To those of us carrying saccular cerebral artery aneurysms,
- and to my family

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The Pathobiology of Saccular Cerebral Artery Aneurysm Rupture and Repair

-a Clinicopathological and Experimental Approach

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ACADEMIC DISSERTATION

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Abbreviations

15-LOX	15-lipoxygenase enzyme
AAA	Abdominal aortic aneurysm
ACA	Anterior cerebral artery
AcomA	Anterior communicating artery
AICA	Anterior inferior cerebellar artery
APKD	Autosomal polycystic kidney disease
ApoB	Apolipoprotein B
ApoE	Apolipoprotein E
BA	Basilar artery
bFGF-R	Receptor for basic fibroblast growth factor
EEL	External elastic lamina
ELISA	Enzyme immunoabsorbent assay
eNOS	Endothelial nitric oxide synthetase
GDCs	Guglielmi detachable coils
HLA	Human leukocytes antigen system
ICA	Internal carotid artery
IEL	Internal elastic lamina
IGF-R	Receptor for insulin-like growth factor
ISAT	International Subarachnoid Aneurysm Trial
ISUIA	International Study of Unruptured Intracranial Aneurysms
LDL	Low density lipoprotein
MCA	Middle cerebral artery
MHC I	Major histocompatibility complex I
MHC II	Major histocompatibility complex II
MHOT	Myointimal hyperplasia / organizing thrombosis
MMPs	Matrix metalloproteinases
NF-kB	Nuclear factor -kappa beta (a transcription factor activated by inflammation)
oxLDL	Oxidatively modified low density lipoprotein
PCA	Posterior cerebral artery
PcomA	Posterior communicating artery
PCR	Polymerase chain reaction
PDGF-Rα / β	Receptor for platelet derived growth factor alpha / beta
PICA	Posterior inferior cerebellar artery
SAH	Subarachnoid hemorrhage
SCA	Superior cerebellar artery
SCAA	Saccular cerebral artery aneurysm
SMC	Smooth muscle cell
TGFβ-R	Receptor for transforming growth factor beta
Th1 -cell	Helper T-cell type 1
Th2 –cell	Helper T-cell type 2
VEGF-R	Receptor for vascular endothelial growth factor

Original publications

I. Remodeling of saccular cerebral artery aneurysm wall is associated with rupture. Histological analysis of 24 unruptured and 42 ruptured cases
Juhana Frösen, MD, Anna Piippo, MB, Anders Paetau, MD PhD, Marko Kangasniemi MD PhD, Mika Niemelä, MD PhD, Juha Hernesniemi, MD PhD, Juha Jääskeläinen, MD PhD Stroke 2004;35:2287-2293.

II. OxLDL accumulates in cerebral aneurysm and is targeted by plasma antibodies in SAH patients
Juhana Frösen MD, Riikka Tulamo MB, Tommi Heikura MSc, Outi Närvänen MSc, Ayse Karatas MD, Mika Niemelä MD PhD, Juha Hernesniemi MD PhD, Juha Jääskeläinen MD PhD, Anna-Liisa Levonen MD PhD, Seppo Ylä-Herttuala MD PhD. (Submitted)

III. Growth factor receptor expression and remodeling of saccular cerebral artery aneurysm wall -Implications for biological therapy preventing rupture
Juhana Frösen, MD, Anna Piippo, MB, Anders Paetau, MD PhD, Marko Kangasniemi MD PhD, Mika Niemelä, MD PhD, Juha Hernesniemi, MD PhD, Juha Jääskeläinen, MD PhD Neurosurgery 2006;58:534-541.

IV. Contribution of mural and bone marrow-derived myointimal cells to thrombus organization and wall remodeling in a microsurgical murine saccular aneurysm model
Juhana Frösen, MD, Johan Marjamaa, MD, Marjukka Myllärniemi, MD, PhD, Usama Abo-Ramadan, PhD, Riikka Tulamo, MB, Mika Niemelä, MD, PhD, Juha Hernesniemi, MD, PhD, Juha Jääskeläinen, MD, PhD Neurosurgery 2006;58:936-944.

And additional unpublished data: Transfused undifferentiated mononuclear cells from healthy human donors do not integrate to the balloon injured aorta of immunotolerant rats

Contents

Abstract

Backround and Purpose
The often fatal (in 50-35%) subarachnoid hemorrhage SAH caused by saccular cerebral artery aneurysm SCAA rupture affects mainly the working aged population. The incidence of SAH is 10-11 / 100 000 in Western countries and twice as high in Finland and Japan. The estimated prevalence of SCAAs is around 2%. Many of those never rupture. However, currently there are, however, no diagnostic methods to identify rupture-prone SCAAs from quiescent, (dormant) ones. Since SCAA rupture has such a sinister outcome, and all current treatment modalities are associated with morbidity and mortality, finding diagnostic markers for rupture-prone SCAAs is of primary importance since a SCAA rupture has such a sinister outcome, and all current treatment modalities are associated with morbidity and mortality. Also the therapies that prevent SCAA rupture need to be developed to as minimally invasive as possible.

Although the clinical risk factors for SCAA rupture have been extensively studied and documented in large patient series, the cellular and molecular mechanisms how these risk factors lead to SCAA wall rupture remain incompletely known. Elucidation of the molecular and cellular pathobiology of the SCAA wall is needed in order to develop i) novel diagnostic tools that could identify rupture-prone SCAAs or patients at risk of SAH, and to ii) develop novel biological therapies that prevent SCAA wall rupture.

Materials and Methods
In this study, histological samples from unruptured and ruptured SCAAs and plasma samples from SCAA carriers were compared in order to identify structural changes, cell populations, growth factor receptors, or other molecular markers that would associate with SCAA wall rupture. In addition, experimental saccular aneurysm models and experimental models of mechanical vascular injury were used to study the cellular mechanisms of scar formation in the arterial wall, and the adaptation of the arterial wall to increased mechanical stress.

Results and Interpretation
Inflammation and degeneration of the SCAA wall, namely loss of mural cells and degradation of the wall matrix, were found to associate with rupture. Unruptured SCAA walls had structural resemblance with pads of myointimal hyperplasia or so called neointima that characterizes early athero-

sclerotic lesions, and is the repair and adaptation mechanism of the arterial wall after injury or increased mechanical stress. As in pads of myointimal hyperplasia elsewhere in the vasculature, oxidated LDL was found in the SCAA walls. Immunity against OxLDL was demonstrated in SAH patients with detection of circulating anti-oxidized LDL antibodies, which were a significantly associated with the risk of rupture in patients with solitary SCAAs.

Growth factor receptors associated with arterial wall remodeling and angiogenesis were more expressed in ruptured SCAA walls. In experimental saccular aneurysm models, capillary growth, arterial wall remodeling and neointima formation were found. The neointimal cells were shown to originate from the experimental aneurysm wall with minor contribution from the adjacent artery, and a negligible contribution of bone marrow-derived neointimal cells. Since loss of mural cells characterizes ruptured human SCAAs and likely impairs the adaptation and repair mechanism of ruptured or rupture-prone SCAAs, we investigated also the hypothesis that bone marrow-derived or circulating neointimal precursor cells could be used to enhance neointima formation and compensate the impaired repair capacity in ruptured SCAA walls. However, significant contribution of bone marrow cells or circulating mononuclear cells to neointima formation was not found.

Introduction

Rupture of a saccular cerebral artery aneurysm (SCAA) is the main cause (in 85%) of the non-traumatic subarachnoid hemorrhage (SAH), fatal in approximately 50% of all cases (111; 332). SAH caused by a SCAA rupture mostly affects previously healthy working aged population (median age 40-60) and has a yearly incidence of around 1000 cases in Finland (123). Based on angiography studies and a prospective autopsy series, it has been estimated that around 2% of the population are SCAA carriers (259).

SAH can be prevented by either microsurgical ligation of the SCAA neck (clipping) or by endovascular embolization of the SCAA lumen (coiling). In experienced hands both therapies have similar success rates in preventing SAH during the one year follow-up (166). Although clipping is associated with a risk of brain lesions due to craniotomy and brain retraction (166) and is dependent on the experience of the operator, in addition to being associated with a higher mortality of some types of anterior circulation SCAAs in a large multicenter study (ISAT) (195). In the long term follow-up, however, clipping remains a superior treatment option because some coiled SCAAs may grow and rupture during long term follow-up (249). This may occur because of residual filling, recanalization of the embolized aneurysm, or compaction of the coils that result from insufficient fibrosis of the coil induced thrombus in the SCAA lumen (247; 249; 253). Bioactive embolization devices that release growth factors, radioactivity, or gene therapy vectors that would increase fibrosis of embolized aneurysms and re-endothelialization of the aneurysm orifice, are being developed to improve the long term results of endovascular therapy (60; 149; 189; 252; 258) .

Most SCAA carriers are asymptomatic, and based on the similar prevalence of SCAAs in prospective obduction series (3-4%) and angiography studies (5-7%) (259) compared to the incidence of SAH (10-22/100 000) (123), it seems that many SCAAs do not rupture during the lifetime of their carriers. Due to morbidity and mortality associated with SCAA treatment, and because of the high fatality and disability rates caused by a SCAA rupture, diagnostic means that distinguish SCAAs with imminent rupture risk are needed in order to better focus invasive therapy (clipping or coiling) for patients with rupture-prone SCAAs. Also SCAAs that have already ruptured have a high risk of re-bleedings and need to be isolated from the cerebral circulation (137; 152-154; 226).

Knowledge of the pathobiology of SCAA formation, growth, rupture, and repair is essential in identification of SCAA carriers with a high risk of

rupture and for rationally designing novel bioactive therapies. Known risk factors for SAH include SCAA size, growth, location, presence of multiple SCAAs, smoking, untreated hypertension, alcohol, caffeine or cocaine abuse, female gender, and familial background (259; 332; 351) (27; 136; 138; 141; 163; 271). Around 10% of Finnish SCAAs have a familiar background (262), with the genetic defect located in the 19q13.3 region in the Finnish population (331; 354). Also some monogenic hereditary diseases (287) as well as some genetic polymorphisms (159; 300), including that of the HLA-region (215; 225; 276), is associated with SCAAs and SAH.

Although the risk factors for SAH have been well documented by several large patient series and meta-analysis, it still remains unknown how the known SAH risk factors affect the SCAA wall. Clinicopathological series of SCAA walls that also describe clinical and radiological data are scarce due to the difficulty of acquiring SCAA samples from patients. The few prior histological series published have described structural fragility, apoptosis, increased inflammation, and increased matrix metalloproteinase (MMPs) activity in the ruptured SCAA wall (30; 42; 133; 155; 169; 208; 234). In addition, angiogenic factors have been investigated in the SCAA wall (160; 299). The mechanisms and effectors of SCAA wall rupture and repair need, however, to be further elucidated to develop novel diagnostic methods that discriminate SCAAs in risk of imminent rupture, and to acquire data for rational design of bioactive therapies.

Review of the literature

Figure 1. Saccular cerebral artery aneurysm (SCAA)

Aneurysms are divided according to their morphology into: i) saccular aneurysms that are pouch-like protrusions of the vessel wall, usually arising in cerebral artery bifurcations (A); ii) fusiform aneurysm that are dilatations of the vessel wall that do not lead to formation of a separate saccular pouch (B); and iii) dolichoectasias that are elongated, tortuous, and some times dilated vessel segments (C). Furthermore, a rare fourth type of cerebral artery aneurysm is the dissecting aneurysm that usually has a fusiform shape (B), but originates from acute dissection or tearing of the arterial wall layers. This study was limited to saccular cerebral artery aneurysms (SCAA). SCAAs come in various shapes (angiographies D-G). The classical "berry"-shape of a SCAA is demonstrated with 3D-DSA in D (AcomA aneurysm), and a more elongated shape (high aspect ratio) with regular DSA in E (MCA bifurcation aneurysm). High aspect-ratio (E) and multilobular shape or the presence of so called secondary pouches (F) associates with high risk of rupture. Aspect ratio is the ratio of fundus length and neck width, measured as demonstrated in G. The usual location of the SCAAs near the Circle of Willis and the skull base is demonstrated with reconstructions of CTA that show a right multilobular MCA aneurysm and the skull base (H).

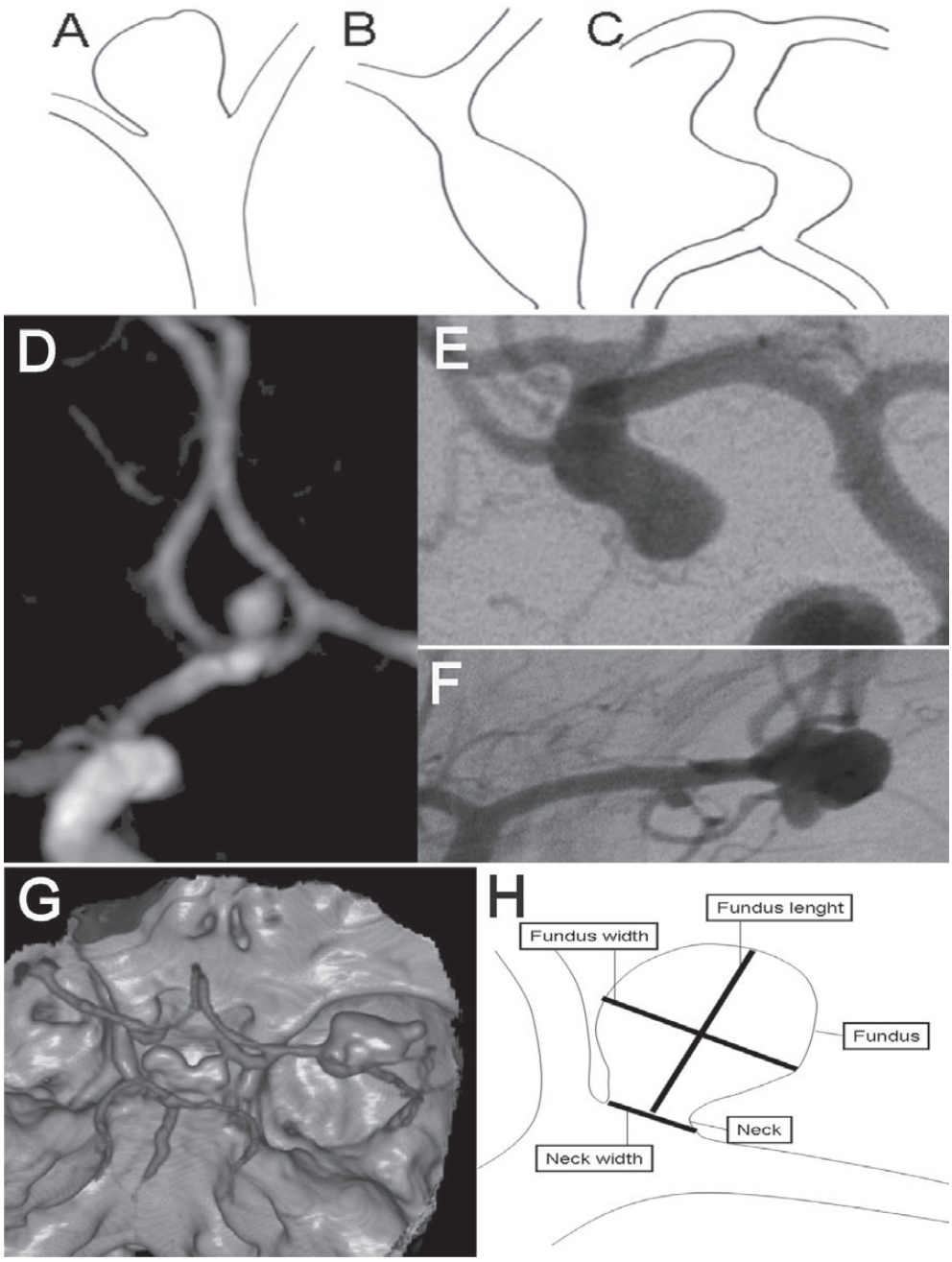

Figure 1. Saccular cerebral artery aneurysm (SCAA)

Figure 2. Subarachnoid hemorrhage

Rupture of a SCAA leads to high pressure hemorrhage to the subarachnoid space surrounding the brain, or occasionally into the brain parenchyme. Blood in the basal cisterns (A), fissura Sylvi (A), and elsewhere in the subarachnoid space (B) is detected with computed tomography (CT) (white in CT). When blood in the subarachnoid space obstructs the physiological flow of CSF, the accumulation of CSF may lead to increased ICP and dilation of the cerebral ventricles (so called hydrocephalia). Normal ventricles are shown in C, post-SAH hydrocephalia in D. In addition, damage caused by an already resorbed ICH (marked with ∗) can be seen in the right frontal lobe under a craniotomy bone flap in D.

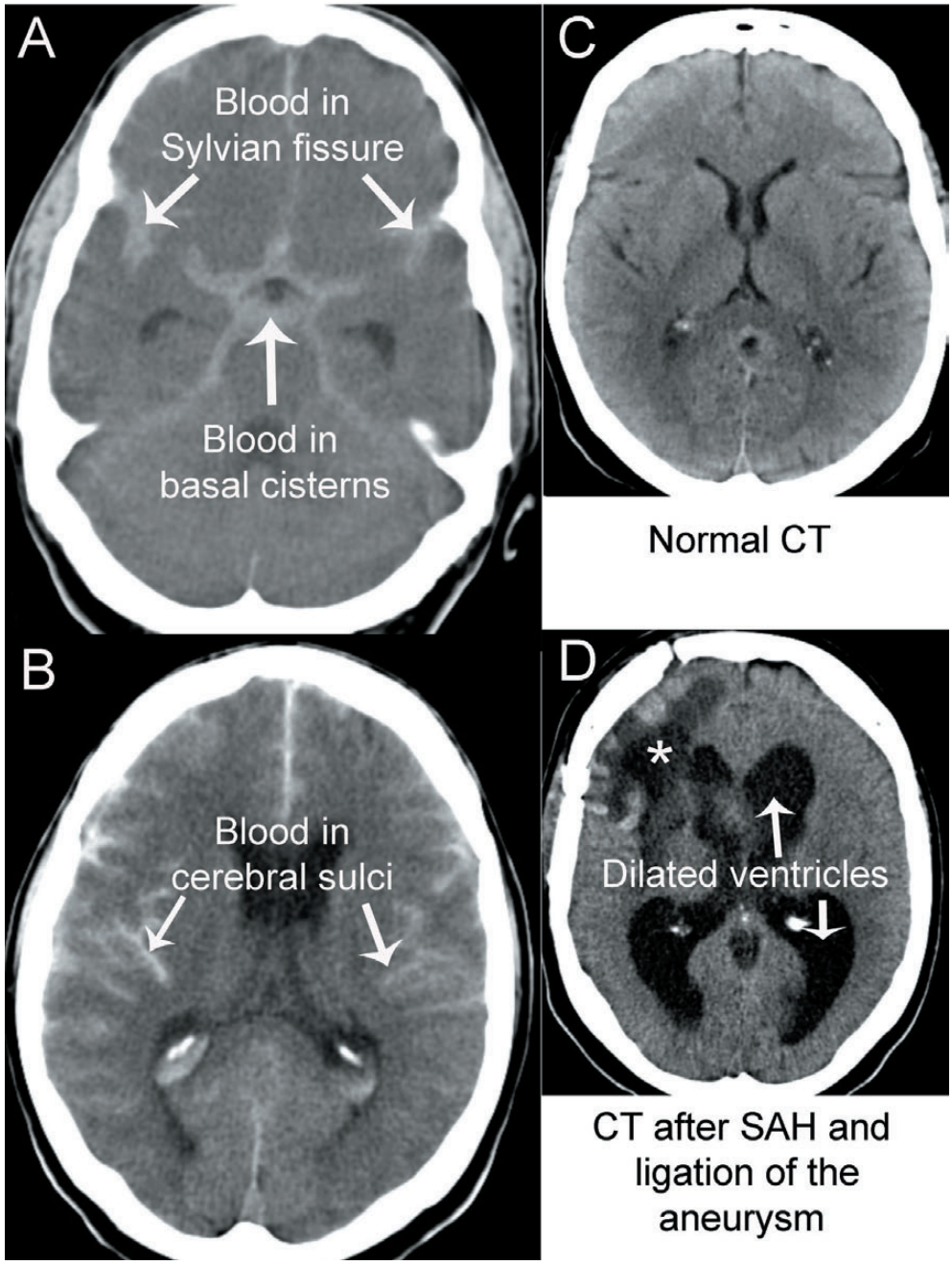

Figure 2. Subarachnoid hemorrhage

Table 1. Aneurysm (A) and patient (B) related risk factors for subarachnoid hemorrhage

Risk factor	Odds ratio or Relative risk	Reference		
			Number of Patients	*Remarks*
A. Aneurysm related				
Size (reported threshold size for increased risk from 7mm to 13mm, or no threshold)	not reported	Tsutsumi (2000)	62	
	not reported	Juvela (1993)	142	
	not reported	Juvela (2003)	142	
	not reported	Wiebers (1981)	65	
>10mm	5.5 (3.3-9.5)	Rinkel (1998)	675	Meta-analysis
7-12mm	3.3 (1.3-8.2)	ISUIA 2	1692	
>12mm	17.0 (8.0-36.1)	ISUIA 2	1692	
Growth	not reported	Juvela (1993)	142	
Morphology	not reported	Beck	147	
	not reported	Ujie	207	
	not reported	Weir	774	
	not reported	Nader-Sepachi	182	
	not reported	Raghavan	27	
and flow dynamics	not reported	Hassan	68	
Location				
Posterior Circulation	4.1 (3.3-9.5)	Rinkel (1998)	434	Meta-analysis
Basilar tip	2.3 (1.1-4.8)	ISUIA 2	1692	
PcomA	2.1 (1.1-4.2)	ISUIA 2	1692	
Presence of symptoms	8.2 (3.9-17)	Rinkel (1998)	463	Meta-analysis

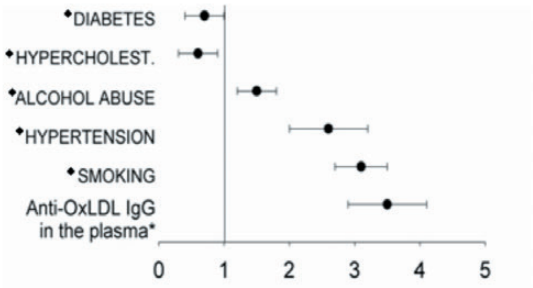

Figure 3. Cardiovascular risk factors and the risk of SAH

The odds ratios for SAH with 95% confidence intervals are presented for the major risk factors of cardiovascular disease. For comparison, odds for circulating anti-oxidized LDL antibodies that were studied in Publication 2 are also shown (marked with ∗). Data of the odds ratios is derived from the systematic review by Feigin et al. in 2005 (marked with ♦).

Risk factor	Odds ratio or Relative risk	Reference		
			Number of Patients	*Remarks*
B. Patient related				
Female gender	not reported	Kissela (2002)	107	
	not reported	Inagawa (2005)	247	
early menarche (before 13y)	3.24 (1.25-4.03)	Okamoto (2001)	124	
nulligradivity	4.23 (1.05-7.56)	Okamoto (2001)	124	
Hormone replacement therapy	0.6 (0.2-1.5) (RR)	Feigin (2005)	3936	Meta-analysis
	0.6 (0.4-0.8) (OR)	Feigin (2005)	3936	Meta-analysis
Age	increasing cumulatively with age not reported	Juvela (2003)	142	
		Inagawa (2005)	247	
Smoking	3.04 (1.12-7.66)	Juvela (2002)	89	
	3.73 (2.67-5.12)	Broderick (2003)	312	
	2.4 (not reported)	Matsumoto (1999)	182	
	not reported	Kissela (2002)	107	
	4.55 (1.08-19.30)	Isaksen (2005)	27 161	
	not reported	Inagawa (2005)	247	
	2.2 (1.3-3.6) (RR)	Feigin (2005)	3936	Meta-analysis
	3.1 (2.7-3.5) (OR)	Feigin (2005)	3936	Meta-analysis
Hypertension	2.21 (1.48-3.29)	Broderick (2003)	312	
	1.46 (1.01-2.11)	Taylor (1995)	20 767	
	not reported	Kissela (2002)	107	
	2.46 (1.52-3.97)	Isaksen (2005)	27 161	
	12.67 (1.53-104.70)	Juvela (2003)	142	
	not reported	Inagawa (2005)	247	
	2.5 (2.0-3.1) (RR)	Feigin (2005)	3936	Meta-analysis
	2.6 (2.0-3.1) (OR)	Feigin (2005)	3936	Meta-analysis
Hypercholesterolemia	not reported	Inagawa (2005)	247	
	0.8 (0.6-1.2) (RR)	Feigin (2005)	3936	Meta-analysis
	0.6 (0.4-0.9) (OR)	Feigin (2005)	3936	Meta-analysis
Body mass index	1.59 (1.08-2.35)	Broderick (2003)	312	
	not reported	Kissela (2002)	107	
Diabetes	0.3 (0.0-2.2) (RR)	Feigin (2005)	3936	Meta-analysis
	0.7 (0.5-0.8) (OR)	Feigin (2005)	3936	Meta-analysis
Cocaine use	24.97 (3.95-unknown)	Broderick (2003)	312	
High alcohol consumption	not reported	Kissela (2002)	107	
	2.1 (1.5-2.8) (RR)	Feigin (2005)	3936	Meta-analysis
	1.5 (1.3-1.8) (OR)	Feigin (2005)	3936	Meta-analysis
Caffeine use	2.48 (1.19-5.20)	Broderick (2003)	312	
	3.86 (1.01-14.73)	Isaksen (2005)	27 161	
Prior corticosteroid use	1.7 (1.1-2.7)	Ruigrok (2005)	1158	
Prior SAH	not reported	ISUIA 1 (NEJM 1998)		
Familial history	3.83 (1.73-8.46)	Broderick (2003)	312	
	4.0 (2.6-5.8) for SCAAs	Raaymakers (1999)	626	
	not reported	Kissela (2002)	107	
	-	Bromberg (1995)		

Figure 4. Histology of extracranial and intracranial arteries

Normal extracranial arterial wall is composed of three layers: endothelium and / or intima on the luminal side, the muscular media, and the adventitia on the outer side. These layers are separated by the internal and external elastic laminas (IEL and EEL). Other elastic laminas also occur between the IEL and EEL in the media. Migration of smooth muscle cells between the endothelium and the IEL and subsequent proliferation is called myointimal hyperplasia, or neointima in animal in which no muscular layer normaly exists between the IEL and the endothelium. Cerebral arteries differ from extracranial arteries in that they do not have the EEL, only an IEL with little elastic laminas in the media layer.

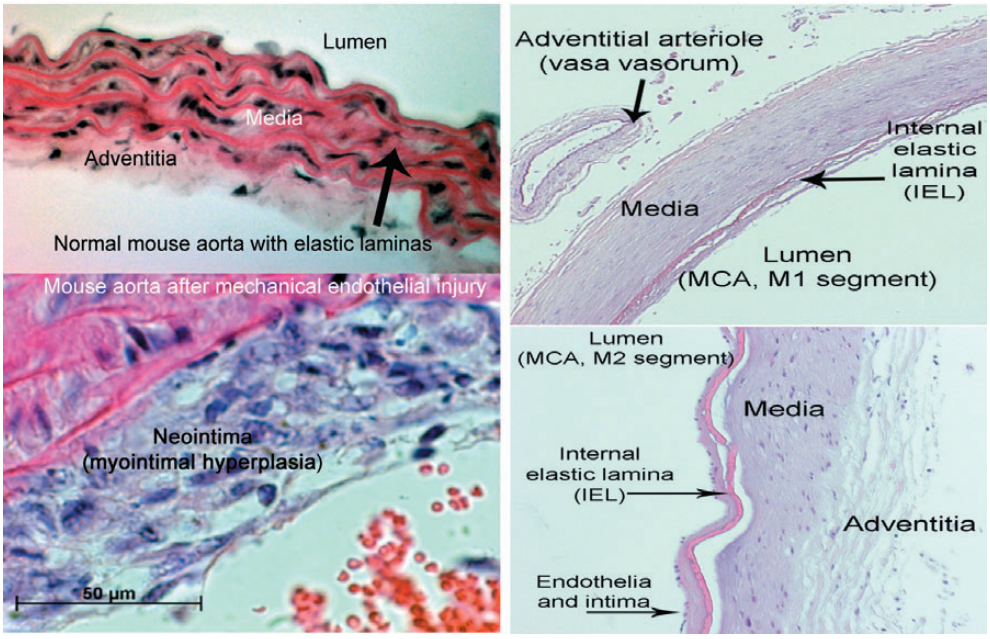

Figure 4. Histology of extracranial and intracranial arteries

Figure 5. Histology of the cerebral arteries

Unlike extracranial arteries, cerebral arteries have gap in their muscular media layer in bifurcations. Although these "gaps" seems as weak points, they seem to be very resistant to mechanical stress due to tendon-like connective tissue that bridges the discontinuity of the media (see ref. Finlay et al.). Myointimal hyperplasia and lipid accumulation occurs in the cerebral artery bifurcations, as in extracranial arteries.

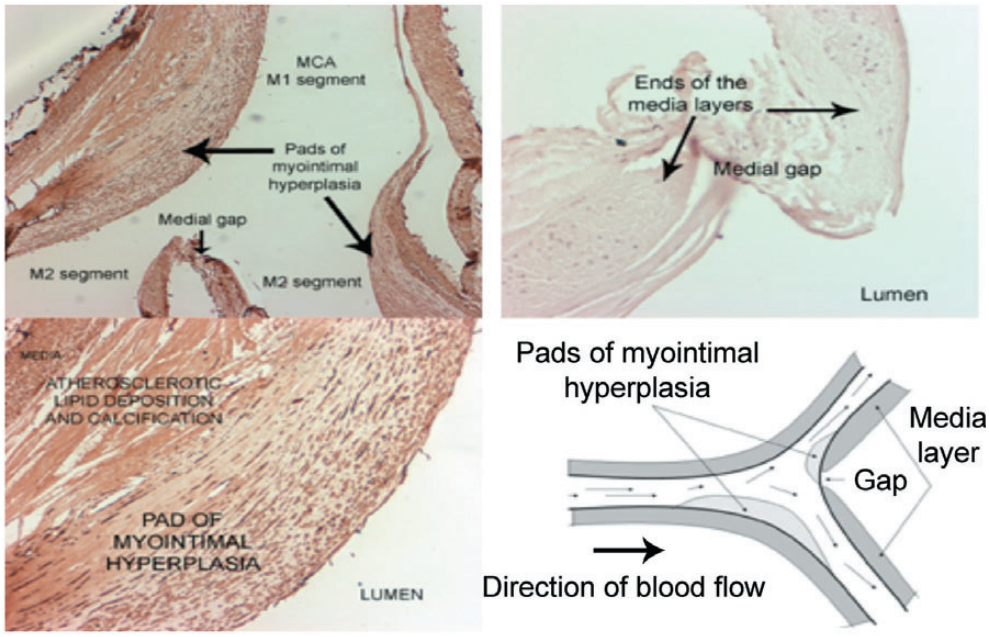

Figure 5. Histology of the cerebral arteries

6.1 Saccular cerebral artery aneurysms (SCAA) and subarachnoid hemorrhage (SAH)

6.1.1 SAH caused by SCAA rupture

Saccular cerebral artery aneurysms (SCAAs) are pathological pouch-like dilatations of the arterial wall (Fig.1), described accurately for the first time probably in 1765 by the milanese physician Francisci Biuni, but even before that as vascular dilation or tumours that may rupture and cause cerebral apoplexy (337). SCAAs are mostly found in proximal bifurcations of the cerebral arteries near the Circle of Willis. Rupture of the SCAA wall was associated with hemorrhage to the brain parenchyma, or to the subarachnoid space between the brain and the surrounding meninges (Fig.2), already in 17th century (337).

6.1.1.1 Subtypes of cerebral artery aneurysms

Aneurysms are described as saccular, fusiform, or dolicoecthatic depending on their shape. Saccular aneurysms are pouch- or berry like protrusions that arise from vascular bifurcations or from the side of a vessel and often have an irregular shape with secondary protrusions (Fig.1). Dilations or pathological increase in vessel caliber that do not lead to formation of a separate saccular pouch are usually called fusiform aneurysms, where as tortuous, dilated elongations of vessels are referred to as dolicoecthatic (Fig.1) (338). Although the great majority of SCAAs arise from the large elastic cerebral arteries that follow the outer surface of the brain in the subarachnoid space, SCAAs may also arise from arterio-venous malformations, spinal arteries, or from the venous system (332).

6.1.1.2 Damage caused by the acute and delayed effects of SCAA rupture and SAH on the brain

Since SCAAs arise from the large cerebral arteries that follow the outer surface of the brain in the subarachnoid space, blood is usually spilled to the subarachnoid space when the SCAA wall ruptures (Fig.2). However, SCAAs may also rupture directly to the brain parenchyma causing intracerebral hemorrhage (ICH) or through the brain parenchyma to the lateral ventricles causing intraventricular hemorrhage (IVH) (121; 190).

6.1.1.2.1 SCAA rupture and brain injury caused by sudden increase in intracranial pressure

After SCAA wall rupture, the high pressure arterial blood that spills to the CSF filled subarachnoid space produces a sudden, temporary increase in ICP that may cause impact injury on the brain parenchyma (88).

6.1.1.2.2 Hydrocephalia caused by SAH

Normally the subarachnoid space is filled with cerebrospinal fluid (CSF) that is constantly produced in the lateral ventricles of the brain, and via the cerebral ventricular system flows to the subarachnoid space from which it is absorbed back to the cerebral venous circulation via the arachnoid villi. After SAH, blood in the subarachnoid space obstructs the physiological circulation of CSF, which may lead to increased intracranial pressure (ICP) due to accumulation of CSF in the cranial vault (so called hydrocephalia) (332). Obstruction of the CSF circulation may occur acutely after the hemorrhage or develop with a long delay of several weeks or months (332).

6.1.1.2.3 Vasospasm after SAH

After SAH, resolution of the blood clot in the subarachnoid space induces a spasm in the cerebral arteries (so called vasospasm) that may lead to critical ischemia in the brain regions supplied by the affected artery. SAH associated cerebral vasospasm is thought to be mediated in large extent by activation of the inflammatory system (65). Vasospasm of the cerebral arteries usually develops during first and second week after SAH, and is a major cause of mortality and morbidity of SAH patients (65; 332)

6.1.1.2.4 Inflammation of the brain parenchyme

After SAH, inflammatory activation occurs also in the brain parenchyme (242) and may be related to the otherwise unexplained clinical deterioration of some SAH patients (186; 187).

6.1.1.3 Causes of subarachnoid hemorrhage

Severe head injury is a common cause of blood in the subarachnoi space. Rupture of a saccular cerebral artery aneurysm (SCAA) is the main cause (in 80-85%) of the non-traumatic SAH (153; 346). Other common causes of non-traumatic SAH include 1) a non-aneurysmatic or so called perimesencephalic bleed in which no vascular or other anomaly is detected (approx.10%), 2) rupture of an arteriovenous malformation (AVM), 3) bleeding of cerebral tumours, 4) or rupture of spinal, fusiform or venous aneurysms (332). In this study SAH refers to non-traumatic aneurysmal SAH caused by SCAA rupture.

6.1.1.4 Incidence and fatality of aneurysmal SAH

The incidence of aneurysmal SAH is 10-11 per 100 000 in most Western countries, whereas in Finland and Japan it is twice as high (123; 283). Aneurysmal SAH accounts for 7-11% of all strokes in Finland (283; 298). In a meta-analysis of worldwide published SAH patient series from 1962 to 1992 the fatality rate was 32-67% with a weighted average of 51% (111; 332). Of those that survive SAH, one third remain severely or moderately disabled (111; 332), and the quality of life of all survivors is significantly

decreased (66). Improvements in SCAA diagnostics and SCAA / SAH patient management have led to decreasing trends in SCAA / SAH mortality (244; 305).

6.1.1.5 Prevalence of SCAAs

In a meta-analysis of autopsy series and angiography studies, the prevalence of SCAAs in the general population without known SAH risk factors, was estimated to be around 2% (259). This meta-analysis also concluded that in prospective autopsy series the prevalence of unruptured SCAAs is around 3-4%, where as in prospective angiography series of living patients the prevalence of SCAAs was around 5-7% (259).

Many SCAAs never rupture during their carriers lifetime, since the prevalence of SCAAs is a hundred times the incidence of SAH (=SCAA rupture) and the prevalence of unruptured SCAAs in autopsy and angiography series is close to the estimated prevalence in the general population. This issue remains controversial despite support by data from the largest prospective and retrospective clinical series (ISUIA I and II, (128;351)). Moreover, factors that characterize those SCAAs that do rupture remain incompletely known.

6.1.2 Diagnostics of SAH and SCAAs

6.1.2.1 Clinical symptoms of SAH

The most frequent clinical presentation of SAH is sudden explosive and persistent headache, often followed by decrease in the level of consciousness and focal neurological symptoms, such as paresis (190; 332). SAH may also manifest as sudden death (332). Some SAH cases are, however, almost symptom free, or with only a minor headache (332), which may be misdiagnosed for e.g. migraine. Approximately one third of patients with fulminant SAH have had minor headaches or "strange intracranial feelings" prior to the SAH, suggesting that many SCAAs undergo so called "minor leaks" prior to fulminant rupture (142). SAH should be suspected always when headache abruptly starts in a patient without other diagnosed causes of headaches, and in the context of sudden loss of consciousness (332). SCAAs may also present with other symptoms than rupture due to either i) local compression of the cranial nerves (usually palsies of III or IV cranial nerves) or ii) due to embolism that originate from partially thrombosed SCAAs (75; 190).

6.1.2.2 Laboratory and radiological presentation of SAH

SAH is diagnosed primarily with a CT-scan that shows blood in the subarachnoid space (Fig.2) in 90% of SAH cases, if performed within 24 hours of the hemorrhage (4). In addition to blood in the subarachnoid space, CT-scan also shows intraparenchymal hematoma or hydrocephalus that may

be caused by the SCAA rupture (3), and may also show ischemic defects that are caused by cerebral vasospasm occurring after SAH. The sensitivity of CT in the diagnosis of SAH declines with increasing time from the hemorrhage, and after 5 days only 60% of cases are detected with CT (4; 153) . Old, and some recent but minor SAH may not be visible with CT, and if the clinical symptoms and history suggest SAH despite a negative CT-scan, the result should be verified by spinal tab. In SAH patients with a recent bleed, the CSF sample obtained from the spinal tab is visibly red, or at least has an increased number of erythrocytes in microscopic examination. In patients with older SAH, the CSF sample is usually yellowish due to breakdown products of haemoglobin (xanthocromia). Diagnostic spinal tab is considered reliable, if performed before two weeks from the onset of symptoms (348)

6.1.2.3 Detection of SCAAs by radiological means

In patients with SAH diagnosed by CT or spinal tab, angiography of the cerebral vessels should be performed to exclude SCAAs as the etiology of the hemorrhage, since ruptured SCAAs have a high risk of rebleeding (137; 152-154; 226). Rebleeding can be prevented if the bled aneurysm is isolated from cerebral circulation. Moreover, SAH is often complicated by cerebral vasospasm that is currently treated with medically induced hypertension, hypervolemia and hemodilution (so called triple-H therapy) (223; 332). Triple-H therapy may provoke rupture of an unsecured weak walled aneurysm, and therefore isolation of the bled aneurysm is necessary before triple-H therapy can be fully started.

6.1.2.3.1 Cerebral angiography techniques for detection of SCAAs

Cerebral angiography can be performed using I) magnetic resonance imaging (MRI / MRA) techniques (e.g. time-of-flight sequence), II) with helical CT and iv-injected iodine-based contrast agent (CT-angiography, CTA), or III) with series of digital X-ray images taken after iodine-based contrast agent is injected to the cerebral arterial tree via intra-arterial catheterization of the target vessel (digital substraction angiography, DSA).

MRA is the least invasive method of cerebral angiography, but may produce false netigative findings of aneurysms (315; 335), and currently is not as accurate in detailed anatomy. In patients with suspected aneurysms in MRI / MRA, an additional DSA or CTA study often has to be performed before treatment of the lesion. Because of its non-invasive nature, MRA is very useful in repeated follow-up angiograms of embolized SCAAs (349) and may show residual flow even better than conventional DSA (357). DSA remains the golden standard of cerebral angiography, and is still superior to other methods in diagnostic and anatomic accuracy. It is, however, the most invasive method and associated with a 1.8% morbidity / mortality rate in SAH patients and a 0.3% morbidity / mortality rate

in patients with unruptured SCAAs (45). CTA is less invasive and quicker than DSA, and is comparable in sensitivity and specificity to DSA (7; 374). However, in small SCAAs (<5mm) CTA is considered less reliable than DSA (112) and near the skull base the CTA image may be disturbed by artefact from the bony prominences of the skull base. However, the visibility of bony landmarks in CTA may also be a benefit that can help surgical planning.

6.1.2.3.2 Current imaging techniques and prediction of SCAA rupture risk

None of the currently available imaging methods for SCAAs can reliably identify those SCAAs which have ruptured or are about to rupture, although some types of radiological presentations (growth, large size, secondary pouches or irregular shape) are frequently associated with ruptured SCAAs (see Table 1) (342) (18; 205; 245; 328). Moreover, distribution of blood in the subarachnoid space may suggest bleeding from a specific anatomical location. However, current imaging methods cannot reliably detect structural weakness or pathobiological changes in the SCAA wall that lead to SCAA rupture. For this, 4D-CTA seems a promising novel technique that can show the dynamic movement of the SCAA wall in function of time, and thus can detect protrusion of weak parts during systole (101; 126). Also novel MRI methods that use iron-oxide or gadolinium conjugated molecules as cell or molecule spesific contrast agents (194; 291; 320), could possibly be used in diagnostics of rupture-prone SCAA. However, before the novel MRI-contrast agents can be used in the diagnostics of SCAAs at risk of rupture, the pathological mechanisms that lead to SCAA rupture have to be described.

6.1.3 Risk factors for SCAA formation and rupture

Risk factors for SCAA rupture need to be known to i) identify patients at increased risk of rupture and ii) to decrease the rupture risk in individual patients by medical therapy and correction of living habits. Known risk factors for SCAA rupture (Table 1, Fig.3) can be divided into those related to the aneurysm and to those related to the patient.

6.1.3.1 Aneurysm related risk factors for rupture

6.1.3.1.1 Size and growth

Risk of rupture increases with SCAA size and is an independent risk factor after adjustment for other varibles (141). Several authors have reported a significantly higher risk of rupture in SCAAs larger than 10mm, with very low risk of rupture in smaller SCAAs (128; 259; 352). In the thus far largest study on unruptured SCAAs (ISUIA), the cumulative rate of rupture was significantly lower in less than 7mm SCAAs (0.05%) compared to larger

than 7mm SCAAs (1%), provided that the patient did not have a history of prior SAH (351). However, clinical observations suggest that many SCAAs below the 10 or 7mm threshold rupture (206; 323; 341; 361). Moreover, in an obduction series of 109 ruptured SCAAs, Inagawa et al. found that 17% of ruptured SCAAs were 4mm or less in diameter, and only 38% 10mm or larger (121), which clearly shows that also SCAAs smaller than the ISUIA threshold may rupture.

Although SCAA size clearly associates with rupture risk, it is still controversial whether a single threshold value for increased rupture risk exists and can be determined. Moreover, accumulating data suggests that decision on interventions should not be based on size alone, since multiple patient and aneurysm related factors contribute to the risk of rupture (206; 341). A recent multivariate analysis in a small retrospective series of 100 SAH patients with smaller than 7mm SCAAs reported that hypertension, age, and location in the anterior circulation all significantly increased the risk of rupture in small (less than 7mm) SCAAs (206). Besides large size, growth of the SCAA during follow-up seems to be associated with rupture risk (143).

6.1.3.1.2 Morphology of the SCAA

In addition to size and growth, also other morphological / radiological features are associated with increased risk of rupture, such as the presence of daughter sacs (so called secondary pouches, Fig. 1) (18) and the ratio of fundus depth and neck width (so called aspect-ratio, Fig. 1) (205; 328; 342). The possible association of secondary pouches with SCAA wall degeneration and weakness is still unconfirmed by histological studies. To explain the association of rupture risk with aspect-ratio, Ujie et al. have presented a hypothesis based on experimental aneurysms sacs constructed in rabbits. According to Ujie et al. a high aspect-ratio over 1.6 leads to slower flow conditions in the SCAA apex and promotes thrombosis that is invaded by mural smooth muscle cells (SMCs) (327). Migration of SMCs from the SCAA wall involves increased activity of matrix metalloproteinases (MMPs) and other proteolytic enzymes, and would thus degenerate and weaken the apical SCAA wall (327). Although Ujie et al. were able to demonstrate slow flow in the apex of high aspect-ratio experimental aneurysms (327), their hypothesis has still to be confirmed by histological studies in experimental or human aneurysm. Besides secondary pouches and aspect-ratio, other types of SCAA morphologies and morphological indices may be associated with increased risk of rupture (99; 245). Large enough clinical series are not yet available to evaluate the real predictive value of other morphological indices than secondary pouches and aspect-ratio.

6.1.3.1.3 Location of the SCAA in the cerebral vasculature

SCAAs located in different parts of the Circe of Willis and the cerebral vascular tree, are thought to have different risks of rupture. In a large meta-analysis of SCAA rupture risk factors and in the ISUIA series, SCAAs of the posterior circulation (basilar tip, SCA, AICA, PICA, and vertebral arteries) and arising from the PcomA, have been reported to have an increased risk of rupture compared to anterior circulation SCAAs (259; 351).

In the anterior circulation (except for AcomA SCAAs), the general rule is that the more proximal the SCAA is, the more prone it is to rupture (341). In obduction series SCAAs arising from the MCA bifurcation are most frequent (306) where as in clinical series MCA aneurysm account for only 20-30% of ruptured SCAAs and the most common site of ruptured aneurysm is the AcomA or anterior cerebral artery (40%) (343) This controversy suggests that AcomA aneurysm may be more prone to rupture than MCA aneurysms, although they are classified as more distal than MCA bifurcation aneurysms.

6.1.3.2 Patient related risk factors for SAH

6.1.3.2.1 Age and gender

The risk of SAH in patients harboring unruptured SCAAs increases cumulatively with age (141), with an annual rupture risk of 1.1% in the Finnish population (141). The peak incidence of SAH is between 40-65 years of age (283), and the majority of SAH patients are younger than 65 years (181). SAH patients are predominantly females (3:2 female to male ratio) (259) and the risk of SAH seems to be associated with the menstrual and reproductive history of the female patients (69; 219), suggesting a possible role of estrogens in the pathobiology of the SCAA wall.

6.1.3.2.2 Medical risk factors and living habits

Health habits affect significantly the risk of SAH. Unlike for occlusive and ectatic atherosclerotic vascular diseases (e.g. aortic aneurysms), hypercholesterolemia, type II diabetes, and obesity are not significant risk factors for SAH (69; 259), although in one study hypercholesterolemia seemed to have some association with increased SAH risk in older women (119) and SAH patients tend to have blood cholesterol values in the upper tertile of the general population (5). Untreated hypertension that is a risk factor for atherosclerotic diseases, is also one of the strongest risk factors for SCAA formation, SCAA rupture in patients harboring unruptured SCAAs, and occurence of fatal SAH in patients harboring unruptured SCAAs (27; 69; 125; 259) (119; 136). Another common risk factor for atherosclerosis and SAH is smoking, which is the strongest known acquired risk factor for SAH (69; 143; 259; 271; 344).

In addition, high alcohol consumption is an independent risk factor for SAH (69; 140; 163), as seem to be also caffeine and cocaine use (27; 125). Prior corticosteroid use has been suggested to be a risk factor for aneurysmal SAH, but this finding is still lacking confirmation by other series (272). Cessation of smoking and medical control of hypertension in SCAA patients is clearly of paramount importance to reduce the risk of SAH. Reduction of alcohol consumption might also be important, as well as limited use of caffeine or other stimulants.

Ruigrok et al. has estimated the attributable risk of known SAH risk factors to the incidence of SAH in the general population. Smoking accounts for approximately 20% of SAH cases, hypertension for 17%, alcohol abuse for 11-21%, and familial background for roughly 11% (271).

6.1.3.2.3 Tendency for multiple SCAAs

Around 20-34% of Finnish SCAA patients have multiple SCAAs (260; 261). The main risk factor for multiple SCAAs are smoking, hypertension, and female gender (139) – the same risk factors as for SCAA development in general.

6.1.3.3 Familial SCAAs

Familial aggregation of SCAAs is well documented in different populations (28; 59; 262; 289). Family history of SCAAs or SAH is found in approximately 10-11% of European SAH patients (271). Familial SCAAs seem to have a higher risk of rupture or rupture at an earlier age than sporadic ones (29; 182). Carriers of familial SCAAs also seem to be younger than SCAA patients in general (214; 264), and also seem to have more often multiple SCAAs (214; 273). Relatives of familial SAH patients have 7-10 times higher risk of SAH than the general population (28; 263; 265). In Finland, screening of first degree relatives of SCAA patients is therefore considered to be indicated in families with more than two verified SCAA or SAH cases (265).

6.1.4 Current therapies to prevent SCAA rupture and subsequent SAH

Because of the sinister outcome of SCAA rupture, invasive therapy to prevent SAH is indicated. To prevent SAH, unruptured SCAAs have to be isolated from the cerebral circulation. Already ruptured SCAAs are at a high risk of rebleeding that is even more often fatal than the first SAH (137; 152-154; 226). Therefore also ruptured SCAAs have to be isolated from the circulation.

6.1.4.1 Exovascular or endovascular occlusion – clipping and coiling

Currently SCAAs can be isolated from the cerebral circulation by i) microsurgical ligation (clipping, introduced in its current form by Yasargil, (360), ii) by endovascular embolization of the SCAA fundi (coiling) introduced by Gugliehlmi (89; 90), or in the rare cases of otherwise untreatble SCAAs, iii) by microsurgical ligation or endovascular occlusion of the feeding parent arteries (trapping) (146; 183). Ligation of the parent artery may necessitate a distal by-pass procedure to ensure sufficient blood flow to the territory supplied by the sacrificed artery (8; 146). In addition to the above mentioned methods, formerly in cases in which the wall of SCAAs could not be totally isolated from the circulation, the SCAA wall was reinforced by muscle fascia or fat grafts wrapped around the SCAA sac (wrapping) (40). Most SCAAs are currently treated by clipping or coiling.

Of the above mentioned methods that can be used to isolate the SCAA from the circulation, only microsurgical clipping and successful reconstruction of the cerebral artery bifurcation / vessel wall with aneurysm clips removes the SCAA totally. Endovascular embolization as well as trapping, leave the SCAA sac in place but lead to its occlusion due to luminal thrombus formation and subsequent fibrosis.

6.1.4.2. Initial results of endovascular therapy

Endovascular therapy leads to good initial radiological and clinical outcome that is comparable to surgery (167). Despite the seemingly high risk of recurrence during the first two years (102; 249), the initial good clinical outcome of endovascular treatment seems to persist longer than the radiological outcome (167). In a series of 160 embolized SCAAs with a median follow-up of 18.2 months, good or moderate outcome on Glascow outcome scale was found in 73%, although many of the embolized SCAAs were unstable and required re-intervention (167). Annual rebleeding in this series was 0.45% (167).

6.1.4.3 Lack of long term efficacy of the endovascular therapy

In a series of 203 GDC embolized SCAAs on 148 patients, Cognard et al. found flow recurrence in the embolized SCAA fundus in altogether 14% of cases during a follow-up from 3 months to 3-4 years (49). In this series recurrences were more common on ruptured and large size SCAAs (49). Only 5 out of 203 embolized SCAAs required a second treatment attempt (49). Despite not achieving complete occlusion in all cases, no rebleedings occurred in this series (49). In a retrospective analysis by Raymond et al. of a series of 501 SCAAs embolized from 1992 to 2002, recurrence of SCAA filling in one year follow-up was found in 33.6% of cases (249). In a series of 178 embolized SCAAs with neck remnants by Hayakawa et al., small SCAAs with small necks had a recurrence rate of 17% during median follow-up of 17 months, where as in small SCAAs with large necks it was

42%, and in large SCAAs 82% (102). To reliably assess long term efficacy of endovascular therapy and the rebleeding and SCAA recurrence rates, large patient series with complete follow-up data from an extensive time period are needed. Although new series are constantly being published, such large long term follow-up series of endovascularly treated SCAA patients are still few. Based on the current data, it seems that recurrences occur rather frequently, more so in ruptured SCAAs and in large size SCAAs, but do not necessarily require a second embolization attempt. In addition it seems that the long term results of coiling vary between centers.

6.1.4.4 Randomized studies comparing the outcome after clipping or coiling

The first prospective randomized study (109 patients) comparing outcomes after SCAA clipping or coiling concluded that in one year follow-up both treatment modalities had similar clinical and neuropsychological outcomes (79% and 75% had good or moderate recovery in Glascow outcome scale) (166). Following that study, the first large randomized multinational / multicenter study (ISAT, 2143 patients) reported that after one year follow-up, 23.7% of patients allocated to the endovascular group were dependent or dead compared to 30.6% in the surgical group, and the trial was stopped by its ethical steering committee (195). In the ISAT trial the relative and absolute risk reductions in dependency or death after allocation to an endovascular versus neurosurgical treatment were 22.6% (95% CI 8.9-34.2) and 6.9% (2.5-11.3), respectively (195).

Although the ISAT study demonstrated that coiling leads to equal or better results than clipping in a selected population of SCAA patients, the study and its applicability to SCAAs in general, has raised many comments and criticisms (13; 62; 96; 106; 180), mostly related to: i) potential selection bias – of the 9559 patients potentially eligible, only 2143 were enrolled; ii) the low number of posterior circulation and MCA aneurysms – applicability to all SCAAs?; and iii) the heterogeneity of the microsurgeons that performed clipping – it is known that experience and higher case load leads to better outcomes in clipping (54; 302). For the above mentioned reasons, the results of the ISAT study should not be generalized to claim superiority of endovascular therapy vs. microvascular clipping in all SCAAs before other large randomized series of clipping vs. coiling in SCAA patients are available and have confirmed the findings of ISAT.

6.1.4.5 Long term results of clipping – patients still in a higher risk of SAH than the normal population

The risks posed by the uncertainty of long term occlusion after endovascular therapy have to be weighted against the risks related to craniotomy and brain retraction. In a prospective series of 101 microsurgically treated SCAA patients, basal frontotemporal lesions were detected with MRI in one-third of the patients (164). These lesions were not related to vasos-

pasm or damage from the initial SCAA rupture, and where considered to be caused by surgical trauma (164). In a retrospective series of 147 patients that had underwent SCAA clipping, frontal lobe lesions were found in 48% of cases. (165).
In addition, also clipped SCAAs may rebleed due to incomplete clipping and de novo SCAAs arising near the clip or elsewhere in the cerebral vasculature (56; 143; 316; 324; 347). The incidence of neck remnants in clipped SCAAs has been estimated to be around 5% (316). Most cases of recurrent SAH seem to occur, however, from de novo SCAAs (347). In two series of 115 surgically treated Japanese patients with unruptured SCAAs and 220 surgically treated Japanese patients with ruptured SCAAs by Tsutsumi et al., the risk of SAH after surgical treatment of SCAAs was 1.4% in 10 years and appr. 12% in 20 years (325; 326). In a series of 752 surgically treated Dutch SAH patients, the risk of SAH was 3.2% in 10 years (95% CI 1.5-4.9%), with most of the bleedings arising from de novo SCAAs (347). Thus even complete clipping of a ruptured or unruptured SCAA does not remove the risk of SAH permanently (324; 347), but surgically treated SCAA patients have still a higher risk of SAH than the normal population. The incidence of de novo SCAA formation in patients that have underwent clipping, has been estimated to be between 0.5-2% per year (56; 143; 324).

6.1.4.6 Current recommendations for management of SCAA patients

6.1.4.6.1 Ruptured SCAAs
Ruptured SCAAs should be occluded or isolated from the cerebral circulation, in order to prevent rebleedings and to enable efficient treatment of potential ischemic complications with triple-H therapy. As discussed above, both clipping and coiling produce good initial occlusion, but discussion about the preferential use of either method in different clinical situations is still ongoing and conclusive data is still missing. Based on the ISAT study that reported better outcomes in endovascularly treated patients, some authors have recommend that all SCAAs should preferably be treated endovascularly, if the morphology and anatomy of the aneurysm is suitable for endovascular occlusion (330). These authors acknowledge that despite the advances in endovascular tools that enable occlusion of wide necked SCAA using balloons and stents, still some SCAAs will be left outside endovascular therapy for anatomical reasons e.g. arterial branches arising from the SCAA sac, and will require microsurgery (330).

Other authors, especially in Finland, have stressed the importance of continuing SCAA clipping in specialized centers with high enough case load to maintain high level of competence (213). Due to acute hydrocephalus and SAH related parenchymal hematomas, many patients (approx. one third) will require emergency surgery anyway (213) and simultaneous clipping (especially in the context of hematoma removal) seems

better justified than performing two high risk procedures. Furthermore, in experienced hands the results of microsurgical clippping are much better than those reported by the ISAT study (see Koivisto et al., (166)) and ISAT studied only anterior circulation aneurysm and in young good grade patients (so the results should not be directly extrapolated to all SCAA carriers). Interpretations from the ISAT study should be made cautiously and ISAT should not be used as a proof that all SCAAs in all centers should be preferably treated endovascularly (213).

6.1.4.6.2 Unruptured SCAAs

For unruptured SCAAs, the indications of occlusive therapy are not as clear. The ISUIA study, which is thus far the largest and most systematic study of the natural history of SCAAs, clearly shows that the natural history and risk of rupture in SCAA patients with or without prior SAH are different (ISUIA). In contrast to prior studies, ISUIA also reported extremely low rupture risks for SCAAs less than 7mm in patients without prior SAH.

For incidental unruptured SCAAs larger than 7mm, and for all unruptured SCAAs in patients with prior SAH, occlusive therapy is recommended (19; 350). However, based on the apparent rupture risk and the rate of neurological disability (appr. 12%) or mortality (ad 3.8%) reported in ISUIA, some of the authors of the ISUIA study have recommended that less than 7mm SCAAs in patients without prior SAH should be treated conservatively (350; 351).This recommendation has been strongly objected by many neurosurgeons, who say that the low risk reported by ISUIA has to be untrue, since many of the ruptured SCAAs encountered in clinical practice are smaller than 7mm and in patients without prior SAH (341).

Several limitations in the ISUIA study decrease its generalizability (341): 1) ISUIA is a non-randomized study, with high crossover from conservative to operative treatment resulting in selection bias. Selection of low risk patients to the follow-up cohort may explain the discrepancy between the low rupture risk estimate of ISUIA, the observed incidence of SAH, the reported prevalence of SCAA in autopsy or angiography series, and the calculated prevalence that would be necessary to explain the true incidence of SAH, if the risk estimate of ISUIA was valid. 2) The low number of AcomA aneurysms in the ISUIA study (10.3%) and the 0% rupture rate for <7mm AcomA aneurysms is in direct conflict with prior studies in which AcomA SCAAs make up 35 to 50% of all ruptured SCAA (341). Furthermore, AcomA aneurysms tend to make up an even higher percentage of small ruptured SCAAs (<7mm).

The critics of ISUIA study suggest that decisions should not be based on size alone but should take into account a multitudy of other factors, including all known patient and aneurysm related risk factors, and the

experience and results of the treating physician (341). This view is in accordance with the recommendations of an expert panel of the American Heart Association, which recommends that: 1) unruptured SCAAs found in the context of SAH from another SCAA, should be treated because of the high risk of rupture, but 2) operative treatment cannot be recommended on incidental SCAAs smaller than 7mm and in patients without prior SAH, unless the patient is very young, has several known risk factors for SCAA rupture, or the SCAA morphology or anatomical location suggests an increased risk of rupture (19). The expert panel of American Heart Association does not recommend coiling over clipping, or vice versa, because of incomplete data. The panel concludes that besides clipping, also coiling is a safe treatment method for occlusion of SCAAs with good initial results (19).

6.1.4.7 Novel solutions to improve the long term outcome of endovascular SCAA therapy

A meta-analysis encompassing 1383 endovascularly treated patients with both unruptured and ruptured SCAAs reported a 46% rate of incomplete obliteration in follow-up (25), suggesting that the good initial results with current embolization devices are not durable and need significant improvement.

6.1.4.7.1 Healing of embolized SCAAs

Endovascular embolization has been thus far mostly performed with electrically detachable Guglielmi coils (GDCs). GDCs induce thrombus formation in the SCAA fundi, and in animal models the thrombus is gradually infiltrated by SMCs or myofibroblasts that synthesize matrix and organize the thrombus into fibrous tissue that is later covered with and endothelial lining (55; 63; 202; 253; 254). Large histological series of coiled human SCAAs are not available, but based on the cases reported, coiled human SCAAs seem to heal similarly as the embolized experimental aneurysms in animal models (86; 113; 127). Some of the GDC embolized SCAA fundi are re-exposed to blood flow and hemodynamic stress due to recanalization of the GDC induced thrombus, or due to compaction of the GDC loops (192; 249). This is thought to occur because of poor organization or recanalization of the GDC induced thrombus (247; 253; 254). Incomplete GDC embolization may also leave residual filling, especially in the SCAA neck (neck remnants) that may start to grow or rupture.

6.1.4.7.2 Novel endovascular embolization tools

Current tools for endovascular occlusion of SCAAs include coils, stents, liquid embolization materials, and temporary occlusion balloon devices (reviewed by Lanzino et al.) (175). In endovascular therapy for SCAAs, stents and neck bridging devices may be used to facilitate coiling of wide

necked SCAAs from which "herniation" of coil loops to parent artery lumen would otherwise be likely (158; 175; 201; 248). A stent or a neck bridging device is positioned over the SCAA neck, after which coil loops can be introduced to the SCAA lumen through the stent net. Stents designed for coronary use are too stiff for navigation in the tortuous and fragile intracranial vessels, and therefore more subtle, gentle and flexible stents for intracranial use have been introduced (Neuroform from Boston Scientific Corp.). Balloon devices are also used in embolization of SCAAs as temporary remodeling devices that can be used to occlude part of the SCAA neck during the time that the SCAA lumen is packed with coils or liquid embolization material (15; 184). After the SCAA lumen has been filled, the assisting balloon device is emptied and removed.

Platinum coils and their variations still remain the main tool for embolization of the SCAA fundus. Currently available coils are (175): i) Guglielmi detachable coils (GDCs), which are electrically detached bare platinum coils that induce thrombosis inside the aneurysm, ii) Matrix coils, which are a modification of the GDCs with a 90% polyglycolide / 10% polylactide polymer coating (Vicryl suture material) added to the outer surface of the GDC, iii) HydroCoils, which are platinum coils coated with an expanding polymer that reacts to blood, and iv) ACT Microcoils that are a modification of the platinum coil that take a more spherical shape (175). The polymer added to the surface of Matrix coils induces an inflammatory response, which in swine models of experimental side wall aneurysms has led to increased fibrosis and re-endothelialization of the aneurysm neck (203). In HydroCoils, the polymer covering expands for 20 minutes in reaction to contact with blood after a 5 minute latent repositioning time (148; 175). The rationale behind the expanding polymer covering is to improve the packing of the SCAA lumen with coils (148) – due to irregular shape of most SCAAs and microspaces left between coils and coil loops, traditional GDCs occludes less than 50% of the luminal volume in most aneurysms (46; 313). ACT Microcoils use a technology that enables the coils to take a more spherical shape than traditional GDCs (212). This has been thought to improve packing of both irregularly shaped and spherical SCAAs (212)

Clinical follow-up data comparing the novel polymer coated coils (Matrix and HydroCoils) is scarce due to the novelty of the devices. The few patient series published suggest that in human SCAAs the pro-inflammatory effect of the polymer coating in Matrix coils does not lead to increased fibrosis or decreased coil compaction / SCAA recurrence, as reported in the swine model of experimental side-wall aneurysms (203). HydroCoil has been used in human SCAAs, and has been found effective in increasing the packing density of the SCAAs (46). Long term follow-up data of the clinical use of HydroCoils has not yet been reported. However, in a rabbit carotid bifurcation aneurysm model, HydroCoil was found to improve

both immediate angiographic occlusion and long term occlusion rate compared to GDCs or Matrix coils (63).

6.1.4.7.3 Biologically active embolization devices

Besides polymer coatings, biologically active coils and other embolization devices are being developed to improve the safety, initial occlusion, and long term patency of SCAA embolization therapy. GDC embolized SCAAs that after coiling procedure have had postintervention 100% angiographic occlusion, have been shown to have tiny open spaces between coils and coil loops (17). Moreover, re-endothelization of the SCAA neck after coiling with bare GDCs is more an exception than a rule in human SCAAs (17). These features are thought to eventually lead to recanalization and possible rupture of the embolized SCAAs. The goal of bioactive embolization devices is to induce fibrosis in the thrombus formed around the coils, so that the resorbable thrombus is replaced by more permanent fibrotic tissue, and eventually the orifice of the aneurysm sac sealed by growth of endothelia over the fibrotic tissue (re-endothelialization).

Many experimental modifications that make the biologically inert bare platinum coils more active inducers of thrombosis, fibrosis, and re-endothelialization, have been tested in animal models. Besides polymer coverings, these approaches include i) inclusion of matrix proteins (e.g. collagen, fibronectin, tenascin-C) to the outer surface or inside the coil (58; 147; 203; 311; 319), ii) release of bFGF, VEGF, of TGF-beta growth factors (2; 60; 100; 189) from the coil, iii) release of low penetration irradiation from 32P coated coils (250), and iv) coating of the coil with autologous cell grafts that produce matrix components and growth factors (149; 188). In addition to bioactive coils, also collagen sponges that contain growth factors or platelet extracts, and alginate (polymer substance derived from sea-weed) have been studied in experimental aneurysms (251; 254). Gene therapy via direct transduction of aneurysm wall cells by local vector delivery (e.g. with a coil), or indirectly by local transplantation of in vitro genetically engineered cells (257) (also possible with a coil) has been investigated in experimental aneurysm models (258). In summary, many concepts that improve the tissue response to embolization devices have been successfully tested in experimental aneurysm models. However, all these models (microsurgically created vascular pouches or ligated arteries) differ in pathobiology from the human SCAA wall, and whether any of these concepts will improve the efficacy of endovascular therapy in human SCAAs, remains to be seen. Moreover, for further rational design of bioactive embolization therapies and for their rational use in clinics, the pathobiology of the SCAA wall should be further elucidated so that the biological therapies can be targeted at molecular pathways relevant in the human disease and not just in the animal models.

6.1.5 Summary of current problems in the diagnostics and therapy of SCAAs

SAH caused by SCAA rupture has a very high fatality (50%), with most of the survivors left severely disabled. SAH affects mainly the previously healthy working aged population. Best therapy would be to prevent SCAA rupture before the first SAH and subsequent brain damage occurs. However, current methods to prevent SCAA rupture (clipping and coiling) are associated with significant risks of brain injury, morbidity, and even mortality. Since not all SCAAs rupture during the lifetime of their carriers, the most important question in treatment of SAH patients is how to identify those SCAAs that are likely to rupture during the expected lifetime of their carriers. The second important question is how to prevent SCAA rupture with least invasiveness and risk, but with permanent results. Of the current therapeutical options, endovascular coiling is generally considered less invasive than microsurgery (although depens of the aneurysm and of the operator) but in its current form the occlusion results of endovascular embolization are not as permanent as are the results of clipping. To improve the long term results of endovascular embolization (coiling), novel bioactive embolization devices are being developed. However, in order to rationally design and test novel bioactive devices that have positive effects on the SCAA wall, the biology of the SCAA wall needs to be elucidated. Moreover, better knowledge of the SCAA wall pathobiology is needed to develop novel diagnostic tools that could identify SCAA carriers at risk of SCAA rupture and SAH.

6.2 Cellular and molecular pathology of the SCAA wall

Clinical risk factors for SCAA rupture have been extensively studied in large patient series. However, the pathobiological processes ongoing in the SCAA wall and leading to rupture of the SCAA wall are poorly known.

6.2.1 Origin of SCAAs – acquired, not congenital lesions

Because SCAAs often appear with no macroscopically visible degeneration (atherosclerotic or other) of the arterial wall, and may be found in patients with anatomical variations of the Circle of Willis (339), it has been thought that SCAAs arise from pre-existing congenital deformities (reviewed by Stehbens WE, (307)). Furthermore, SCAAs are common in patients with some congenital defects such as polycystic kidneys (81) and aortic coarctations (51), although these congenital defects may lead to SCAA formation because they induce hypertension, and are not known to produce any anatomical, histological, or molecular deficits of the cerebral artery wall.

When 1930 Forbus drew attention to the presence of "medial defects" (medial raphés) in cerebral artery bifurcations, and called them "locus minoris resistentiae" based on the apparent weakness of their histological structure (307; 308), it was assumed that the "medial defect" (medial raphé) would be a congenital weakness that predisposes to aneurysm formation. In addition to the "medial defect" (medial raphé) at some cerebral artery bifurcations the IEL (the main remaining support structure of the wall) is fenestrated, which has been interpreted as further structural weaknes (34). However, as reviewed extensively by Stehbens (307; 308), the hypothesis of congenital origin of SCAAs has not been able to withstand accumulating scientific data from clinical, pathological, and experimental studies. The prevalence of medial raphés in cerebral arteries do not correlate with the prevalence of SCAAs, and the site of the medial raphé is usually included in the SCAA wall instead of being the origin of it (307; 308).

Furthermore, SCAAs or pre-aneurysmatic changes are rare in children (103; 104) and formation of new SCAAs (so called de novo aneurysms) is seen in adults (143; 326; 347). Thus SCAAs seem to be lesions acquired during life due to exposure to risk factors, not congenital defects or malformations. Congenital defects or malformations that alter hemodynamics of the cerebral circulation may, however, predispose to SCAA formation.

6.2.1.1 Increased hemodynamic stress induces SCAAs

SCAAs have been induced in primates and rodents by hypertension and carotid ligation, with or without simultaneous pharmacological inhibition of matrix synthesis (97; 98; 168; 197). These experiments suggest that SCAAs can be induced by increased hemodynamic stress, or mismatch between hemodynamic stress and strength of the cerebral artery wall. To further support this hypothesis, mathematical modelling of flow dynam-

ics and shear stress in human cerebral arteries has shown that sites prone to SCAA formation are exposed to high hemodynamic stress (73). Even in physiological conditions the cerebral vasculature is under high hemodynamic stress, since it receives appr. 15% of the cardiac output to meet the high oxygen consumption of brains (appr. 20% of total body oxygen consumption) (150).

The mechanisms how increased hemodynamic stress leads to aneurysm formation are incompletely known. Unilateral carotid artery ligation and induction of hypertension induces de-endothelialization of the cerebral artery bifurcations (131), followed by destructive changes in the IEL of cerebral artery bifurcations, and eventual SCAA formation (161). This suggests that hemodynamic stress may lead to arterial wall degeneration and aneurysm formation via increased proteolytic activity. Increased elastase and protelytic activity in the plasma or serum of SCAA patients has been described in North American patient series (14; 50) but also controversial results have been reported in Japanese patients (281). The increased serum protease activity in North American patients has been attributed to increased circulating pro-matrix metalloproteinase-2 (MMP-2) (318). Also the levels of MMP-9 have been investigated in the serum of SCAA patients, but these did not differ from controls (162). How increased hemodynamic stress leads to increased proteolytic activity in the cerebral artery bifurcation or in the serum of the SCAA patients, remains to be elucidated.

Arteries adapt to increased shear stress and increased hemodynamic pressure by medial hypertrophy, adventitial fibrosis, and myointimal hyperplasia, which is growth of a subendothelial SMC layer (124). Myointimal hyperplasia pads are often found near cerebral artery bifurcations (307) (Figure 5). The role of myointimal hyperplasia in SCAA formation remains unclear, but due to its usual location close to the bifurcation where the SCAA forms, it seems likely that it either contributes to the weakening of the IEL and the bifurcation wall, or that it is a repair and adaptation mechanism trying to compensate for excessive mechanical stress, or that at least it is partly included in the aneurysm wall protruding out of the bifurcation (hence the histological similarity?). SMCs in pads of myointimal hyperplasia secrete high amounts of matrix degrading proteases (e.g. MMPs) (80; 134), which is a potential mechanism by which myointimal pads at cerebral artery bifurcations may contribute to IEL and matrix degradation leading to SCAA formation.

The lack of IEL in the SCAA wall (80; 134) and the observation that carotid artery ligation in experimental animals induces destructive changes in the IEL preceeding eventual SCAA formation (161), suggest that disruption of the IEL is an early event in the pathological sequence that leads to SCAA formation. Because the half life of elastin is extremely long (approx. 50 years) (293), IEL degradation is with great likelihood the result of increased activity of MMPs or other proteolytic enzymes, and not due

to "physiological" aging or degeneration. An intriguing hypothesis is that SCAA formation could be inhibited by pharmacological inhibition of MMP activity and subsequent IEL degradation. That inhibition of MMP activity with doxicycline in rats with one carotid artery ligated did not reduce SCAA formation (156), suggests that many proteolytic enzymes are involved in IEL degration and SCAA formation. The results of this experiment should not be interpreted as categorical failure of the hypothesis that pharmacologic MMP inhibition could inhibit SCAA formation, since doxicycline is not a universal inhibitor of all proteolytic enzymes.

6.2.2 Infection and inflammation and SCAA formation

In addition to those produced by hemodynamic stress, some SCAA are caused by infection of the cerebral artery wall. These aneurysms are called mycotic, and represent only 2-6% of all SCAAs (44; 74; 172). However, it has been proposed that inflammation or activation of the inflammatory system might also be involved in the formation of SCAAs in general, based on the finding that macrophages, T-cells, B-cells, antibodies and complement activation are found in unruptured SCAA walls (42). Inflammation increases proteolytic activity and causes necrotic and apoptotic cell death that can damage the arterial wall, lead to loss of tensile strength, and predispose to aneurysmatic enlargement.

Although inflammation seems to associate with SCAA formation or rupture in general, SCAAs very seldom associate with systemic arteritis or vasculitis syndromes (340).

6.2.3 Molecular genetics of SCAAs

Study of the genetic aberrations associated with SCAA formation or rupture will identify genes and gene products that are important for SCAA wall homeostasis and repair, and will improve our understanding of the pathobiology of the SCAA wall. Furthermore, identification of the genetic defects that are frequent in familial SCAAs, or of genetic polymorphisms associated with increased rupture risk in sporadic SCAA cases, could have diagnostic use in screening family members in risk of SCAA formation or sporadic SCAA carriers that have an increased risk of SAH.

6.2.3.1 Genetic aberrations in familial SCAAs

A number of monogenic diseases with a predisposition to form SCAAs exist (complete list to be found in the "Online Mendelian Inheritance in Man", www.ncbi.nlm.nih.gov/). The most common monogenic disease associated with SCAAs is autosomal polycystic kidney disease (APCKD) which is found in appr. 0.3 % of SCAA patients (71; 288). Other relatively frequent monogenic diseases that been traditionnally associated with SCAAs (286; 287) include: i) Ehler-Danhlos syndrome type IV (mutation in collagen III) (239; 270), ii) Marfan syndrome (mutation in fibrillin-1, a

component of the elastic laminas) (71; 288), although also a report describing no association with SCAAs has been published (52), iii) fibromuscular dysplasia (excess myointimal hyperplasia formation, mutation unknown) (47), and iv) pseudoxanthoma elasticum, which however did not seem to really associate with SCAAs in a recent patient series (329).

In addition to monogenic diseases, approximately 10% of SAH patients have a familial background (271). The exact inheritance pattern and penetrance of familial SCAAs indifferent populations have not yet been established. The Finnish familial SCAAs have been associated in genome wide linkage analysis to a 6.6 cM region of the chromosome 19q13.3 (331; 354), but the Finnish aneurysm gene remains still to be found. In the Japanese population, several genome wide linkage analysis of familial SCAAs have revealed associations with the 7q11 region (222), the 7q22 region (367), the 17cen, 19q13, and Xp22 regions (358) and a weaker association with the 5q22-31 region (222; 367). The Japanese aneurysm gene has neither been found yet. The association of the 7q11 locus with SCAAs has been replicated in white population (68), but was not replicated by all Japanese studies (358; 359). In the North American population a mendelian autosomal dominant form of familial SCAAs has been linked with the 1p34.3-p36.13 locus (207). In addition, the chromosomal area 2p13 has been linked with SCAAs in a large consanguineous Dutch family (267).

Although the genetic defects that cause familial SCAAs remain still unknown, it can be assumed from known SAH risk factors and known monogenic disorders and genetic polymorphisms associated with SCAAs that all genetic defects causing a predisposition to SCAAs either: i) lead to increased hemodynamic stress in cerebral artery bifurcations, ii) lead to structural weakness of the arterial wall, iii) modify the repair and adaptation mechanisms of arterial wall, or iv) modify systemic inflammatory response or adaptive immunity in the cerebral artery wall. Moreover, it can be deduced that acquired environmental factors (e.g. smoking, hypertension, infections of the cerebral artery wall) that lead to similar consequences also increase the risk of SCAA formation and rupture and should be avoided in patients with a risk of SAH, especially in those with a genetic predisposition.

6.2.3.2 Genetic aberrations in sporadic SCAAs

Genetic polymorphisms affect the risk of SAH also in sporadic SCAAs (= non-familial SCAAs). These polymorphisms are mostly variations in the promoter regions or exon regions of the gene that either affect the expression level of the gene or the function of the gene product. These polymorphisms do not necessarily lead to familial aggregation of SCAAs.

In Japanese population, polymorphisms of endoglin have been reported to associate with SCAAs in one series (312), but the association has not been replicated in other studies (221). Endoglin is a TGF-β receptor ex-

pressed on endothelial cells, and plays a central role in vascular growth and development (238).

In North American population, polymorphisms of eNOS and MMP-9 have been associated with SCAA rupture (159; 237). Endothelial nitric oxide synthetase (eNOS) is an enzyme that produces nitric oxide (NO), an important regulator of vascular SMC tone (277) and inhibitor of SMC proliferation (277; 285). MMPs are enzymes that degrade collagen, elastin, and other structural proteins of the vascular wall matrix, and are needed for tissue remodeling and turnover also in the vascular wall (174). In addition, polymorphisms of endoglin have been studied in North American population, but no association was found (238).

In different European populations polymorphisms of angiotensin convertase enzyme (ACE) (300), alpha-1-chymotrypsin gene (301), elastin gene (274) and of the human leukocyte antigen (HLA)-region have been linked with SCAAs or SAH (215; 225; 276). The end-products of ACE (end-product is activated angiotensin) regulate vascular tonus and blood pressure, as well as SMC proliferation and vascular wall remodelling (240; 241). Alpha-1-chymotrypsin is a protease that degrades vascular wall matrix (301). Elastin is the main component of the elastic laminas of the arterial wall. HLA region regulates the adaptive immune system. In addition, also polymorphisms of matrix metalloproteinases (MMPs) and endoglin have been studied in European SAH and SCAA patients, but no association has been found (170; 235; 370).

The reports that the distribution of human leukocyte antigen (HLA) –alleles differs between SAH patients and normal population are very interesting (215; 225; 276). HLA-alleles encode the major histocompatibility complex (MHC) molecules that present potentially antigenic peptides to the immune system (114). Polymorphisms of the HLA-alleles affect how the immune system "sees" various peptides and against which antigens the patients is prone to develop acquired immunity (114). The observation that the risk of SAH is associated with HLA-alleles could suggest that in some SAH patients, exposure to foreign antigens has created an immune response that also targets the cerebral artery or SCAA wall and facilitates either the formation or rupture of SCAAs. Alternatively genetic polymorphisms linked with HLA-haplotypes may increase the risk of SAH.

6.2.4 Histology of SCAA walls

6.2.4.1 Histology of the normal extracranial and intracranial arteries

The layers of a normal elastic or muscular artery are: i) outer loose connective tissue layer (adventitia) that limits to the external elastic lamina (EEL), ii) the muscular layer (media) composed of smooth muscle cells (SMCs) situated between the external and internal elastic laminas (IEL), and iii) the inner layer (intima) that is composed of SMCs and an endothelial layer

(268). In many animals (e.g. rat and mouse, Fig.4), the normal intimal layer consists of only endothelial cells and their basal lamina. In humans, a layer of SMCs that increases with age develops between the internal elastic lamina and the endothelia (268), especially near the branching sites of vessels (Fig. 4 and 5).

Where as the wall of extracranial arteries contains an elastic lamina on both the outer and inner limits of the media layer (the EEL and IEL), the intracranial arteries have no EEL (Fig.4). Furthermore, the cerebral arteries have a gap in the continuity of the muscular media layer at bifurcation sites (so called medial gaps or raphés) (Fig.5) (70; 307; 308). The medial raphés have been interpreted as innate or acquired "defects" in the cerebral artery wall that would have less tensile strength and therefore predispose to SCAA formation at the bifurcation sites (307; 308). However, ultrastructural examination of the medial raphés has revealed collagen fibers organized in a tendon-like manner that is highly resistent to mechanical strecth (70). Thus the normal cerebral artery does not have any "physiological" weak points.

6.2.4.2 Histology of aneurysms

Where as a normal arterial wall is composed of three separate histological layers (see Fig. 4), an aneurysm is a dilated segment of a vessel (artery, capillary, or vein) that has a disorganized wall structure with no clearly defined layers (171). Besides a disorganized wall structure, lack of elastic laminas is a common feature to SCAA walls (155; 169). Many SCAA walls are rich in disorganized smooth muscle cells (SMCs) and resemble histologically intimal thickening (myointimal hyperplasia or neointima) or early atherosclerotic lesions (Fig.5) (169).

6.2.4.3 Histology of unruptured and ruptured SCAAs

It seems clear that the wall of unruptured and ruptured SCAAs differ in structure, but there are only a few histological series that focus on characterization of the morphology and histology of the SCAA wall. In a series of 27 unruptured and 44 ruptured SCAAs, Kataoka et al. found that SCAAs with thick, myointimal hyperplasia-like walls were mostly unruptured, where as ruptured SCAAs had a thin, hyalinized and decellularized wall (155). In an autopsy series of 102 unruptured SCAAs, Inagawa and Hirano found no correlation between SCAA location and wall thickness or SCAA size and wall thickness (120). Thus it seems that some pathobiological processes ongoing in the SCAA walls lead to thinning of the SCAA wall that makes it more rupture-prone, regarless of location or size.

6.2.4.3.1 Inflammation in the SCAA wall

In addition to the structural differences, Kataoka et al. also found more infiltrating inflammatory cells in ruptured SCAAs walls (155). Inflam-

matory cells (T-cells, B-cells, macrophages), as well as antibodies (IgM and IgG) and complement activation have been described in unruptured SCAA walls by Chyatte et al. (42), but no comparison in antibody deposition and complement activation between unruptured and ruptured SCAAs has been made. Nevertheless these finding show that many SCAA walls are at a state of ongoing inflammation that may associate with degeneration of the SCAA wall towards a rupture-prone type.

6.2.3.4.2 Smooth muscle cell phenotype and apoptosis in the SCAA wall
Changes in smooth muscle cell phenotype and signs of programmed cell death, or so called apoptosis, have also been shown in the SCAA wall (208; 234). In the series of Pentimalli et al. apoptosis in ruptured SCAA walls was associated with SCAA shape (234), suggesting that hemodynamic factors or flow dynamics may be associated with apoptosis in the SCAA wall. However, this interpretation has not been confirmed by other series. Neither has the histopathology of the SCAA wall been compared with the SCAA shape or the flow dynamics in the SCAA fundus.

Prior histological series suggest that where as the wall of unruptured SCAAs is thick and rich in disorganized SMCs and resembles mostly pads of myointimal hyperplasia or neointima, ruptured SCAA walls are mostly thin and decellularized and in a state of chronic inflammation.

6.2.5 Mechanisms of SCAA wall degeneration and rupture

Histological studies suggest that SCAA walls rupture because the aneurysm wall becomes decellularized (increased apoptosis) and the matrix degenerates (155; 169). How the epidemiological risk factors of SAH (smoking, hypertension, female gender, prior SAH, age) affect the SCAA wall and lead to loss of cells, matrix degeneration, and eventual rupture, remains unknown.

Although SCAAs seem to form at areas of high hemodynamic stress (73; 296), interestingly enough computational modelling has shown that the SCAA fundus (especially the apex) is exposed to less hemodynamic stress than the normal parent vessel wall (295; 296; 327). Thus it seems that hemodynamic load is not the primary factor triggering SCAA wall rupture, but the remodeling and degeneration of the SCAA fundi wall.

6.2.5.1 Resemblance of SCAA wall, atherosclerotic lesions, and aortic aneurysms – similar pathobiology?

The strongest risk factors for SAH, namely smoking and hypertension, are also risk factors for other vascular diseases, e.g. manifestations of atherosclerosis (236; 334) and abdominal aortic aneurysms (AAAs) (22; 293; 336).

AAAs are typically fusiform and associated with atherosclerosis (231; 293) although AAAs may also occur in patients without other manifesta-

tions of atherosclerosis and most patients with aortic atherosclerosis do not have AAAs (293). AAA have some common risk factors with SCAAs, namely smoking, hypertension, advanced age, familial background, and reported HLA associations (22; 336). Conversely to SCAAs, AAAs are more frequent in males, although they rupture more often in females (293).

The characteristic histological finding in AAAs are: i) loss of medial SMCs, ii) degradation of elastic laminas and arterial wall matrix, and iii) chronic inflammation of the medial and adventitial layers (293). In the series of Kataoka et al., the same histological features characterized ruptured SCAA walls (155). What triggers the loss of mural SMCs, matrix degeneration, chronic inflammation, and aneurysm formation in the aortic wall is still incompletely known. However, it seems clear that AAA formation is related to inflammation in the aortic wall (293). Also occlusive atherosclerotic disease is characterized by an inflammatory reaction (20; 94), but accumulating evidence suggests that the inflammatory response in occlusive and ectatic (aneurysmatic) arterial disease differ, with predominance of a Th2-type response in ectatic arterial disease vs. Th1-type response in occlusive disease (293). Studies on the pathobiology of AAA walls show that inflammatory reaction triggered by atherosclerosis or other factors in the arterial wall may lead to i) aneurysm formation, and ii) to the histological features that characterize also ruptured SCAA walls.

The wall of unruptured SCAAs is characterized histologically by myointimal hyperplasia (155; 169), which is also the characteristic histological finding induced by hypertension (124) and early atherosclerosis in the arterial wall (209). Due to the structural resemblance of unruptured SCAA wall with early atherosclerotic lesions, it seems possible that the systemic factors that lead to growth and progression of the early atherosclerotic lesion from a fibrotic fatty-streak to a large, rupture-prone atherosclerotic plaque (or to an ectatic dilation and eventual aneurysm formation), could similarly affect the unruptured SCAA wall, possibly triggering the processes that lead to loss of mural cells and degeneration of the wall matrix.

SAH survivors have been reported to have increased cardiovascular mortality (266), supporting the hypothesis that SCAA wall rupture could in part be related to early manifestations of a general cardiovascular disease that for its other manifestations still remains subclinical. Moreover, although hypercholesterolemia is not a significant risk factor for SAH (69), SAH patients tend to have blood cholesterol values in the highest tertile of normal population (5).

Several histopathological and biochemical features associated with rupture of atherosclerotic plaques (211) (94; 157; 355), are also found in the SCAA walls, e.g. i) matrix metalloproteinase (MMP) expression (30), ii) apoptosis (208; 234), iii) angiogenic growth factors (299), and iv) chronic inflammation (macrophages, T-cells, B-cells, antibodies, complement activation) (42; 155; 169).

6.2.5.2 Proteolytic activity in the SCAA wall

Increased proteolytic activity and MMP expression is associated with arterial wall remodeling in myointimal or atherosclerotic lesions (185; 369) and in expanding abdominal aortic aneurysms (231). Inhibition of MMP-9 and MMP-12 activity by knock out mutation in ApoE knock out mice that develop atherosclerosis, protects the atherosclerotic arterial media layer from transmedial elastin degradation and aneurysmatic enlargement or so called ectasia (185).

Increased proteolytic activity by serine proteases and matrix metalloproteinases -1,-2, and -9 is found in ruptured SCAA walls compared to basilar arteries (30) or to superior temporal artery branches (162). Increased plasma levels of pro-MMP-2 have been found in SCAA patients (318), where as MMP-9 levels are similar to controls (162). These findings suggest that MMP-2 in the SCAA wall may at least partly originate from the circulation, where as MMP-9 seems to be produced locally. However, thus far the expression of MMP genes in the SCAA wall has not been studied at the mRNA level, and the cell population that produces these enzymes and is responsible for the increased proteolytic activity is unknown.

6.2.5.3 Cell death in the SCAA wall

Many cells in the SCAA wall are undergoing programmed cell death (apoptosis) (133; 234) where as a normal arterial wall has practically no or extremely few apoptotic cell. Increased apoptosis eventually leads to loss of cells from the SCAA wall, unless compensated by increased proliferation. The reasons for apoptotic activity in the SCAA wall are not well known, although recent observations suggest that cytokines released from SCAA wall infiltrating inflammatory cells, especially tumour necrosis factor alpha, induce apoptosis in the SCAA wall (133). However, also other mechanisms seem likely. In atherosclerotic lesions, accumulation of oxidized lipids in SMCs can direclty induce cell death (107), as can also local hypoxia (304) that may results from growth of the atheroma plaque if the neocapillaries supplying the vascular wall are not able to grow as fast (199). Due to the structural resemblance of the SCAA wall with early atherosclerotic lesions, it seems likely that these same mechanisms are at play in the SCAA wall. However, thus far there are no studies about oxidized LDL or other oxidized epitopes, or of hypoxia in the SCAA wall

6.2.5.4 Hypoxia and angiogenesis in the SCAA wall

The wall of large arteries, such as the aorta, elastic arteries, and large muscular arteries, receive their supply of oxygen and nutrients in the inner layer of the wall (intima) by diffusion from the lumen, and to the outer layers (media and adventitia) via small capillaries that infiltrate the arterial wall (so called vasa vasorum) (105; 304). The vasa vasorum are situated in

the adventitia, from which layer they send branches that supply the medial layer. Stripping of the adventitia along with its vasa vasorum, leads to hypoxia and necrosis of the medial layer (105; 304). Medial necrosis and degeneration of the medial layer due to insufficient microcirculation has been proposed to contribute to the genesis of SCAAs (122).

Growth of adventitial capillaries, or vasa vasorum, occurs during growth of neointima (myointimal hyperplasia) after mechanical injury (balloon dilatation injury models) fast (233). Inhibition of vasa vasorum growth also decreases the growth of atheroma plaques (199; 200). Since the SCAA wall histologically resembles myointimal hyperplasia and atheroma plaque, it seems likely that the vasa vasorum are also crucial in the maintenance of a thick, myointima-like wall of the unruptured SCAA wall. Constant distension of the SCAA wall due to systolic blood pressure may via mechanical compression reduce circulation in the SCAA vasa vasorum in a similar fashion that blood flow is blocked in the myocardial capillaries during systole (122). This may lead to hypoxia, degeneration, and rupture of the SCAA wall. It seems also likely that luminal thrombosis that limits diffusion from the lumen, and thus predisposes the inner part of the SCAA wall to hypoxia.

Hypoxia and decreased microcirculation as a cause of SCAA wall rupture has not been studied. Hypoxia would lead to increased expression of angiogenic growth factor receptors and ligands, and to increased neocapillary formation in the SCAA wall. Histopathological studies have found expression of angiogenic factors in SCAA walls (299). Although conclusive data is lacking, decreased microcirculation and local hypoxia may contribute to the cell loss and wall degeneration that leads to SCAA wall rupture. The hypothesis merits experimental testing.

Hypoxia induces changes in angiogenic activity and growth receptor and ligand expression (39; 64), which may affect the myointima or SCAA wall in several ways. In models of immunological vascular injury (transplantation arteriosclerosis) , the angiogenic growth factor VEGF increases myointimal hyperplasia and neointima formation (177). VEGF-receptors 1 and 2 mediate SMC migration (87) and VEGF-receptor 1 (VEGF-R1) activation increases matrix metalloproteinase (MMP) activity in SMCs (333), a requirement for SMC migration (76; 369). VEGF-R1 may also potentiate the mitogenic effect of bFGF on SMCs (53). In neointima formation after vascular injury, bFGF increases SMC proliferation, migration, and neointima growth (179). In contrast, increased expression of the angiogenic growth factor angiopoietin-1 can decreased neointimal hyperplasia in models immunological vascular injury (217).

In addition to the effects of hypoxia on growth factor receptors and ligands, many intracellular signaling pathways are oxygen dependent (91) and therefore hypoxia may alter or modify the effects of inflammatory cytokine and growth factor stimuli on cells. An important example is the

signaling triggered by the inflammatory cytokine tumour necrosis factor alpha, which activates a non-oxygen dependent pro-apoptotic pathway, and a pro-survival pathway that is oxygen dependent because it is mediated by NF-kB (82; 91). Thus, in hypoxic conditions the pro-apoptotic effects of tumour necrosis factor alpha (and of other cytokines) may become more pronounced.

6.2.5.5 Immune response in the SCAA wall

It has been proposed that degeneration of the SCAA wall may be caused by an immune reaction against SCAA wall components (42). This hypothesis is supported by the observations that inflammatory cell infiltration is increased in ruptured SCAA walls (155) and that the pro-apoptotic inflammatory cytokine, TNFa, is synthesized in ruptured SCAA walls in which also signs of inflammatory cell induced apoptosis (Fas-ligand induced cell death) are found (133). Presence of macrophages, T-cells, B-cells, IgM and IgG -antibodies, and of activated complement in the SCAA wall (42; 155), confirms an ongoing inflammatory reaction mediated by the innate and adaptive immune systems. What triggers this immune reaction and what is its effect on the complex biology of the SCAA wall, remains to be elucidated.

6.2.5.5.1 Innate and adaptive (acquired) immunity

The immune system is divided into the innate and adaptive (acquired) arms. The innate arm is fast but unspesific and unable to adapt, where as the adaptive part is slower but able to adapt and develop spesific immune recognition of previously encountered antigens (acquired immunity or so called immunological memory). IgG and IgA -class antibodies, B-cells, and T-cells are mediators of the adaptive immune system, where as the innate immunity is mediated by natural IgM-class antibodies, the complement system, acute phase proteins (e.g. CRP), Toll-receptors, NK-cells, and macrophages (20; 94; 109; 129). Along with complement and Toll-receptors, macrophages act as a link between the innate and adaptive immune systems via presentation of encountered antigens to T-cells, which are then activated if a foreign antigen is recognized (109).

In macrophages this antigen presentation occurs via the major histocompatibility complex II (MHC II) molecules that bind proteins from cytosolic compartments to a spesific groove to which receptors of CD4+ T-cells then bind (109; 372). MHC II molecules are expressed on the surface of macrophages (and other antigen presenting cells) and encoded in the human leukocyte antigen (HLA) -region of the genome (114). If the receptors of the helper T-cells recognize the peptide presented by MHC II as foreign, helper T-cells start to express co-stimulatory surface receptors and secrete inflammatory cytokines that activate antibody production in B-cells and cytoxic response in T-cells and NK-cells (109). Antigen presen-

tation occurs also via MHC I molecules, which are expressed in all normal cells (72). Recognition of an antigen in the groove of the MHC I molecule by a T-cell receptor leads to T-cell induced death of the MHC I expressing cell (72) . MHC I molecules are also encoded in the HLA-region of the genome (114). Activated naïve B-cells (without prior antigen encounter) produce IgM or IgA class antibodies, where as activated memory B-cells (result from prior immunization) produce IgG, IgD, or IgE class antibodies (21). Thus, the presence of IgG antibodies against an antigen in the serum is a sign of prior encounter and immunization against this antigen, a so called "serological scar".

6.2.5.6 Inflammation in atherosclerosis and plaque rupture

Atherosclerotic changes are characterized by chronic inflammation, in which both the innate and adaptive arms of the immune system are activated (20; 94; 129). Inflammation in atherosclerotic lesions manifests as infiltration of macrophages, T-cells (93; 135; 310), B-cells (115), and mast cells (145; 176), in addition to infiltration of natural killer cells in rupture-prone shoulder regions of the atherosclerotic plaque (24). Also binding of antibodies (363), acute phase proteins (e.g. CRP) and activation of the complement system (193; 220) is seen atherosclerotic lesions. Even the earliest fatty streak precursors of atherosclerotic plaques in fetal arteries contain macrophages (209), which is a sign of ongoing inflammation. Besides inflammatory cells, the atheroma plaque consists of SMCs, endothelial cells, and fibroblasts / myofibroblasts, and of a varying amount of lipids that may be extra-or intracellularly located (e.g. foam cells that are macrophages filled with phagocytosed lipids).

Inflammation affects the atheroma plaque in several ways (reviewed by Hansson et al., (94)). Macrophages that are recruited to the site, become activated, and start to secrete inflammatory cytokines, of which some (e.g. TNFa) sensitize SMCs to apoptosis. In addition, infiltrating macrophages may produce significant amounts of MMPs that degrade the matrix. Some of the macrophages also act as antigen presenting cells, and activate and recruit T-cells and B-cells that produce more inflammatory cytokines and antibodies against the modified lipids deposited in the vascular wall. Activated macrophages and T-cells do not only secrete inflammatory cytokines, but also growth factors (e.g. TGF-B, PDGFs, and FGFs) that affect the phenotype, apoptosis, proliferation, matrix synthesis and matrix degrading activity of SMCs. Activation of the immune system in the atheroma is thought to sensitize the plaque for rupture via increased cell death and matrix destruction. However, inflammation simultaneously stimulates fibrotic proliferation, as in wound healing.

In addition to inflammatory cell infiltration, the atherosclerotic lesions also show signs of activation of the adaptive humoral immune system. The atherosclerotic plaques often contain IgM and IgG class antibodies. Some

of the IgM antibodies may represent naturally occurring antibodies (without any exogenous antigenic stimulation) that are "polyreactive" and recognize in a broad manner foreign and self antigens (21) The physiological function of these polyreactive IgM antibodies is to contribute to the first line of defence (prior to adaptation and generation of more specific IgG antibodies) against micro-organisms, and possibly to remove senescent, damaged, or apoptotic cells (21). In the atherosclerotic lesions the polyreactive IgM antibodies bind to oxidized and modified lipids, to apoptotic cells, and to pathogens that might infiltrate the atherosclerotic lesion. After prolonged or repeated antigen exposure, the antigen activated B-cells producing the polyreactive IgM-antibodies undergoe a process of maturation and selection that leads to production of more specific IgG-class antibodies. Binding of IgM or IgG class antibodies on the cell surface leads to cell lysis or induction of apoptosis by the complement system, or to targeting of the cell by the cytotoxic inflammatory cell response (20; 21).

Although activation of the humoral immune response in general leads to cell death and tissue destruction, the antibodies of the humoral immune system may also have a "protective" house keeping role, by removal or antigenic debris from the circulation and tissues. It has been suggested that in atherosclerosis, antibodies generated against modified lipid particles might have a "protective" role via reduction of antigenic and cytotoxic material from the arterial wall (43; 290). Thus some components of the inflammatory reaction in atherosclerotic arterial wall may actually reduce the progression of degeneration of the arterial wall.

6.2.5.7 Potential inducers of immune response in the atherosclerotic plaque

In atherosclerosis, accumulation of lipids (especially LDL) to the arterial wall, followed by oxidative or enzymatic modification, is thought to be the main trigger of inflammation (84). The biochemically altered lipids and the modified LDL associated proteins (e.g. ApoB100) form neoepitopes that are recognized by the immune system and bind naturally occurring antibodies, activate the complement system, and are recognized by phagocytosing cells, which leads to cell death, tissue destruction and inflammation (20; 21; 94).

Immunohistochemistry, Western blotting, and biochemical studies have shown that the atherosclerotic plaque contains so-called neoepitopes, generated from the ApoB protein core and lipids of low density lipoprotein (LDL) particles by oxidative stress or enzymatic cleavage (20; 196; 228; 309; 321; 364). Antibodies and T-cells that recognize these neoepitopes generated from LDL, have been shown from atherosclerotic plaques (363) (310) and from the serum of patients with clinical manifestations of atherosclerosis (67; 282; 356). Besides oxidated LDL (oxLDL), oxidative stress, free radicals, and related enzymatic activity in the atherosclerotic wall may also

render other proteins and components of the vascular wall antigenic, and it should be remembered that many of the classic neoepitopes of oxLDL can be induced to other proteins as well (209; 321).

Besides proteins and lipids modified by oxidative stress, free radicals, and related enzymatic activity, also local or systemic Chlamydia pneumoniae infection has been postulated to be the cause of acquired immune reaction in the atherosclerotic vascular wall (36; 130). Antibodies against Chlamydia pneumoniae are found in many patients with atherosclerosis (118; 243; 279). Also the microbe along with T-cells immunized against it, has been detected in atherosclerotic lesions (57; 144; 173; 198). However, whether Chlamydia pneumoniae is just an innocent bystander, a modulator of the immune response triggered by other antigens, or the primary culprit, remains a debate. Chlamydia pneumoniae infection does not seem to increase or accelerate atherosclerosis in ApoE or in wild type mice (1; 33), although also controversial reports have been published (23). Chlamydia pneumoniae infection may lead or predispose to atherosclerosis by several mechanisms (278), which include immunization to heat shock proteins and other auto-antigens via molecular mimicry and change in serum lipid profiles via interaction of anti-chlamydia antibodies and lipoprotein complexes (83).

6.2.5.8 Potential inducers of acquired immune reaction in the SCAA wall

Apoptosis is found in the SCAA wall (234), and naturally occurring IgM antibodies that bind to damaged or apoptotic cells are likely to bind to these cells. However, to which antigens or components of the SCAA wall the IgM antibodies specifically bind, has not yet been studied.

Due to the structural resemblance of early atherosclerotic lesions and unruptured SCAA walls, it seems likely that LDL might accumulate also in the SCAA wall, become modified, and trigger an inflammatory reaction as in atheroma. The IgM antibodies and especially the acquired IgG class antibodies found in the SCAA wall may be generated against neoepitopes (e.g.OxLDL) created by oxidative and enzymatic stress. Neither oxidative stress nor the presence of neoepitopes in the SCAA wall has been studied thus far. The presence of Chlamydia pneumoniae in the SCAA wall has been investigated, with negative results (32). However, a recent patient series suggests that circulating antibodies against Chlamydia pneumonia would significantly contribute to the risk of SAH (366). As discussed in the context of atheroma plaques, the possible contribution of Chlamydia infection to SCAA rupture may occur via generation of Chlamydia pneumoniae reactive antibodies that due to molecular mimicry also identify components of the SCAA wall, or via modification of lipoproteins.

6.2.6 Adaptation and repair mechanisms of the SCAA wall

Despite the SCAA wall is constantly subjected to hemodynamic stress, many SCAAs never rupture (128), suggesting that the SCAA wall has some mechanisms of adaptation and repair that are in balance with hemodynamic and other stress factors in many SCAAs. The adaptation mechanism of normal arteries to hemodynamic stress is myointimal hyperplasia (124). SCAA walls resemble histopathologically myointimal hyperplasia (169). Due to the histopathological resemblance between the unruptured SCAA wall and myointimal hyperplasia, is seems likely that the SCAA wall reacts to increased hemodynamic stress by increased cell proliferation and matrix synthesis as myointimal hyperplasia elsewhere (204; 303).

6.2.6.1 Formation of myointimal hyperplasia / neointima in response to stress or injury of the vascular wall

Mechanical injury or increased shear-stress that leads to endothelial damage, induces in medial SMCs a change in phenotype, followed by migration of the SMCs to the de-dendothelialized luminal surface of the vessel, with subsequent SMC proliferation and matrix synthesis (48; 76; 95; 204; 303). This migration and proliferation of SMCs leads to the formation of a pad of myointimal hyperplasia, or so called neointima in animal models (48; 76; 95; 204; 303). Formation of local fibrosis and SMC overgrowth in the injured vascular wall has been compared to fibrosis in wound healing, and has been thought to have a similar physiologic role in the injured vascular wall. Like wound healing, formation of neointima or myointimal hyperplasia is associated with inflammation (204; 218) and matrix metalloproteinase production (80; 134).

6.2.6.2 Origin of myointimal / neointimal cells

According to the classic "response to injury" hypothesis presented by Russel Ross, endothelial injury of any kind leads to activation and proliferation of medial SMCs that migrate to the luminal surface and produce intimal thickening or so called myointimal hyperplasia (269). Since medial and intimal SMCs differ in phenotype, a switch of phenotype from a quiescent, contractile phenotype to a proliferating, synthetic phenotype has been thought to occur (35; 317).

Besides medial SMCs, myointimal cells seem to also have other origins. Studies that quantitated SMC proliferation and migration after mechanical vascular injury have revealed a discrepancy between medial SMC proliferation in the injured vascular wall and increase in myointimal cell numbers (76; 292). Moreover, DNA analysis of myointimal SMCs revealed that they are oligoclonal (41), suggesting that many of the myointimal SMCs have originated from a small population of progenitor cells that have migrated to the injured luminal surface. Myointimal hyperplasia also occurs in models of decelularized vascular grafts (9) and fulminant vascular al-

lograft rejection (191) in which the immune system kills all donor cells in host-versus-graft rejection. It is logical to assume that the myointimal cells in these models are recipient-derived, as demonstrated by Saiura et al. (280).

6.2.6.2.1 Bone marrow origin of vascular wall cells

The major arteries of the body (aorta, etc.) have a common developmental origin with the hematopoietic system (230) and it is currently thought that a progenitor cell capable of vascular or hematopoietic differentiation (so called hemangioblast) are found also in post natal life (230; 232). The hematopoietic tissue is located in the bone marrow in adult animals and contains pluri-or multipotent stem cells that constantly proliferate and produce new cells that differentiate and mature into circulating cells of different hematopoietic lineages. Recently several reports have suggested that the bone marrow of adult animals would contain also multipotent primitive stem cells capable of differentiating into many mesenchymal tissue types (37) (224).

Bone marrow-derived myointimal cells were first reported by Han and Campbell in a mouse vascular injury model (92) and later confirmed by Sata et al. and several other authors (108; 256; 294; 314). Sata et al. were also the first to demonstrate that the bone marrow-derived myointimal cells differentiated from a CD34+ progenitor cell population considered to be hematopoietic stem cells (284). Despite reports that failed to find any bone marrow-derived myointimal cells despite recipient origin of the myointima in allografts (116; 178), the contribution of bone marrow-derived myointimal cells into vascular wall remodeling has become a widely accepted concept (108; 365). Bone marrow-derived myointimal cells have been also reported in autopsy studies of humans that have underwent sex-mismatched bone marrow transplantation (38).

In addition to myointimal cells, bone marrow-derived cells have been also reported to differentiate into endothelial cells and to contribute to pathological and physiological angiogenesis in tumors, ischemic tissues, and after mechanical injury (10-12; 117). However, recent reports using genetic labels targeted at endothelial cells (85) or high magnification confocal microscopy (246; 373) have challenged the concepted of bone marrow-derived endothelial cells by demonstrating that some of the observed bone marrow-derived endothelial cells may have infact been misinterpretation of two closely situated cells. Bone marrow-derived myointimal cells have not been confirmed with confocal microscopy.

6.2.6.2.2 Endothelial or myointimal / neointimal precursor cells in the peripheral circulation

Circulating human mononuclear cells differentiate in vitro into myointimal or endothelial cells (255; 297) as suggested by the previously discussed observations in animal models. Differentiation of circulating human

mononuclear cells in myointimal or vascular cells has not been, however, confirmed in vivo.

6.2.7 Summary of the putative cellular and molecular mechanisms of rupture and repair of the SCAA wall

Prior histopathological series of SCAA fundi suggest that the walls of unruptured SCAAs bear histological features resembling those of atherosclerotic lesions and aortic aneurysms. SCAAs do not, however, seem to be manifestations of atherosclerotic disease since they can be induced in experimental animals with hypertension and altered cerebrovascular flow conditions, without the contribution of other risk factors for atherosclerosis. As in atherosclerotic lesions and aortic aneurysms, signs of protease activity, cell death, and chronic inflammation have been described in the SCAA wall in which they seem to associate with wall degeneration and rupture. The factors that trigger and maintain protease activity, cell death, and inflammation in the SCAA wall remain unknown, but due to the histopathological resemblance with early atherosclerotic lesions and aortic aneurysms, it seems possible that the same factors that induce inflammation and degeneration in atherosclerotic lesions and aortic aneurysms, could also work in the SCAA wall. Similarly, it seems likely that the mechanisms of adaptation and repair of the normal and atherosclerotic arterial wall also work in the SCAA wall. Recently contribution of bone marrow-derived myointimal cells to fibrotic lesion formation after mechanical or immunological vascular injury has been described. These cells might be used as local or systemic transplants that repopulate and strengthen the decellularized and degenerated SCAA wall. The concept remains controversial, but is especially interesting to those trying to increase fibrosis in thin, decellularized SCAA walls with ongoing chronic inflammation. The mechanisms leading to degeneration of the SCAA wall and inflammation need to be elucidated to understand why and how SCAA walls rupture and in order to develop experimental models that accurately reproduce parts of the pathobiology of the SCAA wall. Such models are needed to test novel therapeutic concepts, e.g. contribution of bone marrow-derived myointimal cells to the repair and adaptation of untreated and treated SCAAs, or bioactive embolization devices that likely perform differently in real human SCAAs and in many of the experimental models currently used.

Aims of the study

The pathobiology of SCAA wall rupture and repair needs to be elucidated in order to:

i) develop novel diagnostic tools that recognize SCAAs in risk of imminent rupture,
ii) develop novel biological therapies that prevent SCAA wall rupture, and
iii) develop experimental models that reproduce, at least in part, the pathobiology of human SCAA wall and could thus be used to test and develop novel therapies.

The specific aims of this study were to:

I) Characterize the histopathological differences of unruptured and ruptured SCAA walls and investigate the hypothesis that SCAA wall is undergoing active remodeling that in some cases leads to a rupture-prone wall

II) Investigate the hypothesis that as in atherosclerotic lesions and abdominal aortic aneurysms, LDL accumulates also in the SCAA wall, becomes oxidized, and triggers a humoral immune response that associates with the risk of SCAA wall rupture

III) Identify growth factor receptors involved in remodeling of SCAA wall towards a rupture-prone type

IV) Investigate the origin of myointimal cells in an experimental saccular aneurysm model and the potential contribution of bone marrow-derived cells to thrombus organization and neointima formation

V) Investigate the possible use of transfused circulating human mononuclear cells to enhance myointima formation and the repair of the SCAA wall

Patients, Materials and Methods

8.1 Histopathological characterization of unruptured and ruptured SCAA walls (publications I, II, III)

8.1.1 Tissue samples of saccular cerebral artery aneurysm fundi

Tissue samples from the wall of human saccular cerebral aneurysm fundi were collected during microsurgical ligation (clipping) of the aneurysm neck (prof. Juha Hernesniemi, and Dr. Mika Niemelä, Dpt. of Neurosurgery, Helsinki University Central Hospital), and snap frozen in liquid nitrogen in the operating room. Some of the tissue samples were put immediately after resection to 4% paraformaldehyde for fixing.

Collection of the tissue samples and the study protocols were approved by the Ethical committee of the Dpts. of Neurology, Neurosurgery, Opthalmology, and Otorhinolaryngology, of Helsinki University Central Hospital.

Clinical and radiological data was collected retrospectively by reviewing the medical records and preoperative imaging studies of the patients from which tissue samples were obtained.

8.1.2 Histology and immunohistochemistry

For histological studies, snap frozen SCAA wall samples were embedded in OCT Tissue Tek compound (Sakura, Torrance, CA, USA) and cryosectioned at 4um. The sections were stained with Mayer hematoxylin and eosin G, or with Weigert-van Gieson staing for detection of elastic laminas. Some samples were also fixed with 4% paraformaldehyde (PFA) directly after resection or when the snap frozen sample was processed for RNA and protein isolation with TRIZOL (data not included). PFA fixed samples were embedded in paraffin and sectioned at 2-5um for histological stainings and immunohistochemistry.

For immunohistochemistry, 4um cryosections from the SCAA wall were fixed with either -20 degrees cold acetone or with room temperature 4% PFA, after which the samples underwent serum block (1.5-3% normal horse serum in PBS for monoclonal antibodies, and 3-5% rabbit or goat serum in PBS for polyclonal antibodies) for 30 min in room temperature. Serum block was followed by either 1 hour incubation with the primary antibody at room temperature or overnight incubation at +4 °C (antibo-

dies, clones, dilutions, and manufacturers are found in Tables 2-4). After washing three times 5 min in PBS, bound primary antibody was detected by incubating the tissue sections with biotinylated anti-mouse, anti-rabbit, anti-goat, or anti-guinea pig secondary antibody (Vector Vectastain Elite-kits, Vector, Burlingame, CA) as appropriate. After washing the unbound secondary antibody with PBS (3 x PBS for 5min), endogenous peroxidase activity was blocked by incubating the sections in 3%-0.1% H2O2 in PBS or methanol for 20min. Alternatively the sections underwent peroxidase block with 3% H2O2 in methanol before serum block.

Following incubation with the secondary antibody, the sections were washed again three times in PBS, after which they were incubated for 30 min with a horse-radish peroxidase or alkaline phosphatase conjugated avidin-biotin complex (Vector Vectastain-kit) that binds specifically to the biotinylated secondary antibody. The peroxidase activity was then visualized using diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA) or AEC (Vector) chromogens, and alkaline phosphatase activity with Vector Blue substrate (Vector).

Sections were counterstained with Mayer's hematoxylin or Nuclear fast -red and mounted with either water soluble mounting media, or after dehydration with an alcohol series and xylene, with a xylene based mounting media as appropriate.

Immunohistochemistry and -fluorescence were also made from SCAA samples fixed with 4% paraformaldehyde and embedded in paraffin. These samples were sectioned at 2-4 um, deparaffinized in xylene, rehydrated with an alcohol series followed by antigen retrieval with heated 0.01-0.1M citrate buffer (pH 6), after which immunostaining was performed as described above.

For double immunostaining, the first sections underwent immunostaining with one antibody as described above, followed by a second serum block for 1h or overnight, and immunostaining with a second primary antibody as described above, or with a fluorochrome conjugated secondary antibody (1:200, Alexa Fluor 488 green conjugated goat anti-mouse IgG, Molecular Probes Inc., Eugene, OR) that was used to detect anti-alfa smooth muscle actin primary antibody (clone 1A4). Sections stained with fluorochrome conjugated secondary antibody were mounted with Vectashield containing DAPI (Vector).

8.1.2.1 Analysis and quantitation of histology

Two blinded observers performed classification of the SCAA wall structure. In addition, preservation of elastic laminas and endothelium, as well as the presence of atherosclerotic calcifications, medial layer degeneration, myointimal hyperplasia (MH), and endoluminal thrombosis, were evaluated.

To quantitate matrix synthesis and proliferation, the number of proline hydroxylase positive cells and the number of Ki67 positive cells was

counted at 40 magnification from 2 active areas. Results are given as the mean value of the ratio of these cells and total cell number in the 2 active areas.

To quantitate inflammatory cell infiltration, the number of the CD45+, CD3+, CD68+, CD163+, and CD11b+ inflammatory cells was counted from a standardized grid area using 40 magnification (0.0625 mm2) from 2 active areas of the SCAA wall. The mean value for the density of positive cells (number of cells / grid area) was calculated. The ratios and densities were calculated separately for the SCAA wall and for the areas of myointimal hyperplasia / organizing thrombosis (MHOT) when present.

Immunostainings for ApoB, epitopes of oxidized LDL, or IgG were scored as positive or negative and for the locatization of immunopositivity as follows: i) in the matrix, ii) in mural cells, iii) in mononuclear cells, iv) in the luminal thrombus, or v) in fatty streaks of the wall.

Expression of growth factor receptors in the SCAA wall was scored as follows: negative = no staining; moderate expression = less than third of cells positive; strong expression = more than one third of cells positive. Visual scoring was used in the analyses. Image analysis (KS300 software, Carl Zeiss GmbH., Oberkochen, Germany), performed for the 12 receptors in 10 of the 66 samples (120 sections), was found unreliable because of extracellular matrix staining, heterogenous structure, and variable cell sizes.

Microphotographs were taken with an Axiovision light and epifluorescent microscope (Carl Zeiss GmbH, Oberkochen, Germany) or with an Olympus AX70 (Olympus Optical, Japan) light and epifluorescent microscope using microscope mounted digital cameras and AxioVision 3.0 (Carl Zeiss) and analySIS (SoftImagingSystem GmbH, Germany) softwares. Overlay images and figure panels were created with Image J (NIH software) and Adobe Photoshop 8.0 software (Adobe Systems).

8.1.3. Detection of antibodies against OxLDL from serum samples

8.1.3.1 Plasma samples

Blood samples were obtained by venipuncture on EDTA coated tubes on the 4th or 5th postoperative day from patients that underwent microsurgical clipping of a SCAA. Collection of blood samples was approved by the ethical committee. The blood samples were stored at -20 degrees. EDTA plasma was isolated by centrifugation.

8.1.3.2 Detection of anti-Oxidized LDL IgG antibodies with ELISA

The plasma samples of SCAA patients were studied for the presence of anti-oxidized LDL IgG class antibodies with ELISA. A commercial anti-oxidized LDL IgG ELISA kit with a peroxidase conjugated anti-IgG secondary reagent was used

(Ark Therapeutics Group Inc., London, UK). The ELISA plates in the kit were coated with a peptide (p244) sequence derived from OxLDL, corresponding to the amino acids 3144-3163 of ApoB-100. After incubation with peroxidase conjugated anti-IgG reagent and subsequent washes, the plates were incubated with peroxidase substrate (H202) and TMB chromogen for 30 min. Color development was stopped with 0.5 mol/L H_2SO_4, and absorbances were measured at 450 nm with a Multiskan microplate reader (Thermo LabSystems, Waltham, MA). In the peptide ELISA, samples with a mean absorbance above 0.4 and inhibition% with the same peptide of more than 40%, were considered positive.

The results of the above assay were compared to ELISA using native and copper-oxidized LDL as antigens (210). Samples in which the absorbance ratios of OxLDL and native LDL was between 2-3 and inhibition% at least 40%, were graded as positive. All measurements in both types of ELISA were performed as duplicates in each plates, and to control for variance between the plates, 34% of the samples were tested in two or three plates.

8.1.4 Statistics

Proportions, medians, and range were calculated for categorical and continuous variables and compared with Chi-Square or Mann-Whitney U-test between two categorical groups. Logistic regression was used to search for independent predictors of rupture. NCSS 2000 statistical software (NCSS 2000, NCSS Statistical Software, Kaysville, UT) and SPSS 12.0.1 statistical software (Apache Software Foundation) were used. Alpha-level was 0.05.

Table 2. Monoclonal mouse anti-human antibodies used in Publication 1.

Antigen	Clone	Dilution	Source	Ref.
CD31 (PECAM 1: platelet endothelial cell adhesion molecule 1)	JC70A	1:200	DAKO	20
Alfa-smooth muscle actin	1A4	1:500	Sigma	21
Myosin heavy chain	SMMS-1	1:200	DAKO	22
Fibroblast-marker	5B5	1:200	DAKO	23
Ki67	MIB-1	1:250	DAKO	24
CD45 (LCA: leukocyte common antigen)	2B11+PD7/26	1:200	DAKO	25
CD3 (TCR: T-cell receptor)	PC3/188A	1:200	DAKO	-
CD11b (MAC 1: macrophage adhesion molecule 1)	2LPM19c	1:200	DAKO	26
CD68 (macrophage marker)	PG-M1	1:200	DAKO	27
CD163 (macrophage marker)	Ber-MAC3	1:200	DAKO	28

Table 3. Antibodies and dilutions used in Publication 2.

Sigma refers to Sigma-Aldrich Inc., St.Louis, MO. For Anti-ApoB and oxidized LDL antibodies the references were the antibodies were originally characterized, are given in parentheses.

Antigens	Type	Clone / Name	Dilut.	Origin	Used in double stains with
α-smooth muscle actin	mouse monoclonal	1A4	1:500	Sigma	HNE
15-Lipoxygenase	rabbit polyclonal	CheY	1:1000	(362)	-
Anti-native or oxidized LDL					
ApoB-100	mouse monoclonal	MB47	1:500	(368)	-
Minimally modified LDL	mouse monoclonal	Ox4E6	1:200-1:1000	(110)	1A4, YE
Copper oxidized LDL	goat polyclonal	YE	1:500	(353)	Ox4E6
Hydroxynonenal (OxLDL)	guinea-pig polyclon.	HNE	1:1000	(229)	1A4, MDA-2
Malondialdehyde (OxLDL)	mouse monoclonal	MDA-2	1:50	(229)	EO6
Natural anti-OxLDL IgM from ApoE mice	mouse monoclonal	EO6	1:500-1:1000	(227)	1A4, MDA2

Table 4. Antibodies, dilutions, and controls used in Publication 3.

Locus refers to the chromosomal location of the studied growth factor receptor (NCBL Database). PECAM 1 stands for platelet endothelial cell adhesion molecule 1, LCA for leukocyte common antigen, TCR for T-cell receptor, MAC 1 for macrophage adhesion molecule 1, and S-C for Santa-Cruz Inc. For negative control the primary antibody was either replaced with an irrelevant antibody of the same IgG subtype (monoclonals) or incubated with a blocking peptide prior to administration on tissue sections (polyclonals).

Monoclonal antibodies

Antigens	Clone	Dilut.	Manufacturer	Pos. Controls
CD31 (PECAM 1)	JC70A	1:200	DAKO	Perforators, AVMs
α-smooth muscle actin	1A4	1:500	Sigma	Perforators, AVMs
Myosin heavy chain	SMMS-1	1:200	DAKO	Perforators, AVMs
Proline hydroxylase (matrix synthesis)	5B5	1:200	DAKO	Skin, AVMs
Ki67 (proliferating cells)	MIB-1	1:250	DAKO	Tonsil
CD45 (LCA)	2B11+PD7/26	1:200	DAKO	Tonsil, lymph node
CD3 (TCR)	PC3/188A	1:200	DAKO	Tonsil, lymph node
CD11b (MAC 1)	2LPM19c	1:200	DAKO	Tonsil, lymph node
CD68 (macrophages)	PG-M1	1:200	DAKO	Tonsil, lymph node
CD163 (macrophages)	Ber-MAC3	1:200	DAKO	Tonsil, lymph node

Polyclonal antibodies

Receptors	Locus	Blocking peptide	Dilut.	Source and manufacturer		Pos. Controls
IGF-R1alfa	-	Yes	1:100	Rabbit	S-C	Perforators, AVMs
bFGF-R1	8p11	Yes	1:100	Goat	S-C	Angiosarcoma, AVMs
bFGF-R2	10q26	Yes	1:100	Goat	S-C	Angiosarcoma, AVMs
bFGF-R3	4p16	Yes	1:100	Rabbit	S-C	Angiosarcoma, AVMs
bFGF-R4	5q35	Yes	1:100	Rabbit	S-C	Angiosarcoma, AVMs
TGFbeta-R1	9q22	Yes	1:100	Goat	S-C	Skin, AVMs
TGFbeta-R2	3p22	Yes	1:100	Rabbit	S-C	Skin, AVMs
TGFbeta-R3	1p33-32	Yes	1:100	Goat	S-C	Skin, AVMs
PDGF-Ralfa	4q11-13	Yes	1:100	Rabbit	S-C	Hemangioblastomas, AVMs
PDGF-Rbeta	5q31-32	Yes	1:100	Goat	S-C	Hemangioblastomas, AVMs
VEGF-R1	13q12	Yes	1:100	Goat	S-C	Hemangioblastomas, AVMs
VEGF-R2	4q11-12	No	1:100	Rabbit	S-C	Hemangioblastomas, AVMs

8.2 Experimental microsurgical animal models (publication IV+ additional unpublished data)

Figure 6 Microsurgical experimental saccular aneurysm model in rats and mice

The commonly used experimental saccular aneurysm model of ligated vascular graft was applied to rats and mice. A segment of the thoracic aorta was dissected free from between the left subclavian artery and the first intercostal arteries, and ligated distally (A). The ligated aortic segment was sutured end-to-side (B) to the abdominal aortas of rats (2.5-1.5mm) and mice (1-0.5mm). This resulted in saccular arterial pouches (C) that stayed patent and were covered with peritoneum (D, experimental aneurysm 1 month postop.).

Patency of the grafts was followed with magnetic resonance angiography (MRA) in mice (E) and rats (F). In rats the constructed experimental aneurysms were large enough (3x5mm) to be coiled with GDCs in clinical use. Coiled experimental rat aneurysms were followed with MRA (G and H) that showed neck remnants left intentionally for testing of bioactive coils.

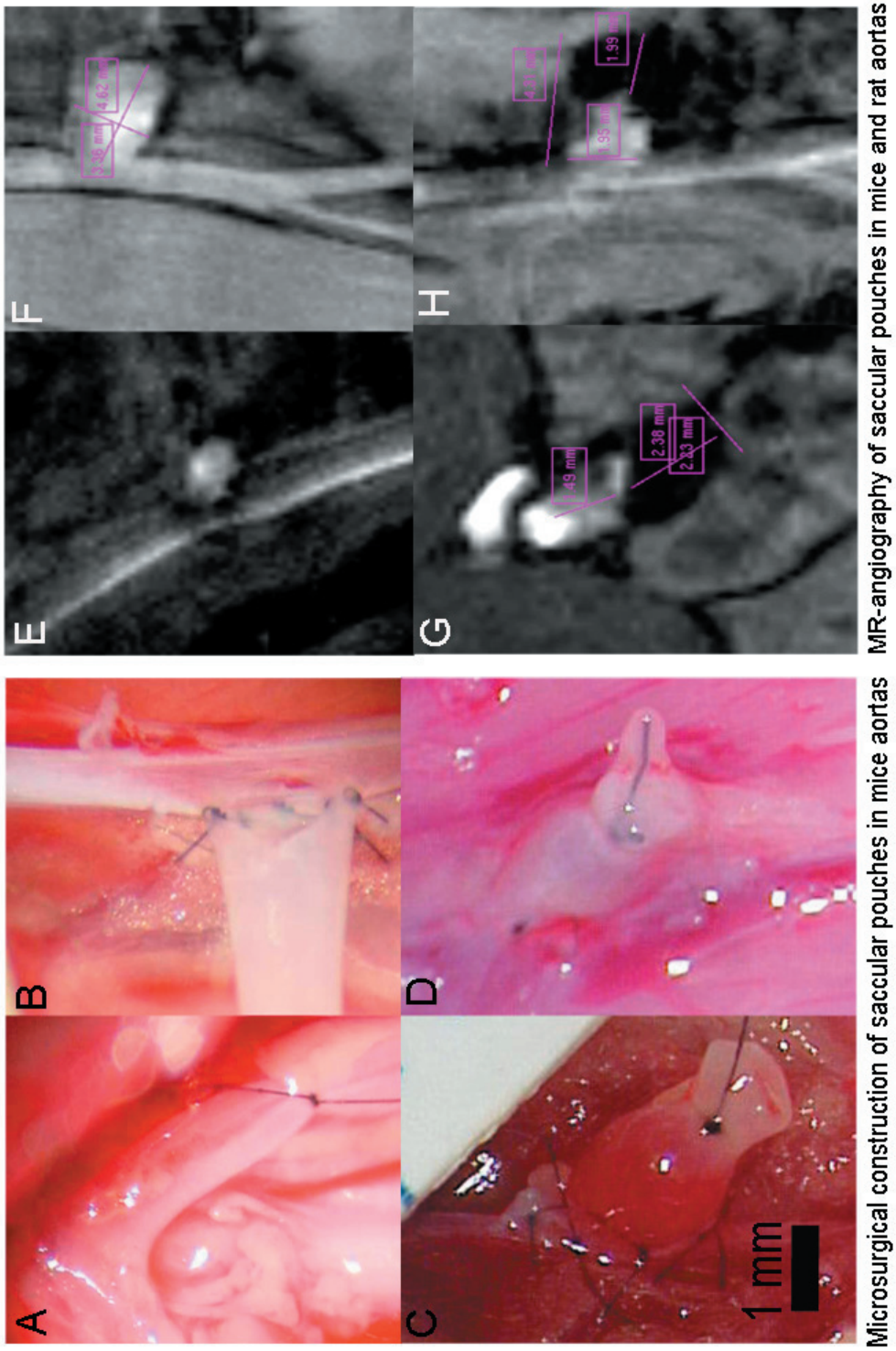

Figure 6 Microsurgical experimental saccular aneurysm model in rats and mice

8.2.1 Microsurgial saccular arterial aneurysm model

The commonly used experimental saccular aneurysm model of microsurgically created vascular pouch was applied to rodents (rats and mice). The end-to-side aortic graft model was first tested in rats (n=14) and then applied to mice (n= 55, survival rate 2/3, most mice lost to complications in fluid balance and anesthesia), in which transgenic strains (ApoE, ROSA, GFP) were used to study the effect of atherosclerosis and the origin of neointimal cells. Male Wistar rats (300-400g) (Harlan, Horst, The Netherlands), C57/B6 wild type mice, transgenic C57/B6 ApoE knockouts, and C57/B6 mice with either beta-galactosidase reporter gene (ROSA) or green fluorescent protein (GFP) reporter gene expressed ubiquitously. Wild type mice were purchased from Biomedicum Helsinki (Helsinki, Finland) and the transgenic strains from Jackson Laboratories Inc. (Bar Harbor, Maine, USA).

Animals were kept on regular tap water and pellet diet, and received humane care in compliance with institutional guidelines. The experiments were reviewed and approved by the municipal veterinarian of the County of Uusimaa, Finland. The rats were anesthesized with medetomidine (0.5mg/kg sc.) and ketamine (60mg/kg ip.), and the mice with a 1:1:2 mixture of midazolam (5mg/ml) and fentanylfluanisone (Hypnorm, Janssen-Cilag, Birkerod, Denmark) diluted in sterile aqua (0.7ml /100g sc.). Buprenorphine (Temgesic, Schering-Plough Oy, Espoo, Finland) was used for analgesia (0.3mg/kg sc. for rats, 5mg/100g sc. for mice).

8.2.1.1 The microsurgical procedure

A segment of donor descending thoracic aorta was sutured end-to-side with continuous 9-0 nylon (rats) or interrupted 11-0 nylon (mice) to the infrarenal abdominal aorta (diameter approximately 1.5-2mm in rats, 0.4-1mm in mice) of a syngenic recipient (Fig.6, please see Videoclip 1 of publication 5. for complete presentation of the procedure). The distal end of the graft was ligated, creating a pulsating saccular pouch (experimental aneurysm, 2-3 x 4-5mm in rats, 1-1.5 x 2.5-3mm in mice). Pulsation of the grafts was inspected before closing the laparotomy. Median ischemia time of the experimental aneurysm was 25min (range 15-40min) in rats and 24min (range 8-55min) in mice.

8.2.1.2 Endovascular GDC placement to the rat experimental aneurysms

The suitability of the murine side wall experimental saccular aneurysm model to embolization with endovascular devices in clinical use was tested in rats (n=6). Guglielmi detachable coils (GDCs) with a loop
diameter of 2mm and lenght of 2cm were placed endovascularly to the experimental rat aneurysms (MRI images, Fig.6). A bilateral thoraco-ab-

dominal incision was made to the donor animal, the abdominal aorta canulated in retrograde direction, and an incision made to the root of the ascending aorta. The cannulated aorta was then flushed with physiological saline, and GDCs introduced to the aorta via the canule. The GDC loops were placed to the aortic segment that was used to construct the saccular aneurysm, and fixed with a silk ligature. The coils were electrically detached and the pusher wire withdrawn. The graft with the GDCs in place was then dissected free and used to construct a saccular aneurysm in the recipient animal (please see Videoclip 2 of publication 5. for complete presentation of the coiling procedure).

8.2.1.3 Experimental aneurysms in ApoE mice and ROSA mice

To investigate whether atherosclerotic degeneration of the aneurysm wall would induce growth of the experimental aneurysms, and to investigate the origin of smooth muscle cells / myofibroblasts (alfa-smooth muscle actin+ cells) that contribute to thrombus organization and remodeling in the experimental aneurysm wall, we performed transplantations between old ApoE mice with atherosclerotic changes (aged over 40 weeks) and ROSA (beta-galactosidase reporter gene) mice (n=4, ApoE-donors and ApoE-recipients; n=4, ApoE-donors and ROSA-recipients; n=3, ROSA-donors and ApoE-recipients). The arterial grafts derived from ApoE –donors were atherosclerotic.

8.2.1.4 Experimental aneurysms in bone marrow transplantated mice

To investigate the hypothesis that some of the recipient-derived neointimal cells originate from the bone marrow, experimental aneurysms were constructed in chimeric C57 mice with syngenic ROSA (n=12) orGFP (n=3) labelled bone marrow. The mice underwent 4Gy total body irradiation as preconditioning to bone marrow transplantation, and after 24 hours received an infusion of 2-5 million ROSA or GFP-labelled donor bone marrow cells via the tail vein. This protocol has led to a bone marrow chimerism of 36-43% and to a peripheral blood chimerism of 80-90% (246). In this study succesful engraftment of the bone marrow was verified from histological sections of the recipient animal spleen. Experimental aneurysms were transplanted 10-20 weeks after bone marrow transplantation.

8.2.1.5 Magnetic resonance angiography (MRA) of the experimental aneurysms

Magnetic resonance angiography (MRA) was used to follow graft (experimental aneurysm) patency after surgery. For MRA in rats, (4.7T PharmaScan, Bruker BioSpin GmbH, Ettlingen, Germany), 3D-time of flight (3D-TOF, TR 30.0ms, Echo-Time (TE) 4.0ms, pulse angle 30) and PHASE-

contrast (TR 60ms, TE 9.9ms, pulse angle 40) MRA sequences were used. For anatomical magnetic resonance imaging (MRI), Gradient-Echo sequence comprising first order flow compensation (GEFC, TR 1000.0ms, TE 4.0ms, Pulse angle 90) and Rapid acquisition with relaxation enhancement (RARE, TR 6103.0, effective TE 38.6) sequences were used. Slice thickness ranged from 1-2mm. In mice, MRA was used to verify patency of the anastomosis one week after operation (Fig.1). In 2D-sequences two field of views (5cm / 5cm or 3 cm / 3cm) and two matrix settings (256 / 256 or 256 / 192) were used. In 3D-TOF field of view was 3cm / 3cm / 2.5cm and matrix was either 256 / 256 / 32 or 256 / 192 / 32. In 70% of mice that had visible flow signal in the experimental aneurysm with GEFC or PHASE-contrast sequences and axial sections of 3D-TOF, the ligated arterial graft was also visible in 3D-time of flight reconstructions. In rats, we used field of views of 10cm / 10cm, 7cm / 7cm, and 5cm / 5cm in 2D-sequences depending on the size of the rat, and a matrix of 256 / 192 or 256 / 256. In 3D-TOF sequence, field of view of 7cm / 7cm / 3cm and a matrix of 256 / 256 / 32 or 256 / 192 / 32 were used.

8.2.1.6 Histology and reporter gene detection in experimental rat and mouse aneurysms

Rats were perfusion fixed through left ventricle with 3% paraformaldehyde (PFA), followed by 6h incubation in 3% PFA and paraffin embedding. The samples were sectioned at 4 μm and stained with Mayer'shematoxylin-eosin (HE). Immunohistochemistry was performed as described previously above for human SCAA samples, after deparaffinization and antigen retrieval in heated citrate buffer (pH 6.0) or heated Tris-EDTA (pH 8-9) buffer. Monoclonal mouse antibodies were used to detect alfa-smooth muscle actin (aSMA, clone 1A4, Sigma), anti-rat CD68 (clone ED1, Serotec, Oxford, UK) and CD3 (T-cell receptor, clone PC3/188A, DAKO, Glostrup, Denmark). Tunel reactions to detect apoptotic cells were performed with an InSituCell Death detection kit (Roche-Diagnostics, Roche, Basel, Switzerland).

Mice were perfusion fixed through the left ventricle, followed by overnight incubation in 0.25% glutaraldehyde. Beta-galactosidase (ROSA) was visualized by incubating the fixed tissues for 72 hours at +37°C in 0.8 mg/ml X-gal solution with potassium ferricyanide, potassium ferrocyanide, and magnesium chloride. The tissues were then paraffin embedded and sectioned into 4μm thickness. Sections were stained with HE and nuclear fast red (Vector, Burlingame, CA). In double staining for X-gal (ROSA) and aSMA, immunostaining was done using a mouse monoclonal antibody against aSMA (clone 1A4, Sigma-Aldrich Co., Saint-Louis, MI) and a Vector mouse-on-mouse kit (Vector). Nuclear fast red was used as background stain. Tissues from GFP bone marrow transplanted animals were incubated overnight in 2% sucrose and fixed with 2.5% glutaraldehyde, af-

ter which they were snap frozen and cryosectioned into 4 or 15 μm thickness. The cryosections underwent acetone fixation and serum block with 5% normal horse serum, followed by 1h or 24h incubation with a Cy3 conjugated aSMA antibody (clone 1A4, Sigma). The sections were background stained with DAPI.

Histopathological changes were examined from the fundus of the experimental aneurysm, distally to the anastomosis which showed inflammation and fibrosis. The following parameters were assessed from HE-stained and alfa-smooth muscle actin immunostained sections: luminal endothelial layer; luminal thrombosis / organizing luminal thrombosis; neointimal hyperplasia; intimal capillaries; and inflammatory cell infiltration in the lumimal surface or luminal organizing thrombus, and in the adventitia. In rats, the presence of apoptosis, macrophages (CD68+) or T-cells (CD3+) in the luminal surface, neointima, media, or adventitiawas qualitatively scored (positive cells present / no positive cells found). In mice, the thickness of neointima was measured from serial sections with MrGrab 1.0 software (Carl Zeiss GmbH., Oberkochen, Germany) and the ratio of X-gal stained (donor or recipient –derived) area in the total neointimal area was quantitated by image analysis (KS300 software, Carl Zeiss). The number of bone marrow derived X-gal (ROSA)+ / aSMA+ cells was counted in 2-3 longitudinal sections per saccular graft under 40x magnification. Immunofluorescence was detected using an Axiovision fluorescence microscope (Carl Zeiss) and confocal microscopy (Nikon Eclipse and UltraVIEW Confocal Imaging System, PerkinElmer Life Sciences, Boston, MA) with Imaging Suite 5.5 (PerkinElmer Life Sciences) and Image J 1.34 (National Institute of Health, USA) softwares.

8.2.1.7 Statistics

Medians and ranges, and proportions were calculated, and Fisher exact test was used (NCSS 2000, NCSS Statistical Software, Kaysville, UT).

8.2.2 Aortic balloon injury model and transplantation of human peripheral mononuclear cells (PBMCs) into immunodeficient rats

To investigate the hypothesis that human peripheral mononuclear cells might contribute to neointima formation via homing into sites of ongoing vascular remodeling and differentiation into neointimal cells, PBMCs isolated from the circulation of healthy humans were transplanted into immunodeficient rats that had underwent balloon injury of the aorta.

8.2.2.1 The aortic balloon injury model

Athymic immunodeficient male RNU-/- rats rats weighing 350-450g were used (Harlan Inc, Horst, The Netherlands). Animals were kept on regular tab water and pellet diet, and received humane care in compliance with institutional guidelines and the Principles of Laboratory Animal Care and

the Guide for the Care and Use of Laboratory Animals, prepared and formulated by the National Institutes of Health (NIH Publication No. 86–23, revised 1985). A permit to perform animal experiments was received from the County of Uusimaa. Medetomidine (0.5mg/kg sc.) and ketamine (60mg/kg ip.) were used for anesthesia and buprenorphine (0.3mg/kg sc.) for analgesia.

The thoracic aorta was denuded with a 2F Fogarty embolectomy catheter by passing the inflated (0.3ml) catheter 4 times through the aorta. The catheter was introduced via the right common iliac artery which was subsequently ligated. The artery was exposed via laparotomy.

8.2.2.2 Isolation and transplantation of circulating peripheral mononuclear cells

Circulating mononuclear cells were isolated with density gradient centrifugation (Ficoll-Paque, Amersham Pharmacia Biotech, Uppsala, Sweden) from 50ml of peripheral blood obtained by venipuncture from 3 healthy male donors (age 25-30) and from 3 healthy female donors (age 22-35). The cells were resuspended in Dulbecco's Eagle cell culture Medium (DMEM) and counted with Burker chamber. 40-60 million human PBMCs were transfused via the penile vein immediately after aortic balloon injury (n=4) or at postoperative days 4 and day 7 (n=8). Three rats died and were harvested during the first postoperative week, others were harvested at postoperative day 14.

8.2.2.3 Isolation and transplantation of mouse bone marrow

Bone marrow cells were isolated from male ROSA-mouse expressing ubiquitously the beta-galactosidase reporter gene (Jackson Laboratories Inc, Bar Harbor, ME) by dissecting the femurs, and flushing the marrow cavity with DMEM. After washing the cells with sterile standard PBS, the cells were resuspended in DMEM and counted. 2-5 million ROSA-mouse bone marrow cells were transfused to the circulation of RNU-/- rats 3 days after balloon injury (n=3).

8.2.2.4 Detection of human PBMCs and mouse BM cells transplanted into Nude-rats

8.2.2.4.1 PCR of human alpha-satelite DNA

DNA was isolated from paraffin embedded tissue sections using High Pure PCR Template Preparation–kit (Roche Diagnostics, Penzberg, Germany). Species spesific alpha-satellite DNA was amplified with 30-35 PCR cycles using GAAGCTTA(A/T)(C/G)T(C/A)ACAGAGTT(G/T)AA and GCTGCAGATC(A/C)C(A/C)AAG(A/T/C)AGTTTC -primers and Fast start Taq polymerase (Roche).

8.2.2.4.2 Histology and immunohistochemistry

Tissue samples from all RNU-/- rats were both snap frozen and cryosectioned at 4um and fixed with 3% paraformaldehyde for 6 hours, paraffin embedded and sectioned at 4um. Immunohistochemistry with human A-blood group specific antibody (clone T36, ID Labs Inc, London, ON, Canada) and against the ubiquitously expressed human major histocompability complex I (MHC I, clone W6/32, DAKO, Denmark) and against human major histocompatibility complex II (MHC II, clone CR3/43, DAKO) were used to localize human PBMCs in RNU-/- rat tissues. The paraffin embedded sections underwent first deparaffinization and antigen retrieval in heated citrate buffer (pH 6), followed by 5% serum block with normal horse serum, and overnight incubation at +4 with anti-A antibody diluted 1:100 in PBS containing 1.5% normal horse serum. Cryosections underwent first serum block followed by overnight incubation with anti-MHC I antibody (1:100 dilution). The bound primary antibody was detected with Vectastain anti-mouse kit (Vector, Burlingame, CA) with AEC or DAB as substrate (Vector and Sigma-Aldrich). Endogenous peroxidase was blocked with 20min incubation in 0.1% hydrogen peroxide. Beta-galactosidase activity (ROSA mouse bone marrow cells) was detected by incubating the cryosections overnight in +37 in X-gal (Sigma-Aldrich) solution with potasisum ferricyanide, potassium ferrocyanide, and magnesium chloride. X-gal incubated sections were background stained with Nuclear Fast red (Vector).

Results and Discussion

Figure 7 Histology of the SCAA wall and growth factor receptor expression

SCAA walls were heterogenous in histological structure. A classification of four dominant wall types was in this study: A) endothelialized wall with linearly organized SMC (microphotograph A), B) thickened wall with disorganized SMC (microphotograph E), C) hypocellular wall with fresh or organizing thrombosis (microphotographs B-C), and D) an extremely thin thrombosis-lined hypocellular wall (D). In addition to the histology, expression of growth factor receptors was studied with immunohistochemistry. Some of the receptors were expressed ubiquitously by the mural cells (like IGF-R1 in microphotograph F or bFGF-R2 in G), whereas some were expressed only in a few neointimal cells (PDGF-Rβ in H) or predominantly in capillaries (TGFβ-R3 in I).

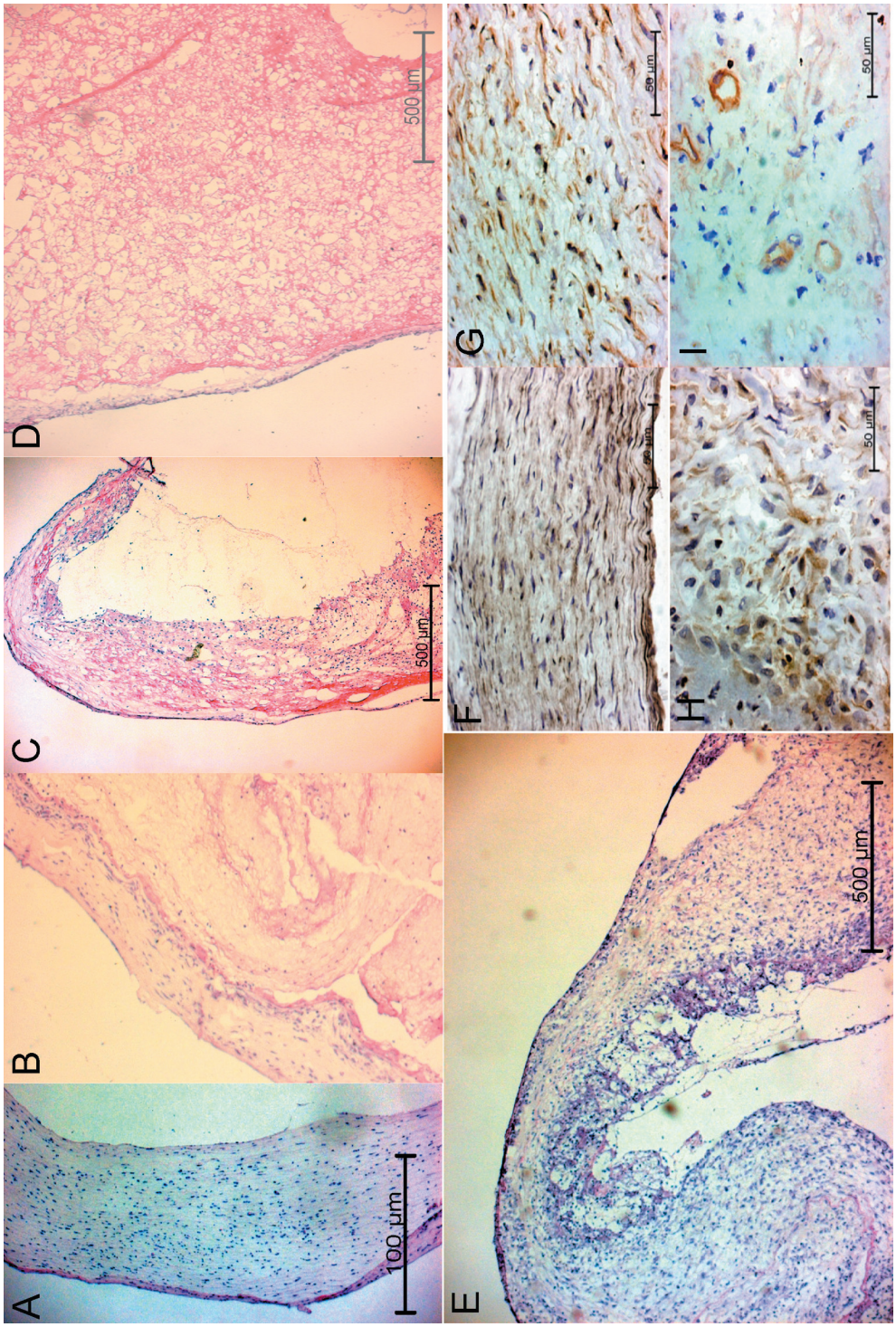

Figure 7 Histology of the SCAA wall and growth factor receptor expression

Figure 8 Oxidized LDL in the SCAA wall and anti-OxLDL antibodies in plasma of SCAA carriers

ApoB100, the core protein of LDL particles, was found in the matrix (A) and in the mural cells (B) of the SCAA wall (scale bars 100 um). Besides the core protein of native LDL (ApoB100), also epitopes characteristic of oxidized LDL were found in the mural smooth muscle cells (HNE red in overlay image C, alfa-smooth muscle actin green in C and in D) and in the SCAA wall matrix (E-G, scale bar 50um). Minimally modified OxLDL (Ox4E6, brown) was found in large areas of the SCAA wall (E), of which some parts stained also with anti-copper oxidized LDL antibody (YE, blue, E). Anti-hydroxynonenyl (HNE, brown) and anti-malondialdehyde modified LDL (blue) immunostainings were also found in the SCAA wall (F). Anti-malondialdehyde immunostaining (blue) colocalized with staining with natural anti-OxLDL antibodies from ApoE-knock out mice (EO6, brown) (G).

In addition to epitopes of native and oxidized LDL in the SCAA wall, IgG antibodies against oxidized LDL were found in the plasma of SCAA patients, and associated with a history of prior SAH, although not statistically significantly. Anti-oxidized LDL antibodies were measured with ELISA using either a peptide of the ApoB100 protein or copper oxidized LDL.

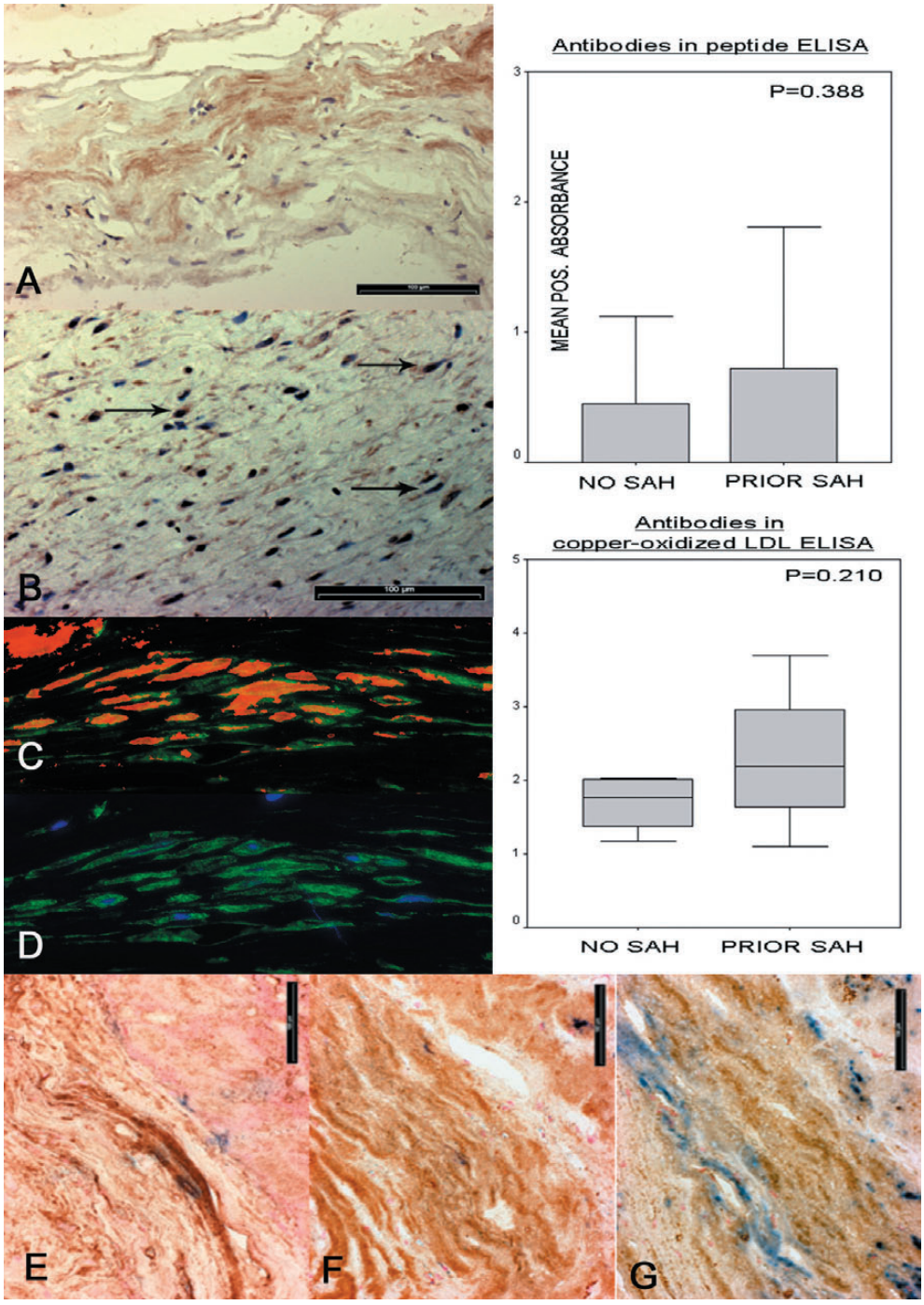

Figure 8 Oxidized LDL in the SCAA wall and anti-OxLDL antibodies in plasma of SCAA carriers

Figure 9 Origin of neointimal cells in mouse saccular aneurysm model

The great majority of neointimal cells in the microsurgical saccular arterial aneurysm model in mice were from the parietal wall of the experimental aneurysm (blue in A and B), but neointimal cells originating outside the graft were also found (B-C, non-blue cells covering the ostium at 4 weeks in B and blue cells that form a neointimal pad in the fundus at 1 week in C). To investigate the potential origin of the neointimal cells that originated outside the experimental aneurysm wall, aneurysms were constructed in bone marrow transplanted mice (chimerism verified from spleen, figure D, transplanted cells blue). Although an influx of bone marrow derived cells was observed, only a few were alfa-smooth muscle actin positive (brown) neointimal cells (E). Confocal microscopy showed bone marrow derived cells (green) immediately adjacent to alfa-smooth muscle actin+ neointimal cells (red) (F), but also true double positive cells (yellow in G-I. G is a digital magnification of a single bone marrow-derived neointimal cell, in fig.I the pseudocolors are inverted and alfa-actin is green). However, bone marrow-derived neointimal cells were extremely rare and had a negligible contribution to thrombus organization and neointima formation in this experimental model.

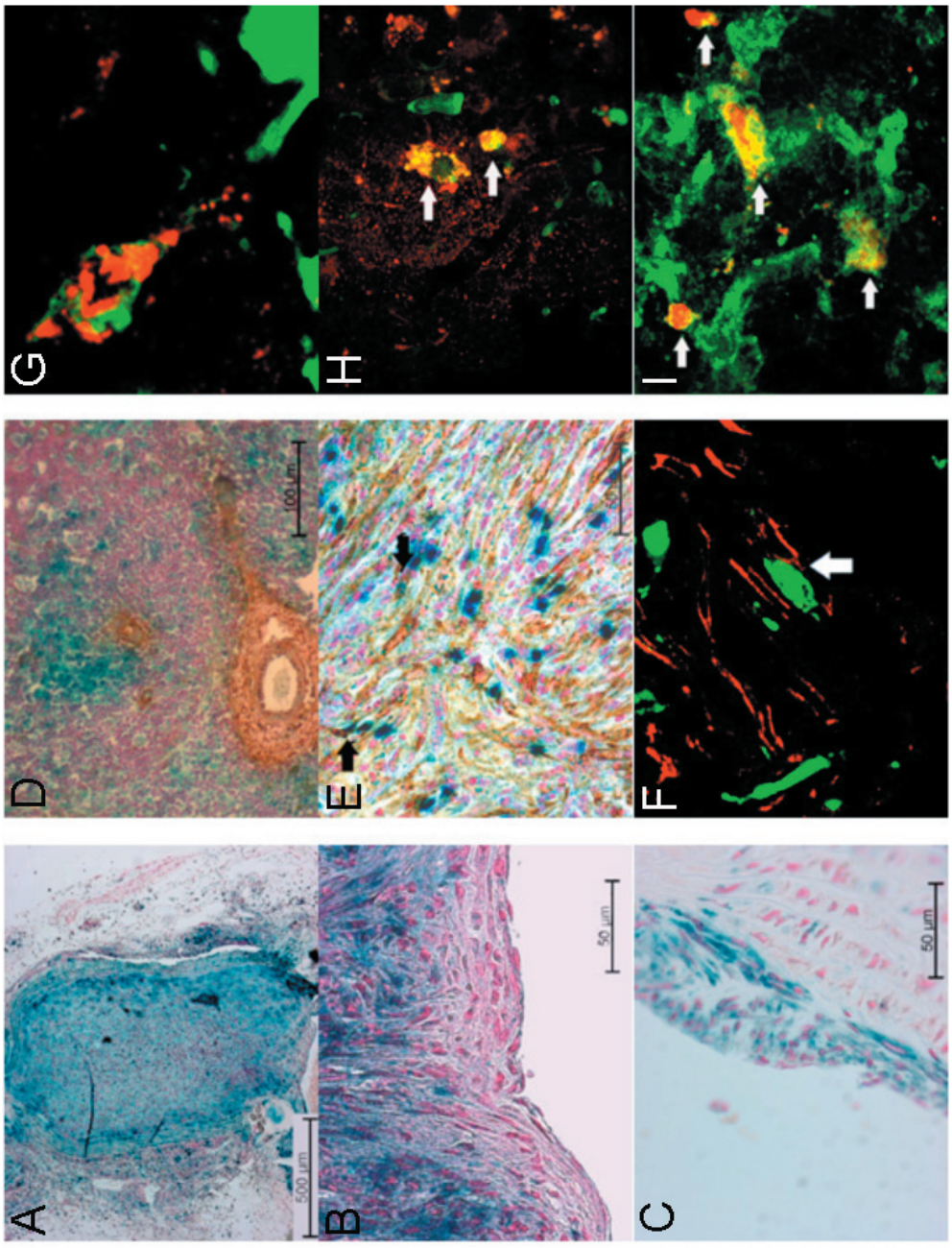

Figure 9 Origin of neointimal cells in mouse saccular aneurysm model

Figure 10 Transfused circulating human peripheral mononuclear cells in immunodeficient rats

Although the survival of transfused human peripheral mononuclear cells in immunodeficient rats was confirmed with PCR of human alfa-satellite DNA from the tissues of immunodeficient rats, and by immunostainings of human specific antigens from rat lymph nodes (A-B), spleen (C), and aortic adventitia (D), the human peripheral blood mononuclear cells did not contribute to neointima formation in the balloon injured rat aorta (E). Some human derived cells were seen in neocapillaries (F) formed to the hind limb that was in transient ischemia due to ligation of the right common iliac artery during aortic balloon injury procedure. Also mouse bone marrow cells (blue) were transfused to balloon injured immunodeficient rats (mouse cells in rat spleen in G) but these did not contribute either to neointima formation (H). Instead, mouse bone marrow cells (turquoise) were found in the healing operation wound among alfa-smooth muscle actin+ myofibroblasts or smooth muscle cells (I).

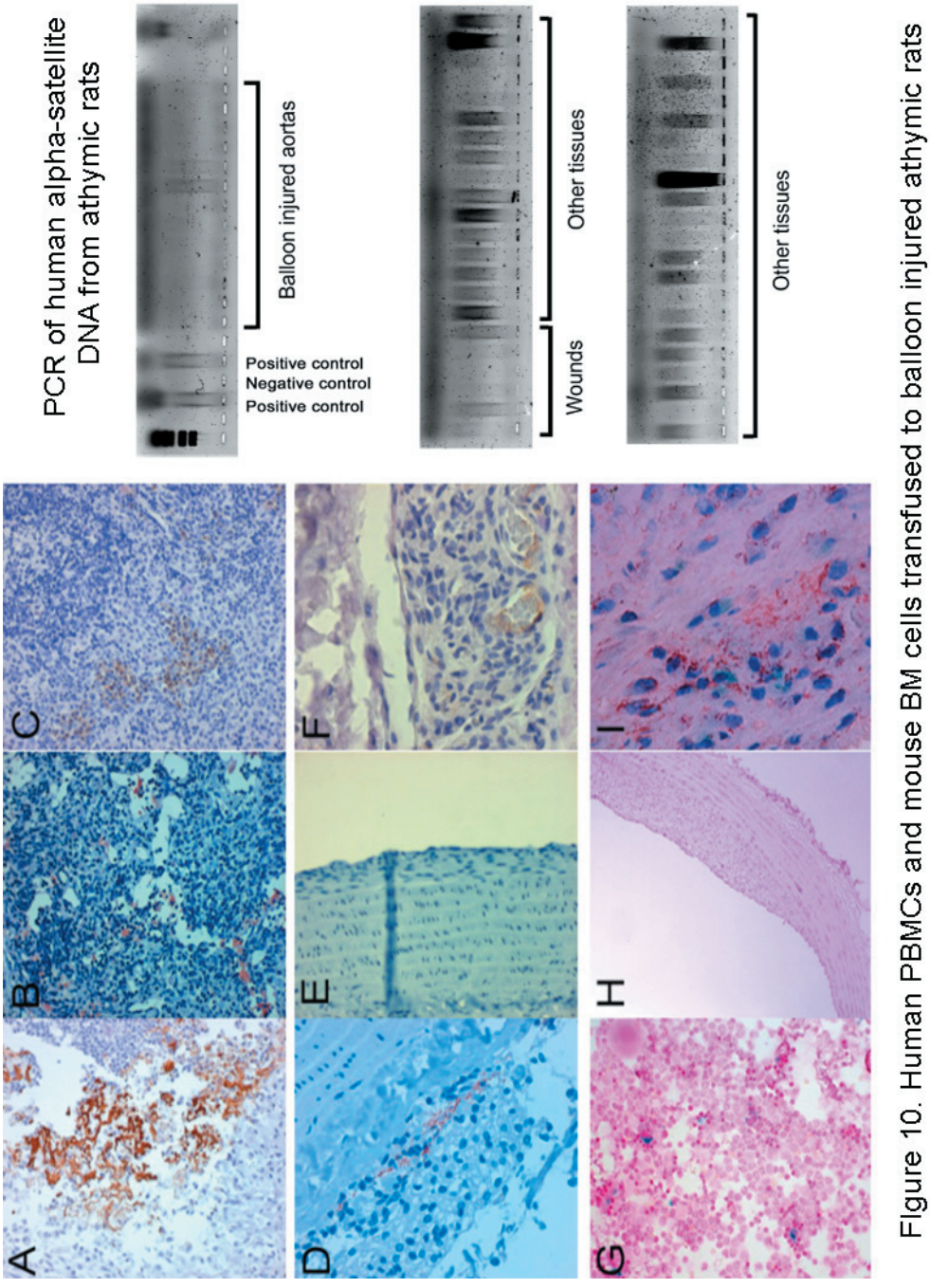

Figure 10. Human PBMCs and mouse BM cells transfused to balloon injured athymic rats

Figure 10 Transfused circulating human peripheral mononuclear cells in immunodeficient rats

9.1 Histopathological comparison of unruptured and ruptured SCAA walls and analysis of histological factors associated with SCAA wall rupture (publication I)

9.1.1 Main findings of the histological comparison of unruptured and ruptured SCAA walls

Histopathological comparison of samples from 24 unruptured and 42 ruptured SCAA walls showed that increased matrix synthesis, proliferation, apoptosis, and infiltration of macrophages and T-cells associate with rupture. In addition, four different types of wall structure were observed (Fig.7): A) endothelialized wall with linearly organized SMC, B) thickened wall with disorganized SMC, C) hypocellular wall with fresh or organizing thrombosis, and D) an extremely thin thrombosis-lined hypocellular wall. The prevailing wall type in the sample associated with rupture (P=0.004). The ratios of ruptured SCAAs were: 42% (7/17) in type A; 55% (11/20) in type B; 64% (9/14) in type C; 100% (15/15) in type D.

9.1.1.1 Repair and adaptation of the SCAA wall

SCAAs are subjected to constant hemodynamic stress and may grow over the years (143), which leads to wall rupture in some but not all cases. It seems therefore likely that the SCAA wall undergoes morphological changes that reflect the adaptation and repair mechanisms, as well as the degeneration that eventually leads to rupture. Endothelialized walls rich in organized SMCs (type A) and walls resembling thick pads of myointimal hyperplasia (type B), were mostly unruptured. Wall thickness and myoinitimal hyperplasia were inversely associated with rupture, as in the series of Kataoka et al. (155). Myointimal hyperplasia resulting from luminal migration and proliferation of mesenchymal cells is the repair and adaptation mechanism of healthy arterial walls (124; 204). An already formed myointimal pad (or neointima) reacts also to mechanical injury or stress by increased proliferation and luminal migration of mesenchymal cells (204). It seems that increased myointimal hyperplasia is an adaptation mechanism of the enlarging SCAA wall. Interestingly, in our series B-type (thick intima-like) walls occurred in younger patients (median 47 years) than A-type (organized) (median 61 years) or C-type (hypocellular with luminal thrombosis) (median 58 years) walls (P=0.021). Possible association between age and SCAA wall maintenance and repair capacity warrants further studies.

9.1.1.2 Thrombosis and fibrosis

Fresh thrombosis or organizing thrombosis (OT) lined the luminal aspect in 25% of unruptured and in 70% of ruptured SCAAs. Thrombosis did not associate with size or shape of the SCAA in this series, although hemody-

namic factors and size and shape related flow conditions in the SCAA fundus are known to predispose to thrombus formation (31; 327). SMC were seen more frequently in luminal OT of ruptured SCAAs. OT often had areas so fibrotic that it was difficult to distinguish them from neighboring MH pads, and they are collectively termed as MH/OT areas in further analysis. Thrombus formation and subsequent organization that leads to formation of myointimal pads is likely a repair mechanisms of tears, dissections, and small ruptures in the SCAA wall.

9.1.2 SCAA wall degeneration and rupture-prone SCAA wall

In a prior series of 27 unruptured and 44 ruptured SCAA fundi, Kataoka et al. found that thick intima-like walls are mostly unruptured and very thin and degenerated walls with hyaline deposits mostly ruptured (155). We identified four histological SCAA wall types that likely reflect consecutive stages (A-D) of wall degeneration proceeding to rupture (A-D, Fig.7). As in the series of Kataoka et al., we also found that aneurysms with thin hyalinized walls (D-type wall in our series) were ruptured. In our series, however, also as many as 55% (11/20) of thick intima-like walls (B-type) had ruptured. This may reflect differences in Finnish and Japanese SCAA populations, or be related to the observation that several aneurysm walls were heterogeneous with gradual change from types A or B to types C or D, mostly in the neck to fundus direction.

Thus some of the SCAAs that were categorized as B-type, thick myointimal wall, might have had undetected small areas of D-type thin wall from which they bled.

Symptoms suggestive of minor leaks before diagnosed SAH were recorded in 12 patients (29%), and minor leaks were associated to the thin, decellularized D-type wall (P=0.011). The wall type was not associated with aneurysm size (P>0.384) or location (P=0.426), or presence of secondary pouches (P=0.795). SCAAs smaller or larger than 7mm or 10mm (the ISUIA thresholds) did not differ in the distribution of different wall types.

9.1.3 Inflammation and the SCAA wall

Ruptured SCAA walls show inflammation (42; 133; 155). It is not known whether inflammation triggers SCAA wall rupture. In our series, ruptured SCAA walls showed increased leukocyte infiltration (CD45, CD3, CD11b, CD68, CD163). Leukocyte density in MH/OT areas and organizing thrombosis were independent risk factors for rupture in logistic regression analysis (R2=0.46, P<0.001 for the model). Of the 42 ruptured SCAAs, 35 had been resected between 3.5h and 48h after rupture. That T-cell and macrophage infiltration were increased in samples resected less than 12 hours from rupture, suggests that these changes were to some extent present prior to rupture since in healthy arterial wall they occur in response to injury during the first 24 hours or later (218).

Infiltrating leukocytes, mainly T-cells and macrophages stimulate SMC proliferation in areas of vascular wall thickening (94). We found that T-cell and macrophage infiltration associate with rupture and furthermore, macrophage infiltration associates with SMC proliferation in the SCAA wall. Density of CD11b+ cells and CD163+ cells in the MH/OT areas and density of CD68+ cells in other parts significantly associated to proliferation in MH/OT areas in multiple linear regression analysis ($R2=0.82$, $P<0.001$ for the model).

It seems possible that in the SCAA wall macrophages stimulate SMC proliferation and thus promote fibrosis in addition to some other possibly deleterious effects. Association of macrophage or T-cells infiltration with apoptosis was not found in our series. However, macrophages and T-cells may induce apoptosis in SMCs via several ways that are not dependent of their numbers (e.g. secretion of the pro-apoptotic cytokine TNFa, (94)) so lack of association in the number of T-cells, macrophages, and apoptotic cells in the SCAA wall cannot be considered as evidence that inflammatory cells do not induce apoptosis or other forms of cell death in the SCAA wall.

9.1.4 Summary of SCAA wall remodeling and rupture risk

The wall of unruptured SCAAs may remain intact for years (128). Thus strong maintenance and repair mechanisms are mandatory. Our results suggest that prior to rupture the SCAA wall becomes unstable and undergoes morphological changes that start at an undefined time interval before rupture. These changes reflect the effect of risk factors that predispose to rupture as well as the maintenance and repair mechanisms trying to prevent rupture. Factors that distinguish unruptured and ruptured SCAAs in our series were: decellularization, apoptosis and degeneration of wall matrix; de-endothelialization; thrombus organization; proliferation; and inflammatory infiltration. Most of these are features related to myointimal hyperplasia that is the mechanism how generally the arterial wall responds to injury or hemodynamic stress (48; 76; 124; 204). In myointimal hyperplasia, proliferation and migration of vascular SMC leads to formation of a thickened fibroid layer on the luminal surface of the vessel (48; 76; 204). During the formation of myointimal hyperplasia, the SMC that migrate from the vascular wall to the luminal surface secrete matrix metalloproteinases that destroy parts of the wall matrix and make the migration of SMCs possible (369). The morphological changes that result from the myointimal hyperplasia and matrix destruction are collectively referred to as remodeling of the vascular wall. Although myointimal hyperplasia is an adaptation mechanism of arteries to hemodynamic stress, in SAH patients, for undefined reasons, vascular wall remodeling was insufficient to prevent SCAA rupture. Paradoxically, in SCAAs remodeling might even facilitate rupture due to increased matrix proteolysis associated with myointimal

hyperplasia (80; 134; 369). In order to better understand the healing mechanisms of ruptured SCAAs, it would be important to study aneurysms at a few weeks after rupture. In our series all but 6 aneurysms were clipped within 48 hour.

Table 5. Patients and Histology of the SCAA wall

Patients and saccular cerebral artery aneurysms (SCAAs) studied in Publication 1. Median and range are given for continuous variables. P-values <0.05 are indicated with ∗ (Chi-Square or Mann-Whitney U-test).

Variables	Bleeding status		P-value
	Unruptured SCAAs (n=24)	*Ruptured SCAAs (n=42)*	
A.Patients			
Age	55 years (38-68)	52 years (13-76)	0.641
Gender	Males 29% (7 / 24)	Males 43% (18 / 42)	0.270
Familial background ∗	21% (5 / 24)	2% (1 / 42)	0.012
Patients with multiple SCAAs (≥2)	46% (11 / 24)	31% (13 / 42)	0.227
Patients with prior aneurysmal SAH	21% (5 / 24)	100% (42 / 42)	
B.Aneurysms resected for study			
Number of known ruptures	0	1 (1-4)	
Time from rupture to resection	-	18 h (3.5 h –183 days) (38 / 42)	
Neck diameter	4 mm (2-10)	3.5 mm (2-10)	0.758
Width of fundus	6 mm (2-34)	7 mm (3-15)	0.308
Lenght of fundus	7.5 mm (3-29)	8 mm (3-18)	0.542
C.Histology of aneurysm wall			
Atherosclerotic calcifications∗	21% (5 / 24)	5% (2 / 40)	0.049
Intact elastic lamina	0% (0 / 24)	0% (0 / 40)	
Remnants of elastic laminae	17% (4/ 24)	15% (6 / 40)	0.859
Endothelial lining absent ∗	30% (7 / 23)	62% (25 / 40)	0.014
Pads of intimal hyperplasia	42% (10 / 24)	48% (19 / 40)	0.650
Organizing thrombosis lining the wall ∗	21% (5 / 24)	60% (24 / 40)	0.002
Infiltrating myosin heavy chain + cells	20% (1 / 5)	67% (16 / 24)	
Fresh thrombosis lining the wall∗	17% (4 / 24)	20% (18 / 40)	0.021

9.2 Accumulation of OxLDL in the SCAA wall and immunization against OxLDL in SCAA carriers (publication II)

9.2.1 Main findings of immunostaining for oxidized LDL in the SCAA wall and serology for oxidized LDL in SCAA carriers and SAH patients

Since unruptured SCAA walls bear histological similarities with early atherosclerotic lesions, we hypothesized that accumulation and oxidation of LDL might occur also in the SCAA wall. As in atherosclerotic lesions and aortic aneurysms, neoepitopes of the oxidized or modified LDL might trigger a chronic inflammatory response that leads to wall degeneration and loss of mural cells, and thus predisposes to rupture.

ApoB100 (the core protein of LDL) and neoepitopes characteristic of oxidized LDL were demonstrated in SCAA walls with immunohistochemistry (see Table 4 for antibodies and Figure 8 for the stainings). Oxidized LDL contains neoepitopes that activate the innate and acquired immune systems (20) (94). Immunization of SCAA patients was demonstrated by showing anti-oxidized LDL IgG-class antibodies in the plasma of the patients with ELISA. Presence of acquired IgG class antibodies against oxidized LDL in the plasma associated with SCAA rupture in carrieris of single SCAAs (P=0.048). These findings support the hypothesis that accumulation and oxidation of LDL in the SCAA wall may be a factor that triggers and maintains chronic inflammation in the SCAA wall.

9.2.2 Accumulation and oxidation of LDL in the saccular aneurysm wall

Immunopositivity for both ApoB (the protein core of LDL particles) and the 15-lipoxygenase enzyme (one of the oxidizers of LDL) was found in all (10 / 10) of the studied SCAA walls. Immunopositivity for various epitopes of OxLDL (Table 4, Fig.8) was found in 96% (26 / 27) of the studied samples. Oxidized epitopes characteristic of OxLDL localized mainly to the SCAA wall matrix and to smooth muscle cells (Fig.8). To ensure that the immunostaining was endeed from oxidized neoepitopes, double stainings for different epitopes characteristic of OxLDL were made (Table 4). In double stainings (n=20 SCAA wall samples), areas showing immunopositivity for copper oxidized LDL (YE antibody) colocalized with immunopositivity for minimally oxidized LDL (Ox4E6 antibody) that stained larger wall areas. Immunostaining for malondialdehyde colocalized with staining for hydroxynonenal (both oxidized epitopes characteristic of OxLDL) and with staining with natural anti-oxLDL antibodies (EO6) in 90% (18 / 20) of the samples (Fig.7). Immunostaining for ApoB or OxLDL epitopes was similar in unruptured and ruptured SCAAs.

9.2.2.1 Humoral immunity triggered by OxLDL in the SCAA wall

In a series of 21 SCAA wall samples Chyatte et al. previously showed the presence of IgM and IgG antibodies in the SCAA wall, along with complement activation (42). They also reported the presence of B-cells in SCAA walls (42), along with T-cells and macrophages that were later investigated in a larger histological series ((42; 155), and publication 1) and found to associate with SCAA wall rupture. Thus the prior histopathological studies suggested activation of the humoral immune system in addition to the activation of the cellular immune response in the SCAA wall. The antigens that trigger the immune response in the SCAA wall remained unknown, however. We were the first to identify an antigen that triggers inflammation in the SCAA wall by showing accumulation and oxidation of LDL in the SCAA wall, and, the immunity of SCAA carriers against OxLDL. It should, however, be remembered that other antigens besides OxLDL may also elicit an inflammatory reaction in the SCAA wall."

9.2.2.2 OxLDL and inflammation in the SCAA wall – similarities with atherosclerosis and aortic aneurysms?

Chronic inflammation is found in atherosclerotic lesions, where it associates with plaque rupture (20; 94). It is currently a widely accepted hypothesis that chronic inflammation in the atheroma plaques is triggered and maintained by accumulation and subsequent oxidation of LDL in the arterial wall (20; 84). The oxidation of LDL components creates neoepitopes that are recognized by the immune system as foreign (20; 84). This leads to activation of the innate and acquired immune systems in the atheroma (20; 84; 94), and increased cell death and proteolytic activity.

We showed with immunohistochemistry that LDL accumulates and becomes oxidized also in the SCAA wall. Since unruptured SCAA walls resemble structurally to early atherosclerotic lesions (77; 169) and ruptured SCAA walls to degenerated AAA walls (publication 1, (155) and (293)), it seems likely that oxidized LDL triggers similar reactions in the SCAA wall as it does in the atherosclerotic lesions.

In atherosclerotic lesions OxLDL triggers infiltration of cytotoxic macrophages and T-cells that also produce cytokines and growth factors that modulate the number and phenotype of mural smooth muscle cells (94). In addition, natural IgM antibodies bind to OxLDL, activating the complement system and opsonizing wall components for phagocytosis by inflammatory cells (20; 21). Macrophages that have phagocytosed OxLDL act as antigen presenting cells and activate further the inflammatory system, with subsequent production of IgG class antibodies and further recruitment of cytotoxic T-cells (20; 21; 94). Binding of IgM or IgG class antibodies on the surface of mural cells that have engulfed OxLDL leads to cell lysis or induction of apoptosis by the complement system, or to targeting of the cell by the cytotoxic inflammatory cell response (20; 21).

The inflammatory response triggered by OxLDL is associated with the ruptured of atherosclerotic plaques (20; 94), and may lead in some cases to loss of mural smooth muscle cells, degeneration of the wall matrix, and aneurysm formation (84). In addition to the effects of OxLDL triggered inflammation, accumulation of OxLDL in mural smooth muscle cells is also toxic in itself and leads to apoptosis of the smooth muscle cells (107).

Although activation of the humoral immune response in general leads to cell death and tissue destruction, the antibodies of the humoral immune system may also have a "protective" house keeping role, by removal or antigenic debris from the circulation and tissues. As suggested in atherosclerosis (43; 290) antibodies generated against modified lipid particles could also have a "protective" role in the SCAA wall by "cleaning up" the OxLDL that has accumulated in the SCAA wall. This interpretation merits further studies.

9.2.3 Seropositivity against OxLDL antigens

Plasma IgG antibodies against OxLDL as measured with the peptide ELISA were found in 43% (40 / 92) of the SCAA carriers, with an increasing trend in patients with a history of SAH (33% vs. 50%, P=0.189, Fig.8). Anti-OxLDL antibodies measured with the peptide ELISA did not associate with age (P=0.747), or gender (P=0.825) but had an increasing trend in smokers (21% in non-smokers vs. 51% in smokers, P=0.066) and patients with hypertension (23% vs. 50%, P=0.178).

Plasma IgG antibodies against OxLDL as measured with copper-oxidized LDL ELISA tended to be elevated in patients with a history of SAH (Fig.8, P=0.213). Antibodies against copper-oxidized LDL did not associate with age (P=0.258), gender (P=0.621), smoking (P=0.820), or hypertension (P=0.966), but were significantly higher in patients with single SCAAs (2.5±0.9 SD) compared to multiple SCAAs (1.8±0.6 SD, P=0.003).

Altogether plasma anti-OxLDL IgG antibodies detected with either ELISA method were more frequent in patients with SAH (62%, 38 /61) than in carriers of unruptured SCAAs (45%, 15 /33; P=0.132). In patients with only one SCAA (n=56), plasma anti-OxLDL antibodies associated significantly with rupture (76% vs 47%, P=0.048). In single SCAA patients, anti-OxLDL antibodies was a risk factor for rupture regardless of age or gender (OR 3.5, 95%CI: 1.9-4.1, P=0.039), but was not independent of smoking and hypertension.

9.2.3.1 Immunity against oxidized LDL - diagnostic implications in SCAA carriers

Becoming immunized against oxidized LDL associates with an increased risk of cardiovascular mortality (20; 282; 322). Clinical series have reported increased mortality in SAH survivors (26; 266), some of which is related to cardiovascular disease (266). This could be explained in part by

the acquired immunity against OxLDL and presence of anti-oxidized LDL antibodies we observed in 62% of SAH patients and in 76% of patients with a single ruptured SCAA.

Screening for anti-oxidized LDL antibodies has been suggested to be a useful diagnostic tool to detect patients at an increased risk of cardiovascular events (282; 322). The value of anti-oxidized LDL antibodies to detect patients at risk of hemorrhagic stroke has been investigated by one study (6). This study found a negative result (6). However, in that study two etiologically different forms of hemorrhagic stroke were grouped together (spontaneous intracerebral hemorrhage and aneurysmal hemorrhage) (6), so the validity of the conclusions is questionable.

In our series circulating anti-OxLDL IgG antibodies were a risk factor for rupture in carriers of single SCAAs (OR 3.5), suggesting that anti-OxLDL serology might have diagnostic potential in the assessment of rupture risk in those patients. The risk of SAH associated with circulating anti-OxLDL IgG antibodies was independent of age or gender, but seemed to be associated with smoking and hypertension. Smoking and hypertension are among the strongest risk factors for SAH (69; 271). Anti-OxLDL antibodies may be a mechanism by which smoking and hypertension affect the SCAA wall and predispose to rupture.

The observation that patients with multiple SCAAs had significantly less plasma anti-OxLDL antibodies suggests that in multianeurysm patients other mechanisms than immunization against OxLDL are more important in the degeneration of the SCAA wall towards a rupture-prone type. These still unknown mechanisms may be related to the factors that initially predisposed the patient to formation of multiple SCAAs.

9.2.3.2 Limitations of the study

Our case-control type comparison of unruptured and ruptured SCAA walls and patients allow us to make associations but not definite conclusions about causality. Although our results strongly suggest that accumulation and oxidation of LDL in the SCAA walls is associated with chronic inflammation that may predispose to rupture, this hypothesis should be tested and confirmed by experimental studies. Moreover, our series does not represent an unselected, continuous series of SAH or SCAA patients, and due to possible bias, the evaluation of the diagnostic potential of anti-oxidized LDL antibody screening in SCAA patients at risk needs further studies. Because SAH is a major systemic stress factor affecting the homeostasis and regulation of multiple functions of the body, it may also be possible that IgG antibody titers in the plasma would rise as a reaction to rupture. Studies that investigate the titers of anti-OxLDL antibodies and other antibodies in the plasma in function of time after the SAH, as well as experimental studies that investigate the effect of OxLDL and anti-OxLDL antibody exposure in experimental aneurysms, will further elucidate the

role of plasma anti-OxLDL antibodies in the diagnostics of SCAA rupture risk.

9.2.4 Summary of oxidized LDL, acquired humoral immunity against it, and the risk of SCAA rupture

Oxidized LDL seems to accumulate into the SCAA wall. The majority of SCAA carriers in which SCAAs rupture, have developed acquired immunity against OxLDL epitopes. Immunization against OxLDL associates with risk of SCAA rupture in carriers of single unruptured SCAA. It seems likely that OxLDL may trigger and maintain chronic inflammation in the SCAA wall. This chronic inflammation may predispose to wall degeneration and eventual rupture, depending on the microenvironment of the mural smooth muscle cells.

Table 6. Plasma anti-OxLDL IgG antibodies in carriers of unruptured and ruptured SCAAs.

Mean and range are given for continuous variables. P-values <0.05 are indicated with∗(Chi-Square or Mann-Whitney U-test).

Variables	History of SAH		P-value
	No SAH (n=33)	*Prior SAH* (n=61)	
A.Patients			
Age (years)	52 (28-79)	55 (22-77)	0.539
Gender (males)	70% (23/33)	67% (41/61)	0.805
Patients with multiple SCAAs (≥2)	31% (10/32)	47% (28/59)	0.134
Patients with more than one aneurysmal SAH	0 (-)	10% (6/59)	-
Minor leak symptoms	27% (9/33)	21% (13/61)	0.644
Familial background	71% (10/14)	52% (11/21)	0.260
Smoking	68% (17/25)	78% (21/27)	0.427
Hypertension	67% (16/24)	76% (16/21)	0.482
B.Aneurysms			
Number of known ruptures	0 (-)	1 (1-3)	-
Time from rupture to resection of SCAA wall sample	0 (-)	34 hours (3hrs – 317 d)	-
Neck diameter (mm)	4 (2-10)	4 (2-10)	0.349
Width of fundus (mm)	6 (3-34)	7 (2-16)	0.020∗
Lenght of fundus (mm)	6 (3-29)	8.5 (2-19)	0.007∗
C.Serology *(Plasma anti-OxLDL IgG antibodies)*			
All SCAA carriers	45% (15/33)	62% (38/61)	0.132
Patients with multiple SCAAs	40% (4/10)	46% (13/28)	0.726
Patients with single SCAAs	48% (11/23)	76% (25/33)	0.048∗

9.3 Growth factor receptors, remodeling, and rupture of the SCAA wall (publication III)

9.3.1 Main findings of the growth factor receptor immunostainings

Of the 12 studied receptors, 11 were expressed in the SCAA wall (Table 5). TGFβ-R1 was absent in the walls of SCAAs and AVMs (n=5). One receptor (IGF-R1) was expressed ubiquitously in all SCAA walls. PDGF-Rβ and VEGF-R1 were expressed almost exclusively in ruptured SCAA walls, although their expression in ruptured SCAA walls was far from universal (15% and 33%). VEGF-R1 associated also with change of wall type from A to D (P=0.027), with endoluminal organizing thrombosis (P=0.019), increased infiltration of T-cells (CD3+) and activated macrophages (CD163+, CD11b+). Other receptors were found in both unruptured and ruptured SCAAs, although they were more frequent in ruptured SCAA walls. In the studied series of 56 SCAAs, unruptured and ruptured SCAAs did not differ by age, gender, or size (Table 5). SCAAs larger or smaller than 7mm - ISUIA cutpoint for occlusive therapy in unruptured aneurysms (351) - did not differ in growth factor receptor expression. Warning leaks were associated with increased bFGF-R1 expression (P=0.018).

9.3.2 Rationale for the study of growth factor receptors in the SCAA wall – Therapeutic implications

Drugs or growth factors that stimulate myointimal hyperplasia and inhibit degenerative remodeling might re-enforce the SCAA wall and prevent rupture. These drugs or growth factors could be delivered either systemically or locally via coated, bioactive endovascular implants. Endovascular implants that deliver growth factors e.g. bFGF, TGF-β, or VEGF, as well as gene therapy constructs, have been tested in animal models (2; 60; 100; 189; 257; 258). However, although the rational design and clinical use of these bioactive endovascular implants requires knowledge of growth factor receptor expression in human SCAA wall, it has not been previously characterized.

9.3.2.1 bFGF receptors in the SCAA wall - therapeutic implications

Upregulation of bFGF receptors in SCAA wall seems to occur in response to risk factors for rupture (minor leaks, wall remodeling). Arterial injury increases bFGF signaling and leads to myointimal hyperplasia (179). bFGF stimulates myointimal hyperplasia in rat intracranial saccular aneurysms, leading to occlusion of the aneurysm lumen (79). Systemic bFGF administration might have deleterious side effects in humans, such as arterial occlusions, but locally delivered bFGF could strenghten the SCAA wall and occlude its lumen. bFGF releasing endovascular implants have been tested

in extracranial saccular aneurysm models (147; 189), but not in humans, so far.

9.3.2.2 TGFβ-receptors in the SCAA wall – therapeutic implications

TGFβ-R1 (isoforms ALK-2 and 5) and -R2 are upregulated in injured arterial wall and form a heterodimer that increases matrix synthesis when activated by TGFβ1 (151; 345). Blocking TGFβ1 signaling with soluble TGFβ-R2 leads to enlargement of the injured vessel via decreased matrix synthesis (275). Unlike in injured extracranial arteries, TGFβ-R1 (ALK-5) was not found in the SCAA walls. Instead, upregulation of TGFβ-receptors 2 and 3 seems to occur in response to risk factors for rupture. Lack of TGFβ-R1 blocks TGFβ1 mediated matrix synthesis and might be a factor causing SCAA enlargement. TGFβ releasing endovascular implants have been tested in extracranial saccular aneurysm models (60) in which TGFβ-R1 is likely expressed. The effect of TGFβ releasing endovascular implants probably differs in human SCAAs.

9.3.2.3 PDGF-receptors in the SCAA wall

Of the published genome-wide linkage analysis studies of SCAAs, one found an association with the 5q 31-35 locus (367) containing PDGF-Rβ. PDGF-Rβ activation leads to SMC proliferation and myointimal thickening (16). PDGF-Rβ expression was weak in the SCAA walls although it correlated with wall remodeling. PDGF-Rβ is induced by TGFβ signaling (132), and TGFβ-receptor deficiency might downregulate PDGF receptors and the mitogenic effect of PDGFs on SCAA wall.

9.3.2.4 VEGF-receptors in the SCAA wall - therapeutic implications

VEGF-receptors were involved in SCAA wall remodeling. VEGF-receptors 1 and 2 mediate SMC migration (87) and VEGF-R1 activation increases matrix metalloproteinase (MMP) activity in SMCs (333), a requirement for SMC migration (76; 369). VEGF-R1 likely increases MMP activity and remodeling also in the SCAA wall, and VEGF-R1 inhibition might decrease extracellular matrix destruction leading to SCAA rupture. However, because of complex and incompletely known interactions between VEGF signalling and other systems, the net effect of VEGF-receptor inhibition or stimulation is difficult to predict: VEGF-R1 potentiates the mitogenic effect of bFGF after vascular injury (53), and VEGF signaling is coupled with angiotensin signalling that regulates vascular remodeling (371). Although successful in experimental extracranial aneurysms (2), VEGF releasing coils have not been tested in humans.

9.3.3 Limitations of immunohistochemistry

Immunohistochemistry is a simple method to localize protein expression to different cell populations and structures in histological sections. Like

all scientific methods, immunohistochemistry needs to be very rigorously controlled and has multiple potential sources of false positive or negative result. False positive signal in immunohistochemical staining may be obtained due to unspesific binding of primary antibodies i) to matrix because of electrostatic forces or ii) to other than the studied protein because of unspesificity of the primary antibody, or iii) because of endogenous enzyme activity in the tissue sections (e.g. endogenous peroxidase in red blood cells vs. horse radish peroxidase used in detection of immunohistochemistry signal). False negative results may be obtained in immunohistochemistry due to i) insensitive primary antibody, ii) excessive dilution of the primary antibody, or iii) insensitive detection system. In order to get proper results, immunohistochemistry protocols and detection methods should be trimmed and optimized for each type of tissue sample and primary antibody individually.

Immunohistochemistry is also very difficult to standardize so that the signal intensity would be truly quantitative – due to the multiple amplification steps and enzyme mediated detection reactions, signal intensity is often more dependent of incubation times than of the amount of studied protein. Instead of assessing the intensity of the signal, most commonly used approaches to quantitate immunohistochemistry rely on i) quantitation of positive cells (counting the absolute number or ratio of positive cells) or ii) quantitation of positive surface area (e.g. ratio of positive area from total surface area).

Because of the categorical nature of immunohistochemistry data (yes or no for positivity) and its inherent methodological caveats, in optimal setting data from immunohistochemistry is confirmed with i) blotting studies (dot, slot, or western) in which protein extracts from tissues are blotted on nitrocellulose membranes and immunostained (quantitative method independent from the artifact sources of immunohistochemistry, but with other potential artifact sources), or with ii) RNA studies which should confirm the expression of the gene encoding the protein that has been immunostained. However, blotting or RNA studies parallel to immunohistochemistry are not always possible. Blotting experiments require disintegration and dissolving the tissue sample which requires inevitably steps leading to denaturation of secondary or tertiary structures of proteins and possibly changes in their immunogenicity (and the ability of the primary antibody to detect the studied protein). RNA studies require were carefully conserved tissue samples in order to preserve the often minute amounts of mRNA that needs to be detected, and often, especially in surgical samples, the quality or amount of isolated RNA is not sufficient for adequate analysis where as proteins maybe very well conserved.

Moreover, one should remember that expression of a gene may have large quantitative differences in the RNA and protein level due to posttranscriptional regulation of gene expression.

Despite the limitations of immunohistochemistry, it remains the most frequently used and one of the best characterized methods to demonstrate expression of specific proteins (e.g. cell surface receptors) in tissue samples. Immunohistochemistry is also the method of choice of modern histopathology to demonstrate specific cell populations, growth factors, or other proteins in diseased tissues, and as such can be considered reliable in itself even without other confirmatory methods when the staining is adequately performed and the antibodies are well characterized.

Our results from immunohistochemistry of SCAA wall samples with a vast panel of antibodies against histological markers, antigenic oxidized neoepitopes, and various vascular growth factor receptors identified a number of changes that discriminate unruptured and ruptured SCAA walls. This study did not include blotting experiments or RNA analysis, which would be complimentary to the now presented data, but paves the way for further analysis at the protein and RNA level by identifying a number of previously undescribed biological differences in unruptured and ruptured SCAA walls that merit the effort of further experiments.

Table 7. Patients and Expression of growth factor receptors in unruptured and ruptured SCAA walls

Patients, aneurysms, and expression of growth factor receptors studied in Publication 3. Age, neck diameter, and fundus width and lenght are given as medians and range. Other variables are expressed as proportions. P-values were determined with Mann-Whitney U-test or Chi-Square test as appropriate. The proportion of SCAAs with strong or moderate receptor expression is given. P-values were calculated using Chi-Square test. P-values <0.05 are marked with ∗.

Variables	Bleeding status		P-values
	Unruptured (n=21)	*Ruptured (n=35)*	
Patients			
Age	56 years (38-68)	52 years (13-76)	0.812
Gender	Males 29% (6 / 21)	Males 46% (16 / 35)	0.203
Minor leaks	0% (0 / 21)	26% (9 / 35)	0.011
Aneurysms			
Neck	4 mm (2-10)	4 mm (2-10)	0.934
Width	6 mm (4-34)	7 mm (4-15)	0.207
Lenght	8 mm (3-29)	8 mm (4-18)	0.535
Secondary pouches	35% (7 / 20)	71% (25 / 35)	0.008∗
Histology (+/-)			
Endothelium	70% (14 / 20)	41% (14 / 34)	0.041∗
Fresh thrombosis	19% (4 / 21)	44% (15 / 34)	0.057
Organizing thrombosis	19% (4 / 21)	65% (22 / 34)	<0.001∗
Myointimal hyperplasia	43% (9 / 21)	47% (16 / 34)	0.761
Receptors			
IGF-R1α	100% (21 / 21)	100% (31 / 31)	-
bFGF-R1	75% (15 / 20)	71% (22 / 31)	0.757
bFGF-R2	47% (9 / 19)	51% (18 / 35)	0.776
bFGF-R3	90% (18 / 20)	97% (30 / 31)	0.315
bFGF-R4	76% (16 / 21)	94% (30 / 32)	0.065
TGFβ-R1 (ALK-5)	0% (0 / 21)	0% (0 / 32)	-
TGFβ-R2	50% (10 / 20)	77% (24 / 31)	0.043∗
TGFβ-R3	43% (9 / 21)	45% (14 / 31)	0.870
PDGF-Rα	75% (14 / 20)	87% (27 / 31)	0.133
PDGF-Rβ	5% (1 / 21)	16% (5 / 32)	0.222
VEGF-R1	5% (1 / 19)	33% (11 / 34)	0.024∗
VEGF-R2	74% (14 / 19)	83% (29 / 35)	0.424

9.4 Contribution of bone marrow-derived cells to thrombus organization and neointima formation in experimental aneurysms (publication IV)

9.4.1 Main findings of the experimental murine microsurgical saccular aneurysm models

Experimental saccular aneurysms constructed to the abdominal aortas of rats or mice by end-to-side aortic transplantation and distal ligation of the aortic graft (Fig.6) remain patent and develop similar histological changes that characterize the wall of SCAAs: i) de-endothelialization, ii) luminal thrombosis, iii) organization of the luminal thrombosis and formation of neointima (myointimal hyperplasia), and iv) inflammation. Decellularization and degeneration that characterizes ruptured human SCAA walls was not seen in the experimental aneurysms, even after induction of severe atherosclerotic changes with ApoE gene knock out.

Of the neointimal area, a median of 58% (42-81%) was derived from the experimental aneurysm wall, and a median of 32% (5-81%) from elsewhere depending on the site of measurement (different tissue sections). Bone marrow-derived alfa smooth muscle actin positive myointimal cells were found, but were exceedingly few and did not contribute significantly to the fibrosis in the experimental aneurysm wall.

9.4.2 Significance of the findings

Endovascular occlusive therapy may remain incomplete and leave neck remnants that may grow and rupture (102), or a complete primary occlusion may fail in the long run (249). A major cause of recurrence after embolization seems to be recanalization of the thrombus by endothelial cells (247; 253). Organization of the thrombus into fibrous tissue (neointima) by infiltration of matrix synthesizing smooth muscle cells (SMCs),reduces aneurysm recurrence in experimental models (258). To goal of endovascular devices is to induce thrombus formation in the acute phase, followed by organization of the thrombus in to fibrosis (neointima formation) to ensure long term patency of the initial occlusion (258)..

9.4.2.1 Atherosclerosis does not lead to decellularization and degeneration of the experimental aneurysms

We did not observed significant aneurysmatic growth or dilatation of the experimental aneurysms, except in a single case. In an attempt to induce aneurysmatic dilatation in the experimental model, we constructed experimental aneurysms from atherosclerotic arteries into atherosclerotic recipient mice. Atherosclerosis did not, however, lead to aneurysmatic dilatation. This may be because the animals were not immunized against oxidized LDL particles as many human SCAA carriers seem to be. Our results

also suggest that mere accumulation of LDL or atherosclerotic changes in the aneurysm wall are not sufficient to trigger decellularization, degeneration, and distension of the aneurysm wall.

9.4.2.2 Myointimal cells (luminal thrombus organization and fibrosis) are mainly derived from the wall of the experimental aneurysm – comparison with decellularized, ruptured human SCAAs?

Arterial or venous pouches that are created either by microsurgery or by ligation of arterial branches, are commonly used as experimental models to develop and test novel bioactive endovascular devices (2; 60; 149; 189; 252) (202; 258). We applied the commonly used model to rats and mice. In our mouse model, the major source of thrombus organizing neointimal cells was the adjacent mural smooth muscle cell population.

Our results in mice suggest that in microsurgically constructed experimental aneurysm models, the majority of thrombus organizing cells originate from the parietal wall of the experimental aneurysm. However, human SCAAs, the ruptured ones in particular, often have thin decellularized walls (155), and the capacity of mural cells to infiltrate and organize induced thrombus in such SCAAs may be less than in the experimental models currently used. The growth factors, drugs, or other bioactive coatings that would recruit circulating intimal precursor cells and stimulate them to contribute to thrombus organization, may be very different from those that yield optimal results in an experimental model in which the thrombus organizing and neointimal cells are mostly derived from the healthy, well cellularized experimental aneurysm wall. Furthermore, the lack of mural cells that could be recruited to organization of luminal thrombus in the ruptured human SCAA wall, may well be the cause of recanalizations and recurrences observed in some of the coiled SCAAs.

9.4.2.3 Bone marrow-derived myointimal cells as a solution to enhance fibrosis in the saccular aneurysm wall?

Both endothelial cells and neointimal SMCs (neointimal cells) can originate from bone marrow-derived circulating precursor cells (12; 61; 216; 284) (108; 314), and their role in the recanalization or organization of thrombosis in human SCAAs needs to be investigated. Such data might reveal novel target sites for bioactive endovascular implants, cell based therapy, or systemic drug therapy that in endovascularly treated SCAAs would either inhibit the recanalization of thrombus or mobilize / stimulate neointimal precursor cells to facilite organization of the thrombus. We investigated the role of bone marrow-derived neointimal cells in thrombus organization and neointima formation in arterial pouches constructed microsurgically in mice. In our model only a negligible contribution of bone marrow-derived myointimal cells was found. However, our results do suggest that the concept of bone marrow-derived myointimal cells is valid,

and thus might have potential in occlusion of aneurysms e.g. as local cell transplants or if suitable growth factors or drugs mobilizing these cells to the circulation are found.

9.5 Use of transfused circulating human mononuclear cells to enhance neointimal thickening –implications for the repair of the SCAA wall (unpublished data)

9.5.1 Rationale of the experiments

Growth of intimal hyperplasia (neointima in rats and mice) that would prevent SCAA recurrence, is the goal of endovascular occlusive therapy in humans. Enhancement of neointima formation is the goal of novel bioactive embolization devices that are being developed. Bone marrow –derived progenitor cells have been shown to contribute to vascular remodeling and intimal hyperplasia in various experimental vascular injury models (284; 294; 314), and in humans (38). Intimal cells and endothelial cells have also been cultured in vitro from circulating human peripheral blood mononuclear cells (PBMCs) (216; 255; 297). Furthermore, endothelial cells grown in vitro from human PBMCs and transplanted locally into injured immunodeficient rat arteries, integrated to the injured vascular wall of immunodeficient rats (78).

Neontimal or endothelial progenitor cells that could be isolated from the circulating PBMC population of healthy humans, expanded and genetically engineered in vitro, followed with autologous retransfusion, might provide novel means to target distal surgically or endovascularly inaccessible occlusive or aneurysmatic vascular disease. We investigated whether such cells are found in the circulation of healthy humans.

9.5.2 Main findings

9.5.2.1 Transfused human PBMCs home into lymphatic tissues in immunodeficient rats, not to neointima

Human DNA was found in immunodeficient rat wound (2 / 5), aorta (3 / 13), and other tissues (34 / 38, spleen, liver, kidney, gut, lungs, heart, muscle) (Fig.10). Human cells were detected with immunohistochemistry in 10/12 of the transplanted rats. The human cells localized to wound, aortic adventitia, para-aortic lymph nodes, spleen, and were occasionally detected in sections of other organs (Fig.10). Capillaries with human cells were found in the adventitia of ligated distal iliac artery of 3 / 12 rats (Fig.10). Asymmetric myointimal hyperplasia (neointima) was found in cross sections and longitudinal sections of all the balloon injured rat aortas (Fig.10), but human cells were not found in the neointima .

9.5.2.2 Mouse bone marrow cells infused to balloon injured rats do not integrate to the neointima

Transfused reporter gene (ROSA mouse) labelled mouse bone marrow cells were found in the rat bone marrow, spleen, and wounds (Fig.10). No mouse-derived cells were found in the neointima (Fig.10).

9.5.3 Significance of the findings – need for in vitro manipulation or local transplantation?

Vascular progenitor cells isolated from the circulating PBMC pool that after genetical engineering in vitro and autologous transfusion, would home to sites of vascular remodeling, could provide a novel basis for gene therapy of distant, surgically or endovascularly inaccessible vascular disease. We tested the hypothesis that transfused PBMCs isolated from the circulation of healthy humans would home to sites of vascular remodeling, but were unable to confirm this hypothesis. Transfused PBMCs from healthy humans homed into lymphatic tissues and sites of inflammation, but not to the injured arterial wall. Neither transfused bone marrow cells integrated to the injured vascular wall.

The concept of circulating vascular endothelial and neointimal progenitor cells has been confirmed in several laboratories, and is widely accepted (216; 255; 284; 294; 297; 314). Endothelial cells cultured from human PBMCs and transplanted locally into immunodeficient rats were reported to interact with the rat tissues and integrated to the rat artery (78). We transfused uncultured human PBMCs and mouse bone marrow cells into similar immunodeficient rats, but were unable to detect these cells in the neointima of injured arteries. The amount of transfused cells (40-60 million per rat) seems sufficient (rat blood volume 20ml, leukocytes 1-10 x million/ml). That transfused human PBMCs integrated to the neointima but turned apoptotic before the 14 days harvesting point is unlikely, since no human cells were detected in the neointima of those rat that died prematurely (n=3), and the cells were detected in the vascular adventitia. That transfused PBMCs isolated from the circulation of healthy humans did not home to injured arteries, could suggest that i) vascular progenitor cells in the circulation of healthy humans are extremely rare, ii) (that) circulating putative endothelial or myointimal progenitor cells have to be primed by a combination of cytokines before they contribute to vascular remodeling, or iii) (that) the expression of adhesion molecules changes during isolation and re-transfusion of PBMCs. Altered expression of adhesion / surface molecules may cause PBMCs to home to other tissues. Our results suggest that activation and mobilization of vascular progenitor cells to the circulation, or cytokine priming of the isolated circulating PBMCs, seems necessary before these cells are able to integrate to sites of vascular remodeling after transfusion.

Conclusions

1) SCAA rupture is associated with decellularization and thinning (degenerative remodeling) of SCAA wall. Rupture is also associated with increased proliferation, luminal thrombosis, organization of luminal thrombosis, and T-cell and macrophage infiltration of the wall. Some of these histopathological changes may reflect ongoing repair and adaptation attempts. The structure of unruptured SCAAs resembles myointimal hyperplasia, and it seems likely that the pathobiology of the SCAA wall is similar to that of myointimal hyperplasia elsewhere. As in aortic aneurysms, loss of mural smooth muscle cells and myointimal hyperplasia, along with chronic inflammation, characterize the rupture-prone SCAA wall.

2) Oxidized LDL accumulates into the SCAA wall, and the majority of SAH patients have developed acquired immunity against OxLDL epitopes. It seems likely that OxLDL in the SCAA wall may trigger and maintain chronic inflammation that predisposes to wall degeneration and eventual rupture, suggesting that SCAA wall degeneration and rupture may be related with atherosclerosis. In patients with single SCAAs, immunity against OxLDL (IgG antibodies) is associated with an increased risk of rupture, where as in patients with multiple SCAAs other factors seem more important in the degeneration and rupture of the SCAA wall.

3) SCAA wall remodeling is associated with changes in growth factor receptor expression. IGF-R1 seems to be ubiquitously expressed in SCAA walls, where as receptors for bFGF, TGFβ, and VEGF seem to be upregulated during SCAA wall remodeling towards rupture-prone type –possibly regulating the processes that lead to remodeling of the SCAA wall. Our immunohistochemistry data suggest that the role of these receptors in the regulation of mural cell migration, proliferation, apoptosis, and matrix and MMP synthesis in the SCAA wall merits investigation with further experiments

4) Neointima formation in end-to-side arterial saccular graft models seems to occur almost exclusively from migration and proliferation of mural cells of the graft and of the adjacent parent artery wall. Contribution of bone marrow-derived neointimal cells was negligible, although a few were found. The contribution of mural cells may be different in human SCAA, since the wall of human SCAAs (especially ruptured ones) is often degenerated and decellularized. Origin of neointimal cells should be investigated

in human SCAAs and in other experimental saccular aneurysm models that are used to test endovascular therapies.

5) Use of circulating human peripheral blood mononuclear cells to reinforce neointima formation was also investigated. Transfused unstimulated human peripheral blood mononuclear cells do not integrate to injured vascular wall. Further experiments with local cell transplantation, modulation of cell phenotype in vitro, or mobilization of vascular progenitors to the circulation may reveal mechanisms how bone marrow-derived neointimal progenitor cells could be used to enhance neointima formation and wall thickening in injured vascular wall or in the human SCAA wall.

Summary

The often fatal (in 50-35%) SAH caused by SCAA rupture affects mainly the working aged population. The incidence of SAH is 10-11 / 100 000 in Western countries and twice as high in Finland and Japan. The estimated prevalence of SCAAs is 2-5%. Many of those never rupture. However, currently there are no diagnostic methods to identify rupture-prone SCAAs from quiescent, dormant ones. Since SCAA rupture has such a sinister outcome, and all treatment modalities are associated with morbidity and mortality, finding diagnostic markers for rupture-prone SCAAs is of primary importance. Also the therapies that prevent SCAA rupture need to be developed to as minimally invasive as possible. A pharmaceutical therapy that would prevent degenerative remodeling of the SCAA wall towards a rupture-prone type, or at least slow the progression considerably, would be of considerable value to those patients that are at a high risk of morbidity or mortality from current therapies, or that carry multiple SCAAs. Moreover, the current methods of endovascular therapy should be improved to achieve better long term durability.

Although the clinical risk factors for SCAA rupture have been extensively studied and documented in large patient series, the cellular and molecular mechanisms how these risk factors lead to SCAA wall rupture remain incompletely known. Elucidation of the molecular and cellular pathobiology of the SCAA wall is needed in order to develop i) novel diagnostic tools that could identify SCAAs or patients at risk of SAH, and to ii) develop novel biological therapies that prevent SCAA wall rupture.

Attempts to identify rupture-prone SCAAs have classically focused mainly on SCAA morphology related parameters (e.g. size, shape, location) based on the assumption that these factors affect flow conditions and the degree of hemodynamic stress that eventually leads to SCAA rupture. Altered hemodynamic conditions in the cerebral vasculature are sufficient to induce SCAAs in experimental aneurysms. Moreover, mathematical modeling of flow conditions in human cerebral artery bifurcations with SCAAs, does show increase stress at the site of SCAA formation. However, SCAA formed in experimental animals by altered hemodynamic conditions do not spontaneously rupture and human SCAAs that rupture do not show uniform size, shape, location, or other flow related parameters. It is therefore likely that also other factors, namely the condition of the SCAA wall, affect the risk of rupture in addition to the hemodynamic load.

In this study, histological samples from unruptured and ruptured SCAAs were compared in order to identify structural changes, cell popu-

lations, growth factor receptors, or other molecular markers that would associate with SCAA wall degeneration and rupture. We found that unruptured SCAA walls resemble in histopathology to pads of myointimal hyperplasia, which are found in arteries that are under high mechanical or immunological stress. Unruptured SCAA walls also resembled in histology to so called "fibrofatty streaks" that represent the early manifestations of atherosclerosis. As in myointimal hyperplasia and in atherosclerotic lesions, inflammation and activation of the acquired immune system were found in the SCAA wall. Increased inflammation associated with SCAA wall rupture, as did the gradual degeneration of the SCAA wall that was characterized by loss of cells and myointimal hyperplasia. Ruptured SCAA walls had histological features similar to the wall of abdominal aortic aneurysms, namely loss of mural cells, degeneration of matrix, and transmural inflammatory infiltration.

In atherosclerotic vascular lesions accumulation and oxidation of LDL particles is the main factor that triggers and maintains chronic inflammation. We investigated the hypothesis that accumulation and oxidation of LDL would also occur in the SCAA wall that has some histological similarities with early atherosclerotic lesions. Oxidation of LDL could trigger and maintain inflammation also in the SCAA wall. We found that the SCAA walls generally contain oxidized LDL, and that having plasma IgG antibodies against oxidized LDL increases the risk of SCAA rupture in carriers of single SCAAs. These findings suggest that accumulation and oxidation of LDL is ongoing in the SCAA wall before it ruptures, and that immunization against oxidized LDL epitopes may increase the risk of SAH in carriers of single SCAAs. Accumulation and oxidation of LDL may trigger activation of humoral and cellular immune reaction in the SCAA wall. We cannot, however, exclude the possibility that also other epitopes that accumulate in the SCAA wall may trigger the chronic inflammation that we and other have observed to associate with SCAA wall rupture. Our results suggest that anti-OxLDL serology could be used to asses SAH risk in carriers of single, unruptured SCAAs. Our findings merit and require further investigation and validation in larger, unselected patient series.

Remodeling of the SCAA wall towards a rupture-prone type was associated with changes in growth factor receptor expression, but we found no receptor that would categorically distinguish unruptured and ruptured SCAAs. From the point of view of pharmaceutical or other biological therapy targeting the mural cells in the SCAA wall, the observations that IGF-receptor 1 was ubiquitously expressed in the SCAA wall and that VEGF-receptor 1 was associated with remodeling and rupture of the SCAA wall, were especially interesting. However, these observations need confirmation in larger series and with also other methods of molecular biology. Moreover, before bioactive therapy can be engineered, the role of these receptors in the signaling cascades of the SCAA wall cells needs to be investigated in depth.

In addition, we investigated the mechanisms of vascular wall healing in experimental models of saccular aneurysms and of mechanical injury leading to neointima (=myointimal hyperplasia) formation. Myointimal hyperplasia, luminal thrombosis, migration of mural cells to the luminal thrombosis, and subsequent organization of the luminal thrombosis into myointimal hyperplasia, seem to be the adaptation and repair mechanism of experimental side-wall saccular aneurysms. Myointimal hyperplasia and luminal, organized thrombosis are mostly derived from mural cells although also a negligible contribution of bone marrow derived myointimal cells was found. These observations suggest that the repair and adaptation mechanisms are impaired in decellularized and degenerated human SCAA walls, since the main source of mural hyperplasia and luminal fibrosis is lost. This may be a reason why the success of endovascular therapies is better in experimental models than in real human SCAAs. The models used to test endovascular devices should be improved so that they would reproduce the pathobiological mechanisms that lead to chronic inflammation and wall degeneration in the human SCAA walls. In an attempt to simulate this, we used in our experimental model ApoE knock out mice that develop spontaneous atherosclerosis. However, even severe atherosclerosis was not sufficient to cause aneurysmatic enlargement and wall degeneration alone.

Finally, we investigate the possibility that intravenously transfused bone marrow-derived cells or circulating peripheral mononuclear cells could be used to enhance neointima formation. Several reports have described contribution of these cells to fibrosis and neointima formation in models of mechanical and immunological vascular injury, and hence we hypothesized that these cells might potentially be used to compensate for the impaired neointima formation in decellularized and degenerated SCAAs. However, we were unable to document contribution of these cells to neointima formation, although the transfused cells seemed to survive in the recipient animal. Our results suggest that if indeed bone marrow derived neointimal cells exist, both bone marrow-derived and circulating peripheral mononuclear cells need to be "activated" or require some other forms of stimulation before they contribute to the neointima formation in injured vascular wall. It is also possible that after systemic transfusion these cells primarily home to other organs than the injured vascular wall, where as after local transplantation the cells might integrate to the injured vascular wall. Further experiments using activation and mobilization of bone marrow cell populations, enrichment of cell populations, in vitro differentiated cells, or local cell transplantation may enlighten the question whether bone marrow-derived cells have potential in occlusive therapy of SCAAs.

Future goals for basic research on the SCAA wall

In order to fully elucidate the pathobiology of SCAA wall rupture and repair, ideally studies that investigate simultaneously the expression of the comprehensive human genome should be performed. Ideally these studies should be performed both at the protein and RNA level. Studies involving the expression of single genes or a set of genes at the protein or RNA level are of course of great value when practically no prior information exists, but these studies have inevitable a very limited view to the hole involved pathobiology, and the inferences and conclusions of such studies are easily misled when the whole setting, or the "whole picture" in which the expression of a set of genes occurs, is neglected. Currently powerful techniques using cDNA or protein microarrays are emerging, and will hopefully provide in the near future methods to study the gene and protein expression associated with the rupture and repair of the SCAA wall in a comprehensive "genome wide" approach.

In addition to the gene expression patterns (at the RNA and protein level) of unruptured and ruptured SCAA, also the intracellular signaling cascades involved should be studied. Activation of many growth factor or cytokine receptors in the cell membrane or in the nucleus is subjected to further regulation by intracellular "secondary mediators and signaling cascades". Activation of these intracellular signaling cascades should be studied to demonstrate together with the expression of so called downstream genes (genes regulated by the activation of a receptor) that a certain receptor actually is activated and has role in the regulation of the SCAA wall pathobiology.

And most importantly of all things, researchers interested in SCAAs need to develop experimental models that reproduce not just the shape and histology of the SCAA wall, but also the pathobiology of the human SCAA wall. This is needed i) to establish the causality of changes associated with rupture in case-control type comparisons of unruptured and ruptured SCAA walls, and to ii) enable interventional studies, e.g. novel pharmaceuticals or gene therapy to modulate the degeneration and repair of the SCAA wall, or bioactive endovascular devices or cellular transplants that secure the rupture-prone SCAA fundus from the cerebral circulation. When such models are available to test the various hypothesis inevitably created by the comparative histological or molecular biology studies of unruptured and ruptured SCAA walls, the possibilities to develop truly new advances in the diagnostics and therapy of SCAAs are there.

Yhteenveto

Aivovaltimoaneurysma (AA), ja sen puhkeamisen aiheuttama hengenvaarallinen lukinkalvonalainen verenvuoto (SAV), on suomalaisessa väestössä poikkeuksellisen yleinen sairaus. Suomessa on vuosittain noin 1000 uutta SAV potilasta, joista lähes puolet menehtyy. AA:n kantajia arvioidaan olevan huomattavasti enemmän, arviolta noin 2% väestöstä. Toistaiseksi ei tiedetä miksi osa AA:ista puhkeaa kun suuri osa säilyy puhkeamattomina läpi kantajiensa elämän. Vaikka AA:n ja SAV:n riskitekijät (tärkeimpinä tupakointi ja korkea verenpaine) ovat hyvin tiedossa, AA:n seinämän biologia ja sen puhkeamiseen johtavat prosessit ovat puutteellisesti tunnettuja. AA:n seinämän puhkeamiseen johtavien solutason tapahtumien selvittäminen on ensiarvoisen tärkeää i) jotta voitaisiin kehittää uusia diagnostisia menetelmiä joiden avulla voidaan erottaa vuotoriskissä olevat AA:t niistä jotka eivät koskaan vuoda, sekä ii) jotta voitaisiin kehittää AA:n seinämän puhkeamisen ehkäiseviä lääkehoitoja tai muita vähän invasiivisia biologisia hoitoja. Nykyisin AA:n seinämän puhkeaminen voidaan ehkäistä kallonsisäisellä mikrokirurgisella ligaatiolla tai tukkimalla AA suonensisäisesti katetrin kautta viedyillä metallikierukoilla. Molempiin hoitoihin liittyy vammautumisen riski, ja lisäksi etenkin suonensisäisesti hoidetut AA:t saattavat uusiutua.

Tämän tutkimuksen tarkoituksena oli selvittää vuotaneiden ja vuotamattomien AA:n seinämien rakenteellisia eroja, sekä tutkia niihin liittyviä biologisia mekanismeja. Lisäksi tutkittiin kokeellisessa aneurysmamallissa aneurysman seinämän rakenteellisia muutoksia sekä niihin osallistuvien solujen alkuperää. Tutkimuksessa todettiin, että vuotamattomien AA:n seinämä on useimmiten tukikudokseltaan ehyempi ja solurikkaampi kuin puhjenneen AA:n seinämä ja että puhjenneen AA:n seinämässä on enemmän tulehdussoluja kuin vuotamattoman AA:n seinämässä. Vuotamattoman AA:n seinämä todettiin muistuttavan rakenteeltaan korkean verenpaineen, mekaanisen stressin, ja valtimokovettumataudin valtimoihin aiheuttamia muutoksia. Tutkimuksessa todettiinkin, että samoin kuin edellä mainituissa valtimon seinämän muutoksissa, myös AA:n seinämään kertyy LDL-kolesteroli partikkeleita, jotka hapettuvat ja muunnuttuaan saattavat käynnistää AA:n seinämässä tulehdusreaktion. Potilailla, joilla AA oli puhjennut, todettiin verenkierrossa useammin ja enemmän vasta-aineita hapettunutta LDL-kolesterolia vastaan kuin vuotamattoman AA:n kantajilla.

Lisäksi kartoitettiin AA:n seinämän soluja sääteleviä kasvutekijäreseptoreita, joista ainoastaan IGF-kasvutekijän reseptori 1 löytyi lähes kaikkien AA:ien seinämistä ja lähes kaikista AA:n seinämän soluista. Reseptoreista

VEGF-kasvutekijän reseptori 1 näytti assosioituvan AA:n seinämän puhkeamiseen. Näiden kasvutekijäreseptoreiden toiminnan tarkempi selvittäminen AA:n seinämässä vaikuttaa AA:n seinämään vaikuttavan lääkehoidon kannalta lupaavalta kohteelta, joskin lisätutkimuksia ja vahvistavia tuloksia tarvitaan.

Kokeellisessa aneurysmamallissa tutkittiin seinämän uudelleen muovautumiseen ja arpeutumiseen osallistuvien solujen alkuperää. Mallissa todettiin rakenteellisia muutoksia, jotka muistuttivat vuotamattoman AA:n seinämää. Kokeellisessa mallissa seinämän uudelleen muovautumiseen osallistuneet solut olivat peräisin lähes täysin aneurysman seinämästä. Koska ihmisen puhjenneen AA:n seinämä on usein hyvin vähäsoluinen, vaikuttaa kokeellisessa mallissa tehtyjen havaintojen perusteella, että puhjennen AA:n seinämän arpeutumis- ja paranemiskyky on vuotamattomaan AA:aan nähden vajavainen. Muualta kehosta peräisin olevilla sidekudossoluilla saatettaisiin voida kompensoida puhjenneen AA:n heikkoa seinämää ja arpeutumista. Tässä tutkimuksessa todettiin luuydinperäisten sidekudossolujen osuuden kokeellisen AA:n seinämän arpeutumiseen olevan erittäin niukkaa. Lisäksi tutkittiin ihmisen verenkierrosta eristettyjen solujen käyttöä mekaanisesti vaurioitetun valtimon seinämän arpeutumisen lisäämisessä. Ihmisen verenkierrosta peräisin olevin stimuloimattomien solujen ei kuitenkaan havaittu osallistuvan valtimon seinämän vaurion paranemiseen.

Sammandrag

Subaraknoidal blödning (SAB) förorsakad av aneurysmruptur är en typ av akut hjärnblödning med dödlig utgång i nästan 50% av fallen. I Finland förekommer det årligen cirka 1000 nya fall av SAB, mest bland annars friska medelålders personer. Cirka hälften av de som överlever SAB får svåra neurologiska komplikationer, till exempel förlamade extremiteter eller neuropsykologiska svårigheter. Alla aneurysm på hjärnblodkärl blöder inte, och en stor del av blöder aldrig. Tyvärr finns det i dag inga metoder att identifiera de aneurysm som eventuellt kommer att blöda.

Nuförtiden kan man förhindra SAB genom att isolera aneurysm från hjärnans blodcirkulation utnyttjande mikroneurokirurgiska eller endovaskulära metoder. Båda metoderna förorsakar mortalitet och morbiditet. I dag finns det ingen farmakologisk terapi för att förhindra aneurysmruptur.

Man känner inte till de patobiologiska processer i aneurysmväggen som leder till ruptur. Vetenskapen om de cellulära och molekylära mekanismerna som ligger bakom rupturer i aneurysmens vägg är nödvändig av flera orsaker: 1) För att kunna kunna utveckla nya metoder att upptäcka aneurysm som kommer att blöda. 2) För att utveckla nya säkra och minimalt invasiva metoder att förhindra aneurysmruptur, till exempel farmakologisk terapi eller stamcellsterapi. Hittills finns det endast begränsat med information och få utförda studier om de histologiska och molekulära skillnaderna mellan rupturerade och orupturerade hjärnaneurysm. Jämförande studier är nödvändiga för att kunna formulera vetenskapliga hypoteser om de cellulära och molekylära processer som leder till aneurysmväggens ruptur. Dessa hypoteser kan man sen pröva i experimentella aneurysmmodeller.

Avsikten med denna doktorsavhandling har varit att i detalj studera de histologiska skillnaderna mellan hjärnaneurysm som har blött och sådana som inte har blött, och att utforska några av de molekylära mekanismer som troligen påverkar de cellulära processer som leder till dessa histologiska skillnader. Den andra avsikten har varit att i experimentella aneurysmmodeller utforska möjligheterna att använda stamceller från benmärg eller cirkulerande mononukleära celler för att läka strukturella förändringar som leder till ruptur i aneurysmväggen.

Denna doktorsavhandlingen visar att rupturerade hjärnaneurysm har en mera degenererad väggstruktur, med minskad cellularitet och ökad infiltration av inflammtoriska celler. Därtill påvisar vi ackumulering av oxiderad LDL på aneurysmväggen och en humoral immunologisk reaktion till

oxiderad LDL hos SAB- patienter. Oxiderad LDL är en av de mest kända orsakerna till inflammation i artärer.

Vi har också utforskat receptorer till vaskulära tillväxtsfaktorer på aneurysmväggen, och påvisar förändringarna på de receptorer som är sammankopplade med aneurysmruptur. I denna doktorsavhandling beskrivs också en ny experimentell aneurysmmodell som användes för att utforska vilken roll stamceller från benmärg har vid förtjockning av artärväggen. Metoden kunde möjligen användas för att förhindra rupturer i aneurysmväggen. Mesenkymala celler härstammande från benmärg spelade en minimal roll vid det experimentella aneurysmets förtjockning, och transplanterade cirkulerande mononukleära celler ökade inte förtjockingen av artärväggen.

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