Chapter 4

Arterial wall thickness and the risk of recurrent ischemic events in carriers of the prothrombin G20210A mutation with clinical manifestations of atherosclerosis

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Abstract

The G20210A mutation in the prothrombin gene is an established risk factor for venous thrombosis. There is controversy about the role of this mutation in arterial thrombotic disease and atherosclerosis. We determined the presence of the prothrombin mutation and examined its influence on carotid and femoral artery intima-media thickness (IMT) and the occurrence of new ischemic events during follow-up in 277 patients with clinically manifest atherosclerotic disease: ischemic stroke, myocardial infarction or peripheral arterial disease. The mean age at entry was 63 years. Mean IMT was significantly higher in carriers of the prothrombin mutation [1.17 (SD0.29)mm versus 0.97 (SD0.25)mm: \( \delta \text{IMT} = 0.20, p = 0.02 \)]. The increase in IMT was not attributable to differences in age, type of arterial disease or cardiovascular risk factors between carriers and non-carriers. During a mean follow-up of 3.5 years, a strong trend for more ischemic events was observed: 4 of the 11 carriers suffered from a recurrent ischemic event, compared with 30 of the 164 male non-carriers (36% versus 18%; \( p = 0.06 \)). These results suggest that the G20210A mutation contributes to the process of arterial wall thickening and is associated with the occurrence of ischemic events in a cohort of elderly persons with established atherosclerosis.
Introduction

Atherosclerosis is a multifactorial process. In its pathogenesis many inherited and acquired risk factors are thought to have a causal role. The atherosclerotic process may manifest as coronary, cerebral and peripheral artery stenosis and occlusion. The distribution of risk factors may vary among patients with these different manifestations and factors with a limited influence in early stages of the atherosclerotic process may have a relevant role in advanced stages.

The coagulation system is important in the pathogenesis of clinical manifestations of atherosclerosis. Thrombotic occlusion of coronary and cerebral arteries is the cause of myocardial and cerebral infarctions. However, the role of the coagulation system in the process of atherosclerotic wall thickening is uncertain, although increased levels of coagulation factors and enhanced coagulation activity have been associated with increased carotid artery wall thickness and coronary artery stenosis. Pathologic and experimental observations indicate that microfissures in the atherosclerotic vessel wall with subsequent mural thrombus formation and fibrotic organization contribute to the process of wall thickening. Thrombin is thought to be an important factor in this process. Thrombin is essential in arterial thrombus formation, but appeared to be a growth factor for vascular smooth muscle cells as well, either directly or by stimulating other growth factors. Whether higher (local) thrombin concentration leads to increased wall thickness in patients with advanced atherosclerotic lesions needs to be elucidated.

Mutations in certain genes of coagulation factors may lead to higher levels or an altered function of these coagulation proteins. In this way, these mutations might be risk factors for atherosclerosis and its clinical complications. One of these potential risk factors is a mutation in the 3' untranslated region of the prothrombin gene. A G→A transition of nucleotide 20210 is associated with higher prothrombin clotting activity and a 2 to 4-fold higher risk for venous thrombosis.

There is considerable controversy about the role of the G20210A mutation in arterial vascular disease. An increased prevalence has been reported in patients with myocardial infarction and cerebral artery disease, but other studies could not confirm these findings. In apparently healthy men in the Physicians' Health Study the presence of the G20210A mutation was not associated with the future occurrence of myocardial infarction or stroke. These conflicting results suggest that the importance as a risk factor could be different in different patient groups and populations. The influence of the G20210A polymorphism on arterial wall thickness is unclear and prospective studies on patients with established atherosclerosis are lacking.

We hypothesized that the G20210A mutation may be a relevant factor in patients with established atherosclerosis, but may have a limited role in healthy individuals. To test the first hypothesis we investigated whether the presence of the G20210A mutation is associated with increased arterial wall thickness, determined by B-mode ultrasound intima-media thickness (IMT) measurement, and a higher incidence of ischemic events during follow-up, in a cohort of patients with clinically manifest atherosclerosis.

Methods

Patients and follow-up

Consecutive patients with clinical manifestations of atherosclerosis were recruited in two teaching hospitals and classified according to previous event: myocardial infarction, ischemic stroke or peripheral arterial disease. Only patients with a recent onset of myocardial infarction (≤ 1 month) or ischemic stroke (≤ 6 months) were included. Myocardial infarction was defined as typical chest pain for more than 20 minutes in combination with laboratory- or
ECG findings consistent with myocardial infarction. Ischemic stroke was defined as acute neurologic deficit persisting at least one week. Intracerebral hemorrhage was ruled out by an early CT-scan. Peripheral arterial disease was defined as typical leg pain on walking and an ankle/arm blood pressure ratio lower than 0.85 in either leg at rest or a history of surgery for intermittent claudication. Smoking status, hypertension defined as a diastolic blood pressure of 95 mmHg or higher or current treatment, and hypercholesterolemia defined as a total plasma cholesterol level of 7 mmol/L or higher or current treatment were registered. Patients provided a peripheral blood sample for DNA analysis.

During prospective follow-up new vascular events were registered. Every six months events were assessed using a questionnaire regarding vascular complications and reported cardiovascular endpoints were confirmed through a review of hospital records, discharge letters from the hospital and autopsy reports. For myocardial infarction and stroke we utilized the same definitions as mentioned for the qualifying event.

The study was approved by the medical ethics committees and all patients gave informed consent to participate.

**B-Mode ultrasound**

The B-mode ultrasound IMT measurement procedures have been described elsewhere\(^{24,25}\). B-mode ultrasound scans were performed by one sonographer. An ATL Ultramark IV (Advanced Technology Laboratories, Bothell, Washington) with a High Resolution Linear Array 7.5 MHz transducer was used. Subjects were scanned in the reclined position. Three right carotid, three left carotid, two right femoral and two left femoral artery wall segments were scanned. Images of each arterial wall segment were stored on S-VHS video tape. IMT of the posterior wall segments was measured off-line\(^{26}\). The sonographer and image analyst were blinded to the clinical status of the subjects.

**G20210A mutation**

Extraction of genomic DNA from peripheral leukocytes in citrated blood was performed using a QIAamp blood kit (QIAGEN, Germany). Digestion with the restriction enzyme Hind III was used to identify the variant allele at position 20210 of the prothrombin gene. The primers and PCR conditions used were previously described\(^{12}\).

**Statistical analyses**

Calculations were performed with SPSS, version 6.1. Frequencies were compared with Chi-square tests or Fisher exact tests when applicable. IMT measurements of investigated artery wall segments were combined on a per subject basis. This was allowed for in this investigation since analyses of the separate arterial wall segments showed a similar percentage of available measurements in the subpopulations in each of the segments. For comparisons, Student’s t-test was used. A p-value of 0.05 was considered statistically significant. Kaplan-Meier estimates were used for comparison of events during follow up.

**Results**

**Patient characteristics and prevalence of the prothrombin G20210A mutation**

A total of 307 consecutive patients were included. The presence of the G20210A mutation was determined in 277 patients of whom DNA samples were available. Of these 277 patients 93 patients had a recent ischemic stroke, 86 a recent myocardial infarction and 98 had peripheral arterial disease. Characteristics of the study cohort and the subgroup with and without the prothrombin mutation are described in Table 1. Except for gender, there were no clinically relevant differences between patients with or without the prothrombin mutation. All
the carriers were male, which may be partially explained by the high percentage males in the myocardial infarction (81%) and peripheral arterial disease (66%) groups. Most mutations were confined to patients with myocardial infarction (5 out of 86) or peripheral arterial disease (4 out of 98), while 2 stroke patients had the mutation.

**Table 1** Characteristics of all patients and characteristics according to genotype

<table>
<thead>
<tr>
<th></th>
<th>All patients</th>
<th>GG</th>
<th>AG</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>277</td>
<td>266</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>male (%)</td>
<td>175 (63)</td>
<td>164 (62)</td>
<td>11 (100)</td>
<td>0.01</td>
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<tr>
<td>age (years)</td>
<td>63</td>
<td>63</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>hypertension (%)</td>
<td>117 (42)</td>
<td>111 (42)</td>
<td>6 (55)</td>
<td></td>
</tr>
<tr>
<td>DM (%)</td>
<td>34 (12)</td>
<td>33 (12)</td>
<td>1 (9)</td>
<td></td>
</tr>
<tr>
<td>hyperchol (%)</td>
<td>126 (48)</td>
<td>121 (48)</td>
<td>5 (45)</td>
<td></td>
</tr>
<tr>
<td>homocysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (μmol/l)</td>
<td>15.1</td>
<td>15.1</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td>smoking (%)</td>
<td>233 (84)</td>
<td>224 (84)</td>
<td>9 (82)</td>
<td></td>
</tr>
</tbody>
</table>

GG = non-carrier of G20210A mutation, AG = heterozygote for the G20210A mutation, DM = diabetes mellitus, hyperchol=hypercholesterolemia

**Intima Media Thickness**

IMT measurements of 143 males and 82 females were available. Since the G20210A mutation was present in males only, we limited the analysis to the male data. In the 134 males without the G20210A mutation 1177 IMT measurements and in the 9 males with the mutation 77 IMT measurements were available. The IMT in subjects with the G20210A mutation was increased compared to subjects without the mutation [IMT<sub>AG</sub>= 1.17 (SD 0.29, range 0.40 - 3.82)mm; IMT<sub>GG</sub> = 0.97 (SD 0.25, range 0.31 - 3.97)mm: ΔIMT=0.20 (95%CI 0.370 - 0.028)mm: p=0.02]. The distribution of factors associated with arterial wall thickness (age, type of arterial disease, cardiovascular risk factors) was similar in carriers and male non-carriers of the mutation so the observed difference cannot be attributed to these factors.

**Ischemic events**

During a mean follow-up of 3.5 years, 4 of the 11 carriers suffered an ischemic event. Two of these carriers had a stroke and two had a myocardial infarction. For comparison only male non-carriers were used. Of these male non-carriers 12 persons suffered from a stroke while 18 had a myocardial infarction. After two years this difference was significant (4/11 versus 17/164, p=0.03, stroke or myocardial infarction) but Kaplan-Meier estimates for the entire follow-up period were not statistically significant for the composite endpoint stroke or myocardial infarction (p=0.06), stroke (p=0.11) or myocardial infarction (p=0.29), although a strong trend was noted (Figure 1).
Discussion

We found an increased carotid and femoral intima-media thickness in carriers of the prothrombin mutation with clinical manifestations of atherosclerosis. B-Mode ultrasound IMT-measurement is a non-invasive way of quantifying atherosclerosis. An increase in IMT has been associated with various risk factors of atherosclerotic disease as well as the occurrence of myocardial infarction and stroke during long term follow-up\(^\text{27-40}\). Since there were no major differences in patient characteristics between carriers and non-carriers of the prothrombin mutation the well known risk factors cannot account for the higher IMT observed in carriers.

In previous reports a correlation between the G20210A mutation and levels of prothrombin, prothrombin activation peptide F1+2 and thrombin anti-thrombin complexes was described suggesting that the mutation leads to increased rates of thrombin generation\(^\text{12,18,41,42}\). Thrombin is thought to be an important factor in arterial wall thickening, either by its procoagulant properties, by initiating the proliferation of smooth muscle cells at sites of vascular injury, or by regulating inflammatory processes\(^\text{6-11}\). The G20210A mutation may contribute to the progression of atherosclerosis in individuals with established atherosclerosis by causing more local thrombin generation.

We found a higher prevalence of the G20210A prothrombin mutation in our patient population compared to published prevalence data in Northwestern Europe\(^\text{43}\) (4.0% [11/277] versus 1.6% [45/2756] odds ratio 2.5 [95%CI: 1.2-5.1]), especially among patients with a myocardial infarction (5.8% [5/86]) and to a lesser extent among patients with peripheral arterial disease (4.1% [4/98]). This comparison was not the primary aim of the study, but the results indicate that within our patient cohort of elderly individuals with clinical manifestations of atherosclerosis the G20210A prothrombin mutation is a moderate risk factor for myocardial infarction and possibly for peripheral arterial disease. The observed higher prevalence supports previous findings in patients with premature coronary artery disease and individuals with a first myocardial infarction before the age of 70 years\(^\text{15,17-19}\). In contrast, in a prospective study with healthy elderly individuals and in other case control studies the same prevalence in patients with a myocardial infarction and control persons was found\(^\text{16,22,23}\).

The number of ischemic events during follow up was higher in carriers of the prothrombin mutation (36% versus 18%). The high rate of ischemic recurrences indicates that the
mutation might also be a prognostic factor in patients with established atherosclerosis. The difference did not reach statistical significance, but only a very strong risk factor would cause a detectable difference in events in such a small group of carriers of a mutation. With an expected prevalence of the mutation in this population of 3% to 4% the study was powered to detect considerable differences in ischemic events only. Since an increase in IMT is associated with the occurrence of myocardial infarction and stroke, the high event rate in carriers compared to non-carriers is consistent with the higher IMT values observed in carriers.

In conclusion, in patients with established atherosclerotic disease, IMT is increased in carriers of the prothrombin G20210A mutation. In addition, we observed a strong trend for more ischemic events in carriers of the mutation. These findings may encourage the design of an adequately powered prospective study in patients with established atherosclerotic disease.

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