Intracranial aneurysms and connective tissue disorders

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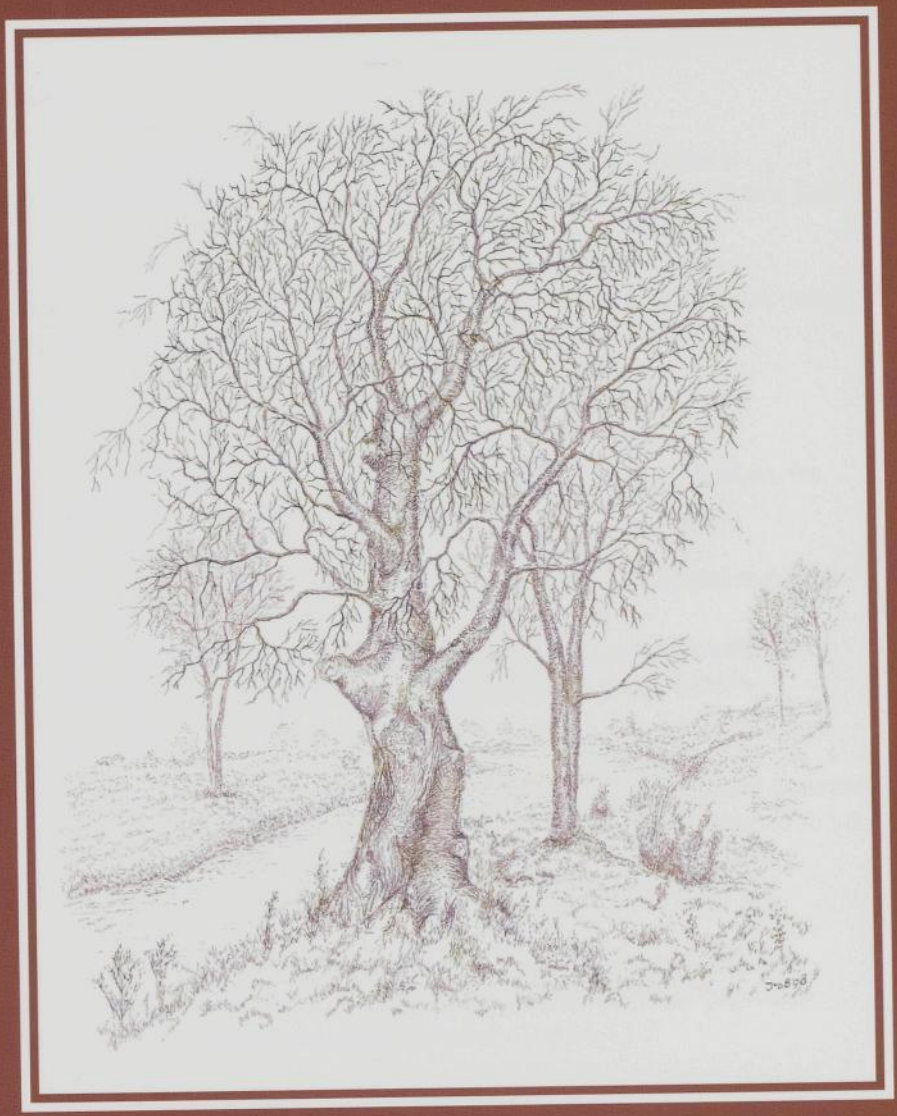

Jan Simon Peter van den Berg

Intracranial Aneurysms and Connective Tissue Disorders

Corrections

Page 36

Line 23-25. " In general population the rate of ischemic strokes is 22/100,000/year.[9] The relative risk in PXE was 21.7 (95% confidence limits 10.8/39.1). "
Should be read as:
The rate of ischemic stroke in the general population is 174/100,000/year.[9]
The relative risk in PXE was 2.74 (95% confidence limits 2.5/3.0).

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Line 15-16. " PXE patients have a clear excess of ischaemic strokes, we estimated a relative risk of 21.7. "
Should be read as:
The relative risk of ischemic stroke in PXE patients is estimated to be 2.74.

Line 22-24. " The relative risk of ischaemic stroke in pseudoxanthoma elasticum patients compared with normal population was 22 (95% confidence interval 10.8-39.1). "
Should be read as:
The relative risk of ischaemic stroke in pseudoxanthoma elasticum patients compared with normal population was 2.74 (95% confidence interval 2.5-3.0).

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Line 6-8. " Het relatieve risico op een herseninfarct bij patiënten met pseudoxanthoma elasticum is in vergelijking met de normale populatie ongeveer 22 (95% betrouwbaarheidsinterval 10.8-39.1). "
Should be read as:
Het relatieve risico op een herseninfarct bij patiënten met pseudoxanthoma elasticum is in vergelijking met de normale populatie ongeveer 2.74 (95% betrouwbaarheidsinterval 2.5/3.0).

Intracranial Aneurysms and Connective Tissue Disorders

Stellingen

1. Hypertensie is een overschatte risico-factor bij de ontstaanswijze van intracraniele aneurysmata.
2. Marfan syndroom is niet geassocieerd met intracraniele aneurysmata.
3. Pseudoxanthoma elasticum is niet geassocieerd met intracraniele aneurysmata.
4. Pseudoxanthoma elasticum is geassocieerd met herseninfarcten.
5. Meer kennis over de onstaanswijze van intracraniele aneurysmata werpt meer vragen op.
6. Voorzichtigheid is geboden bij het voorschrijven van trombocyten- aggregatie remmende medicatie bij patiënten met pseudoxanthoma elasticum.
7. Deficiëntie van type III collageen leidt bij sommige patiënten tot een intracraniëel aneurysma.
8. De verpleging is de ogen en oren van de arts op de afdeling.
9. Het enige milieuvriendelijk aanvliegen van Schiphol gebeurt door actiegroepen.
10. Luchtvaartmaatschappijen die transatlantische vluchten maken dienen als symbool in reclameboodschappen geen watervogel te kiezen.

J.S. Peter van den Berg, 24 juni 1999.

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Voor mijn ouders

Contents

General introduction

Introduction

Rupture of an intracranial aneurysm results in a haemorrhage with often devastating effects. The haemorrhage is usually located in the subarachnoidal space but it may also be present in the ventricles, intracerebrally or in the subdural space.[1] A ruptured intracranial aneurysm frequently leads to death (in approximately 50% of cases), and a large proportion of the survivors (10-20%) remains disabled.[2] In the last decades these figures did hardly change despite developments in neuro-anaesthesia and neurosurgery.[1,3] The high mortality is for a large proportion attributable to severe brain damage caused by the initial haemorrhage.[1,4] Improvement in outcome may therefore be achieved by early detection and obliteration of the aneurysm before rupture. Figures of complications (death or neurological deficits) after surgery for unruptured intracranial aneurysms are considerably higher than previously thought. A meta-analysis showed figures for mortality of 2.6% and for morbidity of 10.9%.[5] Similar figures were reported recently by an international study group.[6] Therefore it is important to investigate if better results can be achieved by using other techniques such as endovascular occlusion of the aneurysm with coils.

The formation of intracranial aneurysms has been related to "medial defects",[7] and hemodynamic stress.[8] At present the development of intracranial aneurysms is regarded as a multifactorial process in which both intrinsic (vessel wall weakness) and extrinsic (hemodynamic) factors play a role. Intrinsic factors may be related to connective tissue disorders since associations have been reported between intracranial aneurysms and Marfan syndrome, pseudoxanthoma elasticum, and Ehlers Danlos syndrome type IV.[9] The latter is a connective tissue disease which is characterized by genetically determined defects of type III collagen due to a decreased type III procollagen production or a production of structurally altered type III procollagen.[10,11] Ehlers Danlos syndrome type IV has been reported to be associated with carotid-cavernous fistulas,[12-16] arterial dissections,[14,17] and intracranial aneurysms [15,17]. Type III collagen is very firm and responsible for the tensile strength of the arteries, especially when the strain on the vessel wall becomes high.[18-20] Several small studies have demonstrated a possible relationship between type III collagen deficiency or unstable type III collagen and intracranial aneurysms.[21-23]

About 5-10% of patients have a positive family history for intracranial aneurysms.[24] The intrinsic factor in the pathogenesis of intracranial aneurysms may be a type III collagen deficiency, and this may also be the familial factor.

Aims and outlines of this thesis

The subject of this thesis was to investigate the intrinsic factors in the pathogenesis of intracranial aneurysms. Therefore the relationship between intracranial aneurysms and the connective tissue disorders pseudoxanthoma elasticum, and Marfan syndrome was studied. Furthermore studies were performed to determine if a type III collagen deficiency is related to the formation of intracranial aneurysms.

Chapter 1 is a review of the pathogenesis of intracranial aneurysms. In 135 patients with Marfan syndrome (**chapter 2**) and in 100 patients with pseudoxanthoma elasticum (**chapter 3**) a follow-up study was performed to investigate the association between these connective tissue disorders and symptomatic intracranial aneurysms.

In **chapter 4** the biochemical properties of type III collagen are discussed, and the possible relationship between type III collagen deficiency and intracranial aneurysms is reviewed. Protein analysis was performed of skin fibroblasts from 41 consecutively admitted patients with intracranial aneurysms, and 41 healthy volunteers to investigate if the production of type III collagen is lower in patients (**chapter 5**). Subsequently, the type III collagen gene in the group of 41 consecutive patients with intracranial aneurysms was analysed for mutations or other defects e.g. null alleles. (**chapter 6**).

In **chapter 7** a family is described of which three members had an intracranial aneurysm. Protein and DNA analysis was performed for the presence of a type III collagen deficiency, and subsequently the underlying cause of the deficiency was investigated.

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The pathogenesis of intracranial aneurysms

JSP van den Berg, M Limburg, and PM Struycken

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Introduction

Every year in the Netherlands approximately 900 people have a subarachnoid haemorrhage;[1] the majority is caused by rupture of an intracranial aneurysm. [2] Mortality of the subarachnoid haemorrhages is high (± 50%) during the first month, and approximately 10% die before hospital admission.[3] A large proportion of survivors remains disabled.[4] Despite developments in the areas of diagnostics, neurosurgery, and neuroanesthetics the mortality of aneurysmal subarachnoid haemorrhages has hardly decreased.[5] Health benefit can be achieved by detection and treatment of intracranial aneurysms before rupture, but only if mortality and morbidity after treatment for unruptured intracranial aneurysms is low. Knowledge of the pathogenesis of intracranial aneurysms and risk factors for development of intracranial aneurysms may improve the detection of people who are at high risk of harboring an intracranial aneurysm.

The structure of an intracranial aneurysm

The wall of an intracranial artery consists of an inner (intima), middle (media) and an outer (adventitia) layer. The internal elastic membrane lies between the intima and media. This layer is important when the strain on the arterial wall becomes high.[7] The media consists of smooth muscle cells and connective tissue. The adventitia is composed of loose connective tissue containing small vessels. In contrast to extracranial arteries, intracranial arteries have no external elastic membrane, a thinner media, and a thinner and looser adventitia.[8]

The wall of an intracranial aneurysm has hardly any structure, consists mainly of connective tissue with an absent or fragmented internal elastic membrane.[9]

Risk factors

Epidemiological studies have identified several risk factors for the development of intracranial aneurysms such as cigarette smoking, a positive family history, hypertension, postmenopause and heavy drinking.[10,11]

Several retrospective patient based,[12,13] and prospective population based studies[14,15] emphasize the importance of hypertension as a risk factor for development of intracranial aneurysms. However, during the last decades,

when there was a substantial reduction in the incidence of strokes in general, perhaps due to improved treatment of hypertension, no reduction in the occurrence of subarachnoid haemorrhages was observed.[16,17] Furthermore, aneurysmal subarachnoid haemorrhages is often observed in the absence of hypertension.

Cigarette smoking reduces the effectiveness of α_1-antitrypsin, an important inhibitor of proteolytic enzymes (e.g. elastase), which degrades amongst others the connective tissue of the arterial vessel wall.[18] Heavy drinking is associated with subarachnoid haemorrhages; one hypothesis suggests a causal relation through hypertension.[19]

A positive family history for subarachnoid haemorrhages is associated with a four times increased risk for having a subarachnoid haemorrhage.[20] It is uncertain if this increased risk is caused by hereditary (e.g. arterial wall defect) or environmental (e.g. toxic influence) factors.

Diseases associated with intracranial aneurysm

In some patients with arteriovenous malformations angiography may also reveal an intracranial aneurysm. The majority of these intracranial aneurysms is located on the arterial route supplying the arteriovenous malformation, suggesting that increased blood flow is relevant in the development of intracranial aneurysms.[21-24]

Intracranial aneurysms have been observed in patients with connective tissue disorders such as Marfan syndrome,[25,26] Ehlers Danlos Syndrome (EDS) type IV[27,28], and Pseudoxanthoma Elasticum.[29] In EDS type IV and Marfan syndrome the defective protein has been identified as respectively type III collagen and fibrillin[30,31], which are constituents of the arterial vessel wall.[32-33] Therefore connective tissue disorders may cause structural (intrinsic) defects in the vessel wall leading to the formation of intracranial aneurysms.

Asymptomatic intracranial aneurysms are found in 5 to 10% of patients with autosomal dominant polycystic kidney disease (ADKP).[34-36] In ADKP, hypertension is not the only factor in the formation of intracranial aneurysms. Some patients with ADKP have an aneurysmal SAH before hypertension develops.[37] An intrinsic factor or a vessel wall defect may therefor also be associated with the formation of intracranial aneurysm in these patients. Polycystin-1 (the product of the PKD1 gene; the gene most frequently involved

in ADKP) has been demonstrated, using antibodies, in the vessel wall of intracranial arteries.[38]

Experimental approach for the induction of intracranial aneurysms

Ferguson investigated the role of turbulence in aneurysm formation using a model with glass "cerebral" vessels.[39] In normal bifurcations turbulence was not a factor leading to hemodynamic stress at the apex, where most aneurysms arise, but impingement of the central fluid streams may cause local vessel weakness.[39]

Hassler was the first to induce cerebral aneurysms by increasing hemodynamic stress in animals through carotid ligation.[40] Subsequently, in addition to carotid ligation, arterial hypertension was induced by ligating the branches of the renal arteries, and by feeding the animals with β-aminopropionitrile (BAPN) which inhibits cross-link formation of elastin and collagen in tissue.[41,42] With this method, formation of intracranial aneurysms was induced in 6 of 7 monkeys (5 saccular, 5 fusiform, and 3 aneurysmal dilatations). Vessel wall changes were classified based on light microscopic findings into three stages: sequential disappearance of the internal elastic lamina, followed by thinning and dilatation of the wall and finally formation of an aneurysm.[41,42]

Aneurysms can also be induced, without hemodynamic stress, using local application of elastase. Histologically these aneurysms resemble intracranial aneurysms in man.[43]

Hypotheses on the pathogenesis of intracranial aneurysms

Several hypotheses on the development of intracranial aneurysms have been formulated. The formation of an intracranial aneurysm has been related to "medial defects", hemodynamic stress, but is nowadays regarded as a multifactorial process.

Intracranial aneurysms have been thought to be formed in the region of the "medial gaps" and were therefore considered to be "congenital". These medial defects are gaps in the media at bifurcations of normal arteries of the

circle of Willis.[44] However, these "media defects" were also encountered in other extracranial arteries,[45,46] and the distribution of these defects is different from that of intracranial aneurysms, since the defects are usually located at the lateral angles whereas intracranial aneurysms predominantly arise from the apices of the bifurcation.[46,47] Nowadays, these medial defects are regarded as part of the normal ontogenesis of vessel walls.[48]

Another hypothesis is that the development of intracranial aneurysms is dependent on a degenerative ("acquired") process, probably hemodynamic stress, possibly arterial hypertension.[49] The experiments of Hassler,[40] the relationship between arteriovenous malformations and intracranial aneurysms,[21-24] the frequency of intracranial aneurysms increasing with age,[50] and the association with hypertension[11] are compatible with this concept. However, the importance of arterial hypertension as a risk factor for the development of intracranial aneurysms appears to be modest since not all patients with subarachnoid hemorrhages have arterial hypertension, with frequencies of hypertension ranging between 39 and 92%.[13-15]

Nowadays the development of an intracranial aneurysm is regarded as a multifactorial process in which both intrinsic (vessel wall abnormalities) and extrinsic (hemodynamic) factors play a role.[51] The following sequence of events is the most likely; hemodynamic stress causes degenerative changes in the endothelial layer (hypertension may increase the damage). Subsequently, the internal lamina elastica degenerates which results in pressure on the arterial wall and a reduced strength of the arterial wall increases the propensity for aneurysm formation which finally occurs.

In some patients the reduced strength of the arterial vessel wall is possibly caused by a reduced type III collagen production.[52] However, in the majority of patients with an intracranial aneurysm this "intrinsic factor" is unknown. Increased activity of proteolytic enzymes e.g. elastase [53] is another candidate for the role of intrinsic factor.

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Is Marfan syndrome associated with symptomatic intracranial aneurysms?

JSP van den Berg, M Limburg, and RCM Hennekam

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Introduction

Marfan syndrome is an inherited disorder of connective tissue, which is characterized by manifestations of the musculoskeletal, ocular and cardiovascular systems, and occurs with an estimated prevalence of 1:10,000.[1] Marfan syndrome is diagnosed on clinical manifestations which are strictly defined.[2,3] The cause is a defect in fibrillin, a glycoprotein which is a structural component of microfibrils found in many tissues.[1,4,5] Several text books and other publications have associated Marfan syndrome with intracranial aneurysms.[6-11] This relationship, if valid, may be of pathogenetic and clinical significance. Studies should be performed to elucidate the possible role of fibrillin in the pathogenesis of intracranial aneurysms, and screening of patients with Marfan syndrome in order to detect asymptomatic intracranial aneurysms might be warranted.

The purpose of this study was to seek evidence for an increased incidence of intracranial aneurysms in patients with Marfan syndrome. We examined the prevalence of symptomatic intracranial aneurysms in a group of patients with Marfan syndrome, investigated the incidence of symptomatic intracranial aneurysms during follow-up, examined the prevalence of Marfan syndrome in patients with intracranial aneurysms admitted to Departments of Neurology and Neurosurgery, and reviewed the literature of the possible relationship between intracranial aneurysms and Marfan syndrome.

Patients and Methods

In 1982 an outpatient clinic in Amsterdam was instituted for patients with Marfan syndrome and other connective tissue disorders (for both children and adults). At the first and subsequent visits, all patients are examined by a (pediatric) cardiologist, an ophthalmologist, an orthopedic surgeon, and a clinical geneticist. Over the years all patients were examined by a stable team of investigators using a standard protocol. In the period 1982-1986 Marfan syndrome was diagnosed using the criteria defined by Pyeritz and McKusick.[3] Thereafter the "Berlin nosology" was followed.[2] There are no large differences between these criteria sets, and in retrospect we have applied the Berlin criteria also to the group of patients seen before 1986. All patients fulfilled these criteria. The records of all patients attending the Marfan clinic between January 1, 1982 and January 1, 1994, and classified as having Marfan syndrome were

retrieved. We collected data on patient`s age, gender, and clinical manifestations. During the follow-up period we searched for new manifestations of the disease.

In addition we retrieved the records over the same period of all patients with Marfan syndrome who visited or were admitted to the Departments of Neurology and Neurosurgery, both inpatients and outpatients.

For the literature review, we performed a Medline Search using the following key words: Marfan syndrome, intracranial aneurysm, connective tissue disorder, and subarachnoid hemorrhage. We also followed all references from the papers thus found, and traced the references on this topic from several text books.[6-8,10,11]

Results

The patient group comprised 135 patients, 63 male and 72 female. The mean age at first presentation was 21.1 years, ranging from 1 to 69 years of age. The presenting manifestations of all patients are listed in Table 1. No patient with Marfan syndrome had a history of a subarachnoid hemorrhage or had presented symptoms relating to an intracranial aneurysm at the first visit to the Marfan clinic.

In 129 patients, with a mean age of 21.3 years, we obtained a follow-up. The mean follow-up was 4.5 years (range 7 months to 12 years) in 129 patients. This resulted in a total number of 581 observation years. Developing complications during the follow-up are listed in Table 2.

A 37 year old patient was admitted because of an alcohol and barbiturates intoxication. At the age of 34 years Marfan syndrome was diagnosed on the basis of an anterior chest deformity, scoliosis, dolichostenomelia, striae distensae, spontaneous pneumothorax, and mitral valve prolapse. During admission he became drowsy. A CT scan of the brain showed an intracerebral hemorrhage of the left hemisphere. Coagulation tests were normal. Cerebral angiography did not reveal an intracranial aneurysm or other vascular abnormalities. During the follow-up 6 patients died, in 4 this was caused by a dissection of the ascending aorta, and in 2 the cause was unknown.

In none of the 826 patients admitted to the Departments of Neurology or Neurosurgery with subarachnoid hemorrhage or intracranial aneurysm the diagnosis of Marfan syndrome was made.

Table 1. Major presenting manifestations in 135 consecutive patients with Marfan Syndrome.

	Number of patients
Skeletal	
Arachnodactyly	69
Anterior chest deformity	86
Scoliosis	39
Dolichostenomelia	47
Tall stature (Height > P 98)	77
High arched palate	12
Hypermobile joints	30
Ocular	
Ectopia lentis	
Unilateral	13
Bilateral	55
Myopia	38
Iridodonesis	33
Iris transillumination	29
Retinal detachment	38
Cardiovascular	
Mitral valve prolapse	95
Aortic root dilatation	88
Dissection of the aorta	13
Dissection of the subclavian artery	1
Pulmonary	
Spontaneous pneumothorax	8
Skin and integument	
Striae distensae	49
Inguinal hernia	11
Umbilical hernia	2
Diaphragmatic hernia	1
Nervous system	
Lumbosacral meningocele	2

Table 2. Complications during follow-up in 129 patients with Marfan Syndrome.

	Number of patients
surgical correction of extreme aortic dilatation	5
spontaneous pneumothorax	5
progression of anterior chest deformity	3
dissection of ascending aorta	4
progressive scoliosis	2
intracerebral hemorrhage	1
surgical correction of an atrial septum defect II	1

Our review of the literature revealed 10 cases of patients with Marfan syndrome and intracranial aneurysm.[12-21] Eight female and 2 male patients were described with a median age of 41.3 years.

Discussion

No symptomatic intracranial aneurysm occurred in our population of patients with Marfan syndrome or became clinically overt during the follow up. In our population of patients with intracranial aneurysm Marfan syndrome was not diagnosed.

The relationship between Marfan syndrome and intracranial aneurysm, as suggested in the literature, [6-11] is probably based on several case reports of intracranial aneurysms in patients with Marfan syndrome,[12-21] an autopsy report of a young women with Marfan syndrome,[22] and a family of which several members had intracranial aneurysms and one other member had Marfan syndrome[23].

Ten cases have been reported of patients with Marfan syndrome and an intracranial aneurysm.[12-21] In all these the diagnosis of Marfan syndrome was based on clinical manifestations. However, in some patients these manifestations were incompletely mentioned; Higashida et al. for instance reported no manifestations at all,[17] and Rose and Pretorius mentioned only the medical history that revealed repair of an ascending aortic aneurysm and replacement of an aortic valve.[19] The diagnosis of Marfan syndrome in some of these patients may be questioned. It is well known that Marfan syndrome can be erroneously

diagnosed, especially in patients with homocystinuria [24] or Ehlers Danlos syndrome type IV[25].

Ter Berg et al. described a family in which 7 members presented with intracranial aneurysms, and one member with subarachnoid hemorrhage. One further family member was said to have Marfan syndrome, without further details. The patient with Marfan syndrome underwent cerebral angiography which disclosed no intracranial aneurysm.[23] Thus, this report provides no further evidence for the co-occurrence of intracranial aneurysms and Marfan syndrome.

Stehbens examined cerebral arterial forks of a 33-year-old woman with Marfan syndrome who died of septicemia following cardiac surgery. He found no intracranial aneurysms, but described atrophic changes and a small evaginated pouch supposedly associated with early aneurysm formation.[22] No control observations in patients without Marfan syndrome were performed, and on basis of this single patient he concluded that the development of intracranial aneurysms in both patients with and without Marfan syndrome was similar.

In several large series of patients with Marfan syndrome with comprehensive descriptions of the clinical manifestations, the occurrence of intracranial aneurysms is not mentioned.[3,26-28] If there were no intracranial aneurysms at presentation, this does not exclude a future development of intracranial aneurysms. However, during 581 patient observation years no symptomatic intracranial aneurysm developed, while the majority of the known complications of Marfan syndrome did occur. Of course we cannot exclude the presence of asymptomatic intracranial aneurysms. In autopsy studies unruptured intracranial aneurysms are found in 0.8 to 2.0% of cases.[29-31] Our Marfan patients had an average age of 21.1 years at presentation. We found no evidence of intracranial aneurysms during a total retrospective (2850 years) and prospective follow-up period of 3431 years. The 95% confidence limits of these findings are 0 to 0.001 events per year. In population studies the incidence is about 0.001 subarachnoid hemorrhages per year.[32,33] This does not refute any correlation between the two entities, but is certainly not suggestive of a strong relation.

At present there is insufficient evidence to postulate an association between Marfan syndrome and intracranial aneurysms. Investigations into a possible pathogenic role of fibrillin deficiency in the development of intracranial aneurysms are not warranted.

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Prevalence of symptomatic intracranial aneurysms and ischaemic strokes in pseudoxanthoma elasticum.

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Submitted for publication

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Introduction

In pseudoxanthoma elasticum (PXE), a heritable connective tissue disorder, the elastic fibres of the skin, eyes, and cardiovascular system become slowly calcified leading to symptoms in these organs.[1] The prevalence is estimated to be approximately 1 per 100,000.[1] The mode of transmission can be both autosomal recessive and autosomal dominant [1], indicating that it is a heterogeneous entity. The cause is unknown, although the locus for PXE has been mapped to chromosome 16p13.1.[2]

PXE has been associated with intracranial aneurysms in several text books and reviews.[3-6] Such a relationship may be of great pathogenetic and clinical significance. This association might elucidate the pathogenesis of intracranial aneurysms, and screening of patients with PXE might be warranted in order to detect asymptomatic intracranial aneurysm. In addition, PXE may cause cerebral ischaemia by premature arterial occlusive disease.[7]

After reviewing the literature on the possible relationship between PXE and symptomatic intracranial aneurysms or ischaemic stroke we performed this study to seek evidence for an increased prevalence of both in patients with PXE.

Patients and Methods

The records of all patients diagnosed with PXE at two major institutes caring for a large group of patients with PXE, the Netherlands Ophthalmic Institute in Amsterdam, and the departments of Dermatology and Ophthalmology at the University Hospital Nijmegen, were retrieved. Furthermore, during the annual meeting of the Dutch PXE Patient Society subjects not attending the Departments in Amsterdam or Nijmegen were invited to participate in the study. PXE was diagnosed on clinical symptoms following the Berlin Nosology [8], and in some verified by a skin biopsy. All patients had been examined by an experienced ophthalmologist and dermatologist. We collected data on patient age, gender, and clinical manifestations. During the follow-up period, all patients were contacted by telephone, we investigated new manifestations of the disease, and inquired about newly developed symptoms and signs attributable to intracranial aneurysms, subarachnoid haemorrhages, or ischaemic stroke. Further details of medical events were retrieved by contacting the responsible physician.

For the literature review, we performed a Medline Search using the following key words in various combinations: PXE, Grönblad-Strandberg syndrome, (ischaemic) stroke, cerebral infarction, (a)symptomatic intracranial aneurysm, connective tissue disorder, and subarachnoid haemorrhage. We also followed all references from papers thus found, and traced references on this topic from several text books.[3-6]

This study was approved by the institutional review boards of the participating centres, and the Medical Ethical Committee of the Academic Medical Centre of the University of Amsterdam. The 95% confidence limits of the number of events during follow-up was calculated using the Poisson method.

Results

The patient group comprised 100 patients, 37 male and 63 female. The mean age at first presentation was 31.7 years, ranging from 2 to 61 years of age. The presenting manifestations of all patients are listed in table 1. None of the patient with PXE had a history of subarachnoid haemorrhage or had presented symptoms or signs relating to an intracranial aneurysm when diagnosed with PXE. One patient presented with an ischaemic stroke at the age of 55 years. Skin biopsies, all diagnostic of PXE, were performed in 46 patients (46%). A family history positive for PXE was present in 47 patients (47%).

Table 1. Major presenting manifestations in 100 consecutive patients with pseudoxanthoma elasticum.

	Number of patients (%)
Impaired vision	69 (69%)
Typical skin lesion*	93 (93%)
Claudication	3 (3%)
Myocardial infarction	3 (3%)
Hypertension	2 (2%)
Gastro-intestinal haemorrhage	2 (2%)
Ischaemic stroke	1 (1%)

*Lax, redundant, and relatively inelastic skin in flexures, exaggeration of nasolabial folds and chin.

In 94 patients (94%), with a mean age of 31.4 years, we obtained a follow-up. The mean follow-up was 17.1 years (range 1 to 49 years). This resulted in a total number of 1602 observation years. Complications that developed during the follow-up period are listed in table 2. One patient developed a gastric haemorrhage when using aspirin. In one patient hypertension resulted in renal failure. Four of the 15 patients with intermittent claudication had surgery for a femoral artery bypass graft, and coronary artery bypass grafting was performed in five of 15 patients with angina pectoris. In 14 patients a computerized tomography (CT) scan of the brain was made, showing cerebral infarction in eight patients, and no abnormalities in six patients (in these last patients the CT was made because of headache). Additional magnetic resonance imaging (MRI) of the brain was performed in three patients showing cerebral infarction. Two patients, with ischaemic strokes, had a cerebral angiography which revealed no aneurysms or other vascular abnormalities. Altogether seven patients developed an ischaemic stroke (rate 77/100,000/year, 95% confidence limits 238-861/100,000/year). For patient characteristics see table 3. None of the patients had symptoms or clinical evidence of large cerebral vessel disease. The cerebrovascular pathology was confined to the small vessels. Additional investigations revealed, besides a mild hyperhomocysteinaemia in one patient (patient 2), no other haematological abnormalities or vasculopathies as a cause for the ischaemic stroke. One patient had ipsilateral transient ischemic attacks before an ischemic stroke developed, and another patient had the ischemic stroke when already using aspirin. In general population the rate of ischemic strokes is 22/100,000/year.[9] The relative risk in PXE was 21.7 (95% confidence limits 10.8/39.1). Five patients died; four (aged 50, 53, 59 and 61 years, respectively) of a myocardial infarction, and one of a major gastric haemorrhage (48 years of age). In 6 patients we were unable to collect follow-up data. Three had moved to an unknown location, and three did not want to participate in the study.

Our review of the pertinent literature revealed four patients with PXE and intracranial aneurysm.[10-13] The characteristics of these patients are summarized in table 4.

The frequency of ischemic stroke in PXE is difficult to determine, since many reports of the patients are sketchy, do not specify, and were published a long time ago and so lack sufficient neuroimaging. The youngest patient described was an 11 year old girl[14], most patients were 45 years or older.[7,15-18] In

a series of 106 patients with PXE five had suffered a stroke.[19] A study investigating the death of 12 patients with PXE revealed ischemic stroke as cause in four, and cerebral haemorrhage as cause in two patients.[20] The cerebral haemorrhage was not further specified.

Table 2. Complications that developed during a mean follow-up period of 17.1 years in 94 patients with pseudoxanthoma elasticum.

	Number of patients (%)
Further impairment of vision	52 (55%)
Exacerbation of skin symptoms	22 (23%)
Hypertension	18 (19%)
Gastro-intestinal haemorrhage	17 (18%)
Angina pectoris	15 (16%)
Claudication	15 (16%)
Ischaemic stroke	7 (7%)
Myocardial infarction	2 (2%)
Severe skin haemorrhage	2 (2%)
Severe nose bleeding	1 (1%)
Severe uterine haemorrhage	1 (1%)
Death	5 (5%)
Cholecystectomy	4 (4%)
Pyrosis	4 (4%)

Table 3. Characteristics of ischaemic strokes in 7 patients with PXE.

Patient	Age*	Sex	Hemiparese s	Hypertension	Cigarette smoking	Cardiac diseases	Peripheral vascular disease	Brain imaging
1	51	M	Left	no	yes	no	no	CT: no abnormalities
2	59	F	Right	no	yes	no	yes	MRI: MIVL bilateral
3	38	F	Right	no	yes	no	no	CT: MIVL bilateral
4	58	M	Left	no	no	yes: ischaemia	yes	MRI: MIVL bilateral
5	61	F	Left	no	no	yes; ischaemia	yes	MRI: MIVL bilateral
6	56	M	Right	yes	no	no	no	CT: MIVL bilateral
7	48	F	Left	no	no	no	no	MRI:no abnormalities

*Age at which stroke occurred; CT = computerized tomography; MRI = magnetic resonance imaging; MIVL = Multiple ischaemic vascular lesions.

Table 4. Characteristics of the patients with PXE and an intracranial aneurysm as described in literature.

Ref	Sex	Age*	Angioid streaks	Typical skin lesions	Localization aneurysm
10	Female	29	yes	not mentioned	subclinoid aneurysm right ICA
11	Female	66	yes	not mentioned	right ICA parasellar
12	Male	20	yes	yes	right cavernous sinus (arteriovenous fistula)
13	Female	66	not mentioned	yes	aneurysm cavernous portion right ICA

Ref = reference number; *Age of patient when intracranial aneurysm became symptomatic; ICA=internal carotid artery.

Discussion

In our population of patients with PXE no symptomatic intracranial aneurysm occurred or became clinically overt during follow-up.

The relationship between PXE and intracranial aneurysm, as suggested in the literature, [3-6] is based on a small number of case reports of intracranial aneurysms in patients with PXE.[10-13] In these cases no pathological data or other evidence was presented pointing to a causal relation. In addition to these four patients with PXE and an intracranial aneurysm, Sharma described a patient with PXE and subarachnoid haemorrhage in whom cerebral angiography showed no intracranial aneurysm.[21] Goto described a patient with PXE and a fusiform aneurysmal dilatation of the basilar artery and left ophthalmic artery, with complete occlusion of the left internal carotid artery.[22] It is conceivable that this obstruction of the left internal carotid artery may have increased the strain in the vertebrobasilar system. Other craniocervical vascular malformations described in patients with PXE are fusiform cervical aneurysm [23], pontine arteriovenous malformation [24], bilateral calcified common carotid aneurysms [11], carotid rete mirabile [25], and an aneurysm of the anterior spinal artery [26]. The last patient had stenosis of the abdominal aorta, and dilatation of internal thoracic arteries. As the anterior spinal artery is an important collateral pathway, this aneurysm may have developed secondarily to haemodynamic stress.

Brain autopsy reports of two patients with PXE have not shown macroscopic intracranial aneurysms or pouch formation of cerebral vessels [18,27], and cerebral angiograms in three patients with PXE showed no intracranial aneurysm [14,21,28]. Also, one large clinical and descriptive series of 106 patients,

and a review of 204 patients with PXE do not mention symptomatic intracranial aneurysms.[19,29]

The absence of intracranial aneurysms in our patient series does not exclude future development of intracranial aneurysms. Our PXE patients had an average age of 31.9 years at presentation. We found no evidence of intracranial aneurysms over a total retrospective (3168 years) and prospective (1602 years) follow-up period of 4770 years. The 95% confidence limits of these findings are 0 to 0.0008 events per year. In population studies the incidence is about 0.001 subarachnoid haemorrhages per year.[30] This does not refute any correlation between the two entities, but is certainly not suggestive of a strong relation. The case histories in the literature probably represent mere fortuitous coincidence.

Ischaemic stroke was present in one patient as a presenting manifestation, recurred in the same patient, and developed during follow-up in six other patients. PXE patients have a clear excess of ischaemic strokes, we estimated a relative risk of 21.7. Focal cerebral ischaemia in PXE is predominantly caused by small-vessel occlusive disease, with hypertension, also common in PXE, as an accelerating factor as was suggested before.[7,18,27] Our findings strongly confirm this relationship. Preventive treatment in patients with PXE and an ischaemic stroke is controversial. After acute ischemic stroke, antiplatelet treatment (aspirin) is advised for secondary prevention, and it should be administered within 48 hours of the stroke onset.[31] However, our data show that gastro-intestinal haemorrhage, even fatal, is a common complication in PXE, and constitutes a firm contraindication for the use of aspirin.

In conclusion, there is at present insufficient evidence to postulate an association between PXE and intracranial aneurysms. There is suggestive evidence of a significant association between PXE and ischaemic stroke. The use of aspirin after ischaemic strokes is controversial in this entity in view of frequent gastrointestinal complications.

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Type III collagen and intracranial aneurysms

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Type III collagen: biochemistry and genetics

Type III collagen deficiency is possibly related to the formation of intracranial aneurysms. At least 16 different types of collagen have been identified. The major fibrillar collagens are type I, II, and III. Type I is present in most connective tissues, type II primarily in cartilage, and type III is mainly found in skin, gut, placental membranes and is a major component of the blood vessel wall.[1-3]

A type III collagen molecule consists of three identical pro-α1(III) chains produced by the rough endoplasmic reticulum of the cell. Each procollagen chain contains a strand of about 1000 residues long in an alpha(α)-helix, rich in glycine (every third residu) and proline, and two globular parts on the amino(N)- and carboxyl(C)-terminal ends. The pro-collagen chains associate at the C-terminal end, prior to spontaneous triple helix formation.[3,4] The stability of the triple helix depends on the locking effect of proline-hydroxyproline residues and on the hydrogen bonding by the hydroxyl group of hydroxyproline. Subsequently the procollagen undergoes post-translational modifications. The N- and C-propeptides are cleaved by specific endopeptidases. The collagen then spontaneously assembles into fibrils.[2,3]

Type III collagen deficiency can result from a variety of mechanisms, e.g. genomic deletion of one of the type III collagen alleles,[5] point mutations leading to abnormal splicing (i.e. exon skipping and insertions),[4-6] substitutions in the α-helix (i.e. a point mutation replacing an amino acid residue often glycine),[2,4-6] or a dysfunction during the post-translational modifications.[3]

Relationship between type III collagen deficiency and intracranial aneurysms?

Type III collagen is an attractive candidate to play an important role in the pathogenesis of intracranial aneurysms. It is very firm and responsible for the tensile strength of the arteries, especially when the strain on the vessel wall becomes high.[7-9] Ehlers Danlos Syndrome (EDS) type IV is a connective tissue disease which is characterized by a genetically determined defect of type III collagen due to a decreased type III procollagen production or a production of structurally altered type III collagen.[2] Patients with this syndrome have an

increased propensity for carotid-cavernous fistulas,[10-15] arterial dissections,[13,16] and intracranial aneurysms.[14,16,17] The vascular fragility in EDS type IV is a feared problem in surgery and arteriography.[18-20]

Several studies have demonstrated a relationship between type III collagen deficiency or unstable type III collagen and intracranial aneurysms.[21-24] Neil-Dwyer et al. obtained skin and arterial fibroblasts from 17 patients undergoing surgery for ruptured intracranial aneurysms and from 6 age- and sex-matched controls. The type III / type I collagen ratios were measured by carboxymethyl cellulose chromatography (CMC) or gel scanner, two different methods of collagen determination. CMC was performed in 11 patients and gel scanning in another 11 patients. In 11 patients (64%) a type III collagen deficiency was found while all of the 6 controls had normal type III / type I collagen ratios.[21] Østergaard et al. used post-mortem samples of the middle cerebral artery of 14 patients who died of a ruptured intracranial aneurysm and from a control group of 14 patients who died of unrelated causes. Through electrophoresis and densitometry a type III collagen deficiency was detected in 43% (6 patients) of the ruptured aneurysm group.[22] This study suffers from a selection bias as only deceased patients were included. There was a fairly large difference between type III / type I collagen ratios from the middle cerebral artery and the brachial artery in several patients. An explanation is that Østergaard et al. used biopsies of the whole artery wall resulting in a mixture of endothelial and medial smooth muscle cells. This may influence the type III / type I collagen ratio since there is a variation in collagen synthesis in endothelial and smooth-muscle cells as was shown in cultures from pig aortas.[25] Although these studies were small, they indicate a relation between type III collagen deficiency and intracranial aneurysms.[21,22]

However, in another small study Leblanc et al., using gel electrophoresis, showed no type III collagen deficiency in skin fibroblasts of five female patients with intracranial aneurysms who all had a positive family history for intracranial aneurysms.[26,27] Majamaa et al. using gel electrophoresis and densitometry, could not demonstrate type III collagen deficiency in 11 patients with intracranial aneurysms, but found a production of unstable type III collagen in skin fibroblasts of 2 patients.[24] In this study six patients (55%) had a relative with an intracranial aneurysm including the 2 patients with unstable type III collagen production.

It is not clear whether the type III collagen gene is involved in the demonstrated type III collagen deficiency.[21,22] Kuivaniemi et al. performed DNA sequence analysis of type III collagen cDNA in 40 patients with intracranial aneurysms and found no mutations in the part of the gene encoding the α-helix and the telo-peptides.[28] Therefore it is not likely that this part of the gene is often involved in the formation of intracranial aneurysms. However, the 3' end of the gene, encoding the C-pro-peptide, has not been analyzed in patients with intracranial aneurysms. The globular part of the C-propeptide is essential in formation of the triple helix of all fibrillar collagens.[3,4] A mutation in the C-terminal part may theoretically lead to a failure of association of the triple helix with an intracellular breakdown of the mutated proα1chain or it may lead to an abnormal association with all three α1chains being destroyed.[6]

Mutations in the C-propeptide of type III collagen, defects in gene regulation or in collagen processing may cause a deficiency of type III collagen.

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Some patients with intracranial aneurysms have a reduced Type III / Type I collagen ratio. A case-control study.

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Introduction

Subarachnoid haemorrhages represent approximately 7% of all strokes,[1] and account for 45% of the strokes between the ages of 35 and 44 years.[2] Over the last four decades the incidence of subarachnoid haemorrhages has not changed, and the mortality rate has remained high.[1,3] A large proportion of the survivors remain in a disabled state.[1] The majority of the subarachnoid haemorrhages is caused by rupture of an intracranial aneurysm.[4] Early detection and preventive surgery of an intracranial aneurysm may lead to an increased live span.[5] Understanding of the underlying mechanisms leading to the formation of intracranial aneurysms is important in view of the development of preventive strategies.

One hypothesis considers the pathogenesis of intracranial aneurysms to be a multifactorial process in which both intrinsic factors, such as vessel wall abnormalities, and extrinsic factors, such as hypertension, play a role.[6-8] A reduced production of type III collagen is a possible candidate for such an intrinsic factor. Several observations suggest a relationship between a defect of type III collagen and the pathogenesis of intracranial aneurysms. Type III collagen is responsible for the tensile strength of the arteries, especially when the strain on the vessel wall becomes high.[9-11] Mutations in the type III collagen gene, encoding the type III procollagen, produces the Ehlers Danlos type IV phenotype.[12] Ehlers Danlos type IV patients show a diversity of clinical symptoms including skin laxity, and ruptured intracranial aneurysms.[13,14]

Several protein based studies have revealed a relationship between a reduced production of type III collagen and intracranial aneurysms.[15-20] However, Kuivaniemi et al, using DNA sequence analysis of part of the gene encoding the triple-helical domain of type III collagen in 40 patients with intracranial aneurysms, did not observe mutations, and concluded that structural mutations in the gene encoding for type III collagen are not a common cause of intracranial aneurysms.[21]

In order to investigate a possible significance of a reduced production of type III collagen on the protein level, we determined the type III / type I collagen ratios from skin fibroblasts of patients with intracranial aneurysms, and compared these to type III / type I collagen ratios in control persons. Our study questions were: is the presence of intracranial aneurysms associated with

a reduced production of type III collagen, and, if so, is there a difference in the clinical features of these patients with a normal and reduced production of type III collagen?

Patients and methods

In a prospective study we included all consecutive patients with an intracranial aneurysm admitted to the Department of Neurosurgery of the Academic Medical Center in Amsterdam. The diagnosis had to be confirmed by a three vessel intra-arterial digital subtraction angiography or at craniotomy. From the medical history the following factors and disorders were recorded: hypertension, polycystic kidney disease or other renal abnormalities, symptoms fitting Marfan syndrome, Ehlers Danlos syndrome type IV, pseudoxanthoma elasticum, and the family history of the same factors. Patients were examined by two experienced clinical geneticists (RCMH and the late JW Oorthuys) for signs of Ehlers Danlos syndrome type IV, Marfan syndrome, and pseudoxanthoma elasticum. The cerebral angiograms were independently studied by two investigators who scored the localization and size of the aneurysm. The largest measured diameter in any projection was taken as the size of the aneurysm. In case of any discrepancy the angiograms were discussed, and a consensus between the two observers was obtained. The control group consisted of 41 healthy volunteers, personnel from the Academic Medical Center, age- and sex-matched, and without a history of intracranial aneurysms. The study was approved by the Hospital Medical Ethical Committee.

Biochemical and molecular studies. After informed consent a skin biopsy was taken for fibroblast culture. When the cells had reached confluence, the medium was removed, and metabolic labelling was performed for 18 hours in medium which contained antibiotics, ascorbic acid, aminopropiontrinil, and ^{14}C proline. A sample of the medium was taken for protein analysis. The proteins were isolated through ethanol precipitation, acetic acid was added to dissolve collagen. Polyacrylamide gel electrophoresis was performed after pepsin digestion, and the proteins were reduced in the gel after one hour. The amount of radioactivity used for electrophoresis was standardized: for each sample the amount was 20.000 cpm. The volume needed for a sample varied between 5 and 10 μl. The signals from the

radioactively labelled collagen chains were quantified in arbitrary but linear units using a PhosphorImager™ (Molecular Dynamics, Sunny Valley, California, USA).[22] The ratio of type III / type I collagen, defined as the "αl(III)/ [α1(I) + α2(I) + α1(III)]" ratio, was then computed. In all subjects the final ratios are the average results of 3 independent separate labelling experiments. All collagen determinations were done within 4 passages of the biopsy.

All type III / type I collagen ratios encountered in the control group were considered to represent normal values. As a result all values found in the patient group that were below the lowest value of the control group were considered to represent abnormally low type III / type I ratios. In patients with abnormally low ratios of type III / type I collagen, intracellular type III procollagen was assessed by electrophoretic analysis of the content of lysed cells. Structurally abnormal type III procollagen has an abnormal electrophoretic mobility.[23]

In the statistical analyses Fisher's exact test and Wilcoxon Rank Sum test were used.

Results

The greater majority of patients (38 of 41) presented with a ruptured intracranial aneurysm. In all patients except one a cerebral angiography was performed. This patient was operated immediately because of a rapid clinical deterioration, and the presence of an aneurysm was confirmed. In the 3 patients without presenting subarachnoid haemorrhage the indications for a cerebral angiography were: intracerebral haemorrhage; atypical facial pain with a positive family history for intracranial haemorrhages; and headache with transient neurological signs of visual disturbance and dysarthria. In this patient the computed tomography scan of the brain showed a lesion compatible with an intracranial aneurysm. The median age of the patients was 49 years (ranging from 26 to 79 years). Other characteristics of the patients are shown in table 1. The 3 patients with a positive family history for polycystic kidney disease also had a positive family history for hypertension. Cerebral angiography in 1 patient also showed a small arteriovenous malformation. The median age of the controls was 43 years (ranging from 26 to 56 years), there were 16 men and 25 women.

Table 1. Characteristics of 41 consecutive patients with intracranial aneurysms.

	Number of patients (% of total)
Men: Women	16 : 25
Polycystic kidney disease	2 (5%)
Hypertension	19 (46%)
Family history	
subarachnoid hemorrhage	4 (10%)
hypertension	20 (49%)
polycystic kidney disease	3 (7%)
Multiple aneurysms	6 (15%)
Localization of aneurysms	
ACA left / right	0 / 4
AcoA	14
ICA left / right	4 / 5
MCA left / right	6 / 6
Basilar artery	5
PICA left / right	2 / 1
Clinical outcome	
discharge to rehabilitation center	2 (5%)
discharge to nursing home	2 (5%)
home	31 (75%)
deceased	6 (15%)

ACA=Anterior Cerebral Artery; ACoA=Anterior Communicating Artery; ICA=Internal Carotid Artery; MCA=Middle Cerebral Artery; PICA= Posterior Inferior Cerebellar Artery.

The results of the protein analysis are presented in figure 1. The type III / type I collagen ratios in the controls ranged from 5.5% to 19.8%, with a median of 10%. The resulting cut-off value below which type III / type I collagen ratios will be considered abnormally low was 5.5%. The type III / type I collagen ratios in the patients ranged from 1.1% to 25.1%, with a median of 10.5%, and 8 patients (19.5%) had a very low ($< 5.5\%$) ratio. This difference is statistically significant ($p = 0.005$, Fisher`s exact test). The ratios of the type III / type I collagen in patients and controls were compared on a group level and there was no evidence that the samples of the ratios of the patients and controls were derived from different distributions (two-tailed p value 0.51, Wilcoxon Rank Sum Test).

The patient with the small arteriovenous malformation had a normal type III / type I collagen ratio. In fibroblasts from the 8 patients with type III /

type I collagen ratios of less than 5.5% intracellular type III procollagen was found to be low, and there were no indications of an excess of structurally abnormal type III procollagen.

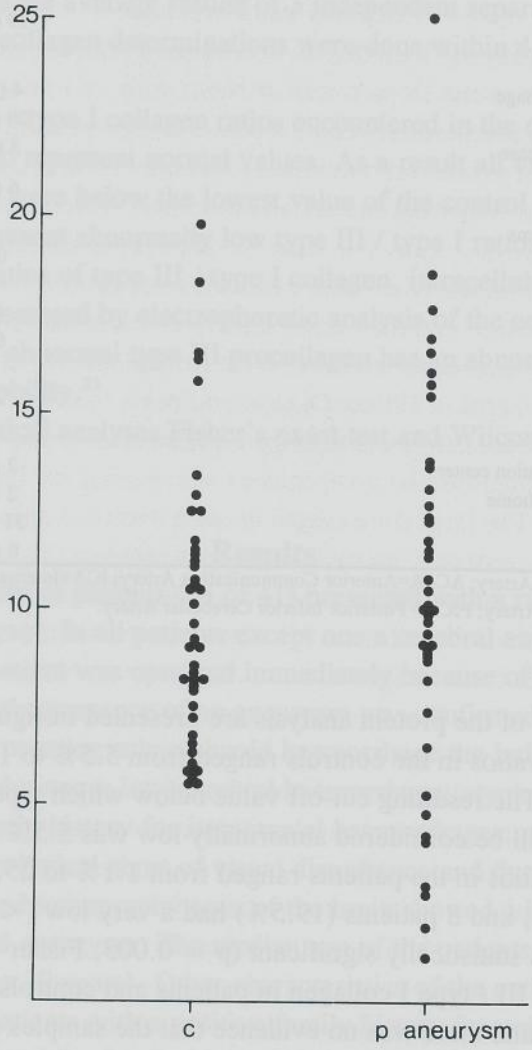

Figure 1. Type III / type I collagen ratios (Col.III %) in 41 controls and 41 consecutive patients with intracranial aneurysms.

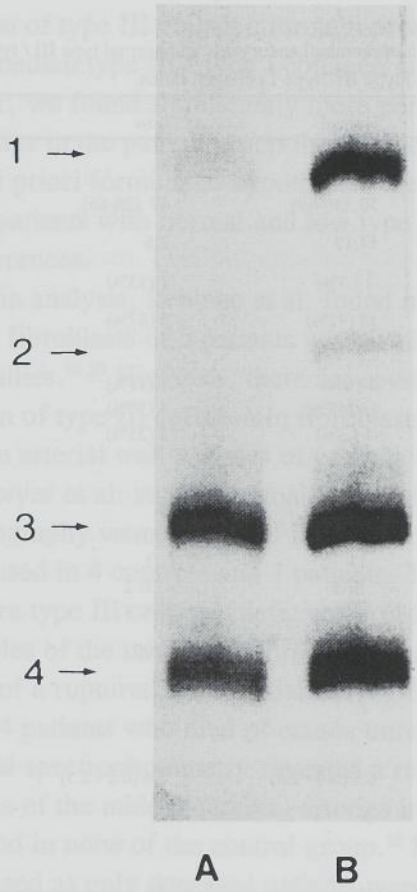

Figure 2. Polyacrylamide gel electrophoresis of a patient with a low type III collagen (A), and a control with a normal type III collagen (B); arrow 1 = type III collagen α1 (αl(III)); arrow 2 = type V collagen α1 (α1(V); arrow 3 = type I collagen α1 (α1(I)); arrow 4 = type I collagen α2 (α2(I)).

We examined whether patients with a very low type III / type I collagen ratio (< 5.5%) differed in clinical characteristics from the patients with a normal type III / type I collagen ratio (table 2). Apart from a trend favouring patients with a very low type III / type I collagen ratio to have a positive

family history for polycystic kidney disease, no statistically significant differences were found.

Table 2. Characteristics of patients with intracranial aneurysms and normal type III / type I collagen ratios compared to patients with low (< 5.5%) type III / type I collagen ratios.

Type III / type I collagen ratio	Normal	Low	p*
Number	33	8	
Median age (and range in years)	50 (30-69)	47 (26-66)	0.64
Sex M:F	14:19	2:6	0.45
Polycystic kidney disease	1 (3%)	1 (13%)	0.36
Hypertension	14 (42%)	5 (67%)	0.23
Family history			
subarachnoid hemorrhage	3 (9%)	1 (17%)	1.00
hypertension	16 (48%)	4 (50%)	1.00
polycystic kidney disease	1 (3%)	2 (25%)	0.11
Localization of aneurysms			
Anterior circulation	30	9	
ACA left/ right	0 / 4	0 / 0	
AcoA	13	1	
ICA left/right	3/ 3	1/ 2	
MCA left/ right	2/ 5	4/ 1	
Posterior circulation	8	0.00	0.19**
Basilar artery	5	0	
PICA left/right	2 / 1	0	
Size of aneurysms			
median (and range in cm)	0.7 (0.4 - 2.0)	0.6 (0.2 - 2.5)	0.64
Multiple aneurysms	5	1	1.00
Outcome			
good	26	7	
home	24 (73%)	7 (88%)	
rehabilitation	2 (6%)	0 (0%)	
poor	7	1	0.68***
nursing home	2 (6%)	0 (0%)	
deceased	5 (15%)	1 (12%)	

*, Fisher's Exact Test, two tailed (for Age and Size, Wilcoxon Rank Sum Test); **, comparison between anterior and posterior circulation; ***, comparison between good and poor outcome. ACA, Anterior Cerebral Artery; AcoA, Anterior Communicating Artery; ICA, Internal Carotid Artery; MCA, Middle Cerebral Artery; PICA, Posterior Inferior Cerebellar Artery.

Discussion

We examined a possible relationship between intracranial aneurysms and a reduced production of type III collagen through protein analysis. We found no difference in the median type III / type I collagen ratios between patients and controls. However, we found significantly more persons with low type III / type I collagen ratios in the patient group than in the control group. This is in keeping with the a priori formulated hypothesis. We examined the clinical characteristics of patients with normal and low type III /type I collagen ratios and found no differences.

Using protein analysis, Leblanc et al. found normal type III collagen production in skin fibroblasts of 5 patients with familial intracranial aneurysms from different families.[24,25] However, there are several reports finding a reduced production of type III collagen in fibroblasts or low amounts of collagen type III in arterial wall samples of patients with intracranial aneurysms.[15-20] Neil-Dwyer et al. studied a small population. Carboxymethyl cellulose chromatography was performed in 4 controls and in 7 patients, and gel scanning was used in 4 controls and 7 patients. They concluded that 11 of the 17 patients were type III collagen deficient.[15] Østergaard and Oxlund used post-mortem samples of the middle cerebral and brachial arteries of 14 patients who died of a ruptured intracranial aneurysm and similar samples of a control group of 14 patients who died of causes unrelated to aneurysm rupture. Electrophoresis and spectrophotometry revealed a reduced amount of type III collagen in samples of the middle cerebral arteries in 6 patients of the ruptured aneurysm group and in none of the control group.[16] However, the studied population was biased as only deceased patients were included. There was a difference between the type III / type I collagen ratios determined from the middle cerebral artery and the brachial artery taken from the same patient. The authors did not supply explanation for this disparity. Majamaa et al. determined the synthesis of type III collagen in skin fibroblast cultures from 11 patients with an intracranial aneurysm (plus 9 controls), and found no reduced production of type III collagen, although they reported a production of unstable type III collagen in 2 patients.[17] In this study six patients had a first or second degree relative with an intracranial aneurysm including the 2 patients with unstable type III collagen production. In another study by Majamaa et al. in 12 patients with aneurysmal subarachnoid haemorrhages (plus 8 controls)

protein analysis showed combined procollagen production in two cell lines to be reduced.[18] Despite widely varying methodology and several possible flaws in design and conduct, these studies indicated a role of reduced production of type III collagen in the formation of intracranial aneurysms.

Two patients with a low type III / type I collagen ratio (< 5.5%) from our study also had a positive family history of polycystic kidney disease, and one of them had polycystic kidney disease himself. Adult polycystic kidney disease (ADPKD) is a disease with a firmly established association with intracranial aneurysms.[26-28] Two loci for ADPKD are known, and have previously been mapped (PKD1 on chromosome 16 and PKD2 on chromosome 4).[29,30] At least a third locus for ADPKD is still unmapped.[31] The gene for type III collagen is located on chromosome 2.[32] A genetic relation between ADPKD and EDS type IV is possible.

Five of the 8 patients with a low type III collagen had one or more risk factors for intracranial aneurysms. This is in keeping with the theory that intracranial aneurysms have a multifactorial cause. This theory is based on the following clinical and epidemiological data. Independent acquired risk factors for intracranial aneurysms are smoking, hypertension and age.[6-8] If twins have intracranial aneurysms, they often occur in analogous or mirrored locations.[33] Familial aggregation of intracranial aneurysms has been described on several occasions. First degree relatives of patients with an intracranial aneurysm in particular have a two- to threefold increased chance of harboring an aneurysm.[34,35] Familial aneurysms have characteristics different from those that occur spontaneously. They occur at a lower age, are located less often in the anterior cerebral artery ramifications, and may rupture at a smaller size. Among family members aneurysms arise more often from the same arterial distribution and rupture more often within the same decade of life.[36,37] However, indications exist for genetic heterogeneity. In a recent review on the pattern of inheritance in all published families no single genetic mode could be identified.[38] Other links to the genetic nature of intracranial aneurysms are the above mentioned association ADPKD, and with heritable connective tissue disorders.[26-28,39] Intracranial aneurysms occur in the setting of pseudoxanthoma elasticum, osteogenesis imperfecta, Ehlers-Danlos Syndrome Type IV, and neurofibromatosus type 1. The suggestion of Adamson et al. on atherosclerosis being a single cause seems less likely.[40]

Our determinations of collagen production in cell cultures will give a rough indication of the various processes happening in the arterial walls. However, our data suggest that a defect in the production of type III collagen plays a role in the pathogenesis of intracranial aneurysms. Although the reduced type III / type I collagen ratios are likely related to type III collagen deficiency, we have not demonstrated this. Another explanation may be an increased production of type I collagen. However, the amount of radioactivity was standardized by adjusting the volume of the samples applied to the electrophoresis. Consequently, a substantial increase of type I collagen should have resulted in a higher specific activity, and lower sample volume, which was not the case. Therefore a decreased type III collagen production is the most likely explanation for the decreased ratio in these samples. Whether the low type III collagen production is a result of dysfunctional enzymes during collagen processing (e.g. defects during the post-translational modification), or a mutation in the gene has to be elucidated. We found no indications for intracellular accumulation of structurally abnormal type III procollagen in cultured skin fibroblasts with a type III / type I collagen ratio of 5.5% or less.

Kuivaniemi et al. performed DNA sequencing analysis on type III collagen cDNA in 40 patients with a high prevalence of familial aneurysms (29 out of 55 = 53%). They found no mutations. Protein analysis was performed in one individual with a variant at aminoacid 435. The protein production was normal. Unfortunately the collagen production in the other patients was not estimated. Only the part of the gene coding for the triple-helical domain of collagen III was studied.[21] This leaves open the possibility of mutations in the remainder of the gene e.g. the region coding for the C-propeptide, and the possibility of defects during the post-translational modification of type III collagen.

The results of the present study support the theory that reduced production of type III collagen is involved in the pathogenesis of intracranial aneurysms in some patients.

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Type III Collagen deficiency in Saccular Intracranial Aneurysms: defect in gene regulation?

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Introduction

Subarachnoid haemorrhages have a high mortality.[1,2] The majority of subarachnoid haemorrhages is caused by rupture of an intracranial saccular aneurysm.[3] The pathogenesis of intracranial aneurysms has not been elucidated, but is thought to be a multifactorial process.[4,5] Several factors, such as smoking, and hypertension, have been associated with the formation of intracranial aneurysms.[6] The formation of intracranial aneurysms has been associated amongst others with pseudoxanthoma elasticum, autosomal dominant polycystic kidney disease, and Marfan syndrome.[7-9] However, the relation with Marfan syndrome may be fortuitous as was recently demonstrated in a follow up study.[10]

No deficiency of type III collagen production was observed in 5 patients with familial intracranial aneurysms.[11,12] However, several studies have suggested that type III collagen deficiency is a risk factor for intracranial aneurysms.[13-17] In none of these studies there was a molecular analysis of the type III collagen gene, except for the study performed by Kuivaniemi and coworkers. They analysed part of the type III procollagen gene encoding the triple-helix. In 40 patients no mutations were found in this part of the gene.[18]

In a previous protein study we observed a decreased production of type III collagen in cultured skin fibroblasts from 6 of 41 consecutive patients with intracranial aneurysms and in none of a group of 41 age- and sex-matched healthy controls.[19] Here we present molecular analysis of the complete COL3A1 gene, including the regions encoding the globular N- and C-terminal parts of the protein. Mutations in the C-propeptide may affect or even prevent triple helix formation as well as protein stability, and mutations in the N-propeptide may affect the function of type III collagen by preventing removal of the N-propeptide. The propeptide regions have not been analysed in the previous study by Kuvaniemi et al.[18]

Patients with a normal level of type III collagen were also studied. It is possible that mild forms of Ehlers Danlos syndrome vascular type have a normal production of a structurally altered type III collagen, due to a mutation of the COL3A1 gene.[20]

In Ehlers Danlos syndrome vascular type inactive gene copies (null alleles) of COL3A1 result in a mild form of the disorder.[21] To determine if both COL3A1 alleles were active we investigated if polymorphisms in the 3'-

untranslated part of type III collagen were present. Null alleles, that do not produce stable mRNA, lead to a low level of gene product.

Brega et al. investigated allele frequencies for type III collagen gene using an Ava II polymorphism (RFLP) in 19 patients with an intracranial aneurysm, and in 15 controls. A diallelic polymorphism with fragments of sizes 5.7 kilobase (allele A) and 4.3 kilobase (allele B) was found. Allele B was demonstrated in 11 patients and only in 2 controls.[22] This polymorphism is located in an intron of the gene and has probably no biological effect. This association could indicate linkage disequilibrium with a mutation in the COL3A1 gene. This may occur when a mutation has arisen in a gene, close to a DNA marker with a certain allele. If the mutation spreads through the population it remains associated with this allele. If this would be the case, a subgroup of patients, in which type III collagen is involved in the formation of intracranial aneurysms, should be identified by a specific allele of the linked polymorphism. These disequilibrium data suggest that an abnormal type III collagen is involved in the formation of some intracranial aneurysms. We studied a more polymorphic, and informative DNA tandem repeat polymorphism in the COL3A1 gene.[23]

Subjects and Methods

Patient population. Forty-one consecutively patients with an intracranial saccular aneurysm admitted to the Department of Neurosurgery of the Academic Medical Center in Amsterdam and 41 healthy controls (age- and sex-matched) were included in this study.[19] After informed consent a skin biopsy was taken for fibroblast culture, and type III collagen production was determined.[19]

Reverse transcription. Total RNA was isolated from 1×10^6 cultured fibroblasts using RNAzol (Life Technologies, Bethesda, Maryland, USA) according to the manufacturer`s instructions. First strand cDNA was prepared using oligo-dT coated magnetic beads (Dynal AS, Oslo, Norway) to capture the mRNA according to Raineri et al.[24] Reverse transcription was performed on the captured mRNA after washing of the beads, using the oligo-dT on the beads as primer and Superscript II Reverse transcriptase (Life technologies, Bethesda, Maryland, USA).

DNA analysis. The COL3A1 gene was analysed on complementary DNA (cDNA), produced from cultured skin fibroblasts RNA. In all patients the region of the gene encoding the triple helix was screened with single strand conformation polymorphism (SSCP) and heteroduplex analysis, followed by DNA sequencing of aberrant fragments. The regions encoding the N- and C-terminal parts were sequenced in all patients. SSCP/heteroduplex analysis was also performed on genomic DNA and cDNA in the 3'-untranslated region of COL3A1 to detect polymorphic sites. If a patient was heterozygous for a polymorphism this was used to determine expression of both gene copies in the cDNA.

PCR-SSCP/heteroduplex analysis. PCR reactions were performed on the immobilized cDNA to amplify the type III collagen cDNA in 20 overlapping fragments for SSCP/heteroduplex mutation analysis and DNA sequencing. For SSCP/heteroduplex analysis fragments of about 350 bp were used. These fragments were analysed on polyacrylamide minigels in an automated electrophoresis system (Phastsystem, Pharmacia, Upsala, Sweden).[25] If a sample yielded additional bands in the single stranded (SSCP) or double stranded (heteroduplex) area of the gel the region of the gene containing this fragment was subjected to sequence analysis. PCR primer sequences and reaction conditions are available upon request from one of the authors (GP).

DNA sequencing. For DNA sequence analysis PCR products were cDNA or/and genomic DNA made with primersets, of which the primer was 5'-biotin labelled. Single stranded DNA was prepared with streptavidin coated Dynabeads (Dynal, Oslo, Norway) according to the manufacturer`s instructions. Dideoxy sequencing reactions with a Pharmacia T7 sequencing kit (Pharmacia, Upsula, Sweden) were performed on the sense strand of the PCR products on the Dynabeads, and on the antisense strand that was washed off the Dynabeads and ethanol precipitated. Specific sequencing primers were used for each PCR fragment. ^{35}S-labelled dCTP was used to detect the products by autoradiography after electrophoresis on 6% denaturing polyacrylamide gels.

C to T polymorphism exon 33 of the COL3A1 gene. To detect if polymorphisms were present in exon 33 a restriction analysis according to Tromp et al was used.[26]

DNA marker in intron 25 COL3A1 gene. Mays et al described a 15-base DNA tandem repeat marker in intron 25 of COL3A1,[23] and we studied this

marker using CY5 labeled dCTP in the PCR reaction to label the PCR products. The estimated length of the alleles was assessed on an ALFExpress automated DNA sequencer (Pharmacia, Upsala, Sweden), and corresponded to an apparent repeat length of 16 bp.

Statistical analysis. For comparison of allele frequencies we calculated the difference, with 95% confidence interval limits, using the Fisher`s exact test.[27]

Results

Thirty-eight patients had a ruptured, and three an unruptured intracranial aneurysm. In all patients, except one, cerebral angiography was performed. This patient was operated immediately because of a rapid clinical deterioration, and the presence of an aneurysm was confirmed during surgery. A positive family history for subarachnoid haemorrhages was present in 4 patients (10%). DNA sequence analysis of the complete N-propeptide and C-propeptide of type III collagen demonstrated no DNA sequence variations in any of the 41 patients.

By carrying out SSCP/heteroduplex analysis of PCR-amplified fragments from the part of the cDNA encoding the triple-helix of type III collagen of all patients we detected in only one patient a fragment with an altered electrophoretic mobility. The cultured fibroblasts of this patient previously showed a decreased synthesis of type III collagen.[19] In order to characterize the nucleotide sequence of the altered fragment of this patient, DNA sequencing was performed. The data showed a T → C change at position 2793 (Numbering according to Ala Kokko et al.[28]). This was confirmed in genomic DNA. However, the T → C change is a silent mutation and does not lead to an amino acid substitution of type III procollagen. Polymorphisms in exon 33 were detected in 16 of the 40 patients using a restriction analysis according to Tromp et al.[26]

To determine if both copies of the gene produced stable RNA we looked for polymorphisms in the 3'-untranslated part of type III collagen. SSCP/heteroduplex analysis of PCR-amplified fragments of untranslated part of type III collagen showed heterozygosity in 25 of the 41 patients; one of them was shown earlier to have a decreased type III collagen production.[19]

In all 25 patients the heterozygous polymorphism could also be

demonstrated in the cDNA, indicating that both alleles of COL3A1 were expressed and produced stable mRNA.

In the study for abnormal allele frequency in the type III collagen gene using a highly variable, tandem repeat marker in intron 25 of the COL3A1 gene, with 6 alleles, no difference in allele frequency was found in our patients compared to the control group (Table 1), nor was this found in the patients with a decreased type III collagen production compared to a normal production.

Table 1. Allele frequency using 16 base tandem repeat marker in intron 25 COL3A1 gene in 41 controls and 41 consecutive patients with intracranial aneurysms.

Allele	Controls	Patients	Difference (%)	95% confidence limits (%)
226	0	1	+1.2	-1.2 / 3.6
242	34	37	+3.7	-18.8 / 11.5
258	20	20	0	-13.1 / 13.1
274	24	21	-3.7	-17.3 / 10.0
290	3	3	0	-5.8 / 5.8
306	1	0	-1.2	-3.6 / 1.2

Discussion

Several studies have shown a decreased level of type III collagen in patients with intracranial aneurysms.[13-17] Our earlier study of type III collagen protein analysis in diploid fibroblast cultures obtained from 41 patients with intracranial aneurysms also showed a significantly decreased level in 6 patients,[19] supporting the hypothesis that a decreased production of type III collagen plays a role in the formation of intracranial aneurysms in some patients.

SSCP/heteroduplex analysis, screening the complete type III collagen coding sequence, showed only one silent mutation (2793T → C), which does not

lead to a change in the amino acid sequence. This result is in agreement with data presented by Kuivaniemi et al,[18] who observed no mutations when sequencing the triple-helix encoding part of the type III procollagen gene in 40 patients with intracranial aneurysms. However, in this study the N-propeptide part or the C-propeptide part of the type III collagen gene were not analysed. The globular part of the C-propeptide is essential for the formation of the triple helix in fibrillar collagens.[29,30] A mutation in the C-terminal part may theoretically lead to a failure of association of the procollagen monomers with an intracellular breakdown of the mutated proα1 chain or it may lead to an abnormal association with all three α1 chains being destroyed.[31] Therefore, the C-propeptide of the type III collagen gene was sequenced, showing no changes. Our data strengthen the conclusion of the Kuivaniemi study that the type III collagen gene is not likely to be involved in the formation of intracranial aneurysms. In the collagen mutation database 3% of the mutations are large deletions.[32] Single or multi-exon deletions can be demonstrated in cDNA as we found in our collagen mutation studies (unpublished results). Large PCR products from cDNA in these patients did not show evidence for deletions or exon skipping. However, we may have missed any large deletions that encompass the entire gene or lead to unstable messenger RNA.

Null alleles leading to a reduced type III collagen secretion have been described.[21] These patients showed a normal or mild clinical phenotype.[21] In 25 patients, including one patient with a decreased production of type III collagen, the presence of a null allele could be excluded, making it unlikely to be a major contributant to the phenotype in this study group.

In none of the 6 patients with an intracranial aneurysm and a decreased level of type III collagen a mutation in the type III collagen was found using SSCP/heteroduplex. SSCP analysis, with PCR fragments of 350 nucleotides, detects more than 80 % of the mutations.[33,34] Combining SSCP with heteroduplex analysis will lead to a higher detection rate. However, polymorphisms in exon 33 were detected with a restriction analysis according to Tromp et al.[26] and not with SSCP/heteroduplex analysis.

The Ehlers Danlos syndrome vascular type phenotype varies from classical ("acrogeric") presentation to almost no visible abnormalities ("atypical").[35] In patients with classical Ehlers-Danlos syndrome vascular type mutations in the type III collagen gene are frequently detected, but they are

often not found in the atypical form of the disease.[36] Our patients with decreased production of type III collagen may be considered as having atypical Ehlers Danlos syndrome vascular type. The decreased production of type III collagen in the patients with "atypical" Ehlers Danlos Syndrome vascular type may be due to defects during post-translational modicification or an altered collagen metabolism e.g. elevated gelatinase activity.[37]

The reported association of intracranial aneurysms with a type III collagen polymorphism [22] was not confirmed in the present study, as none of the 6 alleles of a highly variable marker showed a difference in frequency in the patients compared to the control group. This shows that, at least in the Dutch population, there is no indication of linkage disequilibrium in the region of COL3A1. It is therefore unlikely that a single mutation in this gene plays a role in susceptibility to intracranial aneurysms.

Although a reduced production of type III collagen is a contributive factor to the formation of intracranial aneurysms in some patients, the exact causative molecular mechanisms of this aberration await further studies.

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Type III collagen deficiency in a family with intracranial aneurysms

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Submitted for publication

Type III collagen deficiency in a family with intracranial aneurysms

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Introduction

Rupture of an intracranial aneurysm may result in a subarachnoid haemorrhage (SAH) with a high chance of death, or serious sequelae in surviving patients.[1] Formation of intracranial aneurysms appears to be a multifactorial process with intrinsic (e.g. vessel wall weakness) and extrinsic factors (e.g. arterial hypertension). In some patients the intrinsic factor may be a type III collagen deficiency.[2] Patients with Ehlers Danlos Syndrome (EDS) vascular type, a genetically determined type III collagen deficiency, have an increased likelihood of harboring intracranial aneurysms.[3] Type III collagen protein is abundant in the vessel wall, and responsible for the tensile strength of the arteries.[4] About 10% of patients have a positive family history for intracranial - aneuryms.[5] Some of the familial aneurysms may be due to a type III collagen deficiency. However, in 5 patients with familial intracranial aneurysms no deficiency of type III collagen was observed suggesting that other factors play a role.[6,7]

We describe a family with aneurysmal subarachnoid haemorrhages, and a type III collagen deficiency in some members. DNA analysis was performed to investigate the underlying cause of this deficiency.

Patients and methods

After informed consent blood samples were taken from the mother of patient III-24 (II-9), and skin biopsies were taken for fibroblast culture from the three patients (III-9, III-12, III-24) with a ruptured intracranial aneurysm, and the healthy twin sister of patient III-24 (III-25). Type III collagen production (type III / type I collagen ratio) was determined, as described previously.[2]

DNA analysis. DNA extracted from white blood cells or fibroblasts, SSCP/ heteroduplex analysis of PCR-amplified fragments from the α-helix of type III collagen cDNA, and DNA sequence analysis of the complete N-propeptide and C-propeptide of type III collagen were performed as published previously.[8] A DNA tandem repeat polymorphism in the COL3A1 gene was typed to determine the segregation of the COL3A1 alleles in the family.[9]

Results

Three patients had a ruptured intracranial aneurysm (see figure 1). None smoked cigarettes or had polycystic kidney disease. Patient II-9 died of carcinoma of the pancreas one year after blood samples were taken for our study.

One (patient III-9) experienced sudden headache, and drowsiness at the age of 21 years. She was admitted, and underwent surgery. The physicians told her that she had a ruptured intracranial aneurysm. This happened approximately 41 years ago, and unfortunately all medical records have been destroyed.

Patient III-24. A 31 year old woman, without relevant medical history, was admitted with serious headache and loss of consciousness. At examination she was drowsy, without focal neurological deficit. Computed tomography of the brain demonstrated blood in the subararachnoidal spaces, fourth ventricle, and basal cisterns. Angiography revealed an aneurysm of the right carotid artery at the junction of the middle and anterior cerebral arteries. The aneurysm was successfully clipped in an operative procedure. She was discharged in good health.

Patient III-12. A the age of 44 years she was admitted for subarachnoid haemorrhage. She had hypertension for 4 years for which she took medication. Angiography revealed an aneurysm of the right internal carotid artery. The aneurysm was clipped, and she was discharged in good health. Three years later she was readmitted for another subarachnoid hemaorrhage. There was an aneurysm of the left internal carotid artery at the junction of the left posterior communicating artery. The aneurysm was clipped, and she was discharged in good health. After review of the first angiography the second aneurysm could not be detected.

Protein and DNA analysis. The collagen type III / type I ratio was below 0.06 in patients III-12 and III-24 indicating a reduced protein production of type III collagen in these patients (see table 1 and figure 2). In normal controls this ratio ranges from 0.06 to 0.2.[2] SSCP/heteroduplex analysis of PCR-amplified cDNA fragments encoding type III collagen α-helix revealed no fragments with an altered electrophoretic mobility in any patient. Sequence analysis of the

complete
N-propeptide and C-propeptide of type III collagen demonstrated no DNA sequence variations in any of the three patients.

To investigate whether the patients shared one or eventually two type III collagen genes we studied a highly variable 16 base pairs tandem repeat marker in intron 25 of the COL3A1 gene. Typing revealed two similar alleles (242/242) in patient III-24 and her mother (II-9) while the patients III-9 and III-12 had different alleles (256/272).

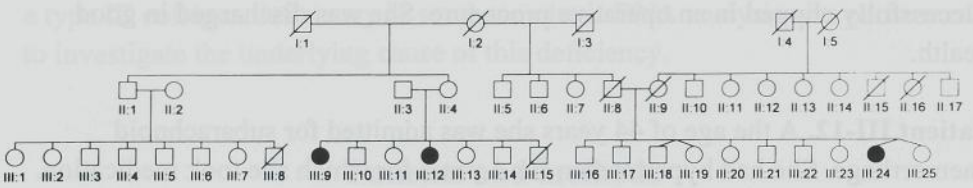

Figure 1. Pedigree of the family.

○, female; □, male; ●, had SAH; ⌀,∅ deceased.

Table 1. Protein and DNA analysis results

	type III / type I collagen ratio	marker intron 25 of COL3A1
III-9	0.139	256/272
III-12	0.045	256/272
II-9	not determined	242/242
1II-24	0.053	242/242
III-25	0.113	242/242

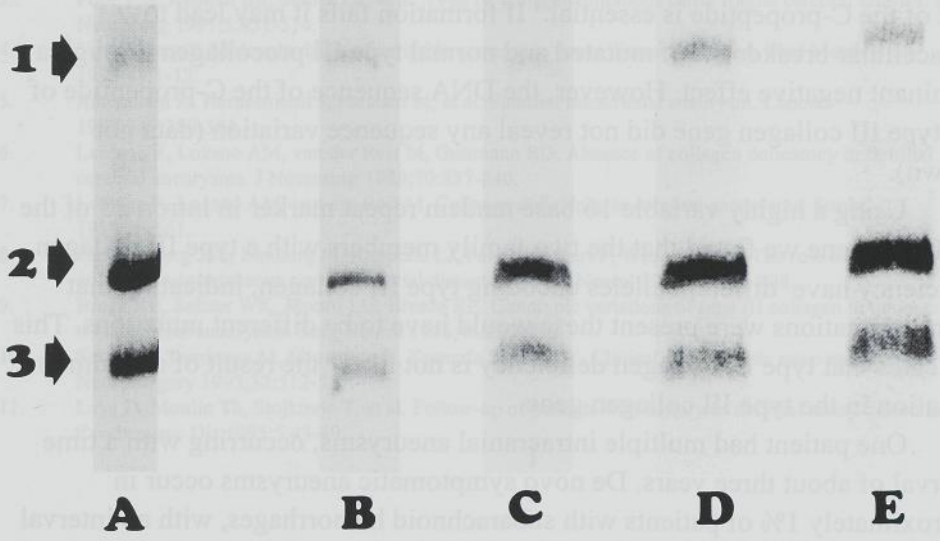

Figure 2. Polyacrylamide gels electrophoresis of patient III-24 (A) with a collagen type III / type I ratio of 0.053, healthy twin sister of patient III-24 (B) with a collagen type III / type I ratio of 0.113, patient III-12 (C) with a collagen type III / type I ratio of 0.045, patient III-9 (D) with a collagen type III / type I ratio of 0.139, and a normal control (E) with a collagen type III / type I ratio of 0,10. Arrow 1 = type III collagen α1 (αl(III)); arrow 2 = type I collagen α1 (α1(I)); arrow 3 = type I collagen α2

Discussion

Type III collagen deficiency has been demonstrated in some patients with sporadic intracranial aneurysms.[2] However, the underlying mechanism has not been elucidated. Leblanc et al did not observe a type III collagen deficiency in 5 patients with familial intracranial aneurysms.[6,7] Now we present two patients with a ruptured intracranial aneurysm from the same family with a type III collagen deficiency.

We screened the complete type III collagen coding sequence for mutations using SSCP/heteroduplex analysis, but found no pathogenetic mutations to explain the type III collagen deficiency. It is noted that SSCP-heteroduplex analysis will detect most but not all sequence variations, missing some mutations.[8]

For the formation of the triple helix in fibrillar collagens the globular part of the C-propeptide is essential.[4] If formation fails it may lead to intracellular breakdown of mutated and normal type III procollagen through a dominant negative effect. However, the DNA sequence of the C-propeptide of the type III collagen gene did not reveal any sequence variation (data not shown).

Using a highly variable 16 base tandem repeat marker in intron 25 of the COL3A1 gene we found that the two family members with a type III collagen deficiency have different alleles encoding type III collagen, indicating that even if mutations were present these would have to be different mutations. This indicates that type III collagen deficiency is not likely the result of a common mutation in the type III collagen gene.

One patient had multiple intracranial aneurysms, occurring with a time interval of about three years. De novo symptomatic aneurysms occur in approximately 1% of patients with subarachnoid hemorrhages, with an interval between first and second bleed ranging from 4 to 34 years.[10] In one study all patients with a recurrent de novo aneurysm had hypertension[10], as did our patient. The patient with a recurrent de novo aneurysm had a type III collagen deficiency. A type III collagen deficiency is possibly associated with recurrent intracranial aneurysms, as has been suggested in the case of recurrent carotid dissections.[11] A study of 105 patients with cervical arterial dissections, demonstrated that 2 of 3 patients with recurrent cervical arterial dissections of the same vessel had EDS vascular type.[11] However, in a previous study we

found no association between type III collagen deficiency and multiple intracranial aneurysms.[2]

In some patients with familial intracranial aneurysms a type III collagen deficiency may be observed. Our data indicate that in the family described here this is probably not due to a mutation in the type III collagen gene, but that it may be related to defects during post-translational modicification or an altered collagen metabolism.

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Summary & Samenvatting

Summary

The purpose of the studies published in this thesis was to identify an intrinsic factor (causing vessel wall weakness) in the multifactorial pathogenesis of intracranial aneurysms. We investigated the possible association between intracranial aneurysms and the connective tissue disorders Marfan syndrome and pseudoxanthoma elasticum. Furthermore the possible role of type III collagen in the formation of intracranial aneurysms was studied.

Chapter 1. Aneurysmal subarachnoidal hemorrhage leads to high mortality and in the survivors to serious morbidity. Improvement of outcome may be achieved by early detection and treatment of unruptured intracranial saccular aneurysms. To accomplish this a better understanding of the pathogenesis of intracranial saccular aneurysms is essential. Associated conditions, risk factors, and pathogenesis of intracranial saccular aneurysms were discussed. Intracranial aneurysms have been thought to be formed in the region of gaps in the media and were therefore considered to be "congenital. Subsequently, the pathogenesis of intracranial aneurysms has been related to hemodynamic stress. Nowodays the formation of intracranial aneurysms is regarded as a multifactorial process with intrinsic (e.g. vessel wall weakness) and extrinsic factors (e.g. hemodynamic stress). The intrinsic factor (e.g. vessel wall weakness) is probably related with a defect of the connective tissue. This resulted in the association of intracranial aneurysms with various connective tissue disorders e.g. Ehlers Danlos syndrome type IV, Marfan syndrome, and pseudoxanthoma elasticum. However, these assumptions have never been investigated.

Chapter 2. The possible relationship between Marfan syndrome and intracranial aneurysms was studied by retrieving the medical records of 135 patients with Marfan syndrome and by performing a follow up study. No patient visiting the clinic had a symptomatic intracranial aneurysm and no symptomatic intracranial aneurysm occurred during follow-up in 129 patients (2850 retrospective patient observation years and 581 prospective patient observation years). The suggested relationship in the literature between Marfan syndrome and intracranial aneurysms is mainly based on 10 case reports and the diagnosis of Marfan syndrome is doubtful in several of these reports.

Furthermore, large studies of patients with Marfan syndrome did not mention ruptured intracranial aneurysms as clinical manifestations. These data indicate that there is insufficient evidence to presume a relationship between symptomatic intracranial aneurysms and Marfan syndrome on the basis of currently available data.

Chapter 3. To investigate if pseudoxanthoma elasticum is associated with intracranial aneurysms we obtained the medical data of 100 patient with pseudoxanthoma elasticum and performed a follow up study. None of them had a symptomatic intracranial aneurysm as presenting symptom. One patient presented with an ischemic stroke. During follow-up of 94 of the 100 patients (mean follow-up 17.1 years, range from 1 to 49 years) none presented with a symptomatic intracranial aneurysm (3168 retrospective patient observation years and 1602 prospective patient observation years). Gastrointestinal bleeding occurred in 18 patients during follow-up, one patient was on aspirin. One patient had an ischaemic stroke as presenting sign and a recurrence during follow-up, and seven patients had ischaemic stroke during follow-up. In these patients computerized tomography or magnetic resonance imaging of the brain showed multiple small ischaemic vascular lesions. Additional investigations revealed, besides a mild hyperhomocysteinaemia in one patient, no other haematological abnormalities or vasculopathies as a cause for the ischaemic stroke. The brain infarction in patients with pseudoxanthoma elasticum is probably caused by small vessel disease. The relative risk of ischaemic stroke in pseudoxanthoma elasticum patients compared with normal population was 22 (95% confidence interval 10.8-39.1). On the basis of the currently available data an association between symptomatic intracranial aneurysms and pseudoxanthoma elasticum is unlikely. However, the incidence of ischaemic stroke, due to small vessel disease, was clearly increased but antiplatelet therapy in patients with pseudoxanthoma elasticum may lead to a high number of gastrointestinal haemorrhages.

Chapter 4 Intracranial aneurysms are known vascular complications of Ehlers Danlos syndrome type IV, a connective tissue disorder with a low or defect production of type III collagen. Type III collagen is a protein of the vessel wall, and is responsible for the tensile strength of arteries. Using protein analysis

several studies in small patient groups demonstrated a low production of type III collagen or a production of a defect type III collagen in some patients with intracranial aneurysms. This led to the assumption that a low or defect production of type III collagen might be the intrinsic factor in the pathogenesis of intracranial aneurysms. However, whether a type III collagen deficiency is a major factor in the pathogenesis of intracranial aneurysms and also the cause of this deficiency remain questionable.

Chapter 5 To investigate if a type III collagen deficiency is involved in the pathogenesis of intracranial aneurysms we performed protein analysis of skin fibroblasts to measure the production of type III collagen (expressed as type III / type I collagen ratio) from 41 consecutively admitted patients with intracranial aneurysms and 41 healthy volunteers (matched for age and sex). The type III / type I collagen ratios in controls ranged from 5.5 to 19.8%, median ratio 10%, and none had a ratio below 5.5%. The type III / type I collagen ratios in patients ranged from 1.1% to 25.1%, median ratio 10.5%, and 8 patients (19.5%) had a low ($< 5.5\%$) ratio.($p = 0.005$, Fisher`s exact test). Our findings support the hypothesis that a reduced production of type III collagen may contribute to the formation of intracranial aneurysms in some patients.

Chapter 6 To order to investigate the cause of type III collagen deficiency we analysed the type III collagen gene in a group of 41 consecutive patients with an intracranial aneurysm of whom 6 patients had a reduced production of type III collagen in cultured diploid fibroblasts from a skin biopsy. Single strand conformation polymorphism (SSCP)/heteroduplex analysis of PCR-amplified fragments from the α-helix of type III collagen cDNA and DNA sequence analysis of the complete N-propeptide and C-propeptide of type III collagen demonstrated no mutations in any of the 41 patients. In 25 patients a hetrozygous polymorphism could be demonstrated in the complementary DNA, indicating both alleles of COL3A1 were expressed and produced stable m RNA. Thus null alleles were excluded in 25 patients including one patient with a low type III collagen production. No differences were found between 41 patients and 41 controls in allele frequencies of a DNA tandem repeat polymorphism located in the COL3A1 gene. It is concluded that the COL3A1

gene is not directly involved in the pathogenesis of most of intracranial aneurysms. The reduced type III collagen production in cultured fibroblasts in some patients with an intracranial aneurysm is not explained by the present study, and needs further exploration, for instance for the presence of posttranslational modifications.

Chapter 7 A positive family history for intracranial aneuryms is found in 10% of patients.
To investigate if familial intracranial aneurysms are related with a type III collagen deficiency we performed type III collagen analysis in three female patients from the same family with an intracranial aneurysm. Two patients had a type III collagen deficiency (type III / type I collagen ratios of 4.5% and 5.3%). Using SSCP/heteroduplex analysis no mutations in the COL3A1 gene were detected, and DNA sequence analysis of the complete C-propeptide of type III collagen revealed no DNA sequence variations. Using a DNA tandem repeat polymorphism in the COL3A1 gene we found that the two patients with low type III collagen production and an intracranial aneurysm had different type III collagen alleles. This suggests that the type III collagen gene is not directly involved in the type III collagen deficiency in this family, and the type III collagen deficiency probably results from defects during post-translational modification or from an altered collagen metabolism.

In conclusion, the pathogenesis of intracranial aneurysms is a multifactorial process with intrinsic (causing vessel wall weakness) and extrinsic (hemodynamic stress) factors. The intrinsic factor may be related to a connective tissue disorder. A causal relation between pseudoxanthoma elasticum or Marfan syndrome with intracranial aneurysms is very unlikely. Consequently the gene responsible for pseudoxanthoma elasticum or the fibillin-gene are probably not involved in the pathogenesis of intracranial aneurysm. In some patients with intracranial aneurysms the intrinsic factor appears to be a type III collagen deficiency. However, the cause of the deficiency remains unclear. Further investigations are necessary to elucidate intrinsic factors associated with the pathogenesis of the intracranial aneurysms.

Samenvatting

Het barsten van een sacculair intracranieel aneurysma (ballonvormige uitstulping van een hersenslagader) leidt tot een bloeding die gepaard gaat met een grote kans op sterfte en bij de overlevenden tot ernstige invaliditeitsverschijnselen. Ondanks ontwikkelingen op het gebied van de neurochirurgie en neuro-anesthesie blijft de prognose na een gebarsten intracranieel aneurysma slecht. Verbetering van dit slechte vooruitzicht kan mogelijk bereikt worden door presymptomatische herkenning en preventieve behandeling van de vaatafwijking. Een beter inzicht in de ontstaanswijze van deze vaatafwijkingen vergemakkelijkt de presymptomatische herkenning. Vorming van deze aneurysmata lijkt een multifactorieel proces te zijn met intrinsieke (leidend tot vaatwand zwakte) en extrinsieke (o.m. hemodynamische) factoren.

De opzet van het onderzoek beschreven in dit proefschrift was om na te gaan in hoeverre de bindweefselziekten Marfan syndroom en pseudoxanthoma elasticum geassocieerd zijn met intracraniale aneurysmata, hetgeen in de literatuur gesuggereerd werd. Verder werd onderzocht of een te lage produktie van type III collageen, een eiwit van de vaatwand, een rol speelt bij de vorming van intracraniele aneurysmata. De mogelijke relatie tussen type III collageen en intracraniale aneurysmata werd onderzocht door middel van eiwit-onderzoek en genetisch onderzoek.

In **hoofdstuk 1** wordt een overzicht gegeven van de ontstaanswijze van intracraniele aneurysmata en van risicofactoren en ziekten geassocieerd met deze vaatafwijkingen. Lange tijd werd verondersteld dat aneurysmata het gevolg waren van de defecten in de tunica media van arteriën en om die reden werden ze beschouwd als aangeboren. Vervolgens werd gedacht dat het ontstaan van de intracraniale sacculaire aneurysmata een degeneratief ("verworven") proces betreft, waarbij hemodynamische stress, waarschijnlijk arteriële hypertensie, een grote rol speelt. Tegenwoordig wordt het ontstaan van intracraniale sacculaire aneurysmata meer gezien als een multifactorieel proces waarbij intrinsieke (congenitale vaatwandafwijkingen) en extrinsieke (hemodynamische) factoren een rol spelen. Het is mogelijk dat een bindweefselstoornis een structureel (intrinsiek) defect van de vaatwand veroorzaakt waardoor deze vaatafwijkingen ontstaan. Dit resulteerde in de

veronderstelling dat intracraniele aneurysmata geassocieerd zouden zijn met bindweefselziekten zoals Ehlers Danlos syndroom type IV, Marfan syndroom en pseudoxanthoma elasticum. Deze relatie is echter nooit onderzocht.

Hoofdstuk 2. Om te onderzoeken of er een relatie bestaat tussen het Marfan syndroom en intracraniele aneurysmata, zijn de medische gegevens achterhaald van 135 patiënten met Marfan syndroom en is een vervolg onderzoek verricht. Geen van de patiënten presenteerde zich met een symptomatisch intracranieel aneurysma, en geen enkel aneurysma leidend tot verschijnselen deed zich voor tijdens vervolg onderzoek van 129 patiënten (2850 retrospectieve patiënt observatie jaren en 581 prospectieve patiënt observatie jaren). De in de literatuur veronderstelde relatie tussen Marfan syndroom en intracraniale aneurysmata is voornamelijk gebaseerd op klinische beschrijvingen van 10 patiënten waarbij in een aantal gevallen de diagnose Marfan syndroom niet duidelijk gesteld is. Verder noemen verscheidene grote patiënten series een gebarsten intracranieel aneurysma niet als klinische presentatie. Op grond van deze gegevens is een relatie tussen het Marfan syndroom en intracraniale aneurysmata onwaarschijnlijk.

Hoofdstuk 3. Bestaat er een associatie tussen pseudoxanthoma elasticum en intracraniele aneurysmata? Hiervoor werd contact opgenomen met 100 patiënten die gediagnostiseerd waren met pseudoxanthoma elasticum. Medische gegevens van deze patiënten werden verzameld en een vervolg onderzoek werd uitgevoerd. Een symptomatisch intracranieel aneurysma deed zich niet voor als eerste klinische verschijnsel. Pseudoxanthoma elasticum werd bij 1 patiënt na een herseninfarct gediagnostiseerd. Gedurende het vervolg onderzoek van 94 patiënten (gemiddelde vervolg periode van 17,1 jaar, variërend van 1 tot 49 jaar) had niemand een symptomatische intracranieel aneurysma (3168 retrospectieve patiënt observatie jaren en 1602 prospectieve patiënt observatie jaren). Achttien patiënten kregen een maag-darm bloeding gedurende het vervolg onderzoek, bij één patiënt tijdens het gebruik van aspirine. De patiënt, die een herseninfarct had als eerste klinische verschijnsel, had tijdens vervolg onderzoek wederom een herseninfarct. In totaal kregen 7 patiënten een herseninfarct tijdens het vervolg onderzoek. Beeldvormend

onderzoek van de hersenen toonde bij deze patiënten meerdere kleine ischemische vasculaire afwijkingen. Verder toonde aanvullend onderzoek, behoudens een milde hyperhomocysteinemie, geen andere hematologische afwijkingen of aanwijzingen voor een vasculopathie als oorzaak voor het herseninfarct. De oorzaak van de herseninfarcten was waarschijnlijk microangiopathie. Het relatieve risico op een herseninfarct bij patiënten met pseudoxanthoma elasticum is in vergelijking met de normale populatie ongeveer 22 (95% betrouwbaarheidsinterval 10.8-39.1). Op grond van de huidige gegevens lijkt er geen verband te zijn tussen symptomatische intracraniale aneurysmata en pseudoxanthoma elasticum. Wel was het aantal herseninfarcten, ten gevolge van microangiopathie, duidelijk verhoogd. Bloedplaatjes aggregatie remmende medicijnen kunnen bij patiënten met pseudoxanthoma elasticum leiden tot maag-darm bloedingen.

Hoofdstuk 4 Intracraniele aneurysmata zijn een bekende vasculaire complicatie bij Ehlers Danlos type IV, een bindweefselziekte veroorzaakt door een te lage of verkeerde produktie van type III collageen. Type III collageen is een eiwit dat zich onder meer bevindt in de vaatwand en is mede-verantwoordelijk voor de elasticiteit van de vaatwand. Uit de resultaten van verschillende studies met kleine groepen patiënten blijkt een relatie tussen type III collageen en intracraniele aneurysmata te bestaan. Echter in hoeverre een deficiëntie van type III collageen een belangrijke factor in de pathogenese van intracraniele aneurysmata is blijft de vraag en wat de onderliggende oorzaak van de type III collageen deficiëntie is.

Hoofdstuk 5. Om na te gaan in hoeverre type III collageen een rol speelt bij de vorming van intracraniele aneurysmata werd eiwit-onderzoek verricht van huidfibroblasten in een groep van 41 patiënten met een intracranieel aneurysma en 41 gezonde vrijwilligers, met dezelfde leeftijd en geslacht verdeling als de patiënten. De type III/ type I ratio (maat voor de produktie van type III collageen) in de controle groep varieerde van 5,5 % tot 19,8% (mediaan 10%), niemand had een ratio onder de 5,5 %. In de patiënten groep varieerde de ratio van 1,1% tot 25,1% (mediaan 10,5%). Verder hadden 8 patiënten (19,5%) een zeer lage ratio (< 5,5%) horend bij een verlaagde produktie van type III collageen type. Bij sommige patiënten lijkt een

verlaagde produktie van type III collageen een rol te spelen bij de vorming van intracraniele aneurysmata.

Hoofdstuk 6. Om te onderzoeken wat de oorzaak is van de type III collageen deficiëntie werd met behulp van DNA-onderzoek onderzocht of in de groep van 41 patiënten met een intracranieel aneurysma mutaties in het COL3A1 gen, dat codeert voor type III collageen, aangetoond konden worden. Zoals vermeldt in hoofdstuk 5 hadden 6 van de 41 patiënten een aangetoonde type III collageen deficiëntie. Met behulp van SSCP (Single strand conformation polymorphism) /heteroduplex analyse konden er geen mutatie`s aangetoond worden in het deel van het chromosoom dat codeert voor de α-helix van type III collageen. Met DNA sequence analyse werd het globulaire N- en C-terminale deel van het type III collagen gen onderzocht en hierbij werden geen mutatie`s gevonden. SSCP/heteroduplex analyse van het niet-coderende 3' terminale deel van type III collageen toonde een heterozygotie in 25 van de 41 patiënten. Hierdoor konden nul allelen bij 25 patiënten uitgesloten worden; één daarvan had een verlaagde collageen type III produktie. De verdeling van lengten van het COL3A1 gen tussen de 41 patiënten en 41 controles was statistisch niet verschillend. Dit bevestigt dat het onwaarschijnlijk is dat mutaties de oorzaak zijn van lage type III collageen produktie. Het COL3A1 gen blijkt niet direct betrokken te zijn bij de pathogenese van intracraniale aneurysmata. Hoe de lage type III collageen produktie, die soms bij patiënten met een intracranieel aneurysma gevonden wordt, ontstaat is niet geheel duidelijk. Mogelijk dat er een stoornis is in het posttranslationele proces of gen-regulering.

Sommige patiënten met een gebarsten intracranieel aneurysma hebben een positieve familie anamnese voor intracraniele sacculaire aneurysmata (twee of meer eerstegraads familieleden met een aneurysmale subarachnoïdale bloeding). Mogelijk dat deze familiaire factor een verlaagde type III collageen produktie is. **(Hoofdstuk 7)** In een familie waarin drie vrouwelijke leden een intracranieel aneurysma hadden is onderzoek verricht om te bepalen of er een type III collageen deficiëntie aanwezig was en indien deze aanwezig was wat de oorzaak ervan was. Twee patiënten met een intracranieel aneurysma hadden een type III collageen deficiëntie (type III / type I collageen ratios van 4,5% en 5,3%). Echter er konden geen mutaties gevonden worden in het gen dat codeert

voor de α-helix van type III collageen en het globulaire N- en C-terminale deel van type III collageen. Met behulp van een DNA marker van het COL3A1 gen werd aangetoond dat de twee patiënten met een type III collageen deficiëntie en een intracranieel aneurysma verschillende type III collageen allelen hadden. Gezien deze resultaten is het waarschijnlijk dat de type III collageen deficiëntie in deze familie het gevolg is van defecten tijdens het post-translationele proces of door een veranderd collageen metabolisme.

Samenvattend, de ontstaanswijze van intracraniale aneurysmata is een multifactorieel proces waarbij intrinsieke (lijdend tot vaatwandzwakte) en extrinsieke (hemodynamische) factoren een rol spelen. De vaatwandzwakte moet mogelijk gezocht worden in een bindweefselstoornis. Bij sommige patiënten met een intracranieel aneurysma lijkt de intrinsieke factor een verminderde type III collageen produktie te zijn, echter het onderliggende verantwoordelijk mechanisme hiervoor is niet geheel duidelijk. Verder onderzoek is nodig om de intrinsieke factor nader te specificeren. Het is niet waarschijnlijk dat het gen van pseudoxanthoma elasticum of het fibrilline gen betrokken zijn bij het ontstaan van deze vaatafwijkingen daar er geen duidelijke relatie bestaat tussen pseudoxanthoma elasticum of Marfan syndroom en intracraniale aneurysmata.

Dankwoord

Curriculum Vitae

List of publications

Appendix

Dankwoord

Dit proefschrift kwam in de loop der jaren tot stand dankzij de medewerking en hulp van velen die ik hierbij bedank.

De patienten en de "controle-groep" die mee hebben gedaan; zonder hen was er geen onderzoek mogelijk geweest.
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Curriculum Vitae

The author was born October 4, 1963 in Abadan (Iran). After secondary school in Haarlem (VWO, Spaarne Scholen Gemeenschap), he started the study of medicine at the University of Amsterdam in 1982. Following his graduation in 1990, he served as a medical officer in the army (Kolonel Six Kazerne, Amsterdam). In 1991 he started to work as a resident in neurology at the Spaarne Ziekenhuis in Haarlem-Heemstede (A Broekers, RH Groen, J Mispelblom Beyer), and subsequently at the department of neurology at the Academic Medical Center in Amsterdam. In September 1994 he started his neurology training at the department of neurology at the University Hospital Nijmegen (Prof.Dr. G.W.A.M. Padberg / Prof.Dr. M.J. Zwarts) which he will finish in March 2000. Currently he is a member of the research project GAIA (Genetic Analysis Intracranial Aneurysms).

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The author was born October 4, 1963 in Aberdeen (Iona). After secondary school in Haarlem (VWO, Spaarne Scholen Gemeenschap), he started the study of medicine at the University of Amsterdam in 1982. Following his graduation in 1990, he served as a medical officer in the army (Colonial Six Kazerne, Amsterdam). In 1991 he started to work as a resident in neurology at the Spaarne Ziekenhuis in Haarlem-Heemstede (A. Broekers, RH Groen, J. Mispelblom Beyer), and subsequently at the department of neurology at the Academic Medical Center in Amsterdam. In September 1994 he started his neurology training at the department of neurology at the University Hospital Nijmegen (Prof.Dr. G.W.A.M. Padberg / Prof.Dr. M.J. Zwarts) which he will finish in March 2000. Currently he is a member of the research project GAIA (Genetic Analysis Inflammatorial Aneurysms).

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Appendix

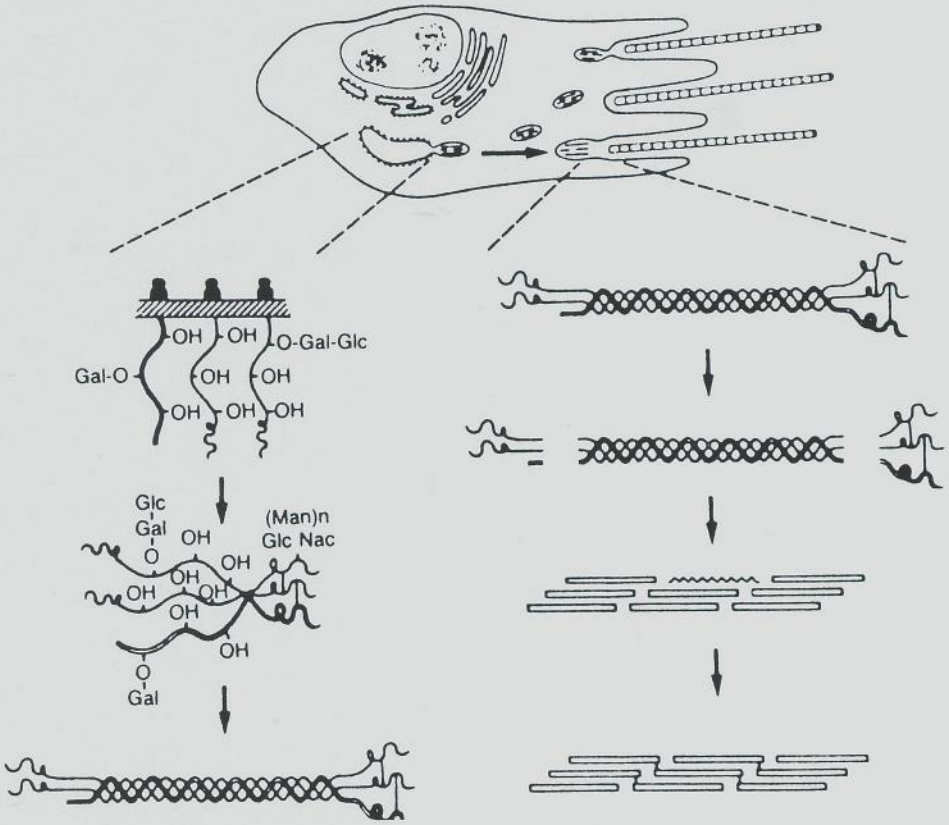

Figure showing the normal synthesis of procollagen and collagen by a fibroblast. On the left is depicted the assembly of proα chains in cisternae of the rough endoplasmic reticulum, posttranslational hydroxylations and glycosylations, the association and disulfide bonding of C-propeptides, and the folding of the triple helix by nucleated growth. On the right is depicted the proteolytic cleavage of procollagen to collagen, the self-assembly of fibrils by nucleated growth, and the covalent cross linking of the fibrils. The collagen molecule is first shown as a triple helix, and then as either a wavy line (representing a molecule assembling on the surface of a fibril) or a rectangle (representing the quarter-staggered array of monomers in a fibril). The proteolytic cleavage of procollagen and the assembly of fibrils may occur within crypts of fibroblasts as shown here or perhaps at some distance from the cell. Gal, galactose; Glc, glucose; Glc Nac, N-acetylglucosamine; (Man)n, Mannose.

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