Evidence in favor of the contribution of genes involved in the maintenance of the extracellular matrix of the arterial wall to the development of intracranial aneurysms

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Received April 11, 2006; Revised and Accepted October 6, 2006

Intracranial aneurysm is probably a complex disease with both genetic and non-genetic or environmental risk factors contributing to the etiology of the disease. A disruption of the extracellular matrix (ECM) of the arterial wall is a likely factor in the pathogenesis of intracranial aneurysms. We analyzed 44 potential candidate genes involved in the maintenance of the integrity of the ECM in 382 Dutch Caucasian patients with intracranial aneurysms and 609 Dutch Caucasian controls for 384 tag single nucleotide polymorphisms (SNPs) using the GoldenGate assay on an Illumina BeadStation 500 GX. We identified SNPs that were associated with intracranial aneurysms \( (P < 0.01) \) in six of these 44 genes: serpine1 \( (\text{SERPINE1}, P = 0.0008) \), transforming growth factor beta induced \( (\text{TGFBI}, P = 0.0026) \), perlecan \( (\text{HSPG2}, P = 0.0044) \), fibronectin \( (\text{FN1}, P = 0.0069) \), fibrillin 2 \( (\text{FBN2}, P = 0.0077) \) and alpha 1 type IV collagen \( (\text{COL4A1}, P = 0.0087) \). In a second independent cohort of 310 Dutch Caucasian intracranial aneurysm patients and 336 Dutch Caucasian controls, the association for the \( \text{HSPG2} \) gene \( \text{[combined odds ratio (OR) 1.33, 95% confidence interval (CI) 1.13–1.57, } P = 6 \times 10^{-4}] \) was replicated. The population attributable risk (PAR) for this SNP is 19%. Combining the two cohorts still showed association for the \( \text{SERPINE1} \) \( \text{[combined OR 1.27, 95% CI 1.07–1.50, } P = 0.004, \text{ PAR 6%}] \), \( \text{FBN2} \) \( \text{[combined OR 1.37, 95% CI 1.07–1.75, } P = 0.01, \text{ PAR 3%}] \) and \( \text{COL4A1} \) \( \text{[combined OR 1.22, 95% CI 1.05–1.42, } P = 0.007, \text{ PAR 7%}] \) genes. These PARs are likely to be overestimates as they are calculated from the joint analyses combining stages 1 and 2 of our association study. Our findings indicate that variation in genes involved in the maintenance of the integrity of the ECM of the arterial wall plays a role in susceptibility to intracranial aneurysms. These findings further support our hypothesis that diminished maintenance of the ECM of the arterial wall is important in the development of intracranial aneurysms.

INTRODUCTION

Intracranial aneurysms \( \text{[ANIB, (MIM 105800)]} \) are found in ~2% of the general population (1). Rupture of an intracranial aneurysm, which is most common between 40 and 60 years of age, causes a subarachnoid hemorrhage (SAH) and prognosis after rupture is poor: half the patients die and 20% remain dependent for activities of daily life (2–3). Although the incidence of aneurysmal SAH is low (approximately eight per 100 000 person-years) (4), because of the young age at onset and the poor prognosis, the loss of productive life years as a consequence of SAH is comparable to that of ischemic stroke (5).

Familial occurrence of intracranial aneurysms suggests genetic factors are involved in the development of intracranial aneurysms. Familial clustering of SAH is found in ~10% of patients with SAH, and the first-degree relatives of patients with SAH have a 2.5 to seven times greater risk of developing...
Intracranial aneurysm is probably a complex disease with both genetic and non-genetic or environmental risk factors contributing to the etiology of the disease (12). Genome-wide linkage studies in patients with intracranial aneurysms have already identified several different loci for intracranial aneurysms (i.e. loci on chromosomes 1p34.3–p36.13 (13), 5q22–31 (14), 7q11 (14–15), 14q22 (14), 17cen (14,16), 19q13.3 (16–18) and Xp22 (16–17)), four of which have been replicated (i.e. 7q11, 17cen, 19q13.3 and Xp22).

A disruption of the extracellular matrix (ECM) of the arterial wall is a likely factor in the pathogenesis of intracranial aneurysms, as a decrease in structural proteins of the ECM has been demonstrated in the intracranial arterial wall of many ruptured intracranial aneurysms and also in skin biopsies and in intra- and extracranial arteries of aneurysm patients (12). Furthermore, the genetic loci identified for intracranial aneurysms include some interesting candidate genes coding for structural proteins of the ECM of the arterial wall.

On the basis of previous studies on the genetic and pathophysiological background of intracranial aneurysms, a list of 44 of the most promising positional and/or functional candidate genes involved in the maintenance of the integrity of the ECM of the arterial wall was selected. The aim of the present study was to investigate whether single nucleotide polymorphisms (SNPs) in these ECM candidate genes are associated with intracranial aneurysms in the Dutch population. For this purpose, we have taken advantage of a comprehensive screen using tag SNPs in those 44 genes by genotyping approximately nine SNPs per gene (range 1–59).

**PATIENTS AND METHODS**

**Design of the study**

An association study of intracranial aneurysms was performed using a two-stage genotyping approach. For stage 1, we genotyped 384 SNPs in cases and controls. As an additional analysis, the SNP frequency between subgroups consisting of patients with familial and non-familial intracranial aneurysms and patients with ruptured and unruptured intracranial aneurysms was compared separately to study whether there are specific SNPs associated with familial intracranial aneurysms and/or rupture of intracranial aneurysms. For stage 2, we genotyped the SNPs yielding the most significant associations in a second cohort of cases and controls to confirm the associations.

**Patients and controls**

For stage 1, DNA, isolated from whole blood, was available from a cohort of 382 Dutch Caucasian patients admitted to the University Medical Center Utrecht and 609 Dutch Caucasian controls. The patients included both ruptured and unruptured and familial and non-familial intracranial aneurysms; the controls were blood bank donors. Ruptured intracranial aneurysms were defined by symptoms suggestive of SAH combined with subarachnoid blood on CT and a proven aneurysm at angiography (conventional angiogram, CT- or MR-angiogram) and unruptured intracranial aneurysms were identified by CT- or MR-angiography or conventional angiography. Patients with familial intracranial aneurysms were defined as having at least two first-degree relatives with ruptured or unruptured intracranial aneurysms. For stage 2, DNA was available from a second, independent cohort of 310 Dutch aneurysmal SAH patients and 336 ethnically matched Dutch Caucasian controls, including spouses and healthy family members of patients with diverse diseases, for example, celiac disease and diabetes mellitus type 2 but not intracranial aneurysms. All patients and controls gave their informed consent. This study was approved by the Medical Ethical Committee of the University Medical Center Utrecht.

**Snp selection and genotyping**

For stage 1, 44 candidate genes involved in the maintenance of the integrity of the ECM were selected on the basis of either (a) localization to an implicated chromosomal region, on the basis of previous linkage studies, (b) association with intracranial aneurysms, (c) expression within intracranial aneurysm tissue based on previous expression studies, (d) evidence from functional studies in intracranial aneurysm patients, (e) disease causing genes of heritable disorders of connective tissue and ECM associated with intracranial aneurysms and (f) membership of the same gene family as already selected candidate genes or a combination of the aforementioned criteria. The 44 candidate genes analyzed are shown in Table 1, whereas a more detailed description of these candidate genes, including reference to previous expression-, functional-, linkage- and association studies from the literature, is provided in Supplementary Material, Table S1. SNPs were selected by downloading all the SNPs typed in the CEPH population (Utah residents with ancestry from northern and western Europe) in our 44 candidate genes from the HapMap database (http://www.hapmap.org/, genome build 34) (19). From these SNPs, tag SNPs (20) were selected using the aggressive tagging option of the Tagger program (Paul de Bakker, http://www.broad.mit.edu/mpg/tagger/) so that all SNPs with a minor allele frequency ≥5% were captured with r² ≥ 0.8. Any SNPs with low Illumina quality design scores were excluded. Such prioritization of tag SNPs can be done with little loss of power (21). Eventually, 384 tag SNPs distributed in 44 candidate genes were derived for genotype analysis (Supplementary Material, Table S2). SNP genotyping was performed using the GoldenGate assay on an Illumina BeadStation 500 GX (Illumina Inc., San Diego, USA). All tag SNPs were examined for their resulting quality and those that had a low signal or those with poor clustering were excluded (n = 22). DNA samples with low signals for most of the SNPs were also excluded (n = 19).

For stage 2, we selected the six genes with SNPs yielding the most significant associations (P < 0.01) in stage 1 and one gene with associated SNPs, for which association with intracranial aneurysms had already been demonstrated in the literature. For these selected SNPs, we obtained Taqman Assays on Demand or Assays by Design (Applied Biosystems, Foster City, USA), which were genotyped on an ABI 7900HT instrument (Applied Biosystems, Foster City, CA, USA).
The AB assay IDs of the Taqman Assays on Demand are provided in Supplementary Material, Table S3.

**Statistical analysis**

Association $\chi^2$ values with two-tailed $P$-values and Hardy–Weinberg equilibriums were calculated using the Haplview program [available at http://www.hapmap.org (22)] for both stages of our study. In stage 1, SNPs with a $P < 0.001$ in the controls were considered not in Hardy–Weinberg equilibrium (22); in stage 2, this threshold was set at 0.05. For the associated SNPs tested in stage 2, we assessed differences in allele frequencies as an odds ratio (OR) with corresponding 95% confidence intervals (CI), using the allele with the lower frequency in the controls as opposed to the allele frequency in patients as the reference allele. The population attributable risk (PAR) for the SNPs for which there was still association after combining stages 1 and 2 was calculated using the following formula: $\text{PAR} = \frac{\text{PF}(\text{RR} - 1)}{[\text{PF}(\text{RR} - 1) + 1]}$, where PF is the population fraction with the risk factor and RR is the relative risk of the risk factor (23), which can be replaced by the odds ratio (24).

**RESULTS**

**Genotyping stage 1**

No tag SNPs showed deviation from the Hardy–Weinberg equilibrium (Supplementary Material, Table S2). After the quality checks, 22 poorly performing tag SNPs were excluded because of too low a signal, scattering or overlap of clusters, leaving 362 tag SNPs for further analysis (conversion rate of 94.5%). The association data of the 362 tag SNPs that passed through the quality check, with $\chi^2$ and corresponding $P$-values, are shown in Supplementary Material, Table S2. Of the DNA samples, 11 of 609 control samples and eight of the 382 patient samples performed poorly because of low signals and were excluded for further analyses; this left 598 controls and 374 patients to be analyzed (conversion rate of 98.1%). The clinical data of the 374 patients are shown in Table 2.

Considering a $P$-value $< 0.05$, we saw association to SNPs in 16 of the 44 analyzed genes (Table 3). Considering a $P$-value $< 0.01$, we saw association for SNPs in six of the analyzed genes: the serpine1 gene ($\text{SERPINE1}$; strongest association for SNP rs6956010, $P = 0.0008$), transforming growth factor beta induced ($\text{TGFBI}$; one associated SNP rs756463, $P = 0.0026$), perlecan ($\text{HSPG2}$; strongest association for SNP rs3767137, $P = 0.0044$), fibronectin ($\text{FNB1}$; strongest association for SNP rs2289202, $P = 0.0069$), fibrillin 2 ($\text{FBN2}$; strongest association for SNP rs331069, $P = 0.0077$) and alpha 1 type IV collagen ($\text{COL4A1}$; strongest association for SNP rs3783107, $P = 0.0087$) (Table 3). Significant association was also found for one SNP in the elastin gene ($\text{ELN}$; rs 4717865, $P = 0.0103$), and association of this gene with intracranial aneurysms had already been demonstrated in the literature (14,25). Analysis revealed no strong linkage disequilibrium (LD) between the two associated SNPs in $\text{SERPINE1}$ (high $D' = 0.9$, but low $r^2 = 0.2$), the two associated SNPs in $\text{HSPG2}$ (low $D' = 0.03$ and low $r^2 = 0.0$) and the four associated SNPs in $\text{FNB1}$ ($D' = 0.9$ between 0.2 and 0.9 with low $r^2$ between 0.03 and 0.4). There was high LD between two associated SNPs in $\text{FBN2}$ (rs28114 and rs331069 with $D' = 0.9$ and $r^2 = 0.8$) but not between the remaining associated SNPs. For $\text{COL4A1}$ high LD between two of the five associated SNPs was observed (rs675605 and rs630943 with $D' = 0.9$ and $r^2 = 0.8$) but not for the remaining ones.

No differences in SNP frequency were observed (a) between patients with familial and non-familial intracranial aneurysms.

**Table 1.** Analyzed candidate genes involved in the maintenance of the integrity of the ECM

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural proteins</td>
<td></td>
</tr>
<tr>
<td>Collagens producing fibers ($n = 5$)</td>
<td>Alpha 1 type I collagen gene, alpha 2 type I collagen gene, alpha 1 type III collagen gene, alpha 1 type V collagen gene, alpha 2 type V collagen gene</td>
</tr>
<tr>
<td>Collagens contributing to ECM structure ($n = 4$)</td>
<td>Alpha 1 type IV collagen gene, alpha 2 type VI collagen gene, alpha 2 type VIII collagen gene and alpha 1 type XVI collagen gene</td>
</tr>
<tr>
<td>Elastic fibers ($n = 1$)</td>
<td>Elastin</td>
</tr>
<tr>
<td>Glycoproteins ($n = 12$)</td>
<td>Fibulin 1 and 2, fibronectin, microfibril-associated protein 1, 2, 3 and 4, fibrin 5, fibromodulin, perlecan, emilin 1 and 2</td>
</tr>
<tr>
<td>Other structural proteins ($n = 2$)</td>
<td>Secreted protein acidic and rich in cysteine, transforming growth factor beta induced</td>
</tr>
<tr>
<td>Proteases</td>
<td></td>
</tr>
<tr>
<td>Metallorproteinases ($n = 3$)</td>
<td>Metalloproteinase 2, 9 and 14</td>
</tr>
<tr>
<td>Other proteases ($n = 4$)</td>
<td>Elastase, cathepsin B and D, plasminogen</td>
</tr>
<tr>
<td>Protease inhibitors</td>
<td></td>
</tr>
<tr>
<td>Tissue inhibitors of metalloproteinase ($n = 3$)</td>
<td>Tissue inhibitor of metalloproteinase 1, 2 and 3</td>
</tr>
<tr>
<td>Other protease inhibitors ($n = 6$)</td>
<td>A1 antitypsine, serpine 3, serpine1, cystatin C, K and S</td>
</tr>
<tr>
<td>Other enzymes ($n = 2$)</td>
<td>Lysyl oxidase, lysyl oxidase 3</td>
</tr>
<tr>
<td>Growth factors ($n = 2$)</td>
<td>Connective tissue growth factor, osteoblast specific factor 2</td>
</tr>
</tbody>
</table>

**Table 2.** Clinical data of the analyzed patients who had intracranial aneurysms for stages 1 and 2

<table>
<thead>
<tr>
<th></th>
<th>Patients stage 1 ($n = 374$)</th>
<th>Patients stage 2 ($n = 310$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women (%)</td>
<td>260 (69.5)</td>
<td>194 (62.6)</td>
</tr>
<tr>
<td>Familial intracranial aneurysms (%)</td>
<td>77 (20.6)</td>
<td>22 (7.2)</td>
</tr>
<tr>
<td>Unruptured intracranial aneurysms (%)</td>
<td>61 (16.3)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Multiple intracranial aneurysms (%)</td>
<td>69 (18.4)</td>
<td>70 (22.5)</td>
</tr>
<tr>
<td>MCA aneurysms (ruptured and unruptured) (%)</td>
<td>90 (24.1)</td>
<td>50 (16.2)</td>
</tr>
<tr>
<td>Mean age at time of SAH (years)</td>
<td>50.7 (range 10–84)</td>
<td>44.6 (range 11–66)</td>
</tr>
</tbody>
</table>

MCA, middle cerebral artery.
nor (b) between patients with ruptured and unruptured intracranial aneurysms (data not shown).

Genotyping stage 2

In stage 2, there was also no evidence of a deviation from the Hardy–Weinberg equilibrium (data not shown). The clinical data of the 310 patients analyzed in stage 2 are shown in Table 2. For SNP rs1561299, the Taqman assay did not have an acceptable quality and was therefore not genotyped in this stage. Additional genotyping of 18 SNPs in the SERPINE1, TGFBI, HSPG2, FN1, FBN2, COL4A1 and ELN genes in the second cohort of 310 patients and 336 controls confirmed the association of the same allele of SNP rs3767137 in HSPG2 with intracranial aneurysms ($P = 0.05$; Table 4). Combining both cohorts (stage 1 and stage 2), the association seen in stage 1 remains (OR 1.33, 95% CI 1.13–1.57, $P = 0.0006$). The PAR for this SNP is 19%. The second SNP in HSPG2 rs7556412, found to be associated in stage 1 and not in LD with SNP rs3767137, showed no association in this stage or in the combined cohort. The association of SNP rs6956010 in SERPINE1 could not be replicated in stage 2, but on combined analyses of both cohorts, the association of this SNP remained statistically significant, although less strong than observed in stage 1 (OR 1.27, 95% CI 1.07–1.50, $P = 0.004$). The PAR for this SNP is 6%. For the other SNP in SERPINE1 rs2070682, which is not in LD with SNP rs6956010, no association in this stage nor in the combined cohorts could be confirmed. The association of the four associated SNPs in FBN2 and the five associated SNPs in COL4A1 could not be replicated in stage 2. However, for rs331079 in FBN2 and SNP rs3783107 in COL4A1, a preponderance of the allele found to be more frequent in the patient group in stage 1 was also observed in the patient group for stage 2, and in combined analyses on both cohorts, the association of these two SNPs remained statistically significant, although less strong than observed in stage 1 (rs331079 in FBN2: OR 1.37, 95% CI 1.07–1.75, $P = 0.01$; rs3783107 in COL4A1: OR 1.22, 95% CI 1.05–1.42, $P = 0.007$). The PAR for the SNP in FBN2 is 3% and for the SNP in COL4A1 7%. SNP rs331079 in FBN2 is not in LD with the other SNPs in this gene that appeared to be associated in stage 1. Also, SNP rs3783107 in COL4A1 is not in LD with the other SNPs in this gene that showed association in stage 1. Associations for the SNPs in the TGFBI, FN1 and ELN genes could not be confirmed. For the SNPs in FN1, it was even the case that the association in stage 2 was with the allele complementary to the allele that was associated in stage 1.

**DISCUSSION**

By analyzing genes that are involved in the maintenance of the integrity of the ECM of the arterial wall and which are therefore candidate genes for intracranial aneurysms, we identified...
Table 4. Analyzing the six genes with SNPs yielding the most significant associations from stage 1 ($P < 0.01$) and the gene (*ELN*) for which association with intracranial aneurysms had already been demonstrated in the literature in a second, independent cohort of 310 intracranial aneurysm patients and 336 controls.

<table>
<thead>
<tr>
<th>Gene</th>
<th>rs number</th>
<th>Stage 1: frequency associated allele</th>
<th>Stage 2: frequency associated allele in stage 1</th>
<th>Stage 1 + 2: allele frequencies combined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
<td>$\text{OR}(95%\ CI)$</td>
<td>$\text{P-value}$</td>
</tr>
<tr>
<td></td>
<td>($n = 374$)</td>
<td>($n = 598$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SERPINE1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>rs6956010</td>
<td>27.5 20.7 1.45 (1.16–1.81) 0.0008</td>
<td>24.9 24.1 1.04 (0.80–1.36) 0.74</td>
<td>26.3 21.9 1.27 (1.07–1.50) 0.004</td>
</tr>
<tr>
<td></td>
<td>rs756463</td>
<td>77.2 70.9 1.39 (1.11–1.73) 0.0026</td>
<td>72.4 72.4 1.00 (0.77–1.29) 1.00</td>
<td>73.0 71.4 1.08 (0.92–1.26) 0.35</td>
</tr>
<tr>
<td><strong>TGFBI</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>rs7556412</td>
<td>66.5 61.0 1.27 (1.04–1.55) 0.0165</td>
<td>60.6 61.5 0.96 (0.76–1.22) 0.75</td>
<td>63.8 61.2 1.12 (0.96–1.30) 0.14</td>
</tr>
<tr>
<td></td>
<td>rs2289202</td>
<td>78.6 73.1 1.35 (1.08–1.70) 0.0069</td>
<td>72.9 75.5 0.87 (0.67–1.14) 0.30</td>
<td>76.0 73.9 1.12 (0.94–1.32) 0.19</td>
</tr>
<tr>
<td></td>
<td>rs2034776</td>
<td>79.7 75.0 1.31 (1.04–1.64) 0.0193</td>
<td>72.3 78.6 0.71 (0.54–0.93) 0.01</td>
<td>76.3 76.2 1.00 (0.85–1.19) 0.97</td>
</tr>
<tr>
<td><strong>HSPG2</strong></td>
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<tr>
<td></td>
<td>rs331069</td>
<td>54.4 48.1 1.29 (1.06–1.56) 0.0077</td>
<td>49.3 50.7 0.95 (0.76–1.18) 0.62</td>
<td>52.1 49.1 1.13 (0.98–1.30) 0.10</td>
</tr>
<tr>
<td></td>
<td>rs1052002</td>
<td>90.4 86.5 1.36 (1.10–1.86) 0.044</td>
<td>89.4 89.5 1.03 (0.71–1.50) 0.86</td>
<td>89.0 88.2 1.22 (0.97–1.55) 0.08</td>
</tr>
<tr>
<td><strong>FN1</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>rs675603</td>
<td>72.8 68.5 1.23 (1.00–1.52) 0.0468</td>
<td>69.3 70.5 0.96 (0.75–1.22) 0.71</td>
<td>71.2 69.2 1.03 (0.92–1.15) 0.60</td>
</tr>
<tr>
<td></td>
<td>rs2391824</td>
<td>40.0 34.5 1.27 (1.04–1.54) 0.0155</td>
<td>35.8 35.0 1.04 (0.82–1.31) 0.76</td>
<td>38.1 34.7 1.16 (1.00–1.35) 0.05</td>
</tr>
</tbody>
</table>

ND, not determined.
a SNP in the perlecan (HSPG2) gene associated with intracranial aneurysms in the Dutch population. In addition, we found evidence that SNPs in the serpine1 (SERPINE1), fibrillin 2 (FBN2) and alpha 1 type IV collagen (COL4A1) genes may also be associated with intracranial aneurysms in the Dutch population.

This study reports an unique analysis of a large set of SNP genotypes in the most promising positional and/or functional candidate genes for intracranial aneurysms of the ECM pathway. Using the HapMap data (19), we achieved great coverage of the analyzed candidate genes with inter-SNP distances of 5–10 kb, which is what is required to conclusively discount these genes from having a role in intracranial aneurysms susceptibility (26). To capture the maximum information from these SNPs as efficiently as possible, we selected tag SNPs on the basis of known patterns of LD. With this approach, four genes proved to be associated with intracranial aneurysms: HSPG2 with an OR of 1.33, SERPINE1 with an OR of 1.27, FBN2 with an OR of 1.37 and COL4A1 with an OR of 1.22. Considering that the first-degree relatives of patients with SAH have a seven times greater risk of developing SAH than the general population (6) and assuming that there are no gene–gene interactions between the genes, these genes can together explain ~70% [(1.33 + 1.27 + 1.37 + 1.22)/7] or less of this increased genetic risk. Our findings that genes involved in the maintenance of the integrity of the ECM of the arterial wall are associated with intracranial aneurysms further strengthens our view that diminished maintenance of the ECM of the arterial wall is an important aspect in the development of intracranial aneurysms.

The HSPG2 gene is located in a previously reported locus for intracranial aneurysms on chromosome 1p34.3–p36.13 (ANIB3; HUGO nomenclature committee) identified in a single North American family (13). HSPG2 codes for a large (467 kDa) heparan sulphate proteoglycan (27). It is expressed in basement membranes, including those of the arterial wall, and is believed to be involved in the stabilization of macromolecules and cell adhesion (28). As a major component of basement membranes, it interacts with other basement membrane components such as laminin, collagen type IV and also with other ECM molecules such as fibronectin (28), which plays a role in enhancing cell adhesion (29–30). Fragmentation of the basement membrane components, collagen type IV and fibronectin, has been observed in intracranial aneurysms (31), and this may be caused by the loss of heparan sulfate HSPG2 to interact with the other components. On the other hand, the fragmentation of collagen type IV in the basement membrane may also partly or fully be explained by genetic variation in COL4A1 leading to disruption of this collagen type, as in this study we also observed association of SNPs in COL4A1 with intracranial aneurysms.

The proteoglycans of the ECM, in general, may play an important role in the pathogenesis of intracranial aneurysms, as in a previous study we already showed association with intracranial aneurysms of SNPs and haplotypes in the versican (CSPG2) gene, which encodes another proteoglycan of the ECM (32). As CSPG2 also interacts with fibronectin, the loss of capacity of both HSPG2 and CSPG2 to interact with fibronectin may contribute to the development of intracranial aneurysms.

SERPINE1 maps to 7q21.3–q22, whose locus lies in the vicinity of a locus for intracranial aneurysms on chromosome 7q11 identified in 104 Japanese affected sib pairs (14) (ANIB1; HUGO nomenclature committee). SERPINE1 inhibits active metalloproteinases (MMPs), which are enzymes that degrade collagen and other ECM molecules, and represses plasmin (33). Plasmin in turn activates inactive zymogens of MMPs (pro-MMPs) by cleavage of the N-terminal predomain (33). In intracranial aneurysms patients, the relation of SERPINE1 to the occurrence of intracranial aneurysms has not yet been investigated, but it is possible that the expression and/or function of SERPINE1 may be diminished in these patients, leading to higher levels of active MMPs and consequently to more degradation of ECM molecules. In patients with abdominal aortic aneurysms (AAA), this hypothesis is already supported as the 4G allele of the deletion/insertion (4G/5G) polymorphism, which is associated with higher levels of SERPINE1, was less common in AAA patients compared to controls (34). Furthermore, SERPINE1 mRNA levels were lower in AAA tissue when compared with athero-occlusive abdominal aortas (35).

FBN2 codes for one of the fibrillins, which are ECM macromolecules and assemble into microfibrils surrounding the elastin fibers (36). The gene maps to 5q23–q31, whose location overlaps with a locus for intracranial aneurysms on chromosome 5q22–31 (14). FBN2 is the disease-causing gene of congenital contractual arachnodactyly, a disease phenotypically similar to the Marfan syndrome and characterized by arachnodactyly, dolichostenomelia, scoliosis, multiple congenital contractures and abnormalities of the external ears (37). Disruption of fibrillin 2 may lead to a diminished assembly and therefore disruption of the elastin fibers. A disruption of internal elastic lamina, which predominantly consists of elastic fibers, has already been demonstrated in intracranial aneurysms (38–39).

In the present study, we identified four risk factors for intracranial aneurysms, with the SNP in HSPG2 accounting for 19%, the one in SERPINE1 for 6%, the one in FBN2 for 3% and the one in COL4A1 for another 7% of the cases. These PARs are likely to be overestimates, as they are calculated from the joint analyses combining stages 1 and 2 of our association study. Previously, we had investigated the PARs of the risk factors known—at that time—for aneurysmal SAH and showed that the modifiable risk factors account for most of the SAH cases, with heavy drinking of alcohol accounting for 21%, smoking for 20%, hypertension for 17% and moderate alcohol drinking for 11%. A further 11% of the cases could be attributed to a positive family history for SAH and 0.3% to autosomal dominant polycystic kidney disease (40). A recent systematic review identified more risk factors for aneurysmal SAH (41), but no PARs have been calculated yet for these factors. Adding up the PARs of all the risk factors identified thus far would lead to a total of >100%, which further underscores the multifactorial origin of the disease.

Analyses of large sets of SNPs for association with intracranial aneurysms may generate false-positive results and therefore raise the question whether multiple testing correction should be applied. As yet, there is no straightforward correction for these highly correlated single tests. The debate on multiple testing correction is further complicated by the fact...
In general, these genes will be the foci of future studies. The ECM pathway will be associated with aneurysm formation. Aortic aneurysms (42–43), we anticipate that genes within SERPINE1, FN2 and COL4A1 identified in the first stage remained significant on combining both stages, which suggests they are true associations. Of course, the ultimate proof of association will be consistent replication in other patient populations and the identification of the functional variant.

In the replication tests, the associations for the SNPs in the TGFBI, FN1 and ELN genes could not be confirmed. For the SNPs in FN1, it was even the case that the association in stage 2 was with the allele complementary to the allele that was associated in the first stage, which is strongly indicative that the observed association of FN1 with intracranial aneurysms is a false-positive result. It may be hypothesized that the observed associations for these genes in stage 1 could be attributed to the inclusion of patients with unruptured intracranial aneurysms in this stage, since only patients with ruptured ones were included in stage 2. However, this hypothesis seems unlikely, as combined analysis of the stage 2 patient cohort with the patients with unruptured intracranial aneurysms from stage 1 did not in fact change our findings (data not shown).

The findings on the ELN gene are in contrast with a previous observation, when we analyzed 18 exonic and intronic SNPs of the ELN gene in 167 aneurysmal SAH patients and 167 age- and sex-matched controls and found significant association of ELN variants with intracranial aneurysms (25). The 18 tested exonic and intronic SNPs are different from the tag SNPs used in this study. The 167 aneurysmal SAH patients were included in the first stage of the present study, and for both the entire patient population of the first stage and this subset of 167 patients, we found association of ELN variants with intracranial aneurysms. As these observations could not be validated in the second stage of this present study, our previous results (25) and the results of the first stage of this study are likely to be false-positive.

In conclusion, the identification of the HSPG2 gene, and possibly the SERPINE1, FBN2 and COL4A1 genes, as susceptibility gene(s) in intracranial aneurysms is an intriguing finding that needs to be further evaluated in order to understand their importance in the development of intracranial aneurysms. Furthermore, the possibility that these genes are likely to be involved in the maintenance of the integrity of the ECM of the arterial wall should be further explored. The possible consequences of the SNPs identified in these genes on the function and expression levels of HSPG2, SERPINE1, FBN2 and COL4A1 remain to be elucidated. Since the involvement of the ECM in the pathogenesis of aneurysm formation has also been suggested for abdominal and thoracic aortic aneurysms (42–43), we anticipate that genes within the ECM pathway will be associated with aneurysm formation in general. These genes will be the foci of future studies.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS
Y.M.R. was supported by the Netherlands Organization for Scientific Research (NWO), project no. 940-37-023. This work was supported by the Netherlands Brain Foundation, project no. 13F05.13.

Conflict of Interest statement. None declared.

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