From Intracranial Aneurysm to Subarachnoid Hemorrhage: Unraveling the Genetics

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FROM INTRACRANIAL ANEURYSM TO SUBARACHNOID HEMORRHAGE: UNRAVELING THE GENETICS

Een onderzoek naar de genetische achtergronden
van intracraniële aneurysmata en
subarachnoïdale bloedingen
(met een samenvatting in het Nederlands)

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Promotoren: Prof.dr. G.J.E. Rinkel
Prof.dr. C. Wijmenga

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Contents

Chapter 1

Preface and Outline of the Thesis

Preface

Approximately 2% of the general population harbor an intracranial aneurysm.[1] Most intracranial aneurysms do not cause symptoms and remain undiagnosed. However, in a small subset of patients the intracranial aneurysm ruptures causing a subarachnoid hemorrhage (SAH). SAH is a subset of stroke. Based on an incidence of approximately 8 per 100,000 person-years,[2] this means that up to 1000 patients have an aneurysmal SAH per year in the Netherlands. It often occurs at a relatively young age and has a poor prognosis. Up to 50% of the patients die from the consequences of the hemorrhage and 30% of the survivors remain dependent for activities of daily life.[3] Patients who survive the initial hours after hemorrhage are threatened by rebleeding as long as the aneurysm is not occluded and by secondary cerebral ischemia in the initial weeks after the hemorrhage.[3] The young age and poor outcome explain why in the general population the loss of productive life years from aneurysmal SAH is as large as that from brain infarcts, the most common type of stroke.[4]

Although little is known about the pathogenesis of intracranial aneurysm and subsequent aneurysmal SAH, an important role for genetic factors is recognized as familial predisposition is the strongest risk factor for aneurysmal SAH.[1] These genetic factors may include genes involved in the maintenance of the integrity of the extracellular matrix (ECM) of the arterial wall, which provides strength and elasticity to intracranial arteries, since a disruption of the ECM is suggested to play a role in the pathogenesis of intracranial aneurysms. This hypothesis is based upon the observation that intracranial aneurysms are associated with heritable disorders of connective tissue and ECM.[5,6] Moreover, 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 intra- and extracranial arteries of aneurysm patients.[7-15] Identifying the genetic factors for intracranial aneurysm and aneurysmal SAH will lead to a better understanding of the pathogenesis of intracranial aneurysms and aneurysmal SAH. The identification may in the future lead to new therapeutic interventions to help prevent the development, growth and/or rupture of intracranial aneurysms. Furthermore, with the identification of the genetic factors involved in the pathogenesis, diagnostic tools using high-risk genotypes can be developed. These diagnostic tools may help to identify first-degree relatives of SAH patients with a high risk of developing one or more intracranial aneurysms. These first-degree relatives can be screened for aneurysms regularly and those not at high risk can be reassured.

The principal aim of this thesis is to identify genes involved in intracranial aneurysms and subsequent aneurysmal SAH. These genes will be identified using a study population with a familial preponderance of the disease. In *Part I* of the thesis this study population and its familial risk factor are described. *Part II* describes the identification of genes, especially those implicated in the maintenance of the integrity of the ECM of the arterial wall, involved in the occurrence of intracranial aneurysms and aneurysmal SAH. *Part III* explores the utilization of genetics in clinical studies on aneurysmal subarachnoid hemorrhage. Especially factors involved in the occurrence of rebleeding and secondary cerebral ischemia after aneurysmal SAH and in the recovery after secondary cerebral ischemia are investigated.

Outline of the Thesis

Part I

Chapter 2 outlines the population attributable risks of the known risk factors, including the risk factor of a positive family history, for aneurysmal SAH. *Chapter 3* describes the comparison of characteristics of intracranial aneurysms (*i.e.* size and number) in patients with a familial form of aneurysmal SAH and those with the non-familial or sporadic form. In *Chapter 4* demographic and clinical features in patients of families with familial intracranial aneurysms and different patterns of inheritance are compared. In addition, the ages of patients with SAH in affected parent-child pairs are compared, since previous studies suggested anticipation. *Chapter 5* describes a pilot study on the comparison of subtle manifestations of connective tissue or ECM disease between patients with aneurysmal SAH and controls to assess whether intracranial aneurysms is not a localized disruption but may rather represent a more general laxity of the connective tissue and ECM.

Part II

Chapter 6 gives an overview of the current knowledge on the genetic background of intracranial aneurysms. The two most common approaches used to identify genes, *i.e.* association studies and genome-wide linkage studies, and the results of these studies in intracranial aneurysms are discussed. It is explained that genes involved in the maintenance of the integrity of the ECM are likely to be involved in the pathogenesis of intracranial aneurysms. *Chapters 7* and *8* describe the results of two association studies analyzing two functional and positional candidate genes both coding for structural proteins of the ECM. In *Chapter 7* the elastin gene is

explored while in *Chapter 8* the versican gene is being investigated. In *Chapter 9* a large set of 44 additional candidate genes involved in the maintenance of the integrity of the ECM is analyzed for association with intracranial aneurysms. *Chapter 10* describes the results of a genome-wide linkage study in a large Dutch family with intracranial aneurysms. *Chapter 11* describes a literature study in which the genomic loci identified in whole genome linkage studies on intracranial, thoracic and abdominal aortic aneurysms are described and compared in search of possible common genetic risk factors for the three types of aneurysms.

Part III

In *Chapter 12* genes that are expressed after ischemia and that influence recovery after ischemia in the animal model of ischemia are explored for a relationship with outcome in patients after aneurysmal SAH. *Chapter 13* analyzes whether genes modifying prothrombotic coagulation are associated with risk of secondary cerebral ischemia and rebleeding after aneurysmal subarachnoid hemorrhage. In *Chapter 14* the implications of the results as described in this thesis are discussed. Also, future perspectives in the research on the genetics of intracranial aneurysms and aneurysmal SAH are described.

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Part I: Clinical Studies on Intracranial Aneurysms and Aneurysmal SAH

Chapter 2

Attributable risk of common and rare determinants of aneurysmal subarachnoid hemorrhage

Ynte M. Ruigrok, Erik Buskens, and Gabriël J.E. Rinkel

*Stroke 2001; **32:** 1173-1175*

Abstract

Background and purpose

Smoking, hypertension, alcohol consumption, autosomal dominant polycystic kidney disease (ADPKD), and positive family history for subarachnoid hemorrhage (SAH) are well-known risk factors for SAH. For effective prevention, knowledge about the contribution of these risk factors to the overall occurrence of SAH in the general population is pivotal. We therefore investigated the population attributable risks of the risk factors for SAH.

Methods

We retrieved the relative risk and prevalence of established risk factors for SAH from the literature and calculated the population attributable risks of these risk factors.

Results

Drinking alcohol 100 to 299 g/wk accounted for 11% of the cases of SAH, drinking alcohol >300 g/wk accounted for 21%, and smoking accounted for 20%. An additional 17% of the cases could be attributed to hypertension, 11% to a positive family history for SAH, and 0.3% to ADPKD.

Conclusions

Screening and preventive treatment of patients with familial preponderance of SAH alone will cause a modest reduction of the incidence of SAH in the general population. Further reduction can be achieved by reducing the prevalence of the modifiable risk factors alcohol consumption, smoking, and hypertension.

Spontaneous subarachnoid hemorrhage (SAH) from rupture of an intracranial saccular aneurysm has an incidence of approximately 6 per 100 000.[1] Although outcome after aneurysmal SAH has improved slightly over the last 3 decades, the prognosis remains poor; half the patients die, and 20% remain dependent for activities of daily life.[2] The best way to reduce morbidity and mortality is prevention of the occurrence of SAH. Prevention can be achieved by reducing risk factors or by screening and preventive treatment of patients at increased risk of aneurysmal rupture.

Smoking, hypertension, and alcohol abuse are established risk factors for SAH.[3] Furthermore, patients with autosomal dominant polycystic kidney disease (ADPKD)[4] and first-degree relatives of patients with SAH[5] have an increased risk for SAH. However, little is known about the contribution of each risk factor to the overall occurrence of SAH in the general population (the so-called population attributable risk [PAR][6]). For effective prevention, knowledge about which risk factors have to be targeted is essential. We therefore investigated the PARs of smoking, hypertension, alcohol consumption, ADPKD, and positive family history for SAH.

Methods

Smoking

The relative risk (RR) of SAH for smoking was retrieved from a systematic review of studies on risk factors for SAH; in the review the RR is 1.9 (95% CI, 1.5 to 2.3).[3] In the Netherlands, 34.7% of the population older than 16 years smokes; the age-adjusted prevalence is 27.9%.[7]

Hypertension

We extracted the estimate of the RR of SAH for hypertension (RR, 2.8; 95% CI, 2.1 to 3.6) from the same systematic review.[3] The age-adjusted prevalence of men and women with hypertensive blood pressure (>160/95 mm Hg) from the Monitoring Trends and Determinants in Cardiovascular Disease (MONICA) survey 1989-1990 is 11.4%.[8]

Alcohol

The estimates on the RRs of SAH for alcohol drinking were retrieved from a longitudinal study on the relationship between alcohol and SAH.[9] We could not use the other studies discussed in the systematic review[3] because it was impossible to recalculate the data of these studies into the categories of drinking alcohol 100 to 299 and >300 g/wk. In the Netherlands, 13.3% of the population consumes >6 alco-

holic drinks per week, which is approximately >100 g/wk.[7] The age-adjusted prevalence of drinking >100 g/wk is 10.7%. Consumption of >3 alcoholic drinks daily (approximately >300 g/wk) occurs in 7% of the population older than 18 years;[10] the age-adjusted prevalence is 5.9%. The age-adjusted prevalence of drinking 100 to 299 g/wk is 10.7% minus 5.9% and equals 4.8%. The RR of SAH for drinking 100 to 299 g/wk is 3.5 (95% CI, 1.1 to 11.0), and that for drinking >300g/wk is 5.6 (95% CI, 1.9 to 16.7).[9]

Positive family history for SAH

For the estimate of the RR of SAH for positive family history, we used data from a recent study performed in the Netherlands; in that study the RR was 6.6 (95% CI, 2.0 to 21.0).[5] Two other studies found similar RRs of 4.1 and 4.5.[11,12] We could not perform a meta-analysis on the RR of familial preponderance for SAH because we could not extract crude data from these two other studies.[11,12]

The incidence of SAH approximates 6 per 100 000 person-years.[1] This means that in the Netherlands, where the population size approximates 15 million people, 900 new patients have a SAH annually. With a life expectancy at birth of 78 years in the Netherlands,[7] lifetime risk of SAH is 1 per 214 [$(6x10^{-5})x(15x10^{6})x78$]. Consequently, in the Netherlands 70 093 [$(15x10^{6})/214$] people had or will have SAH. The mean number of first-degree relatives per family of a patient with SAH is 5.[13] A crude estimate of the total amount of people with a first-degree relative who had or will have SAH is then 350 465 (5x70 093) or 2.3% of the Dutch population.

Autosomal dominant polycystic kidney disease

Because no data are available on the RR of ADPKD for SAH, we calculated this RR as follows: the estimated RR of intracranial aneurysms in patients with ADPKD is 4.4 (95% CI, 2.7 to 7.2).[14] If we assume an equal risk of aneurysm rupture given

Table. *RR, prevalence, and PAR associated with risk factors for SAH*

Risk factor	RR (95% CI)	Prevalence, %	PAR, %
Drinking alcohol 100-299 g/wk	3.5 (1.1-11.0)	4.8	11
Drinking alcohol >300 g/wk	5.6 (1.9-16.7)	5.9	21
Smoking	1.9 (1.5-2.3)	27.9	20
Hypertension	2.8 (2.1-3.6)	11.4	17
Positive family history	6.6 (2.0-21.0)	2.3	11
ADPKD	4.4 (2.7-7.2)	0.1	0.3

the presence of an aneurysm, the RR would be 4.4. The prevalence for ADPKD in the general population is 1 per 1000.[15,16]

Data analysis

The PAR is an estimate of the fraction of the total number of patients with SAH in the population that can be attributed to a particular risk factor. The PAR of a given risk factor is influenced by the prevalence and the RR of this risk factor and is calculated according to the following formula: PAR = PF(RR-1)/[PF(RR-1)+1], where PF is the population fraction with the risk factor.[6]

Results

The PARs associated with the risk factors for SAH are shown in the ***Table.*** Drinking alcohol 100 to 299 g/wk accounted for 11% of the cases of SAH, drinking alcohol >300 g/wk accounted for 21%, and smoking accounted for 20%. An additional 17% of the cases were attributable to hypertension, 11% of the cases to a positive family history for SAH, and 0.3% to ADPKD. If all first-degree relatives of patients with SAH are effectively screened and if all aneurysms found during screening are eliminated, then a reduction in incidence of SAH of a maximum of 11% can be achieved.

Discussion

We found that excessive alcohol consumption and smoking are the major contributors to the incidence of SAH. Secondary contributors are hypertension, modest alcohol consumption, and familial preponderance.

We may have underestimated the PAR of smoking and hypertension for SAH. We extracted the RR of SAH for smoking from a systematic review[3] in which the results of 2 longitudinal cohort studies are combined.[9,17] The diagnosis of SAH was not always confirmed by CT or angiography in these longitudinal studies. Since CT shows sources other than a ruptured aneurysm, most often intracerebral hematomas in up to 20% of patients with the clinical diagnosis of SAH,[18] these studies probably have included patients with primary intracerebral hematoma. Because smoking is a less pronounced risk factor for intracerebral hematoma than for aneurysmal SAH,[19] including patients with intracerebral hematoma dilutes the RR for SAH. Accordingly, the attributable risk of smoking for SAH is probably under-

estimated in our analysis. For the prevalence of hypertension in the population we defined hypertension as either average systolic blood pressure >160 mm Hg or diastolic blood pressure >95 mm Hg. Because the risk of stroke increases with higher blood pressure levels,[20] the actual number of patients with SAH from hypertension is probably larger than we found using a single RR for all people with hypertension. The notion that our results are an underestimation of the PARs of smoking and hypertension for SAH is supported by previous studies that found a PAR of smoking of 40% and a PAR of hypertension of 30% for SAH.[21,22]

We reported the PAR of each risk factor for SAH separately, because the data presented in the various articles did not allow us to perform multivariate analyses. However, smoking and alcohol consumption are often combined, and heavy drinking is known to be a risk factor for hypertension.[23] The PARs presented for smoking, alcohol consumption, and hypertension may therefore not be independent. For this reason, it is not justified to add the percentages for smoking and alcohol to one overall atherosclerotic percentage. However, we believe that the estimates provide a good impression of the relative importance of the various risk factors.

The PARs found in our analysis are overall percentages; it was not possible to differentiate for age and sex. The relative contribution of risk factors for SAH probably differs between young and older patients. Genetic factors probably play a more important role in younger patients, and atherosclerotic risk factors probably play a more important role in older patients. Furthermore, SAH is more common in women,[1] while smoking, hypertension, and alcohol use are more common in men.

PARs may differ between different regions and may change over time. In the present analysis the estimates of prevalence of risk factors for SAH were based on studies conducted in Western European countries. In other populations the prevalence of the risk factors for SAH is different and therefore also the corresponding PARs. For example, the incidence of SAH in Finland is almost 3 times higher than in other parts of the world,[1] which may be partly attributed to the high prevalence of hypertension in this country.[24,25] Two studies performed in the late 1980s and the early 1990s found a PAR of smoking of 40% and a PAR of hypertension of 30% for SAH.[21,22] Since that period the prevalences of smoking and hypertension have decreased,[26] which may explain that our PARs of smoking and hypertension for SAH are lower.

The incidence of SAH has remained stable over the last 3 decades.[1] In contrast, the cardiovascular risk factors smoking and hypertension are reduced and, in accordance, the incidence of stroke in general has declined.[27] The reason for not finding a reduction in the incidence of SAH is probably that the number of patient-

years in SAH incidence studies performed is too small to detect such a decline. The highest number of patient-years in an incidence study was found to be 2 800 000.[1] To demonstrate an incidence reduction from 6 per 100 000 to 5 per 100 000 patient-years, 2 incidence studies with a total of 5 million patient-years are needed. Recent data from Finland suggest a decreasing incidence of SAH,[28,29] in combination with a decline of cardiovascular risk factors.[30,31]

We defined patients with a positive family history for SAH as the total number of people with a first-degree relative who had or will have SAH and found this to be approximately 2.3% of the total Dutch population. Even if screening programs for intracranial aneurysms in first-degree relatives of patients with SAH would result in accurate detection and effective prevention, SAH could only be prevented in those first-degree relatives of whom a family member already suffered SAH. Therefore, the 11% of the SAH cases attributable to a positive family history can never be totally eliminated.

In conclusion, screening and preventive treatment of patients with familial preponderance of SAH alone will cause a modest reduction of the incidence of SAH in the general population. Further reduction can be achieved by reducing the prevalence of the modifiable risk factors alcohol consumption, smoking, and hypertension. Screening programs for intracranial aneurysms in patients with ADPKD will have little influence on the incidence of SAH.

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Chapter 3

Characteristics of intracranial aneurysms in patients with familial subarachnoid hemorrhage

Ynte M. Ruigrok, Gabriël J.E. Rinkel, Ale Algra, Theodora W.M. Raaymakers, and Jan van Gijn

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Abstract

Background and purpose

Compared with sporadic aneurysms, familial aneurysms rupture at an earlier age and are more often located at the middle cerebral artery. Other characteristics of familial aneurysms may also differ from sporadic aneurysms. The authors compared the size of ruptured aneurysms and the number of aneurysms between patients with familial subarachnoid hemorrhage (SAH) and those with sporadic SAH.

Methods

The authors included all patients with familial SAH admitted to the University Medical Center Utrecht (UMCU) and their first-degree relatives with proven aneurysmal SAH, including admissions elsewhere. As reference group the authors used a consecutive series of patients with sporadic SAH admitted to the UMCU from December 1995 to March 1997. Criteria for sporadic SAH were absence of a positive family history and exclusion of aneurysms in first-degree relatives by means of MR angiography. The authors dichotomized sizes of aneurysms into small (<10 mm) and large (>10 mm). Size and number of aneurysms between patients with familial SAH and sporadic SAH were compared with relative risks (RR) with corresponding 95% CI.

Results

The authors found 58 patients with familial SAH (48 with information on aneurysm size) and 88 patients with sporadic SAH. Twenty of 48 patients with familial SAH (41%) had large aneurysms, versus 17 (19%) with sporadic SAH (RR, 2.1; 95% CI, 1.2 to 3.6). Fifteen of 58 patients with familial SAH (26%) had multiple aneurysms, versus 9 (10%) with sporadic SAH (RR, 2.5; 95% CI, 1.2 to 5.4).

Conclusions

Familial aneurysms are generally larger at time of rupture and more likely to be multiple than sporadic aneurysms. The development of large and multiple aneurysms may be related to genetic factors that determine defects of the arterial wall.

A familial predisposition is the strongest risk factor for the development of intracranial aneurysms.[1,2] Familial clustering of subarachnoid hemorrhage (SAH) from an aneurysm is found in approximately 10% of patients with aneurysmal SAH, and first degree relatives of patients with SAH have an up to seven times greater risk of SAH.[3] In comparison with sporadic aneurysms, familial aneurysms rupture at an earlier age and are more often located at the middle cerebral artery.[4-6]

One study found no difference in aneurysm size between familial and sporadic patients,[7] while another found aneurysms to be smaller in patients with familial SAH.[5] Some studies found a higher frequency of multiple aneurysms in familial aneurysms,[5,6] but others did not.[4,7,8] In most of these studies a positive family history for SAH and aneurysms was excluded in the control groups with sporadic patients only by interviewing the patients,[5-8] and not by interviewing[5-8] or screening all first-degree relatives.[4-8] Episodes of aneurysmal SAH or the presence of unruptured aneurysms may therefore have been overlooked in relatives of the patients allocated to the control group. Erroneously including patients with familial aneurysms in the control group may dilute the difference between the two groups.

In this study we compared aneurysm size and number of aneurysms between patients with familial SAH and patients with sporadic SAH in whom a positive family history for SAH and aneurysms was excluded by interviewing the patients and their relatives and by excluding the presence of unruptured aneurysms with MR angiography in the first-degree relatives.[9]

Methods

Patients with ruptured familial aneurysms

Patients with familial aneurysms were defined by an aneurysmal SAH and at least one other first-degree relative with aneurysmal SAH or presence of an unruptured aneurysm. Aneurysmal SAH was defined by symptoms suggestive of SAH combined with subarachnoid blood on CT and a proven aneurysm at angiography (conventional angiogram, CT or MR angiogram). Patients with an unruptured aneurysm were identified by CT or MR angiography, conventional angiography, surgery, or autopsy.

We included all patients with familial aneurysmal SAH admitted to the University Medical Center Utrecht between January 1985 and December 2002. First-degree relatives with aneurysmal SAH of index patients who had been admitted elsewhere were also included. First-degree relatives with SAH who had died be-

fore angiography could be performed were excluded. We also excluded families with autosomal dominant polycystic kidney disease or connective tissue disorders such as Ehlers-Danlos disease.

Patients with ruptured sporadic aneurysms

As reference group we used a consecutive series of patients with sporadic aneurysmal SAH admitted to the University Medical Center from December 1995 to March 1997, who participated in a study on screening relatives for the presence of asymptomatic aneurysms.[9] In this previous study all first-degree relatives between 20 and 70 years of age of SAH patients were invited for screening with MR angiography for the presence of unruptured aneurysms.[9] The 98 patients included in our hospital had 365 first-degree relatives between 20 and 70 years of age who could be screened with MR angiography. In 12 of these 365 relatives (3.3%) unruptured intracranial aneurysms were identified with MR angiography. The 10 index patients with SAH who had relatives with unruptured intracranial aneurysms (nine index patients had one relative and one index patient had three relatives with unruptured intracranial aneurysms) were excluded for the present study. The remaining 88 index patients had 308 first-degree relatives who were screened and in whom no unruptured intracranial aneurysms were identified. Furthermore, in these 88 patients a history of aneurysmal SAH had been excluded by interview of first-degree relatives.[9] Episodes of aneurysmal SAH were excluded by interviewing the patient and their first-degree relatives. Pedigrees of all families were constructed. Whenever an episode of SAH or an aneurysm was suspected in one of the first-degree relatives (if a relative reported to have had a stroke, an intracranial vascular lesion, or intracranial surgery, or if relatives had died from a presumed stroke or had suddenly died) medical records of first-degree relatives were retrieved and reviewed. Diagnostic criteria for SAH in these patients were similar to those for SAH in the patients with familial SAH.

Data collection

Of the patients with familial and sporadic SAH, we collected data on size of the ruptured aneurysm, and on number and location of ruptured and unruptured aneurysms. The size of the ruptured aneurysm was measured on CT angiography or conventional angiography by its greatest diameter, with correction for magnification. In all patients with sporadic SAH and in patients with familial SAH admitted to the University Medical Center Utrecht after 1995 CT angiography had been performed. Conventional angiography had been done in patients with familial SAH admitted to the University Medical Center Utrecht before 1995 and in patients who

had been admitted elsewhere. We recorded how many vessels were visualized during these catheter procedures. If angiograms of patients could no longer be retrieved, these patients were studied only for the number of aneurysms.

Data analyses

We dichotomized size of aneurysm into small (<10 mm) and large (>10 mm). To compare size and number of aneurysms between patients with familial SAH and patients with sporadic SAH we used relative risks (RR) with corresponding 95% CI. The size of the ruptured aneurysms was also compared according to their different locations (*i.e.* anterior communication artery, middle cerebral artery, internal carotid artery, and vertebrobasilar artery) to analyze if any differences in size between patients with familial SAH and sporadic SAH could be attributed to the different locations of the aneurysms.

Results

We found 68 patients with familial SAH. Ten of these were excluded since they died before (CT) angiography could be performed. The mean age at time of SAH was in the remaining 58 patients with familial SAH 47.0 years (SD 12.0 years) and in the 88 patients with sporadic SAH 52.5 years (SD 14.3 years). In the group of patients with familial SAH 44 patients (65%) were women compared with 65 (74%) patients in the group of patients with sporadic SAH.

In all 88 patients with sporadic SAH CT angiography was performed but 61 of these 88 patients (69%) had an additional conventional angiogram. In 40 of the 58 patients with familial SAH CT angiography was performed and in the 18 remaining patients conventional angiography was performed. In 33 of the 40 patients in whom CT angiography was performed an additional conventional angiogram was made. Thus, in 51 of the 58 (88%) patients with familial SAH conventional angiography was performed. Of the 18 remaining patients in whom only conventional angiography was performed, four vessel angiography was performed in 12, angiography of both carotid arteries and one vertebral artery in four, angiography of both carotid arteries in one and angiography of only one carotid artery also in one patient. In nine patients with familial SAH we did not have data on the size of aneurysms since angiograms of these patients could no longer be retrieved, leaving 49 (58 minus 9) patients with familial SAH to be studied for the size of aneurysms.

We could retrieve exact sizes of the ruptured aneurysms in 58 of 88 patients with sporadic SAH and in 44 of 49 patients with familial SAH. In the 58 patients

with familial SAH the size of the ruptured aneurysms ranged from 4 to 30 mm with a mean size of 11.0 mm (95% CI, 9.2 to 12.8), a median of 10.0 mm, and a SD of 5.9 mm. In the 44 patients with sporadic SAH the sizes ranged from 2 to 25 mm with a mean size of 8.1 mm (95% CI, 6.8 to 9.2), a median of 7.0 mm, and a SD of 4.4 mm. The difference of the mean sizes of these two groups was 2.9 mm, 95% CI 0.9 to 4.9. Twenty of the 49 patients (41%) with familial SAH had large aneurysms compared with 17 of 88 patients (19%) with sporadic SAH. In patients with familial SAH the ruptured aneurysm was more often larger than 10 mm (RR, 2.1; 95% CI, 1.2 to 3.6) than in patients with sporadic SAH (***Table 1***). On comparing the size of ruptured aneurysms according to the different aneurysm locations we found trends towards larger aneurysm size for all the different locations in patients with familial SAH.

In the 58 patients with familial SAH we found 86 aneurysms: 43 patients (74%) had one aneurysm, 11 patients had two aneurysms, 1 had three, 2 had four, and 1 had five aneurysms. In the group of patients with sporadic SAH 99 aneurysms

Table 1. *Comparison of size of ruptured aneurysms between patients with familial SAH and those with sporadic SAH.*

Size of IA	Familial SAH	Sporadic SAH	Relative risk (95% CI)
All IA < 10 mm	29 (59)	71 (81)	Reference
All IA > 10 mm	20 (41)	17 (19)	2.1 (1.2-3.6)
At ACA < 10 mm	16 (70)	32 (91)	Reference
At ACA > 10 mm	7 (30)	3 (9)	3.6 (1.0-12.3)
At MCA < 10 mm	7 (54)	15 (68)	Reference
At MCA > 10 mm	6 (46)	7 (32)	1.5 (0.6-3.4)
At ICA < 10 mm	5 (56)	14 (78)	Reference
At ICA > 10 mm	4 (44)	4 (22)	2.0 (0.7-6.2)
At VBA < 10 mm	1 (33)	6 (75)	Reference
At VBA > 10 mm	2 (67)	2 (25)	2.7 (0.6-11.3)

Values are n (%).
SAH: subarachnoid hemorrhage; IA: intracranial aneurysm;
ACA: anterior communicating artery; MCA: middle cerebral artery;
ICA: internal carotid artery; VBA: vertebrobasilar artery.

Table 2. *Comparison of number of aneurysms between patients with familial SAH and those with sporadic SAH.*

IA	Familial SAH	Sporadic SAH	Relative risk (95% CI)
Single	43 (74)	79 (90)	Reference
Multiple	15 (26)	9 (10)	2.5 (1.2-5.4)

Values are n (%).
SAH: subarachnoid hemorrhage; IA: intracranial aneurysm.

were found: 79 patients (90%) had a single aneurysm, 7 patients had two aneurysms, and 2 patients had three aneurysms. Overall, patients with familial SAH more often had multiple aneurysms (RR, 2.5; 95% CI, 1.2 to 5.4) (***Table 2***).

Discussion

We found that ruptured aneurysms are more often large in patients with familial SAH than in patients with sporadic SAH. Furthermore, patients with familial SAH more often have multiple aneurysms than patients with sporadic SAH. The effect of larger aneurysm size in familial SAH is probably not related to the location of the aneurysm on the circle of Willis as we found trends toward larger aneurysm size for all different aneurysm locations in this group.

The most important methodologic difference with other studies that addressed size and number of aneurysms in familial SAH is the way we have sampled our control patients with sporadic SAH. To rule out a positive family history we interviewed not only the patients, but also the first-degree relatives, and we drew complete pedigrees. Drawing pedigrees and interviewing all relatives considerably increases the chance of identifying a positive family history and also increases the accuracy.[10] Furthermore, in our study the presence of unruptured aneurysms was excluded by MR angiography in first-degree relatives of the reference patients.[9] Without such screening for unruptured aneurysms, aneurysms remain undetected in relatives, and patients with seemingly sporadic aneurysms may in fact have had familial aneurysms. Inclusion by mistake of patients with familial SAH and aneurysms in the reference group leads to dilution of any differences with familial aneurysms. This problem has been largely eliminated in our study.

In some patients with familial SAH in our study not all vessels were visualized during angiography. In contrast, in patients with sporadic SAH all intracranial vessels were completely visualized since they were investigated by CT angiography. Incomplete angiography in patients with familial SAH may have left some aneurysms undetected. Consequently, we may still have underestimated the true difference in the proportions of multiple aneurysms between familial and sporadic SAH. On the other hand, the proportion of patients with conventional angiograms was slightly higher in the patients with familial SAH. Because the sensitivity of CT angiography in detecting small, additional aneurysms is somewhat lower than that of conventional angiography,[11,12] relatively more small aneurysms may have remained undetected in the group of patients with sporadic SAH. Therefore, it is also possible that we have slightly overestimated the difference in the proportions of multiple aneurysms between familial and sporadic SAH.

In a Finnish population no difference in aneurysm size between familial and sporadic cases was found.[7] Apart from the definition of sporadic SAH, differences in the study populations may have influenced the results. The incidence of SAH in Finland is almost three times as high as in other parts of the world, in Finland men are more affected, and the proportion of aneurysms of the middle cerebral artery is larger than in other populations.[13-15] Missing data may also have contributed to the apparent lack of difference in aneurysmal size between familial and sporadic SAH. In the Finnish study data on size were lacking for 33% of patients with familial SAH and for only 1% of patients with sporadic SAH. The missing information may well have been caused by early deaths and because poor outcome is associated with aneurysm size,[16] perhaps the results have been biased toward an artificially small size in the group of patients with familial SAH.

In a 1987 review of the literature on familial SAH the authors found that the size of aneurysms in the patients included in the review was smaller than that reported in sporadic patients.[5] This conclusion is probably explained by publication bias and by lack of information on aneurysm size in 76% of the familial cases published at the time.

Previous studies found multiple aneurysms more frequent in familial aneurysms,[5,6] but in most studies the difference was not statistically significant.[4,7,8] Relatively small numbers of patients included in these studies or, again, inclusion of familial cases in the control group may have limited the statistical power of these studies.

When identifying a patient with SAH from a large ruptured aneurysm and multiple aneurysms one should be cautious to conclude that this patient probably has familial SAH. The positive predictive value of familial SAH in case of large or

multiple aneurysms is small because sporadic SAH is much more frequent than familial SAH (90% versus 10%).[3] Therefore, large ruptured aneurysms and multiple aneurysms are more often related to sporadic SAH than familial SAH.

The results of a prospective study in the Netherlands suggested that patients with familial SAH more often have a poor outcome than patients with sporadic SAH.[17] As poor outcome occurs more often in patients with large aneurysms[16] the higher risk of poor outcome in familial cases may, in part, be explained by larger aneurysm size.

The development of large and multiple aneurysms in some families may be related to genetic factors that are determinants for defects of the arterial wall. In previous studies smoking and hypertension have been identified as risk factors for multiple and familial aneurysms.[18-20] The interaction between genetic and environmental factors may predispose to a relative weakness of the arterial wall, with a higher risk of developing large and multiple aneurysms in patients with familial aneurysms than in patients with sporadic aneurysms. Connective tissue defects have been found in patients with multiple aneurysms,[21] which may represent a phenotypic expression of genetic characteristics in familial intracranial aneurysms. Recently, several candidate genes for aneurysms were proposed based on the results of two genome wide linkage studies in affected sibpairs with familial aneurysms.[22,23] One study showed positive evidence for linkage on 7q11 in the vicinity of the elastin gene,[22] while another study found linkage on 19q12-13, which contains several loci related to cerebrovascular disease.[23] Further studies should unravel the precise role of the genetic factors in the development of familial SAH and aneurysms.

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Chapter 4

Anticipation and phenotype in familial intracranial aneurysms

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Abstract

Background and purpose

In familial intracranial aneurysms there is evidence for genetic heterogeneity, probably from mutations at separate loci. To compare demographic and clinical features in patients of families with familial intracranial aneurysm and different patterns of inheritance; and to compare the ages of patients with subarachnoid hemorrhage (SAH) in affected parent-child pairs to determine whether there is anticipation.

Methods

Pedigrees for 53 families with familial intracranial aneurysms were constructed, divided into patterns of inheritance suggestive or not suggestive of autosomal dominant transmission. Demographic and clinical features were compared. The age at time of SAH in affected parent-child pairs was compared using the Wilcoxon test.

Results

No differences in demographic or clinical features were found between families compatible with an autosomal dominant pattern of inheritance and those with a non-dominant pattern. In families with affected members in two successive generations the age at time of SAH in parents was 55.2 years and in children 35.4 years (mean difference, 19.8 years; $p<0.001$).

Conclusions

Phenotypes are similar in families with and without a probable autosomal dominant pattern of inheritance. Thus in future genetic studies on familial intracranial aneurysms, stratification according to phenotype is not likely to be useful. Anticipation probably occurs, as affected parents are significantly older at the time of SAH than their affected children.

Familial intracranial aneurysms are defined by the presence of at least two affected first degree relatives with aneurysmal subarachnoid hemorrhage (SAH) or an unruptured intracranial aneurysm. In comparison with sporadic intracranial aneurysms, familial aneurysms rupture at an earlier age and are more often located at the middle cerebral artery.[1-3]

Some pedigrees of familial intracranial aneurysms seem consistent with an autosomal dominant pattern of inheritance, whereas others suggest a multifactorial or autosomal recessive pattern. Segregation analysis has shown that several mendelian inheritance modes were compatible, but that autosomal dominant and autosomal recessive transmission were the most likely patterns.[4] Thus genetic heterogeneity in familial intracranial aneurysm is possible, either with mutations at separate loci (locus heterogeneity) or with different mutations at the same locus (allelic heterogeneity). Recently, evidence for possible locus heterogeneity in familial intracranial aneurysms was found as one study reported that a genome-wide scan for intracranial aneurysm susceptibility genes showed positive evidence for linkage on 7q11,[5] while another found linkage on 19q.[6] Locus heterogeneity may be characterized by differences in phenotype.

An example of a genetically heterogeneous disorder is Alzheimer's disease. In genetic studies on Alzheimer's disease the patients are dichotomized according to phenotype into those with early onset and those with late onset, and different genetic deficits have been identified in the two distinct subgroups.[7] In case there is a difference in phenotype in familial intracranial aneurysms, future genetic studies on this condition might also use stratification according to phenotype to optimize mutational screening.

We compared demographic and clinical features between patients of families consistent with an autosomal dominant pattern of inheritance with those where the pattern of inheritance was not suggestive of autosomal dominant transmission (non-dominant mode). In addition, in families with an autosomal dominant transmission we compared the ages at the time of SAH in parent-child pairs, because a previous study indicated that in two successively affected generations the age at time of SAH was lower in children than in their parents, which suggests genetic anticipation.[1]

Methods

Ascertainment of families and definition of familial intracranial aneurysms

For this study we had intended to define familial intracranial aneurysms as the presence of at least two first degree relatives with aneurysmal SAH, in contrast to

the commonly used definition (the presence of at least two affected first degree relatives with aneurysmal SAH or an unruptured intracranial aneurysm), as the latter will result in the inclusion of families where only one relative had SAH and one other had an unruptured intracranial aneurysm. Inclusion of such families is likely to lead to bias because many have been ascertained differently from those where two members have had an SAH (for example, by active screening of asymptomatic relatives of an SAH patient). However, because most other genetic studies on familial intracranial aneurysms have included families with one SAH and one unruptured aneurysm, we carried out two separate analyses using both definitions: *(1)* defined as the presence of at least two affected first degree relatives with aneurysmal SAH; *(2)* defined as the presence of at least two affected first degree relatives with aneurysmal SAH or with an unruptured intracranial aneurysm.

We used records of patients with familial intracranial aneurysms known in the University Medical Center Utrecht from two previous studies, collected between September 1991 and October 1992[1] and from December 1995 to March 1997.[8] In the first study patients with at least two first degree relatives with SAH were selected from a prospectively collected series of patients with aneurysmal SAH.[1] The second study used magnetic resonance (MR) angiography to screen for intracranial aneurysms in first degree relatives of a consecutive series of index patients with aneurysmal SAH.[8] We also included families from the outpatient clinic for intracranial aneurysms of the University Medical Center Utrecht. Members of these families visited the outpatient clinic at their own request or after referral by a neurologist or a neurosurgeon. All medical documents on family members with a medical history suggestive of SAH, intracerebral hemorrhage, stroke, or unruptured intracranial aneurysm were reviewed. Asymptomatic individuals were considered eligible for screening if they were related in the first degree to at least two patients with SAH or an unruptured intracranial aneurysm. These persons were screened with MR angiography. First degree relatives who chose not to visit the outpatient clinic were not actively invited to be screened.

Inclusion and exclusion criteria

Index patients with aneurysmal SAH were defined by symptoms suggestive of SAH combined with subarachnoid blood on computed tomography (CT) and a proven aneurysm on CT angiography or conventional angiography. In patients who died before angiography could be done, the pattern of hemorrhage on CT had to be compatible with a ruptured aneurysm. Patients with an unruptured intracranial aneurysm were identified by CT or MR angiography, conventional angiography, surgery, or necropsy. Episodes of SAH in relatives were categorized into ‘definite’

or 'probable' SAH. The diagnosis of definite SAH was based on clinical features suggestive of SAH in combination with either subarachnoid blood (as demonstrated by CT or analysis of the cerebrospinal fluid), or an intracranial aneurysm proved by angiography (conventional angiogram, CT angiogram, or MR angiogram), surgery, or necropsy. Probable SAH was defined as either sudden severe headache in combination with a normal neurological examination, and hemorrhagic CSF followed by sudden deterioration and death within four weeks (consistent with rebleeding), or as a history describing a second ictus followed by death within the first four weeks after 'stroke' in a person aged less than 70 years.

We constructed pedigrees for each family and determined the mode of inheritance. We defined an autosomal dominant pattern of inheritance as the presence of at least two affected relatives (with SAH or an unruptured intracranial aneurysm) in two successive generations, or at least two affected half brothers or half sisters. If only siblings were affected and if one of the parents had died before 60 years of age, while the cause of death was other than SAH or unknown, the parent was considered as being 'non-informative' and the family was excluded from the analysis. If only siblings were affected and both parents were still alive or had reached an age above 60 years without having had an SAH, families were defined as having a pattern of inheritance suggestive of a mode of inheritance other than autosomal dominant. These families will be further referred to as having a non-dominant pattern of inheritance. Families with autosomal dominant polycystic kidney disease (ADPKD) and connective tissue disorders such as Ehlers-Danlos disease were excluded.

Data collected

For the SAH patients, we collected data on age at time of SAH, sex, number and location of ruptured and unruptured intracranial aneurysms, and outcome after SAH (on discharge from the hospital). For outcome on discharge from the hospital we used the modified Glasgow outcome scale (GOS) with three different categories: independent (GOS 4 and 5), dependent (GOS 2 and 3), or dead (GOS 1).[9] Where a patient had a second SAH later of life, we used only the data on the first SAH. In patients with an unruptured intracranial aneurysm we only studied sex and the number and location of any intracranial aneurysms.

Literature search

To compare our data on age, sex, and number and location of ruptured and unruptured intracranial aneurysms with those in earlier studies, we carried out a Medline search for articles in English on familial intracranial aneurysms from 1954 to 2002 using

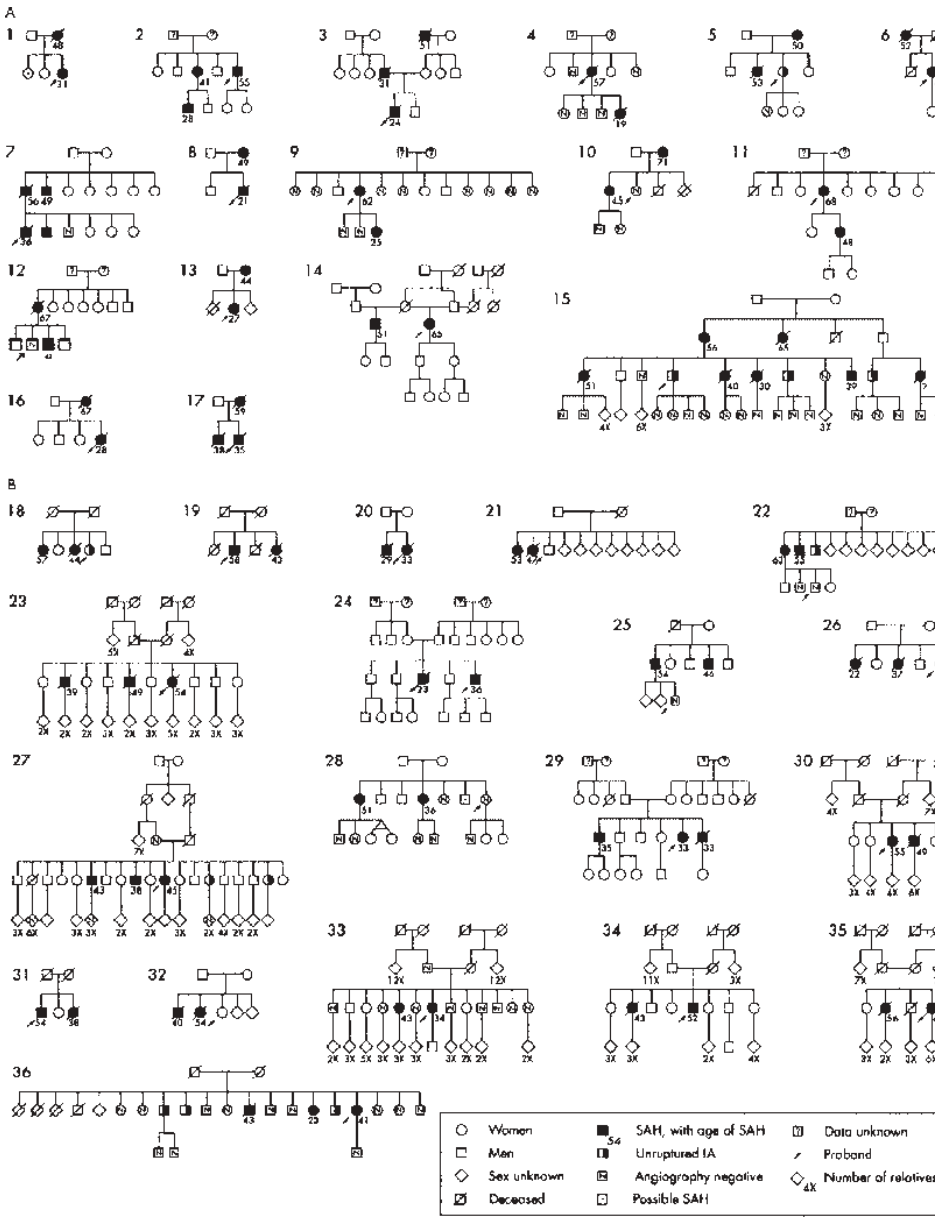

Figure 1. **(A)** Families with familial intracranial aneurysms with an autosomal dominant pattern of inheritance, defined as the presence of at least two affected first degree relatives with aneurysmal subarachnoid hemorrhage (SAH). **(B)** Families with familial intracranial aneurysms with a non-autosomal dominant pattern of inheritance, defined as the presence of at least two affected first degree relatives with aneurysmal SAH.

the key words aneurysm, cerebral, intracranial, subarachnoid hemorrhage, genetics, and familial in different combinations. We also scrutinized the reference lists of all publications retrieved for additional studies. In these families we applied only the strictest definition of familial intracranial aneurysms (the presence of at least two first degree relatives with aneurysmal SAH; see paragraph on ascertainment of families above). We used the same inclusion and exclusion criteria as for our own families with familial intracranial aneurysm, as described above. In addition, we included only families with complete information on age at time of SAH, sex, and number and location of ruptured intracranial aneurysms for all affected subjects. We excluded reports of families with only affected siblings and no information on the parents. Twin studies were excluded.

Data analyses

As described in the paragraph on ascertainment of families, we carried out two separate analyses using the two different definitions of familial intracranial aneurysm. The demographic and clinical features were compared in patients from families with an autosomal dominant pattern of inheritance and with a non-dominant pattern. Age at the time of SAH was compared in the two groups by calculating the difference in mean age with the corresponding 95% confidence interval (CI). This analysis may be influenced by ascertainment bias, as individuals with early onset disease (that is, SAH at a young age) may be referred early to a specialist and be diagnosed as having familial intracranial aneurysm, whereas patients with late onset disease may not come to medical attention. To correct for this possible bias we also conducted this analysis excluding all parent-child pairs involving a proband. For the remaining features we assessed the proportions of the characteristics and calculated the differences between these proportions with corresponding 95% confidence intervals. In the families with an autosomal dominant pattern of inheritance and two successive generations of patients with SAH, the ages at onset of SAH in the different generations were compared using the Wilcoxon test for nonparametric comparison of paired samples. Analysis of variance (ANOVA) was used to test whether the distribution of the difference between ages at onset in parent-child pairs differed according to the sex of the affected parent.

Results

Using the strict definition of familial intracranial aneurysms (presence of at least two first degree relatives with aneurysmal SAH), we included 36 families. Of

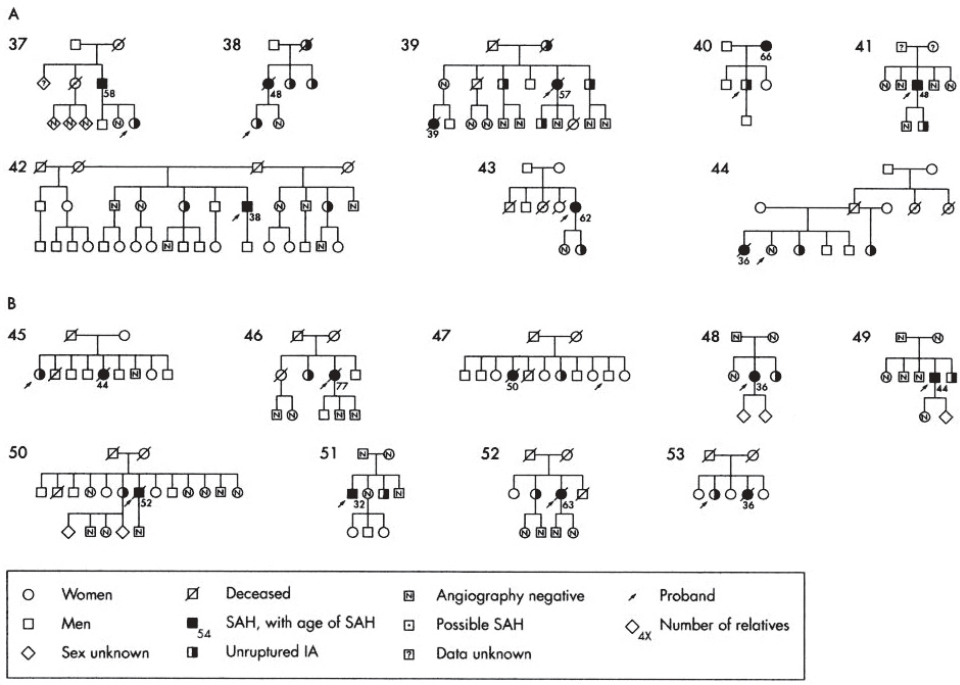

Figure 2. **(A)** Additional families with an autosomal dominant pattern of inheritance.
Family 38: At the age of 48, patient II:1 collapsed and computed tomography (CT) showed a subarachnoid hemorrhage with an intracerebral hematoma suggestive of an aneurysm of the left middle cerebral artery. She died before angiography could be done. Screening with conventional angiography showed an aneurysm of the anterior communicating artery in both her sisters (patients II:2 and II:3) and an aneurysm of the right middle cerebral artery in her mother (patient I:2). Several years later patients III:1 and III:2 were screened for intracranial aneurysms with magnetic resonance angiography (MRA). In patient III:1 an aneurysm of the top of the basilar artery was found.
Family 39: Patient II:5 was 59 years of age when she suddenly collapsed. CT showed a subarachnoid hemorrhage. She died before angiography could be done. Her daughter (patient III:9) was diagnosed with an arteriovenous malformation from the vena magna Galeni, from which she had recurrent intracerebral hemorrhages and died at the age of 24. Screening with MRA showed an aneurysm of the anterior communicating artery in patient II:3, of the left middle cerebral artery in patient II:6, and of the right middle cerebral artery in patient III:7. Patient III:1 at age 39 years was found unconscious approximately three years after screening of her family members. CT showed subarachnoid blood; CT angiography demonstrated an aneurysm of the left middle cerebral artery. Four years after screening patient I:2 died at age 94. Necropsy revealed an unruptured aneurysm of the anterior communicating artery.
(B) Additional families with a non-autosomal dominant pattern of inheritance.

these, 17 had an autosomal dominant pattern of inheritance and 19 a non-dominant pattern (***Figure 1A and 1B***). In these 36 families, 84 members had had an SAH and 11 had been treated for unruptured aneurysms. In all but one of the 17 families with an autosomal dominant inheritance the conclusion was based on subsequent generations being affected, with 41% (95% CI, 28% to 55%) of the siblings in the second generation affected. In the remaining family, half brothers or half sisters were affected. In the 19 families with non-dominant pattern of inheritance, 34% (95% CI, 26% to 42%) of siblings were affected. In one of these families the parents were consanguineous (first cousins), which suggests an autosomal recessive inheritance (***Figure 1B:*** family 27).

When we used the wider definition of familial intracranial aneurysms (presence of at least two affected first degree relatives with aneurysmal SAH or with an unruptured intracranial aneurysm) we were able to include 17 additional families: eight with an autosomal dominant pattern of inheritance and nine with a non-dominant pattern (***Figure 2A and 2B***). In 53 families (36 plus 17), 102 members had had an SAH and 36 had been treated for unruptured aneurysms. Of the 25 families with autosomal dominant inheritance, 22 had two or three affected generations, with 44% (95% CI, 33% to 55%) of the siblings of the second or third generation affected. In the other three families, half brothers or half sisters were affected. In the 28 families with a non-dominant pattern of inheritance 33% (95% CI, 26% to 39%) of siblings were affected.

In two of the 25 families with an autosomal dominant pattern of inheritance three successive generations of patients had intracranial aneurysms (***Figure 2A:*** families 38 and 39). In family 39, patient III-1 had an SAH from an intracranial aneurysm of the middle cerebral artery, while screening with MRA did not show an intracranial aneurysm in her mother. The most likely explanation is a reduced penetrance or anticipation, as the mother may still develop an intracranial aneurysm in the future.

In our literature search for families with familial intracranial aneurysm we identified 34 families.[10-32]

Demographic and clinical features

In the ***Table*** we summarize the demographic and clinical features for patients from families with at least two first degree relatives with aneurysmal SAH (our preferred definition of familial intracranial aneurysm) separately for our own observations and those in previous studies. We found no difference in mean age at the time of SAH between patients with autosomal dominant and non-dominant patterns of inheritance. Also, the proportion of women or multiple intracranial aneurysms did

Table. *Comparison of characteristics of patients with familial intracranial aneurysms (defined hemorrhage) and an autosomal dominant or non-dominant pattern of inheritance*

	This study		
	AD (n)	**Non-AD (n)**	**Difference (95% CI)**
Mean age at time of SAH (including proband) (years)	45.0 (42), range 21 to 71	43.7 (42), range 22 to 63	1.3 (-4.5 to 6.7)
Mean age at time of SAH (excluding proband) (years)	46.7 (29), range 24 to 71	42.5 (31), range 22 to 64	4.2 (-1.8 to 10.0)
Proportion of women	63% (29)	55% (27)	8 (-12 to 28)
Multiple intracranial aneurysms	18% (6)	26% (9)	-8 (-27 to 11)
Outcome			
Dead	43% (18)	43% (17)	0 (-21 to 22)
Dependent	5% (2)	10% (4)	-5 (-17 to 6)
Independent	52% (22)	48% (19)	4 (-17 to 27)
Site of intracranial aneurysms			
ACA	48% (19)	35% (17)	13 (-8 to 33)
MCA	25% (10)	29% (14)	-4 (-22 to 15)
ICA	23% (9)	29% (14)	-6 (-24 to 12)
VBA	5% (2)	8% (4)	-3 (-13 to 7)

ACA: anterior communicating artery complex; AD: autosomal dominant inheritance; non-AD: ICA: internal carotid artery complex (including posterior communicating artery); MCA: middle VBA: vertebrobasilar artery complex.

not differ between the two groups, and we found no differences in the location of the intracranial aneurysms. The outcome after SAH was similar in patients with a dominant and a non-dominant pattern of inheritance. When we used the wider definition of familial intracranial aneurysms, we again found no difference between families with a dominant and a non-dominant pattern of inheritance according to age at time of SAH, sex, and proportion of patients with multiple intracranial aneurysms (data not shown). Also, we did not find differences in the location of ruptured and unruptured intracranial aneurysms in these families.

Tests for anticipation effect

In the families with an autosomal dominant pattern of inheritance and SAH in two successive generations, the age of the child at the time of the hemorrhage was

as the presence of at least two first degree relatives with aneurysmal subarachnoid (families from the present study and from published reports).

	Published reports	
	AD (n)	**Non-AD (n)**	**Difference (95% CI)**
	44.9 (49)	49.5 (29)	4.6 (-2.2 to 11.0)
	65% (33)	62% (21)	3 (-18 to 24)
	11% (6)	14% (5)	-3 (-17 to 11)
	25% (14)	21% (8)	4 (-13 to 21)
	21% (12)	31% (12)	-10 (-28 to 8)
	48% (27)	49% (18)	-1 (-21 to 19)
	7% (4)	3% (1)	4 (-4 to 13)

mode of inheritance other than autosomal dominant; CI: confidence interval; cerebral artery complex; SAH: subarachnoid hemorrhage;

lower than the age of the parent in 19 of the 20 pairs (***Figure 1A***). Seven of these pairs have been reported before.[1] The mean age of the parent at the time of the SAH was 55.2 years and that of the child, 35.4 years (difference 19.8 years, range –3 to 38 years). The difference in mean age between the two generations was statistically significant ($p<0.001$). After exclusion of all parent-child pairs involving a proband from each pedigree, the mean age of the parent at the time of the SAH was 56.8 years and that of the child, 40.2 years (difference 16.6 years, range –3 to 26 years). This difference in mean age between the two generations was again statistically significant ($p=0.007$). In all families from the literature with SAH in two successive generations (27 parent-child pairs, of which 21 have been reviewed before[1]), the age at time of SAH was lower in the child. In these families from previous reports, the mean age of the parent at the time of the SAH was 56.6

years against 34.6 years for the child (difference 22.0 years); this difference was statistically significant ($p<0.001$).

The mean difference in age at onset between parent and child was 20.5 years if the parent was a mother (n=18) and 13.5 years if the parent was a father (n=2). This difference was not statistically significant (p=0.28). In the families reported previously, this mean difference was 21.1 years if the parent was a mother (n=22) and 26.0 years if the parent was a father (n=5) (p=0.38).

Discussion

In this study we report a large number of families with familial intracranial aneurysms. We found no differences in demographic or clinical features between affected members from families with a pattern of inheritance suggestive of autosomal dominant transmission and those from families with a pattern of inheritance not suggestive of autosomal dominant transmission (non-dominant mode). Our results imply that stratification according to phenotype is not possible in future genetic studies on familial intracranial aneurysms. Of course, absence of differences in phenotype between patients with an autosomal dominant pattern and patients with a seemingly non-dominant pattern does not preclude locus heterogeneity.

We extended upon a previous observation, based on smaller numbers, that the mean age of parents at the time of SAH is significantly higher than that of their affected children.[1] This phenomenon was consistent throughout 19 of the 20 families, and corroborates the notion of clinical anticipation. In these analyses the number of male parents was much smaller than the number of affected mothers. We therefore could not demonstrate an effect of the sex of the affected parent on anticipation and childhood onset of intracranial aneurysm. Comparing outcome, number, and size of aneurysms in parent-child pairs probably will not further substantiate the existence of anticipation, as the association between multiple aneurysms and poor outcome has not been demonstrated,[33] and large aneurysms are only associated with a small increase in risk of poor outcome.[34] It is not possible to demonstrate such a small increase in our limited study population of 20 parent-child pairs.

A shortcoming of our study is that no systematic screening of all first degree family members was undertaken, which may have led to some bias. Because screening was incomplete, some families may only show an autosomal dominant pattern of inheritance later on, as the parents or the children of the affected siblings may

harbour undetected intracranial aneurysms. It is also possible that these parents and children will develop intracranial aneurysms in the future. Theoretically, inclusion of families in the group with a non-dominant mode that later appear to have a dominant mode of inheritance may have obscured true differences.

Anticipation can be assumed erroneously, as a result of several kinds of ascertainment bias. As early as 1948 Penrose mentioned three sources of error.[35] The first is selection of parents with late onset by limitation of reproduction in early onset patients. This form of bias might operate in familial intracranial aneurysm. The mean age of SAH in familial (and sporadic) intracranial aneurysm is higher than the reproductive age, but 22% of the female SAH patients are still younger than 45 years of age.[36]

The second potential source of bias is the early diagnosis of severe early onset disease and the late recognition of milder late onset disease. As we analyze only the onset of SAH and as the onset of SAH is sudden and requires prompt medical attention regardless of family history, this source of bias does not seem to play a role here. Furthermore, we attempted to adjust for this tendency by undertaking a separate analysis with exclusion of all parent-child pairs involving an index patient.

The third source of bias is that of index selection, caused by the problem that pairs consisting of a parent with early onset disease and a child with late onset disease are unlikely to be ascertained by a study, given the large span of time separating the two events. To adjust for this type of bias, the optimal design study should be carried out in a stable study population with a high yield of case ascertainment over a long period of time. Unfortunately, it is almost impossible to conduct such a study, as it would last several decades. Another solution might be to consider only the families where the last generation children were born a long time ago, for example before 1920. However, with such an analysis no patients at all would be left in our or in any other.

For our study we excluded six parent-child pairs because the episode suggestive of SAH could not be proven with certainty in the child or the parent. In five of these the parent was older than the child at the time of the episode suggestive of SAH. The differences in age in these excluded parent-child pairs were comparable to those of the parent-child pairs included in our study. Based on these results, the bias of index selection is probably small in our study.

Clinical anticipation may be explained by the transmission of an unstable trinucleotide repeat sequence that increases in size down successive generations. The seven autosomal dominant disorders so far described with unstable mutations (myotonic dystrophy, Huntington disease, spinocerebellar ataxia types 1, 2, 3, and 7, and

dentatorubral pallidolysian atrophy) all show anticipation.[37,38] On the other hand, clinical anticipation may be explained by an increased exposure to risk factors for aneurysmal SAH - such as smoking, alcohol consumption, and hypertension[39] - in affected children compared with their affected parents. Further studies are needed to unravel the cause of the notion of clinical anticipation.

We observed a single family with a pattern suggestive of an autosomal dominant mode of inheritance and an unaffected parent - that is, no aneurysm on MR angiography - with affected offspring. As anticipation is likely to be involved in the genetics of familial intracranial aneurysm, the unaffected parent may still develop an aneurysm in the future. Alternatively, this phenomenon may indicate reduced penetrance.

The families with a pattern of inheritance suggestive of a non-dominant transmission may represent a heterogeneous group. For example, some of these families may turn out to have an autosomal dominant pattern of inheritance if the parents or the children of the patients develop intracranial aneurysms later on. In other families genetic factors may play a minor role in that siblings could have been affected by chance and not so much by genetic factors, given the approximately 2% rate of unruptured intracranial aneurysms in the general population.[40] Also families with polygenetic inheritance may have been included. Furthermore, at least one of the families with a non-dominant transmission may have a transmission mode compatible with an autosomal recessive pattern as the parents of the affected siblings were consanguineous.

Two families with an autosomal dominant pattern of inheritance showed intracranial aneurysms in three successive generations. Such families are rare, probably because SAH and intracranial aneurysms could be diagnosed with certainty only in the past few decades, following the introduction of catheter angiography and CT. Shinton et al[41] reported a family with patients with SAH in three successive generations, but aneurysms were shown in only two generations. In a family described by Schievink *et al*, a patient of the third generation died from an episode suggestive of SAH, but the diagnosis could not be confirmed as necropsy was not carried out.[4]

Conclusions

In familial intracranial aneurysm, phenotypes are similar in families with probable autosomal dominant and non-dominant patterns of inheritance. There is no indication that in future genetic studies on familial intracranial aneurysms stratification according to phenotype can be used. Anticipation is very probable in familial intracranial aneurysm.

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Chapter 5

Searching for subtle features of laxity of connective tissue in patients with ruptured intracranial aneurysms. A pilot study.

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Abstract

Background and purpose

A disruption of the extracellular matrix (ECM) of the intracranial arterial wall is a likely contributing factor in the pathogenesis of intracranial aneurysms. If this is a generalized process, patients with subarachnoid hemorrhage (SAH) may have subtle signs of a general laxity of the connective tissue. We performed a pilot study to assess general laxity in SAH patients and to examine which tests for joint mobility can be best used in these patients.

Methods

In 59 SAH patients and sex- and age-matched controls we compared: *(1)* joint mobility (scores of Beighton and of Bulbena and the total range of joint motion); *(2)* skin extensibility; and *(3)* bone quantity and stiffness. For analyses we used independent samples T-test and linear regression with adjustment for possible confounders.

Results

Patients had a higher degree of joint mobility according to the Beighton criteria (total score 4 in patients versus 2 in controls, p=0.001) and the summation of Z-scores of active range of motion measurements (0.1 in patients versus -0.1 in controls, p=0.0036), and a tendency for a higher degree of joint mobility according to the Bulbena criteria (total score 3 in patients versus 2 in controls, p=0.097) and the summation of active range of joint motion measurements (1292 degrees in patients versus 1275 in controls, p=0.13). No differences were found for the other investigated characteristics.

Conclusions

Our preliminary results suggest that SAH patients have a higher degree of joint mobility than controls. The Beighton score can be best used in our patient population.

Subarachnoid hemorrhage (SAH) is a subtype of stroke caused by rupture of an intracranial aneurysm. A disruption of the extracellular matrix (ECM) of the intracranial arterial wall is a likely contributing factor in the pathogenesis of intracranial aneurysms as these are associated with heritable disorders of connective tissue and ECM such as Ehlers-Danlos type IV.[1] The most common symptoms of the Ehlers-Danlos syndrome (EDS) are skin abnormalities, joint hypermobility, arterial complications and easy bruising.[2] EDS type 4 is caused by mutations in the gene coding for collagen type 3A1 which is an important constituent of the ECM of the arterial wall.[3] In patients with intracranial aneurysms a reduced production of the type III collagen has been found although mutations in its gene could not be demonstrated.[4,5]

Another heritable disorder of connective tissue is Marfan's syndrome, characterized by skeletal, ocular, and cardiovascular complications[6] and caused by mutations in the gene coding for fibrillin-1.[7] This glycoprotein is the most important component of the microfibrillar network surrounding the elastin fibers.

A decrease in structural proteins (collagen and elastin fibres) of the ECM has been demonstrated in the intracranial arterial wall of many ruptured intracranial aneurysms,[8] which further supports the involvement of the ECM in the pathogenesis of intracranial aneurysms. This disruption of the ECM proteins is found not only in aneurysms but also in skin and unaffected intra- and extracranial arteries,[8] which implies that patients with intracranial aneurysms may have a generalized laxity of connective tissue. If so, patients with intracranial aneurysms may have subtle signs of connective tissue disease as seen in patients with Ehlers-Danlos type IV and Marfan's syndrome, such as increased joint mobility and skin extensibility, and a lower bone mass.

We performed a pilot study on signs of general laxity in patients with SAH to examine the feasibility of the tests used to investigate signs of laxity and to examine which tests to determine the degree of joint mobility, *i.e.* Bulbena score, Beighton score and active range of joint motion measurements, can be best used in our patient population.

Patients and Methods

Participants

A group of 59 Caucasian patients with a history of aneurysmal SAH were included. Aneurysmal SAH was defined by symptoms suggestive of SAH combined with subarachnoid blood on CT and a proven aneurysm at angiography (conventional

angiogram, CT- or MR-angiogram). Patients with connective tissue disorders such as EDS or autosomal dominant polycystic kidney disease were excluded.

As a reference group, 59 sex- and age (+/- 3 years) matched healthy Caucasian controls were included. Exclusion criteria for controls were a known history of intracranial aneurysms, EDS and Marfan's syndrome. The Medical Ethics Committee (METC) of the University Medical Center Utrecht approved the study. Informed consent was obtained from all participants.

Clinical characteristics

Weight was measured in a standardized manner without shoes and heavy clothing to the nearest 500 g. Body height, sitting height and arm span were measured in a standardized manner to the nearest cm. The ratio arm span – height was calculated.

The presence of generalized hypermobility of the joints was quantified by means of the active range of joint motion measurements, the hypermobility scores by passive manoeuvres of Beighton[9] and of Bulbena.[10] For the active range of joint motion measurements, used as a measure of stiffness of capsules and ligaments, the active ranges of joint motion of the shoulder (anteflexion), elbow (flexion and extension), wrist (palmar and dorsal flexion), knee (flexion and extension) and ankle (plantar and dorsal extension) were measured bilaterally to the nearest 2 degrees with a standard 2-legged 360 degrees goniometer, using the "anatomical landmark" method.[11] For the measurements, participants were asked to actively stretch or bend the joint maximally without interference by the investigator. Total range of joint motion was a summing-up of range of joint motion of all assessed joints.

The Beighton score assesses the degree of mobility by passive manoeuvres for 8 joints (right and left little finger, thumb, elbow, and knee) and by the ability to reach the floor with the palm of the hands with stretched legs (range: 0 – 9; with 1 point for each hypermobile joint and 1 point for the ability to reach the floor). Generalized hypermobility of the joints was considered present when the score was at least 5 out of 9 points.[9]

The Bulbena score assesses the degree of mobility in 9 joints by passive manoeuvres (thumb, little finger, elbow, shoulder, hip, knee, patella, ankle, and meta-tarso-phalangeal joint), and the presence or absence of ecchymoses (range 0-10; with 1 point for each hypermobile joint and 1 point for the presence of ecchymoses). Generalized hypermobility of the joints was considered present when the score was at least 5 in women and at least 4 in men.[10]

In 10 patients slight paresis or increased muscle tone (spasticity) was found. In these subjects, no measurements were performed in these extremities and the total score was calculated by doubling the excursions of the non-involved extremities.

In the remaining patients and controls no significant differences were found between the left and right extremities and therefore the excursions of the right and left extremities were summed up in these subjects.

Preceding the present study, an intra-observer study regarding range of joint motion was performed for the observer who was going to examine the patients in the present study. One-hundred seventeen joint excursions in 15 healthy participants were measured twice within 24 hours. The mean difference between two measurements was 0.23 degrees with a standard deviation (SD) of 3.8, indicating that 95% (=2 SD) of the differences between the measurements was not exceeding 7.6 degrees. We considered these values acceptable.

Quantitative ultrasound (QUS) measurement was performed as a non-invasive method for assessing bone quantity and bone stiffness. Measurements of the right os calcis were performed with a Sahara ultrasound device (Hologic QDR 4500, Hologic Inc, Waltham, MA) measuring broad band ultrasound attenuation (BUA, dB/MHz) and speed of sound (SOS, m/s). Acoustic phantoms, provided by the manufacturer, were scanned daily and showed no drift over the time span of the study.

Skin extensibility was measured at the ventral part of both forearms and at the medial part of the upper leg, using a vacuum tissue compliance meter.[12] The amount of skin displacement after applying a negative pressure of 10 kPa was indicated in millimeters. The reliability of this instrument was previously shown to be high.[12] Total skin extensibility was analyzed as a summation of all four measurements. The existence of striation and the appearance of scars was recorded.

A questionnaire was filled in by the investigator asking the patients and controls questions concerning skeletal-, skin-, eye-, cardiovascular-, and gynaecological problems, functional health after aneurysmal SAH, dexterity, smoking history, medication, medical history, familial diseases and ethnical background.

Outcome measures and confounders

For outcome measures the total sum of physical activity hours/week (summing-up of hours spent at work, sports, cycling/walking and house-keeping) were recorded. Furthermore, global functional health was measured by means of the modified Rankin Scale.[13]

As possible confounders the cigarette-package-years was calculated. Also muscle strength of the proximal and distal muscles in lower and upper extremities was measured with a hand-held dynamometer (MicroFet2, Hoggan Health Industries Inc., Utah, USA). Measurements were performed two times, and the highest value was registered. In the arms, the shoulder abductors and dorsal flexors of the wrist

joint were measured as well as the grip strength. In the legs, hip flexors and dorsal extensors of the ankle-joints were measured. Total muscle strength was analyzed as a summation of all measurements. Blood pressure was measured twice at the right brachial artery with a manual device [Spreidel + Keller (S+K) 2010, Welch Allyn Medical Products, NY, USA]. The average of the two measurements of systolic and diastolic blood pressure was used for analysis. Mean arterial pressure (MAP) was calculated as two times diastolic blood pressure plus systolic blood pressure divided by 3. The body mass index (BMI) was calculated.

Statistics

Central estimators were calculated as means (SD) or medians (interquartile range) when appropriate. Differences between patients and controls were analyzed using independent samples T-test. Dichotomous variables were compared using the Chi-square test. Clinical characteristics were also analyzed using linear regression with adjustment for possible confounding factors (total physical activity hours per week, Rankin score, smoking, total muscle strength, MAP and BMI). Results are presented as linear regression coefficients with corresponding 95% confidence intervals (95% CI).

To account for large differences in distributions and ranges between the various measurement locations for skin extensibility and joint motion, individual Z-scores of measurements at each location were calculated [Z-score = (observed value - mean value)/SD]. Subsequently, mean Z-scores were calculated for each participant and used for further analysis. For the Z-scores of joint motion a total Z-score was also calculated by summing-up of all the measurements.

Results

Table 1 shows the baseline characteristics of patients and controls. Mean age at examination was 52.2 years (SD 11.7) and 69.5% were female in both groups. Mean age at the time of SAH was 46.8 years (SD 11.3), ranging from 18 till 70 years of age. BMI and total muscle strength were comparable between patients and controls. The MAP and the total of cigarette-package-years were higher while the total sum of physical activity hours per week and global functional health according to the Rankin score were decreased in patients compared to controls.

Patients had a higher degree of joint mobility according to the criteria of Beighton and the summation of Z-scores of active range of motion measurements (***Table 2***), also after adjustment for the possible confounders (total physical activity hours per

Table 1. *Baseline characteristics of patients and controls.*

	Patients (n=59)	**Controls (n=59)**
Age (years)	52.2 (11.8)	52.2 (11.7)
Women (%)	69.5	69.5
BMI (kg / m^2)	25.7 (4.5)	25.6 (4.4)
Mean arterial pressure (mmHg)	109 (13)	104 (14)
Total physical activity hours / week	34.0 (19.3)	42.5 (12.9)
Median Rankin score (interquartile range)	1 (1-2)	0 (0-1)
Median cigarette-package-years (interquartile range)	19.2 (0-31.3)	6 (0-25.5)
Total muscle strength	1691.2 (520.6)	1816.0 (421.8)

Data are presented as mean (standard deviation), or as median (interquartile range) when indicated.
BMI: body mass index.

week, Rankin score, smoking, total muscle strength, MAP and BMI; Beighton criteria: regression coefficient -1.28, 95% CI -12.1 to -0.5, p-value 0.002; summation of Z-scores of active range of motion: regression coefficient -0.34, 95% CI -0.53 to -0.15, p-value 0.01). A tendency towards a higher degree of joint mobility was found according to the Bulbena criteria and the summation of active range of joint motion measurements (***Table 2***). After adjustment for the possible confounders the association of the summation of active range of joint motion measurements became statistically significant. The conclusions for the Bulbena criteria did not change after adjustment for the possible confounders (regression coefficient -0.67, 95% CI -1.40 to 0.06, p-value 0.071), but the higher degree of joint mobility according to the summation of active range of joint motion measurements became statistically significant (regression coefficient -30.8, 95% CI -54.4 to -7.3, p-value 0.01). The absolute difference of the active range of motion between patients and controls was 17 on a total of 1270 degrees (1.3%).

No statistically significant differences were found between patients and controls regarding bone density and laxity of the skin (***Table 2***). Also no differences were found between patients and controls for sitting height and arm span, the presence of striae and scars at physical examination and for the remaining questionnaire items on skeletal-, skin-, eye-, cardiovascular-, and gynaecological problems (data not shown).

Table 2. *Signs of general laxity in patients and controls.*

	Patients (n=59)	Controls (n=59)	p-value
Range of joint motion			
Median Beighton score (interquartile range)	4 (2-5)	2 (1-4)	0.001
Median Bulbena score (interquartile range)	3 (2-4)	2 (1-4)	0.097
Total range of joint motion (degrees)	1292.5 (64.2)	1275.1 (58.6)	0.13
Total Z-score range of joint motion (degrees)	0.1 (0.52)	-0.1 (0.47)	0.036
Bone density			
BUA (dB/MHz)	76.6 (22.1)	76.3 (12.8)	0.93
SOS (m/sec)	1537.1 (40.8)	1536.6 (28.6)	0.94
Skin extensibility			
Total skin extensibility (mm)	39.4 (1.5)	39.3 (1.5)	0.64

Data are presented as mean (standard deviation), or as median (interquartile range) when indicated.
BUA: broadband ultrasound attenuation (dB/MHz); SOS: speed of sound (m/sec).

Discussion

In this pilot study we found that with the tests used to investigate signs of general laxity of the connective tissue, patients with ruptured intracranial aneurysms have a higher degree of joint mobility than healthy controls. The investigation of characteristics of connective tissue and ECM laxity in this study was based on characteristics found in patients with EDS type IV and Marfan's syndrome. Hypermobility is a feature common to both syndromes. Similarities in connective tissue composition between the ligaments, tendons and capsules of joints and intracranial arteries[14] is a likely explanation of our findings with respect to the higher degree of joint mobility in patients with ruptured intracranial aneurysms. We found no differences between patients and controls for any of the other signs of laxity studied. Since our study group was relatively small, small differences can have been missed. The absence of even a tendency of a difference suggests that future studies should concentrate on joint mobility as discriminating feature between SAH patients and controls.

The tests used to determine the degree of joint mobility were the Bulbena score, Beighton score and active range of joint motion measurements. Differences were most pronounced for the Beighton criteria and the summation of Z-scores of total

range of joint motion. A likely explanation that differences were less distinct according to the Bulbena criteria is that the presence or absence of ecchymoses is one of the items of this score. As no differences were found for signs of laxity other than joint mobility, the presence of the ecchymoses item probably dilutes the actual effect of the difference in degree of joint motion. The absolute difference of the active range of joint motion measurements between patients and controls was too small to be meaningful in daily practice to differentiate patients from controls. Therefore in future studies on laxity the Beighton criteria are the most promising. This score is considered a reliable test with a high reproducibility[15] and validity.[16]

In the control subjects the absence of unruptured intracranial aneurysms was not proven, and therefore we may have underestimated the true difference between patients and controls. However, since only 2% of the general population harbours an intracranial aneurysm[17] the influence will be very small. Also, inclusion of SAH patients with subtle deficits such as paresis may have underestimated the results.

A limitation of the study might be that the assessor (RHAW) was not blinded for patients and controls, but given the type of disease blinding is difficult to achieve.

We are not aware of other studies investigating subtle manifestations of connective tissue disease and ECM laxity in intracranial aneurysm patients, but one study analyzed these manifestations in patients with aneurysms of the abdominal aorta.[18] In this study a total score of signs of connective tissue laxity was calculated. This total score was composed of the Beighton sum score and of characteristics of connective tissue laxity such as skin laxity, pes planus or club feet and scoliosis. These total scores were higher in abdominal aortic aneurysm patients than in controls. Because only total scores were used, it can not be assessed whether this difference could be attributed to a higher degree of joint mobility or to presence of the other signs studied. We therefore do not know if the results are in agreement with the results of our study.

We conclude that the data from our study suggest that patients with intracranial aneurysms have an increased joint mobility, and that the Beighton score can be best used to determine degree of joint mobility in our patient population in future studies. It is unlikely that in further studies clinically meaningful differences between patients and controls will be found for other signs of laxity.

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Part II: Genetic Studies on Intracranial Aneurysms and Aneurysmal SAH

Chapter 6

Genetics of intracranial aneurysms

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Abstract

Subarachnoid hemorrhage (SAH) is a subtype of stroke caused by the rupture of an intracranial aneurysm. Genetic factors play an important role in the pathogenesis of SAH and intracranial aneurysms. SAH and intracranial aneurysms are complex in origin, involving the interaction of several genes and environmental factors. A disruption of the extracellular matrix of the arterial wall, which provides strength and elasticity to intracranial arteries, is a likely factor in the pathophysiology of intracranial aneurysms. In this review, we discuss the genetic association, linkage, and gene-expression studies of SAH and intracranial aneurysms. At present, four genome-wide linkage studies have identified genetic loci for intracranial aneurysms. Interestingly, three of these loci include functional candidate genes coding for structural proteins of the extracellular matrix. Of these genes, elastin and collagen type 1A2 are the most promising candidates, because allelic association with intracranial aneurysms has also been shown for these genes. However, variation in these two genes only explains a small proportion of the genetic factors involved in intracranial aneurysms. Future studies need to identify new candidate genes to help unravel the pathophysiology of the disorder.

Subarachnoid hemorrhage (SAH) is a subtype of stroke and most commonly caused by rupture of an aneurysm on an intracranial artery. About 2% of the general population have an intracranial aneurysm.[1] Rupture of an aneurysm is most common between 40 years and 60 years of age and prognosis after rupture is poor: half the patients die within 1 month and 20% remain dependent for activities of daily life.[2-4] The incidence of aneurysmal SAH in the general population is low (about 8 per 100 000 person-years),[5] but 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.[6] In contrast to the high fatality and morbidity in cases of a ruptured aneurysm, the International Study of Unruptured Intracranial Aneurysms Investigators (ISUIA) showed that preventive treatment of an unruptured aneurysm in patients with no history of a ruptured one leads to a better prognosis - 0.6% case fatality and 10% morbidity (defined as moderate to severe neurological disability or serious cognitive abnormality) for surgical clipping, and 0% case fatality and 7% morbidity for endovascular treatment.[7] Preventive treatment should certainly be considered for any patients who have unruptured aneurysms with a substantial risk of rupture.

SAH is 1.6 times more common in women than in men.[1] Hormonal factors probably explain the sex-specific risk of SAH, as this risk is higher in postmenopausal women than premenopausal women.[8] Smoking, alcohol consumption and hypertension are common risk factors for aneurysmal SAH.[9,10] Besides these environmental risk factors, genetic factors also play an important part in the pathogenesis of SAH and intracranial aneurysms - first-degree relatives of patients with the disorder have up to seven times greater risk than the general population,[11-15] and about 10% of patients with aneurysmal SAH have first or second degree relatives with SAH or unruptured intracranial aneurysms.[13-18] Magnetic resonance angiography is not sufficiently effective to screen the first-degree relatives of patients with sporadic SAH for intracranial aneurysms,[19] and repeated screening is necessary to rule out newly developed aneurysms in familial SAH.[20] We therefore need other diagnostic tools that use biomarkers to identify those first-degree relatives at risk who would benefit most from screening for aneurysms. As a familial predisposition is the strongest risk factor for the development of intracranial aneurysms,[1,9] the detection of genetic risk factors could provide further diagnostic capability. In the future, genotype assessment might help to identify first-degree relatives of patients with SAH who have a high risk of developing one or more intracranial aneurysms. These first-degree relatives can be screened for aneurysms regularly and those not at high risk can be reassured. Furthermore, identification of these genetic factors will lead to a better understanding of the pathophysiology of intracranial

aneurysms, about which little is known. The identification of disease-causing genes and increased understanding of the pathophysiology of the disease may lead to new therapeutic interventions to help prevent the development, growth, or rupture of intracranial aneurysms.

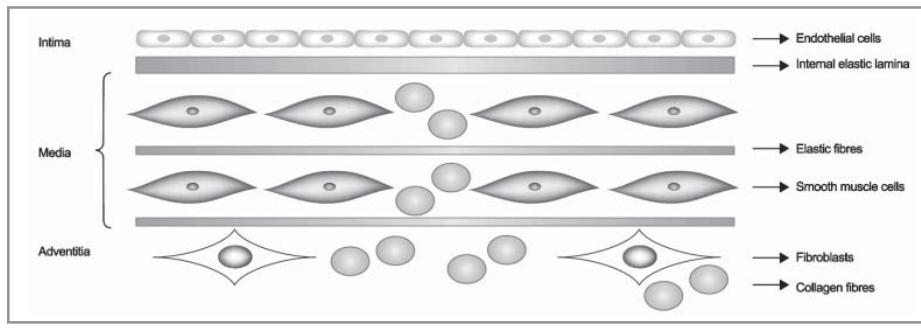
Disruption of the extracellular matrix is likely to be a factor in the pathophysiology as intracranial aneurysms are associated with heritable disorders of connective tissue and extracellular matrix.[21,22] Moreover, a decrease in structural proteins of the extracellular matrix has been shown in the intracranial arterial wall of many ruptured intracranial aneurysms and also in the skin biopsies and intracranial and extracranial arteries of patients with aneurysms.[23-31]

Extracellular Matrix and Intracranial Aneurysms

Cerebral arterial wall

Collagen types 1 and 3 probably represent 80-90% of the total arterial collagen and create most of the tensile strength of arteries (***Figure***). Cerebral arteries have three histological layers: an outer collagenous adventitia, a prominent muscular media, and an inner intima lined by a layer of endothelial cells. An internal elastic lamina separates the intima from the media, but the external elastic lamina seen in extracranial arteries is absent.[23] The extracellular matrix is a stable complex of macromolecules secreted by fibroblasts that form a sheet surrounding cells; in the cerebral arterial wall it is composed of collagen and elastin fibers embedded in glycoproteins and proteoglycans.[23,32] The reticular fibers form a framework around the individual muscle fibers of the media[23] and comprise collagen type 3, glycoproteins and proteoglycans.[33] Collagen type 1 is mainly confined to the adventitia.[34] Collagen types 4, 5 and 6 are also found in the vascular wall: collagen type 4 is a constituent of the basement membrane and forms a sheet-like network; collagen type 5 is associated with the muscle-fiber surface in the media and with the basement membrane; and collagen type 6 is located throughout the matrix of the media without any specific accumulation in the basement membrane.[35] The elastin fibers that are mainly confined to the internal elastic lamina are the principal load-bearing elements and responsible for dilatation and recoil.[36] Elastin makes up 90% of the elastic fibers and microfibrillar glycoproteins make up the remaining 10%. The glycoproteins, fibrillins 1 and 2, are the most important components of the microfibrillar network surrounding the elastin network. The elastic fibers are cross-linked by lysyl oxidase.[37] Elastin is an obvious candidate gene for an association with intracranial aneurysms because intracranial arteries lack an external elastic

Figure: *Intracranial arterial wall.*



lamina[23] and defects in the internal elastic lamina may therefore lead to a major loss of structural support of the arteries.[38,39] Furthermore, because most intracranial aneurysms develop at artery bifurcations where the layer of smooth muscle cells and the internal elastic lamina may already be defective,[40,41] the bifurcations might be more susceptible to aneurysm formation if there are also defects in the elastin.

Involvement of extracellular matrix in pathophysiology of intracranial aneurysms

The most common heritable disorder of connective tissue and extracellular matrix associated with SAH is autosomal dominant polycystic kidney disease (ADPKD),[21,42] but it only accounts for about 0.3% of cases with SAH from a ruptured aneurysm.[9,43] The disease is caused by mutations in the genes *PKD1* and *PKD2*,[44,45] which encode proteins involved in protein-protein and protein-carbohydrate interactions in the extracellular matrix.[46,47] Another heritable disorder of connective tissue associated with aneurysmal SAH is Ehlers-Danlos disease IV, caused by mutations in collagen type 3, an important constituent of the extracellular matrix.[22] However, this association is weaker than that between ADPKD and intracranial aneurysms, and few patients with SAH have the syndrome.[22,48] More fusiform-shaped aneurysms may occur in patients with Ehlers-Danlos disease IV than in those with isolated intracranial aneurysms who typically have saccular aneurysms.[49] Other heritable disorders of connective tissue and extracellular matrix that might be associated with SAH and intracranial aneurysms are Marfan's syndrome,[50] caused by mutations in the fibrillin-1 gene,[51,52] and pseudoxanthoma elasticum,[50] caused by mutations in *ABCC6*, the gene product of which is involved in elastic fiber assembly.[53] However, no association for either of these disorders with SAH and intracranial aneurysms has been proven.[54,55]

The structural proteins of the extracellular matrix that are decreased in the intracranial arterial wall of many ruptured aneurysms, in skin biopsies, and in intracranial and extracranial arteries of patients with aneurysms are collagen type 3 (relative to collagen type 1), collagen type 4 and elastin fibers. Patients with aneurysms have 35% fewer reticular fibers in the arterial medial layer of intracranial and extracranial arteries than controls without intracranial aneurysms.[25] The reticular fibers in patients are irregularly distributed and seem to be shorter than in controls.[25-27] Some patients with aneurysm have a deficiency in collagen type 3 relative to type 1 in skin fibroblasts and in intracranial and temporal arteries,[24,28,29] and collagen type 4 may be structurally disrupted in the aneurysmal wall.[30] There are defects in the internal elastic lamina in some intracranial aneurysms,[23,31] and disruption of collagen and elastin fibers was also found in skin biopsies of some patients.[56] As the disruption of the extracellular matrix proteins was found not only in aneurysms but also in skin and unaffected intracranial and extracranial arteries, intracranial aneurysms might not be a localized disease but rather represent a more general disease of the extracellular matrix.

The decrease in extracellular matrix proteins could be explained by disrupted synthesis. However, because there is constant degradation and synthesis of the constituents of the extracellular matrix regulated by a range of proteases (matrix

Table 1. *Substrates of the proteases and protease inhibitors thought to be involved in the pathophysiology of intracranial aneurysms.*

Enzyme	Function	Substrates
MMP-2	Protease	Collagen types 1, 4, 5, 7, 10, 11, 14; elastin; fibronectin; gelatine; laminin; MMP-1, MMP-9, MMP-13
MMP-9	Protease	Collagen types 4, 5, 7, 10, 14; elastin; fibronectin; gelatine; plasminogen
Elastase	Protease	Elastin; collagen types 1, 2, 4; basement membrane
MT1-MMP	Protease	Collagen types 1, 2, 3; casein; elastin; fibronectin; gelatine; laminin; MMP-2, MMP-13
Plasmin	Protease	Fibrin; fibronectin; laminin; MMP-3, MMP-9, MMP-12, MMP-13
α1 antitrypsin	Protease inhibitor	Elastase
TIMPs	Protease inhibitor	MMPs

MT1-MMP: membrane type-metalloproteinase.

metalloproteinases [MMPs], neutrophil or leukocyte elastase) and their inhibitors (tissue inhibitor of MMPs [TIMPs] and α1 antitrypsin), growth factors, and cytokines (***Table 1***),[36,57] the decrease may also be the result of accelerated protein degradation caused by an imbalance between these proteases and protease inhibitors. Serum elastase concentrations and the ratio of elastase versus α1 antitrypsin were high in patients with ruptured and unruptured aneurysms compared with those in controls.[58,59] Furthermore, compared with unruptured aneurysms, ruptured aneurysms have high elastase activities in the arterial wall.[58] Patients with aneurysms have high expression and activity of MMP-2 and MMP-9.[60-63] Membrane type MMP and plasmin, both involved in the activation of MMP-2, are localized in the aneurysmal wall, which may indicate a localized activation of MMP-2 within the aneurysm.[60] Besides MMP-9, concentrations of its inhibitor, TIMP, are also high in the walls of aneurysms.[63] There are no differences in plasma MMP-9 activity between patients with intracranial aneurysms and controls.[63]

Genetic Studies of Intracranial Aneurysms

The segregation of SAH and intracranial aneurysms does not follow a strict pattern of mendelian inheritance. The complex inheritance pattern is caused by the interaction of several genes and environmental factors, each of which has a different effect on disease susceptibility. Hence, combinations of genetic and environmental factors will lead to the disease but no one factor alone is sufficient to cause it. Therefore, being a carrier of one or more genetic factors will not necessarily lead to an individual becoming affected by the disease, and there may be similar phenotypes caused by different combinations of genetic and environmental factors. The presence of multigenic inheritance, environmental factors, and genetic heterogeneity complicates the identification of the disease-susceptibility genes. Genetic terms used in the discussion below are explained in the ***Panel***.

Candidate gene studies

To identify the genes involved in a disease, genetic association across large groups of patients can be studied. Association studies can assess candidate genes suspected of being involved in the disease because of their function or genes from linkage regions (*i.e.* positional candidate genes) can be scrutinized for genetic associations. Genome-wide screens using all the human genes are even more advanced and have the important advantage that no knowledge of the disease mechanism is needed because every gene in the genome is a candidate gene. These ge-

Panel: *Genetic terms*

Allele
One of the variant forms of a gene at a particular locus or location on a chromosome

Allelic heterogeneity
When a genetic disease is caused by different mutations at the same locus

Association study
A study that investigates an association between a disease and a specific allele in patients and controls

Gene expression study
A study that analyzes the expression of genes in tissues by measuring amounts of mRNA for a gene in patients and comparing these with the amounts in controls

Haplotype
A set of closely linked genetic markers (for example, SNPs) present on one chromosome that tend to be inherited together

Linkage
The association of genes or markers that lie near each other on a chromosome

Linkage disequilibrium
The phenomenon that certain alleles occur together more often than can be accounted for by chance

Linkage study
A study that establishes whether cosegregation of a disease phenotype with DNA markers of known location, dispersed throughout the genome, exists in affected families

Locus
The place on a chromosome where a specific gene is located

Locus heterogeneity
The phenomenon that a genetic disease is caused by different mutations at separate loci

Marker
A segment of DNA at an identifiable location on a chromosome of which the inheritance can be traced; a marker can be a gene or a fragment of DNA

Microarray
A way of studying large numbers of genes simultaneously using miniature arrays of gene fragments attached to glass slides

Microsatellite polymorphism
Repetitive short sequences of DNA used as genetic markers

Reduced penetrance
The phenomenon that family members may carry the disease gene(s) but do not always have signs or symptoms of the disease

Restriction fragment length polymorphism
Genetic variation at the site where a restriction enzyme cuts a piece of DNA; such variations affect the size of the resulting fragments and can be used as genetic markers

Single nucleotide polymorphism
DNA sequence variations that occur when a single nucleotide (A, T, C, or G) in the genome sequence is changed

nome-wide screens are now feasible but are still expensive and raise several statistical issues.

The principle of an association study is to observe differences in the frequencies of specific alleles between patients and controls. The major advantage of genetic association studies lies in the ease of the design: they use independent patients, who can be collected easily. Furthermore, the mode of inheritance does not need to be specified. This type of study can detect genetic factors with only small effects (*e.g.* a relative risk of 1.2) in contrast to linkage studies that mainly detect genetic factors with large effects. Moreover, fine mapping is not possible with linkage studies and the genetic factors thus lie in large regions of several hundreds of genes. Association can be detected either directly with functional genetic variants that have biological consequences related to the disease or indirectly with variants that are in linkage disequilibrium with the associated variants. However, up to now the second type of study has rarely led to the identification of pathogenic mutations. Many association studies are done by use of single-nucleotide polymorphisms (SNPs) and haplotypes. Association studies require particular attention to the statistical power to be achieved and the correct matching of patients and controls as allele frequencies may differ between different ethnic populations. The problems of ethnic mismatching between patients and controls can be overcome with the transmission-disequilibrium test or affected family-based controls.[64] As most genetic factors will only have a small effect, large numbers of case-control pairs are needed (*i.e.* hundreds to thousands). In addition, to explore association to a candidate gene, multiple alleles will be tested, which raises the chance of finding false-positive associations. This problem, however, can be corrected for by use of, for example, a Bonferoni correction, although this might be too stringent to apply in all situations. So far, association studies in intracranial aneurysms have been described only with functional and positional candidate genes. As genes involved in maintaining the integrity of the extracellular matrix have been proposed as the most likely candidates for intracranial aneurysms, these are the genes that have been studied most commonly (***Table 2***).[65-82]

Collagen type 3 A1 (COL3A1)

COL3A1 is located on chromosome 2q31. Mutations in this gene have not been identified in patients with sporadic or familial intracranial aneurysm.[71,72] However, studies have focused only on the coding regions of the gene and on major gene defects. The association between common SNPs and haplotypes in *COL3A1* with intracranial aneurysms has not been studied in a large population. A small study of 19 patients from North America has shown association of an Ava II restriction

Table 2. *Potential intracranial aneurysm candidate genes involved in maintaining the*

Candidate gene	Chromosomal location	Evidence from candidate gene studies	Populations
Elastin	7q11.2	Association haplotype intron20-intron23 ($p=3.81x10^{-6}$)[65]	85 sporadic and 87 familial Japanese patients[65]
		Association haplotype intron4-exon22 (p=0.02) and intron5-exon22 (p=0.002)[67]	167 sporadic Dutch patients[67]
		No association haplotype intron20-intron23[68,69]	120 sporadic German patients;[68] 30 sporadic and 175 familial German patients[69]
Collagen type 3 A1	2q31	Association Ava II site (p=0.01)[70]	19 US patients[70]
		No mutations identified in coding region[71,72]	41 sporadic Dutch patients;[71] 16 sporadic and 24 familial patients, different nationalities[72]
Collagen type 1 A2	7q22.1	Association SNPs (OR 3.19, 95% CI 2.22-6.50, p=0.00087)[74]	145 sporadic and 115 familial Japanese patients[74]
Lysyl oxidase	5q22.3-q31.2	No association SNPs[75,76]	85 sporadic and 87 familial Japanese patients;[75] 25 German familial patients[76]
Fibrillin 2	5q23-q31	No association SNPs[75]	85 sporadic and 87 familial Japanese patients[75]
α1 antitrypsin	14q32.1	Heterozygous α1 anti-trypsin deficiency more common (p=0.005)[77]	100 US patients[77]
MMP-9	20q11.2-q13.1	Association $(CA)_n$ microsatellite poly-morphism (p=0.02)[78]	76 US patients[78]
		No association $(CA)_n$ microsatellite poly-morphism[79,80]	92 patients from UK;[79] 57 Finnish patients[80]
		No association SNPs[81]	40 German patients[81]
MMP-1, *MMP-3*, *MMP-12*	11q22-q23, 11q23 and 11q22.2-22.3	No association SNPs[79,80]	92 patients from UK;[79] 57 Finnish patients[80]
TIMP-1, *TIMP-2*, *TIMP-3*	Xp11.3-p11.23, 17q25 and 22q12.1-q13.2	No association SNPs[82]	44 German patients[82]

SNP: single nucleotide polymorphism; MMP: metalloproteinase; TIMP: tissue inhibitor of

integrity of the extracellular matrix.

Locus identified through linkage studies	Evidence from gene expression studies	Evidence from functional studies
Japanese study[65]	Overexpression[66]	Defective internal elastic lamina intracranial aneurism[23,31] Disruption of elastin fibers in skin biopsies[56]
NA	Overexpression[66] Reduced expression[73]	Reduced or absent in skin and intracranial and temporal arteries[24,28,29] Reduced/irregular/shorter reticular fibers in intracranial and extracranial arteries[25-27]
Japanese study[65]	Overexpression[66]	Not determined
Japanese study[65]	No evidence	Not determined
Japanese study[65]	No evidence	Not determined
NA	No evidence	Ratio elastase *vs* α1 anti-trypsin elevated[59]
NA	No evidence	Increased expression + activity in intracranial aneurism[63]
NA	No evidence	Not determined
NA	Overexpression of TIMP-3[66]	Not determined

metalloproteinase; NA: not applicable; OR: odds ratio; 95% CI: 95% confidence.

fragment length polymorphism in *COL3A1* (*i.e.* when *COL3A1* is treated with the endonuclease Ava II, two distinct restriction fragments occur) with non-familial aneurysms (p=0.01).[70]

Collagen type 1 A2 (COL1A2)

COL1A2 was analyzed for association of 21 SNPs with intracranial aneurysms in 115 Japanese patients with familial and 145 with sporadic disorder.[74] Three different SNPs, of which one results in an alanine to proline amino-acid substitution, showed significant differences in allelic frequencies between cases and controls, especially when testing the SNP resulting in an amino-acid substitution in familial cases (odds ratio, 3.19; 95% CI, 2.22 to 6.50; p=0.00087). This SNP may be a functional variant necessary for the development of intracranial aneurysms. However, as the SNP's allele frequency is low (5.2% in the intracranial aneurysms population versus 2.7% in the control population) it may only account for a small proportion of cases.

Lysyl oxidase (LOX)

Four SNPs in *LOX* were analyzed in 172 Japanese patients with intracranial aneurysm and 192 controls but no associations between these SNPs and intracranial aneurysms were observed.[75] In another study, with only 25 German patients with familial intracranial aneurysm, no association of four SNPs was found.[76]

Fibrillin 2 (FBN2)

FBN2 is the disease-causing gene in congenital contractular arachnodactyly and is closely homologous to fibrillin 1 (*FBN1*).[83] *FBN2* was explored for allelic association with intracranial aneurysms using five SNPs dispersed throughout the gene in 172 Japanese patients with intracranial aneurysm, but no allelic association was observed.[75]

α1 antitrypsin

α1 antitrypsin is an important inhibitor of elastase.[84] Mutations affecting function of the gene product are associated with both emphysema and liver disease; homozygosity for the deficient "Z" allele leads to a high risk of emphysema.[85] The heterozygous α1-antitrypsin deficiency state (*i.e.* heterozygosity for the "Z" and "S" alleles) was more common in 100 patients with intracranial aneurysms from North America (16%) than in 904 controls (7%; p = 0.005).[77] Furthermore, in another North American study, the "Z" allele was eight times more common in a subset of patients with intracranial aneurysm (in 46 patients from London [PA,

USA] but not in 26 patients from Pittsburgh [PA, USA]) than in controls, but this difference was not significant after correction for multiple comparisons.[86]

Metalloproteinases (MMPs)

In a case/control study with 76 North American patients, the *MMP-9* gene $(CA)_n$ microsatellite polymorphism was found to show association with intracranial aneurysms ($p=0.02$).[78] Two other studies (one in 92 patients from the UK and the other with 57 patients from Finland) found no association between this *MMP-9* polymorphism and intracranial aneurysms, nor was there an association of intracranial aneurysms with SNPs in *MMP-1*, *MMP-3*, or *MMP-12*.[79,80] Recently 11 SNPs in *MMP-9* were tested for association with intracranial aneurysms in 40 German patients and 40 controls, but no difference in genotype frequency was observed between them.[81] It is possible that the small size gave the study insufficient power.

Tissue inhibitors of metalloproteinases (TIMPs)

The most important inhibitors of the activity of MMPs are the TIMPs. Three SNPs in *TIMP-1*, four in *TIMP-2*, and two in *TIMP-3* were analyzed in 44 German patients with intracranial aneurysm, but no significant association was found.[82] This study may have had too few patients to show any association.

Other candidate genes

Other candidate genes that are not involved in the maintenance of integrity in the extracellular matrix have been tested for association with intracranial aneurysms, including endoglin,[87-89] angiotensin converting enzyme,[90-92] NADPH oxidase P22PHOX,[93] and phospholipase C.[94] Conflicting or negative results were found for all these genes.

Few of the studies that showed association with SAH and intracranial aneurysms have so far been replicated in subsequent studies. The studies showing association may have been hampered by false-positive findings and small sample sizes in subsequent studies to confirm the association. As intracranial aneurysms is a complex disease, most disease-causing genetic factors will have only small effects. To detect a susceptibility locus with a relative risk of 1.2 or greater at a significance level of 0.05 and a power of 80%, a cohort of at least 3000 case-control pairs with intracranial aneurysms is needed. Detection of loci with larger relative risks can be done with smaller sample sizes but even for a relative risk of 1.5, 600 case-control pairs are required (genetic power calculator, SGDP statistical genetics group, http://statgen.iop.kcl.ac.uk).

Linkage studies

A complementary approach to analyzing the genetic risk factors of a disease is the use of linkage studies in families in which the disease segregates. The functional candidate-gene approach described above is still limited by our knowledge about the underlying disease mechanism and the known pathways, so that genetic association may explain only a small proportion of the risk factors for intracranial aneurysms, and other essential genes involved in unknown pathways may have been overlooked. Linkage studies overcome this problem as these make use of whole-genome screening to localize new genes involved in the pathophysiology of a disease. Linkage studies can establish whether cosegregation of a disease phenotype with DNA markers of known location, dispersed throughout the genome, exists in families with the disease. Linkage of the disease phenotype (and thus the disease-causing gene) with a specific DNA marker means that the marker and the disease-causing gene are located nearby in the DNA. Linkage is typically investigated by analysis of large families, but because intracranial aneurysms have late onset,[4] low prevalence,[1] and high case fatality after rupture,[2-4] large families with SAH are rare. A further problem is that family members who have no intracranial aneurysms on screening and who are therefore registered as unaffected but may still develop intracranial aneurysms later in life. The search for disease-causing genes may be further complicated by locus heterogeneity, which can decrease the power of the analysis.

Another issue in traditional linkage analysis is the presumed mode of inheritance, an important factor that needs to be defined. The traditional type of analysis is called “model-based”. For intracranial aneurysms the mode of inheritance is unknown and is most likely to be heterogeneous.[95] To overcome some of these problems with linkage studies, affected sibling-pair analysis can be used; this is a “model-free” design of linkage analysis in which the mode of inheritance, but also the disease-gene frequency and disease-gene penetrance, do not need to be specified. Important covariates can also be included in this approach that may increase the power to detect linkage.[96] The principle is based on allele-sharing between affected siblings, who, on average, share 50% of their DNA, and looks for any deviation from 50% sharing. Regions with increased sharing of alleles may point towards the disease locus. A disadvantage of this approach is that at least 100 affected sibling pairs are needed to show significant sharing above 50% for a locus with a relative risk of 2.0.[97] The collection of such sib-pairs with SAH and intracranial aneurysms is hampered by the high fatality after SAH.

Thus far, only two whole-genome linkage studies with the affected sibling-pair approach have been done, one in Japan [65] and the other in Finland ,[98] both in popu-

Table 3. *Genetic loci identified for intracranial aneurysms with possible candidate genes found in linkage studies.*

Study population	Genetic loci	Potential candidate genes at these loci	Reference
Japanese	7q11	Elastin (*ELN*); Collagen type 1 A2 (*COL1A2*); Genes for KREV interaction trapped 1 (*KIRT1*)	65
Japanese	14q22	Latent transforming growth factor β-binding protein 2 (*LTBP2*)	65
Japanese	5q22-31	Lysyl oxidase (*LOX*); Fibrillin 2 (*FBN2*); Fibroblast growth factor 1 (*FGF1*)	65
Finnish	19q13.3	D site of the albumin promotor-binding protein (*DBP*); Histidine-rich calcium-binding protein (*HRC*); Nitric oxide synthase-interacting protein (*NOSIP*); Tumour upregulated CARD-containing antagonist of caspase nine (*CARD8*)	98

lations with a high incidence of SAH.[5,99] The Japanese study was done in 104 affected sibling-pairs with a model-free design.[65] Three linkage regions were identified (7q11, 14q22, and 5q22-q31; maximum LOD scores 3.22, 2.31, and 2.24, respectively; ***Table 3***). The strongest linkage was with chromosome region 7q11 near the elastin gene (*ELN*). In a study analyzing linkage to *ELN* in 39 patients with intracranial aneurysm in 13 different families from Utah (USA), linkage to 7q11 was confirmed using a recessive-affected-only model (multipoint TLOD =2.34).[100] *ELN* was further analyzed for allelic and haplotype associations by use of 14 SNPs distributed throughout the gene in a sample of 85 sporadic and 87 familial Japanese patients with SAH. Although there were no allelic association with any of the 14 SNPs, the haplotype between the SNPs at intron 20 and intron 23 was strongly associated with intracranial aneurysms ($p=3.81 \times 10^{-6}$), whereas homozygous patients had a high risk with an odds ratio of 4.39 (95% CI, 2.62 to 12.11; p=0.02), which further supported a locus for familial intracranial aneurysms within or close to *ELN*.[65] In two more studies,[68,69] one analyzing 120 sporadic and the other 30 familial and 175 sporadic German patients with SAH, no allelic association was found with the haplotype associated with intracranial aneurysms in Japanese patients.[65] There may be allelic heterogeneity between the Japanese and

German populations of SAH patients, because it cannot be excluded that other variants in the *ELN* are associated in German patients.[68,69]

Recently, the above conclusion was supported because different haplotypes other than the one in the Japanese study[65] were found to be associated in 167 patients with sporadic SAH in the Dutch population (haplotype between the SNPs at intron 5 and exon 22 [p=0.002]; haplotype between the SNPs at intron 4 and exon 22 [p=0.02]).[67] In another study of linkage to *ELN*, 14 Japanese families were selected with more stringent inclusion criteria (at least three affected members per family)[101] than used in the earlier Japanese genome-wide linkage study (at least two affected members per family).[65] Whereas a model-free sibling-pair analysis was used in the genome-wide linkage study,[65] in the study of 14 families, linkage was tested assuming both an autosomal dominant model (which may be incorrect because the mode of inheritance is unknown and could be heterogeneous[95]) and an inheritance-free mode. Linkage to *ELN* could be excluded in 11 families and was inconclusive in the other three. A possible explanation for the lack of linkage to *ELN* in this study may be locus heterogeneity. In families with more than three affected members, genes other than *ELN* may play a part. Interestingly, the chromosome region 7q11[65] also includes the candidate gene *COL1A2*, which is another important structural protein of the extracellular matrix. Involvement of the extracellular matrix in the pathophysiology of intracranial aneurysms is indeed possible because the other linkage regions also include potential candidate genes involved in the extracellular matrix, for example, lysyl oxidase (*LOX*) and fibrillin 2 (*FBN2*) on 5q22-q31.[65]

A second genome-wide linkage scan was done in 139 affected sibling-pairs together with 83 other affected relative pairs (*i.e.* pairs with affected family members other than siblings) from the Finnish population.[98] Both a model-free design and a model-based analysis assuming a recessive mode of inheritance were used and linkage was shown to chromosome 19q13.3 (maximum LOD score 3.93 with a model-free design and 3.99 with inclusion of covariates; maximum LOD score 3.16 assuming a recessive mode of inheritance; ***Table 3***). This region includes 135 genes of which 102 have been characterized. An explanation for the different regions of linkage identified between the Japanese and Finnish populations may be that replicating the results of linkage studies is commonly difficult and requires many more families to be analyzed. More linkage studies are therefore needed before any definite conclusions can be drawn. The extent to which genes play a part in different populations may also differ considerably, and, finally, the differences may be explained by locus heterogeneity.

Recently, two genome-wide linkage studies were done in two different large

pedigrees with intracranial aneurysms.[102,103] The first study was done in a Dutch consanguineous family and showed linkage on chromosome 2p13 between markers D2S2206 and D2S2977 (maximum multipoint LOD score of 3.55).[102] Several genes in this region were suggested as candidate genes for intracranial aneurysms, such as the smooth muscle actin γ2 and actin-related protein 2 genes, which are genes encoding proteins involved in actin metabolism.[102] In the second study, a Northern American family had aneurysms that segregated as a dominant trait and linkage with a locus on chromosome 1p34.3-p36.13 was found - maximum LOD score of 4.2.[103] Possible candidate genes in this region include polycystic kidney disease-like 1, fibronectin type III domain-containing gene, and collagen type 16 A2, which are all involved in the maintenance of the extracellular matrix.[103]

Gene expression studies

Another way to identify previously unrecognized associations between genes and a disease is to do gene expression studies, an approach that can be used to characterize unrecognized pathophysiological pathways. In these studies, mRNA concentrations of all genes in a tissue are compared with those of normal controls. One complication of a gene-expression approach is that besides the primary specific pathophysiological and genetic factors underlying the disease, secondary molecular factors will be detected that are a consequence of the disease. However, these secondary factors can still be interesting as they may point towards pathways that can be targeted for therapy.

A global gene expression analysis was used to compare the expression of genes in a sample of an intracranial aneurysm of a 3-year-old girl to the expression in her superficial temporal artery.[66] A significant overexpression of many genes was observed including genes encoding extracellular matrix components (fibronectin; collagen types 3A1, 1A1, 1A2, 6A1, and 6A2; and elastin) and genes involved in the turnover of the extracellular matrix (*TIMP-3* and osteoblast-specific factor-2). Although this study provides insight into the molecular pathophysiology of intracranial aneurysms, this girl was not representative of patients with intracranial aneurysm as the disorder is seldom seen in childhood.[104]

In another study, the expression of a selection of genes was analyzed in samples of 24 ruptured and unruptured intracranial aneurysms, superficial temporal arteries of 43 intracranial aneurysm patients, and 19 control patients without intracranial aneurysms.[73] Concentrations of prostacyclin-stimulating factor and the protein RAI, both implicated in the process of tissue repair, were low in samples of patients with ruptured intracranial aneurysms compared with those in samples of superficial temporal arteries of patients with intracranial aneurysms. Furthermore, a decrease of

collagen-type-3 expression was also found in contrast to the previous expression study that showed an increase of expression of this gene.[66] These decreases in gene expression were also observed in samples of unruptured intracranial aneurysms but they were not significant.[73]

Animal Models

Animal models of intracranial aneurysms can help to unravel the pathophysiology and to identify the disease-causing genes. So far, no transgenic animal model with intracranial aneurysms has been developed, although an interesting animal model that may lead to a better understanding of the pathophysiology is the experimentally induced elastase aneurysm model in rats and rabbits.[105-107] Elastase degrades elastin, collagens types 1, 2 and 4, and the basement membrane. In the elastase rodent model saccular aneurysms developed after intravascular application of elastase to arteries of the circle of Willis.[105-107] This elastase model can, for example, be used to analyze gene expression to identify gene and protein functions involved in the disease, which will help in the development of prevention and treatment strategies.

Conclusions

There are no diagnostic tests for specific genetic risk factors to identify first-degree relatives of patients with SAH who are at a high risk of developing intracranial aneurysms and who would benefit most from screening. However, four genome-wide linkage studies have identified genetic loci for intracranial aneurysms on 7q11, 14q22, 5q22-31 in a Japanese study,[65] on 19q13.3 in a Finnish study,[98] on 2p13 in a Dutch family,[102] and on 1p34.3-p36.13 in a US family.[103] These genetic loci include some interesting candidate genes coding for structural proteins of the extracellular matrix. Of those genes, *ELN* and *COL1A2* are the most promising candidates as allelic association with intracranial aneurysms has also been shown for these genes.[65,67,74] In the future, the role of these genes should be studied further. As association studies harbour the risk of false-positive findings the results of such studies should be confirmed in additional samples. In the case of true associations, the genes should be analyzed in different ethnic groups, as there are indications for locus and allelic heterogeneity between different populations. These studies should be combined with investigations of gene function to provide evidence as to why

and how these variants cause intracranial aneurysms.

Genes for structural proteins of the extracellular matrix - other than *ELN* and *COL1A2* - and genes coding for proteases and protease inhibitors involved in the turnover of the extracellular matrix remain other likely candidate genes for intracranial aneurysms, but no specific genes have so far been identified as showing clear association with intracranial aneurysms. However, many of those association studies have probably been underpowered. Therefore, more large-scale population genetic association and linkage studies are needed to unravel the genetic risk factors of intracranial aneurysms. As the case fatality of aneurysmal SAH is high (half the patients die)[2-4] large multicenter cohorts are needed. Finally, the genetic markers confirmed in these large populations may contribute to the development of prognostic scoring rules that will generate risk profiles for different patient groups. Initially, these risk profiles can be used to identify relatives within families segregating intracranial aneurysms who are at high risk of also developing aneurysms. The relatives with the high-risk profiles can be screened regularly by magnetic resonance angiography to identify aneurysms that can be treated preventively. At a later stage such risk profiles may also be developed for population screening. With the identified genetic markers more insight will be gained into the pathophysiology of intracranial aneurysms that may generate new leads for therapeutic interventions for intracranial aneurysms.

As new technologies and statistical methods in the field of genomics are developing fast, new approaches may also be used to identify new candidate genes. One such approach is a genome-wide screen for genetic association considering every gene in the genome to be a potential candidate gene using linkage disequilibrium.[108] The linkage disequilibrium structure of the human genome is currently being described.[109] With this information, SNPs can be chosen in such a way that all genetic information can be captured using the smallest number of SNPs possible.[108] Another direction for future work is to do more large-scale expression studies using microarrays to identify the cellular pathways that are disrupted during the development of intracranial aneurysms.

Search strategy and selection criteria

References for this review were identified by searches of PubMed in November 2004 for articles in English published from 1970 through 2004, using the key words “aneurysm”, “cerebral”, “intracranial”, “subarachnoid hemorrhage”, “genes”, “familial”, and “extracellular matrix” in various combination. We also scrutinized the reference lists of the publications retrieved for other studies.

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Chapter 7

Association of polymorphisms and pairwise haplotypes in the elastin gene in Dutch patients with subarachnoid hemorrhage from non-familial aneurysms

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Abstract

Background and purpose

A locus containing the elastin gene has been linked to familial intracranial aneurysms in 2 distinct populations. We investigated the association of single-nucleotide polymorphisms (SNPs) and haplotypes of SNPs in the elastin gene with the occurrence of subarachnoid hemorrhage (SAH) from sporadic aneurysms in the Netherlands.

Methods

We genotyped 167 SAH patients and 167 matching controls for 18 exonic and intronic SNPs in the elastin gene. A Bonferroni correction was applied for multiple comparisons with all novel associations, with a correction factor derived from the number of SNPs tested (p-value after Bonferroni correction [p_{corr}]).

Results

SAH was statistically significant associated with a SNP in exon 22 of the elastin gene (minor allele frequency was 0.000 in patients and 0.028 in controls; odds ratio [OR], 0.0; 95% CI, 0.0 to 0.7; p=0.004; p_{corr}=0.05) and possibly with an SNP in intron 5 (minor allele frequency was 0.062 in patients and 0.128 in controls; OR, 0.5; 95% CI, 0.2 to 0.8; p=0.007; p_{corr}=0.08). Haplotypes of intron 5/exon 22 (p_{corr} =0.002), intron 4/exon 22 (p_{corr}=0.02), and intron 4/intron 5/exon 22 ($p=9.0x10^{-9}$) were also associated with aneurysmal SAH.

Conclusions

Variants and haplotypes within the elastin gene are associated with the risk of sporadic SAH in Dutch patients. Gradual increase of statistical power with the inclusion of 2 or 3 SNPs in the studied haplotypes supports the validity of our conclusion that the elastin gene is a susceptibility locus for SAH.

Genetic factors are likely to be involved in the development of intracranial aneurysms (IAs) because familial predisposition is the strongest risk factor for aneurysmal subarachnoid hemorrhage (SAH).[1,2] Familial clustering is found in approximately 10% of patients with SAH, and first-degree relatives of patients with SAH have a 3 to 7 times greater risk of developing SAH than the general population.[1]

In many ruptured IAs, the arterial wall contains reduced amounts of extracellular matrix proteins.[3,4] Elastin is an important structural protein of this extracellular matrix and is mainly confined to the internal elastic lamina in intracranial arteries.[5] Elastin has been proposed as a functional candidate gene for IA because defects in the internal elastic lamina have been found in IAs.[6-8] Recently, elastin has also been suggested to be a positional candidate gene for familial IA because a genome-wide and a locus-specific linkage study in affected sib pairs and affected pedigree members, respectively, showed linkage to a region on chromosome 7q11 that includes the elastin gene.[9,10] The gene was analyzed further for allelic and haplotype associations in a sample with equal numbers of sporadic and familial patients with SAH from Japan.[9] Although no allelic association was found with any of the 14 single-nucleotide polymorphisms (SNPs) investigated in the elastin gene, the haplotype constructed from the intron 20 (INT20) and INT23 polymorphisms was strongly associated with IA ($p=3.81 \times 10^{-6}$),[9] which further supported a locus for IA within or near the elastin gene. However, an additional genome-wide and a locus-specific linkage study of IA failed to provide positive results for 7q11.[11,12] Furthermore, the INT20/INT23 haplotype was not associated with IA in a sample from Central Europe.[13] To investigate the role of the elastin gene in sporadic SAH patients further, we studied the association of 18 exonic and intronic SNPs, including the 14 SNPs analyzed previously,[9] and haplotypes of pairwise combinations of these SNPs in the elastin gene with sporadic, aneurysmal SAH in the Dutch population.

Patients and Methods

Patient and control recruitment

We included 167 consecutive Dutch patients with sporadic aneurysmal SAH admitted to the University Medical Center Utrecht and 167 age- and sex-matched Dutch controls. Patients with aneurysmal SAH were defined by symptoms suggestive of SAH combined with subarachnoid blood on computed tomography (CT) and a proven aneurysm on CT angiography or conventional angiography. The matched controls were selected from the database of the Department of Medical

Table 1. *Characteristics of the analyzed polymorphisms in the elastin gene.*

SNP name	Location/Position	Nucleotide change	Amino acid change
PM1	Promoter -1042	C>T	
PM2	Promoter -972	G>A	
PM3	Promoter -38	C>T	
INT1	Intron 1	$(CCTT)_n$ repeat	
INT4	Intron 4 196+71	G>A	
EX5	Exon 5 212	C>T	Ala>Val
INT5	Intron 5 233-94	G>A	
INT6	Intron 6 326-59	G>A	
INT8	Intron 8 427+92	G>C	
INT14	Intron 14 746-28	G>A	
EX20 1	Exon 20 1192	G>C	Gly>Arg
EX20 2	Exon 20 1264	G>A	Gly>Ser
INT20	Intron 20 1315+17	T>C	
EX22	Exon 22 1380	G>A	Leu>Leu
INT23	Intron 23 1501+24	T>C	
EX26	Exon 26 1828	G>C	Gly>Arg
INT26	Intron 26 1934-20	C>T	
INT32	Intron 32 2273-34	C>T	
3UTR	3'-UTR 659	G>C	

Ala: alanine; Val: valine; Gly: glycine; Arg: arginine; Ser: serine; Leu: leucine.

Genetics, which includes healthy family members of patients with diverse diseases. The ethical review board of our hospital approved our study protocol.

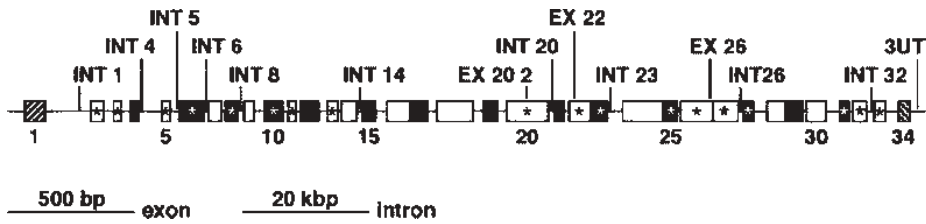
Polymorphisms in the elastin gene

We analyzed 18 exonic and intronic SNPs (***Table 1***), of which 14 were analyzed previously in Japanese SAH patients.[9] We also included 4 previously published SNPs[14] and 1 SNP from the SNP database (ID rs2229427). Furthermore, a tetranucleotide repeat polymorphism within INT1[9,15] was analyzed because this polymorphism showed allelic association with aneurysmal SAH in a previous study.[9] A map of the elastin gene with informative polymorphisms is shown in ***Figure 1***.

Laboratory analyses

Genotyping of the SNPs in the elastin gene was performed with coded genomic DNA samples using a multiplex fluorescent primer extension assay.[16] For all reac-

Figure 1. *Location of informative polymorphisms in the elastin gene.*



Location of informative polymorphisms investigated in this study is shown by tie lines to a schematic representation of the elastin gene. The promoter region, introns and 3'-untranslated region (3'-UTR) of the elastin gene are shown by solid lines. Exons are indicated by boxes on the basis of the nature of domains encoded. Open boxes: hydrophobic domains; full boxes: crosslink domains; hatched boxes: signal peptide and C-terminal cysteine-containing domains. Exons subject to alternative splicing in dermal fibroblasts (Z. Urbán *et al*., unpublished data, 2004) are indicated by asterisks. Different scaling was used for drawing exons and introns as indicated by scale bars below the diagram (bp: base pairs; kbp: kilobase pairs).

tions, we used no template negative controls and sequence-confirmed positive controls for each available genotype. Assay conditions are available upon request. Genotyping results were verified by review of the chromatograms by 2 independent observers. Discordant or missing genotype calls were subjected to genotyping by direct sequence analysis of both strands. The tetranucleotide repeat polymorphism within INT1 was detected by polymerase chain reaction.[15]

Statistical analysis

Statistical analysis of the haplotype frequency and linkage disequilibrium (LD) calculations were conducted using the COCAPHASE option of the software UNPHASED v2.402 which uses likelihood ratio tests in a log-linear model.[17] The calculated LD statistics included global D' and Pearson χ^2 tests.[18] Differences in allele frequencies of each SNP between patients and controls were assessed as an odds ratio (OR) of the minor allele with a corresponding 95% CI and p-value using the major allele as reference. In analyzing haplotypes, the OR of the most frequent haplotype for a given combination of SNPs was assessed by using the remaining haplotypes as reference. A Bonferroni correction (a multiple-comparison correction) was applied to all significant associations, with a correction factor derived from the number of SNPs or haplotypes tested (p-value after Bonferroni correction [p_{corr}]). For the tetranucleotide repeat polymorphism in INT1, differences in allele frequencies between patients and controls were compared using χ^2 test comparing

Table 2. *SNP genotype and allele frequencies in patients with aneurysmal SAH vs controls.*

SNP	Genotype	Patients, n (%)	Controls, n (%)	H-W
INT4 (n=151)	GG	140 (92.7%)	134 (88.8%)	0.94
	GA	10 (6.6%)	15 (9.9%)	
	AA	1 (0.7%)	2 (1.3%)	
INT5 (n=144)	GG	127 (88.2%)	117 (81.3%)	0.87
	GA	16 (11.1%)	17 (11.8%)	
	AA	1 (0.7%)	10 (6.9%)	
INT6 (n=132)	GG	113 (85.6%)	115 (87.1%)	0.92
	GA	18 (13.6%)	14 (10.6%)	
	AA	1 (0.8%)	3 (2.3%)	
INT8 (n=146)	GG	129 (88.4%)	128 (87.7%)	0.93
	GC	17 (11.6%)	17 (11.6%)	
	CC	0 (0%)	1 (0.7%)	
EX20 2 (n=155)	GG	55 (35.5%)	56 (36.1%)	0.62
	GA	69 (44.5%)	79 (51.0%)	
	AA	31 (20.0%)	20 (12.9%)	
INT20 (n=149)	TT	106 (71.2%)	94 (63.1%)	0.80
	TC	37 (24.8%)	52 (34.9%)	
	CC	6 (4.0%)	3 (2.0%)	
EX22 (n=145)	GG	145 (100%)	140 (96.5%)	
	GA	0 (0%)	2 (1.4%)	
	AA	0 (0%)	3 (2.1%)	
INT23 (n=138)	TT	43 (31.1%)	43 (31.1%)	0.57
	TC	68 (49.3%)	72 (52.2%)	
	CC	27 (19.6%)	23 (16.7%)	
EX26 (n=145)	GG	128 (88.4%)	123 (84.8%)	0.92
	GC	16 (11.0%)	22 (15.2%)	
	CC	1 (0.7%)	0 (0%)	
INT26 (n=141)	CC	139 (98.6%)	136 (96.5%)	0.98
	CT	2 (1.4%)	5 (3.5%)	
	TT	0 (0%)	0 (0%)	
INT32 (n=161)	CC	127 (78.9%)	112 (69.6%)	0.57
	CT	30 (18.6%)	49 (30.4%)	
	TT	4 (2.5%)	0 (0%)	
3UTR (n=150)	GG	77 (51.3%)	62 (41.3%)	0.69
	GC	66 (44.0%)	81 (54.0%)	
	CC	7 (4.7%)	7 (4.7%)	

H-W: p-value for χ^2 test of Hardy -Weinberg equilibrium for SNPs with a minor allele

Allele	Patients, n (%)	Controls, n (%)	OR	95% CI	p	p_{corr}
G	290 (96.0%)	283 (93.7%)	0.6	0.3-1.4	0.20	
A	12 (4.0%)	19 (6.3%)				
G	270 (93.8%)	251 (87.2%)	0.5	0.2-0.8	0.007	0.08
A	18 (6.2%)	37 (12.8%)				
G	244 (92.4%)	244 (92.4%)	1.0	0.5-2.0	1.0	
A	20 (7.6%)	20 (7.6%)				
G	275 (94.2%)	273 (93.4%)	0.9	0.4-1.8	0.73	
C	17 (5.8%)	19 (6.6%)				
G	179 (85.2%)	191 (91.0%)	1.2	0.8-1.6	0.33	
A	131 (4.8%)	119 (9.0%)				
T	249 (83.6%)	240 (80.5%)	0.8	0.5-1.3	0.34	
C	49 (16.4%)	58 (19.5%)				
G	290 (100%)	282 (97.2%)	0.0	0.0-0.7	0.004	0.05
A	0 (0%)	8 (2.8%)				
T	154 (55.8%)	158 (57.2%)	1.1	0.8-1.5	0.73	
C	122 (44.2%)	118 (42.8%)				
G	272 (93.8%)	268 (92.4%)	0.8	0.4-1.6	0.51	
C	18 (6.2%)	22 (7.6%)				
C	280 (98.9%)	277 (97.3%)	0.4	0.1-2.3	0.25	
T	2 (1.1%)	5 (2.7%)				
C	284 (88.2%)	273 (84.8%)	0.8	0.5-1.2	0.20	
T	38 (11.8%)	49 (15.2%)				
G	220 (73.3%)	205 (68.3%)	0.8	0.5-1.1	0.17	
C	80 (26.7%)	95 (31.2%)				

frequency of >5% in the control group.

only alleles with frequencies >5.0%. Our study was performed in a paired fashion. Therefore, data were analyzed only if genotypes were available for both individuals in a patient-control pair. Tests for Hardy-Weinberg equilibrium were conducted using χ^2 tests.

Assuming a recessive disease locus,[9] our cohort of 167 cases and 167 controls had an 80% power to detect a susceptibility locus with a relative risk of >1.2 at a significance level of 0.05 when testing SNPs with minor allele frequencies of >0.025 (genetic power calculator, SGDP Statistical Genetics Group).

Results

The SNPs PM1, PM2, PM3, exon 5 (EX5), INT14 and EX20 1 were not polymorphic in our population. Distribution of the genotypes of the remaining 12 SNPs and the tetranucleotide repeat polymorphism was consistent with Hardy-Weinberg equilibrium (***Table 2***).

SAH association with elastin gene alleles

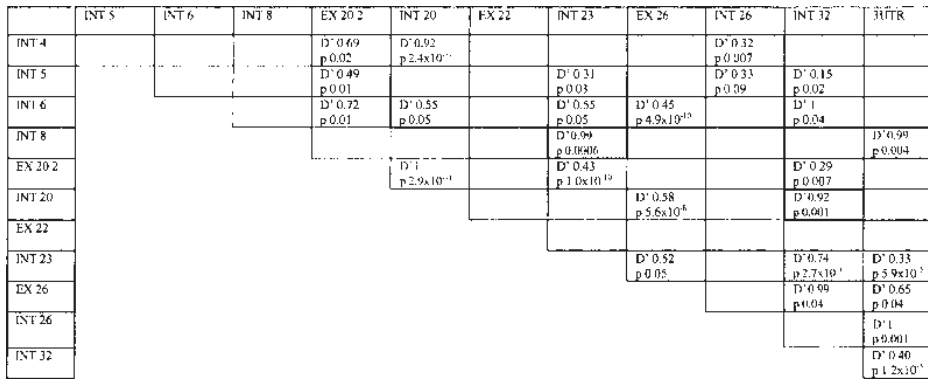
We compared allele frequencies of the remaining 12 polymorphic SNPs between patients and controls (***Table 2***). The EX22 SNP was associated with aneurysmal SAH because 0% of the patients were carriers of the minor allele compared with 2.8% of the controls (OR, 0.0; 95% CI, 0.0 to 0.7; p=0.004). After Bonferroni correction, the association remained statistically significant (p_{corr}=0.05). The INT

Table 3. *Association study with haplotypes consisting of pairwise combination of alleles of SNPs EX 22, INT4 and INT 5 in SAH patients vs controls.*

Haplotype	Patients (%)	Controls (%)	p*	p_{corr}
INT4 EX22	(G,G) 95.8% (A,G) 4.2% (G,A) 0%	(G,G) 89.7% (A,G) 7.2% (G,A) 3.1%	0.001	0.02
INT5 EX22	(G,G) 93.4% (A,G) 6.6% (G,A) 0%	(G,G) 83.3% (A,G) 13.6% (G,A) 3.1%	$7.7x10^{-5}$	0.002

*: p-value for Pearson's χ^2 statistical comparison of haplotype frequencies of patients *vs* controls.
p_{corr}: p-value after Bonferroni correction.

Figure 2. *Pairwise LD between SNPs in the elastin gene in control individuals.*



D' value is a measure of LD with values between 0 and 1; D' values between 0.7 and 1.0 are considered to be an evidence of LD. Shading indicates D'>0.70 and p<0.05.

5 SNP showed association with aneurysmal SAH with 6.2 % carriers of the minor allele in the patient group versus 12.8% in the control group (OR, 0.5; 95% CI, 0.2 to 0.8, p=0.007). After applying Bonferroni correction, this p-value was no longer statistically significant (p_{corr} =0.08). The remaining 10 SNPs were not associated with aneurysmal SAH. Allele frequencies of the tetranucleotide repeat polymorphism in INT 1 were not significantly different in patients with aneurysmal SAH and controls (p=0.37; 4 *df*, data not shown).

SAH association with elastin gene haplotypes

We constructed haplotypes using all 21 possible pairwise SNP combinations that included SNPs EX22 and INT5. Haplotype association with SAH was found for all haplotypes involving SNP EX22 and almost all haplotypes involving SNP INT5 (except for INT5/INT6, INT5/INT8, and INT5/INT23). After Bonferroni correction, association with haplotypes of INT5/EX22 remained statistically significant (p_{corr}=0.002; ***Table 3***). The G,G haplotype (major alleles for both INT5 and EX22) was more prevalent in patients than in controls (OR, 2.6; 95% CI, 1.2 to 5.8). In addition, association with haplotypes of INT4/EX22 also remained significant after correction (p_{corr}=0.02; ***Table 3***). The G,G haplotype (major alleles for INT4 and EX22) was also more prevalent in patients than in controls (OR, 2.8; 95% CI, 1.5 to 5.4). As expected, haplotypes of SNP combination INT4/INT5/EX22 were even more strongly associated with SAH ($p=9.0x10^{-9}$) with the G,G,G haplotype being more prevalent in patients than in controls (90% versus 76%, OR, 2.9; 95% CI, 1.7 to 4.8).

LD pattern within the elastin gene

Because many SNPs in the elastin gene have relatively low minor allele frequencies, many LD analyses showed high p-values. In our LD analyses, we only show the results with a p-value <0.05 (***Figure 2***). Pairwise analysis showed an irregular pattern of LD between SNPs in the control patients with an overall weak LD (***Figure 2***). A possible ancestral haplotype INT20/INT23/INT32/3UTR did not show haplotype association in patients with aneurysmal SAH and controls. The LD pattern was similar in controls and SAH patients (data not shown).

Discussion

In a series of Dutch patients with sporadic aneurysmal SAH, we found a significant association with an SNP EX22 with more carriers of the minor allele in the control group. An explanation for this finding may be that the minor allele or an allele in disequilibrium with it is protective of SAH. Furthermore, we found that the haplotypes INT5/EX22, INT4/EX22, and haplotype INT4/INT5/EX22 also showed significant association with aneurysmal SAH. Gradual increase of statistical power with the inclusion of 2 or 3 SNPs in the studied haplotypes supports the validity of our conclusion that the elastin gene is a susceptibility locus for SAH.

Allele frequencies of the elastin gene differ between Dutch and Japanese populations.[9] Six of the SNPs described in the Japanese patients were not polymorphic in the Dutch population. Moreover, the association of aneurysmal SAH with the haplotype between the INT20/INT23 polymorphism and the (CCTT) repeat microsatellite in INT1 of the elastin gene[9] was not confirmed in our study. Differences in study populations may in part explain the differences found. We only included patients without a known positive family history for IA, whereas the Japanese study population consisted of approximately 50% of patients with a positive family history. In addition, we used a clinically homogeneous population of only patients with aneurysmal SAH, whereas the Japanese study included not only patients with aneurysmal SAH but also patients with unruptured IA. Another explanation for the differences between the studies is that historical isolation has led to different allele frequencies and haplotype structure across populations.[19] If this is true, population-specific variants may contribute to the risk of SAH and IA. Such variations may, for example, play a role in the difference in SAH incidence, which is 3x higher in Japan (and in Finland) than in other parts of the world.[20,21]

Our results also replicate the findings that in 30 familial and 175 sporadic SAH patients from Central Europe, no allellic association of the haplotype between the

INT20/INT23 polymorphism was found.[13] These authors also suggested allelic heterogeneity between Japanese and European populations of SAH patients. Further indication of possible population differences is that linkage to chromosome 7q11 demonstrated in Japanese[9] and North American[10] SAH patients was not confirmed in 2 other linkage-mapping studies.[11,12]

A strength of our study was that we used patient-control pairs matched by age and sex to minimize differences in SAH risk between cases and controls. In addition, to prevent genotyping bias, the study was conducted in a blinded fashion. We investigated a large number of SNPs, which increase the risk of finding a false-positive association of a genotype with aneurysmal SAH by chance. However, in this study, analyses with a large number of SNPs were necessary because LD between the SNPs was generally low. Furthermore, we applied a Bonferroni correction to all novel associations to reduce the risk of finding false-positive associations.

The analyzed SNPs in the elastin gene did not show strong LD. These results are consistent with the LD analysis in the Japanese population, in which the LD for SNPs in the elastin gene was also very weak.[9] Boundaries between haplotype blocks correlate with meiotic recombination hot spots.[22] Although recombination rates within the elastin gene locus have not been investigated directly, a previous report of a *de novo* recombination between 2 mutations in the elastin gene[23] suggested that the elastin gene may be a recombination hot spot, which would explain the lack of LD in this locus.

The elastin protein consists of lysine-rich cross-linking domains and hydrophobic domains responsible for elastic properties. The domain structure of the protein is a reflection of the exon organization of the gene because the hydrophobic and cross-linking domains are encoded by separate exons. The primary transcript of the gene is alternatively spliced.[24,25] Exonic SNPs or intronic polymorphisms located close to exons may alter efficiency of the splicing and thus change the domain content of the resulting polypeptide. SNPs INT4, INT5, and EX22 are flanking or are located within such alternatively spliced exons. Altered domain content of the corresponding elastin may confer resistance to the pathogenic mechanism leading to IA rupture.

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Chapter 8

The versican gene and the risk of intracranial aneurysms

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Abstract

Background and purpose

The proteoglycan versican is an excellent candidate gene for intracranial aneurysms (IAs) as it plays an important role in extracellular matrix assembly and is localized in a previously implicated locus for IAs on chromosome 5q.

Methods

We analyzed all the common variations using 16 tag single nucleotide polymorphisms (SNPs) and haplotypes in the versican gene using a two-stage genotyping approach. For stage 1, 16 SNPs were genotyped in 307 cases and 639 controls. For stage 2, the two SNPs yielding the most significant associations ($p<0.01$) were genotyped in a second independent cohort of 310 cases for confirmation of the associations.

Results

In stage 1 we found several SNPs in strong linkage disequilibrium (LD) and haplotypes constituting these SNPs associated with IAs in the Dutch population (strongest SNP association for rs173686 with OR, 1.34; 95% CI, 1.09 to 1.65; p=0.004). In stage 2 we confirmed association for the two SNPs with the most significant associations (strongest SNP association for rs173686 with OR, 1.36; 95% CI, 1.11 to 1.67; p=0.003).

Conclusion

SNPs in strong LD and haplotypes constituting these SNPs in the versican gene are associated with IAs, suggesting that variation in or near the versican gene plays a role in susceptibility to IAs.

We hypothesized that disruption of the extracellular matrix (ECM) of the arterial wall is a likely factor in the pathogenesis of intracranial aneurysms (IAs).[1] Interesting candidate genes are structural genes of the ECM and genes involved in ECM assembly. The proteoglycan versican plays an important role in the ECM assembly process and is localized close to a previously implicated locus for IAs on chromosome 5q in a Japanese cohort.[2]

We performed a comprehensive genetic association study analyzing all the common variants using tag single nucleotide polymorphisms (SNPs) and haplotypes between these SNPs in the versican gene in a Dutch case-control population.

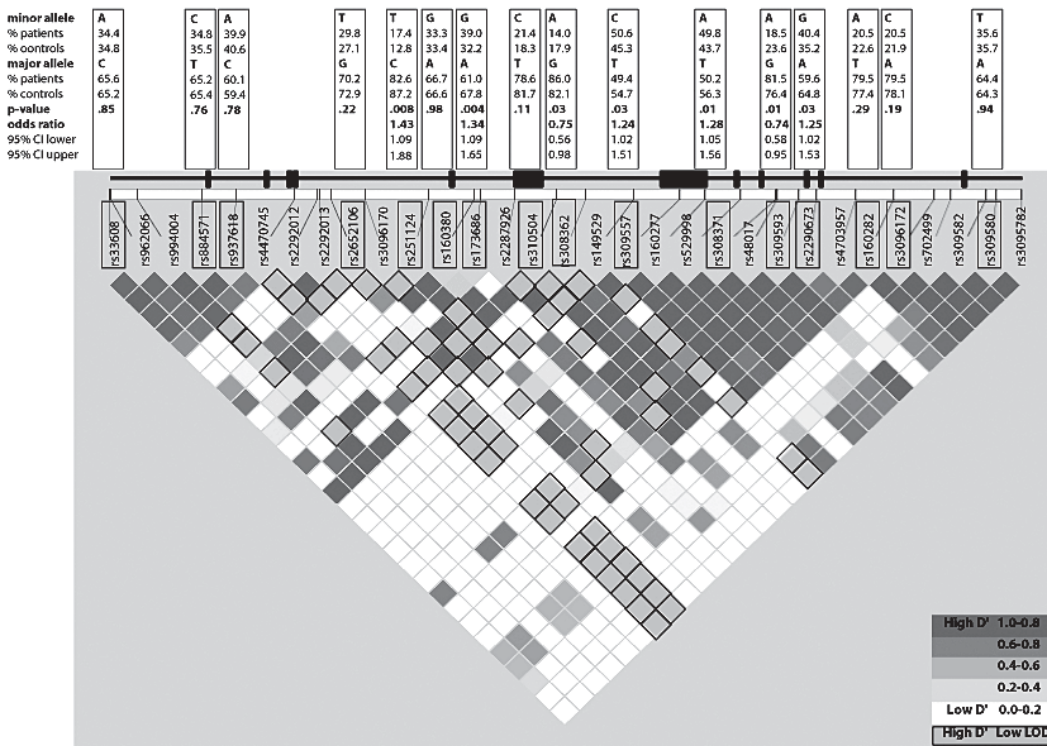
Methods

We used a two-stage genotyping approach. For stage 1, 16 SNPs were genotyped in 307 cases and 639 controls. For stage 2, the two SNPs yielding the most significant associations ($p<0.01$) were genotyped in a second cohort of 310 cases for confirmation of the associations.

For stage 1, we included 307 prospectively collected Dutch Caucasian patients with ruptured and unruptured IAs admitted to the University Medical Center Utrecht (UMC) and 639 ethnically matched Dutch Caucasian controls, comprising blood bank volunteers (n=460) and unrelated controls selected from a database of healthy family members of UMC patients with various diseases other than IAs (n=179). Ruptured IAs were defined by symptoms suggestive of subarachnoid hemorrhage (SAH) combined with subarachnoid blood on CT and a proven aneurysm at angiography (conventional angiogram, CT-angiogram or MR-angiogram), and unruptured IAs were identified by CT or MR angiography, conventional angiography, surgery or autopsy. For stage 2, DNA was available from a second independent cohort of 310 Dutch aneurysmal SAH patients also from the UMC Utrecht. Patients of stage 2 were retrospectively collected as these patients, who have been treated for aneurysmal SAH in the past by surgical clipping of the aneurysms, participated in a screening study for the formation of new aneurysms by means of CT-angiography.[3] During this screening these patients were asked to give blood for DNA analysis. The UMC Utrecht ethical review board approved our study protocol.

Sixteen tag SNPs in the versican gene were selected from the International HapMap Project (www.hapmap.org) using Tagger (Paul de Bakker, http://www.broad.mit.edu/mpg/tagger/). The pairwise linkage disequilibrium (LD) between the SNPs in the gene is shown in the ***Figure***. Genotyping was performed

Figure. *The pairwise linkage disequilibrium between all single nucleotide polymorphisms in the versican gene and comparison of the allelic frequencies of the 16 tag SNPs between 307 intracranial aneurysms (IA) patients of stage 1 and 639 healthy controls.*



95% CI lower: 95% confidence interval lower limit; 95% CI upper: 95% confidence interval upper limit. The exons of the gene are shown as black squares.

using Taqman assays on coded genomic DNA samples (*i.e.* blind to the diagnostic group).

Differences in allele and haplotype SNP frequencies between patients and controls were assessed as odds ratios (OR) using the χ^2 test with corresponding 95% confidence intervals (CI) and p-values. Haplotype frequencies were analyzed using Haploview.[4]

Assuming an autosomal recessive model[2] and a 5% high-risk allele frequency, our results had 80% power for detecting a susceptibility locus with a relative risk >1.7 at a significance level of 0.05 (Genetic power calculator, SGDP statistical genetics group, http://statgen.iop.kcl.ac.uk).

Table 1. *Characteristics of the analyzed patients with intracranial aneurysms of stage 1 and 2 and controls.*

	Patients stage 1 (n=307)	**Patients stage 2 (n=310)**	**Controls (n=639)**
Women	217 (70.7%)	194 (62.6%)	329 (51.5%)
Mean age (years)	58.0 (range 20-93)	55.1 (range 26-72)	51.6 (range 18-91)
Mean age SAH patients (years)	52.3 (range 12-85)	44.6 (range 11-66)	
Familial aneurysms	56 (18.2%)	22 (7.2%)	
Unruptured aneurysms (no SAH)	36 (11.7%)	0 (0%)	
Multiple aneurysms	76 (24.8%)	50 (16.2%)	
MCA aneurysms	72 (23.4%)	70 (22.5%)	

SAH: subarachnoid hemorrhage; MCA: middle cerebral artery complex.

Results

The characteristics of patients analyzed in stage 1 and 2 and of controls are shown in ***Table 1***. The distribution of the SNP genotypes was consistent with Hardy-Weinberg equilibrium. We had 0.2% missing genotypes. Analyzing patients of stage 1 versus controls, the rs251124, rs173686, rs308362, rs309557, rs308371, rs309593 and rs2290673 SNPs were associated with IAs (p-values<0.05), with rs251124 (OR, 1.43; 95% CI, 1.09 to 1.88; p=0.008) and rs173686 (OR, 1.34; 95% CI, 1.09 to 1.65; p=0.004) having the strongest association (***Figure***). The SNPs rs308362, rs309557, rs308371, rs309593 and rs2290673 are all on the same strong LD block with high D' values (>0.8). The SNPs rs251124 and rs173686 are in high LD with each other with a high D' value of 1.0 and are in high LD with most of the SNPs in the block including the previous five SNPs (D' values >0.7). We constructed haplotypes using the SNPs (rs251124 and rs173686) most strongly associated with IAs and then using all the SNPs associated with IAs (***Table 2***). The strongest haplotype association with IAs was found for the rs251124 and rs173686 SNPs (C,A) (*i.e.* C allele SNP rs251124, A allele for SNP rs173686; OR, 0.74; 95% CI, 0.60 to 0.91; p=0.003) and (T,G) (OR, 1.40; 95% CI, 1.06 to 1.84, p=0.02).

In stage 2, the SNPs rs251124 and rs173686 yielding the most significant associations were genotyped in the second cohort of patients. Comparing the allele frequency of these SNPs in this patient group to the allele frequency of the control

group, again significant associations with IAs are found for both SNPs rs251124 (T allele 18.1% in patients versus 12.8% in controls; OR, 1.50; 95% CI, 1.14 to 1.96; p=0.002) and rs173686 (G allele 39.3% in patients versus 32.2% in controls; OR, 1.36; 95% CI, 1.11 to 1.67; p=0.003). When combining the patients of stage 1 and stage 2 the associations with IAs become stronger for both SNPs (rs251124 OR, 1.47; 95% CI, 1.17 to 1.84; p=0.0006; rs173686 OR, 1.35; 95% CI, 1.14 to 1.60; p=0.0003). Adjustment for age and sex using logistic regression did not change our conclusions.

Discussion

We found that SNPs in strong LD and haplotypes constituting these SNPs in the versican gene are associated with IAs. We replicated our findings in a second independent cohort of IA patients. Our findings suggest that variation in or near the versican gene plays a role in susceptibility to IAs, which would then confirm our hypothesis that diminished maintenance of the ECM is important in the development of IA.

Our results are consistent with the linkage findings of a study in a Japanese cohort showing linkage to 5q22-31 (this locus lies in the vicinity of the versican gene),[2] as we also observed increased allele sharing in the versican gene. However, our sample of affected sib pairs with IAs was very small (14 pairs), resulting in p-

Table 2. *Association of haplotypes from the SNPs showing the strongest association with intracranial aneurysms (IAs) (rs251124 and rs173686 SNPs; haplotype 1) and from all the SNPs associated with IAs (rs251124, rs173686, rs308362, rs309557, rs308371, rs309593 and rs2290673; haplotype 2) in IA patients versus healthy controls.*

Haplotype	Patients	Controls	OR	95% CI	p-value
Haplotype 1					
(C,A)*	60.6%	67.6%	0.74	0.60-0.91	0.003
(T,G)	16.8%	12.7%	1.40	1.06-1.84	0.02
Haplotype 2					
(C,A,A,T,T,A,A)	9.6%	13.4%	0.69	0.50-0.95	0.02
(T,G,G,C,A,G,G)	12.1%	8.8%	1.42	1.03-1.96	0.03

*: C allele of SNP rs251124, A allele of SNP rs173686; OR: odds ratio; 95% CI: 95% confidence interval.

values below the threshold levels for linkage in genome-wide screens (data not shown). Interestingly, this IA locus on chromosome 5q[2] lies close to a locus identified in thoracic aortic aneurysms (TAA) on 5q13-14, which may suggest a common genetic factor for the two types of aneurysms.[5] Moreover, as versican has already been suggested as a positional candidate gene in TAA,[5] variation in the versican gene may be a common genetic risk factor for both these diseases.

The SNPs showing association with IAs lie in a region of the versican gene that includes the two largest exons (6 and 7) where alternative RNA splicing occurs.[6] These alternatively spliced exons encode glycosaminoglycan (GAG) attachment sites, which can bind chrondroitin sulphate (CS) chains[6] that are believed to have an anti-adhesive function. The largest spliced variant (V0) includes the two largest exons while the smallest variant (V3) lacks these exons. Consequently V3 has no GAG attachment sites and therefore a lower number of CS chains attached.[6] Overexpression of V3 in arterial smooth muscle cells enhances cell adhesion, reduces growth and migration, and induces tropoelastin synthesis.[6] In this way V3 may influence the accumulation of ECM components. In IA patients, the splicing process of versican may be altered resulting in a higher proportion of larger isoforms, and thus in diminished ECM assembly.

The demonstrated association of versican with IAs should be replicated by studies in other populations. These should include large numbers of patients as IA is a complex disease and most of the genetic factors will therefore have only a small effect. Indeed, the odds ratios of the associated SNPs and haplotypes that we found are relatively low. The same holds true for the previously reported linkage data as the 5q linkage peak appeared moderately high, with a maximum LOD score of 2.24.[2] After the association has been confirmed, the causal variant in the versican gene should be identified by sequencing and functional analysis.

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Chapter 9

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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Abstract

Background and purpose

Development of intracranial aneurysms is probably caused by interaction of several genes and environmental factors. Previously, we hypothesized that a disruption of the extracellular matrix (ECM) of the arterial wall is a likely factor in the pathogenesis of intracranial aneurysms.

Methods and results

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. In six of these 44 genes SNPs that were associated with intracranial aneurysms ($p<0.01$) were identified: serpine1 (*PAI1*, p=0.0008), transforming growth factor, beta induced (*TGFBI*, p=0.0026), perlecan (*HSPG2*, p=0.0044), fibronectin (*FN1*, p=0.0069), fibrillin 2 (*FBN2*, p=0.0077) and collagen 4A1 (*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 *HSPG2* gene [combined odds ratio (OR), 1.33; 95% confidence interval (CI), 1.13 to 1.57; p=0.0006] was replicated. The population attributable risk (PAR) for this SNP is 19%. Combining the two cohorts, the association for the *PAI1* (combined OR, 1.27; 95% CI, 1.07 to 1.50; p=0.004; PAR of 6%), *FBN2* (combined OR, 1.37; 95% CI, 1.07 to 1.75; p=0.01; PAR of 3%) and *COL4A1* (combined OR, 1.22; 95% CI, 1.05 to 1.42; p=0.007; PAR of 7%) genes remained.

Conclusions

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.

Intracranial aneurysms (ANIB, [MIM 105800]) are found in approximately 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 8 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 to be involved in the development of intracranial aneurysms. Familial clustering of SAH is found in approximately 10% of patients with SAH, and first-degree relatives of patients with SAH have a two and a half to seven times greater risk of developing SAH than the general population.[6-11] Intracranial aneurysms are likely a complex disease caused by interaction of several genes and environmental factors.[12] Up to now several 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).

Recently, we hypothesized that 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 intra- and extracranial arteries of aneurysm patients.[12] Furthermore, the identified genetic loci for intracranial aneurysms include some interesting candidate genes coding for structural proteins of the ECM of the arterial wall.

Based on previous studies on the genetic and pathophysiological background of intracranial aneurysms, a list of the most promising positional and/or functional candidate genes for intracranial aneurysms 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.

Patients and Methods

Design of the study

An association study of intracranial aneurysms was performed using a two-stage

genotyping approach. For stage 1, 384 single nucleotide polymorphisms (SNPs) were genotyped in cases and controls. As an additional analysis, the SNP frequency between subgroups, consisting of patients with familial and non-familial intracranial aneurysms and of patients with ruptured and unruptured intracranial aneurysms, was compared separately to study whether there are specific SNPs that are associated with familial intracranial aneurysms and/or rupture of intracranial aneurysms. For stage 2, the SNPs yielding the most significant associations were genotyped in a second cohort of cases and controls for confirmation of the associations.

Patients and controls

For stage 1, DNA, isolated from whole blood, was available for a cohort of 382 Dutch Caucasian patients with ruptured and unruptured, familial and non-familial intracranial aneurysms admitted to the University Medical Center Utrecht, and 609 Dutch Caucasian controls, comprising 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 by 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 based on previous candidate gene studies, *(c)* expression within intracranial aneurysmal tissue based on previous expression studies, *(d)* evidence from functional studies in intracranial aneurysm patients, *(e)* disease causing genes of heritable disorder of connective tissue and ECM associated with intracranial aneurysms, and *(f)* membership of the same gene family of already selected candidate genes, or a combination of the above criteria. The analyzed 44 candidate

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

Gene products	Genes
Structural proteins	
Collagen fibers (n=9)	Collagen 1A1, 1A2, 3A1, 4A1, 5A1, 5A2, 6A2, 8A2 and 16 A1
Elastic fibers (n=1)	Elastin
Glycoproteins (n=12)	Fibrillin 1and 2; Fibronectin; Microfibril-associated protein 1, 2, 3 and 4; Fibulin 5; Fibromodulin; Perlecan; Emilin 1 and 2
Other structural proteins (n=2)	Secreted protein, acidic, cystein-rich; Transforming growth factor, beta induced
Proteases	
Metalloproteinases (n=3)	Metalloproteinase 2, 9 and 14
Other proteases (n=4)	Elastase; Cathepsin B and D; Plasminogen
Protease inhibitors	
Tissue inhibitors of metallo-proteinase (n=3)	Tissue inhibitor of metalloproteinase 1, 2 and 3
Other protease inhibitors (n=6)	A1 antitrypsine; Serpina 3; Plasminogen activator inhibitor 1/serpine 1; Cystatin C, K and S
Other enzymes (n=2)	Lysyl oxidase; Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3
Growth factors (n=2)	Connective tissue growth factor; Osteoblast specific factor 2

genes are shown in ***Table 1***, while 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 ***Table A***. SNPs were selected by downloading all the SNPs typed in the CEPH (Utah residents with ancestry from northern and western Europe) population in our 44 candidate genes from the HapMap database (http://www.hapmap.org/).[19] From these SNPs, tag SNPs[20] were selected using the aggressive tagging option of the program Tagger (Paul de Bakker, http://www. broad.mit.edu/mpg/tagger/) so that all SNPs with a minor allele frequency >5% were captured with $r^2 > 0.8$. 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 ***Table B***). 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 all those that had a low signal or too wide clusters 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 ($p<0.01$) and one gene with associated SNPs for which association with intracranial aneurysms was already 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 ABI7900HT instrument (Applied Biosystems, Foster City, CA). The AB assay IDs of the Taqman Assays on Demand are provided in Supplementary ***Table C***.

Statistical analysis

Association chi-squares with two-tailed p-values and Hardy-Weinberg equilibriums were calculated using the Haploview program (available at http://www.hapmap.org [22]), for both stages of our study. For the associated SNPs tested in stage 2, differences in allele frequencies were assessed 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 the association remained on combining stage 1 and 2 was calculated using the following formula: PAR = PF(RR-1)/[PF(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 Hardy-Weinberg equilibrium (Supplementary ***Table B***). After the quality checks, 22 poorly performing tag SNPs were excluded because of too low signal, scattering or overlap of clusters, leaving 362 tag SNPs for further analysis (conversion rate of 94.3%). The association data of the 362 tag SNPs that passed through the quality check with chi-square and corresponding p-values are shown in Supplementary ***Table B***. Of the DNA samples, 11 of 609 control samples and eight of the 382 patient samples performed poorly due to low signals and were excluded for further analyses, leaving 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, association to SNPs in 16 of the 44 analyzed genes

was observed (***Table 3***) while considering a p-value <0.01, association was shown for SNPs in 6 of the analyzed genes: the serpine1 gene (*PAI1*; strongest association for SNP rs6956010; p=0.0008), transforming growth factor, beta induced (*TGFBI*; one associated SNP rs756463; p=0.0026), perlecan (*HSPG2*; strongest association for SNP rs3767137; p=0.0044), fibronectin (*FN1*, strongest association for SNP rs2289202; p=0.0069), fibrillin 2 (*FBN2*, strongest association for SNP rs331069; p=0.0077), collagen 4A1 (*COL4A1*, strongest association for SNP rs3783107; p=0.0087) (***Table 3***). Significant association was also found for one SNP in the elastin gene (*ELN*, rs 4717865; p=0.0103) for which gene association 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 *PAI1* (high D'=0.9, but low r^2=0.2), the two associated SNPs in *HSPG2* (low D'=0.03 and low r^2=0.0), and the four associated SNPs in *FN1* (D' 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 *FBN2* (rs28114 and rs331069 with D'=0.9 and r^2=0.8) but not between the remaining associated SNPs. For *COL4A1* high LD between two of the five associated SNPs was observed (rs675605 and rs630943 with D'=0.9 and r^2=0.8) and not for the remaining ones.

No differences in SNP frequency were observed between patients with familial and non-familial intracranial aneurysms and 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 Hardy–Weinberg equilibrium (data not shown). The clinical data of the 310 patients analyzed in stage 2 are

Table 2. *Clinical data of the analyzed patients with intracranial aneurysms of stage 1 and 2.*

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

MCA: middle cerebral artery; SAH: subarachnoid hemorrhage.

Table 3. *Genes with SNPs associated (p-value < 0.05) with intracranial aneurysms in stage 1*

Gene	Locus	No of tested tag SNPs	No of SNPs with p-value <0.05	Lowest p-value
Plasminogen activator inhibitor 1/serpine 1	*SERPINE1/PAI1* 7q11	3	2	0.0008
Transforming growth factor, beta induced	*TGFBI* 5q31	5	1	0.0026
Perlecan	*HSPG2/PLC* 1p36.1	11	2	0.0044
Fibronectin	*FN1* 2q34	25	4	0.0069
Fibrillin 2	*FBN2* 5q23-31	41	4	0.0077
Collagen 4A1	*COL4A1* 13q34	28	5	0.0087
Elastin	*ELN* 7q11	2	1	0.0103
Fibulin 5	*FBLN5* 14q22	18	1	0.0106
Tissue inhibitor of metalloproteinase 3	*TIMP3* 22q12.1-q13.2	21	1	0.0114
Collagen 5A1	*COL5A1* 9q34.2-q34.3	59	2	0.0117
Microfibril-associated protein 1	*MFAP1* 15q15-q12	3	2	0.016
Microfibril-associated protein 4	*MFAP4* 17cen	1	1	0.0219
Collagen 1A1	*COL1A1* 17q21.31-q22	2	1	0.0238
Lysyl oxidase	*LOX* 5q23.3q31.2	2	1	0.0374
SPARC	*SPARC* 5q31.3-q32	7	1	0.0402

SNP: single nucleotide polymorphism.

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 *PAI1*, *TGFBI*, *HSPG2*, *FN1*, *FBN2*, *COL4A1* and *ELN* in the second cohort of 310 patients and 336 controls confirmed association for 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 of stage 1 was strength-

analyzing 598 controls and 374 patients.

Evidence from linkage studies	Evidence from gene expression studies	Evidence from functional analyses	Evidence from association studies
(14,15)			
(14)	(44)		
(13)			
	(44)	(31-45)	
(14)			
	(44)	(31,45)	
(14,15)	(44)	(38,39)	(14,25)
(14)			
	(44)		
(14,16)			
(14,16)	(44)	(46)	
(14)			
(14)	(44)		

ened (OR, 1.33; 95% CI, 1.13 to 1.57; p=0.0006). The population attributable risk 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 cohorts. The association of SNP rs6956010 in *PAI1* 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

Table 4. *Analyzing the six genes with SNPs yielding the most significant associations of stage demonstrated in the literature in an additional independent cohort of 310 intracranial*

		Stage 1: Frequency associated allele			
Gene	**rs number**	**patients (n=374)**	**controls (n=598)**	**OR (95% CI)**	**p-value**
PAI1	rs6956010	27.5%	20.7%	1.45 (1.16-1.81)	0.0008
	rs2070682	58.5%	53.8%	1.21 (1.00-1.47)	0.0431
TGFBI	rs756463	77.2%	70.9%	1.39 (1.11-1.73)	0.0026
HSPG2	rs3767137	77.2%	71.3%	1.37 (1.10-1.70)	0.0044
	rs7556412	66.5%	61.0%	1.27 (1.04-1.55)	0.0165
FN1	rs2289202	78.6%	73.1%	1.35 (1.08-1.70)	0.0069
	rs1561299	63.0%	58.1%	1.23 (1.01-1.50)	0.0322
	rs2043776	79.7%	75.0%	1.31 (1.04-1.64)	0.0193
	rs4673999	56.7%	52.0%	1.21 (1.00-1.47)	0.0431
FBN2	rs28114	53.4%	48.4%	1.23 (1.01-1.48)	0.0318
	rs331069	54.4%	48.1%	1.29 (1.06-1.56)	0.0077
	rs331079	11.4%	8.0%	1.48 (1.07-2.04)	0.0134
	rs10520002	90.4%	86.5%	1.36 (1.00-1.86)	0.044
COL4A1	rs12017058	55.6%	49.9%	1.25 (1.03-1.51)	0.018
	rs3783107	40.9%	34.9%	1.29 (1.06-1.57)	0.0087
	rs675605	72.8%	68.5%	1.23 (1.00-1.52)	0.0468
	rs630943	75.3%	70.6%	1.27 (1.02-1.58)	0.0259
	rs2391824	40.0%	34.5%	1.27 (1.04-1.54)	0.0155
ELN	rs868005	43.5%	37.6%	1.28 (1.05-1.55)	0.0103

OR: odds ratio; 95% CI: 95% confidence interval; ND: not determined.

1 (p<0.01) and the gene (ELN), for which gene association with intracranial aneurysms was already aneurysm patients and 336 controls.

Stage 2: Frequency allele associated in stage 1				**Stage2 1+2: Allele frequencies combined**			
patients (n=310)	**controls (n=336)**	**OR (95% CI)**	**p-value**	**patients (n=684)**	**controls (n=934)**	**OR (95% CI)**	**p-value**
24.9%	24.1%	1.04 (0.80-1.36)	0.74	26.3%	21.9%	1.27 (1.07-1.50)	0.004
53.7%	54.5%	0.97 (0.77-1.22)	0.78	56.3%	54.0%	1.10 (0.95-1.27)	0.20
72.4%	72.4%	1.00 (0.77-1.29)	1.00	73.0%	71.4%	1.08 (0.92-1.28)	0.35
76.7%	71.9%	1.28 (0.99-1.67)	0.05	76.9%	71.5%	1.33 (1.13-1.57)	0.0006
60.6%	61.5%	0.96 (0.76-1.22)	0.75	63.8%	61.2%	1.12 (0.96-1.30)	0.14
72.9%	75.5%	0.87 (0.67-1.14)	0.30	76.0%	73.9%	1.12 (0.94-1.32)	0.19
ND	ND	ND	ND	ND	ND	ND	ND
72.3%	78.6%	0.71 (0.54-0.93)	0.01	76.3%	76.2%	1.00 (0.85-1.19)	0.97
52.1%	57.9%	0.79 (0.63-1.00)	0.04	54.6%	54.0%	1.02 (0.89-1.18)	0.74
49.8%	50.6%	0.97 (0.77-1.22)	0.79	51.8%	49.2%	1.11 (0.96-1.29)	0.14
49.3%	50.7%	0.95 (0.76-1.18)	0.62	52.1%	49.1%	1.13 (0.98-1.30)	0.10
9.8%	8.1%	1.21 (0.82-1.79)	0.32	10.7%	8.0%	1.37 (1.07-1.75)	0.01
89.4%	89.5%	1.03 (0.71-1.50)	0.86	89.0%	88.2%	1.22 (0.97-1.55)	0.08
51.6%	52.0%	0.99 (0.79-1.23)	0.90	53.8%	50.7%	1.13 (0.98-1.30)	0.09
39.0%	36.0%	1.13 (0.90-1.43)	0.27	40.1%	35.4%	1.22 (1.05-1.42)	0.007
69.3%	70.5%	0.96 (0.75-1.22)	0.71	71.2%	69.2%	1.03 (0.92-1.15)	0.60
71.7%	72.7%	0.95 (0.74-1.22)	0.69	73.7%	71.4%	1.12 (0.95-1.32)	0.15
35.8%	35.0%	1.04 (0.82-1.31)	0.76	38.1%	34.7%	1.16 (1.00-1.35)	0.05
40.4%	44.0%	0.86 (0.68-1.09)	0.20	42.1%	39.8%	1.10 (0.95-1.27)	0.19

stage 1 (OR, 1.27; 95% CI, 1.07 to 1.50; p=0.004). The population attributable risk for this SNP is 6%. For the other SNP in *PAI1* rs2070682, which is not in LD with SNP rs6956010, association in this stage or in the combined cohorts could not 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 predominance of the allele found to be more frequent in the patient group in stage 1 was also observed in the patient group of this stage and on combined analyses of both cohorts the association of these two SNPs remained statistically significant (rs331079 in *FBN2* OR, 1.37; 95% CI, 1.07 to 1.75; p=0.01; rs3783107 in *COL4A1* OR, 1.22; 95% CI, 1.05 to 1.42; p=0.007). The population attributable risk 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 associated in stage 1. Also, SNP rs3783107 in *COL4A1* is not in LD with the other SNPs in *COL4A1* that showed association in stage 1. For the SNPs in the *TGFBI*, *FN1* and *ELN* genes, the associations could not be confirmed. For the SNPs in *FN1* even association with the opposite alleles compared to the alleles associated in stage 1 was observed.

Discussion

By analyzing genes that are involved in the maintenance of the integrity of the ECM of the arterial wall and therefore candidate genes for intracranial aneurysms, SNPs in the perlecan (*HSPG2*), serpine1 (*PAI1*), fibrillin 2 (*FBN2*) and collagen type 4A1 (*COL4A1*) genes associated with intracranial aneurysms in the Dutch population, were identified.

This study reports a 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] great coverage of the analyzed candidate genes was achieved with inter-SNP distances of 5–10 kb, which is required to conclusively discount these genes having a role in intracranial aneurysms susceptibility.[26] To capture the maximum information of these SNPs as efficiently as possible, tag SNPs were selected on the basis of known patterns of LD. With this approach four genes proved associated with intracranial aneurysms: *HSPG2* with an OR of 1.33, *PAI1* with an OR of 1.27, *FBN2* with an OR of 1.37 and *COL4A1* with an OR of 1.22. Considering that 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 approximately 70% 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 important 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 sulfate 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] In intracranial aneurysms, fragmentation of the basement membrane components collagen type IV and fibronectin has been observed[31] and this may be caused by loss of the capacity of HSPG2 to interact with the other components. On the other hand, fragmentation of collagen type 4 in the basement membrane[31] may also (partly) 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.

PAI1 maps to 7q21.3-q22 which 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). PAI1 inhibits active metalloproteinases (MMPs), which are enzymes that degrade collagens 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 aneurysm patients the relation of *PAI1* to the occurrence of intracranial aneurysms has not been investigated yet, but it is possible that in these patients the expression and/or function of PAI1 may be diminished, 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 associ-

ated with higher levels of PAI1, was less common in AAA patients compared to controls.[34] Furthermore, PAI1 mRNA levels were lower in AAA tissue compared to 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 which location overlaps with a locus for intracranial aneurysms on chromosome 5q22-31.[14] *FBN2* is the disease-causing gene of congenital contractural arachnodactyly, a disease phenotypically similar to Marfan's syndrome and characterized by arachnodactyly, dolichostenomelia, scoliosis, multiple congenital contractures and abnormalities of the external ears.[37] Disruption of FBN2 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, four risk factors for intracranial aneurysms were identified with the SNP in *HSPG2* accounting for 19%, the one in *PAI1* for 6%, the one in *FBN2* for 3%, and the one in *COL4A1* for another 7% of the cases. Previously, we already investigated the population attributable risks of the at that time known risk factors for aneurysmal SAH, and showed that the modifiable risk factors account for most of the SAH cases, with heavy alcohol drinking 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 (ADPKD).[40] A recent systematic review identified more risk factors for aneurysmal SAH[41] and for these risk factors no population attributable risks have been calculated yet. Adding up the population attributable risks of all the risk factors identified thus far, will lead to a total of >100%, which further delineates 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 that the genes included in our screen were all based on prior evidence but with different prior probabilities: some were based on prior linkage and association reports, some on functional or gene expression reports and some on biological plausibility. To overcome the problem of multiple testing correction, consistent replication addressing the same variant and phenotype is now considered as the best test to correct for false positive results. In our study, the association of the SNP in *HSPG2* found in the first stage of our study, was repli-

cated in the second stage, and the associations of SNPs in *PAI1, FBN2* and *COL4A1* identified in the first stage remained significant on combining both stages suggesting 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, FBN2, COL4A1* and *ELN* genes could not be confirmed. For the SNPs in *FN1* even association with the opposite alleles compared to the alleles associated in the first stage was observed, which is strongly indicative that the observed association *FN1* with intracranial aneurysms is a false positive result. It may be hypothesized that the observed associations for these genes in stage 1 are attributed to the inclusion of patients with unruptured intracranial aneurysms in this stage, as in stage 2 only patients with ruptured ones were included. However, this hypothesis seems unlikely as combined analysis of patient cohort of stage 2 with the patients with unruptured intracranial aneurysms of stage 1 did not change our findings (data not shown).

The findings on the *ELN* gene are in contrast with a previous observation, where 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 are part of the patients included in the first stage of the present study and for both the entire patients population of the first stage and the subset of the 167 patients association of *ELN* variants with intracranial aneurysms was found. As these observations could not be validated in the second stage of the present study, our previous results[25] and the results of the first stage of the present study are likely to be false positive.

In conclusion, the identification of *HSPG2, PAI1, FBN2* and *COL4A1* as susceptibility genes in intracranial aneurysms, is an intriguing finding that needs to be further evaluated 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 identified SNPs in these genes on the function and expression levels of HSPG2, PAI1, 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 are associated with aneurysm formation in general. These will be the foci of future studies.

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Supplementary Table A. *Description of the selected 44 candidate genes involved in the maintenance of the integrity of the extracellular matrix.*

	Gene	Locus	Evidence from linkage studies	Evidence from gene expression studies	Evidence from functional analyses	Evidence from association studies	Evidence from heritable disorders associated with intracranial aneurysms
1	Elastin	*ELN* 7q11	(1,2)	(3)	(4,5)	(1,6)	
2	Collagen 3A1	COL3A1 2q31		(3,7)	(8-10)		Ehlers Danlos type IV (11)
3	Collagen 1A1	COL1A1 17q21.31-q22	(1,12)	(3)	(13)		
4	Collagen 1A2	*COL1A2* 7q22.1	(1)	(3)	(13)	(14)	
5	Fibrillin 2	*FBN2* 5q23-31	(1)				
6	A1 antitrypsine	*PI/SERPINA1* 14q32.1	(1)			(15,16)	
7	Serpina 3	AACT/SERPINA3 14q32.1	(1)			(17)	
8	Lysyl oxidase	LOX 5q23.3q31.2	(1)				
9	Metalloproteinase 9	*MMP9* 20q11.2q31.2			(18)	(19)	
10	Tissue inhibitor of metalloproteinase 3	*TIMP3* 22q12.1-q13.2		(3)			
11	Transforming growth factor, beta induced	*TGFBI* 5q31	(1)	(3)			
12	Collagen 4A1	*COL4A1* 13q34		(3)	(10,20)		
13	Fibronectin	*FN1* 2q34		(3)	(10,12,20)		
14	Metalloproteinase 14	*MMP14* 14q11	(1)		(21)		
15	Secreted protein,	*SPARC* 5q31.3-q32	(1)	(3)			
16	Microfibril-associated protein 4	*MFAP4* 17cen	(1,12)				
17	Collagen 16 A1	*COL16A1* 1p34.3-p36.13	(22)				

(continued on page 129)

18	Metalloproteinase 2	*MMP2* *16q13*			(23,24)	
19	Elastase	*ELA2* 19p13.3			(25,26)	
20	Plasminogen	*PLG* 6q26			(21)	
21	Connective tissue growth factor	*CTGF* 6q23.1		(3)		
22	Osteoblast specific factor 2	*OSF2* 13q13.3		(3)		
23	Cathepsin B	*CTSB* 8p22		(3)		
24	Cathepsin D	CTSD 11p15.5		(3)		
25	Collagen 6A2	*COL6A2* 21q22.3		(3)		
26	Fibrillin 1	*FBN1* 15q21.1				Marfan (11)
27	Fibulin 5	*FBLN5* 14q22	(1)			
28	Tissue inhibitor of metalloproteinase 2	*TIMP2* 4q22	(1)			
29	Tissue inhibitor of metalloproteinase 1	*TIMP1* Xp22	(12,27)			
30	Microfibril-associated protein 3	*MFAP3* 5q22	(1)			
31	Procollagen-lysine, 2-oxoglutarate 5-dioxygenase 3	*PLOD3* 7q11	(1,2)			
32	Plasminogen activator inhibitor 1/serpine 1	*SERPINE1* */PAI1* 7q11	(1,2)			
33	Fibromodulin	*FMOD* 1p34	(22)			
34	Collagen 8A2	*COL8A2* 1p34	(22)			
35	Perlecan	*HSPG2/* *PLC* 1p36.1	(22)			
36	Microfibril-associated protein 2	*MFAP2* 1p36.1-p35	(22)			
37	Microfibril-associated protein 1	*MFAP1* 15q15-q12				

(continued on page 130)

38	Emilin 1	*EMILIN1* 2p23
39	Emilin 2	*EMILIN2* 18p11.3
40	Cystatin C	*CTSC* 20p11.2
41	Cathepsin S	*CTSS* 1q21
42	Cathepsin K	*CTSK* 1q21
43	Collagen 5A1	*COL5A1* 9q34.2-q34.3
44	Collagen 5A2	*COL5A2* 2q31

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Supplementary Table B. *The association data of the 378 tag SNPs that passed through the quality check.*

Rs number	Major allele	Gene	HWE cases	HWE controls	Case-control frequency
rs761423	T, T	*MFAP2*	1	0,67	0.538, 0.523
rs2284746	T, T	*MFAP2*	1	0,634	0.537, 0.554
rs3754511	T, T	*MFAP2*	0,206	0,786	0.819, 0.836
rs2270701	G, G	*HSPG2*	1	0,738	0.822, 0.803
rs3767137	C, C	*HSPG2*	0,319	0,464	0.772, 0.713
rs7556412	A, A	*HSPG2*	0,267	0,907	0.665, 0.610
rs2290501	A, A	*HSPG2*	0,484	0,872	0.691, 0.657
rs3767138	G, G	*HSPG2*	0,057	0,242	0.812, 0.792
rs2305562	G, G	*HSPG2*	0,822	0,15	0.523, 0.509
rs4654991	A, A	*HSPG2*	0,732	0,409	0.806, 0.812
rs2254358	T, T	*HSPG2*	0,573	1	0.663, 0.639
rs6698486	C, C	*HSPG2*	0,947	0,843	0.731, 0.705
rs9426785	A, A	*HSPG2*	1	0,221	0.617, 0.590
rs4654773	G, G	*HSPG2*	0,022	0,764	0.839, 0.852
rs7545321	G, G	*COL16A1*	0,729	0,954	0.587, 0.628
rs2271928	C, C	*COL16A1*	0,52	0,81	0.556, 0.583
rs1474182	T, T	*COL16A1*	0,785	0,867	0.530, 0.573
rs909002	T, T	*COL16A1*	0,933	0,667	0.525, 0.565
rs4949459	C, A	*COL16A1*	0,373	0,889	0.533, 0.508
rs2297684	A, A	*COL16A1*	0,179	0,254	0.547, 0.577
rs2297682	G, G	*COL16A1*	0,944	0,579	0.554, 0.561
rs2297680	G, G	*COL16A1*	0,752	0,157	0.868, 0.877
rs3818788	G, G	*COL16A1*	0,877	0,529	0.652, 0.655
rs10493049	T, T	*COL16A1*	0,827	1	0.860, 0.863
rs4295917	A, A	*COL16A1*	0,234	0,302	0.666, 0.676
rs7550047	T, T	*COL8A2*	1	0,307	0.915, 0.911
rs7553155	T, T	*COL8A2*	0,225	0,257	0.885, 0.898
rs10888390	G, G	*CTSS*	0,58	0,831	0.677, 0.647
rs12085336	A, A	*CTSK*	0,39	0,708	0.672, 0.639
rs3820224	G, G	*FMOD*	1	1	0.880, 0.881
rs10800913	T, T	*FMOD*	0,124	0,709	0.876, 0.878
rs2011616	C, C	*EMILIN1*	0,752	0,215	0.636, 0.644
rs2138533	C, C	*COL3A1*	0,905	0,862	0.632, 0.607
rs1516454	T, T	*COL3A1*	0,126	0,443	0.863, 0.857
rs1914037	T, T	*COL3A1*	0,755	0,548	0.745, 0.736
rs2203601	T, T	*COL3A1*	0,746	0,047	0.555, 0.554
rs2271682	T, T	*COL3A1*	0,863	0,61	0.699, 0.709
rs6715268	T, T	*COL3A1*	0,322	0,573	0.760, 0.744
rs6434318	A, A	*COL5A2*	0,681	0,453	0.820, 0.806
rs7425297	C, C	*COL5A2*	0,481	0,654	0.880, 0.855
rs7561002	T, T	*COL5A2*	0,485	0,885	0.670, 0.641
rs4417749	T, T	*COL5A2*	0,669	0,903	0.924, 0.904
rs1263	G, G	*FN1*	0,365	0,604	0.638, 0.658
rs3817500	T, T	*FN1*	0,203	0,044	0.636, 0.651
rs10498037	C, C	*FN1*	0,626	1	0.920, 0.924
rs2289200	C, C	*FN1*	1	0,658	0.717, 0.736
rs2304573	A, A	*FN1*	0,227	0,202	0.767, 0.798

Case-control ratio	Chi-square	p-value
392:336, 608:554	0,416	0,5187
389:335, 644:518	0,516	0,4727
596:132, 972:190	1,004	0,3163
597:129, 925:227	1,087	0,2972
562:166, 828:334	8,121	0,0044
484:244, 709:453	5,749	0,0165
503:225, 763:399	2,382	0,1227
591:137, 920:242	1,125	0,2888
381:347, 591:571	0,39	0,5325
587:141, 943:219	0,079	0,7788
483:245, 740:418	1,17	0,2794
532:196, 819:343	1,479	0,224
449:279, 686:476	1,3	0,2542
611:117, 990:172	0,557	0,4556
427:301, 730:432	3,276	0,0703
405:323, 677:485	1,265	0,2608
386:342, 665:495	3,36	0,0668
382:346, 656:506	2,866	0,0904
388:340, 590:572	2,968	0,0849
396:328, 669:491	1,607	0,2049
402:324, 652:510	0,099	0,7533
632:96, 1019:143	0,314	0,5752
475:253, 761:401	0,012	0,9138
626:102, 1003:159	0,04	0,8407
485:243, 785:377	0,178	0,6735
666:62, 1059:103	0,068	0,7945
644:84, 1044:118	0,898	0,3434
493:235, 750:410	1,868	0,1717
489:239, 743:419	2,056	0,1516
641:87, 1024:138	0,002	0,9612
638:90, 1020:142	0,008	0,9269
463:265, 748:414	0,116	0,7333
459:267, 704:456	1,212	0,2709
628:100, 996:166	0,112	0,7382
542:186, 852:306	0,178	0,6734
404:324, 644:518	0,001	0,9753
509:219, 824:338	0,213	0,6444
553:175, 864:298	0,616	0,4325
597:131, 937:225	0,548	0,459
641:87, 993:169	2,571	0,1089
488:240, 745:417	1,682	0,1946
673:55, 1050:112	2,412	0,1204
463:263, 765:397	0,834	0,361
462:264, 757:405	0,445	0,5046
670:58, 1074:88	0,097	0,755
522:206, 855:307	0,797	0,3719
557:169, 926:234	2,564	0,1094

rs13652	A, A	*FN1*	0,471	0,802	0.853, 0.581
rs1250215	C, C	*FN1*	0,945	0,724	0.621, 0.646
rs10498038	G, G	*FN1*	1	0,062	0.927, 0.943
rs1968510	G, G	*FN1*	1	0,877	0.891, 0.898
rs2372544	C, C	*FN1*	1	0,089	0.582, 0.604
rs724617	G, G	*FN1*	0,537	0,158	0.544, 0.559
rs1250250	C, C	*FN1*	0,223	0,011	0.780, 0.803
rs2289202	C, C	*FN1*	0,526	0,602	0.786, 0.731
rs1250229	C, C	*FN1*	0,401	0,98	0.698, 0.726
rs1250233	C, C	*FN1*	0,321	0,086	0.782, 0.786
rs1250234	T, T	*FN1*	0,684	0,859	0.798, 0.819
rs6753702	A, A	*FN1*	0,9	0,275	0.702, 0.724
rs1250217	C, T	*FN1*	0,25	0,538	0.516, 0.512
rs1250223	G, G	*FN1*	0,352	0,873	0.849, 0.867
rs10498039	C, C	*FN1*	0,747	0,637	0.702, 0.716
rs1250225	G, G	*FN1*	0,274	0,356	0.761, 0.779
rs1561299	C, C	*FN1*	0,268	0,788	0.630, 0.581
rs1437790	A, A	*FN1*	0,198	0,327	0.841, 0.841
rs2043776	A, A	*FN1*	0,608	0,72	0.797, 0.750
rs4673999	A, A	*FN1*	0,483	0,784	0.567, 0.520
rs10040971	A, A	*LOX*	0,024	0,211	0.868, 0.852
rs840464	A, C	*LOX*	0,215	0,83	0.537, 0.512
rs3853401	C, C	*LOX*	0,71	0,828	0.707, 0.675
rs7288	G, G	*FBN2*	1	1	0.870, 0.857
rs1366455	A, A	*FBN2*	0,502	0,53	0.713, 0.693
rs3805620	A, A	*FBN2*	0,786	0,46	0.926, 0.915
rs2291628	C, C	*FBN2*	1	0,823	0.935, 0.916
rs190450	C, C	*FBN2*	0,261	0,815	0.694, 0.701
rs39937	T, T	*FBN2*	1	0,34	0.937, 0.938
rs27855	C, C	*FBN2*	0,736	0,762	0.593, 0.579
rs255725	A, A	*FBN2*	0,698	0,992	0.829, 0.803
rs32215	C, C	*FBN2*	0,328	0,081	0.696, 0.674
rs467610	C, C	*FBN2*	0,1	0,326	0.764, 0.766
rs32223	C, C	*FBN2*	1	0,042	0.766, 0.777
rs28114	C, T	*FBN2*	0,842	0,061	0.534, 0.516
rs154001	T, T	*FBN2*	0,806	0,976	0.679, 0.671
rs27754	G, G	*FBN2*	1	0,292	0.643, 0.680
rs26024	T, T	*FBN2*	0,686	0,15	0.595, 0.637
rs10519991	T, T	*FBN2*	0,045	1	0.902, 0.878
rs552850	A, A	*FBN2*	1	0,686	0.764, 0.739
rs432792	A, A	*FBN2*	0,522	0,673	0.860, 0.849
rs331097	A, A	*FBN2*	0,296	0,845	0.777, 0.755
rs331095	A, A	*FBN2*	0,25	0,37	0.610, 0.608
rs331076	G, G	*FBN2*	0,008	0,386	0.640, 0.596
rs331069	C, T	*FBN2*	0,861	0,193	0.544, 0.519
rs4492151	A, A	*FBN2*	1	0,891	0.893, 0.903
rs331068	T, T	*FBN2*	0,541	0,644	0.552, 0.564
rs331086	G, G	*FBN2*	0,721	0,678	0.779, 0.783
rs3805652	T, T	*FBN2*	1	1	0.893, 0.862
rs331083	C, C	*FBN2*	0,442	0,871	0.565, 0.576
rs331079	C, C	*FBN2*	0,562	0,196	0.886, 0.920
rs1561004	G, G	*FBN2*	0,659	0,518	0.764, 0.749
rs4836373	A, G	*FBN2*	0,901	0,747	0.510, 0.528
rs6595827	A, A	*FBN2*	0,937	0,583	0.695, 0.662

619:107, 989:173	0,008	0,929
452:276, 751:411	1,25	0,2635
675:53, 1096:66	1,943	0,1633
649:79, 1044:118	0,233	0,6295
424:304, 701:459	0,89	0,3454
396:332, 649:513	0,384	0,5355
568:160, 933:229	1,412	0,2348
569:155, 849:313	7,305	0,0069
508:220, 844:318	1,789	0,181
569:159, 910:248	0,048	0,8273
579:147, 950:210	1,338	0,2474
510:216, 841:321	0,993	0,3189
376:352, 595:567	1,457	0,2273
618:110, 1008:154	1,284	0,2571
511:217, 830:330	0,402	0,5262
554:174, 905:257	0,809	0,3683
459:269, 675:487	4,588	0,0322
612:116, 977:185	0	0,9939
580:148, 870:290	5,476	0,0193
412:314, 604:558	4,09	0,0431
630:96, 990:172	0,915	0,3389
390:336, 595:567	4,334	0,0374
515:213, 784:378	2,229	0,1354
633:95, 996:166	0,575	0,4484
519:209, 805:357	0,865	0,3522
672:54, 1063:99	0,702	0,4021
681:47, 1061:97	2,338	0,1263
505:223, 815:347	0,126	0,7228
682:46, 1090:72	0,011	0,9147
432:296, 671:487	0,359	0,5492
602:124, 931:229	2,079	0,1493
507:221, 783:379	1,054	0,3046
555:171, 890:272	0,005	0,942
558:170, 903:259	0,288	0,5915
388:338, 600:562	4,61	0,0318
494:234, 780:382	0,109	0,7413
467:259, 788:370	2,782	0,0954
433:295, 740:422	3,362	0,0667
657:71, 1020:142	2,725	0,0988
556:172, 859:303	1,427	0,2322
626:102, 986:176	0,46	0,4977
566:162, 876:284	1,233	0,2669
444:284, 706:456	0,01	0,92
466:262, 693:469	3,608	0,0575
395:331, 603:559	7,097	0,0077
645:77, 1037:111	0,486	0,4857
401:325, 654:506	0,238	0,626
567:161, 910:252	0,048	0,8263
650:78, 1002:160	3,795	0,0514
411:317, 669:493	0,228	0,633
645:83, 1069:93	6,118	0,0134
555:171, 870:292	0,599	0,4389
369:355, 613:549	2,472	0,1159
492:216, 763:389	2,122	0,1452

rs6882394	A, A	*FBN2*	0,981	0,557	0.695, 0.662
rs1004965	T, T	*FBN2*	0,08	0,37	0.640, 0.627
rs6595829	G, G	*FBN2*	1	0,834	0.673, 0.642
rs10520002	C, C	*FBN2*	0,658	0,895	0.904, 0.873
rs4836375	G, G	*FBN2*	0,029	0,167	0.657, 0.625
rs764371	T, T	*FBN2*	0,532	0,793	0.901, 0.888
rs1435514	C, C	*FBN2*	0,319	0,5	0.587, 0.548
rs6595837	C, C	*FBN2*	0,763	0,726	0.949, 0.939
rs6865322	G, G	*FBN2*	0,779	0,561	0.691, 0.659
rs6891153	A, A	*FBN2*	0,358	1	0.909, 0.906
rs756463	G, G	*TGFBI*	0,319	0,476	0.772, 0.709
rs756462	T, T	*TGFBI*	0,722	1	0.821, 0.793
rs2282791	T, T	*TGFBI*	0,394	0,229	0.550, 0.546
rs2072239	C, C	*TGFBI*	0,069	0,475	0.749, 0.778
rs2302038	A, A	*TGFBI*	0,483	0,145	0.511, 0.526
rs1054204	C, C	*SPARC*	0,717	0,647	0.560, 0.578
rs2288810	A, A	*SPARC*	1	1	0.934, 0.932
rs3756631	A, A	*SPARC*	0,914	0,233	0.816, 0.851
rs725937	G, G	*SPARC*	0,566	0,906	0.640, 0.648
rs4958486	C, C	*SPARC*	1	1	0.963, 0.957
rs4958487	A, A	*SPARC*	0,121	0,188	0.562, 0.559
rs1432860	G, G	*SPARC*	0,393	0,705	0.694, 0.699
rs816036	G, G	*MFAP3*	0,541	0,527	0.610, 0.606
rs1370912	A, A	*MFAP3*	1	0,095	0.886, 0.887
rs4958692	G, G	*MFAP3*	0,177	0,703	0.834, 0.858
rs928501	C, C	*CTGF*	0,636	0,569	0.723, 0.740
rs10945685	T, T	*PLG*	0,774	0,873	0.786, 0.781
rs1950562	G, G	*PLG*	0,449	0,395	0.566, 0.558
rs4252072	T, T	*PLG*	0,847	0,531	0.695, 0.707
rs4252092	G, G	*PLG*	0,329	0,887	0.647, 0.603
rs813641	C, C	*PLG*	0,034	0,423	0.816, 0.838
rs3757017	G, G	*PLG*	0,206	0,726	0.622, 0.654
rs868005	A, A	*ELN*	1	0,188	0.565, 0.624
rs4717865	G, G	*ELN*	0,017	1	0.926, 0.920
rs3763468	G, G	*COL1A2*	0,971	0,954	0.864, 0.877
rs388625	G, G	*COL1A2*	0,798	1	0.573, 0.594
rs2299418	G, G	*COL1A2*	0,554	0,71	0.805, 0.788
rs1858822	A, A	*COL1A2*	0,204	0,736	0.625, 0.609
rs389328	A, A	*COL1A2*	0,177	0,803	0.875, 0.867
rs42524	C, C	*COL1A2*	0,393	0,748	0.791, 0.795
rs10487254	C, C	*COL1A2*	0,895	0,925	0.802, 0.801
rs2521205	C, C	*COL1A2*	0,055	0,263	0.501, 0.514
rs369982	C, T	*COL1A2*	0,956	1	0.519, 0.502
rs42530	T, T	*COL1A2*	0,121	0,72	0.664, 0.643
rs441051	C, C	*COL1A2*	0,745	0,975	0.799, 0.802
rs400218	C, C	*COL1A2*	0,886	0,521	0.659, 0.683
rs6465412	T, T	*COL1A2*	0,044	1	0.728, 0.750
rs1062394	C, C	*COL1A2*	0,781	0,043	0.908, 0.922
rs6956010	G, G	*PAI1*	0,307	0,248	0.725, 0.793
rs2070682	A, A	*PAI1*	0,414	0,355	0.585, 0.538
rs2227714	G, G	*PAI1*	0,722	0,671	0.948, 0.954
rs7777930	G, G	*PLOD3*	0,448	0,411	0.838, 0.855
rs10487887	G, G	*PLOD3*	0,896	1	0.935, 0.951
rs1736081	A, A	*CTSB*	0,042	1	0.742, 0.752

503:221, 769:393	2,207	0,1374
465:261, 726:432	0,352	0,5527
490:238, 746:416	1,911	0,1669
658:70, 1015:147	4,057	0,044
478:250, 726:436	1,959	0,1617
656:72, 1032:130	0,789	0,3743
427:301, 637:525	2,675	0,1019
691:37, 1091:71	0,877	0,3489
503:225, 766:396	2,042	0,153
662:66, 1053:109	0,053	0,8185
562:166, 824:338	9,043	0,0026
596:130, 921:241	2,273	0,1317
399:327, 628:522	0,022	0,8821
545:183, 904:258	2,154	0,1422
369:353, 608:548	0,394	0,5303
408:320, 672:490	0,584	0,4448
680:48, 1083:79	0,03	0,8623
594:134, 987:173	4,007	0,0453
466:262, 753:409	0,122	0,7265
701:27, 1112:50	0,404	0,5249
409:319, 650:512	0,011	0,9174
505:223, 812:350	0,055	0,8139
444:284, 704:458	0,031	0,8611
645:83, 1031:131	0,007	0,9322
607:121, 993:165	1,955	0,162
525:201, 854:300	0,651	0,4199
572:156, 907:255	0,07	0,7912
410:314, 648:514	0,135	0,7131
506:222, 822:340	0,327	0,5677
471:257, 701:461	3,63	0,0568
594:134, 974:188	1,571	0,21
453:275, 760:402	1,968	0,1607
411:317, 725:437	6,578	0,0103
674:54, 1069:93	0,214	0,6435
629:99, 1019:143	0,67	0,4131
417:311, 690:472	0,814	0,3671
586:142, 912:246	0,826	0,3633
454:272, 708:454	0,486	0,4856
637:91, 1007:155	0,278	0,5978
574:152, 924:238	0,056	0,8123
584:144, 929:231	0,005	0,9436
365:363, 597:565	0,275	0,5999
377:349, 583:579	0,789	0,3745
482:244, 745:413	0,831	0,3621
582:146, 932:230	0,019	0,8898
480:248, 794:368	1,17	0,2794
530:198, 869:289	1,172	0,279
659:67, 1069:91	1,114	0,2912
528:200, 921:241	11,341	0,0008
425:301, 625:537	4,09	0,0431
690:38, 1109:53	0,424	0,5151
610:118, 994:168	1,069	0,3013
681:47, 1105:57	2,07	0,1502
539:187, 874:288	0,225	0,6356

rs1736090	G, G	*CTSB*	0,763	1	0.633, 0.647
rs1293291	G, G	*CTSB*	1	0,989	0.665, 0.669
rs6980952	C, C	*CTSB*	0,056	0,337	0.629, 0.613
rs1293288	C, C	*CTSB*	0,61	0,188	0.565, 0.573
rs1692804	T, T	*CTSB*	0,9	0,905	0.625, 0.628
rs4363274	A, A	*COL5A1*	0,225	1	0.625, 0.638
rs4304399	A, A	*COL5A1*	1	0,78	0.799, 0.807
rs12002679	G, G	*COL5A1*	0,015	0,644	0.722, 0.703
rs10858265	T, T	*COL5A1*	0,496	0,583	0.657, 0.616
rs10046876	G, G	*COL5A1*	0,347	0,568	0.743, 0.741
rs12000599	C, C	*COL5A1*	1	0,637	0.860, 0.849
rs4842139	G, G	*COL5A1*	0,775	0,874	0.882, 0.888
rs12554098	C, C	*COL5A1*	0,966	0,573	0.876, 0.868
rs4842142	G, G	*COL5A1*	0,647	0,587	0.760, 0.757
rs4077962	C, C	*COL5A1*	0,574	0,987	0.900, 0.893
rs10858270	A, A	*COL5A1*	0,922	0,157	0.863, 0.877
rs4596720	T, T	*COL5A1*	0,026	0,235	0.540, 0.529
rs4841924	T, T	*COL5A1*	1	0,954	0.949, 0.948
rs4401947	G, G	*COL5A1*	0,29	0,525	0.745, 0.732
rs4341231	C, C	*COL5A1*	0,733	0,864	0.607, 0.548
rs3124291	G, G	*COL5A1*	0,656	0,23	0.525, 0.541
rs3109671	T, T	*COL5A1*	0,766	0,073	0.511, 0.511
rs4240703	C, C	*COL5A1*	0,605	0,613	0.843, 0.861
rs3922914	T, T	*COL5A1*	0,174	0,824	0.824, 0.845
rs4548258	C, C	*COL5A1*	0,919	0,498	0.644, 0.625
rs3128591	A, A	*COL5A1*	0,193	0,622	0.730, 0.762
rs11103479	G, G	*COL5A1*	0,407	0,756	0.699, 0.731
rs7044312	C, C	*COL5A1*	0,321	0,155	0.600, 0.589
rs7875140	A, A	*COL5A1*	0,808	0,48	0.751, 0.763
rs3128597	C, C	*COL5A1*	0,547	0,472	0.690, 0.674
rs3128606	T, T	*COL5A1*	0,838	0,62	0.576, 0.558
rs3109682	A, A	*COL5A1*	0,918	0,384	0.723, 0.725
rs3109676	G, G	*COL5A1*	0,908	0,453	0.712, 0.716
rs3128616	G, G	*COL5A1*	0,455	0,132	0.611, 0.620
rs4842152	A, A	*COL5A1*	0,056	0,83	0.702, 0.694
rs4842153	A, A	*COL5A1*	0,027	0,626	0.753, 0.753
rs7849193	T, T	*COL5A1*	0,281	0,652	0.905, 0.902
rs11103509	A, A	*COL5A1*	0,848	0,603	0.952, 0.956
rs4842158	G, G	*COL5A1*	0,017	0,663	0.797, 0.800
rs4842161	T, T	*COL5A1*	0,143	0,332	0.544, 0.531
rs10745384	T, T	*COL5A1*	0,375	0,767	0.521, 0.534
rs4842164	A, A	*COL5A1*	0,138	1	0.644, 0.641
rs4563961	T, T	*COL5A1*	0,703	0,734	0.672, 0.676
rs3124932	G, G	*COL5A1*	0,004	0,539	0.558, 0.534
rs6537949	T, T	*COL5A1*	0,14	0,556	0.864, 0.874
rs3124934	G, G	*COL5A1*	1	0,368	0.940, 0.937
rs3128621	C, C	*COL5A1*	0,042	0,884	0.561, 0.529
rs3811162	G, G	*COL5A1*	0,288	0,764	0.618, 0.584
rs3811159	A, A	*COL5A1*	0,194	0,954	0.536, 0.530
rs3811157	C, C	*COL5A1*	0,173	0,544	0.519, 0.507
rs4842168	C, C	*COL5A1*	0,854	1	0.526, 0.531
rs3827851	C, C	*COL5A1*	0,753	0,616	0.587, 0.549
rs7851471	C, C	*COL5A1*	0,839	0,987	0.638, 0.624
rs4531123	G, G	*COL5A1*	0,32	0,329	0.909, 0.926

461:267, 750:410	0,345	0,5572
484:244, 776:384	0,034	0,8529
458:270, 712:450	0,509	0,4754
411:317, 666:496	0,135	0,7136
455:273, 730:432	0,02	0,8877
455:273, 741:421	0,31	0,5775
582:146, 938:224	0,172	0,6783
517:199, 815:345	0,816	0,3663
477:249, 713:445	3,273	0,0704
541:187, 861:301	0,011	0,9165
626:102, 987:175	0,394	0,5302
642:86, 1032:130	0,173	0,6774
638:90, 1009:153	0,258	0,6112
553:175, 880:282	0,013	0,9095
655:73, 1038:124	0,199	0,6558
628:100, 1019:143	0,817	0,3661
393:335, 615:547	0,201	0,6538
689:37, 1101:61	0,021	0,884
542:186, 851:311	0,341	0,5594
442:286, 637:525	6,349	0,0117
376:340, 627:533	0,421	0,5165
372:356, 594:568	0	0,9933
614:114, 999:161	1,139	0,2859
600:128, 982:180	1,436	0,2308
469:259, 726:436	0,728	0,3935
530:196, 886:276	2,51	0,1132
509:219, 849:313	2,19	0,1389
437:291, 684:478	0,251	0,6163
547:181, 887:275	0,35	0,5541
502:226, 783:379	0,508	0,4759
419:309, 648:514	0,583	0,4452
526:202, 843:319	0,019	0,8891
517:209, 830:330	0,025	0,8738
445:283, 720:442	0,132	0,7161
510:216, 806:356	0,166	0,684
548:180, 875:287	0	0,9896
659:69, 1048:114	0,057	0,8119
693:35, 1111:51	0,181	0,6708
563:143, 922:230	0,023	0,8797
396:332, 617:545	0,303	0,582
378:348, 620:542	0,298	0,5848
469:259, 745:417	0,019	0,8913
489:239, 786:376	0,045	0,8313
406:322, 621:541	0,977	0,323
629:99, 1016:146	0,424	0,5147
684:44, 1087:73	0,048	0,827
407:319, 615:547	1,768	0,1836
449:277, 679:483	2,163	0,1414
390:338, 616:546	0,056	0,8125
378:350, 589:573	0,273	0,6013
383:345, 617:545	0,043	0,8361
420:296, 633:521	2,602	0,1067
454:258, 711:429	0,366	0,5453
660:66, 1071:85	1,825	0,1767

rs3811153	T, T	*COL5A1*	1	0,844	0.549, 0.552
rs3811152	G, G	*COL5A1*	0,154	0,472	0.886, 0.897
rs4841933	C, C	*COL5A1*	0,292	0,365	0.610, 0.590
rs3827848	C, C	*COL5A1*	0,76	0,933	0.815, 0.832
rs3811146	G, G	*COL5A1*	0,441	0,037	0.565, 0.576
rs4842172	T, T	*COL5A1*	0,48	0,357	0.674, 0.651
rs4842174	C, C	*COL5A1*	0,77	0,398	0.831, 0.830
rs7864699	A, A	*COL5A1*	1	0,242	0.904, 0.914
rs9410002	T, T	*COL5A1*	0,043	0,681	0.573, 0.606
rs13946	T, T	*COL5A1*	0,476	0,766	0.728, 0.769
rs2292963	C, C	*CTSD*	0,947	0,835	0.801, 0.763
rs2292962	G, G	*CTSD*	0,337	0,111	0.867, 0.848
rs17571	G, G	*CTSD*	0,568	0,812	0.942, 0.928
rs9547952	C, C	*OSF2*	0,189	0,445	0.944, 0.960
rs9603226	C, C	*OSF2*	0,639	1	0.886, 0.895
rs4512969	A, A	*OSF2*	0,87	0,397	0.879, 0.861
rs7323378	T, T	*OSF2*	0,354	0,018	0.528, 0.546
rs3794374	C, C	*OSF2*	0,703	0,403	0.672, 0.660
rs7338244	C, C	*OSF2*	0,271	0,036	0.802, 0.796
rs1028728	T, T	*OSF2*	0,753	0,013	0.709, 0.723
rs13260	C, C	*COL4A1*	0,217	0,269	0.901, 0.898
rs1192198	T, T	*COL4A1*	0,268	0,669	0.809, 0.838
rs1133219	G, G	*COL4A1*	0,422	0,009	0.640, 0.656
rs12017058	T, C	*COL4A1*	0,454	0,633	0.555, 0.501
rs10492497	T, T	*COL4A1*	1	1	0.926, 0.938
rs3783107	C, C	*COL4A1*	0,343	0,674	0.591, 0.651
rs1373749	A, A	*COL4A1*	0,606	0,991	0.696, 0.717
rs496916	C, C	*COL4A1*	0,3	0,359	0.668, 0.625
rs648705	C, C	*COL4A1*	0,588	0,33	0.636, 0.650
rs529041	G, G	*COL4A1*	1	0,447	0.923, 0.931
rs626444	G, G	*COL4A1*	0,883	0,201	0.534, 0.555
rs675605	G, G	*COL4A1*	0,851	0,444	0.728, 0.685
rs630943	G, G	*COL4A1*	1	0,511	0.753, 0.706
rs680484	G, G	*COL4A1*	0,85	0,855	0.602, 0.611
rs913745	A, A	*COL4A1*	0,965	0,015	0.787, 0.801
rs521969	G, G	*COL4A1*	0,185	0,721	0.922, 0.908
rs7320618	A, A	*COL4A1*	0,76	0,885	0.815, 0.818
rs4145072	T, T	*COL4A1*	0,513	0,589	0.504, 0.515
rs562992	T, T	*COL4A1*	1	0,9	0.845, 0.833
rs627527	T, T	*COL4A1*	1	0,817	0.569, 0.551
rs492560	T, T	*COL4A1*	0,175	0,181	0.676, 0.650
rs4773139	C, C	*COL4A1*	1	1	0.777, 0.793
rs561437	G, G	*COL4A1*	0,441	0,755	0.504, 0.502
rs552125	G, G	*COL4A1*	0,621	1	0.864, 0.877
rs1411040	A, A	*COL4A1*	1	0,864	0.842, 0.861
rs7983081	A, A	*COL4A1*	0,974	1	0.828, 0.857
rs7317784	T, T	*COL4A1*	0,483	0,957	0.704, 0.673
rs2391824	C, C	*COL4A1*	0,907	0,369	0.600, 0.655
rs2269213	G, G	*MMP14*	0,796	0,466	0.893, 0.906
rs2236302	C, C	*MMP14*	1	0,001	0.905, 0.912
rs743257	G, G	*MMP14*	0,942	0,63	0.525, 0.520
rs929608	G, A	*FBLN5*	0,466	0,426	0.516, 0.544
rs10484030	G, G	*FBLN5*	0,422	0,444	0.746, 0.728
rs2244158	G, G	*FBLN5*	0,11	0,569	0.891, 0.891

400:328, 641:521	0,009	0,926
645:83, 1042:120	0,539	0,463
444:284, 685:477	0,774	0,3791
593:135, 967:195	0,965	0,326
407:313, 668:492	0,203	0,6521
491:237, 757:405	1,054	0,3045
603:123, 963:197	0,001	0,9818
653:69, 1062:100	0,493	0,4825
416:310, 704:458	1,998	0,1575
530:198, 894:268	4,118	0,0424
583:145, 887:275	3,639	0,0564
631:97, 984:176	1,235	0,2664
684:42, 1078:84	1,495	0,2214
687:41, 1116:46	2,853	0,0912
645:83, 1040:122	0,377	0,5395
640:88, 1001:161	1,222	0,2689
383:343, 633:527	0,591	0,4419
489:239, 767:395	0,272	0,6021
584:144, 923:237	0,118	0,7316
516:212, 839:321	0,463	0,4961
656:72, 1043:119	0,061	0,8055
587:139, 972:188	2,691	0,1009
466:262, 761:399	0,499	0,4802
404:324, 582:580	5,585	0,0181
674:54, 1090:72	1,073	0,3003
430:298, 756:406	6,88	0,0087
507:221, 833:329	0,906	0,3411
486:242, 726:436	3,564	0,0591
463:265, 755:407	0,369	0,5433
670:56, 1082:80	0,459	0,498
389:339, 645:517	0,777	0,3781
530:198, 796:366	3,952	0,0468
548:180, 820:342	4,96	0,0259
438:290, 709:451	0,171	0,6789
573:155, 931:231	0,549	0,4588
671:57, 1055:107	1,073	0,3002
593:135, 951:211	0,045	0,8329
367:361, 598:564	0,198	0,6565
615:113, 966:194	0,475	0,4908
414:314, 640:522	0,582	0,4456
492:236, 755:407	1,356	0,2441
566:162, 922:240	0,683	0,4085
357:351, 577:573	0,011	0,9167
629:99, 1019:143	0,67	0,4131
613:115, 1001:161	1,353	0,2448
603:125, 996:166	2,859	0,0909
511:215, 781:379	1,935	0,1642
437:291, 759:399	5,863	0,0155
650:78, 1053:109	0,893	0,3446
659:69, 1060:102	0,267	0,6057
381:345, 603:557	0,044	0,8336
376:352, 632:530	6,537	0,0106
543:185, 846:316	0,73	0,3929
649:79, 1033:127	0,004	0,9477

rs2017488	T, T	*FBLN5*	0,296	0,999	0.777, 0.812
rs741198	G, G	*FBLN5*	0,469	1	0.938, 0.940
rs1861085	G, G	*FBLN5*	0,681	0,97	0.820, 0.806
rs726063	G, G	*FBLN5*	1	0,678	0.913, 0.902
rs2160079	C, C	*FBLN5*	0,702	1	0.923, 0.928
rs2160080	A, A	*FBLN5*	0,53	1	0.761, 0.787
rs12589592	G, G	*FBLN5*	1	1	0.641, 0.649
rs2284340	G, G	*FBLN5*	0,883	0,282	0.534, 0.547
rs2246416	A, A	*FBLN5*	0,837	0,784	0.716, 0.694
rs2268002	C, C	*FBLN5*	0,974	0,507	0.828, 0.819
rs2474028	G, G	*FBLN5*	0,928	0,126	0.659, 0.652
rs3783937	C, C	*FBLN5*	1	1	0.776, 0.762
rs12432450	A, A	*FBLN5*	0,562	0,555	0.549, 0.532
rs3814835	T, T	*FBLN5*	0,484	0,558	0.915, 0.912
rs2430378	C, C	*FBLN5*	0,433	0,002	0.880, 0.880
rs1303	T, T	*PI*	0,752	0,806	0.739, 0.759
rs17580	A, A	*PI*	1	0,966	0.966, 0.966
rs6647	T, T	*PI*	0,543	0,315	0.755, 0.744
rs2239651	T, T	*PI*	1	0,749	0.734, 0.750
rs709932	C, C	*PI*	0,109	0,672	0.838, 0.823
rs1980618	A, A	*PI*	0,103	0,294	0.650, 0.623
rs2749531	C, C	*PI*	0,252	0,277	0.734, 0.730
rs3748316	G, G	*PI*	0,858	0,811	0.817, 0.837
rs1125782	A, A	*PI*	0,803	0,132	0.563, 0.548
rs4934	C, C	*AACT*	0,799	1	0.540, 0.534
rs663214	C, C	*MFAP1*	0,656	0,411	0.711, 0.657
rs678084	G, G	*MFAP1*	1	0,249	0.795, 0.756
rs3759790	G, G	*MFAP1*	0,747	0,406	0.924, 0.900
rs13598	A, A	*FBN1*	0,974	0,01	0.856, 0.869
rs2015637	T, T	*FBN1*	1	1	0.901, 0.905
rs8029993	A, A	*FBN1*	0,252	0,26	0.734, 0.749
rs1812873	T, T	*FBN1*	0,252	0,277	0.734, 0.749
rs9806323	T, T	*FBN1*	1	0,544	0.857, 0.841
rs243866	C, C	*MMP2*	0,504	1	0.732, 0.771
rs1053605	G, G	*MMP2*	0,535	1	0.941, 0.920
rs9302671	C, C	*MMP2*	0,323	0,542	0.639, 0.650
rs2241145	G, G	*MMP2*	0,528	0,587	0.556, 0.544
rs243842	A, A	*MMP2*	0,549	0,449	0.622, 0.634
rs243840	A, A	*MMP2*	0,259	0,825	0.816, 0.812
rs243836	G, A	*MMP2*	0,391	0,547	0.510, 0.502
rs7501702	A, A	*MFAP4*	0,016	0,878	0.717, 0.764
rs2696247	A, A	*COL1A1*	0,375	0,227	0.865, 0.892
rs2269336	C, C	*COL1A1*	0,305	0,177	0.868, 0.902
rs1384364	C, C	*TIMP2*	0,888	0,76	0.824, 0.806
rs8080307	G, G	*TIMP2*	0,804	0,204	0.920, 0.916
rs9894295	T, T	*TIMP2*	0,695	1	0.874, 0.870
rs2277700	T, T	*TIMP2*	0,79	0,366	0.815, 0.820
rs4796812	C, C	*TIMP2*	0,626	0,543	0.926, 0.922
rs4789939	C, C	*TIMP2*	0,495	1	0.828, 0.810
rs2376999	A, A	*TIMP2*	1	0,976	0.885, 0.885
rs2889529	A, A	*TIMP2*	0,296	0,496	0.574, 0.582
rs7502916	T, T	*TIMP2*	0,843	0,76	0.605, 0.607
rs6501266	G, G	*TIMP2*	0,203	0,502	0.512, 0.524
rs8066695	T, T	*TIMP2*	0,792	0,532	0.571, 0.553

566:162, 943:219	3,226	0,0725
683:45, 1085:69	0,032	0,8579
597:131, 937:225	0,548	0,459
665:63, 1045:113	0,644	0,4222
670:56, 1076:84	0,145	0,7035
554:174, 915:247	1,808	0,1787
467:261, 754:408	0,107	0,7435
389:339, 636:526	0,304	0,5812
521:207, 807:355	0,96	0,3272
603:125, 952:210	0,25	0,6173
480:248, 758:404	0,098	0,7548
565:163, 885:277	0,526	0,4685
400:328, 617:543	0,555	0,4564
659:61, 1056:102	0,063	0,8014
632:86, 1005:137	0	0,9903
538:190, 881:279	1,004	0,3163
701:25, 1123:39	0,01	0,9188
550:178, 864:298	0,339	0,5603
534:194, 872:290	0,672	0,4123
610:118, 955:205	0,676	0,4111
473:255, 724:438	1,37	0,2418
534:194, 848:314	0,032	0,8583
595:133, 969:189	1,198	0,2737
410:318, 637:525	0,407	0,5234
392:334, 620:542	0,073	0,7868
516:210, 764:398	5,805	0,016
579:149, 879:283	3,836	0,0502
673:55, 1046:116	3,206	0,0734
623:105, 1010:152	0,686	0,4074
654:72, 1052:110	0,104	0,7467
534:194, 870:292	0,541	0,4621
534:194, 869:291	0,572	0,4495
624:104, 970:184	0,948	0,3303
533:195, 896:266	3,68	0,0551
685:43, 1069:93	2,947	0,086
464:262, 755:407	0,22	0,6387
405:323, 632:530	0,279	0,5972
453:275, 735:425	0,248	0,6187
594:134, 944:218	0,037	0,8474
371:357, 582:578	0,23	0,6315
522:206, 888:274	5,255	0,0219
628:98, 1037:125	3,224	0,0726
632:96, 1046:114	5,106	0,0238
600:128, 937:225	0,934	0,3337
670:58, 1064:98	0,129	0,7197
636:92, 1011:151	0,051	0,8212
590:134, 953:209	0,082	0,775
674:54, 1071:91	0,108	0,7422
601:125, 941:221	0,969	0,325
644:84, 1028:134	0	0,9965
417:309, 676:486	0,1	0,7522
439:287, 705:457	0,008	0,93
373:355, 609:553	0,247	0,6193
416:312, 643:519	0,593	0,4411

rs4789933	T, T	*TIMP2*	0,606	1	0.944, 0.950
rs4789932	C, C	*TIMP2*	1	0,554	0.515, 0.523
rs3810068	A, A	*EMILIN2*	0,673	0,714	0.637, 0.623
rs684320	A, A	*EMILIN2*	0,873	1	0.681, 0.699
rs506739	C, C	*EMILIN2*	0,942	0,627	0.598, 0.599
rs612071	A, A	*EMILIN2*	0,693	0,787	0.526, 0.536
rs642887	C, C	*EMILIN2*	0,04	0,178	0.880, 0.859
rs6506037	A, A	*EMILIN2*	0,298	0,933	0.672, 0.678
rs637647	G, G	*EMILIN2*	0,711	0,376	0.742, 0.760
rs11080994	A, A	*EMILIN2*	0,445	0,645	0.537, 0.560
rs607411	G, G	*EMILIN2*	0,648	0,754	0.845, 0.830
rs1790994	T, T	*EMILIN2*	0,665	0,248	0.823, 0.826
rs1059281	G, G	*EMILIN2*	0,962	0,872	0.730, 0.691
rs3826946	T, T	*ELA2*	0,071	0,941	0.837, 0.840
rs3761007	C, C	*ELA2*	0,25	0,707	0.903, 0.925
rs6114208	G, G	*CTSC*	0,423	0,23	0.783, 0.764
rs4810482	T, T	*MMP9*	0,446	0,28	0.639, 0.641
rs2250889	C, C	*MMP9*	0,834	0,507	0.962, 0.959
rs3918261	T, T	*MMP9*	0,429	0,337	0.846, 0.875
rs2839108	A, A	*COL6A2*	0,801	0,694	0.757, 0.726
rs7279347	T, T	*COL6A2*	0,244	0,429	0.533, 0.515
rs2839110	T, T	*COL6A2*	0,523	0,389	0.841, 0.816
rs2839112	T, T	*COL6A2*	0,209	0,668	0.915, 0.893
rs2839121	C, C	*COL6A2*	0,664	1	0.814, 0.786
rs5754289	G, G	*TIMP3*	0,685	0,035	0.828, 0.853
rs1962223	G, G	*TIMP3*	0,26	0,981	0.769, 0.782
rs9619311	T, T	*TIMP3*	0,994	0,851	0.719, 0.739
rs5749512	A, A	*TIMP3*	0,405	0,135	0.894, 0.916
rs130274	G, G	*TIMP3*	0,592	0,715	0.705, 0.733
rs713685	C, C	*TIMP3*	0,032	0,311	0.898, 0.912
rs738992	C, T	*TIMP3*	0,876	0,568	0.549, 0.510
rs135026	A, A	*TIMP3*	1	0,481	0.916, 0.923
rs130293	A, A	*TIMP3*	1	0,399	0.919, 0.926
rs80272	T, T	*TIMP3*	1	0,82	0.868, 0.882
rs135028	T, T	*TIMP3*	1	0,847	0.946, 0.950
rs6518799	G, G	*TIMP3*	0,759	0,988	0.922, 0.932
rs242076	C, C	*TIMP3*	0,489	0,399	0.549, 0.586
rs743751	C, C	*TIMP3*	0,762	1	0.921, 0.933
rs715572	C, C	*TIMP3*	0,769	0,944	0.724, 0.728
rs5754312	A, A	*TIMP3*	0,697	0,024	0.563, 0.572
rs2283885	G, G	*TIMP3*	1	0,694	0.830, 0.796
rs137484	T, T	*TIMP3*	0,438	0,745	0.937, 0.926
rs1427378	A, A	*TIMP3*	0,422	0,916	0.746, 0.780
rs9862	T, T	*TIMP3*	0,724	0,3	0.512, 0.515
rs137485	T, T	*TIMP3*	0,325	0,265	0.743, 0.735

P-values were calculated in Haploview using the chi-square test.
Hardy Weinberg equilibrium (HWE) was calculated separately for cases and controls.

687:41, 1104:58	0,37	0,5431
375:353, 608:554	0,118	0,7308
461:263, 724:438	0,357	0,55
496:232, 809:349	0,628	0,4282
435:293, 696:466	0,004	0,9505
383:345, 623:539	0,181	0,6701
641:87, 998:164	1,818	0,1775
489:239, 788:374	0,085	0,7711
540:188, 883:279	0,792	0,3736
391:337, 651:511	0,97	0,3247
615:113, 965:197	0,669	0,4134
599:129, 960:202	0,035	0,8517
530:196, 803:359	3,271	0,0705
609:119, 976:186	0,038	0,8453
650:70, 1071:87	2,828	0,0926
567:157, 886:274	0,947	0,3306
464:262, 744:416	0,01	0,9207
700:28, 1112:48	0,099	0,7536
614:112, 1017:145	3,303	0,0691
551:177, 844:318	2,159	0,1418
387:339, 599:563	0,553	0,4572
612:116, 948:214	1,914	0,1665
666:62, 1038:124	2,342	0,1259
581:133, 909:247	2,046	0,1526
603:125, 991:171	2,041	0,1531
560:168, 909:253	0,44	0,5073
522:204, 859:303	0,931	0,3345
651:77, 1064:98	2,447	0,1178
513:215, 852:310	1,818	0,1775
654:74, 1060:102	1,019	0,3127
400:328, 592:568	6,401	0,0114
667:61, 1073:89	0,317	0,5731
669:59, 1072:86	0,289	0,5906
632:96, 1025:137	0,808	0,3687
689:39, 1104:58	0,123	0,7258
671:57, 1083:79	0,713	0,3986
400:328, 681:481	2,45	0,1175
669:57, 1084:78	0,873	0,3502
527:201, 846:316	0,039	0,8437
410:318, 665:497	0,151	0,6974
604:124, 925:237	3,276	0,0703
682:46, 1076:86	0,807	0,369
543:185, 906:256	2,86	0,0908
372:354, 599:563	0,017	0,8959
541:187, 854:308	0,155	0,6934

Supplementary Table C. *AB assay IDs of the Taqman assay on demand used for genotyping of stage 2.*

Gene	Rs number	Taqman assay on demand: AB assay ID
PAI1	rs6956010	None (Taqman assay by design)
	rs2070682	C__2620939_1_
TGFBI	rs756463	C__2265157_10
HSPG2	rs3767137	C__1603659_10
	rs7556412	C__25471573_10
FN1	rs2289202	C__15880783_10
	rs1561299	C__2110794_10
	rs2043776	C__11469966_10
	rs4673999	C__11734927_10
FBN2	rs28114	C__2950398_1_
	rs331069	None (Taqman assay by design)
	rs331079	C__1561675_10
	rs10520002	C__30283886_20
COL4A1	rs12017058	None (Taqman assay by design)
	rs3783107	None (Taqman assay by design)
	rs675605	C__3147641_10
	rs630943	C__3147643_10
	rs2391824	C__16002102_10
ELN	rs868005	C__1253643_20

Chapter 10

Genome-wide linkage in a large Dutch family with intracranial aneurysms:

Replication of two loci for intracranial aneurysms to chromosome 1p36.11-p36.13 and Xp22.2-p22.32

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Abstract

Background

Approximately 2% of the general population harbor intracranial aneurysms. The prognosis after rupture of an intracranial aneurysm is poor: 50% of the patients die as a result of the rupture. Familial occurrence of intracranial aneurysms suggests there are genetic factors involved in the development of such aneurysms.

Methods and results

A large, consanguineous pedigree with 7 of 20 siblings affected by intracranial aneurysms was compiled and a genome-wide linkage analysis on this family was performed using Illumina's SNP-based linkage panel IV, which includes 5861 SNPs. A non-parametric linkage (NPL) "affecteds-only" approach with Genehunter was used and identified two loci with suggestive linkage (NPL=3.18) on chromosome regions 1p36 and Xp22. Additional microsatellite markers were genotyped in the two candidate loci and showed suggestive linkage to the locus on chromosome 1 with an NPL of 3.18 at 1p36.11-p36.13, and significant linkage to the locus on chromosome X with an NPL of 4.54 at Xp22.2-p22.32.

Conclusions

The two potential loci for intracranial aneurysms, which we identified in this large Dutch family, overlap with loci that have already been identified in previous linkage studies from different populations. Identification of genes from these loci will be important for a better understanding of the disease pathogenesis.

Genetic factors are likely to be involved in the development of intracranial aneurysms (Mendelian Inheritance in Men [MIM] 105800), as familial predisposition is the strongest risk factor for intracranial aneurysms and aneurysmal subarachnoid hemorrhage (SAH).[1,2] Familial clustering of SAH is found in approximately 10% of patients with SAH, and first-degree relatives of patients with SAH have a 2.5 to 7 times greater risk of developing SAH than the general population.[3-8] However, the segregation of SAH and intracranial aneurysms does not follow a strict Mendelian inheritance pattern in families, so the complex inheritance pattern seen is assumed to be caused by the interaction of several genes and environmental factors.[9]

In complex diseases, the identification of susceptibility genes is hampered by their multi-genic origin and genetic heterogeneity.[10] Causal variants of susceptibility genes in complex diseases are expected to be common. Not all carriers of these common causal variants will become affected by the disease, since they may not carry all the remaining disease-causing alleles of the susceptibility genes necessary to develop the disease.[11] In addition, different combinations of genes may lead to the same phenotype in different populations.[11] To overcome these difficulties, large single families can be studied in which the disease is expected to be genetically homogeneous, and the disease-causing genes will lead to the same phenotype.[12,13]

In an analysis of a North American family with intracranial aneurysms that segregated as a dominant trait, linkage was found to a locus on chromosome 1p34.3-p36.13 with a maximum LOD score of 4.2.[14] We previously reported on a genome-wide linkage study in a large, Dutch consanguineous family. Using a recessive mode of inheritance, positive evidence of linkage on chromosome 2p13 was found with a maximum LOD score of 3.55.[15] Because aneurysms develop over time, relatives with familial intracranial aneurysms are often screened at intervals; this yields a considerable benefit, as new aneurysms are detected in approximately 10% of the relatives who previously had a negative screening result.[16] Indeed, since the completion of our genome-wide linkage study in 2004, repeated screening has revealed newly developed aneurysms in two siblings of our family five years after their last negative screening. As these newly affected siblings did not carry the disease haplotype of chromosome 2p13, our previous results of positive linkage, assuming an autosomal recessive mode of inheritance, were no longer significant. A follow-up genome-wide linkage study in this family was therefore decided upon. A model-free, non-parametric linkage, “affecteds-only” approach was used to overcome the possible problem of complex etiology.

Patients and Methods

Family

The family pedigree has already been described in our previous linkage study.[15] Details of the family members are shown in ***Figure 1*** and ***Table 1***. The parents are first cousins and only their children are affected. DNA samples are available for the mother (I-2) and for 16 of her 20 children (see Table 1). The father (I-1) and the mother (I-2) of the children both died but not from aneurysmal SAH. The father died at 62 years of age and the mother at 89 years of age. The children with aneurysmal SAH (II-6, II-9 and II-12) were defined by symptoms suggesting SAH combined with subarachnoid blood on a computerized tomography scan (CT) and a proven aneurysm on CT angiography or conventional angiography. Individual II-2 had an episode suggestive of aneurysmal SAH at the age of 36, but she died before a CT scan or angiography could be performed. As there is no DNA sample available from this patient she was not included in the present analysis. The children II-11, II-15, II-17 and II-19 were diagnosed with an unruptured intracranial aneurysm identified by MR angiography. The intracranial aneurysms in children II-11 and II-17 were identified after the end of our previous linkage study, five years after their last negative MR angiography. In our previous linkage study,[15] individual II-13 was designated as having an unruptured intracranial aneurysm identified by MR angiography. However, at follow-up (MR angiographies done one and six years after the initial positive MR angiography) an aneurysm could no longer be visualized. This possible false-positive finding can be explained by the improved image quality of recent MR angiographies, performed with increasing magnetic field

Figure 1. *Pedigree of the large consanguineous Dutch family with intracranial aneurysms.*

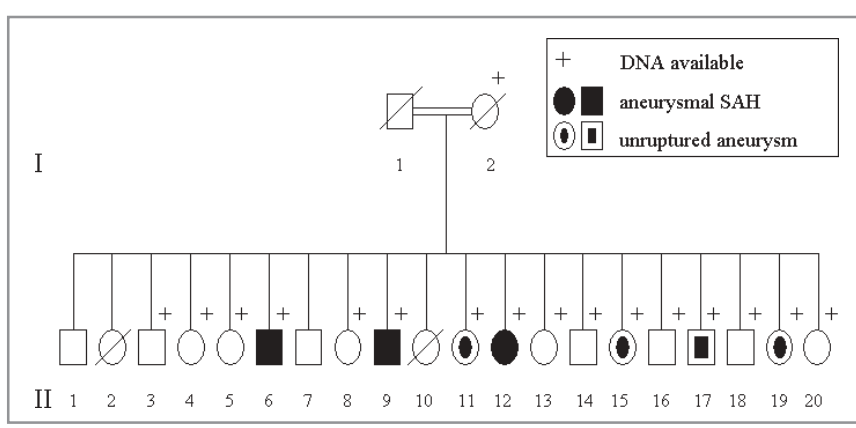


Table 1. *Clinical features of the affected and non-affected family members (revised since our previous study in 2004[15]).*

ID	ID previous study[15]	Year of birth	Age at death (years)	Diagnosis	Aneurysm location	Age at diagnosis (years)	DNA available
I-1	III-1	1908	62				–
I-2	III-2	1911	89				+
II-1	IV-1	1928					–
II-2	IV-2	1931	36	SAH?	unknown	36	–
II-3	IV-4	1932					+
II-4	IV-5	1933					+
II-5	IV-6	1935					+
II-6	IV-7	1936		SAH	ACoP L; ACM R+L	42	+
II-7	IV-9	1938					–
II-8	IV-10	1939					+
II-9	IV-11	1940		SAH	ICA R	37	+
II-10	IV-12	1942	0				–
II-11	**IV-13**	**1943**		**UIA**	**ICA L**	**61**	**+**
II-12	IV-14	1945		SAH	ACoP L	45	+
II-13	**IV-15**	**1946**					**+**
II-14	IV-16	1947					+
II-15	IV-18	1949		UIA	ACoA L; ICA L	45	+
II-16	IV-19	1950					+
II-17	**IV-20**	**1952**		**UIA**	**ACM R**	**52**	**+**
II-18	IV-21	1953					+
II-19	IV-22	1955		UIA	ICA L	38	+
II-20	IV-23	1959					+

The ID numbers of the three individuals that were assigned a different phenotype in our previous linkage study[15] are indicated in bold. SAH?: unconfirmed aneurysmal subarachnoid hemorrhage; SAH: aneurysmal subarachnoid hemorrhage confirmed by radiological imaging; UIA: unruptured intracranial aneurysm; ACoA: anterior communicating artery; ACoP: posterior communicating artery; ICA: internal carotid artery; MCA: middle cerebral artery; R: right; L: left.

strengths and leading to an improved sensitivity and, more importantly, to an improved specificity in detecting intracranial aneurysms.[17] In our current analysis, individual II-13 was therefore designated as being unaffected. In conclusion, seven out of the 16 children participating in this analysis are affected and have been diagnosed with either ruptured or unruptured intracranial aneurysms.

Genotyping

A two-stage design was used for the linkage analysis. Firstly, a whole-genome screen was performed in all the available individuals (n=17) using Illumina's single nucleotide polymorphism (SNP)-based linkage panel IV. Genomic regions of potential interest were then followed up using microsatellite markers.

Genotyping of SNP markers

Illumina's SNP-based linkage panel IV includes 5861 informative SNP markers, distributed evenly across the human genome with an average distance of 0.64 cM.[18] The information content of this linkage panel is comparable to standard 5 cM short-tandem-repeat marker maps. The SNPs were genotyped using the BeadArray™ technology on an Illumina BeadStation following the manufacturer's protocol (www.illumina.com). All SNPs were examined for their resulting quality and those that had a low signal or too wide clusters were excluded (n=109).

Genotyping of microsatellite markers

In potentially interesting regions (see Linkage analysis section), additional microsatellite markers were genotyped (n=24; see Supplementary ***Table A***). The markers were selected from the Marshfield Center for Medical Genetics marker set. Genotyping for the microsatellite analysis was performed by PCR with detection of fluorescent PCR products on a 3700 DNA sequencer (Applied Biosystems), and analyzed with GeneScan and Genotyper software (Applied Biosystems). For details of genotyping with microsatellite markers, see Van Belzen *et al.*[19] A Mendelian inheritance check was performed for both the SNP and microsatellite markers; markers with Mendelian errors were excluded (for SNPs n=41; for microsatellite markers n=0) from the linkage analysis.

Linkage analysis

Although the consanguinity of the parents in our family suggests an autosomal recessive mode of inheritance, other modes of inheritance cannot be ruled out. Therefore, a model-free, non-parametric linkage (NPL) analysis was performed with the linkage program GENEHUNTER,[20] using an "affecteds-only" approach. Using the NPL statistics, allele sharing in all possible pairs of affected individuals was determined and compared to the expected values for allele sharing. All the available 17 individuals who were genotyped, were included in the analysis and the disease status of the 7 patients with proven ruptured or unruptured intracranial aneurysms was set as affected. The other 10 were set as unknown. As we had no DNA sample of individual II-2, who had an episode suggestive of aneurysmal SAH,

she was not included in this analysis. For the analysis of the microsatellite markers on the X-chromosome, the non-parametric analysis of the MAPMAKER/SIBS program[21] was used. As the SNP marker sets per chromosome were too large for the GENEHUNTER program, they were divided into smaller sets of 100 SNPs with a sliding window of 20 SNPs. The family studied was also too large for analysis with either the GENEHUNTER and the MAPMAKER/SIBS programs, and was therefore divided into two smaller families (family 1: IDs I-2, II-3, II-4, II-5, II-6, II-8, II-9, II-11, II-12; family 2: IDs I-2, II-13, II-14, II-15, II-16, II-17, II-18, II-19, II-20). After scanning these two families separately, the NPL scores were combined.

P-values corresponding to the obtained NPL scores were determined according to Lander and Kruglyak.[22] The threshold levels for linkage were determined according to the genome-wide significance levels proposed by Lander and Kruglyak,[22] with "suggestive linkage" as an NPL score >3.18 (p-value of 7.4×10^{-4}), "significant linkage" as an NPL score >4.08 (p-value of 2.2×10^{-5}), and "highly significant linkage" as an NPL score >4.99 (p-value of 3.0×10^{-7}). Chromosomal regions with NPL scores >3.18 were considered as potentially interesting.

Results

Analysis of SNP markers

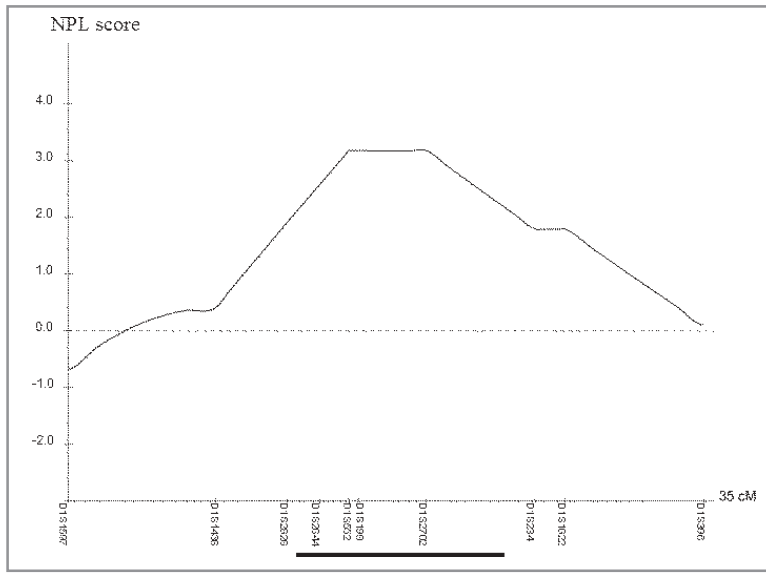
Of the 5861 genotyped SNPs from Illumina's SNP-based linkage panel IV, 109 random SNPs were excluded because of poor quality and 41 random SNPs because of Mendelian errors during the inheritance check, leaving a total of 5711 SNPs for analysis (97.4%). The maximum NPL scores per chromosome are shown in Supplementary ***Figure a***. The analysis of the SNPs identified four intervals of potential interest with maximum NPL scores of 3.18 (p-value 7.4×10^{-4}): 1p36.13-36.21, 4p14-15.1, 21q22.3, and Xp22.2-22.31. The NPL scores in the previously identified locus on chromosome 2p13[14] varied between -0.75 (p-value 0.77) and 0.64 (p-value 0.26; Supplementary ***Figure b***).

Analysis of microsatellite markers

Analysis with two additional microsatellite markers (Supplementary ***Table B***) reduced the evidence of linkage to 4p14-15.1, with NPL scores varying between 0.58 (p-value 0.28) and 0.90 (p-value 0.18), and to 21q22.3, with NPL scores between -0.59 (p-value 0.72) and -0.75 (p-value 0.77).

Analysis of 10 additional microsatellite markers at the 1p36.13-36.21 locus

Figure 2. *Analysis of linkage with microsatellite markers on 1p36.11-36.13.*

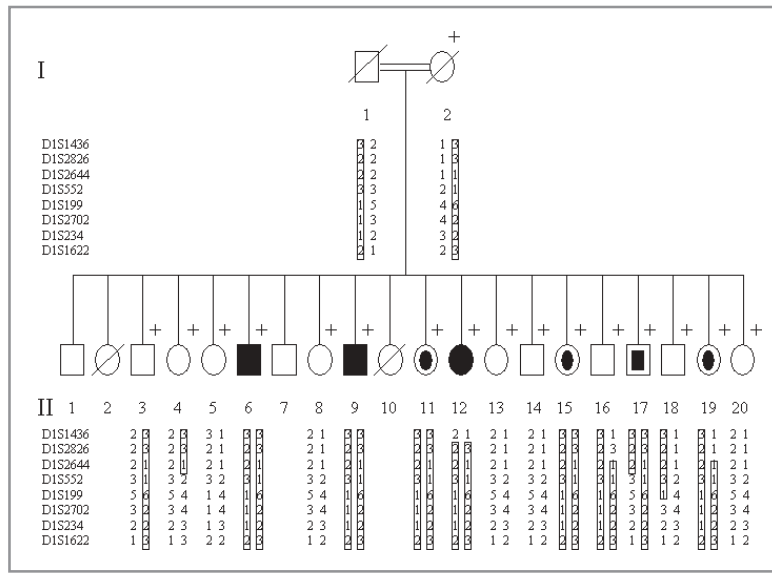


The horizontal bar below the plot indicates the extent of the NPL-1 interval.

showed a maximum NPL score of 3.18 (p-value 7.4 x 10^{-4}) (***Figure 2***). The maximum NPL score peak occurred at markers D1S199, D1S552 and D1S2702, and the NPL-1 interval was flanked by markers D1S2826 and D1S234, which define an 18.05 cM interval. This corresponds to a 6 Mb segment from 18.6 million bp to 24.9 million bp on chromosome 1p36.11-36.13 (***Figure 2***). The haplotypes from this region are shown in ***Figure 3***. Six out of the seven patients share a bi-allelic six-marker haplotype (ranging from D1S2644 to D1S1622) with one chromosome inherited from the father and one from the mother, consistent with an autosomal recessive mode of inheritance. The disease haplotype of the locus on 1p36.13-36.21 identified by the SNP analysis, also consists of one chromosome from the father and one from the mother. One patient (II-17) with an unruptured intracranial aneurysm inherited the whole haplotype from the mother but only part of the haplotype from the father. The as yet unaffected sibling II-16 inherited the disease haplotype from both parents. Analyzing the haplotypes shows that two of the patients are recombinants, which narrows down the critical region to lying between markers D1S2826 and D1S199.

Figure 4 shows that analysis of nine additional microsatellite markers at the Xp22.2-22.31 locus using the MAPMAKER/SIBS program, yielded a maximum NPL score of 4.54 (p-value 2.8 x 10^{-6}). Marker DXS7108 has the maximum NPL

Figure 3. *Chromosome 1p36.11-36.13 haplotypes of microsatellite markers.*



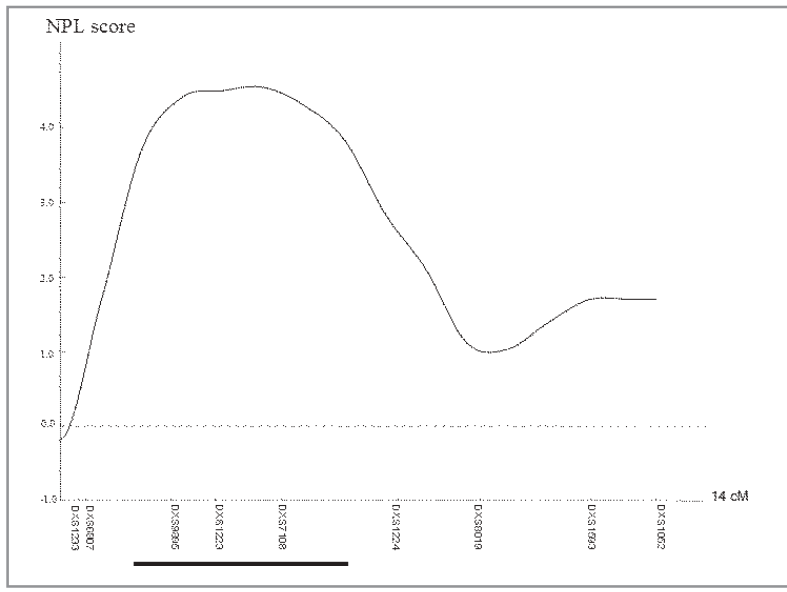
score while the NPL-1 interval is flanked by markers DXS6807 and DXS1224, defining a 7.73 cM interval. This corresponds to an 8 Mb segment from 4.6 million bp to 13.0 million bp on chromosome Xp22.2-p22.32. Six out of the seven patients share a five-marker haplotype ranging from DXS6807 to DXS1224 inherited from the mother (***Figure 5***). However, patient II-15 who has an unruptured intracranial aneurysm did not inherit the disease haplotype from the mother, while sibling II-18 who is as yet unaffected did inherit this haplotype.

The presence of high linkage disequilibrium (LD) between SNP markers can cause inflation of multipoint linkage statistics, which leads to false-positive results.[23] Therefore, the two intervals of potential interest identified by analysis of the SNP markers on chromosome 1 and X were confirmed by analyzing additional microsatellite markers in these regions. In view of the replication of linkage with the microsatellite markers, the NPL scores of the chromosome 1 and X regions are not likely to be inflated due to the presence of LD between the SNP markers analyzed.

Discussion

A follow-up linkage analysis in a large Dutch family with intracranial aneurysms is reported. In this family the disease is expected to be genetically homogeneous in a

Figure 4. *Analysis of linkage with microsatellite markers on Xp22.2-p22.32.*

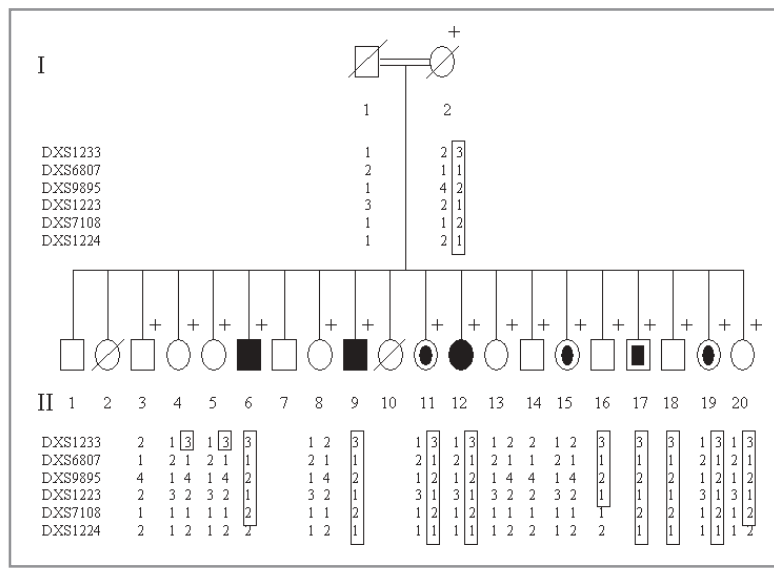


The horizontal bar below the plot indicates the extent of the NPL-1 interval.

single family, but due to the complex etiology of intracranial aneurysms, it is still possible that the disease in this family has a multi-genic origin. Thus, using a model-based, parametric linkage analysis may lead to misclassifying the model. Therefore a model-free, non-parametric linkage analysis was performed and significant linkage to two different loci, at 1p36.11-36.13 and Xp22.2-p22.32 was identified. Transmission of locus 1p36.11-36.13 seems to follow a recessive mode of inheritance, but that of locus Xp22.2-p22.32 seems to be inherited in a dominant mode. Neither of the loci is shared by all the affected patients, but each patient has at least one of the loci. The two loci may therefore compensate each other, which would indeed suggest a multi-genic origin for the disease in this family. Multigenic origin for diseases in single families has been described previously, for example in a family with celiac disease.[19]

The locus at 1p36.11-36.13 overlaps with the previously reported locus on chromosome 1p34.3-p36.13 (ANIB3; HUGO nomenclature committee) identified in a single North American family.[14] This family was analyzed under a dominant model, and a maximum LOD score of 4.2 was found at marker D1S234, which appeared to be located adjacent to the marker generating the highest NPL score in our family (D1S2702). An excellent candidate gene in the overlapping region between the

Figure 5. *Chromosome Xp22.2-p22.32 haplotypes of microsatellite markers.*



two studies is the perlecan gene, which codes for a heparan sulphate proteoglycan involved in the maintenance of the extracellular matrix (ECM) of the arterial wall. It is a major component of basement membranes interacting with other basement membrane components such as laminin, collagen type IV, and other ECM molecules such as fibronectin.[24] Perlecan is believed to be involved in stabilizing macromolecules and in cell adhesion.[24] In a comprehensive case-control study with tag SNPs, we recently demonstrated SNPs in the perlecan gene to be associated with intracranial aneurysms in two independent populations (combined p-value of 0.0005; Ruigrok *et al*, submitted), further emphasizing the possible involvement of this gene in the pathogenesis of intracranial aneurysms.

The second locus on Xp22.2-p22.32 has also been previously reported in the literature.[25,26] A nonparametric linkage analysis in 29 Japanese families, with three or more family members with intracranial aneurysms, identified three chromosomal regions including the Xp22 locus with a maximum NPL score of 2.16.[25] As the authors defined the boundaries of the locus by NPL scores with nominal p-values <0.05, it is not clear if this locus overlaps with our 90% confidence interval defined by the maximum NPL score –1. A Finnish linkage study analyzing affected sib pairs with intracranial aneurysms, also found evidence for linkage to Xp22 with a maximum LOD score of 2.08, but after genotyping additional markers their evidence for linkage was reduced in this region and it was not explored further.[26] A

susceptibility locus for intracranial aneurysms on the X-chromosome may explain the preponderance of affected women with intracranial aneurysms,[1] as many genes on chromosome Xp escape X inactivation.[27]

In our previous linkage study,[15] one of the siblings was assigned as being affected because MR angiography had revealed an unruptured intracranial aneurysm. However, in follow-up MR angiographies this aneurysm could no longer be identified. The misclassification of phenotype in our previous study once and again emphasizes that the definition of the phenotype should meet high standards of reproducibility and validity.[28]

As various genome-wide linkage studies in intracranial aneurysms have already identified several different loci for intracranial aneurysms (*i.e.* loci on chromosomes 1p34.3-p36.13,[14] 5q22-31,[29] 7q11,[29,30] 14q22,[29] 17cen,[25,29] 19q13.3,[25,26,31] and Xp22[25,26]), intracranial aneurysms are a genetically heterogeneous disease. Even in our single family, in which two loci on chromosome 1p36.11-36.13 and Xp22.2-p22.32 were identified, the disease appears to be genetically heterogeneous and multi-genic. As the locus on chromosome 1 had already been identified in another single, large family with intracranial aneurysms from North America,[14] and evidence of linkage to the locus on chromosome Xp22 was obtained in affected sib pairs and multiple families with intracranial aneurysms in Japanese and Finnish populations,[25,26] the two loci may thus be general risk factors for intracranial aneurysms in different populations.

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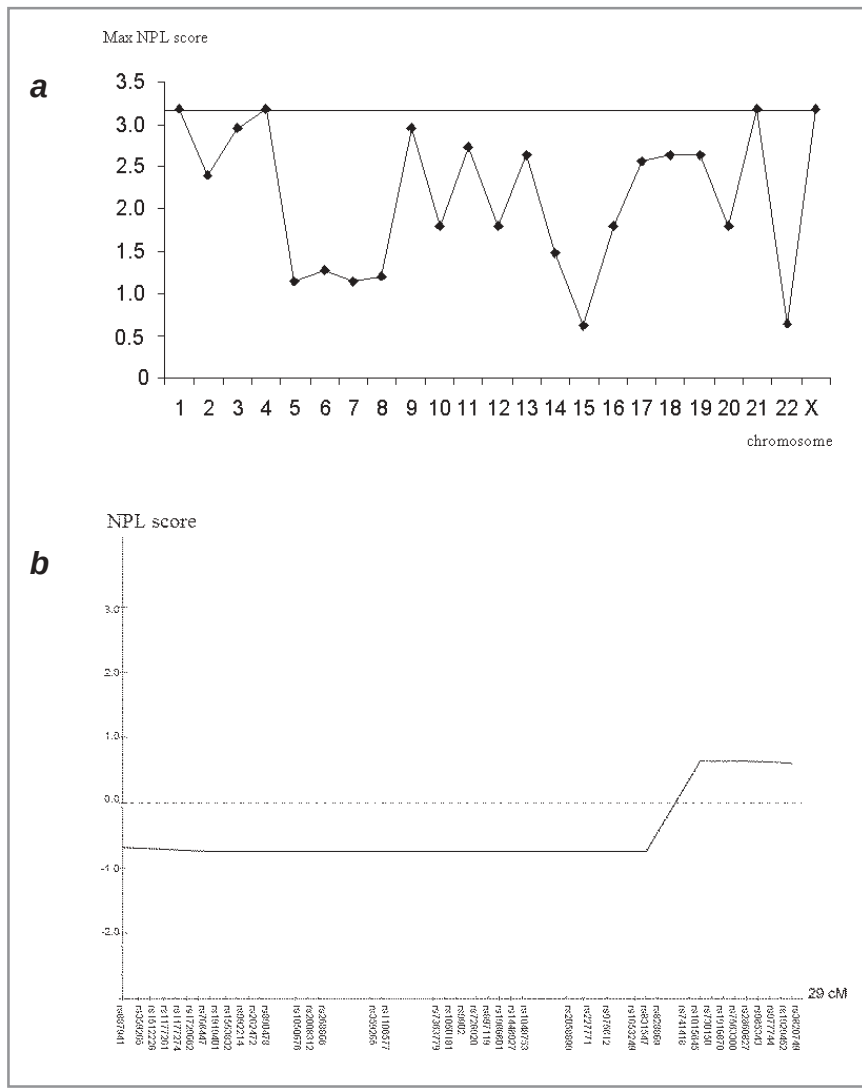
Supplementary Figure. *Non-parametric linkage (NPL) scores in genome-wide screen using Illumina's SNP-based linkage panel IV.*



a: NPL scores per chromosome;
b: NPL scores in the previous identified locus on chromosome 2p13.[15]

Supplementary Table A. *Additionally typed microsatellite markers.*

Chromosome	Microsatellite markers	Position in cM	Position in million bp
1	D1S1597	29.93	13.5
	D1S1436	37.05	18.6
	D1S2826	41.92	18.2
	D1S2644	43.72	18.8
	D1S552	45.33	19.0
	D1S199	45.33	19.7
	D1S2702	49.07	22.3
	D1S234	55.10	24.9
	D1S1622	56.74	30.0
	D1S396	64.38	32.1
4	D4S2408	45.97	31.0
	D4S1627	60.16	44.0
21	D21S1260	46.71	41.7
	D21S112	51.49	46.2
X	DXS1233	13.33	2.9
	DXS6807	13.50	4.6
	DXS9895	15.66	7.2
	DXS1223	16.75	8.3
	DXS7108	18.37	10.0
	DXS1224	21.23	13.0
	DXS8019	23.26	17.5
	DXS7593	25.97	22.2
	DXS1052	27.59	22.3

Supplementary Table B. *Non-parametric linkage (NPL) scores using additional microsatellite markers at the chromosome 4 and 21 regions.*

Chromosome	Position	NPL score	p-value
4	0.00	0.58	0.23
	2.84	0.64	0.23
	5.68	0.70	0.16
	8.51	0.76	0.16
	11.35	0.83	0.16
	14.19	0.91	0.16
21	0.00	-0.75104	0.71
	0.96	-0.71658	0.67
	1.91	-0.68387	0.67
	2.87	-0.65282	0.57
	3.82	-0.62333	0.57
	4.78	-0.59531	0.57

Chapter 11

A comparison of genetic chromosomal loci for intracranial, thoracic aortic and abdominal aortic aneurysms in search of common genetic risk factors

Ynte M. Ruigrok, Rim Elias, Cisca Wijmenga, and Gabriël J.E. Rinkel

Abstract

Objectives

Besides differences in epidemiology and pathology there are also similarities between intracranial aneurysms (IA), aneurysms of the ascending thoracic aorta (TAA) and the infrarenal aortic abdominal aneurysms (AAA). So has a familial preponderance been observed in all three aneurysmal types and therefore genetic factors are likely to be involved in the pathogenesis of all three of them. Common genetic risk factors for the three types of aneurysms have been suggested. This review describes the results of whole genome linkage studies on IA, TAA and AAA, and compares the genomic loci identified in these studies in search of a possible common genetic risk factors for the three aneurysmal types.

Methods

A literature search was performed on all whole genome linkage studies performed on IA, TAA and AAA. The genomic loci identified in these studies were described and compared in search of similarities between them.

Results

Five chromosomal regions on 3p24-25, 4q32-34, 5q, 11q24 and 19q were identified that may play a role in the pathogenesis of two or more aneurysmal types: 3p24-25 for TAA and IA, 4q32-34 for AAA and IA, 5q for TAA and IA, 11q24 for TAA, AAA and IA, and 19q for AAA and IA.

Conclusions

Five chromosomal regions were identified that may include common genetic factors for IA, TAA and AAA. Further studies are needed to explore these chromosomal regions in the different aneurysm patient groups. These studies may further help to unravel the disease pathogenesis of aneurysms in general. The complex nature caused by the interaction between genetic variants and environmental or non-genetic disease risk factors of the three different aneurysm types can explain why, despite differences in epidemiology and pathology, common genetic risk factors are still likely to contribute to the pathogenesis of IA, TAA and AAA.

Normal arteries have three histological layers, that is: an outer collagenous adventitia, a prominent muscular media, and an inner intima lined by a layer of endothelial cells. An internal elastic lamina separates the intima from the media, and the external elastic lamina separates the media from the adventitia. This external elastic lamina is only seen in extracranial arteries, and is absent in intracranial arteries.[1] Dilatation of arteries may lead to aneurysm formation. Most aneurysms develop in the intracranial arteries and the aorta. Intracranial aneurysms (IA) are characteristically saccular shaped, defined by localized outpocketings of the arterial wall occurring at arterial branching points[2] (***Figure A***). In IA intima proliferation and atherosclerosis may occur, but these aneurysms are predominantly characterized by fragmentation of the elastic laminae and loss of smooth muscle cells.[3] Aortic aneurysms are most commonly fusiform, characterized by circumferential widening of the artery.[2] The majority of the aortic aneurysms are found at the ascending thoracic aorta [thoracic aortic aneurysms (TAA)] and the infrarenal abdominal aorta [abdominal aortic aneurysms (AAA)].[4] Therefore, the focus will be on these two types of aortic aneurysms. The ascending aorta is the section between the aortic root and the arch, and no other arteries branch from it (***Figure B***). TAA located at the ascending aorta will eventually lead to dissection of the ascending aorta if left untreated. Thus, TAA and dissections of the ascending aorta are related conditions. TAA result from so-called medial necrosis or 'Erdheim cystic medial necrosis', histologically characterized by loss of smooth muscle cells, elastic fiber degeneration and an accumulation of basophilic ground substance, leading to weakening of the aortic wall and consequently to aneurysm formation.[5] Medial necrosis also occurs in association with connective

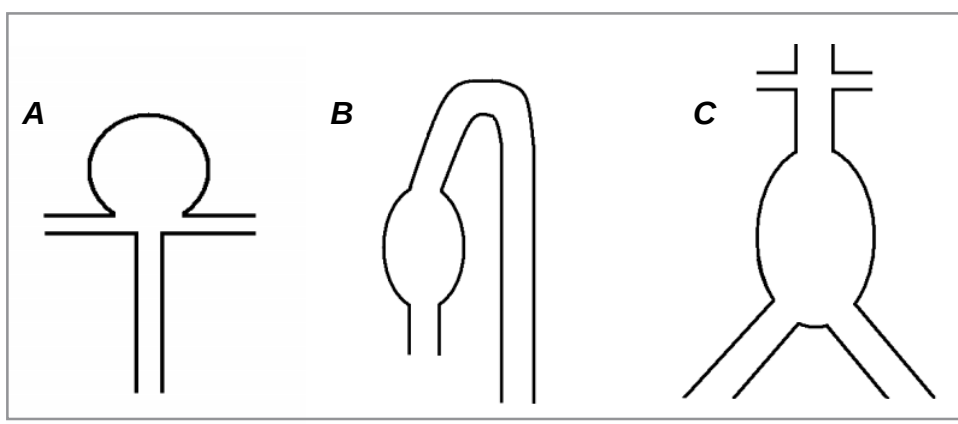


Figure. *Schematic representation of the three aneurysm types: intracranial aneurysm (**A**), thoracic aortic aneurysm (**B**), abdominal aortic aneurysm (**C**).*

tissue diseases such as Marfan's syndrome, and vascular (type IV) Ehlers-Danlos syndrome, but is more frequently found in the absence of such syndromes. AAA are located below the renal arteries and extends to the common iliac arteries, and about half of the AAA also involve these iliac arteries[4] (***Figure C***). In AAA the atherosclerosis is outspoken as these aneurysms are characterized by intimal atherosclerosis and chronic transmural inflammation in combination with destructive remodeling of the elastic media.[6,7]

IA are found in approximately 2% of the general population,[8] and the incidence of rupture of IA leading to subarachnoid hemorrhage (SAH) is 8 per 100,000 person-years.[9] Most patients with SAH from a ruptured IA are younger than 60 years of age and women have a higher risk than men.[8,10,11] Besides female sex risk factors for SAH from IA are smoking, hypertension, excessive alcohol and nonwhite ethnicity.[12] Although an exact estimate of the prevalence of TAA is not available, it has been suggested that AAA are four times as prevalent as TAA[13] with the prevalence of AAA being approximately 5%.[14] The incidence of TAA rupture is approximately 2.7 per 100,000 person-years.[15] These ruptures are commonly seen in the sixth and seventh decade of life[16] and men are affected approximately 2 to 4 times more often than women.[16] The most important risk factor associated with the development of TAA and subsequent dissections is hypertension.[16-20] The prevalence of AAA is approximately 5%, characteristically occurs in people older than 55 years, and is more common in men than in women.[14,21,22] The incidence of AAA rupture is up to 10 per 100,000 person-years.[21,23-25] Besides the risk factors male sex and age, smoking and hypertension are also important risk factors for the development of AAA.[22,26-28]

Despite the apparent epidemiological and pathogenic differences between IA, TAA and AAA described above, there are also similarities between the three aneurysmal types (***Table 1***). Firstly, a co-incidence between the different aneurysm types has been described. The co-incidence between IA and TAA has not systematically been investigated but a co-incidence of the familial forms of IA and TAA has been suggested in two studies describing two families with TAA, in which some family members were also diagnosed with IA while in a third family, family members were diagnosed with carotid aneurysms.[29,30] Further studies are needed to analyze whether a true co-incidence between (the familial forms of) TAA and IA exists. Several reports on the co-existence of AAA and IA in the same patient have been published,[31-35] but only two detailed studies exploring the possibility of a co-existence are known.[36,37] In a retrospective study the percentages of a simultaneous diagnosis of IA in AAA patients and AAA in IA patients are not higher than expected, but it is interesting to observe that most of the patients in whom both types

of aneurysms are diagnosed, had the familial form of IA or AAA.[36] In the second study a positive family history (defined by two or more first-degree relatives with AAA) for AAA was observed in 10.5% of a consecutive series of IA patients.[37] However, only 85% of eligible IA patients and 51% of the relatives of those 85% IA patients were enrolled for this study. Furthermore, a family history for AAA was not verified by reviewing the medical records, which leads to inaccuracies.[38] Because of these biases, the percentage of 10.5% is likely to be an overestimation. Nevertheless, based on the results of this study, a co-incidence of IA and AAA is still possible, but further well-designed studies are needed to assess this issue. In contrast, the co-incidence of AAA and TAA is well defined. In a study reviewing 1,510 patients with aneurysms of the aorta, 12.6% had multiple ones: more than half of TAA patients had other aneurysms, mostly AAA, and 12% of AAA patients had TAA.[39] Another study showed AAA to be present in 28% of TAA patients.[40] Although not systematically analyzed, in the familial forms of TAA and AAA a co-incidence of TAA and AAA has also been reported occasionally. One study reported on 3 families in which both TAA and AAA were found,[41] while in another study on TAA families, in one of these families an additional AAA was diagnosed.[29] On the co-incidence of IA, TAA and AAA not much is known. In the literature only one case report describes a patient in whom an IA, TAA and AAA was diagnosed.[33] In conclusion, co-incidences between IA and AAA, between IA and TAA, and between TAA and AAA have been suggested, but only for TAA and AAA the co-incidence has been proven by well designed systematic studies. A second similarity between the three types of aneurysms is that for all three types of aneurysms a familial preponderance has been demonstrated.[19,20,42-54] Familial clustering of SAH is found in approximately 10% of patients with SAH, and first-degree relatives of patients with SAH have a two and a half to seven times greater risk of developing SAH than the general population.[45-51] Familial IA rupture at a younger age than non-familial ones[47,48,51-53,55] and are generally larger at time of rupture and more likely to be multiple than non-familial aneurysms.[56] As in sporadic IA, women are more affected in familial IA.[47,51-55] The segregation of SAH and intracranial aneurysms does not follow a strict Mendelian inheritance pattern in families consistent with genetic heterogeneity.[57] In patients where TAA is not associated with specific connective tissue diseases such as Marfan's syndrome, up to 20% of the patients have a first-degree relative with the disease.[19,20] The risk of a brother of a TAA patient of also developing a TAA is 11 times higher than the risk in the general population, whereas the risk for sisters is two times higher.[19,20] Compared to sporadic patients, familial patients present at a significantly younger age and their aneurysms have faster growth rates.[20] Pedigree analysis suggests genetic heteroge-

Table 1. *Similarities and differences in epidemiology and pathology of IA, TAA and AAA.*

	IA
Pathology characteristics	Predominantly fragmentation elastic laminae, smooth muscle cell loss; to lesser extent intimal proliferation and atherosclerosis[3]
Prevalence	2%[8]
Incidence of rupture (per 100,000 patient years)	8[9]
Sex distribution	female > male[8,10,11]
Age (years)	50-60[8,10,11]
Atherosclerotic risk factors	+[12]
Co-incidence with other aneurysms	AAA?[36,37]
Prevalence among first degree relatives of patients	10%[45-51]
RR for siblings of patients	2.5-7[45-51]
Sex distribution in familial form	female > male[47,51-55]
Mode of inheritance in familial form	genetic heterogeneity[57]

AAA: abdominal aortic aneurysm; IA: intracranial aneurysm; TAA: thoracic aortic aneurysm; AD: autosomal dominant

neity. The primary mode of inheritance seems to be autosomal dominant with decreased penetrance and variable expression,[30] but in some families the mode of inheritance seems consistent with X-linked dominant and recessive modes.[19,20] At least 18% of patients with AAA have a first-degree relative with AAA, with up to 18% of brothers and 5% of sisters having AAA.[42-44] Persons with an affected first-degree relative have an up to 12-fold increase in AAA risk,[42,58] while the relative risk for male siblings of a male patient is 18.[59] Patients with familial AAA are younger than the patients with sporadic ones.[60,61] Although the prevalence of AAA is lower in women than in men, patients with familial AAA are more likely to be women.[62,63] Almost all possible modes of inheritance have been advocated and it is

TAA	AAA
Erdheim cystic medial necrosis (*i.e.* fragmentation elastic laminae, smooth muscle cell loss, accumulation basophilic ground substance)[5]	intimal atherosclerosis, chronic inflammation, remodelling elastic media[6,7]
+/- 1.25%[13]	5%[14]
2.7[15]	10[21,23-25]
male > female[16]	male > female[14,21,22]
> 60[16]	> 60[14,21,22]
+/-[16-20]	++[22,26-28]
AAA[39,40]	TAA[39,40]
20%[19,20]	18%[42-44]
11[19,20]	12[42,58]
male > female[19,20]	female > male[62,63]
genetic heterogeneity, but AD with decreased penetrance most prevalent[19,20,30]	genetic heterogeneity[64,65]

ADPKD: autosomal dominant polycystic kidney disease; RR: relative risk;

concluded that genetic heterogeneity exists.[64,65]

Based upon the fact that for all three types of aneurysms a familial preponderance has been demonstrated, genetic factors are likely to be involved in their pathogenesis. Indeed, for each type of aneurysm genome-wide linkage studies have already identified several chromosomal loci linked to the disease,[41,66-74] consistent with the presence of genetic heterogeneity proven for the three different aneurysm types. Furthermore, common genetic risk factors for the three types of aneurysms have been suggested.[29,30,36,37,41,75,76] The common genetic factors may include genes involved in the maintenance of the extracellular matrix (ECM) of the arterial wall. In all three types of aneurysms, involvement of genes coding for components and

regulators of the ECM in the pathogenesis of aneurysm formation has been suggested, based upon the association of the three types of aneurysms with heritable disorders of connective tissue, the histological characteristics of the aneurysms, and already identified genes related to the disease.[64,77,78]

This review describes the results of whole genome linkage studies performed on IA, TAA and AAA, and compares the genomic loci identified in these studies in search of possible common genetic risk factors for the three types of aneurysms.

Methods

The electronic database MEDLINE was searched up to December 2005 for all whole genome linkage studies on IA, TAA and AAA, using the text words intracranial aneurysm, cerebral aneurysm, aortic aneurysm, thoracic, abdominal, genes, genetics and linkage, in all relevant combinations. Only linkage studies demonstrating loci with LOD scores >2.2 and non-parametric LOD (NPL) score >3.18 (p-value of 7.4×10^{-4}), *i.e.* threshold levels for "suggestive linkage" according to the genome-wide significance levels proposed by Kruglyak and Lander,[79] were selected. The genomic loci identified in these studies were described and compared in search of similarities between them. The linkage study in Japanese sib pairs with IA performed by Onda *et al* provides crude data of the LOD scores of all markers analyzed.[69] We therefore decided to additionally analyze the linkage scores of the markers as identified in the study by Onda *et al* [69] in loci identified in AAA and TAA to see whether there is evidence for more similarities in loci between AAA, TAA and IA.

Results

Identified loci for IA

In an analysis, a single Northern American family with IA that segregated as a dominant trait linkage, was found to a locus on chromosome 1p34.3-p36.13 with a maximum LOD score of 4.2.[68] A Japanese study was performed in 104 affected sib pairs and three linkage regions were identified (7q11, 14q22 and 5q22-q31; maximum LOD score 3.22, 2.31 and 2.24, respectively).[69] Another genome-wide linkage scan was performed using 139 affected sib pairs together with 83 other affected relative pairs (*i.e.* pairs with affected family members other than siblings) from the Finnish population and linkage was shown to chromosome 19q13.3 (maximum

LOD score 3.99).[70,71] Linkage to this region was confirmed by analyzing 29 Japanese IA families with 3 or more individuals affected by IA, using a non-parametric model-free linkage analysis, but the threshold levels for "suggestive linkage" were not met (NPL score of 2.15).[72]

Identified loci for TAA

Loci for TAA have been mapped to 11q (FAA1), 5q (FAA2), and 3p (FAA3). The first locus was identified in a genome-wide linkage study of 3 families in which TAA was associated, in some individuals, with AAA and aneurysms at other arteries.[41] The TAA in the families segregated as autosomal dominant traits and did not meet the diagnostic criteria for Marfan or Ehlers-Danlos. A locus on chromosome 11q23.3-q24 was found with a maximum LOD score of 4.4.[41] In a second study, fifteen families with TAA and dissections with an apparent dominant inheritance were studied, and linkage analysis showed a major locus for familial TAA and dissections on 5q13-14 (maximum LOD of 4.74).[73] The majority of the families studied (9 of 15) demonstrated evidence of linkage to this locus.[73] The third locus was identified in a genome-wide scan in a single family of Swiss-German heritage with TAA and thoracal aorta dissections without features of Marfan's syndrome and with exclusion of known loci for familial thoracic aneurysm.[74] A critical region at 3p25-p24 was demonstrated with a maximum LOD score of 4.27, which locus overlaps the locus for Marfan's syndrome type II (MFS2). Marfan's syndrome is characteristically caused by mutations in the fibrillin-1 gene (*FBN1*) located on chromosome 15q21.1 (MFS1),[80-82] but a mutation in the TGF-beta receptor 2 gene (*TGFBR2*) located on chromosome 3p24-p25 (MFS2) was also identified in a single family.[83,84] However, whether this single family meets all the diagnostic criteria for Marfan's syndrome is debated.[85,86] In an additional study analyzing 80 families with TAA without Marfan's syndrome, mutations in the *TGFBR2* gene could also be identified.[29] These mutations were found in 4 out of 80 unrelated families with familial TAA who did not have Marfan's syndrome. All 4 mutations affected an arginine residue at position 460 in the intracellular domain of TGFBR2. Remarkably, the affected family members of the four families not only had TAA of the ascending aorta, but also of the descending aorta and of carotid and popliteal arteries. Furthermore, some members had IA.

Identified loci for AAA

A whole-genome scan of AAA using affected-relative-pair (ARP) linkage analysis that included covariates to allow for genetic heterogeneity, identified two susceptibility loci for AAA on chromosomes 19q13 (maximum LOD score of 4.75) and

4q31(maximum LOD score of 3.73).[66] In a genome-wide scan in three Dutch families with AAA, linkage to chromosome 19q was confirmed (NPL score 3.95).[67] The linkage region identified in this latter study was situated just right of the region found in the previous study and the two regions did not overlap. The authors concluded that this difference may be explained by genetic heterogeneity, as the two linkage regions may both include a different gene involved in the pathogenesis of AAA.[67] However, differences in analysis methods of both studies (parametric[66] versus non-parametric analysis,[67] and inclusion[66] versus no inclusion of covariates[67]) may have led to subtle differences in the confidence intervals of the maximum LOD scores. Therefore, it is still possible that the two studies do indeed point towards the same genetic factor.

Comparison of identified loci for IA, AAA and TAA

Comparing the 99% confidence intervals of the loci for IA, AAA and TAA reported in the literature (***Table 2***), the locus on 19q13 for IA overlaps with the locus for AAA. As said, for AAA the locus on 19q13 has been identified in two independent studies,[66,67] but without overlap between the two loci. The IA locus[70,71] only overlaps with the locus identified by one of these studies.[67] The IA locus on 5q22-31[69] lies immediately adjacent to the TAA locus on 5q13-14 (FAA2)[73] but these two loci only just do not overlap. Interestingly, in the first stage of the IA linkage study, two markers on 5q14.3 (p-value 0.001) and 5q15 (p-value 0.018) attained statistically significant linkage, and this region does overlap with the TAA locus on 5q13-14. This IA region was then further explored and subsequent extended multipoint linkage analysis did not suggest linkage to the 5q14.3-q15 locus anymore, but to a region adjacent to the locus on chromosome 5q22-31. The versican gene (*CSPG2*) is an interesting candidate gene located in the TAA locus on 5q13-14 and in the vicinity of the IA locus on 5q22-31. The proteoglycan CSPG2 plays an important role in the ECM assembly process of the arterial wall. Exons 3 to 13 (of a total of 15 exons) of the *CSPG2* gene were studied in detail in TAA family members in whom the 5q13-14 was identified, but no disease-causing mutations could be identified in this region of the gene. Mutations in the remaining part of the gene (exons 1 to 2 and 14 to 15, intronic regions) may have been left unnoted. Recently we showed that single nucleotide polymorphisms (SNPs) in strong linkage disequilibrium (LD) and haplotypes constituting these SNPs in the *CSPG2* gene are associated with IAs, suggesting that variation in or near the gene plays a role in susceptibility to IAs.[87] Further studies, for example association studies of SNPs in *CSPG2* and TAA, should explore whether variation in *CSPG2* is a common genetic risk factor for both IA and TAA.

Table 2. *99% confidence intervals of loci identified in whole-genome linkage studies in IA, TAA and AAA.*

Aneurysm type	Locus	Markers	BP positions (million bp)
IA	1p36.13-p34.3	D1S199-D1S496	19.7-35.1
	5q22-31	D5S2103-D5S436	88.8-145.2
	7q11.2	D7S502-D7S479	66.5-96.0
	14q22	D14S258-D14S74	69.6-77.7
	19q13*	D19S545-D19S246	52.2-55.6
TAA	3p24-25	D3S2303-D3S1619	17.9-34.1
	5q13-q14	D5S253-D5S641	8.1-8.2
	11q23.3-q24	D11S1339-D11S1353	101.5-122.8
AAA	4q31*	D4S1644-D4S1586	142.1-147.1
	19q13	D19S414-D19S197	36.6-46.8
	19q13.3	D19S902-D19S571	53.0-58.0

AAA: abdominal aortic aneurysm; IA: intracranial aneurysm; TAA: thoracic aortic aneurysm; bp: base pair.
* 95% confidence interval reported as 99% confidence interval could not be determined.

Comparison of crude data of IA linkage study[69] with identified loci for TAA and AAA

Borderline significant linkage was observed for a marker at 3p26.3 (p-value 0.073), which marker is located in the TAA locus FAA3 on 3p25-p24.[74] Also, two closely located markers on 4q32-34 show evidence for linkage (marker on 4q32.1, p-value 0.060 and marker on 4q34.1, p-value 0.040) and these markers are located close to the identified locus for AAA on 4q31.[66] Finally, two adjacent markers on 11q24 (p-value 0.060) and 11q25 (p-value 0.023) also show some evidence of linkage, and these markers lie in and close to the identified TAA locus on 11q23.3-q24 (FAA1).[41] In the IA linkage study genomic regions were considered to have suggestive evidence of linkage in this first set of crude data, if a marker was linked with a p-value of <0.01 or if two or more adjacent markers were linked with a p-value <0.05.[69] The markers located in the regions 3p26.3, 4q32-34 and 11q24-q25 did not meet these criteria for suggestive linkage and therefore these regions were not further explored. However, since IA and AAA, and possibly also TAA, are complex diseases caused by the interaction of several genes and environmental factors, most of the genetic factors will only have small effects. Therefore, some genetic factors may not lead to linkage scores with threshold levels for “suggestive linkage” ac-

cording to the genome-wide significance levels, but these genetic factors may be still worth looking at (***Table 3***).

Table 3. *Possible common genetic factors in AAA, IA and TAA.*

Locus	Gene	Aneurysm type	Evidence
3p24-25	*TGFBR2*	TAA and IA	locus for TAA[74]
			mutations *TGFBR2* identified in families with TAA and in some family members also with IA[29]
			crude data of IA linkage study show possible evidence of linkage to IA in this region[69]
4q32-34	?	AAA and IA	locus for AAA[66]
			crude data of IA linkage study show possible evidence of linkage to IA in this region[69]
5q	*CSPG2*?	TAA and IA	locus for TAA and IA[69,73]
			association *CSPG2* and IA[87]
11q24	?	AAA and TAA	locus identified in families with TAA and in some family members also with AAA[41]
		AAA and TAA and IA	crude data of IA linkage study show possible evidence of linkage to IA in this region[69]
19q	?	AAA and IA	locus for AAA and IA[66,70,71]

AAA: abdominal aortic aneurysm; IA: intracranial aneurysm; TAA: thoracic aortic aneurysm.

Conclusions

Besides differences in epidemiology and pathology there are also many similarities between IA, TAA and AAA. For AAA and TAA a co-incidence has been proven, while for AAA and IA a co-incidence has been suggested. Furthermore, in all three aneurysmal types familial clustering is apparent in 10-20% of the patients with first-degree relatives being at high risk of also developing an aneurysm. On analyzing the mode of inheritance in the families with the three different types of aneurysms, genetic heterogeneity is suggested. Because of the familial preponderance, genetic factors are likely to be involved in the pathogenesis of these aneurysms. As for each type of aneurysm genome-wide linkage studies have identified several chromosomal loci linked to the disease, genetic heterogeneity is likely.

Looking at the identified genetic risk factors for the three types of aneurysms, more similarities between AAA, IA and TAA are recognized and common genetic risk factors for the three aneurysms seem plausible. Five chromosomal regions on 3p24-25, 4q32-34, 5q, 11q24 and 19q are identified that may include genes involved in the pathogenesis of two or more aneurysmal types. Further exploration of these regions seems worthwhile, and future studies should explore these possible common genetic risk factors of AAA, IA and TAA. For example, mutations in *TGFBR2* located in the 3p24-25 region have been demonstrated in families with TAA, in which some family members also have IA. To study whether variants in *TGFBR2* also contribute to the development of IA alone, *TGFBR2* may be screened for mutations in IA families, or *TGFBR2* may be tested for association with IA in IA patients. For *CSPG2* positioned in the chromosomal 5q region, association with IA has already been demonstrated. As this chromosomal region is also identified in families with TAA, this gene can also be tested for association in TAA patients.

As for the remaining three chromosomal regions 4q32-34, 11q24 and 19q, no obvious candidate genes have been appointed yet. These three chromosomal regions are large and include hundreds of genes. The identified gene *TGFBR2* located on 3p24-25 and the candidate gene *CSPG2* in the 5q region are both genes related to the ECM of the arterial wall and for all three aneurysmal types involvement of the ECM in the pathogenesis has been suggested.[64,77,78] Therefore, ECM related genes are also plausible candidate genes for the 4q32-34, 11q24 and 19q regions, and the genes related to the ECM located in these regions should be tested for association with the aneurysmal types. Once these disease-causing genes have been identified, it will become evident whether the disease-causing gene of 4q32-34 is indeed the same for AAA and IA, the one of 11q24 for AAA and TAA and

possibly IA, and the one of 19q for AAA and IA.

These suggested studies may further help to unravel the disease pathogenesis of aneurysms in general. IA and AAA, and possibly also TAA, are complex diseases caused by the interaction between genetic variants and environmental or non-genetic disease risk factors.[88] Because of the multi-factorial nature of the disease, each individual genetic variant generally has only a modest effect, and the interaction of genetic variants with each other (gene-gene interactions) or with environmental factors (gene-environment interactions) may attribute to the observed phenotype.[88] Thus, combinations of genetic and environmental factors will lead to the disease. The complex nature of the three different aneurysm types can explain why, despite differences in epidemiology and pathology, common genetic risk factors are still likely to contribute to the pathogenesis of IA, TAA and AAA.

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Part III: Genetic Studies on Secondary Cerebral Ischemia. Rebleeding and Outcome after Aneurysmal SAH

Chapter 12

Genes and outcome after aneurysmal subarachnoid hemorrhage

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Abstract

Background and purpose

Initial and secondary ischemia are important determinants of outcome after subarachnoid hemorrhage (SAH). Cerebral ischemia is a potent stimulus for expression of genes that may influence recovery.
We investigated whether functional polymorphisms in the apolipoprotein E *(APOE),* insulin-like growth factor-1 *(IGF-1),* tumor necrosis factor-A *(TNF-A),* interleukin-1A *(IL-1A),* interleukin-1B *(IL-1B),* and interleukin-6 *(IL-6)* genes are related with outcome after aneurysmal SAH.

Methods

Genotyping of the polymorphisms was performed in a consecutive series of 167 patients with aneurysmal SAH. The risk of a poor outcome was analyzed with logistic regression with adjustment for prognostic factors for outcome after SAH, using the homozygotes for the wild type alleles as a reference.

Results

Patients carrying any *IGF-1* non-wild type allele had a lower risk of a poor outcome (OR, 0.4; 95% CI, 0.2 to 1.0), while carriers of the *TNF-A* non-wild type allele had a higher risk (OR, 2.3; 95% CI, 1.0 to 5.4). We could not demonstrate an association with outcome for *APOE* (*APOE* ε4 OR, 0.4; 95% CI, 0.1 to 1.2; *APOE* ε2 OR, 0.7; 95% CI, 0.2 to 2.4), *IL-1A* (OR, 1.8; 95% CI, 0.8 to 4.0), *IL-1B* (OR, 0.7; 95% CI, 0.3 to 1.5) and *IL-6* (OR, 0.7; 95% CI, 0.3 to 1.8) polymorphisms.

Conclusions

Variation in some genes that are expressed after cerebral ischemia may partly explain the large differences in outcome between patients with aneurysmal SAH. SAH patients homozygote for the *IGF-1* wild type allele or carriers of the *TNF-A* non-wild type allele have a higher risk of poor outcome. Additional studies in other populations are needed to assess the generalizability of our results.

Outcome after aneurysmal subarachnoid hemorrhage (SAH) remains poor; half the patients die and 20% remain dependent for activities of daily life.[21] The initial impact of the hemorrhage, as reflected by the clinical condition on admission and the amount of extravasated blood, is the strongest prognostic factor for a poor outcome.[5,24] These two measures reflect the severity of the initial cerebral ischemia caused by cessation of cerebral blood flow at the time of aneurysmal rupture.[16,19] Secondary cerebral ischemia is the most important complication after treatment of the aneurysm.[5,20] There are large inter-individual differences in recovery after aneurysmal SAH. These may be partly genetic in origin.

Cerebral ischemia is a potent stimulus for gene expression in animal models.[1,26,36] In these laboratory animals, the gene products of the expressed genes influence recovery after ischemia.[17,23,25,43] In man, some of the corresponding genes have different variants (polymorphisms) leading to different protein isoforms or differences in level of protein expression. These include apolipoprotein E *(APOE)*,[27] insulin-like growth factor-1 *(IGF-1)*,[34] tumor necrosis factor-A *(TNF-A)*,[38] interleukin-1A *(IL-1A)*,[31] interleukin-1B *(IL-1B)*,[33] and interleukin-6 *(IL-6)*.[12] Therefore, the different polymorphic variants of *APOE, IGF-1, TNF-A, IL-1A, IL-1B* and *IL-6* may have different effects on recovery after cerebral ischemia.

We hypothesized that in patients with aneurysmal SAH, initial or secondary cerebral ischemia leads to expression of the *APOE, IGF-1, TNF-A, IL-1A, IL-1B* and *IL-6* genes. The aim of our study was to investigate whether the *APOE, IGF-1, TNF-A, IL-1A, IL-1B* and *IL-6* genotypes are associated with outcome after aneurysmal SAH.

Methods

Patient recruitment

From February 1999 to February 2002 we obtained informed consent for genotyping from 167 consecutive patients with aneurysmal SAH admitted to the University Medical Center Utrecht or from their relatives. Patients with aneurysmal SAH were defined by symptoms suggestive of SAH combined with subarachnoid blood on CT and a proven aneurysm on CT angiography or conventional angiography. Blood samples were taken during the first four days of the hospital admission. All patients were treated according to our standard protocol, which includes oral nimodipine, refraining from antihypertensive medication, and intravenous administration of fluid until a positive fluid balance of at least 750 cc. The ethical review board approved our study protocol.

Data collection

We recorded the patient's age at time of SAH, sex, clinical condition on admission, amount of blood on initial CT scan, and any episodes of rebleeding or secondary cerebral ischemia. For the clinical condition on admission we used the World Federation of Neurological Surgeons' (WFNS) scale.[9] The amount of blood on the CT scan on admission (within 72 hours after the initial symptoms) was graded on a scale of 0 to 30 as defined by Hijdra *et al*.[18] Rebleeding was defined as a sudden deterioration in the level of consciousness or a sudden increase in headache, combined with an increase of blood on CT compared with the previous CT. Secondary cerebral ischemia was defined as a gradual decline in the level of consciousness or a gradual development of new focal deficits or both, with confirmation of a new hypodensity on CT, and no evidence for rebleeding, hydrocephalus or metabolic disturbances.

Laboratory analyses

Genotyping of the *APOE (APOE* ε2, ε3 and ε4 alleles; *APOE* ε3 allele wild type),[27] *IGF-1* (variable CA repeat; allele with 19 CA repeats/192 base pairs (bp) wild type),[34] *TNF-A* (-863 C/A),[38] *IL-1A* (-889 C-T),[31] *IL-1B* (-511 C-A),[33] and *IL-6* (-174 G-C)[12] polymorphisms was performed on coded DNA samples so that the patients' characteristics and outcomes remained unknown. Polymerase chain reaction (PCR) was performed using previously described primers for *APOE*,[44] *IGF-1*,[34] *TNF-A*,[38] *IL-1A*,[31] *IL-1B*,[33] and *IL-6*.[12] The assay conditions are available on request. We used well-established negative and positive quality controls for all these reactions.

Outcome assessment

Outcome was assessed approximately three months (with a range from eight to 16 weeks) after SAH by means of the Glasgow Outcome Scale (GOS).[22] Outcome was assessed before the laboratory analyses were done, *i.e.* without knowledge of the patient's genotype.

Data analyses

The clinical condition on admission was determined as 'good' (WFNS I-III) or 'poor' (WFNS IV-V). For the amount of SAH we dichotomized the scores at the median of the Hijdra scores. The amount of subarachnoid blood could not be assessed in 16 patients (10%) because they had not been admitted within 72 hours of the initial symptoms; for them a score was computed instead with the likelihood approach.[35] We split the outcome into 'good' (independent [GOS 4 and 5]) or

'poor' (dependent [GOS 2-3] or dead [GOS 1]). Using logistic regression, we assessed the risk of a poor outcome three months after SAH as an odds ratio (OR) with corresponding 95% confidence intervals (CI).

For the analyses on the *APOE* genotype we grouped carriers of ε2/2 and ε2/3 as well as carriers of ε4/4, ε3/4 and ε2/4. For the analyses of the *IGF-1, TNF-A, IL-1A, IL-1B* and *IL-6* polymorphisms, we compared patients homozygous for the wild type allele (WT) with patients homozygous or heterozygous for the non-wild type allele(s) (non-WT). Since the frequencies of the 9 non-wild type alleles of the *IGF-1* polymorphism are low, these alleles were pooled as described in other studies.[13,34,41] We adjusted for the known prognostic factors for outcome after SAH: sex, age at time of SAH, clinical condition on admission, amount of blood on initial CT scan, and episodes of rebleeding or secondary cerebral ischemia.

Results

The distribution of all genotypes was in Hardy-Weinberg equilibrium. The genotype frequencies are shown in ***Table 1***. The patients' characteristics and outcome are summarized in ***Table 2***. Compared to those carrying the *IL-6* non-WT allele, patients homozygous for the *IL-6* WT allele had less often a poor clinical condition on admission (10% versus 27%, p=0.006) and more often a large amount of cisternal blood (62% versus 45%, p=0.03). The remaining characteristics did not show differences between patients with WT and non-WT alleles (data not shown).

Table 1. *APOE, IGF-1, TNF-A, IL-A, IL-1B and IL-6 genotype frequencies.*

	APOE ε4*	APOE ε2**	IGF-1	TNF-A	IL-1A	IL-1B	IL-6
WT/WT	102 (61%)	102 (61%)	58 (35%)	113 (68%)	78 (47%)	72 (44%)	58 (35%)
WT/non-WT	37 (22%)	21 (12%)	85 (52%)	53 (31%)	76 (45%)	68 (41%)	78 (47%)
non-WT/non-WT	6 (4%)	1 (1%)	22 (13%)	1 (1%)	13 (8%)	24 (15%)	29 (18%)

Genotyping data were available for 167 patients for the *APOE, IL-1A* and *TNF-A* polymorphisms, for 165 patients for the *IGF-1* and *IL-6* polymorphisms, and for 164 patients for the *IL-1B* polymorphism.
*WT/non-WT: ε3/4 or ε2/4; **WT/non-WT: ε2/3.
APOE ε2/ε4: apolipoprotein ε2/ε4 allele; WT: wild type allele; non-WT: non-wild type allele; *IGF-1*: insulin-like growth factor-1; *TNF-A*: tumor necrosis factor-A; *IL-1A*: interleukin-1A; *IL-1B*: interleukin-1B; *IL-6*: interleukin-6.

Table 2. *Patients' baseline characteristics.*

Characteristics	All patients (n=167)
Mean age ± SD	52.6 ± 14.1
% Women	71%
Poor clinical condition on admission	22%
Amount of cisternal blood >median	43%
Rebleeding	14%
Secondary cerebral ischemia	27%
Dead	17%
Vegetative or severe disability*	15%
Moderate disability	25%
Good recovery	43%

*: 1 patient with a Glasgow Outcome Scale 2 (vegetative state).
SD: standard deviation.

Table 3 shows the adjusted and unadjusted odds ratios for poor outcome three months after SAH according to genotype. Patients carrying any *IGF-1* non-wild type allele had a lower risk of a poor outcome (adjusted OR, 0.4; 95% CI, 0.2 to 1.0; p = 0.05; or in other words homozygotes for the *IGF-1* wild type allele had a higher risk), whereas patients carrying the *TNF-A* non-wild type allele had a higher risk (adjusted OR, 2.3; 95% CI, 1.0 to 5.4; p = 0.05). When we made additional adjustments for the covariates pneumonia, hydrocephalus and the intervention clipping versus coiling, the odds ratios did not change. Although the risk of poor outcome was lower for patients carrying the *APOE* ε4 allele and higher for carriers of the *IL-1A* non-wild type allele, these associations were not statistically significant (*APOE* ε4 adjusted OR, 0.4; 95% CI, 0.1 to 1.2; *IL-1A* adjusted OR, 1.8; 95% CI, 0.8 to 4.0). We could not demonstrate any effect of the *APOE* ε2, *IL-1B* and *IL-6* polymorphisms on outcome.

Discussion

We found that some genes expressed after experimental cerebral ischemia may be associated with the outcome of patients with aneurysmal SAH. SAH patients carrying the *TNF-A* non-wild type allele or the *IGF-1* wild type allele have a higher risk of poor outcome. We could not demonstrate an association of the *APOE* ε4, *APOE* ε2, *IL-1A*, *IL-1B* and *IL-6* polymorphisms with outcome after aneurysmal SAH.

Table 3. *Unadjusted and adjusted odds ratios* for poor outcome three months after SAH** according to genotype****

Genotype	Independent (GOS 4 and 5)	Dependent or dead (GOS 1-3)	Crude Odds Ratio (95% CI)	Adjusted Odds Ratio (95% CI)
APOE ε3/3	64% (65)	36% (37)	1 (reference)	1 (reference)
APOE ε4/4 + ε3/4 + ε2/4	74% (32)	26% (11)	0.6 (0.3-1.3)	0.4 (0.1-1.2)
APOE ε3/3	64% (65)	36% (37)	1 (reference)	1 (reference)
APOE ε2/3 + ε2/2	77% (17)	23% (5)	0.5 (0.2-1.5)	0.7 (0.2-2.4)
IGF-1 WT	55% (32)	45% (26)	1 (reference)	1 (reference)
IGF-1 non-WT	76% (81)	24% (26)	0.4 (0.2-0.8)	0.4 (0.2-1.0)
TNF-A WT	72% (81)	28% (32)	1 (reference)	1 (reference)
TNF-A non-WT	61% (33)	39% (21)	1.6 (0.8-3.2)	2.3 (1.0-5.4)
IL-1A WT	73% (57)	27% (21)	1 (reference)	1 (reference)
IL-1A non-WT	64% (57)	36% (32)	1.5 (0.8-3.0)	1.8 (0.8-4.0)
IL-1B WT	68% (49)	32% (23)	1 (reference)	1 (reference)
IL-1B non-WT	68% (63)	32% (29)	1.0 (0.5-1.9)	0.7 (0.3-1.5)
IL-6 WT	70% (41)	30% (17)	1 (reference)	1 (reference)
IL-6 non-WT	66% (71)	34% (36)	1.2 (0.6-2.4)	0.7 (0.6-1.8)

*adjusted for gender, age, clinical condition on admission, amount of blood on initial CT-scan, and episodes of rebleeding or delayed cerebral ischemia; **based on the GOS and split into good (independent [GOS 4 and 5]) and poor (dependent [GOS2-3] or dead [GOS 1]); ***genotyping data were available for 167 patients for the *APOE, IL-1A* and *TNF-A* polymorphisms, for 165 patients for the *IGF-1* and *IL-6* polymorphisms, and for 164 patients for the *IL-1B* polymorphism.
SAH: subarachnoid hemorrhage; GOS: Glasgow Outcome Scale; CI: confidence interval; *APOE* ε2/ε3/ε4: apolipoprotein ε2/ε3/ε4 allele; WT: homozygote WT-allele; non-WT: heterozygote and homozygote non-WT allele; *IGF-1*: insulin-like growth factor-1; *TNF-A*: tumor necrosis factor-A; *IL-1A*: interleukin-1A; *IL-1B*: interleukin-1B; and *IL-6*: interleukin-6.

Since several genes, including *TNF-A* and *IGF-1*, are expressed after experimental cerebral ischemia,[1,26,36] their gene products are likely to influence recovery after acute and secondary cerebral ischemia in SAH patients. However, with our study we can not rule out other mechanisms by which these genes may influence outcome. In our study we analyzed functional polymorphisms leading to different protein isoforms or differences in level of protein expression. People with the

TNF-A non-wild type allele have a lower serum TNF-α concentration than those carrying the wild type allele.[38] This implies that lower TNF-α serum levels may explain the higher risk of poor outcome in our SAH patients carrying the *TNF-A* non-wild type allele. The *IGF-1* wildtype allele was associated with lower serum IGF-1 levels than all other alleles in two studies,[13,34] but another study found the wildtype allele associated with higher levels.[41] This difference in results may be explained by chance or by genetic and environmental differences between the populations studied. According to the findings in two of the three studies, lower IGF-1 levels may explain the higher risk of poor outcome after SAH. To our knowledge, thus far no studies have been performed analysing TNF-α or IGF-1 levels in blood or CSF of patients with SAH and different *TNF-A* or *IGF-1* alleles, respectively.

The elevated expression of the *IGF-1* gene after experimental cerebral ischemia[4] appears to be neuroprotective[17] as the gene product IGF-1 inhibits apoptosis and promotes cell survival.[11,42] This neuroprotective effect is mediated through promoting reorganization of actin, activation of focal adhesion proteins and stimulating neurite outgrowth.[11]

Some laboratory studies suggest that increasing levels of TNF-α (the gene product of *TNF-A*) have a neurotoxic role[3,8] while other studies demonstrated a neuroprotective effect with TNF-α protecting against cell death.[6,7] These heterogeneous effects of TNF-α after ischemia may be explained by a difference in the time of TNF-α activation or the presence of other mediators.[37] However, the overall effect of TNF-α is not yet known and should be subject of further research.

We are not aware of previous studies on the relationship of polymorphisms in the *IGF-1, IL-1A, IL-1B* and *IL-6* genes and outcome after cerebrovascular disease, including aneurysmal SAH. Recently, the *TNF-A* 308 G/A polymorphism was found to be associated with outcome after cerebral infarction.[15] Heterogeneous results have been found in previous studies on the *APOE* ε4 genotype and outcome after aneurysmal SAH: two studies found that the *APOE* ε4 allele is related to poor outcome[28,32] while a third study did not find an association.[10] Possible differences in these findings may be attributable to the small patient groups studied (approximately 100[28,32]) to differences in adjustment for prognostic factors (*e.g.* no adjustment for rebleeds[10,28] or secondary cerebral ischemia[10,28,32]), or to inclusion of patients with non-aneurysmal SAH.[10] The effect of the *APOE* ε4 allele has also been investigated for other forms of central nervous system injury. The *APOE* ε4 allele appeared to be associated with higher case fatality and morbidity in patients with head injury[14,39,40] and nonaneurysmal intracerebral hemorrhage[2,29] but not in patients with ischemic stroke.[30] The effect of the *APOE* ε4 allele may therefore depend on the type of brain damage.

There are some possible shortcomings in the interpretation of our findings. Some patients could not be included because they died soon after admission and before they could be asked to participate in this study. Our results therefore apply to a population of patients who survived the initial hours after the hemorrhage. Another methodological issue in our study is the problem of multiple testing: when many genotypes are investigated, a false-positive association of a genotype with outcome may be found by chance. Since our sample size is relatively small, our results should be interpreted with caution. Moreover, further studies in other populations are needed to assess the external validity of our findings. We adjusted for the known prognostic factors for outcome after SAH: sex, the three baseline prognostic factors most closely related to poor outcome in aneurysmal SAH being the neurological condition of the patient on admission, age and the amount of subarachnoid blood on the initial CT scan,[20,24] and the two most important complications leading to poor outcome after the initial hemorrhage being episodes of rebleeding or secondary cerebral ischemia. This list of prognostic values influencing outcome is not complete, but a priori we did not expect a relationship between the studied genetic factors and adverse events and interventions, making confounding unlikely and statistical adjustment not necessary. Indeed, when we made additional adjustments for these covariates the odds ratios did not change. The main advantages of our study are that outcome was assessed independently of the patient's genotype and that we had a large patient group with proven aneurysmal SAH and a complete follow-up.

We found that genetic variation in the *TNF-A* and *IGF-1* genes may be associated with outcome in aneurysmal SAH patients. In the future, these findings may lead to a better understanding of the differences in recovery from initial or secondary cerebral ischemia after aneurysmal SAH. Furthermore, knowledge on genetic variation and variation in outcome from ischemia after SAH may eventually lead to new treatment strategies, including genotype-specific intervention. Before we reach this stage, further studies are needed to confirm that genetic variation in genes expressed after cerebral ischemia is associated with altered outcome after aneurysmal SAH. In future studies the association between genetic variation in the *TNF-A* and *IGF-1* genes and the risk of a poor outcome could be assessed in patients with acute and secondary cerebral ischemia to further explore the hypothesis that these genes are associated with the outcome of patients with aneurysmal SAH by expression after cerebral ischemia.

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Chapter 13

Genes influencing coagulation and risk of secondary cerebral ischemia and rebleeding after aneurysmal subarachnoid hemorrhage

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Abstract

Objectives

We investigated whether genes influencing coagulation are associated with the occurrence of secondary cerebral ischemia and rebleeding in patients with aneurysmal subarachnoid hemorrhage (SAH).

Methods

Genotyping of the factor V Leiden, prothrombin G20210A, methylene-tetrahydrofolate reductase (*MTHFR*) C677T, Val34Leu, Tyr204Phe and Pro564Leu factor XIII subunit A, and His95Arg factor XIII subunit B mutations was performed in 208 patients with aneurysmal SAH. Secondary cerebral ischemia occurred in 49 (23.6%) patients and rebleeding in 28 (13.5%). The patients were followed up for three months. The risk of secondary cerebral ischemia and rebleeding was assessed as hazard ratio (HR) with 95% confidence intervals (95% CI) using Cox regression.

Results

For secondary cerebral ischemia, the HR was 1.8 (95% CI, 0.6 to 4.9) for factor V Leiden, 0.54 (95% CI, 0.1 to 3.9) for prothrombin G20210A, 0.4 (95% CI, 0.1 to 1.5) for MTHFR, 0.9 (95% CI, 0.5 to 1.6) for Val34Leu, 0.9 (95% CI, 0.3 to 2.4) for Tyr204Phe and 0.8 (95% CI, 1.4 to 1.4) for Pro564Leu factor XIII subunit A, and 1.54 (95% CI, 0.8 to 2.8) for His95Arg factor XIII subunit B variant. For rebleeding, the HR was 0.6 (95% CI, 0.1 to 4.45) for factor V Leiden, 0.7 (95% CI, 0.1 to 5.4) for prothrombin G20210A, 0.6 (95% CI, 0.2 to 1.7) for *MTHFR*, 1.2 (95% CI, 0.5 to 2.6) for Val34Leu, 0.3 (95% CI, 0.04 to 2.5) for Tyr204Phe and 1.8 (95% CI, 0.8 to 4.2) for Pro564Leu factor XIII subunit A, and 1.1 (95% CI, 0.4 to 2.9) for His95Arg factor XIII subunit B variant.

Conclusions

No large effects of the investigated mutations in the genes influencing coagulation were found on the occurrence of secondary cerebral ischemia and rebleeding with aneurysmal SAH.

In patients who survive the initial hours after aneurysmal subarachnoid hemorrhage (SAH), secondary cerebral ischemia and rebleeding are major causes of death and disability.[1-4] Secondary cerebral ischemia occurs even with the current treatment in approximately 25% to 35% of patients.[1-3] Rebleeding occurs in 40% if the aneurysm is not treated[3] and remains an important cause of death even if the aim is to occlude the aneurysm early after the bleeding.[4]

Coagulation factors may be involved in these complications as antifibrinolytic treatment reduces the occurrence of rebleeding but increases the risk of secondary cerebral ischemia.[5,6] A direct analysis of these factors is hampered by early activation of the coagulation and fibrinolytic system following SAH.[7] Therefore, studying genetic factors may be a better approach. A genetic study in SAH patients already suggested that the plasminogen activator inhibitor-1 (*PAI-1*) gene modifying coagulation is indeed involved in the occurrence of secondary cerebral ischemia.[8] The role of mutations in coagulation factor V,[9] prothrombin,[10] methylenetetrahydrofolate reductase (*MTHFR*),[11] coagulation factor XIII subunit A[12,13] and subunit B[14] genes is currently unclear. The factor V Leiden and prothrombin G20210A mutations are associated with an increased risk of thrombosis[9,10] and may therefore be associated with an increased risk of secondary cerebral ischemia in SAH patients. In contrast, Val34Leu, Tyr204Phe and Pro564Leu factor XIII subunit A mutations are associated with an increased risk of bleeding[12,13] and may increase the risk for rebleeding in SAH patients. The role of the *MTHFR* C677T mutation and His95Arg factor XIII subunit B muatation in coagulation is not (yet) clear.[11,14]

The aim of this study was to investigate whether the above mentioned mutations are associated with the occurrence of secondary cerebral ischemia and rebleeding in patients with aneurysmal SAH.

Methods

Patient recruitment

208 Dutch patients with aneurysmal SAH admitted to the University Medical Center Utrecht were included. Aneurysmal SAH was defined by symptoms suggestive of SAH, combined with subarachnoid blood on CT and a proven aneurysm on CT angiography or conventional angiography. The ethical review board of the University Medical Center Utrecht approved our study protocol.

Table 1. *Patients' baseline characteristics.*

Characteristics	All patients (n=208)
Mean age ± SD	59.5 ± 14.9
Women	149 (71.6%)
Poor clinical condition on admission	41 (19.7%)
Amount of cisternal blood > median	74 (35.6%)
Rebleeding	28 (13.5%)
Secondary cerebral ischemia	49 (23.6%)

SD: standard deviation

Data collection

The patients' age at time of SAH, sex, clinical condition on admission, amount of blood on initial CT scan, and any episodes of rebleeding or secondary cerebral ischemia were recorded. For the clinical condition on admission, the 'World Federation of Neurological Surgeons' (WFNS) scale was used.[15] The amount of blood on the CT scan on admission (within 72 hours after the initial symptoms) was graded on a scale of 0 to 30, as defined by Hijdra et al.[16] Rebleeding was defined as a sudden deterioration in the level of consciousness or a sudden increase in headache, combined with an increase of blood on CT compared with the previous CT. Secondary cerebral ischemia was defined as a gradual decline in the level of con-

Table 2. *Genotype frequencies of polymorphisms analyzed.*

	FV Leiden (n=207)	Prothrombin G20210A (n=207)	*MTHFR* C677T (n=207)
Homozygote wildtype	196 (94.7%)	200 (96.6%)	97 (46.9%)
Heterozygous	10 (4.8%)	7 (3.4%)	88 (42.5%)
Homozygous variant	1 (0.5%)	0 (0%)	22 (10.6%)

* not in Hardy Weinberg equilibrium.

sciousness or a gradual development of new focal deficits or both, with confirmation of a new hypodensity on CT, and no evidence for rebleeding, hydrocephalus or metabolic disturbances. The patients were followed up for three months.

Laboratory analyses

DNA was isolated from whole venous blood. Genotyping was performed using polymerase chain reaction (PCR), using previously described primers and assay conditions for the factor V Leiden (factor V G1691A),[9] prothrombin G20210A,[10] *MTHFR* C677T,[11] Val34Leu,[17] Tyr204Phe and Pro564Leu factor XIII subunit A,[18] and His95Arg factor XIII subunit B[14] mutations. Genotyping of the variants was performed on coded DNA samples so that the patients' characteristics remained unknown for the person who performed the laboratory analyses.

Data analyses

Tests for Hardy-Weinberg equilibrium were conducted using χ^2 tests. The risk of secondary cerebral ischemia and rebleeding was assessed as a hazard ratio (HR) using Cox regression with corresponding 95% confidence intervals (CI). Patients homozygous for the wild type allele were compared with patients homozygous and heterozygous for the variant allele. For *MTHFR* C677T, an additional analysis was performed, comparing patients homozygous for the wild type or heterozygous for the variant allele with patients homozygous for the variant allele. For the assessment of the risk of secondary cerebral ischemia, patients were censored in case they had a rebleeding, and patients were censored in the analysis on the risk of rebleeding in case the aneurysm was treated by means of clipping or coiling. The clinical condition on admission was determined as 'good' (WFNS I-III) or 'poor' (WFNS IV-V). For the amount of cisternal blood, the scores were dichotomized at

Val34Leu FXIII A (n=208)	Tyr204Phe FXIII A* (n=207)	Pro564Leu FXIII A (n=194)	His95Arg FXIII B* (n=196)
122 (58.7%)	188 (90.8%)	114 (58.8%)	151 (77.0%)
71 (34.1%)	14 (6.8%)	64 (33.0%)	37 (18.9%)
15 (7.2)	5 (2.4%)	16 (8.2%)	8 (4.1%)

Table 3. *Hazard ratio's for risk of secondary cerebral ischemia and rebleeding after sub-*

Genotype	Secondary cerebral ischemia or rebleeding	No secondary cerebral ischemia or rebleeding
FV Leiden	**Secondary cerebral ischemia**	**No secondary cerebral ischemia**
GG	45 (23.0%)	151 (77.0%)
GA and AA	4 (36.4%)	7 (63.6%)
	Rebleeding	**No rebleeding**
GG	26 (13.2%)	171 (86.8%)
GA and AA	1 (9.1%)	10 (90.9%)
prothrombin G20210A	**Secondary cerebral ischemia**	**No secondary cerebral ischemia**
GG	48 (24.0%)	152 (76.0%)
GA and AA	1 (14.3%)	6 (85.7%)
	Rebleeding	**No rebleeding**
GG	27 (13.4%)	174 (86.6%)
GA and AA	0 (0%)	7 (100%)
***MTHFR* C677T**	**Secondary cerebral ischemia**	**No secondary cerebral ischemia**
CC	28 (28.9%)	69 (71.1%)
CT and TT	21 (19.1%)	89 (80.9%)
	Rebleeding	**No rebleeding**
CC	17 (17.3%)	81 (82.7%)
CT and TT	10 (9.1%)	100 (90.9%)
	Secondary cerebral ischemia	**No secondary cerebral ischemia**
CC and CT	47 (25.4%)	138 (74.6%)
TT	2 (9.1%)	20 (90.9%)
	Rebleeding	**No rebleeding**
CC and CT	22 (11.9%)	163 (88.1%)
TT	4 (18.2%)	18 (81.8%)
Val34Leu FXIII A	**Secondary cerebral ischemia**	**No secondary cerebral ischemia**
ValVal	30 (24.6%)	92 (75.4%)
ValLeu and LeuLeu	18 (20.9%)	68 (79.1%)
	Rebleeding	**No rebleeding**
ValVal	14 (11.4%)	109 (88.6%)
ValLeu and LeuLeu	14 (16.3%)	72 (83.7%)
Tyr204Phe FXIII A	**Secondary cerebral ischemia**	**No secondary cerebral ischemia**
TyrTyr	44 (23.4%)	144 (76.6%)
TyrPhe and PhePhe	4 (21.1%)	15 (78.9%)
	Rebleeding	**No rebleeding**
TyrTyr	27 (14.3%)	162 (85.7%)
TyrPhe and PhePhe	1 (5.3%)	18 (94.7%)

arachnoid hemorrhage according to genotype.

Crude hazard ratio (95% CI)	Adjusted hazard ratio (95% CI)
1 (reference)	1 (reference)
1.8 (0.6-4.9)	1.8 (0.6-5.0)
1 (reference)	
0.6 (0.1-4.45)	NA
1 (reference)	1 (reference)
0.5 (0.1-3.9)	0.5 (0.1-3.9)
1 (reference)	
0.7 (0.1-5.4)	NA
1 (reference)	1 (reference)
0.6 (0.3-1.0)	0.6 (0.3-1.0)
1 (reference)	
0.5 (0.2-1.1)	NA
1 (reference)	1 (reference)
0.4 (0.1-1.5)	0.4 (0.1-1.5)
1 (reference)	
0.6 (0.2-1.7)	NA
1 (reference)	1 (reference)
0.9 (0.5-1.6)	0.9 (0.5-1.6)
1 (reference)	
1.2 (0.5-2.6)	NA
1 (reference)	1 (reference)
0.9 (0.3-2.4)	0.9 (0.3-2.4)
1 (reference)	
0.3 (0.04-2.5)	NA

(continued on page 202)

Pro564Leu FXIII A	**Secondary cerebral ischemia**	**No secondary cerebral ischemia**
ProPro	31 (27.2%)	83 (72.8%)
ProLeu and LeuLeu	17 (21.3%)	63 (78.8%)
	Rebleeding	**No rebleeding**
ProPro	12 (10.5%)	102 (89.5%)
ProLeu and LeuLeu	14 (17.5%)	66 (82.5%)
His95Arg FXIII B	**Secondary cerebral ischemia**	**No secondary cerebral ischemia**
HisHis	33 (21.9%)	118 (78.1%)
HisArg and ArgArg	14 (31.1%)	31 (68.9%)
	Rebleeding	**No rebleeding**
HisHis	18 (11.8%)	134 (88.2%)
HisArg and ArgArg	8 (17.8%)	37 (82.2%)

CI: confidence interval; NA: not applicable.

the median of the Hijdra scores. As a poor clinical condition on admission and a large amount of extravasated blood increase the risk of secondary cerebral ischemia,[19,20] we adjusted for these prognostic factors using their dichotomized values.

Results

The patients' characteristics are summarized in ***Table 1***. Secondary cerebral ischemia occurred in 49 (23.6%) patients and rebleeding in 28 (13.5%). The genotype frequencies are shown in ***Table 2***. The distribution of the Tyr204Phe factor XIII A and His95Arg factor XIII subunit B genotype frequencies were not in Hardy-Weinberg equilibrium.

Table 3 shows the HR for the risk of secondary cerebral ischemia and rebleeding according to genotype. The risk of secondary cerebral ischemia was lower for patients carrying the CC genotype on comparing patients carrying the CT and TT genotype with a crude HR of 0.6 (95% CI, 0.3 to 1.0) and an adjusted HR of 0.6 (95% CI, 0.3 to 1.0). However, this lower risk of secondary cerebral ischemia for patients carrying the *MTHFR* 677T variant is not seen on comparing patients with CC and CT genotype with patients with the TT genotype [Crude HR 0.4 (95% CI, 0.1 to 1.5) and adjusted HR 0.4 (95% CI, 0.1 to 1.5)].

Also no statistically significant effect of the remaining mutations on the risk of secondary cerebral ischemia and rebleeding could be demonstrated. Adjustment

1 (reference) 0.8 (0.4-1.4)	1 (reference) 0.8 (0.4-1.4)
1 (reference) 1.8 (0.8-4.2)	NA
1 (reference) 1.5 (0.8-2.8)	1.6 (0.8-2.9)
1 (reference) 1.1 (0.4-2.9)	NA

for prognostic factors for the risk of secondary cerebral ischemia after SAH did not change our observations.

Discussion

This study was performed to analyze whether the factor V Leiden, prothrombin G20210A, *MTHFR* C677T, Val34Leu, Tyr204Phe and Pro564Leu factor XIII subunit A, and His95Arg factor XIII subunit B mutations are associated with the occurrence of secondary cerebral ischemia and rebleeding in patients with aneurysmal SAH. We found no large effects of these investigated mutations in the genes influencing coagulation on the occurrence of secondary cerebral ischemia and rebleeding with aneurysmal SAH.

The risk of secondary cerebral ischemia was lower for patients carrying the *MTHFR* 677T variant on comparing patients with the CC genotype with patients with the CT and TT genotypes, but this effect could not be demonstrated anymore on comparing patients with the CC and CT genotypes with patients with the TT genotype. Previous studies showed that the *MTHFR* 677T mutation has no or a slightly increased risk of thrombosis. The TT genotype of the *MTHFR* 677T mutation is associated with higher homocysteine levels and thus believed associated with thrombosis and not with bleeding.[22] We therefore believe that the first observation is an unlikely finding and that the risk of secondary cerebral ischemia may not be influenced by the *MTHFR* C677T variant.

The distribution of the Tyr204Phe factor XIII A genotype frequencies was not in Hardy-Weinberg equilibrium. A possible explanation for this observation may be that Tyr204Phe factor XIII A polymorphism is associated with the risk of aneurysmal SAH. A case-control study of 42 women with non-fatal hemorrhagic stroke including 23 aneurysmal SAH patients and 345 controls found the Phe204 variant of Tyr204Phe factor XIII A to be associated with an increased risk of non-fatal hemorrhagic stroke.[18] Although the number of patients included in this study is small,[18] its results indeed support the hypothesis of an association of the Tyr204Phe factor XIII A polymorphism with the risk of aneurysmal SAH. However, future studies should further explore this possible association.

Aneurysmal SAH patients who died soon after admission could not be included in the present study, because they could not be asked to participate in this study. Our results therefore apply to a population of patients who survived the initial hours after the hemorrhage. Also, exclusion of these patients may have biased our results, as some of these patients may have died because of early rebleeding within hours of the initial hemorrhage,[23,24] leading to an under-representation of patients with rebleeding in our study.

A role for genetic factors involved in coagulation in the occurrence of secondary cerebral ischemia after SAH has been suggested in a previous study, showing the 4G genotype of the *PAI-1* 4G/5G polymorphism to be associated with the occurrence of secondary cerebral ischemia after SAH.[8] This effect was thought to be mediated through the higher PAI-1 levels associated with the 4G genotype, leading to formation of microthrombi and subsequent secondary cerebral ischemia.[8] In this study we were not able to further delineate the role of genetic factors involved in coagulation in the occurrence of secondary cerebral ischemia, as we could not demonstrate any large effects of several well-known and frequent mutations in other genes modifying coagulation on the occurrence of secondary cerebral ischemia nor on the occurrence of rebleeding after aneurysmal SAH.

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Chapter 14

General Discussion

The studies described in this thesis were carried out to identify genes involved in intracranial aneurysms and subsequent aneurysmal subarachnoid hemorrhage (SAH). This chapter discusses the results of these studies, relates them to findings by others and describes how, based on these results, future studies should be designed. In the first part of this chapter the disease intracranial aneurysms and aneurysmal subarachnoid hemorrhage (SAH) is discussed being a genetic disorder. In the second part the performance of genetic studies in general is discussed followed by a section on the evidence for the involvement of extracellular matrix (ECM) in the pathogenesis of intracranial aneurysms and aneurysmal SAH (part 3). In part 4 intracranial aneurysms and aneurysmal SAH as a complex disease is discussed with genetic risk factors interacting with environmental risk factors. In the fifth part of this chapter the involvement of genetic factors in complications and outcome after aneurysmal subarachnoid hemorrhage is described. Then whole genome association studies are being discussed as the future for genetic studies (part 6) and finally, general conclusions are drawn (part 7).

1. Intracranial aneurysms and aneurysmal subarachnoid hemorrhage (SAH) as a genetic disorder

1.1. Genetic risk factors in intracranial aneurysms and aneurysmal subarachnoid hemorrhage (SAH)

A higher concordance of a disease among relatives of patients than among relatives of controls or the general population are observations that lead to the conclusion that a disease is familial and possibly under genetic influence. Further support for a genetic influence in developing a disease may come from twin studies in which concordance rates for the disease are higher for monozyous than for dizygous twins.[1] Up to now, such twin studies have not been performed in intracranial aneurysms and aneurysmal SAH. However, a higher concordance among relatives has been demonstrated with first-degree relatives of patients with SAH having a two and a half to seven times greater risk of developing SAH than the general population.[2-7] Although it is known that the risk of having an aneurysm is the highest for siblings of SAH patients,[8] not much is known on the exact relative risk for these siblings. For the calculation of the sib relative risk (λ_s), which is a commonly used measure to indicate the genetic influence for a disease, this relative risk for these siblings is needed. One screening study identified intracranial aneurysms in 6% of the siblings of SAH patients.[8] Based on this percentage of 6% and a population prevalence of 2%,[9] the sib relative risk (λ_s) for developing intracranial aneurysms can be

estimated and is 3. Therefore, for this disease a genetic influence was also suggested and genetic studies have been undertaken to search for the genetic factors underlying the disease.

Genome wide linkage studies have up to now identified several suggestive loci for intracranial aneurysms (*i.e.* loci on chromosomes 1p34.3-p36.13,[10] 5q22-31,[11] 7q11,[11,12] 14q22,[11] 17cen,[11,13] 19q13.3,[14,15] and Xp22[13,14]). Although results of linkage studies generally appear difficult to replicate,[16] four of the identified loci for intracranial aneurysms have been replicated thus far (*i.e.* 7q11, 17cen, 19q13.3 and Xp22). In one of our studies of this thesis we were also able to replicate two of these intracranial aneurysm loci by performing a genome-wide linkage study in a single large pedigree with intracranial aneurysms: we observed suggestive linkage to a locus at 1p36.11-p36.13, and significant linkage to a locus at Xp22.2-p22.32 (*Chapter 10*). Interestingly, the chromosomal region 5q has also been identified in families with thoracic aortic aneurysms while the region 19q in families with abdominal aortic ones (*Chapter 11*). It may be worthwhile further exploring these regions in the different aneurysmal patients groups as common genetic risk factors for the different aneurysmal types may be possible (*Chapter 11*).

A genome-wide linkage study only points towards the regions in the genome that contain the disease-causing genes and not to these genes itself.[17] Such chromosomal regions often contain hundreds of genes, many of which may be plausible candidate genes for the disease. Thus, additional studies are needed to identify the disease-causing gene from such a region.[17] One of the most commonly used additional studies is an association study to test genetic variants in candidate genes of the chromosomal region (*i.e.* positional candidate gene). However, such association studies are not only used as complementary studies to linkage studies. They can also be performed as independent studies using candidate genes suspected of being involved in the disease based on their function (*i.e.* functional candidate gene). Based on linkage regions described by us and others, we tested 46 positional and functional candidate genes for their involvement in intracranial aneurysms and aneurysmal SAH described in this thesis. The versican (*CSPG2*) gene, located close to the chromosomal region at 5q22-31 (*Chapter 8*), the perlecan (*HSPG2*) gene, located in the region at 1p36.11-p36.13 (*Chapter 9*), the fibronectin (*FBN2*) gene, located in the 5q22-31 region (*Chapter 9*), the collagen type 4A1 (*COL4A1*) gene, found fragmentized in intracranial aneurysms, and the elastin (*ELN*) gene (*Chapter 7*) and the serpine1 (*PAI1*) gene, both located in the region at 7q11 (*Chapter 9*) appeared associated with intracranial aneurysms and aneurysmal SAH (although later the association for *ELN* was found to be false positive (*Chapter 9*)). Another possibility for the identification of disease-causing genes in chromosomal

regions identified by linkage studies is to screen candidate genes from this region for disease-causing mutations. Using this approach we plan to screen the coding regions and the intron-exon boundaries of the perlecan (*HSPG2*) gene for mutations in affected family members of the large family with intracranial aneurysms in *Chapter 10*.

1.2. Genetic heterogeneity and phenotype in intracranial aneurysms

Genetic complex diseases result from an interaction between genetic variants and environmental or non-genetic disease risk factors.[18] Because of the multi-factorial nature of the disease, each individual genetic variant generally has only a modest effect, and the interaction of genetic variants with each other (gene-gene interactions) or with environmental factors (gene-environment interactions) may attribute to the observed phenotype.[18] Thus, combinations of genetic and environmental factors will lead to the disease, but no factor alone is sufficient to cause it. Therefore, being a carrier of one or more genetic factors will not necessarily lead to an individual becoming affected by the disease, and there may be similar phenotypes caused by different combinations of genetic and environmental factors. The development of intracranial aneurysms and aneurysmal SAH is a complex disease, in which the genetic risk factors and environmental or non-genetic disease risk factors jointly contribute to the development of the disease (*Chapter 2*).

The search for disease-causing genes in complex diseases may be complicated by its genetic heterogeneity as these diseases are caused by variants in multiple genes. In intracranial aneurysms and aneurysmal SAH, genetic heterogeneity also plays a role since its segregation does not follow one strict Mendelian inheritance pattern in families,[19] and several different loci for intracranial aneurysms have already been identified in genome wide linkage studies.[10-15] Even in our single family analyzed in a whole genome study in which two loci (on chromosome 1p36.11-36.13 and Xp22.2-p22.32) were identified, the disease appears to be genetically heterogeneous and multi-genic (*Chapter 10*). Transmission of the locus 1p36.11-36.13 seems to follow a recessive mode of inheritance, while the locus Xp22.2-p22.32 seems to be inherited in a dominant mode.

Accurate definition of the phenotype of the disease is important in the performance of genetic studies.[18,20] In the genetic studies described in this thesis we tried to define a phenotype as homogeneous as possible with clear diagnostic criteria. Ruptured aneurysms are 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 aneurysms are identified by CT or MR angiography or conventional angiography. As approximately 85% of all

cases of SAH are caused by rupture of intracranial aneurysms, the remaining 15% of patients with other causes for their SAH such as trauma or infection were not included in our study.[21] Patients with underlying genetic syndromes associated with intracranial aneurysms, such as autosomal dominant polycystic kidney disease or connective tissue disorders such as Ehlers-Danlos disease,[22,23] were also not included. Despite defining this homogeneous phenotype for intracranial aneurysms, at this point we do not yet know whether all intracranial aneurysms may indeed be considered as having the same phenotype and thus the same genetic background. In *Chapter 4* of this study we investigated whether a difference in phenotype in patients with familial intracranial aneurysms exists between patients of families consistent with an autosomal dominant (AD) pattern of inheritance and patients of families where the pattern of inheritance was not suggestive of an AD transmission. However, phenotypes appeared similar in both types of families. Therefore, in future genetic studies on familial intracranial aneurysms, stratification according to this type of phenotype is not likely to be useful. Further questions on whether all intracranial aneurysms may be considered as the same genetic background do remain. For example, do the same genetic factors play a role in familial and non-familial or sporadic intracranial aneurysms and in ruptured and unruptured intracranial aneurysms? These questions will be addressed in the next two paragraphs.

The controls used in our genetic studies were ethnically matched Dutch Caucasian controls, comprising blood bank volunteers and unrelated controls selected from a database of healthy family members of patients with various diseases other than intracranial aneurysms. The control subjects have not been screened for unruptured intracranial aneurysms. As approximately 2% of the general population harbor an intracranial aneurysm,[9] undetected intracranial aneurysms are present in the control group. Inclusion by mistake of patients with aneurysms in the control group leads to dilution of any differences. Consequently, we may have underestimated the true difference of the found associations for the versican (*CSPG2*), perlecan (*HSPG2*), serpine1 (*PAI1*), fibronectin (*FBN2*) and the collagen type 4A1 (*COL4A1*) gene in patients compared to controls. Otherwise, in our study analyzing a large set of candidate genes for association with intracranial aneurysms, genes with relatively small effects may have been overlooked (*Chapter 9*).

1.3. Genetic heterogeneity between familial and non-familial or sporadic intracranial aneurysms?

Clinically, patients with non-familial intracranial aneurysms can be distinguished from patients with familial ones. Compared to non-familial intracranial aneurysms, patients with familial aneurysms have a hemorrhage from the aneurysm at a younger

age.[24] Furthermore, familial aneurysms are generally larger at time of rupture and more often multiple (*Chapter 3*) and located at the middle cerebral artery than sporadic aneurysms.[24] The fact that familial intracranial aneurysms differ clinically from non-familial ones, raises the question whether genetic factors also differ between familial and non-familial intracranial aneurysms. However, up to now, we do not know whether genetic heterogeneity exists between patients with familial and non-familial or sporadic intracranial aneurysms. In the Japanese population three different SNPs in the collagen type 1A2 gene showed significant differences in allelic frequencies between cases and controls with these differences in allelic frequency being more evident in patients with a positive family history.[25] In contrast, in the genetic study described in *Chapter 9* of this thesis, no large differences in allele frequencies of the genes that showed association could be observed between the Dutch patients with non-familial intracranial aneurysms and with familial ones. It is possible that the genes analyzed in our study indeed play a comparable role in non-familial and familial intracranial aneurysms. Otherwise, since patients that are now defined as non-familial may become familial cases in the future (as they may have first degree relatives who may harbor undetected intracranial aneurysms or may develop them in the future), theoretically, inclusion of these patients in the group of non-familial cases may have obscured true differences. Future studies should further assess the possible existence of genetic heterogeneity between familial and non-familial intracranial aneurysms. Ideally, to lower the bias of inclusion of familial patients in the non-familial group, the first degree relatives of a cohort of intracranial aneurysm patients should be screened for the presence of intracranial aneurysms. Based on the family history for intracranial aneurysms and the results of this screening study, the intracranial aneurysm patients can be divided in two groups of familial and non-familial patients, and genetic association studies can be performed comparing allele frequencies between the two groups.

1.4. Genetic heterogeneity between ruptured and unruptured intracranial aneurysms?

The discrepancy between the prevalence of intracranial aneurysms (approximately 2% in the general population[9]) and the incidence of aneurysmal rupture (based on an incidence of SAH of 8 per 100,000 person-years[26] and a population size in the Netherlands of approximately 16 million people, up to 1000 patients have an aneurysmal SAH per year), may lead to the hypothesis that some intracranial aneurysms are more prone to rupture than others. The ‘International Study of Unruptured Intracranial Aneurysms’ (ISUIA) provided clinical evidence for this hypothesis as

this study showed that risks of rupture were higher in large aneurysms (>7 mm), in aneurysms of the posterior circulation, and in patients with a previous episode of SAH from another aneurysm.[27] However, the results of this study are debated due to insufficient power of the study caused by small numbers in subgroups (*e.g.* the absence of rupture in some subgroups, such as small aneurysms of the anterior communicating artery).[28-30] Not much more is known about possible differences in pathogenesis, including genetic factors, between ruptured and unruptured aneurysms. The pathogenesis of the two different types of aneurysms may be different, resulting on the one hand in aneurysms that will eventually rupture, and on the other hand in aneurysms that remain intact and will not cause hemorrhage. Otherwise, the way the two types of aneurysms develop may be identical, and additional (possibly genetic) factors may increase the susceptibility of rupture of some of these aneurysms. A small study already suggested that polymorphisms in the endothelial nitric oxide synthase (*eNOS*) gene may identify intracranial aneurysms that are more prone to rupture.[31] Therefore, genetic heterogeneity may exist between ruptured and unruptured intracranial aneurysms. No differences in SNP frequency were observed in our association study between patients with ruptured and unruptured intracranial aneurysms (*Chapter 9*), but compared to the proportion of patients with ruptured aneurysms (84%), the proportion of patients with unruptured ones was low (16%). Thus, true differences in genetic factors contributing to the pathogenesis of the two types of aneurysms may not have been detected. Genetic association studies including large numbers of patients with ruptured as well as unruptured intracranial aneurysms, should further unravel the possible genetic differences between ruptured and unruptured aneurysms. Especially identification of genetic factors that increase the chance of aneurysms to rupture, will have great implications for the general practice of aneurysm patients. In case an unruptured intracranial aneurysm is identified, these genetic factors can then be used to estimate the risk of rupture of aneurysms, and based on this estimated risk decisions can be made whether or not preventive occlusion of the aneurysm is needed.

2. The performance of genetic studies

2.1. Genetic studies: use of single nucleotide polymorphisms (SNPs)

In general, there are two types of markers available for association and linkage studies: the microsatellites and single nucleotide polymorphisms (SNPs), with each type of marker having advantages as well as disadvantages. The current trend is to

Box: *Explanation of inflation of linkage scores in whole genome linkage study by high linkage disequilibrium between SNP markers.*

When SNPs are highly correlated they are called, in genetic terms, to be in linkage disequilibrium (LD). SNPs close to each other will generally be in high LD, and SNPs in high LD form so-called haplotypes. Within these haplotypes there is redundant information as by knowing an SNP in a haplotype, a prediction can be made about another highly correlated SNP in the same haplotype. Because of the high density of SNPs used in whole genome linkage studies, LD and areas of conserved haplotypes across the genome are observed. Since the programs used to calculate linkage scores assume linkage equilibrium between markers, the presence of LD may influence the results. If a proportion of SNPs in the marker set are in LD, one might expect that the programs used to calculate linkage scores would improve the accuracy of the linkage probabilities, resulting in higher linkage scores.

use the SNP markers with an estimated 10 million common SNPs present in the human genome. Although SNPs are biallelic and usually have lower heterozygosities than microsatellite markers, they are present at a greater density throughout the genome and are associated with lower genotyping error rates than the microsatellite markers.[18] Recent developments have made it possible to perform high-throughput and cost-effective SNP typing. Of these developments, the most relevant include the completion of the human genome sequence,[32,33] the identification of large numbers of SNPs with completion of linkage disequilibrium patterns between them by the human 'Haplotype Map' (HapMap) project,[34] and the development of rapid high-throughput methods, enabling to type thousands of SNPs across the genome quickly and economically. The assessment of the linkage disequilibrium patterns enables the selection of efficient sets of markers. In this study we also successfully performed an association study in which we analyzed an efficiently chosen set of 384 tag SNP markers, using a high-throughput SNP genotyping facility (*Chapter 9*). As in this study a total of almost 1,000 intracranial aneurysm patients and controls were included, nearly 400,000 genotypes were generated and analyzed with ease and within a reasonable amount of time.

Currently, such large sets of SNP markers are predominantly used for the performance of association studies, but it is also becoming apparent that compared with maps composed of microsatellite markers, maps of SNPs offer superior power to detect linkage.[35,36] We also performed a whole genome linkage study, using SNPs in a large pedigree with intracranial aneurysms identifying two chromosomal regions (*Chapter 10*). When performing such whole genome linkage study with SNPs, one has to be cautious in interpreting the results as the presence of high

linkage disequilibrium between SNP markers can cause inflation of linkage scores (see ***Box*** for further explanation), which may lead to false-positive results.[37] On the contrary, the linkage disequilibrium between SNP markers also generates advantages. The information on the linkage disequilibrium between the SNPs genotyped in a whole-genome linkage study, can subsequently be applied in further studies that use linkage disequilibrium mapping, to search for the causal variant in the regions of interest identified in the linkage study.[36]

2.2. Genetic studies: chance of finding false-positive results

An important problem in association studies is the chance of finding false-positive results, and consequently failure to replicate the identified association between variants and disease.[38] False positive results may for example arise from biases in the study design or from statistical fluctuation. With the use of p-values below 0.05 as a criterion for statistical significance, 5% of the studies are expected to have a p-value below 0.05 in the absence of a true association. With each test, the possibility of a false-positive result increases. Most methods used to correct for multiple comparisons, such as the Bonferroni correction, are considered too stringent. Therefore, it has been proposed that study designs should include a replication set of samples, designed to eliminate false positive findings and highlight true findings.[35,39] The importance of replication of positive results to confirm true findings also became apparent in our study. The observed association of the elastin gene with aneurysmal SAH (*Chapter 7*) could not be confirmed in a large replication cohort (*Chapter 9*), and therefore the observed association is a likely false positive finding. In contrast, the association of the versican (*CSPG2*) (*Chapter 8*), perlecan (*HSPG2*), serpine1 (*PAI1*), fibronectin (*FBN2*) and collagen type 4A1 (*COL4A1*) (*Chapter 9*) genes with intracranial aneurysms could be confirmed in a replication cohort, emphasizing that these observed associations may indeed be true findings.

3. Evidence for the involvement of extracellular matrix (ECM) in the pathogenesis of intracranial aneurysms and aneurysmal SAH

In association studies one can use a hypothesis-based approach analyzing functional and positional candidate genes. Otherwise, a genome-wide association study is an hypothesis-free approach, for which no knowledge on the disease mechanism is needed. In this kind of studies, every gene in the genome is considered as a potential candidate gene. At the start of our studies as presented in this thesis, this

kind of genome-wide association studies was not yet feasible as it was still expensive and raised a number of statistical issues. Therefore, we decided upon association studies analyzing functional and positional candidate genes, based upon the hypothesis that diminished maintenance of the ECM of the arterial wall is important in the development of intracranial aneurysms. In the genetic studies of this thesis this hypothesis is strengthened. We found genes involved in the maintenance of the integrity of the ECM of the arterial wall associated with intracranial aneurysms (*Chapters 8 and 9*). Besides the ECM genes serpine1 (*PAI1*), fibronectin (*FBN2*) and collagen type 4A1 (*COL4A1*), two genes coding for ECM proteoglycans, versican (*CSPG2*) and perlecan (*HSPG2*), also appeared to be associated with intracranial aneurysms. The ECM is composed of collagen and elastin fibers embedded in glycoproteins and proteoglycans.[40] The two largest groups of proteoglycans of the ECM are the chondroitin sulfate proteoglycans to which versican belongs, and the heparan sulfate proteoglycans which includes perlecan.[41] Both groups of proteoglycans have various important protein binding domains with which they are able to interact with diverse molecules, including key components of the ECM.[41,42] The proteoglycans of the ECM in general may play an important role in the pathogenesis of intracranial aneurysms. Its contribution to the pathogenesis may be explained by the loss of function of the proteoglycans to interact with other ECM components, leading to weakening of the arterial wall and consequently to aneurysm formation (*Chapter 6*). In the genetic studies of this thesis we have identified SNPs in versican (*CSPG2*) and perlecan (*HSPG2*) that are associated with intracranial aneurysms, but by doing that we have not yet identified the causal variants in these genes. To search for these causal variants many polymorphisms should be tested in these genes, as linkage disequilibrium between the polymorphisms and the causal variant may exist over long regions. Therefore, the association pattern in the gene and the region surrounding the gene should be determined before the causal variant can be identified. Otherwise these genes may be sequenced directly in search of the causal variant, as is already discussed for the perlecan (*HSPG2*) gene in the first paragraph of the general discussion 'Genetic risk factors in intracranial aneurysms and aneurysmal subarachnoid hemorrhage (SAH)'.

Once the genetic variants are identified in the genes coding for the proteoglycans, the consequences of these variants on its function and binding capacities should be elucidated in future functional studies. However, it is not easy to perform these functional studies in intracranial aneurysms, as the affected tissue of intracranial aneurysms is not easily accessible. Biopsies of intracranial aneurysms can only be obtained during surgery. Furthermore, during surgery these biopsies are not routinely taken and as intracranial aneurysms are generally small in size (<10 mm)[9],

taking biopsies is not always possible. Instead of the intracranial aneurysm biopsies, the proteoglycan functions may possibly be studied in skin biopsies of intracranial aneurysm patients. Connective tissue alterations have already been shown in skin biopsies from patients with intracranial aneurysms,[43] which may suggest that intracranial aneurysms is not a localized but a generalized disruption of the connective tissue and the ECM. In a clinical study of this thesis, describing the comparison of subtle manifestations of connective tissue or ECM disease between patients with aneurysmal SAH and controls (*Chapter 5*), further evidence is found that aneurysm patients have a generalized disruption of the connective tissue. Patients with ruptured intracranial aneurysms appeared to have a higher degree of joint mobility compared to healthy controls. Similarities in connective tissue composition between the ligaments, tendons and capsules of joints and intracranial arteries[44] may explain the findings with respect to the higher degree of joint mobility of patients with ruptured intracranial aneurysms. Future studies exploring the changes of the connective tissue and ECM in skin biopsies of intracranial aneurysm patients, are warranted to further test the hypothesis of a general disruption of the ECM.

4. Intracranial aneurysms and aneurysmal SAH as a complex disease: genetic risk factors interacting with environmental risk factors

The significance of the contribution of environmental risk factors to complex diseases is well recognized.[45] The public health significance of a particular risk factor can be determined by its population attributable risk, defined by the proportion of disease incidence that can be reduced if a specific genetic or environmental risk factor is eliminated. The population attributable risk is influenced by the prevalence and relative risk of the risk factor. In *Chapter 2* the population attributable risks of the risk factors known at the start of the study were calculated, and it was concluded that reduction of the incidence of aneurysmal SAH seemed best achieved by influencing the modifiable environmental risk factors, *i.e.* alcohol consumption, smoking, and hypertension, since these are the major contributors to aneurysmal SAH in the general population, with a combined population attributable risk for these three factors of almost 50%. Approximately 10% of the aneurysmal SAH cases could be attributed to a positive family history for aneurysmal SAH. This increased familial risk suggests genetic factors to be involved and some of these genetic factors were further defined in the course of this study. For these factors the population attributable risks were also calculated. These risks are 10% for the

versican (*CSPG2*) gene (*Chapter 8*), 19% for the perlecan (*HSPG2*) gene, 6% for the serpine1 (*PAI1*) gene, 3% for the fibrillin 2 (*FBN2*) gene and 7% for the collagen type 4A1 (*COL4A1*) gene (*Chapter 9*). Adding up the population attributable risks of all the genetic and environmental risk factors identified thus far leads to a total of >100%, which further supports the notion that intracranial aneurysms and aneurysmal SAH is complex in origin and suggests gene-gene, gene-environment and/or environment-environment interactions to be involved.

It is important to further explore these possible interactions; especially the knowledge on the interaction between genes and environment will have great implications for the treatment and counseling of intracranial aneurysm patients and their relatives. This can be illustrated by a hypothetical case in which a high risk genotype will only lead to the development of intracranial aneurysms in case of the additional environmental risk factor hypertension. Relatives of intracranial aneurysm patients who have this high risk genotype and who do not have been diagnosed with intracranial aneurysms yet should then be controlled for their blood pressure. To study these gene-environment interactions, case-control studies may be used comparing cases and controls with different combinations of the genotype under study and exposure to the environmental risk factor (*i.e.* with the genotype and the exposure, with the genotype but without the exposure, without the genotype but with the exposure, and without genotype and exposure).[46] Although there is an ongoing debate on the power of these case-control studies,[47,48] it is well known that a large number of cases and controls are needed. A simple rule that can be used to get an impression of the number of patients needed, says that the sample size necessary is at least four times the sample size needed to study the effect of one of the risk factors.[49] Given the fact that environmental risk factors are often measured with some error, the sample size should be even larger.[50,51] Therefore, to explore the gene-environment interactions in intracranial aneurysm patients large multicentre patient cohorts are needed.

5. Genetic factors in complications and outcome after aneurysmal subarachnoid hemorrhage

Genetic factors may not only play an important role in the development of intracranial aneurysms and subsequent hemorrhage, but also in the occurrence of frequent and life threatening complications after aneurysmal rupture, such as secondary cerebral ischemia and rebleeding and in outcome after SAH. For example, in patients with the same amount of SAH on computer tomography (CT) scanning of the brain

(a large amount of SAH on CT is an important risk factor for the development of secondary cerebral ischemia[52,53]), there is a large variability as to whether or not secondary cerebral ischemia will develop. This raises the possibility that genetic factors are involved. Also, the fact that there are large inter-individual differences in recovery after aneurysmal SAH, although aneurysmal SAH patients are treated the same, may suggest the involvement of genetic factors. Up to now, relatively few studies have been performed assessing this possible genetic involvement. Two previous studies suggested genetic variants to be involved in the development of secondary cerebral ischemia.[5455] One study suggested a polymorphism in the plasminogen activator inhibitor-1 (*PAI1*) gene, associated with the occurrence of secondary cerebral ischemia after SAH,[54] while the other showed a polymorphism in the endothelial isoform of nitric oxide synthase (*eNOS*) to be associated.[55] As discussed in *Chapter 12* of this thesis, we found indication that variation in some genes that are expressed after cerebral ischemia, may partly explain the large differences in outcome between patients with aneurysmal SAH. SAH patients, homozygous for the insulin-like growth factor-1 (*IGF-1*) wild type allele and carriers of the tumor necrosis factor-A (*TNF-A*) non-wild type allele, both had a higher risk of poor outcome. We found no large effects of genetic parameters influencing coagulation, on the occurrence of secondary cerebral ischemia and rebleeding in patients with aneurysmal SAH (*Chapter 13*).

However, the results should be interpreted with caution since the sample size was relatively small in both our studies, and this may have prevented us to identify genetic factors with small effects. The results of a recent meta-analysis with pooled data, including the data described in *Chapter 12*, on the effect of the apolipoprotein E (*APOE*) genotype on recovery after aneurysmal SAH, support this concern.[56] In this meta-analysis a harmful effect of the *APOE* E4 allele in patients with SAH was found, while we were not able to demonstrate this effect analyzing the data described in *Chapter 12* alone. Thus, further studies are needed to assess the validity of our findings. Nevertheless, our studies and the previous studies published in the literature[54,55] do underline the possibility of a genetic involvement in the development of complications of the hemorrhage and recovery after aneurysmal SAH. These genetic factors should be further explored in studies including larger samples of aneurysmal SAH patients. These studies may increase the knowledge on the pathogenesis of the complications and the recovery after aneurysmal SAH. This knowledge may contribute to the optimization of treatment strategies, aiming to improve the recovery after aneurysmal SAH and to prevent secondary cerebral ischemia and rebleeding, as these complications are still major causes of death and disability after aneurysmal SAH.[57-60] For example, if in a SAH patient genetic factors, in-

cluding the *IGF-1*, *TNF-A* and *APOE* genotypes, are identified that together can predict a poor outcome, this patient should be treated more aggressively compared to a patient in whom a good outcome is predicted. Otherwise, the identification of high-risk genotypes for rebleeding may help in the decision making for early occlusion of ruptured intracranial aneurysms.

6. The future: whole genome association studies

Although genome-wide association studies using SNPs were not yet feasible at the start of the studies as presented in this thesis, they are now being used as a powerful approach to identify the genes that underlie complex diseases.[61-64] These genome-wide association studies can be performed because of the recent developments already discussed, such as the completion of the human genome sequence, the completion of linkage disequilibrium patterns between SNPs, and the development of rapid high-throughput SNP genotyping methods. Approximately 300,000 to 500,000 SNPs will provide genome wide coverage.[65] Naturally, such a large number of SNPs that needs to be tested for association, again raises the statistical issue of multiple comparison. Consistent replication addressing the same variant and phenotype, is also for genome-wide association studies considered the best test to correct for false positive results.[16] Performing genome-wide association studies in intracranial aneurysms, may point to additional genetic factors associated with the pathogenesis of intracranial aneurysms. These genetic factors may include other important key players involved in the ECM. Otherwise, these studies may identify factors of other important pathways also involved in the development of intracranial aneurysms. For example, the inflammatory cascade has recently been proposed as a possible contributor to the intracranial aneurysmal pathogenesis as well.[66] To perform these genome-wide association studies large number of patients and controls are needed. The HumanHap500 Genotyping BeadChip of Illumina including 500 K SNPs will become available soon (www.illumina.org). Using this chip and expecting to identify genes with moderate size effects (relative risks of approximately 1.4) with 85% power, then at least 1000 cases and controls should be included in such a genome-wide association study.[67] Again, large multicentre patient cohorts are needed to make this kind of study possible.

7. Conclusion

The main outcome of the studies described in this thesis is that more insight in the genes involved in the pathogenesis of intracranial aneurysms and subsequent aneurysmal SAH has been obtained. By the identification of these genetic factors, an increasing understanding of the pathogenesis of the disease has been realized. The genes identified so far to be associated with intracranial aneurysms and aneurysmal SAH are involved in the maintenance of the integrity of the ECM of the arterial wall. In a clinical part of this study it is suggested that intracranial aneurysms are not a localized disease, but rather represent a more general disease of the ECM, which suggestion may open possibilities to further study the biological consequences of the identified genetic variants. The identification of these genetic factors may in the future lead to new therapeutic interventions to help prevent the development, growth and/or rupture of intracranial aneurysms. The identification of genetic factors that increase the chance of aneurysms to rupture, will help to differentiate between high and low risk unruptured intracranial aneurysms. These risk profiles can help in the decision making for preventive treatment of unruptured aneurysms. Before this is possible, complete understanding of the genetic factors with their biological functions is needed. Finally, with the identification of the genetic factors involved, diagnostic tools using high-risk genotypes can be developed to identify first-degree relatives of SAH patients with a high risk of developing one or more intracranial aneurysms. These first-degree relatives can be screened for aneurysms regularly and those not at high risk can be reassured.

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Summary

The principal aim of this thesis was to identify genes involved in intracranial aneurysms and subsequent aneurysmal subarachnoid hemorrhage (SAH). These genes were identified using a study population with a familial preponderance of the disease. In *Part I* of the thesis this study population and its familial risk factor are described. *Part II* describes the identification of genes, especially those implicated in the maintenance of the integrity of the extracellular matrix (ECM) of the arterial wall, that are involved in the occurrence of intracranial aneurysms and aneurysmal SAH. *Part III* explored the utilization of genetic factors in clinical studies on aneurysmal SAH. Especially factors involved in the occurrence of rebleeding and secondary cerebral ischemia after aneurysmal SAH and in outcome after secondary cerebral ischemia were investigated.

Part I: Clinical Studies on Intracranial Aneurysms and Aneurysmal SAH

Chapter 2 outlined the population attributable risks (PAR) of the known risk factors, including the risk factor of a positive family history, for aneurysmal SAH. The modifiable risk factors alcohol consumption (PAR of 11%), smoking (20%), and hypertension (17%) were the major contributors to SAH in the general population. Patients with familial preponderance of SAH (11%) and with autosomal dominant polycystic kidney disease (ADPKD) (0.3%) had less influence on the incidence of SAH.

Chapter 3 describes the comparison of characteristics of intracranial aneurysms (*i.e.* size and number) in patients with a familial form of aneurysmal SAH and those with the non-familial or sporadic form. Familial aneurysms were generally larger at time of rupture (RR, 2.1; 95% CI, 1.2 to 3.6) and more often multiple (RR, 2.5; 95% CI, 1.2 to 5.4) than non-familial or sporadic aneurysms.

In *Chapter 4*, demographic and clinical features in patients of families with familial intracranial aneurysms and different patterns of inheritance were compared. In addition, the ages of patients with SAH in affected parent-child pairs were compared, since previous studies suggested anticipation. Between families, compatible with an autosomal dominant (AD) pattern of inheritance, and those not compatible with an AD pattern of inheritance, no differences in demographic or clinical features were found. In families with affected members in two successive generations, the age at time of SAH in parents was significantly higher than in their children (55.2 versus 35.4 years, mean difference 19.8 years, $p<0.001$).

Chapter 5 describes the comparison of subtle manifestations of connective tissue or the ECM disease between patients with aneurysmal SAH and controls, to assess whether intracranial aneurysms is not a localized disruption, but may rather represent a more general laxity of the connective tissue and ECM. Patients with

ruptured intracranial aneurysms had a higher degree of joint mobility compared to controls. No differences were found between patients and controls for the other investigated subtle manifestations of connective tissue or ECM disease.

Part II: Genetic Studies on Intracranial Aneurysms and Aneurysmal SAH

Chapter 6 gives an overview of the current knowledge on the genetic background of intracranial aneurysms from the literature. The two most common approaches used to identify genes, *i.e.* association studies and genome-wide linkage studies, and the results of these studies in intracranial aneurysms were discussed. It was explained that genes involved in the maintenance of the integrity of the ECM are likely to be involved in the pathogenesis of intracranial aneurysms. Up to now, four genome-wide linkage studies have identified genetic loci for intracranial aneurysms. Three of these loci include interesting functional candidate genes coding for structural proteins of the ECM. Some of those candidate genes have been further explored and allelic association with intracranial aneurysms has been demonstrated.

Chapter 7 describes the results of an association study analyzing the functional and positional candidate gene elastin (*ELN*), coding for a structural protein of the ECM, for association with aneurysmal SAH. Haplotypes constructed of single nucleotide polymorphisms (SNP) in intron 5/exon 22 (OR, 2.6; 95% CI, 1.2 to 5.8; p=0.002), intron 4/exon 22 (OR, 2.8; 95% CI, 1.5 to 5.4; p=0.02), and intron 4/ intron 5/exon 22 (OR, 2.9; 95% CI, 1.7 to 4.8; $p=9.0x10^{-9}$) were found to be associated with aneurysmal SAH.

In *Chapter 8*, another functional and positional candidate gene versican (*CSPG2*), also coding for a structural protein of the ECM, was explored for association with intracranial aneurysms. Several SNPs in strong linkage disequilibrium and haplotypes constituting these SNPs, were found to be associated with intracranial aneurysms (strongest SNP association OR, 1.34; 95% CI, 1.09 to 1.65; p=0.004). The association for the two SNPs with the most significant associations was confirmed in a second independent cohort of patients (strongest SNP association OR, 1.36; 95% CI, 1.11 to 1.67; p=0.003).

In *Chapter 9*, a large set of 44 additional candidate genes, involved in the maintenance of the integrity of the ECM, was analyzed for association with intracranial aneurysms. In fifteen of these 44 genes, SNPs associated with intracranial aneurysms ($p<0.05$) were found with SNPs in serpine1 (*PAI1*, p=0.0008), transforming growth factor, beta induced (*TGFBI*, p=0.0026) and perlecan (*HSPG2*, p=0.0044) having the strongest associations. In a second independent cohort, the association for *HSPG2* (combined OR, 1.33; 95% CI, 1.13 to 1.57; p=0.0006) was replicated.

Combining the two cohorts, the associations for the *PAI1* (combined OR, 1.27; 95% CI, 1.07 to 1.50; p=0.004), *FBN2* (combined OR, 1.37; 95% CI, 1.07 to 1.75; p=0.01) and *COL4A1* (combined OR, 1.22; 95% CI, 1.05 to 1.42; p=0.007) genes remained. The association of SNPs in elastin (*ELN*), as found in *Chapter 7*, could not be confirmed.

Chapter 10 describes the results of a genome-wide linkage study in a large Dutch family with intracranial aneurysms. Suggestive linkage was shown to a locus on chromosome 1 with a non-parametric linkage score (NPL) of 3.18 at 1p36.11-p36.13, and significant linkage was shown to a locus on chromosome X with an NPL of 4.54 at Xp22.2-p22.32. These loci overlap with loci that have already been identified in previous linkage studies from different populations.

Chapter 11 describes a literature study, in which the genomic loci identified in whole genome linkage studies on intracranial, thoracic and abdominal aortic aneurysms were described and compared, in search of possible common genetic risk factors for the three types of aneurysms. Five chromosomal regions on 3p24-25, 4q32-34, 5q, 11q24 and 19q were identified that may play a role in the pathogenesis of two or more aneurysmal types.

Part III: Genetic Studies on Secondary Cerebral Ischemia. Rebleeding and Outcome after Aneurysmal SAH

In *Chapter 12*, genes that are expressed after ischemia and that influence recovery after ischemia in the animal model of ischemia, were explored for a relationship with outcome in patients after aneurysmal SAH. Patients carrying any insulin-like growth factor-1 (*IGF-1*) non-wild type allele, had a lower risk of a poor outcome (OR, 0.4; 95% CI, 0.2 to 1.0), while carriers of the tumor necrosis factor-A (*TNF-A*) non-wild type allele, had a higher risk (OR, 2.3; 95% CI, 1.0 to 5.4).

Chapter 13 analyzed whether genes modifying coagulation were associated with risk of secondary cerebral ischemia and rebleeding after aneurysmal SAH. No association of the factor V Leiden, prothrombin G20210A, methylenetetrahydrofolate reductase (*MTHFR*), Val34Leu, Tyr204Phe and Pro564Leu factor XIII subunit A, and His95Arg factor XIII subunit B mutations and polymorphisms, could be demonstrated with the occurrence of secondary cerebral ischemia and rebleeding in patients with aneurysmal SAH.

Samenvatting

Een bloeding tussen de hersenvliezen (subarachnoïdale bloeding of SAB) is een vorm van beroerte die vaak op jonge leeftijd optreedt en meestal ernstige gevolgen heeft: 50% van alle patiënten overlijdt en van de patiënten die overleven, houdt de helft invaliderende restverschijnselen. Een dergelijke bloeding ontstaat meestal uit een gebarsten bloedvat-uitstulping (aneurysma) in het hoofd. Het is niet goed bekend hoe dergelijke aneurysmata ontstaan. We weten wel dat naast hoge bloeddruk en roken, ook erfelijke factoren een belangrijke rol spelen. Eerste graads familieleden van een patiënt met een SAB uit een aneurysma, hebben een tot 7 maal verhoogde kans om ook zo'n bloeding te krijgen. Bovendien komt bij 10% van de patiënten de aandoening in de familie voor. Er zijn aanwijzingen dat binnen de groep van erfelijke factoren, een rol wordt gespeeld door factoren die betrokken zijn bij de opbouw en afbraak van de extracellulaire matrix (ECM) van de bloedvaten. Deze ECM zorgt voor stevigheid en soepelheid van de bloedvaten. Wanneer bekend is welke erfelijke factoren een rol spelen bij het ontstaan van SAB en aneurysmata in het hoofd, zal men beter kunnen begrijpen hoe deze bloedingen en aneurysmata precies ontstaan. Met die kennis kunnen mogelijk in de toekomst nieuwe behandelingen worden ontwikkeld, waarmee het ontstaan, de groei en/of bloeding van aneurysmata voorkomen kan worden. Als bekend is welke stoornissen in het erfelijke materiaal een rol spelen bij aneurysmata in het hoofd, kunnen bovendien familieleden van patiënten met een SAB uit een aneurysma op een eenvoudige manier onderzocht worden. Met het onderzoek kan vastgesteld worden of deze familieleden een verhoogde kans hebben op het ontwikkelen van aneurysmata. De familieleden met een verhoogde kans kunnen vervolgens regelmatig een röntgenonderzoek krijgen, waarbij gezocht wordt naar aneurysmata. Wanneer op deze manier een aneurysma tijdig ontdekt wordt, kan het behandeld worden voordat een bloeding optreedt.

Het belangrijkste doel van de studies beschreven in dit proefschrift was het in kaart brengen van de erfelijke factoren die betrokken zijn bij aneurysmata in het hoofd en SAB uit een aneurysma. Deze erfelijke factoren werden bestudeerd met behulp van een groep patiënten bij wie aneurysmata familiair voorkomen. In *Deel I* van dit proefschrift wordt deze groep patiënten en de familiaire risicofactor beschreven. *Deel II* beschrijft het in kaart brengen van de erfelijke factoren die een rol spelen bij het ontstaan van aneurysmata en SAB uit aneurysmata . Hierbij werden met name die factoren, die betrokken zijn bij de opbouw en afbraak van de ECM van de bloedvaten, bestudeerd. *Deel III* exploreert het gebruik van erfelijke factoren in klinische studies met SAB patiënten. Met name die erfelijke factoren werden onderzocht, die betrokken zijn bij het optreden van complicaties na SAB uit een aneurysma en bij het herstel na het doormaken van secundaire cerebrale

ischemie. De complicaties die we bestudeerden zijn een nieuwe bloeding na de SAB, ook wel recidief bloeding genoemd, en samenkramping van de vaten in reactie op de bloeding, wat een verminderde doorbloeding van het hersenweefsel tot gevolg heeft (secundaire cerebrale ischemie).

Deel I: Klinische Studies bij Aneurysmata in het Hoofd en SAB uit een Aneurysma

Hoofdstuk 2 beschrijft de risicofactoren die bijdragen aan het ontstaan van SAB uit een aneurysma. Voor deze risicofactoren (inclusief de familiaire risicofactor) zijn in dit hoofdstuk de zogenaamde populatie attributieve risico's (PAR) berekend. Met een PAR van een risicofactor wordt aangegeven welk percentage van de ziekte door deze risicofactor wordt veroorzaakt. De te modificeren risicofactoren alcohol consumptie (PAR van 11%), roken (20%) en hypertensie (17%) blijken de voornaamste factoren voor het ontstaan van SAB in de algemene bevolking. Het familiair voorkomen van SAB (11%) of het voorkomen van de nierziekte "autosomal dominant polycystic kidney disease (ADPKD)" (0.3%) draagt minder duidelijk bij aan het ontstaan van SAB.

Hoofdstuk 3 beschrijft het vergelijken van kenmerken (namelijk grootte en aantal) van aneurysmata in het hoofd bij patiënten met een familiaire vorm van SAB en bij patiënten met een niet-familiaire vorm. Familiaire aneurysmata blijken over het algemeen groter wanneer ze barsten en een bloeding veroorzaken. Daarnaast blijkt dat bij patienten met een familiair aneurysma vaker meerdere aneurysmata tegelijkertijd aanwezig zijn, dan bij patienten met een niet-familiair aneurysma.

In *Hoofdstuk 4* werden de demografische en klinische kenmerken van patiënten met familiaire aneurysmata in het hoofd vergeleken bij families waarin de ziekte op een verschillende manier overerft. Hierbij werden de kenmerken van patiënten vergeleken tussen families waarin de ziekte op een zogenaamde autosomaal dominante (AD) manier (een aangedane ouder geeft de ziekte aan 50% van de kinderen door) overerft en families waarin de ziekte op een andere manier overerft. Omdat eerdere studies aanwijzingen vonden dat kinderen op een jongere leeftijd een SAB kregen dan hun aangedane ouder, werden bovendien de leeftijden vergeleken waarop de patiënten in deze families een SAB kregen in aangedane ouder-kind paren. Het steeds vroeger in het leven optreden van een erfelijke ziekte wordt anticipatie genoemd. Tussen families met een AD manier van overerving en families met een andere manier van overerving werden geen verschillen in demografische en klinische karakteristieken gevonden. In families met aangedane familieleden in twee opeenvolgende generaties (de aangedane ouder-kind paren) blijkt de leeftijd waarop de SAB optreedt hoger in de aangedane ouders dan in de

kinderen. In deze studie werden dus aanwijzingen voor anticipatie gevonden.

Hoofdstuk 5 beschrijft het vergelijken van subtiele uitingen van bindweefselaandoeningen tussen patiënten met een SAB uit een aneurysma en gezonde controles, om te onderzoeken of een aneurysma in het hoofd een lokale aandoening van de hersenvaten is, of een meer gegeneraliseerde aandoening van het bindweefsel in het hele lichaam. Patiënten met een SAB uit een aneurysma bleken soepelere gewrichten te hebben vergeleken met gezonde controles. De andere onderzochte subtiele uitingen van bindweefselaandoeningen lieten geen verschil zien tussen patiënten en controles.

Deel II: Studies naar de Erfelijke Factoren van Aneurysmata in het Hoofd en SAB uit een Aneurysma

Hoofdstuk 6 geeft een literatuuroverzicht van de huidige kennis over de erfelijke achtergrond van aneurysmata in het hoofd. De twee meest gebruikte methoden om erfelijke factoren in kaart te brengen, zijn de associatie studies en genoom-wijde linkage of koppelings-onderzoeken. In een associatie studie werd de associatie tussen een ziekte en een specifieke variant in een gen tussen patiënten en gezonde controles onderzocht. Wanneer associatie van een gen met een ziekte wordt aangetoond, houdt dat in dat een bepaalde variant in een gen vaker voorkomt bij patiënten met de ziekte dan bij gezonde controles. In een koppelings-onderzoek werd binnen een familie waarin een ziekte voorkomt de overerving van diverse genetische varianten met de ziekte onderzocht. Als een bepaalde variant met de ziekte overerft ligt deze variant waarschijnlijk in de buurt van het gen dat de ziekte veroorzaakt. De resultaten van deze studies, uitgevoerd bij patiënten met aneurysmata in het hoofd, worden besproken. Bovendien wordt in dit hoofdstuk uitgelegd dat het aannemelijk is dat erfelijke factoren die betrokken zijn bij de opbouw en afbraak van de ECM van de bloedvaten, ook betrokken zijn bij het ontstaan van aneurysmata in het hoofd. Tot nu toe hebben vier koppelings-onderzoeken meerdere gebieden in het erfelijk materiaal geïdentificeerd die mogelijk betrokken zijn bij het ontstaan van aneurysmata in het hoofd. Drie van deze geïdentificeerde gebieden in het erfelijk materiaal bevatten interessante genen die betrokken zijn bij de opbouw en afbraak van de ECM. Een aantal van deze genen zijn al verder onderzocht op betrokkenheid bij aneurysmata in het hoofd. Associatie met aneurysmata in het hoofd is aangetoond voor enkele van deze genen.

Hoofdstuk 7 beschrijft de resultaten van een associatie studie waarin het gen elastine (*ELN*), dat codeert voor een belangrijk eiwit van de ECM, werd onderzocht op associatie met SAB uit een aneurysma. Bepaalde varianten in dit gen (zogenaamde single nucleotide polymorphisms of SNPs) en combinaties van deze

varianten (zogenaamde haplotypes) blijken een associatie te hebben met SAB uit een aneurysma.

In *Hoofdstuk 8* werd een ander gen versican (*CSPG2*), dat ook codeert voor een belangrijk eiwit van de ECM, onderzocht op associatie met aneurysmata in het hoofd. Verschillende SNPs die nauw met elkaar gecorreleerd zijn en haplotypes van deze SNPs blijken geassocieerd met aneurysmata in het hoofd. De associatie van de twee SNPs die het sterkst geassocieerd zijn werd bevestigd in een tweede onafhankelijke groep van patiënten.

In *Hoofdstuk 9* werden 44 andere genen, die mogelijk ook betrokken zijn bij de opbouw en afbraak van de ECM, onderzocht op associatie met aneurysmata in het hoofd. In 15 van deze 44 genen werden SNPs geïdentificeerd die geassocieerd blijken met aneurysmata in het hoofd, waarbij de sterkste associaties werden gevonden voor SNPs in serpine1 (*PAI1*), transforming growth factor, beta induced (*TGFBI*) en perlecan (*HSPG2*), fibronectin (*FN1*), fibrillin 2 (*FBN2*) en collagen 4A1 (*COL4A1*). In een tweede onafhankelijke groep van patiënten en controles werd de associatie voor *HSPG2* bevestigd. Bij het gezamenlijk analyseren van de twee groepen patiënten en controles bleven de associaties voor *PAI1, FBN2* en *COL4A1* bestaan. De eerder gevonden associatie van SNPs in elastine (*ELN*) (*Hoofdstuk 7*) kon in deze studie niet worden bevestigd.

Hoofdstuk 10 beschrijft de resultaten van een koppelings-onderzoek in een grote familie waarbij meerdere familieleden aneurysmata in het hoofd hebben. Er werden twee gebieden in het erfelijk materiaal geïdentificeerd die een rol lijken te spelen bij het ontstaan van aneurysmata in het hoofd in deze familie. Het eerste gebied werd gevonden op chromosoom 1 en het tweede gebied op het X chromosoom. Deze twee gebieden overlappen met gebieden in het erfelijk materiaal die al eerder in andere studies waren geïdentificeerd.

Hoofdstuk 11 bevat een literatuurstudie waarin de gebieden in het erfelijk materiaal geïdentificeerd in koppelings-onderzoeken worden beschreven bij drie groepen patiënten: patienten met aneurysmata in het hoofd, patienten met aneurysmata aan de grote lichaamsslagader (aorta) in de borstholte en patienten met aneurysmata aan de grote lichaamsslagader in de buikholte. Deze gebieden werden tussen de drie verschillende patiënten groepen vergeleken, met als doel mogelijke overeenkomstige erfelijke risicofactoren te identificeren voor de drie typen aneurysmata. Er werden vijf gebieden in het erfelijk materiaal (op chromosoom 3, 4, 5, 11 en 19) geïdentificeerd die mogelijk een rol spelen bij het ontstaan van twee of meer typen aneurysmata.

Deel III: Studies naar de Erfelijke Factoren betrokken bij Complicaties en Herstel na SAB uit een Aneurysma

In *Hoofdstuk 12* werd onderzocht of er genen geassocieerd zijn met het herstel van patienten die secundaire ischemie doormaken als complicatie van een SAB uit een aneurysma. Patiënten die een bepaalde variant in het insulin-like growth factor-1 (*IGF-1*) gen hebben, blijken een lager risico op een slecht herstel te hebben, terwijl patiënten die een bepaalde variant in het tumor necrosis factor-A (*TNF-A*) gen hebben een hoger risico op een slecht herstel hebben.

In *Hoofdstuk 13* werd onderzocht of er een associatie bestaat tussen genen die betrokken zijn bij de stolling en het risico op de complicaties secundaire cerebrale ischemie en recidief bloedingen na SAB uit een aneurysma. Er kon geen associatie aangetoond worden tussen de varianten in de factor V Leiden, prothrombin G20210A, methylenetetrahydrofolate reductase (*MTHFR*), Val34Leu, Tyr204Phe en Pro564Leu factor XIII subunit A, en His95Arg factor XIII subunit B genen en het risico op secundaire cerebrale ischemie en recidief bloedingen in patiënten met SAB uit een aneurysma.

Publications

This thesis

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(William Arthur Ward)

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Curriculum Vitae

Ynte Ruigrok werd geboren op 15 juli 1973 te Wijk bij Duurstede. Na het eindexamen aan het Christelijk Gymnasium te Utrecht begon zij in 1991 aan de studie Medische Biologie aan de Universiteit Utrecht. Tijdens deze studie heeft ze student-assistentschappen farmacologie/toxicologie gedaan. Ook verrichtte zij een onderzoeksstage bij de vakgroep Moleculaire Celbiologie en Elektronen-Microscopische Structuuranalyse van de Faculteit Biologie van de Universiteit Utrecht. Na het afronden van deze stage begon zij in 1995 met de studie Geneeskunde aan de Universiteit Utrecht. Alvorens zij in 1997 met haar co-schappen begon heeft zij wetenschappelijk onderzoek verricht naar genetische risico-factoren bij herseninfarcten bij het Department of Clinical Neuroscience, King's College School of Medicine and Dentistry and Institute of Psychiatry in Londen, UK (supervisor prof.dr H.S. Markus).
In 1999 behaalde zij het artsexamen waarna zij gedurende een jaar werkzaam was als arts-onderzoeker en AGNIO op de afdeling Neurologie van het Universitair Medisch Centrum te Utrecht. In 2000 begon zij op diezelfde afdeling met de opleiding tot neuroloog (opleider prof.dr J. van Gijn). In 2001 was zij werkzaam als trial coördinator van de *European/Australian Stroke Prevention in Reversible Ischaemia Trial (ESPRIT) Study Group.* In januari 2002 kon zij met behulp van een AGIKO subsidie van NWO starten met het promotie-onderzoek waarvan de resultaten opgeschreven zijn in dit proefschrift. In 2005 kreeg zij de subsidie "Arts in opleiding tot specialist" 2005 van de Nederlandse Hartstichting, waarmee zij het wetenschappelijk onderzoek naar de genetische achtergronden van intracraniële aneurysmata en subarachnoïdale bloedingen kan continueren nu haar promotie-onderzoek afgerond is.