant presence of other major risk factors (e.g., smoking, hypertension, and obesity), at variance with data previously reported by others.

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On the other hand, the extreme tertiles of prothrombin activity distribution are considered, the association between prothrombin and CAD was found to be independent of all the risk factors included in the logistic regression model, including all the risk factors that were associated with prothrombin activity in the multiple linear regression model.

The group with higher prothrombin activity included more carriers of the 20210A allele. However, the similar distribution of genotypes between patients and control subjects suggests that prothrombin activity might be only one of the effectors and that other, at present unknown, environmental and/or genetic factors, besides the 20210A allele, may be responsible for high prothrombin activity.

The present study does not explain the mechanism underlying the association between CAD and high prothrombin activity, which may be merely a marker rather than a cause of the disease. An increased propensity to thrombosis does not seem convincing, because in this case, the association should be stronger for acute ischemic events, and prothrombin activity was not associated with the risk of MI. Apart from its effect on blood coagulation, thrombin is involved in the regulation of endothelial cell proliferation and fibroblast mitogenesis. Thus, it would seem more plausible to

## TABLE 3. Prothrombin Activity in the Study Population as a Function of Prothrombin Genetic Markers

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Prothrombin Activity, %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>GG (n=473)</td>
<td>122.2±28.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AG (n=23)</td>
<td>153.2±38.6</td>
<td>...</td>
</tr>
</tbody>
</table>

Values are mean±SD. Genotype-phenotype correlation study was performed on 496 subjects, i.e., whole population after exclusion of subjects taking anticoagulant drugs at time of blood sampling or during previous week.
attribute the development and propagation of atherosclerotic lesions to these additional properties.

One limitation of the present study is its case-control design. Thus, our findings need to be confirmed by prospective studies, which constitute the most effective means of obtaining reliable information on the clinical utility of this new marker of cardiovascular disease.

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